Université Lille 1 – PRES Lille Nord de France

Mémoire en vue de l'obtention de l'Habilitation à Diriger des Recherches

Robert-Alain Toillon

Contrôle de la croissance des cellules cancéreuses de sein :

Aspects fondamentaux et applications thérapeutiques

Soutenue le 18 juin 2009 devant la commission d'examen

Président du jury :	Pr. Philippe Delannoy
Rapporteurs :	Pr. Olivier Coqueret
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Résumé

Les progrès de la recherche et de la médecine en oncologie résultent en l'amélioration de la survie des patientes atteintes d'un cancer du sein. La compréhension des mécanismes qui gouvernent la croissance tumorale a ainsi permis de développer des thérapies ciblées (Tamoxifène, Fulvestran, Herceptine). Néanmoins, le cancer du sein entraîne, toujours, le décès d'environ 30% des malades. Il est donc impératif de poursuivre l'étude des mécanismes de cancérisation dans leur globalité afin d'accroître l'arsenal thérapeutique et son efficacité. L'ensemble de mes travaux de recherche s'inscrit dans cette thématique. Durant ma thèse, j'ai plus particulièrement étudié les mécanismes qui concourent à l'homéostasie tissulaire et inhibent l'oncogenèse. J'ai démontré que le développement des cellules cancéreuses mammaires est contrôlé par les cellules épithéliales mammaires normales qui produisent des facteurs apoptogènes, notamment l'IGFBP3 et la maspine. J'ai ensuite développé des recherches translationnelles à l'Institut Jules Bordet qui visent à rationaliser l'usage des différentes thérapies dans le cancer du sein (notamment en ce qui concerne la radiothérapie et l'hormonothérapie). Ainsi, j'ai pu montrer que la radiothérapie peut avoir un impact sur l'efficacité de l'hormonothérapie. D'autre part, j'ai étudié les effets sur la croissance des cellules cancéreuses de sein d'une nouvelle molécule dérivée du Tamoxifène. Depuis 2006, au sein de l'unité du Pr Hondermarck, mes travaux se sont orientés vers la compréhension de la signalisation des facteurs de croissance dans le cancer du sein et plus particulièrement du NGF et de son récepteur TrkA. En effet, le NGF et ses récepteurs sont exprimés et modulés dans le cancer du sein. A l'instar des cellules neuronales où les mécanismes d'activation de ces complexes sont mieux décrits, j'ai plus particulièrement entrepris de définir par protéomique fonctionnelle les premières étapes de l'activation du récepteur TrkA et du recrutement de ses adaptateurs. L'objectif de ce travail est de dégager les différences observées entre cellules neuronales et cancéreuses de sein dans un but ultime de développement de molécules thérapeutiques impactant TrkA et ses effets sur la croissance des cellules cancéreuses de sein.

Sommaire

Curriculum vitae

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Formation

1997-2001	Doctorat de Sciences de la Vie et de la Santé. Titre : « Caractérisation de l'effet pro-apoptogène des cellules épithéliales mammaires normales sur les cellules cancéreuses de sein », USTL (Mention Très Honorable avec félicitations). Directeur de thèse : Pr. X. Le Bourhis ; Jury : Pr. H. Hondermarck, Pr. G. Leclercq, Pr. E. Solary, Pr. F. Calvo, Dr. J.-P. Peyrat.
1996-97	DEA de Biologie-Santé, option Biologie Cellulaire, USTL (Mention Bien).

1994-95 Maîtrise de Biologie Cellulaire, USTL (Mention Assez-Bien).

1993-94 Licence de Biologie Cellulaire, USTL.

1991-93 DEUG Sciences de la Nature et de la Vie, option Chimie-Biologie, USTL.

1991 Baccalauréat série D. Académie de Lille (Mention Assez-Bien).

Activités de Recherche

2006 Maître de conférences de l'université Lille 1. « Signalisation de TrkA dans les cellules cancéreuses de sein ». Laboratoire INSERM U908, JE 2488. Signalisation des facteurs de croissance dans les cancers du sein. Protéomique fonctionnelle. Directeur : Pr. H. Hondermarck.

2002-06 Chargé de Recherche. « Développement des études de la croissance et des voies de signalisation des (anti)-œstrogènes ». Laboratoire J-C Heuson de Cancérologie mammaire, Institut Jules Bordet, Bruxelles, Belgique. Directeur: Pr. G. Leclercq.

Participation au groupe de recherche européen COST-D20 « Metal compound in the treatment of cancer and viral diseases ».

2001-02 Attaché Temporaire d'Enseignement et de Recherche. « Purification et identification des facteurs pro-apoptogènes produits par les cellules épithéliales mammaires normales ». Laboratoire de Biologie du Développement, Université Lille 1.

1997-2001 Doctorat Sciences de la Vie et de la Santé. « Caractérisation de l'effet pro-apoptogène des cellules épithéliales mammaires normales sur les cellules cancéreuses de sein ». Laboratoire de Biologie du Développement, USTL. Directeur de thèse: Pr. X. Le Bourhis.

1996-97 DEA Biologie-Santé. « Mise en évidence des effets des cellules épithéliales mammaires normales sur la croissance, la migration et l'invasion des lignées cancéreuses de sein ». Laboratoire de Biologie du Développement, USTL. Directeur de stage: Dr. X. Le Bourhis.

Encadrements

2008- M. Cyril Corbet, Doctorant (Boursier MRT). Ecole doctorale Biologie Santé de Lille. «Implication de la phosphorylation du récepteur TrkA dans le cancer du sein».

2007-08 M. Cyril Corbet, Master II, Ecole doctorale Biologie Santé de Lille. « Etude de la phosphorylation du récepteur TrkA dans les cellules cancéreuses de sein».

2006-07 M^{elle} Olympe Tomavo, Master II, Ecole doctorale Sciences de la Matière, du Rayonnement et de l'Environnement, Lille. « Synthèse de nouveaux inhibiteurs de la croissance des cellules cancéreuses de sein : dérivés de l'acide caféique »

2005 M. Nicolas Loriers, « Graduat » (Master II pro). Haute Ecole Rennequin Sualem, Liège, Belgique. « Etude de la réponse au Tamoxifène dans les cellules irradiées. »

2004 M^{elle} Muriel Depuidt, Ingénieure. Haute école Lucia de Brouckère, Bruxelles, Belgique. « Etude du rôle de la liaison Calmoduline/ERα ».

Publications

Articles originaux :

1. Lambrecht V, Le Bourhis X, Toillon RA, Boilly B, Hondermarck H (1998). Alterations in both heparan sulfate proteoglycans and mitogenic activity of Fibroblast Growth Factor-2 are

triggered by inhibitors of proliferation in normal and breast cancer epithelial cells. ***Experimental Cell Research***, **245**: 239-44.

2. Toillon RA, Adriaenssens E, Wouters D, Lottin S, Boilly B, Hondermarck H, Le Bourhis X (2000). Normal Breast Epithelial Cells induced apoptosis of breast cancer cell line MCF-7 through p53 pathway. ***Molecular and Cellular Biology Research Communication***, **3**: 338-44.
3. Ouadid-Ahidouch H, Le Bourhis X, Roudbaraki M, Toillon RA, Delcourt P, Prevarskaya N (2001). Changes in the K+ current-density of MCF-7 cells during progression through the cell cycle: possible involvement of an h-ether a gogo K+ channel. ***Receptors Channels* 7**: 345-56.
4. Descamps S, Toillon RA, Adriaenssens E, Pawlowski V, Le Bourhis X, Boilly B, Peyrat JP (2001). Nerve growth factor stimulates proliferation and survival of human breast cancer cells through two distinct signalling pathways. ***Journal of Biological Chemistry* 276**: 17864-70.
5. Chopin V, Toillon RA, Jouy N, Le Bourhis X. Sodium butyrate induces P53-independent, Fas-mediated apoptosis in MCF-7 human breast cancer cells (2002). ***British Journal of Pharmacology***, **135**: 79-86.
6. Toillon RA, Chopin V, Jouy N, Fauquette W, Boilly B, Le Bourhis X (2002). Normal breast epithelial cells induce p53-dependent apoptosis and p53-independent cell cycle arrest of breast cancer cells. ***Breast Cancer Research and Treatment***, **71**: 269-80.
7. Toillon RA, Descamps S, Adriaenssens E, Ricort JM, Bernard D, Boilly B, Le Bourhis X (2002). Normal Breast Epithelial Cells induce apoptosis of breast cancer cells via Fas signaling. ***Experimental Cell Research***, **275**: 31-43.
8. Vergote D, Cren-Olive C, Chopin V, Toillon RA, Rolando C, Hondermarck H, Le Bourhis X (2002). (-)-Epigallocatechin (EGC) of green tea induces apoptosis of human breast cancer cells but not their normal counterparts. ***Breast Cancer Research and Treatment***, **76**: 195-201.

9. Laïos I, Journé F, Laurent G, Nonclercq D, Toillon RA, Seo HS, Leclercq G (2003). Mechanisms governing the accumulation of estrogen receptor alpha in MCF-7 breast cancer cells treated with hydroxytamoxifen and related anti-estrogens. ***Journal of Steroid Biochemistry and Molecular Biology***, **87**: 207-21.
10. Chopin V, Toillon RA, Jouy N, Le Bourhis X (2004). P21WAF1/CIP1 is dispensable for G1 arrest, but indispensable for apoptosis induced by sodium butyrate in MCF-7 breast cancer cells. ***Oncogene***, **23**: 21-9.
11. Magné N, Toillon RA, Laïos I, Lacroix M, Van Houtte P, Leclercq G (2004). Effects of ionizing radiation on oestrogen receptor in MCF-7 breast cancer cells. ESTRO, Amsterdam. Meeting abstract, ***Radiotherapy and Oncology,*** **73**: S235-S235.
12. Laïos I, Journé F, Nonclercq D, Salazar Vidal D, Toillon RA, Laurent G, Leclercq G (2005). Role of the proteasome in the regulation of oestrogen receptor turn over and function in MCF-7 breast carcinoma cells. ***Journal of Steroid Biochemistry and Molecular Biology***, **94**: 347-59.
13. Toillon RA, Magné N, Laïos I, Lacroix M, Devriendt D, Van Houtte P, Leclercq G (2005). Effects of low gamma-ray irradiation doses on sensitivity of MCF-7 breast cancer cells to estrogenic and anti-estrogenic stimuli, ***Breast Cancer Research and Treatment***, **93**: 207-15.
14. Magné N, Toillon RA, Castadot P, Ramaioli A, Namer M (2006). Different clinical impact of oestradiol receptor determination according the analytical method. A study on 1940 breast cancer patients over a period of 16 consecutive years, ***Breast Cancer Research and Treatment, 95*** : 179-84.
15. Toillon RA, Magné N, Laios I, Castadot P, Kinnaert E, Van Houtte P, Desmedt C, Leclercq G, Lacroix M (2007). Estrogens decrease gamma-ray-induced senescence and maintain cell cycle progression in breast cancer cells independently of p53. ***International Journal Radiation Oncology Biology Physic,*** **67**: 1187-200.

16. Toillon RA, Lagadec C, Page A, Chopin V, Sautière PE, Ricort JM, Lemoine J, Zhang M, Hondermarck H, Le Bourhis X (2007). Proteomic Demonstration that Normal Breast Epithelial Cells Induce Apoptosis of Breast Cancer Cells through Insulin-Like Growth Factor Binding Protein-3 and Maspin. ***Molecular and Cellular Proteomics***, **6**: 1239-47.
17. Nonclercq D, Journé F, Laïos I, Chaboteaux C, Toillon RA, Leclercq G, Laurent G (2007). Effect of nuclear export inhibition on oestrogen receptor regulation in breast cancer cells. ***Journal of Molecular Endocrinology***, **39**: 105-18.
18. Lagadec C, Adriaenssens E, Toillon RA, Chopin V, Romon R, Van Coppenolle F, Hondermarck H, Le Bourhis X (2008). Tamoxifen and TRAIL synergistically induce apoptosis in breast cancer cells. ***Oncogene***, **27**:1472-7.
19. Lagadec C, Meignan S, Adriaenssens E, Foveau B, Vanhecke E, Romon R, Toillon RA, Oxombre B, Hondermarck H, Le Bourhis X (2009). TrkA overexpression enhances growth and metastasis of breast cancer cells. ***Oncogene***, *sous presse*.

Articles de revues:

20. Le Bourhis X, Toillon RA, Boilly B, Hondermarck H (2000). Autocrine and paracrine growth inhibitors of breast cancer cells. ***Breast Cancer Research and Treatment***, **60**: 251-8.
21. Magné N, Toillon RA (2004). Ciblage du récepteur aux facteurs de croissance épithéliaux (REGF) par les inhibiteurs de tyrosine kinase et applications dans les cancers colorectaux. ***Oncologie***, **6**: 41-5.
22. Lacroix M, Toillon RA, Leclercq G (2004). Stable "portrait" of ductal breast tumors during progression. Data from biology, pathology, and genetics. ***Endocrine Related Cancer***, **11**: 497-523.

23. Magné N, Didelot C, Toillon RA, Van Houtte P, Peyron JF (2004). Biomodulation of transcription factor NF-kappaB by ionizing radiation. ***Cancer/Radiotherapie***, **8**: 315-21.
24. Magné N, Toillon RA, Bourgeois N, Moretti L, Castadot P, Simon S, Philippson C, Van Houtte P (2005). Une nouvelle ère pour la radiothérapie avec des avancées technologiques, biologiques et cliniques prometteuses. ***Revue Médicale de Bruxelles***, **26**: 21-6.
25. Magné N, Toillon RA, Van Houtte P, Gérard J-P, Peyron J-F (2006). NF-kappaB modulation and ionizing radiation: mechanisms and future directions for cancer treatment. ***Cancer letters***, **231**: 158-68.
26. Lacroix M, Toillon RA, Leclercq G (2006). P53 and breast cancer: an update. ***Endocrine Related Cancer***, **13**: 293-325.
27. Hondermarck H, Tastet C, El Yazidi-Belkoura I, Toillon RA, Le Bourhis X (2008). Proteomics of breast cancer: the quest for markers and therapeutic targets. ***Journal of Proteome Research***, **7**: 1403-11.
28. Chargari C, Toillon RA, MacDermed D, Castadot P, Magné N (2009). Concurrent hormonal and radiation therapy in breast cancer patients: what is the rationale? ***The Lancet Oncology***, **10**: 53-60.

Posters-Congrès-Séminaires

1. Ouadid-Ahidouch H, Le Bourhis X, Toillon RA, Prevarskaya N (1998). Membrane potential and k+ currents in mammary cancer cells. Physiological society congress, Southampton, Royaume-Uni.
2. Toillon RA, Adriaenssens E, Wouters D, Lottin S, Boilly B, Hondermarck H, Le Bourhis X (1999). Normal Breast Epithelial Cells induced apoptosis of breast cancer cells through p53

and tyrosine phosphatase pathways. COST Biology of mammary gland conference, Tours, France.

3. Vercoutter AS, Lemoine J, Hornez L, Le Bourhis X, Toillon RA, Boilly B, Peyrat JP, Hondermarck H (1999). Proteomic analysis of cancer versus normal breast epithelial cells. COST Biology of mammary gland conference, Tours, France.
4. Toillon RA, Chopin V, Adriaenssens E, Lottin S, Hondermarck H, Boilly B, Le Bourhis X (2000). Identification des facteurs produits par les cellules épithéliales mammaires normales induisant l'apoptose des cellules cancéreuses de sein. Journée des boursiers de l'Association pour la Recherche sur le Cancer, Paris, France.
5. Toillon RA, Leclercq G (2003). Régulation du Récepteur aux œstrogènes alphas par les thérapies adjuvantes dans le cancer du sein. Séminaire de la recherche Télévie 2003. Université de Liège, Belgique.
6. Toillon RA, Magné N, Leclercq G (2004). Régulation du Récepteur aux œstrogènes alphas par les thérapies adjuvantes dans le cancer du sein. Séminaire de la recherche Télévie. Université Catholique de Bruxelles, Belgique.
7. Toillon RA, Magné N, Laïos I, Leclercq G (2004). Estrogens impact growth-inhibitory efficiency of radiotherapy in MCF-7 breast cancer cells. Journée Lilloise de Cancérologie (5ème éd). Université des Sciences et Technologie de Lille.
8. Gallo D, Toillon RA, Jacquemotte F, Laurent G and Leclercq G (2004). Estrogen Receptor alpha derived peptide (P295-T311) down-regulates Estrogen Receptor alpha in MCF-7 cells through abrogating its association with Calmodulin. Université Libre de Bruxelles, Belgique.
9. Magné N, Toillon RA, Laïos I, Lacroix M, Van Houtte P, Leclercq G (2004). Effects of ionizing radiation on estrogen receptor in MCF-7 breast cancer cells. ESTRO, Amsterdam, Pays-Bas.

10. Toillon RA, Laïos I, Leclercq G, Vessières A, Jaouen G (2005). Dual effects of organometallics SERMs in breast cancer cells COST D20 WG 007-01 meeting, Paris.
11. Haddad I, Toillon RA, Leclercq G, Magné N, Vinh J (2005). Analyse protéomique dans le cancer du sein : rôle des récepteurs aux œstrogènes dans la résistance à la radiothérapie. 1^{ER} Symposium de Chimie et Biologie Analytiques, Montpellier.
12. Toillon RA, Laïos I, Leclercq G, Vessières A (2005). Dual effects of organometallics SERMs in breast cancer cells. FECS meeting, Rimini, Italie.
13. Toillon RA (2007). Proteomic demonstration that normal breast epithelial cells can induce apoptosis of breast cancer cells through insulin-like growth factor binding protein-3 and maspin. SMAP meeting, Pau.

Obtention de financements de recherche

2008 **La Ligue contre le cancer** (France), **40 000 euros,** « Etude de la phosphorylation du récepteur TrkA et implication dans le cancer du sein ». (acquisition d'un système d'acquisition d'image type Chemidoc).

Gefluc (France), **10 000 euros,** « Etude de la phosphorylation du récepteur TrkA et implication dans le cancer du sein »**.** (acquisition d'un lecteur de plaque).

2007 **Université Lille 1** (Villeneuve d'Ascq, France), **44 000 euros**, BQR, « Etudes ex-vivo des effets du NGF sur le contrôle de la prolifération des cellules tumorales mammaires ». (aide à l'installation, achat de petits appareils de laboratoire et informatique).

2006 **Amis de l'Institut Bordet** (Bruxelles, Belgique), **60 000 euros**, « Régulation de l'impact de la radiothérapie par les œstrogènes dans le cancer du sein ». (fonctionnement).

Fonds National de la Recherche Scientifique (FNRS), **10 000 euros**, Crédit aux chercheurs, «SERMs (Selective Estrogen Receptor Modulators) organométalliques à dualité fonctionnelle. Etude des mécanismes d'action de ces composés originaux ». (fonctionnement).

2005 **Accord de coopération CGRI-FNRS-CNRS** (Belgique/ France), 2005-2006, « Exploration pharmacologique et synthèse d'inhibiteurs des interactions récepteurs d'œstrogènes / PNRC ». (déplacement).

Amis de l'Institut Bordet (Bruxelles, Belgique), **52 500 euros**, « Régulation de l'impact de la radiothérapie par les œstrogènes dans le cancer du sein ». (fonctionnement).

2004 **Fonds National de la Recherche Scientifique** (FNRS, Belgique), **± 180 000 euros,** obtention d'un mandat de chargé de recherche (3 ans). « Impact de la radiothérapie sur la régulation des récepteurs aux œstrogènes alpha dans les cellules cancéreuses du sein et modulations thérapeutiques ». (salaire).

Amis de l'Institut Bordet (Bruxelles, Belgique), **25 000 euros**, « Etude des mécanismes de régulation du récepteur aux œstrogènes alphas dans la lignée cancéreuse de sein MCF-7 sous l'influence d'anti-œstrogènes et de la radiothérapie». (Achat d'appareil d'électrophorèse 2-D).

Fonds National de la Recherche Scientifique/Amis de l'Institut Bordet (Bruxelles, Belgique), **108 990 euros**, « Recherche multidisciplinaire en cancérologie : utilisation d'une technique d'imagerie pour analyse de gels d'électrophorèse ». (acquisition d'un scanner Typhoon 9400 et d'un système d'analyse d'image Fuji LAS 3000)(projet associé de 4 laboratoires).

Bilan détaillé des travaux

Le cancer du sein reste, à nos jours, la première pathologie tumorale chez la femme, avec environ 50.000 nouveaux cas et 12.000 décès en France (en 2005, selon les données de l'InVS). Après une augmentation de l'incidence, imputable à la généralisation du dépistage et au vieillissement de la population, ces chiffres ont tendance à se stabiliser. Avec 30% d'échecs thérapeutiques (Gonzalez-Angulo *et al*, 2007) et une survie à 5 ans estimée à 79,8% (Coleman *et al*, 2008), le développement de thérapies ciblées et adaptées à chaque patiente semble nécessaire. En effet, l'essor de la génomique (microarrays) a permis d'affiner la classification des tumeurs en quelques grandes catégories (luminal A et B, basal, normal like, ErbB2 positive) mais confirme l'hétérogénéité des tumeurs mammaires (Lacroix *et al*, 2004, **annexe, article 1**; Blick *et al*, 2008). Dans ce contexte, les travaux que je poursuis depuis mon doctorat s'inscrivent dans la nécessité d'améliorer notre connaissance du développement tumoral dans le but d'accroître l'arsenal thérapeutique. Ces travaux s'articulent selon trois axes :

- Homéostasie et cancer: Etude de la régulation de la croissance des cellules cancéreuses de sein par les cellules épithéliales normales,
- Recherches pré-cliniques et translationnelles: Nouvelles combinaisons thérapeutiques permettant d'accroître l'efficacité des anti-œstrogènes dans le cancer du sein,
- Etude de la signalisation de TrkA dans le cancer du sein.

I- Homéostasie et cancer: Etude de la régulation de la croissance des cellules cancéreuses de sein par les cellules épithéliales normales.

L'évolution de la tumeur mammaire est extrêmement longue (6 à 8 ans) (Oudard, 1997). Elle est soumise à des interactions complexes entre les cellules tumorales et le tissu normal environnant (Bissell et Radisky, 2001 ; Allinen *et al*, 2004 ; Ingber, 2008 ; Gatenby et Gillies, 2008). Ainsi des contrôles intercellulaires provenant des cellules normales peuvent pendant un certain temps limiter le développement tumoral, donc freiner l'étape de promotion infra-clinique des cellules tumorales dans le tissu normal. L'identification de facteurs provenant du tissu normal, capables de ralentir la croissance et l'invasion des cellules cancéreuses du sein ainsi que la compréhension de leurs mécanismes d'action constituent une thématique essentielle dans la compréhension des mécanismes d'oncogenèse et le développement de futures approches thérapeutiques plus efficaces. Au début de notre étude, un certain nombre de travaux indiquaient que les cellules épithéliales mammaires normales (CEMN) sécrètent des facteurs inhibiteurs tels que le « mammary derived growth factor » (MDGI) (Böehmer *et al*, 1987), la mammastatine (Ervin *et al*, 1989), ou encore le TNFalpha (Varela et Ip, 1997 ; Le Bourhis *et al*, 2000). Cependant, l'étude de leur action inhibitrice sur la croissance cellulaire restait fragmentaire et leurs mécanismes d'action étaient encore mal connus. Dong-Le Bourhis *et al* (1997) avaient également montré que les CEMN inhibent la croissance des cellules cancéreuses du sein, et que ce processus ne semble pas impliquer des facteurs inhibiteurs déjà connus. Ces études étaient focalisées sur le contrôle de la prolifération, mais il a été démontré que les processus entraînant la mort cellulaire par apoptose étaient tout aussi importants dans l'inhibition de la croissance des cellules cancéreuses (Hale *et al*, 1996 ; Tang et Porter, 1997). Les travaux initiés au cours de ma thèse ont

montré que le milieu conditionné de CEMN induit l'apoptose des cellules MCF-7 et T-47D (cellules cancéreuses de sein hormono-dépendantes) mais n'a pas d'effet sur les cellules MDA-MB-231 (cellules cancéreuses de sein hormono-indépendantes). L'induction de l'apoptose dans les cellules MCF-7 s'accompagne d'une augmentation de l'expression des protéines $P21^{WAF1/CIP1}$, P53 et du ratio Bax/Bcl-2. La protéine P53 est par ailleurs impliquée dans l'induction de l'apoptose des cellules MCF-7 et T-47D. De plus, l'activité apoptogène du milieu conditionné par les CEMN dépend également de la phosphorylation de protéines dans les cellules cancéreuses de sein. L'ensemble de ces résultats a fait l'objet de deux publications (Toillon *et al*, 2000 ; Toillon *et al*, 2002a). En parallèle, les mécanismes de survie mis en place par les cellules cancéreuses de sein ont été étudiés. Ainsi, nos travaux ont permis d'établir que l'activation constitutive de la PI3 kinase et de NF-kappaB est responsable de la résistance des cellules MDA-MB-231 à l'action apoptogène des CEMN. Par ailleurs, nous avons montré que l'apoptose des cellules cancéreuses de sein induite par les CEMN est liée à l'activation du système Fas/Fas Ligand (Toillon *et al*, 2002b).

Néanmoins, à la fin de ma thèse, nous n'avions que peu d'indices sur la nature des facteurs apoptogènes produits par les CEMN dans le milieu de culture : Facteurs de nature protéique, thermolabiles (inactivation par chauffage à 56°C) et de masse moléculaire supérieure à 30 kDa. A partir de ces propriétés, nous avons défini un protocole de purification des facteurs apoptogènes. En résumé, dix litres de milieu conditionné ont été concentrés par ultrafiltration tangentielle sur cassette (ultrasette Pal-Gelmann, seuil de coupure de 30 kDa). Puis une première séparation est effectuée sur cartouche Sep-Pak CM. Les fractions sont éluées de la cartouche dans des tampons de plus en plus hydrophobes (contenant 50, 80 et 100% d'acétonitrile). Elles sont ensuite

analysées en HPLC préparatrice puis en HPLC analytique. Nous avons dès lors identifié par spectrométrie de masse deux facteurs pro-apoptogènes produits par les CEMN : l'IGFBP-3 (Insulin Growth Factor Binding Protein-3) et la maspine (Toillon *et al*, 2007, **annexe, article 2**).

L'IGFBP-3 fait partie de la famille des IGFBPs qui comportent 6 membres. L'IGFBP-3 est la plus abondante. Elle se retrouve essentiellement dans le plasma où elle est complexée avec l'IGF-1 et 2 (Baxter, 1993) et contrôle leurs biodisponibilités. Au niveau des cellules cancéreuses de sein, il semble que son expression soit inversement corrélée à celle du récepteur aux œstrogènes (Figueroa *et al*, 1993 ; Rocha *et al*, 1996). Néanmoins, il n'existe pas de corrélation entre une élévation du taux plasmatique d'IGFBP-3 et sa production par les tumeurs mammaires *in vivo*. Il semble alors que l'IGFBP-3 produit agisse de façon locorégionale (revue dans Perks et Holly, 2008; Yamada et Lee, 2009) : l'IGFBP-3 stimule la croissance des cellules épithéliales normales (Strange *et al*, 2002 ; Burrows *et al*, 2006) et inhibe celle des cellules cancéreuses de sein (Salahifar et al, 2000 ; Burrows *et al*, 2006). Nos résultats étayent cette hypothèse et mettent en lumière l'effet pro-apoptogène de la sécrétion de l'IGFBP-3 par les cellules épithéliales mammaires normales sur les cellules cancéreuses de sein. Nous avons observé que cet effet pro-apoptogène est indépendant de la liaison de l'IGFBP-3 aux IGFs. Deux questions restent en suspens : quelle est la cible moléculaire (récepteur) des IGFBP-3 sur les cellules cancéreuses de sein ? Comment l'IGFBP-3 induit-elle l'apoptose dans les cellules cancéreuses de sein ? A ce jour, plusieurs pistes ont été abordées. En ce qui concerne les cibles moléculaires, de nombreux travaux reportent l'existence de sites de liaison membranaire pour l'IGFBP-3, néanmoins, leur nature est inconnue. L'IGFBP-3 pourrait s'associer aux récepteurs du TGF-beta ou encore agir en combinaison avec des

intégrines (Leal *et al*, 1997 ; Kuemmerle *et al*, 2004). L'hypothèse d'une activation des complexes RXR par les IGFBP-3 (Liu *et al*, 2000) semble, quant à elle, être remise en cause par l'étude de Zappala *et al* (2008). D'autre part, en absence de sites de liaison clairement identifiés aux IGFBP-3, les mécanismes d'induction de l'apoptose restent hypothétiques. Néanmoins, nous avons démontré que l'apoptose induite par les CEMN dépend de l'augmentation de Fas et de son récepteur et que l'activation constitutive de NF-kappa B l'inhibe (Toillon *et al*, 2002b). Ces résultats sont en accord avec les travaux de Kim *et al* (2004), qui démontrent l'implication de la voie des récepteurs de mort dans l'apoptose induite par les IGFBP3.

Le deuxième facteur pro-apoptogène identifié est la maspine (mammary serine protease inhibitor). Elle appartient à la famille des serpines (serine protease inhibitor) qui est composée entre autres membres des plasminogen activator inhibitors 1 et 2, ou encore de α-1 anti trypsin. Comme nous avons pu le vérifier sur les cellules MCF-7, son expression est diminuée dans la plupart des cancers du sein. Cette diminution résulte le plus souvent de la méthylation de son promoteur (revue dans Khalkhali-Ellis, 2006). Il a également été reporté que son expression serait affectée au niveau post-transcriptionnelle par le micro ARN mir-21 (Zhu *et al*, 2008). Dans notre étude, nous avons confirmé le rôle apoptotique de la maspine. En effet, les travaux de Latha *et al* (2005) et Zhang *et al* (2005) ont démontré que la surexpression de la maspine induit l'apoptose des cellules cancéreuses. Pour autant, le mécanisme d'induction de l'apoptose n'est pas à ce jour clairement établi : les mécanismes qui régissent l'apoptose induite par la maspine sont-ils identiques entre les formes intra-cellulaires ou sécrétées ?

L'ensemble de mes travaux sur les interactions cellules épithéliales mammaires normales / cellules cancéreuses montrent donc que :

- Les cellules normales exercent un rétrocontrôle puissant sur la croissance des cellules cancéreuses de sein, *via* l'IGFBP3 et la maspine,
- Cette homéostasie cellulaire est néanmoins altérée par des mécanismes d'échappement mis en place lors de la progression tumorale qui impliquent notamment NF-kappaB,
- Néanmoins ces mécanismes d'échappement sont réversibles et ouvrent des pistes thérapeutiques qu'il conviendrait d'exploiter.

II- Recherches pré-cliniques et translationnelles : Recherche de nouvelles combinaisons thérapeutiques permettant d'accroître l'efficacité thérapeutique des anti-œstrogènes dans le cancer du sein.

Les œstrogènes jouent un rôle majeur dans l'initiation et la promotion des cancers du sein (Russo et Russo, 2006; Lacroix *et al*, 2004). Au cours de ces dernières années, d'importants progrès ont permis de mieux comprendre leurs mécanismes d'action (Heldring *et al*, 2007). Ainsi, ils agissent principalement *via* deux types de récepteurs aux œstrogènes alpha (ERα) et beta (ERβ). Ce sont des récepteurs nucléaires. Ils présentent classiquement 5 domaines fonctionnels dont un domaine de liaison à l'ADN et deux domaines d'activation (AF1 et AF2). Ces domaines définissent l'activité de facteur de transcription ligand-inductibles des récepteurs aux œstrogènes : en présence de 17-beta œstradiol (E2, ligand naturel), ils se fixent, au niveau des promoteurs de gènes cibles, sur des séquences consensus (Estrogen Response Element ou ERE). Ils s'associent également, dans le noyau ou la mitochondrie, à d'autres facteurs de transcription pour agir sur des sites AP1, SP1 entre autres (Pearce et Jordan, 2004 ; Klinge, 2008). D'autre part, les récepteurs aux œstrogènes interfèrent avec les voies de signalisation de la croissance cellulaire ; leurs effets sont alors qualifiés de «non génomiques». Il a été décrit que l'E2 peut également agir

indépendamment de ces récepteurs en activant un récepteur à 7 domaines transmembranaires (GPR 30) (revue dans Prossnitz *et al*, 2007). Cette activation serait, en partie, responsable de l'induction des MAP-kinases. L'ensemble de ces effets régule la prolifération, la survie et la différenciation des cellules cancéreuses de sein. Un pourcentage élevé (60 à 70%) des tumeurs mammaires exprime la forme alpha du récepteur aux œstrogènes et il semblerait que l'expression de l'ERβ soit diminuée voire abolie au cours du développement tumoral (Bardin *et al*, 2004). Cependant, environ 30% des tumeurs échappent à l'hormonothérapie d'où l'importance de développer de nouvelles stratégies thérapeutiques (Gonzalez-Angulo, 2007).

Dans le cadre de mon post-doctorat (Institut Jules Bordet, Université Libre de Bruxelles, Belgique), mes travaux ont participé au développement des axes de recherche sur l'amélioration du diagnostic, des traitements et la recherche de nouveaux anti-œstrogènes dans le cancer du sein. J'ai tout d'abord défini au laboratoire des axes technologiques permettant d'apprécier la croissance (prolifération, apoptose, sénescence, tests clonogéniques) mais aussi d'étudier les voies de signalisation cellulaire induites par le récepteur: protéomique et transcriptomique (microarrays). Pour les études de microarrays, le choix s'est porté sur l'analyse avec des puces Affymetrix, Human Genome U133 plus 2.0 Array, permettant d'étudier la totalité du génome (environ 47.000 transcripts, correspondant à 38.500 gènes). L'interprétation des données obtenues par microarrays Affymetrix s'est focalisée sur les gènes régulés ou non par les œstrogènes relevant des grandes fonctions biologiques de la cellule qui concourent au développement tumoral (prolifération, apoptose, migration, invasion). Cependant, conscient des limites de l'analyse transcriptomique (prix, temps, interprétations délicates et parcellaires…), j'ai, en parallèle, opté pour la réalisation d'analyse protéomique. Cette approche permet la mise en évidence

des co-régulateurs associés aux récepteurs aux œstrogènes et l'analyse des voies de signalisation qui modulent et/ou que module le récepteur aux œstrogènes. Outre les collaborations au sein de l'équipe (Laïos *et al*, 2003 et 2005 ; Magné *et al*, 2006b ; Nonclercq *et al*, 2007), j'ai donc entièrement pris en charge le développement de deux programmes de recherche.

1- Impact de la radiothérapie sur la régulation du récepteur aux œstrogènes alpha dans les cellules cancéreuses de sein et modification du protocole thérapeutique.

A l'inverse des agents hormonaux ou chimio-thérapeutiques, les interactions entre œstrogènes et radiothérapie ont été peu étudiés. Ces études ne permettaient pas de conclure quant à l'existence potentielle d'effets agonistes, synergiques ou antagonistes de ces deux thérapies (Schmidberger *et al*, 2003). Nous avons donc entrepris l'étude de la croissance des cellules cancéreuses de sein sous l'effet des radiations ionisantes. Nous avons, pour cela, utilisé un panel de lignées représentatives du cancer du sein (Lacroix *et al*, 2004 ; Blick *et al*, 2008) dont les différents statuts vis à vis des récepteurs aux œstrogènes ont été vérifié par RT-qPCR (Patricia de Crémoux, Institut Curie, Paris, communication personnelle) et qui appartiennent aux différents groupes de cellules cancéreuses de sein comme décrit dans les classifications moléculaires (Tableau 1).

	Luminal [a)]	Basal [a)]	ErbB2 [b)]	ERα [b,c)]	ERβ [b,c)]
MCF-7	+			+	+
T-47D	+			+	+
ZR-75-1	+			+	+
MDA-MB-231		+		-	+
SKBR3	+		+++	-	-
Hs578-T		+		-	-

Tableau 1: Classification moléculaire des lignées cancéreuses de sein sélectionnées pour notre étude. L'expression des récepteurs aux œstrogènes α et β a été vérifiée par RT-qPCR. (a) Lacroix *et al*, 2004; (b) Blick *et al*, 2008 (c) De Crémoux, communication personnelle.

Nous avons, tout d'abord, observé que les cellules cancéreuses de sein hormono-dépendantes (MCF-7, T47-D, ZR-75-1) sont très sensibles à la radiothérapie, contrairement aux hormono-indépendantes (MDA-MB-231, SKBR3). Cependant, cette sensibilité peut en grande partie s'expliquer par leur statut vis-à-vis de P53 (Lacroix *et al*, 2006, **annexe, article 3**). En effet, P53 est un acteur central dans la détermination de la réponse au stress des cellules. Dans les cancers du sein, ses mutations participent à l'oncogenèse. L'activité de P53 est modulée par des modifications post-transcriptionnelles qui permettent de stabiliser (activation) ou déstabiliser (inactivation) la protéine. Outre l'aspect quantitatif des modifications de p53, l'aspect qualitatif (phosphorylation, acétylation, ubiquitination) permettra d'orienter les réponses cellulaires vers un arrêt de la prolifération, l'apoptose ou la sénescence.

Dans ce contexte, nous avons étudié l'impact des radiations sur la régulation et la fonction du récepteur aux œstrogènes dans les cellules MCF-7 (Toillon *et al*, 2005, **annexe, article 4**). De façon intéressante, nous avons reporté que les faibles doses d'irradiation (≤8 Gy) entraînent, dès 72h, une perte d'ERα par une diminution de sa synthèse. Pour autant, la réponse vis-à-vis de l'E2 des récepteurs résiduels ne semble pas être inactivée (maintien de l'effet sur la croissance et des activités transcriptionnelles de l'ERα). Si l'on s'intéresse plus particulièrement à la croissance cellulaire, l'E2 contrecarre de façon importante les effets anti-prolifératifs des rayonnements ionisants (tests de viabilité MTT, cristal violet, cytométrie en flux), diminuant potentiellement leur efficacité thérapeutique. Nos résultats montrent que la croissance des cellules MCF-7 est abolie par une irradiation à la dose de 8 Gy sans toutefois observer de perte cellulaire ce qui suggère un arrêt de prolifération sans induction de mort cellulaire (détection de l'apoptose par marquage à

l'annexine V). Cet effet radio-protecteur de l'E2 est supprimé par les anti-œstrogènes (4-OH-TAM ou ICI 182780).

Poursuivant ce travail, j'ai recherché par analyses transcriptomique (microarrays) et protéomique les voies de signalisation induites par l'irradiation modulées par les œstrogènes et l'implication de p53 dans cette réponse. Ainsi, l'irradiation des cellules MCF-7 induit la sénescence *via* l'activation de la voie p53/$P21^{waf1/cip1}$/Rb. Par contre, l'E2 inhibe cette induction en augmentant la liaison de la protéine $p21^{waf1/cip1}$ avec la cycline D1 qui libère alors la cycline E2 ; ce qui entraîne une augmentation de la phosphorylation de Rb. Cette inactivation de Rb serait à l'origine de la diminution de la sénescence et l'augmentation de la croissance (clonogénicité) de cellules irradiées. D'autre part, malgré les nombreuses interactions entre l'ERα et P53 (Lacroix *et al*, 2006) notamment dans les cellules irradiées (Liu *et al*, 2006), nous n'avons pas établi, pour de faibles doses, d'effet des œstrogènes sur l'expression (western blot), la localisation (immuno-cytochimie) ou l'activité transcriptionnelle (ELISA trans-AM) de p53 (Toillon *et al*, 2007, **annexe, article 5**). L'ensemble de nos résultats suggère que les œstrogènes maintiennent la croissance des cellules en interférant avec les voies de signalisation induites par l'irradiation en aval de p53. D'autres mécanismes ne sont cependant pas à exclure. Nous avons, par exemple, démontré que l'effet de l'E2 dépend également de l'activation des voies des facteurs de croissance (Ras-Raf-MAP-kinase ; PI3-kinase-Akt). Dans les cellules, ces voies concourent à l'activation de nombreuses protéines et notamment NF-kappaB qui est un facteur déterminant de la radiorésistance des cellules cancéreuses de sein (Magné *et al*, 2006b, **annexe, article 6**).

Si les œstrogènes diminuent potentiellement l'efficacité thérapeutique de la radiothérapie, l'association de la radiothérapie avec un traitement anti-œstrogène est elle envisageable ? J'ai ainsi pu observer qu'aux doses utilisées en clinique, l'utilisation d'un anti-

œstrogène pur comme le Fulvestrant (ICI 182780), présente un effet synergique sur l'inhibition de croissance induite par l'irradiation. Par contre, le 4-OH-Tamoxifène (métabolite actif du Tamoxifène, médicament le plus prescrit (Nolvadex)) présente des effets synergique ou agoniste faible. Pour des faibles doses (<=2 Gy ; doses utilisées en clinique), l'irradiation renforcerait, au contraire, les effets agonistes du 4-OH-TAM sur la croissance diminuant son activité inhibitrice à long terme (clonogénicité) ce qui est corrélé avec une diminution de l'induction de la sénescence par le 4-OH-TAM. Plus précisément, nous avons observé que l'irradiation induit de façon spécifique une voie de survie à l'origine des résistances au 4-OH-TAM. Ces études devront être approfondies pour décrypter les mécanismes moléculaires à l'origine de ces effets opposés. De plus la transposition sur des modèles animaux précliniques est nécessaire. En effet, nous avons reporté qu'il existe de nombreuses contradictions entre les différentes études qui peuvent être expliquées par les différences de protocoles expérimentaux ce qui souligne l'importance des facteurs exogènes dans les réponses radiothérapie/Tamoxifène (revue dans Chargari *et al*, 2009, **annexe, article 7**).

Nos résultats indiquent que la sénescence est un mécanisme essentiel mis en jeu dans l'arrêt de croissance après irradiation. La croissance est contrôlée de façon fine par les anti-(œstrogènes) ce qui peut expliquer le rôle dans la promotion tumorale des œstrogènes et dans la résistance au Tamoxifène. Les travaux futurs devront s'orienter donc vers :

- les mécanismes de contrôle de la sénescence par les œstrogènes,
- l'impact de ce contrôle sur l'apparition des résistances aux thérapies endocrines et notamment les nouvelles thérapeutiques (anti-aromatase).

2- Effets de nouveaux complexes organométalliques sur la croissance des cellules cancéreuses de sein.

Les traitements des cancers du sein hormono-dépendants visent essentiellement à inhiber les effets de l'activation de l'ERα. Les approches les plus répandues font appel à des anti-œstrogènes pour bloquer les interactions entre le récepteur alpha et ses co-activateurs mais d'autres études ont, depuis longtemps mais sans succès, cherché à vectoriser des agents cytotoxiques inorganiques tels que le cis-platine (von Angerer 1995; Chesne *et al,* 1986) ou plus récemment l'oxaliplatine (Top *et al*, 2003) en les greffant sur les (anti)-œstrogènes. L'équipe du Pr. G. Jaouen (ENSCP, Paris) développe depuis plusieurs années des dérivés d'œstrogènes couplés à différents complexes organométalliques de Ru, Ti, Fe, Co (Jaouen *et al*, 2004) et a acquis une expertise internationale dans la chimie bio-organométallique (Jaouen, 2005). Parmi tous ces agents, ils ont montré que le ferrocifène (Figure 1), dérivé de ferrocène et du Tamoxifène, a un potentiel thérapeutique intéressant (Jaouen *et al*, 2004 ; Vessières *et al*, 2005).

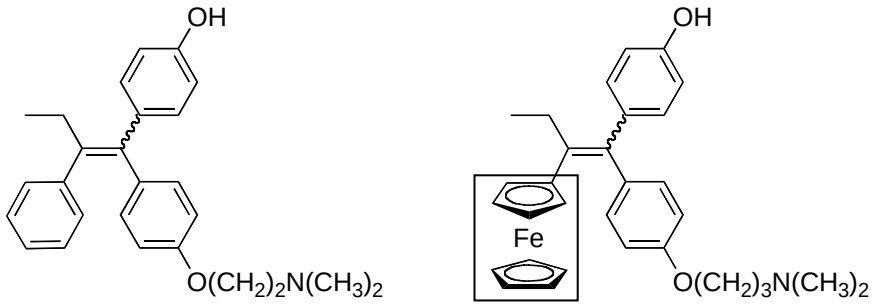
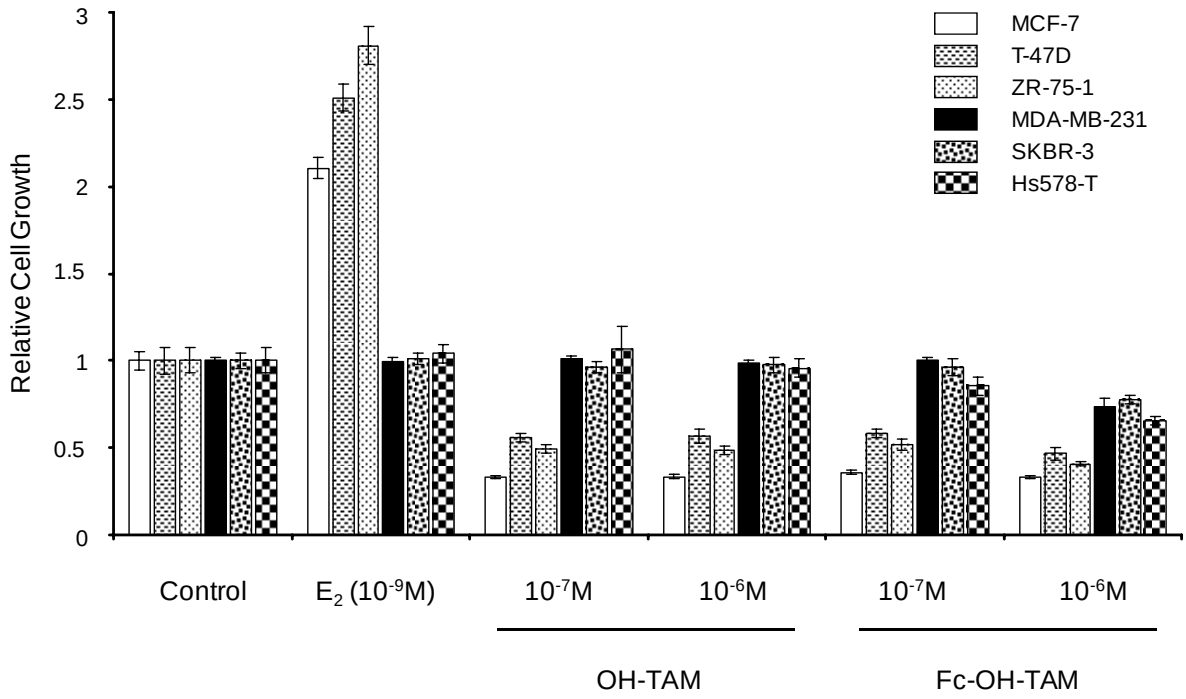


Figure 1: Formules développées du 4-OH-Tamoxifène (OH-TAM) et du Fc-OH-TAM. Le Fc-OH-TAM est dérivé de l'OH-TAM par la substitution d'un noyau aromatique par un ferrocène (encadré).

Les études réalisées jusqu'à présent n'avaient pas permis de définir précisément les effets sur la croissance et les mécanismes d'action de cette nouvelle famille de complexe dans les cellules cancéreuses de sein. Dans le cadre du programme COST D20 et de mes recherches sur les voies de signalisation des (anti)-œstrogènes, j'ai entrepris de poursuivre la collaboration entre les laboratoires du Pr Guy Leclercq (Université Libre de Bruxelles, Belgique) et du Pr Gérard Jaouen (ENSCP, Paris) concernant le ferrocifène.

2.1-<u>Effets du ferrocifène sur la croissance des cellules cancéreuses de sein.</u>

Les effets sur la croissance ont été mesurés sur un panel de lignées dont les phénotypes sont repris dans le tableau 1 (page 20). J'ai ainsi pu observer que le Fc-TAM inhibe la croissance de toutes les lignées testées (figure 2). Cette inhibition est plus forte dans les cellules ERα-positives.



<u>Figure 2:</u> Effets de l'OH-TAM et du Fc-OH-TAM sur la croissance des cellules cancéreuses de sein. Vingt quatre heures après ensemencement, les cellules sont placées dans un milieu dépourvu en œstrogène (EMEM ou DMEM-10% sérum déstéroïdé) et traitées pendant 96 h en présence du solvant (contrôle, éthanol, 1/1000^{ième}), E2 (10^{-9}M), OH-TAM (10^{-6}M, 10^{-7}M) ou Fc-OH-TAM (10^{-6}M, 10^{-7}M). A la fin des expériences, les cellules sont fixées (PAF 4%, PBS, pH 7,2) et soumises à une coloration au cristal violet. L'absorbance est mesurée à 540 nm. Les résultats sont la moyenne de 3 expériences réalisées sur 6 puits/conditions.

Si l'on analyse les processus biologiques à l'origine de cet arrêt de croissance, on observe que le Fc-OH-TAM n'induit pas d'apoptose des cellules cancéreuses de sein (analyses effectuées en cytométrie de flux avec un marquage Annexine V-FITC ; données non montrées). En revanche, cette inhibition de croissance semble liée à une modification du cycle cellulaire. Ainsi, on observe comme pour les cellules MDA-MB-231 (Figure 3), une augmentation rapide (dans les premières heures) du nombre de cellules en phase S en présence du Fc-OH-TAM.

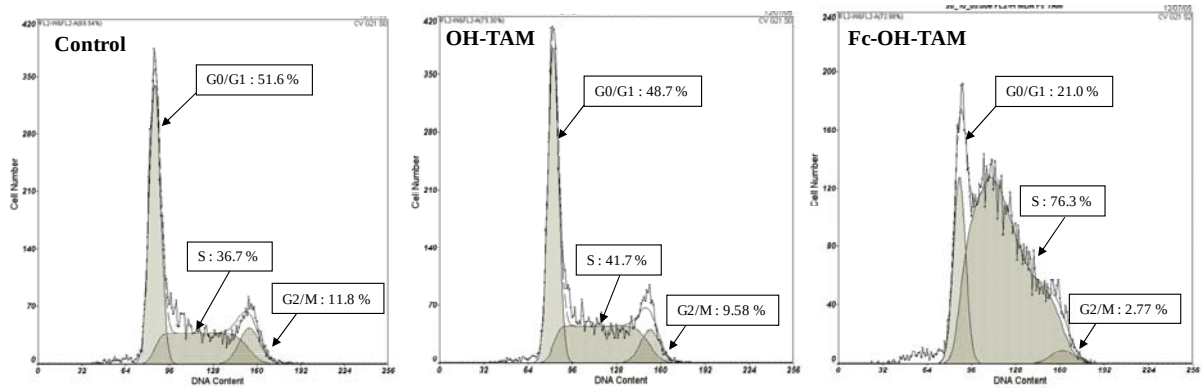


Figure 3: Répartition des cellules dans les différentes phases du cycle cellulaire observée en cytométrie en flux. Vingt quatre heures après ensemencement (sérum déstéroïdé) les cellules sont incubées avec solvant (contrôle, éthanol, 1/1000^{ième}), l'OH-TAM (10^{-6}M) ou le Fc-OH-TAM (10^{-6}M). Après 24h d'incubation, les cellules sont fixées et l'incorporation de l'iodure de propidium est effectuée selon les instructions du fabricant (DNA prep reagent, Beckman Coulter). Les histogrammes sont représentatifs de 3 expériences indépendantes.

Cette accumulation de cellules en phase S semble être transitoire dans les cellules exprimant l'ERα (MCF-7 et ZR-75-1; Tableau 2), alors qu'elle se maintient dans les cellules MDA-MB-231 et SKBR-3 (Tableau 2). En terme de cinétique, cette accumulation est plus lente dans les cellules SKBR-3 mais tout aussi sensible à 48 heures.

24 H													
		MCF-7			ZR-75-1			MDA-MB-231			SKBR-3		
		G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M
Control		70,6	18,5	10,9	71	23.5	5.55	56,9	31,5	11,6	73.8	19.7	6.57
E2		55	36,7	8,27	53.7	37	9.32	55,3	33,1	11,6	75.1	18.1	6.82
0H-TAM	10^{-7}M	60,8	28,9	10,4	77.1	18.8	4.04	ND	ND	ND	ND	ND	ND
	10^{-6}M	58,1	34,2	7,69	77.3	18.3	4.43	61,7	31	7,24	77	15.9	7.12
Fc-OH-TAM	10^{-7}M	53,7	36,2	10,1	38.3	9.51	52.1	ND	ND	ND	ND	ND	ND
	10^{-6}M	17,2	77,5	5,27	22.5	67.8	9.71	16,1	77,4	6,45	76.7	19.7	3.58
48 H													
		MCF-7			ZR-75-1			MDA-MB-231			SKBR-3		
		G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M
Control		70,4	25,27	4,03	61.6	28.8	9.69	50,1	41,6	8,3	70.0	20.4	9.6
E2		58,8	33,3	7,9	41.4	47	11.3	56,3	32,4	11,3	71	20.8	8.14
0H-TAM	10^{-7}M	84,5	13	2,47	75.7	19.2	5.12	ND	ND	ND	ND	ND	ND
	10^{-6}M	85,3	11,2	3,53	80.1	19.1	0.78	57,4	32	10,6	70.6	20.4	8.91
Fc-OH-TAM	10^{-7}M	84,2	12,8	3,05	80.6	15	4.45	ND	ND	ND	ND	ND	ND
	10^{-6}M	76,5	10,5	12,9	82.1	17.9	0	1,13	95,2	3,64	48.7	48.0	3.35

Tableau 2 : Répartition des cellules dans les différentes phases du cycle cellulaire observée en cytométrie en flux. Vingt quatre ou 48 heures après ensemencement (sérum déstéroidé) les cellules sont incubées avec solvant (contrôle, éthanol, 1/1000^{ième}), l'OH-TAM (10^{-7}M, 10^{-6}M) ou le Fc-OH-TAM (10^{-7}M, 10^{-6}M). Après 24 heures d'incubation les cellules sont fixées et l'incorporation de l'iodure de propidium est effectuée selon les instructions du fabricant (DNA prep reagent, Beckman Coulter). Les résultats sont la moyenne de 3 expériences indépendantes ; par soucis de clarté les écarts à la moyenne ne sont exceptionnellement pas reportés. ND : Non déterminé.

Par incorporation de BrdU, nous avons pu vérifier dans les cellules MDA-MB-231 que le Fc-OH-TAM induit une entrée précipitée des cellules en phase S (Figure 4A). De plus, en faisant une expérience de pulse et chasse (Figure 4B), nous observons que les cellules qui entrent en phase S sous l'effet du Fc-OH-TAM ne sont pas capables de continuer leur progression à travers le cycle cellulaire. En effet, les cellules BrdU+ (région R2) montrent, au bout de 24h de pulse, un profil en contenu d'ADN représentatif d'une accumulation en S (Figure 4C).

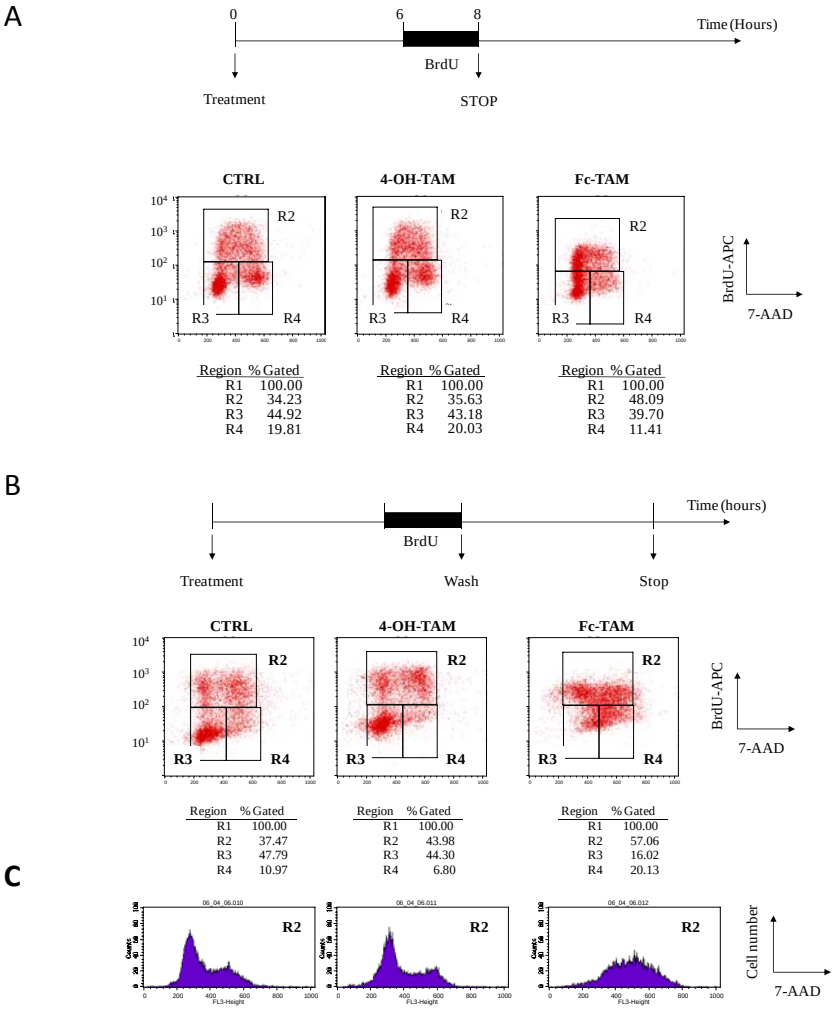


Figure 4: Analyse du blocage en phase S par incorporation de BrdU dans les cellules MDA-MB-231. A) Six heures après traitements (Contrôle, OH-TAM, ou Fc-OH-TAM), les cellules sont incubées en présence de BrdU pendant 2 heures. **B)** Six heures après traitements (Contrôle, OH-TAM, ou Fc-OH-TAM), les cellules sont incubées en présence de BrdU pendant 2 heures (pulse) puis rincées (chasse) et remises en culture 24H. A la fin des expériences les cellules sont fixées et marquées par un anticorps anti-BrdU complexé à l'APC et par le 7-AAD. L'incroporation de BrdU et le contenu en ADN sont alors mesurés par cytométrie en flux. 3 régions sont définies : la région R2 englobe les cellules BrdU positives tandis que les régions R3 et R4 contiennent les cellules BrdU négatives. **C)** Analyse du cycle cellulaire des cellules de la région R2, 24 heures après la chasse de BrdU.

De plus, le Fc-OH-TAM induit un arrêt de croissance également caractérisé par une diminution de la clonogénicité des cellules (Figure 5A) et une induction de la sénescence (Figure 5B) accrue par rapport à l'OH-TAM.

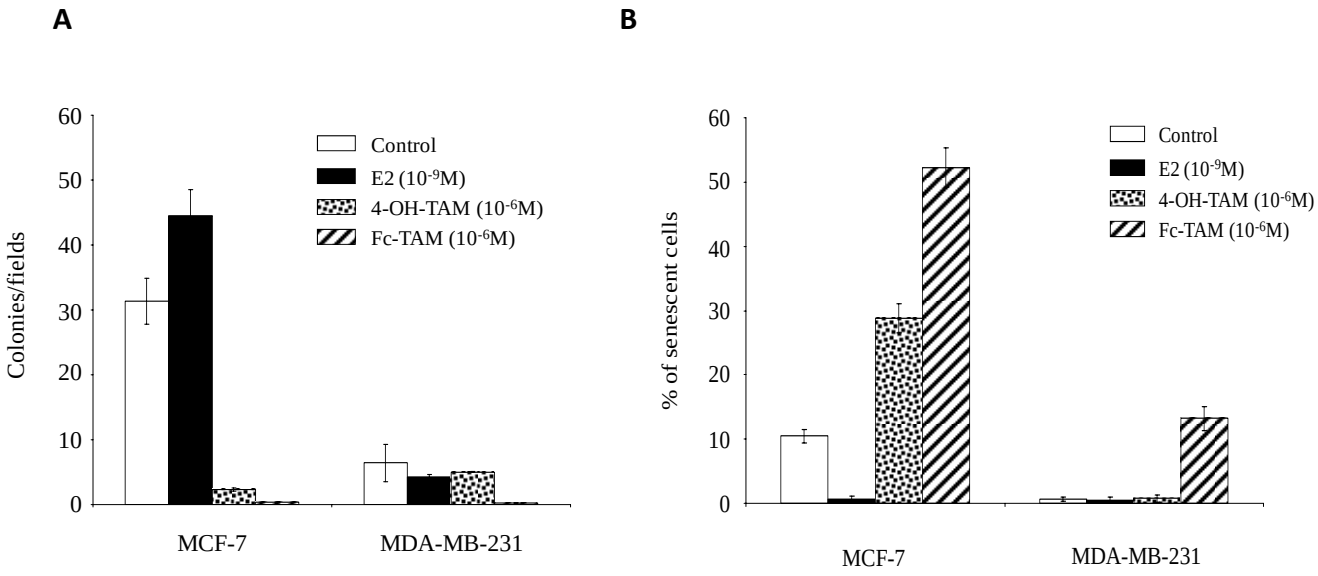


Figure 5: Effet du Fc-OH-TAM sur la clonogénicité et la sénescence des cellules cancéreuses de sein. A) La clonogénicité a été mesuré par comptage des colonies formées après 3 semaines de culture en agar mou, les cellules sont cultivées dans un milieu dépourvu en œstrogène (1% sérum strippées) **B)** la sénescence est appréciée après 96 heures de traitement, par la mesure de l'activité SA-Beta galactosidase (voir article Toillon *et al*, 2007). Les résultats sont représentatifs de 3 expériences indépendantes réalisées en triplicate.

2.2-Mécanismes d'action des ferrocifènes.

Compte tenu des effets sur le cycle cellulaire et en particulier l'arrêt de croissance en phase S, nous avons tout d'abord étudié les effets du Fc-OH-TAM sur la synthèse et l'intégrité de l'ADN. Nous avons observé que l'apport exogène de nucléotides ne permet pas la reprise du cycle cellulaire ce qui indique que le Fc-OH-TAM ne perturbe pas la synthèse des nucléotides par la dihydrofolate réductase (données non montrées). De plus, les effets du Fc-OH-TAM ne semblent pas liés à une interaction directe avec l'ADN qui entraînerait des cassures comme pour le cisplatine (test COMET) (Vessières, manuscrit en préparation).

D'autre part, la présence du greffon ferrocène qui est caractérisé par des capacités d'oxydoréduction particulières de ces complexes (Hillard *et al*, 2006), nous ont conduit à étudier le rôle de la production de radicaux libres (ROS) dans les effets du Fc-OH-TAM. En effet, les ROS ont des fonctions diverses dans les cellules et interviennent dans les voies de signalisation pour activer la croissance lorsqu'ils sont produits à faible dose mais ils ont aussi

un effet inhibiteur à forte concentration. Ainsi, nous avons montré que le traitement des cellules par la N-acétyl-cystéine inhibe partiellement les effets du Fc-OH-TAM sur le cycle cellulaire et la sénescence. L'ensemble de nos résultats démontre que l'action du Fc-OH-TAM sur la croissance est en partie liée à ses capacités d'oxydoréduction et la production de ROS.

Ces résultats font l'objet d'un manuscrit en préparation qui établit que :

- La liaison du ferrocifène précipite l'entrée en phase S ce qui détermine un mécanisme d'action potentiel de ce récepteur dans les résistances au Tamoxifène,
- La production de ROS induite par le greffon ferrocène conditionne la réponse cellulaire vis à vis du vecteur (Tamoxifène).

Néanmoins, les cibles moléculaires du Fc-OH-Tam restent inconnues à ce jour. Le Fc-OH-TAM pourrait exacerber les effets inhibiteurs sur la croissance du Tamoxifène. En effet, nous avons démontré, avec d'autres équipes, que le Tamoxifène à forte concentration présente des effets indépendants du récepteur aux œstrogènes (Lagadec *et al*, 2008 ; **annexe, article 8**). Plusieurs hypothèses sur les effets ER-indépendants du Tamoxifène sont d'ailleurs en étude (Klinge *et al*, 2008). Pour autant, nous pouvons écarter ces dernières car nous avons observé que le Fc-OH-TAM agit en absence d'expression de l'ERβ et de la GPR30 (site secondaire potentiel de liaison du Tamoxifène). D'autre part, contrairement au Tamoxifène qui, par le biais de ses métabolites, entraîne des dommages à l'ADN, le test COMET nous permet d'infirmer cette seconde piste de recherche. Il semble donc intéressant de rechercher les cibles moléculaires du Fc-OH-TAM et d'aller plus loin dans la compréhension de ces mécanismes d'action. L'ensemble de ces études pourraient à terme définir des nouvelles pistes de recherche sur les actions ER-indépendantes des

triphényléthylènes et comprendre les mécanismes d'échappement et/ou au contraire d'initiation de la cancérogenèse qu'ils engendrent.

III- Signalisation de TrkA dans les cancers du sein.

Notre laboratoire a été le premier à démontrer l'implication du NGF (Nerve Growth Factor) dans le cancer du sein. En effet, nous avons mis en évidence que le NGF est un puissant facteur mitogène et anti-apoptotique pour les cellules cancéreuses de sein, alors qu'il est sans effet sur la croissance des cellules épithéliales mammaires normales (Descamps *et al*, 1998, 2001b ; Com *et al*, 2007). Le NGF est surexprimé dans les cellules cancéreuses, alors qu'aucune expression n'est détectée dans les cellules mammaires normales, ce qui aboutit à une stimulation autocrine de la croissance tumorale (Dollé *et al*, 2003). Des travaux récents ont, par ailleurs, montré que le NGF participe au développement tumoral *in vivo* (en souris SCID ; Severe Combined ImmunoDeficiency) et est une cible thérapeutique potentielle (Adriaenssens *et al*, 2008). Le NGF interagit avec les cellules cibles par l'intermédiaire de deux récepteurs membranaires : le récepteur à activité tyrosine kinase TrkA (Tropomyosin related kinase A) et le récepteur p75NTR (p75 Neurotrophin Receptor) appartenant à la famille des récepteurs du TNF (Tumor Necrosis Factor). L'effet mitogène du NGF implique TrkA et la voie des MAP-kinases (Mitogen Activated Protein-kinases) tandis que l'effet anti-apoptotique passe par le récepteur p75NTR (Descamps *et al*, 2001b ; El Yazidi-Belkoura *et al*, 2003). TrkA et p75NTR sont exprimés dans la majorité des tumeurs mammaires et sont liés au pronostic (Descamps *et al*, 2001a ; Aragona *et al*, 2001 ; Davidson *et al*, 2004). De plus, la surexpression du récepteur TrkA dans les cellules cancéreuses de sein augmente leur croissance et leur capacité métastatique (Lagadec *et al*, 2009, **annexe, article 9**). Les résultats que nous avons obtenus sont confortés par les travaux d'autres équipes. Ainsi, il a

été montré que la suppression de l'activité de TrkA par des endocannabinoïdes résulte en une inhibition de la croissance des cellules mammaires (Melck *et al*, 2000). De plus, TrkA coopère avec HER-2 pour activer la croissance des cellules de cancer du sein (Tagliabue *et al*, 2000) et la drogue de référence en hormonothérapie du cancer du sein, le Tamoxifène, inhibe l'effet mitogène du NGF (Chiarenza *et al*, 2001). Enfin, l'implication du NGF dans le développement tumoral de tissus non neuronaux a, par ailleurs, été décrite. Outre le sein (Dollé *et al*, 2004; Davidson *et al*, 2004), le NGF est en effet impliqué dans la cancérogenèse de la glande thyroïde (Páez Pereda *et al*, 2000), de l'ovaire (Davidson *et al*, 2003) et du pancréas (Zhu *et al*, 2002). L'ensemble de ces travaux indique que le NGF, par son action sur la prolifération, la survie, la migration et l'invasion des cellules cancéreuses, joue un rôle important dans le développement tumoral. Les voies de signalisation intracellulaires décrites pour TrkA impliquent de nombreuses protéines. Ces partenaires interagissent avec des résidus tyrosine (Tyr) phosphorylés du récepteur. A ce jour, seuls sept résidus phospho-tyrosine (pTyr) ont été décrits pour moduler la signalisation de TrkA, sur les 11 Tyr contenus dans le domaine intracellulaire du récepteur. Les résidus Tyr-670, Tyr-674/675 sont impliqués dans l'autophosphorylation du récepteur, Tyr-490 lie Shc/FRS2, Tyr-785 active la PLC-γ1, Tyr-751 et Tyr-695 sont potentiellement impliqués dans l'activation de la PI3-kinase et le trafic du récepteur, respectivement (Reichardt, 2006 ; de Pablo *et al*, 2008) (Figure 6). Jusqu'à présent, aucune étude n'a mis en avant l'interaction de partenaires sur des résidus phospho-sérine (pSer) et phospho-thréonine (pThr) en absence d'outils moléculaires fiables permettant l'analyse (anticorps anti-pSer et -pThr).

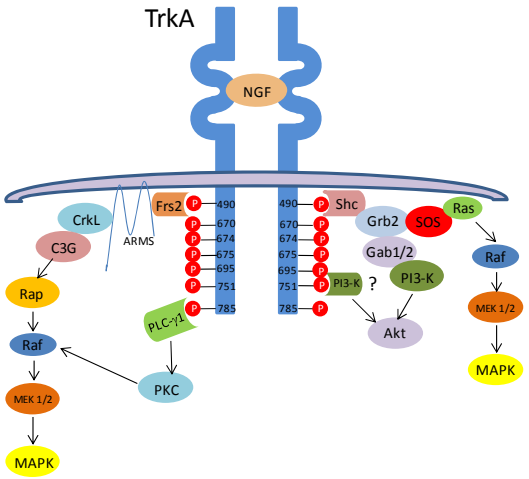


Figure 6 : **Représentation schématique de la signalisation du récepteur TrkA**. La phosphorylation de résidus tyrosine du domaine intracellulaire, suite à la fixation du NGF, permet le recrutement de partenaires protéiques et l'induction de différentes voies de signalisation (d'après Reichardt, 2006). N-ter : extrémité N-terminale, C-ter : extrémité C-terminale.

Cependant, la prédiction informatique des modifications post-traductionnelles (http://www.cbs.dtu.dk/services/NetPhos/) indique que TrkA est potentiellement phosphorylé sur 5 Ser et 2 Thr de son domaine intracellulaire et que certains de ces sites sont contenus dans des motifs consensus pour des protéines kinases comme la PKA (Protein kinase A), PKC (Protein kinase C), cdc2 (cyclin-dependent kinase 2), CKI (Casein kinase I) et p38MAPK (www.cbs.dtu.dk/services/NetPhosK; www.phosida.com). Le récepteur TrkA pourrait donc être régulé par des protéines kinases endogènes comme cela a déjà été décrit pour l'EGFR (Boeri Erba *et al*, 2007). Des études de marquage au ^{32}P suivies d'une analyse des acides aminés confirment la phosphorylation de résidus sérine de TrkA après stimulation des cellules PC12 par le NGF (MacPhee et Barker, 1997). Par ailleurs, la phosphorylation de résidus sérine et thréonine a déjà été mise en évidence sur l'EGFR (Wu *et al*, 2006), après stimulation par l'EGF, par une analyse en spectrométrie de masse, avec cinq résidus pSer et une pThr identifiés. Dans une autre étude, la phosphorylation du résidu Thr-654 de l'EGFR par la PKC est décrite pour intervenir dans la régulation de l'activation du récepteur via l'inhibition du recrutement de la calmoduline (Aifa *et al*, 2006). Plus récemment, Van Kanegan et Strack (2009) ont montré que la phosphatase PP2A régule l'activation de TrkA

dans les cellules PC12 en modulant la phosphorylation de résidus sérine et thréonine. Afin d'étudier le rôle de la phosphorylation du récepteur TrkA dans le cancer du sein, nous avons entrepris une analyse de protéomique fonctionnelle axée sur l'étude de la phosphorylation de TrkA et l'identification des adaptateurs sur les résidus phosphorylés (travaux de M. Cyril Corbet, étudiant en 1^{ière} année de Doctorat que j'encadre). Pour étudier TrkA et ses adaptateurs, nous avons donc du définir toutes les étapes permettant l'isolement, l'enrichissement et la purification du récepteur et de ses adaptateurs en vue de leur analyse en spectrométrie de masse. En effet, l'essor des technologies de spectrométrie de masse a considérablement accru les possibilités d'étude en analyse protéomique. Ce développement de la protéomique a notamment permis de dresser des cartes protéiques, dans de nombreux modèles, qui couvrent actuellement plusieurs milliers de protéines obtenues à partir d'extraits complexes (Strande *et al*, 2009). Néanmoins, si l'on considère que le génome comporte un peu plus de 30 000 gènes et code plusieurs millions de formes protéiques (en incluant toutes les modifications post-traductionnelles et les épissages alternatifs…), seule la partie émergée de l'iceberg a été, à ce jour, découverte (Hondermarck *et al*, 2008, **annexe, article 10**). Ces différences flagrantes entre la puissance analytique des spectromètres de masse et le faible nombre d'identifications dans les banques de données s'expliquent par la rareté de certaines protéines, leur abondance relative par rapport aux protéines majoritaires et les difficultés d'extraction et de conservation des modifications post-traductionnelles (phosphorylation labile et transitoire). Ces difficultés sont accrues dans le cas des protéines membranaires (Josic et Clifton, 2007).

1- Mise au point de l'immuno-précipitation sur le TrkA natif

Pour isoler le récepteur et enrichir les extraits en protéines d'intérêt, nous avons, tout d'abord, défini les critères pour les expériences d'immuno-précipitation. Sur la base d'un tampon RIPA classique, nous avons établi 15 autres combinaisons en modulant le tampon (Tris ou HEPES) mais aussi les détergents (NP-40, Triton X-100, déoxycholate de sodium et SDS). Il en résulte que l'utilisation de Triton X-100 permet une extraction efficace du récepteur (Figure 7, condition n°4).

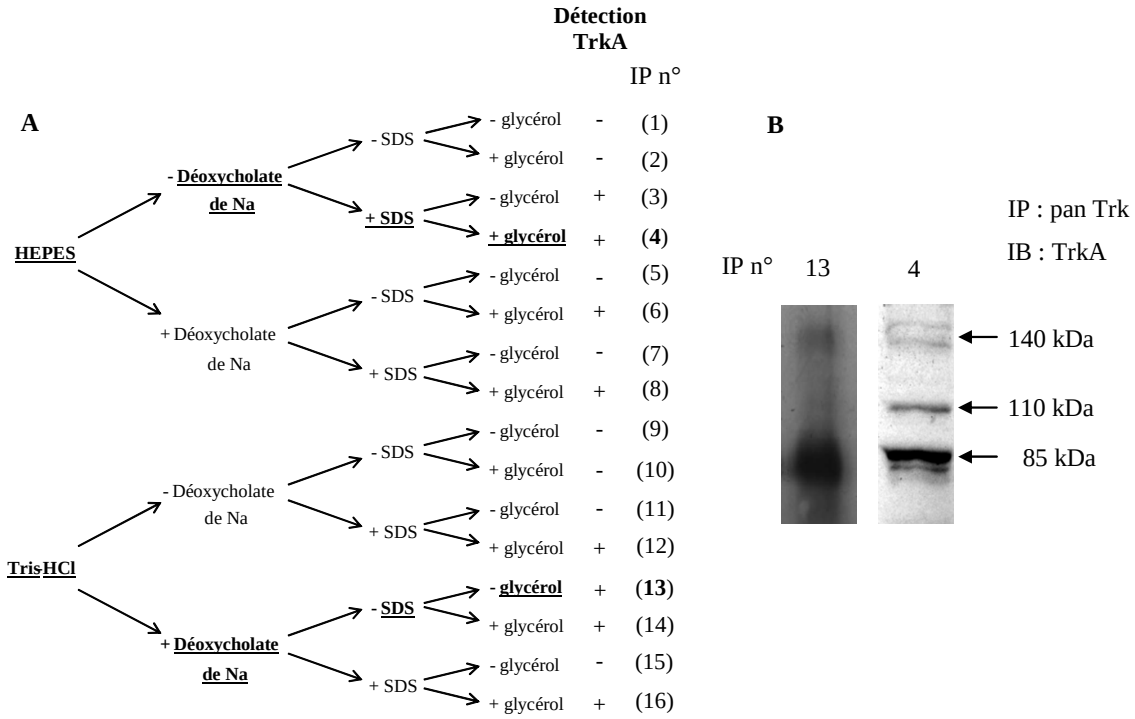


Figure 7 : **Test de différents tampons de lyse en vue de l'extraction de TrkA dans les cellules MDA-MB-231**.**A**) Différents tampons de lyse testés : tampon HEPES (HEPES 40 mM pH 7.5, 120 mM NaCl, 1 mM EDTA, 1% Triton X-100) et tampon Tris (Tris-HCl 50 mM pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40) supplémentés ou non de 0,25% de déoxycholate de Na, 0,1% de SDS et 10% de glycérol. Le lysat de cellules MDA-MB-231 (1 mg) a été immunoprécipité avec l'anticorps anti-pan Trk couplé à des billes de Protéine G-Agarose et la totalité de l'immunoprécipitat a été déposé sur gel pour une révélation anti-TrkA. La détection (+) ou l'absence (-) de visualisation de TrkA par Western-blot est indiquée. **B**) Exemples de résultats d'immunoprécipitation. IP n°13 : tampon RIPA : (Tris-HCl 50 mM pH 7.4, 1% NP-40, 0,25% déoxycholate de sodium, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM NaF, 1/100ème d'inhibiteur de protéases, PhosStop (1 pastille pour 10 ml de tampon ; Roche)). IP n°4 : tampon de lyse : (HEPES 40 mM pH 7.5, 1 mM EDTA, 120 mM NaCl, 10 mM NaPPi, 50 mM NaF, 1,5 mM Na_3VO_4, 1% Triton X-100, 0,1% SDS, 1 mM PMSF, 10% glycérol et 1/100ème de cocktail d'inhibiteurs de protéases (Sigma)). IP : immunoprécipitation ; IB : immunoblot.

De plus, les travaux de Thelemann *et al* (2005) sur l'EGFR montrent que le Triton X 100 (1%) permet également une bonne conservation des interactions récepteur/adaptateurs. Dans ce travail, les auteurs soulignent aussi que ces interactions sont diminuées par la présence de déoxycholate de sodium.

2- Surexpression de TrkA et analyses en spectrométrie de masse

Malgré les mises au point effectuées sur les tampons d'immunoprécipitation, la quantité de TrkA produite par les cellules ne permet pas son analyse en spectrométrie de masse (données non montrées). L'enrichissement des extraits protéiques en TrkA semble donc être déterminant. Pour accroître les chances de visualiser TrkA, nous avons donc surexprimé le récepteur. En effet, les études réalisées sur le FGFR (Fibroblast Growth Factor Receptor) ou l'EGFR montrent l'importance de la quantité de récepteurs dans la cellule pour assurer sa détection par MS et utilisent pour cela des types cellulaires surexprimant le récepteur ou des systèmes de surexpression transitoire (Hinsby *et al*, 2004 ; Schuchardt et Borlak, 2008).

Nos résultats montrent que la transfection transitoire du vecteur pcDNA3-TrkA permet d'augmenter fortement l'expression du récepteur dans les cellules MDA-MB-231. Cette augmentation s'observe à la fois en RT-PCR quantitative (Figure 8, Tableau 3) et Western-blot (Figure 9) ce qui indique que le récepteur surexprimé subit toutes les étapes de maturation post-transcriptionnelle. Nous avons donc réalisé deux immuno-précipitations de TrkA à partir de 5 mg de protéines totales de cellules MDA-MB-231 sur exprimant le récepteur (Figure 10). Néanmoins, nous n'avons pas identifié de peptides correspondant à TrkA dans les analyses réalisées en spectrométrie de masse (tableau 4).

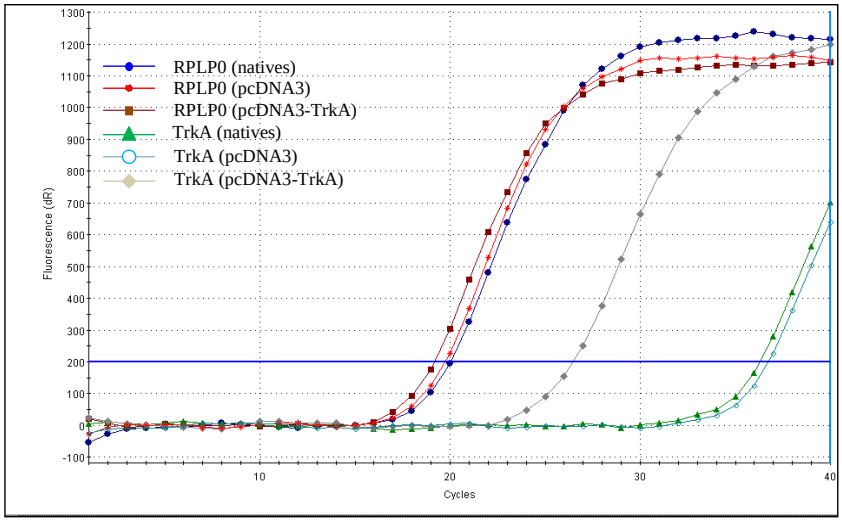


Figure 8 : Expression de TrkA dans les cellules MDA-MB-231 analysée par RT-PCR en temps réel. L'expression de TrkA et RPLP0 (gène de référence) a été mesurée dans les cellules MDA-MB-231 natives et/ou 48 h après transfection transitoire du vecteur pcDNA3.1/Hygro vide ou contenant la séquence de TrkA.

Condition	Ct (TrkA)	Ct ($RPLP_0$)
MDA-MB-231 natives	36,31	20,02
MDA-MB-231-pcDNA3	36,75	19,78
MDA-MB-231-TrkA	26,55	19,12

Tableau 3 : Valeurs de Ct (*Cycle threshold*) des différents systèmes d'expression pour les gènes TrkA et $RPLP_0$. L'expression de TrkA et $RPLP_0$ (gène de référence) a été mesurée dans les cellules MDA-MB-231 natives et/ou 48 h après transfection transitoire du vecteur pcDNA3.1/Hygro vide ou contenant la séquence de TrkA.

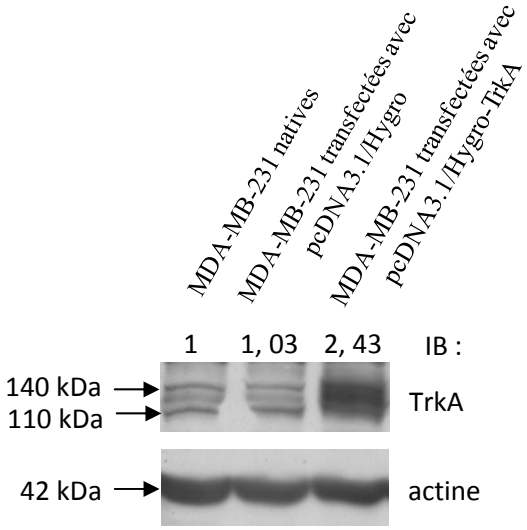


Figure 9 : Mesure de l'expression de TrkA par Western-blot dans les cellules MDA-MB-231 natives et transfectées transitoirement. L'expression de TrkA a été appréciée par Western-blot anti-TrkA dans les cellules MDA-MB-231 natives et/ou 48 h après transfection transitoire du vecteur pcDNA3.1/Hygro vide ou contenant la séquence de TrkA. L'actine est utilisée comme témoin de charge. Les valeurs du rapport densitométrique TrkA/actine (logiciel Quantity One) sont indiquées au dessus du blot.

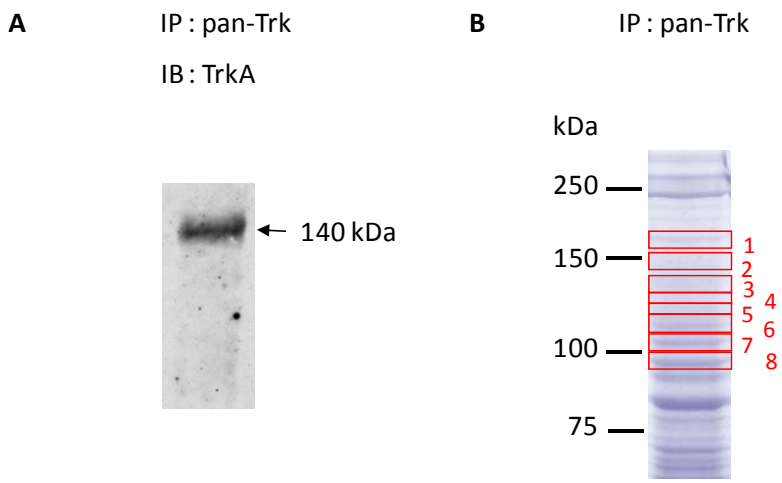


Figure 10 : **Immunoprécipitation anti-pan Trk dans les cellules MDA-MB-231-TrkA**. 5 mg de protéines totales ont été immunoprécipitées avec l'anticorps anti-pan Trk et 1/10ème de l'immunoprécipitat a été soumis à une analyse en Western-blot. **A** : Détection de TrkA après immunoprécipitation. **B** : Gel coloré au bleu de Coomassie colloïdal après immunoprécipitation anti-pan Trk. Les bandes 1 à 8 ont été excisées et soumises à une analyse LC-MS/MS. IP : immunoprécipitation, IB : immunoblot, PM : poids moléculaire.

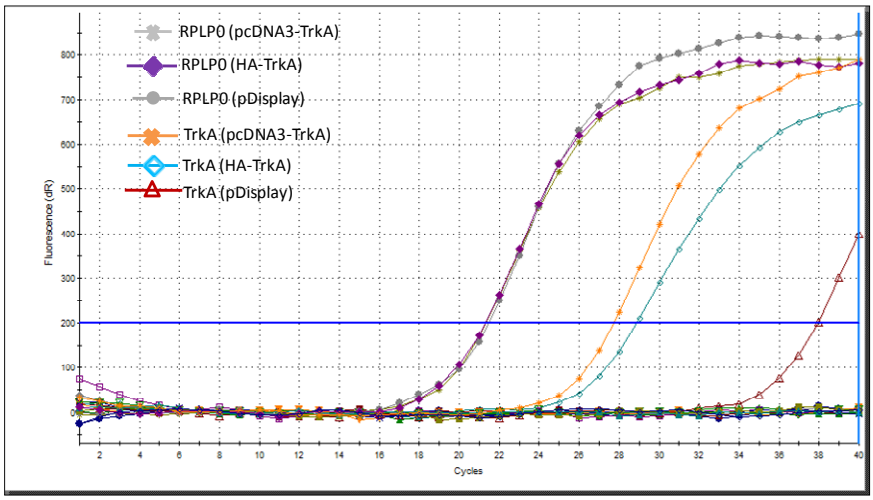
Numéro du spot	Nom de la protéine	Numéro d'accession Swiss-Prot	Peptides identifiés (nombre)	Score Mascot	Masse (Da)
1	Clathrin heavy chain 1	Q00610	8	163	191493
4	LRP 130	P42704	4	100	157805
	ELKS/RAB6-interacting	Q8IUD2	2	43	128008
5	hnRNP U	Q00839	4	99	90457
6	Vinculin	P18206	9	180	123722
	Protein transport protein Sec24C	P53992	2	42	118239
7A	Ubiquitin-activating enzyme 1	P23314	3	125	111774
	Heat shock 70 kDa protein 4	P34932	6	103	94240
	Hexokinase-1	P19367	2	91	102420
	SND-1	Q7KZF4	3	63	101934
8A	Alpha-actinin-4	O43707	9	215	104788
	Alpha-actinin-1	P12814	8	172	102993
	Endoplasmin precursor	P14625	8	89	92411
	Hp95	Q8WUM4	2	88	95963
	Elongation factor 2	P13639	4	86	95277

Tableau 4 : **Identification des protéines après immunoprécipitation anti-pan Trk dans les cellules MDA-MB-231-TrkA**. Le numéro du spot, le nom de la protéine et son numéro d'accession dans la banque de données Swiss-Prot, le nombre de peptides identifiés, le score Mascot et la masse moléculaire des protéines identifiées sont reportés dans le tableau.

Au vu de ces résultats, nous aurions pu changer le tampon (ajout de DTT, changement de détergents) afin d'augmenter la spécificité de l'immunoprécipitation.

Néanmoins, nous avons écarté cette option pour ne pas perdre les interactions TrkA/adaptateurs. L'analyse des spectres obtenus par LC-MS-MS nous révèle, en effet, que nous avons identifié des partenaires potentiels de TrkA, impliqués dans le trafic vésiculaire. De ce fait, il est fort probable que ces protéines majoritaires masquent les peptides du récepteur. Nous avons, donc, opté, pour l'utilisation d'une protéine chimérique HA-TrkA qui permet d'accroître l'efficacité et le rendement de l'immunoprécipitation par l'utilisation des anticorps anti-HA (plus sensibles et spécifiques que les anticorps anti-TrkA). D'autre part, différentes études montrent que l'adjonction d'un tag HA (séquence: YPYDVPDYA) ou myc (séquence: EQKLISEEDL) à l'extrémité N-terminale ou C-terminale de TrkA ne modifie pas ses propriétés de liaison au NGF ni sa réponse biologique (Arevalo *et al*, 2000 ; Robinson *et al*, 2005).

La séquence de TrkA issue du pcDNA3 a été introduite dans le vecteur pDisplay ce qui permet d'ajouter le tag HA à l'extrémité N-terminale de TrkA. Nous avons observé comme avec le vecteur pcDNA3 Trka une forte augmentation de l'expression des récepteurs dans les cellules MDA-MB-231 transfectées par le vecteur pDisplay-HA-TrkA (HA-TrkA)(Figures 11-12 ; Tableau 5).



<u>Figure 11:</u> Expression de HA-TrkA dans les cellules MDA-MB-231 analysée par RT-PCR en temps réel. L'expression de TrkA, HA-TrkA et RPLP0 (gène de référence) a été mesurée dans les cellules MDA-MB-231 natives et/ou 48 h après transfection transitoire du vecteur pcDNA3.1/Hygro vide ou pDisplay contenant la séquence de TrkA ou HA-TrkA respectivement.

Condition	Ct (TrkA)	Ct ($RPLP_0$)
MDA-MB-231-pDisplay	38,96	22.45
MDA-MB-231-TrkA	28,84	22.01
MDA-MB-231-HA-TrkA	28.36	22.13

Tableau 5: Valeurs de Ct (*Cycle threshold*) des différents systèmes d'expression pour TrkA. L'expression de TrkA, HA-TrkA a été mesurée dans les cellules MDA-MB-231 natives et/ou 48 h après transfection transitoire du vecteur pcDNA3.1/Hygro vide ou pDisplay contenant la séquence de TrkA ou HA-TrkA respectivement.

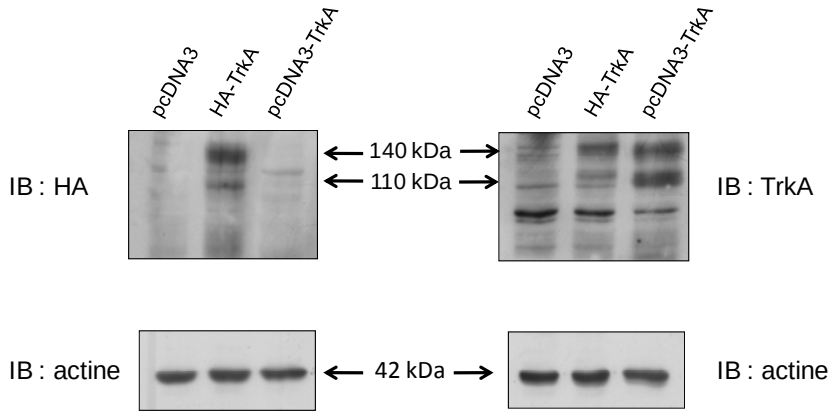


Figure 12: Expression de HA-TrkA visualisée par Western-blot dans les cellules MDA-MB-231 natives et transfectées transitoirement. L'expression de TrkA a été appréciée par Western-blot anti-HA ou anti TrKA dans les cellules MDA-MB-231 natives et/ou 48 h après transfection transitoire du pcDNA3.1/Hygro vide ou pDisplay contenant la séquence de TrkA ou HA-TrkA respectivement. L'actine est utilisée comme témoin de charge.

Nous avons donc recherché TrkA en spectrométrie de masse après immuno-précipitation du HA-TrkA par les anticorps anti-HA (Figure 13).

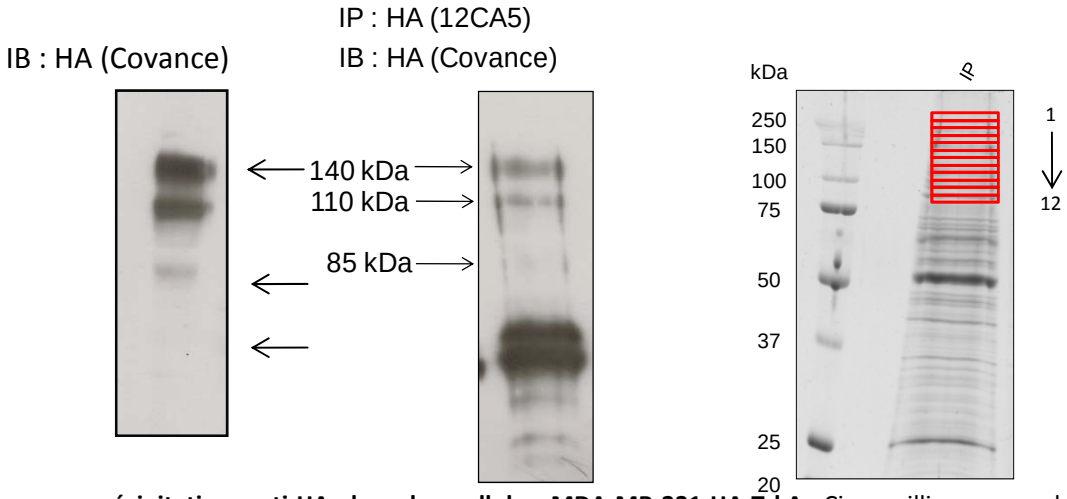


Figure 13: Immunoprécipitation anti-HA dans les cellules MDA-MB-231-HA-TrkA. Cinq milligrammes de protéines totales ont été immunoprécipitées avec l'anticorps anti-HA et 1/10ème de l'immunoprécipitat a été soumis à une analyse en Western-blot. **A**) Détection de HA-TrkA dans le lysat total avant immunoprécipitation. **B**) Détection de HA-TrkA après immunoprécipitation. **C**) Gel coloré au bleu de Coomassie colloïdal après immunoprécipitation anti-HA. Les bandes 1 à 12 ont été excisées et soumises à une analyse LC-MS/MS. IP : immunoprécipitation, IB : immunoblot.

Les résultats des identifications obtenues sont résumés dans le tableau 6. Nous avons ainsi détecté 7 peptides (Tableau 7) appartenant à TrkA dans la bande 3 (qui se situe à 140 kDa).

Numéro du spot	Protéine identifiée	Numéro d'accession Swiss-Prot	Peptides identifiés (nombre)	Score Mascot	Masse (Da)
1	Filamin-A	P21333	63	185	280564
	Filamin-B	O75369	45	170	278021
	Filamin-C	Q14315	18	39	290778
	C2D2A	Q9P2K1	2	37	179422
	Plectin-1	Q15149	10	46	531408
2	Myosin-9	P35579	78	258	226392
	CAD protein	P27708	19	59	242829
	Myosin-10	P35580	16	54	228798
	Myosin-14	Q7Z406	9	39	227863
3	Glycogen debranching enzyme	P35573	15	103	174652
	Clathrin heavy chain 1	Q00610	29	89	191493
	TrkA	**P04629**	**7**	**57**	**140000**
	Vigilin	Q00341	7	46	141352
	UDP-glucose:glycoprotein glucosyltransferase 1	Q9NYU2	6	40	174867
4	ATP-dependent RNA helicase A	Q08211	37	172	140869
	Leucine-rich PPR motif-containing protein (LRP130)	P42704	16	53	157805
	Valyl-tRNA synthetase	P26640	4	52	140387
5	Regulator of nonsense transcripts 1	Q92900	16	69	124267
	Putative ATP-dependent RNA helicase DHX30	Q7L2E3	10	58	133854
	Cullin-associated NEDD8-dissociated protein 1	Q86VP6	17	45	136289
	Importin-4	Q8TEX9	2	38	118640
	Leucyl-tRNA synthetase	Q9P2J5	7	35	134379
6	hnRNP U	Q00839	14	53	90457
	eIF3c	Q99613	4	42	105278
7	hnRNP U	Q00839	33	185	90457
	Sec24D	O94855	13	72	112926
	Importin subunit beta-3	O00410	9	67	123550
	Major vault protein	Q14764	12	55	99266
	hnRNP U-like protein 2	Q1KMD3	2	45	85052
	hnRNP U-like protein 1	Q9BUJ2	8	38	95679
8	HSP 74	P34932	9	78	94240
	Nucleolin	P19338	15	77	76568
	Major vault protein	Q14764	23	56	99266
	C-1 tetrahydrofolate synthase	P11586	8	55	101495
	HSP 105	Q92598	14	54	96804
	ILF3	Q12906	8	53	95279
	Lon protease homolog	P36776	3	45	106422
	Ubiquitin-like modifier-activating enzyme 1	P22314	9	41	117774
9	Alpha-actinin-4	O43707	52	322	104788
	Alpha-actinin-1	P12814	60	301	102993
	Endoplasmin precursor	P14625	22	102	92411
	Caprin-1	Q14444	6	94	78318
	Alpha-actinin-2	P35609	19	89	103788
	HSP 90-beta	P08238	3	76	83212
	Alpha-actinin-3	Q08043	12	70	103229
	Puromycin-sensitive aminopeptidase	P55786	8	51	103211
	Programmed cell death 6-interacting protein	Q8WUM4	4	44	95963
	Dynamin-1 (-2)	Q05193	2	36	97347

	Dynamin-3	Q9UQ16	2	36	96621
10	Elongation factor 2	P13639	43	270	95277
	Valosin-containing protein	P55072	23	118	89266
	Glycogen phosphorylase, liver form	P06737	12	52	97087
	Glycogen phosphorylase, brain form	P11216	9	51	96635
	Glycogen phosphorylase, muscle form	P11217	5	50	97031
	Transferrin receptor protein 1	P02786	8	48	84818
	hnRNP U-like protein 1	Q9BUJ2	8	44	95679
	26S proteasome non-ATPase regulatory subunit 2	Q13200	9	43	100136
	Nucleolar RNA helicase 2	Q9NR30	10	43	87290
11	HSP 90-alpha	P07900	18	119	84607
	HSP 90-beta	P08238	10	97	83212
	Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15	O43143	4	58	90875
	Transportin-1	Q92973	4	42	101244
	Transportin-2	O14787	3	42	101321
12	HSP 90-beta	P08238	54	402	83212
	HSP 90-alpha	P07900	56	359	84607
	ATP-dependent RNA helicase DDX1	Q92499	12	63	82380
	ILF3	Q12906	8	60	95279

Tableau 6: Identification des protéines après immunoprécipitation anti-HA dans les cellules MDA-MB-231-HA-TrkA. Le numéro du spot, le nom de la protéine et son numéro d'accession dans la banque de données Swiss-Prot, le nombre de peptides identifiés, le score Mascot et la masse moléculaire des protéines identifiées sont reportés dans le tableau.

Masse attendue	Masse calculée	Erreur (Da)	Séquence
881,4781	881,4065	0,0716	^{637}IGDFGMSR^{644}
1092,5076	1092,524	-0,0164	^{485}WELGEGAFGK^{494}
1185,7207	1185,654	0,0667	^{626}NCLVGQGLVVK^{636}
1194,5219	1194,5193	0,00266	^{645}DIYSTDYYR^{653}
1290,7354	1290,6431	0,0923	^{664}WMPPESILYR^{673}
1403,8286	1403,6986	0,13	^{82}FVAPDAFHFTPR^{93}
1811,8857	1811,8876	-0,0019	^{495}VFLAECHNLLPEQDK^{509}

Tableau 7: Les masses attendue et calculée des peptides trypsiques chargés correspondant aux peptides de la séquence de TrkA sont listées et indiquées en Da. Les séquences correspondant à chaque peptide sont indiquées avec les positions du premier et du dernier acide aminé (position des résidus dans la séquence du HA-TrkA).

Outre l'identification de TrkA, cette seconde série d'analyse a permis d'identifier 65 protéines contre 15 avec une Ip anti-pan Trk. Cette différence s'explique probablement par le rendement de l'immuno-précipitation plus élevée avec l'anticorps anti-HA. Seules trois protéines ne sont pas retrouvées dans cette identification (vinculin, hexokinase-1 et SND-1). L'analyse des fonctions biologiques de ces protéines immuno-précipitées révèle qu'il s'agit

avant tout de protéines associées au cycle de vie du récepteur (shuttling). En effet, nous trouvons des protéines endoplasmiques (UDP-glucose:glycoprotein glucosyltransferase 1, sec 24D, Endoplasmin precursor), associées au trafic vésiculaire (LRP130, clathrin, filamin, myosin, plectin, Transferrin receptor protein 1). Cependant on retrouve également des protéines qui sont associés à d'autres processus biologiques comme la maturation des ARNm (ATP-dependent RNA helicase DDX1, hnRNP U-like protein 1, Vigilin, ILF3, Caprin-1…), le repliement des protéines (HSP 90-beta, HSP 74, HSP 105…). Après avoir vérifié l'interaction de ces protéines avec TrkA, il serait intéressant de replacer celles-ci dans la signalisation du récepteur et déterminer leurs implications dans les réponses biologiques.

3- Incidence de la surexpression de HA-TrkA sur la réponse biologique

Dans le but d'étudier TrkA, nous avons donc artificiellement induit sa surexpression. De ce fait, il n'est pas exclu que cette augmentation de TrkA modifie la réponse biologique des cellules cancéreuses de sein (Yan et al, 2002). En effet, la surexpression des récepteurs à activité tyrosine kinase peut induire leur auto-activation (Di Fiore *et al*, 1987 ; Hinsby *et al*, 2003). Dans le cas de TrkA, nous avons observé que la surexpression stable dans les cellules cancéreuses de sein MDA-MB-231 entraîne une modification de la réponse biologique partiellement insensible au ligand (Lagadec *et al*, 2009). Ainsi, la surexpression de TrkA exerce des effets pléiotropes sur la croissance, la clonogénicité, l'apoptose, la migration/invasion, la métastase, et l'angiogenèse tumorale. Ces effets sont liés à l'hyper-phosphorylation du récepteur consécutive à son auto-activation. TrkA activé (pTrkA) induit alors l'activation des voies de transduction dépendantes de la Pi3-kinase et des MAP-kinases. Dans d'autres modèles, cette surexpression modifie également cette réponse (Jung et Kim, 2009 ; Yan *et al*, 2002). Ces effets sont probablement liés à un déséquilibre des

complexes formés par TrkA (Rabizadeh et Bredesen, 2003 ; Jung et Kim, 2008). Nous avons donc vérifié que la surexpression transitoire du HA-TrkA entraîne une réponse biologique comparable à celle obtenue par la surexpression de TrkA. Plus particulièrement, nous avons recherché l'impact de la surexpression sur la phosphorylation du récepteur et la migration cellulaire.

Pour étudier la phosphorylation, les cellules MDA-MB-231 transfectées par le HA-Trka ont été stimulées par le NGF en absence ou en présence de l'inhibiteur pharmacologique de TrkA (K252A). Les lysats cellulaires sont alors soumis à une immuno-précipitation anti-HA et un western blot anti-pTyr (utilisation de l'anticorps PY-20). Comme le montre la figure 14, En absence de NGF, TrkA est phosphorylé et cette phosphorylation est légèrement augmentée par le traitement au NGF. Dans tout les cas ces phosphorylations sont inhibées par le K252a.

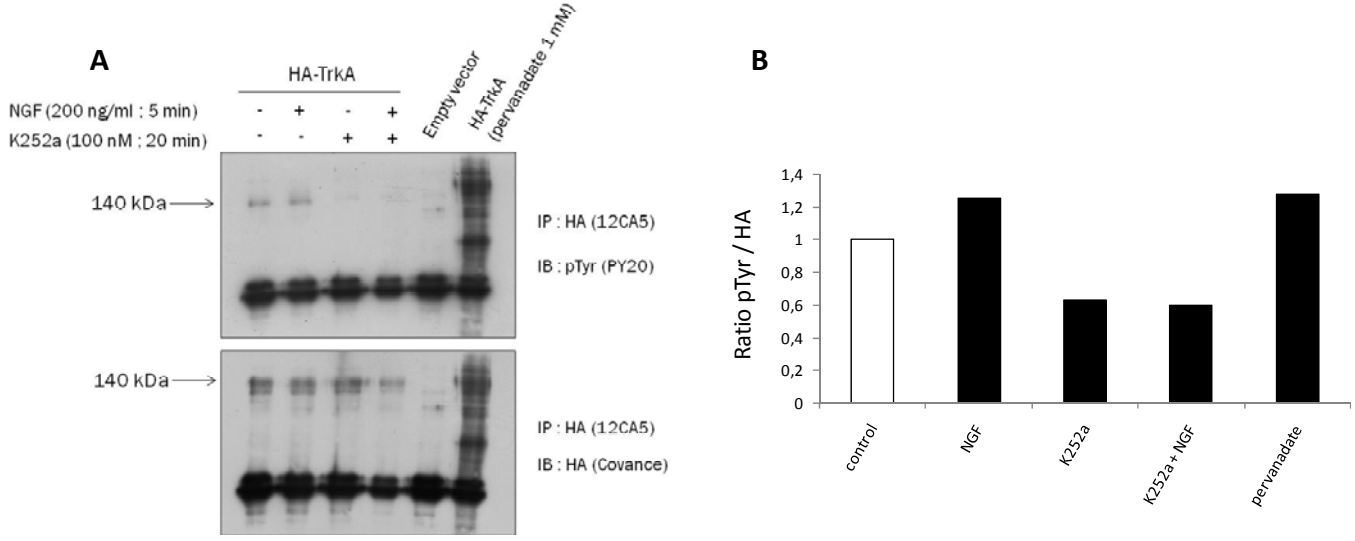


Figure 14: Effet du NGF sur la phosphorylation du HA-TrkA. Un milligramme de protéines totales a été immunoprécipité avec l'anticorps anti-HA et soumis à une analyse en Western-blot. **A** : Détection de tyrosines phosphorylées avec l'anticorps PY20 (panel haut) et détection de HA-TrkA (panel bas). **B** : Analyse densitométrique réalisée sur le panel haut (en A) (logiciel Quantity One, Biorad). IP : immunoprécipitation, IB : immunoblot.

L'effet du NGF sur la migration a par ailleurs été démontré dans de nombreuses études sur les cancers du sein (Lagadec *et al*, 2009), de la prostate (Festuccia *et al*, 2007) et

du pancréas (Okada *et al*, 2004) et serait lié à l'induction des voies MAPK/ERK et PI3K/Akt (Lagadec *et al*, 2009; Okada *et al*, 2004 ; Rahbek *et al*, 2005). Cette surexpression n'altère pas l'activation du récepteur par le NGF et son inhibition par le K252a. D'autre part, le niveau d'expression induit par la surexpression est compatible avec la quantité de TrkA mesurée dans les biopsies tumorales mammaires (Descamps *et al*, 2001b). L'amplification et/ou la surexpression des récepteurs aux facteurs de croissance sont d'ailleurs impliquées dans de nombreux cancers (Arteaga et Johnson, 2001 ; Nahta *et al*, 2003). L'ensemble de ces résultats suggère donc que la surexpression transitoire ne modifie pas l'activation de TrkA, dans nos conditions expérimentales. De ce fait, il nous sera possible d'étudier la dynamique des phosphorylations dans les cellules. En effet, il a été décrit que les cinétiques d'activation/inactivation sont à l'origine des réponses biologiques induites par le NGF (Jullien *et al*, 2002). Cette stratégie a été utilisée avec succès pour l'analyse de la phosphorylation de l'EGFR (Boeri Erba *et al*, 2007). Dans cette étude, les auteurs ont quantifié la phosphorylation des différents sites de phosphorylation du récepteur en utilisant un traitement au pervanadate de sodium comme méthode de normalisation. Une cinétique de traitement avec l'EGF a permis d'établir un profil dynamique de la phosphorylation du récepteur (Wu *et al*, 2006).

En conclusion, nos travaux permettent d'établir que la surexpression transitoire de HA-TrkA peut être utilisée pour étudier les phosphorylations de TrkA et le recrutement de ses adaptateurs. De plus, l'amélioration des conditions d'extraction, de purification et d'analyse protéomique devrait nous permettre d'appréhender le rôle des phosphorylations TrkA dans les réponses biologiques.

Perspectives

Nous disposons aujourd'hui des outils moléculaires permettant d'étudier les phosphorylations de TrkA sous l'effet du NGF dans les cellules mammaires et leurs implications dans le développement tumoral. Nous aborderons ces différents points selon 3 grands axes :

-Cartographie des sites de phosphorylation de TrkA sous l'effet du NGF,

-Identification des partenaires protéiques associés aux sites de phosphorylation de TrkA,

-Implication des phosphorylations de TrkA dans le développement tumoral.

1- Cartographie des sites de phosphorylation de TrkA sous l'effet du NGF

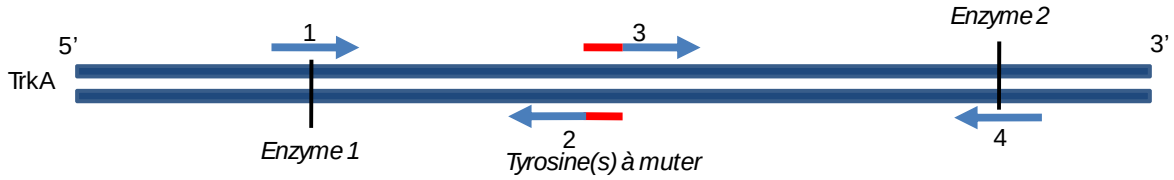
La détection sélective des phosphorylations des résidus sérine, thréonine et tyrosine sera réalisée sur un spectromètre de masse hybride « Triple quadripôle-Trappe ionique linéaire » couplé en amont à un système nano-LC. L'intérêt du spectromètre de masse hybride est de pouvoir combiner les hautes sélectivité et sensibilité du scan d'ions précurseurs et la haute sensibilité du mode MS/MS d'une trappe ionique (Williamson *et al*, 2006). La définition des critères de l'analyse en MS peut être également déterminante. En absence de stimulation du récepteur par le NGF, la digestion *in silico* de la séquence de TrkA (www.expasy.org/tools/peptidecutter) permettra de générer une liste de peptides théoriques dont les masses sont calculées (incluant les modifications variables) et inclues pour une recherche préférentielle lors de l'analyse MS. D'autre part, la phosphorylation des récepteurs et de ses adaptateurs est un phénomène labile et transitoire. La plupart des phosphoprotéines sont exprimées en faible abondance et sont retrouvées à de faibles quantités (picomole). Les signaux des phosphopeptides dans les spectres MS sont donc, en général, cachés par la haute concentration relative des peptides non phosphorylés dans les

digestats protéolytiques des échantillons (Mann *et al*, 2002). Lors du développement des analyses du phosphoprotéome, ces contraintes ont nécessité le développement de méthodes préparatrices puissantes pour détecter les phosphopeptides. Ainsi, la détection sélective des phosphopeptides en MS est possible par un scan d'ions précurseurs qui identifie les ions PO^{3-} de m/z 79 libérés par les résidus sérine, thréonine et tyrosine phosphorylés. Les phosphopeptides détectés sont ensuite séquencés par MS/MS. Ce scan couplé en amont d'une séparation en LC permet de minimiser les risques de non-détection de peptides minoritaires. De plus, nous réaliserons un pré-enrichissement des phosphoprotéines/phosphopeptides en amont de l'analyse MS par des systèmes PMAC (Phosphoprotein enrichment by Metal-Affinity Chromatography), IMAC (Immobilized Metal Affinity Chromatography) et colonnes TiO_2. La combinaison des enrichissements PMAC et TiO_2 a été utilisée efficacement pour l'analyse du phosphoprotéome après stimulation de cellules neuronales par l'IGF-1 (Insulin-like Growth Factor-1) et le PACAP (Pituitary Adenylyl Cyclase Activating Polypeptide) (Delcourt *et al*, 2007). Il est donc envisageable que l'utilisation de ces techniques d'enrichissement, d'analyse et le changement de spectromètre de masse pour des appareils plus sensibles (Orbitrap, FT-ICR) nous permettent d'identifier les phosphorylations de TrkA et ses protéines adaptatrices.

De plus, nous déterminerons la dépendance des phosphorylations en fonction du modèle cellulaire ce qui nous renseignera sur l'importance de la régulation de TrkA par les récepteurs aux œstrogènes et/ou de la famille de l'EGFR qui sont deux déterminants majeurs des phénotypes tumoraux mammaires (Lacroix *et al*, 2004). Ces études seront effectuées en surexprimant TrkA-HA et le récepteur sera isolé par immuno-précipitation avant analyse en spectrométrie de masse dans les lignées MCF-7, MDA-MB-231 et SKBR3.

2- Identification des partenaires protéiques associés aux sites de phosphorylation de TrkA

Il est admis que le NGF est un facteur pléiotrope qui agit sur les cellules PC-12 et certains neurones comme un facteur de différenciation, alors que sur les cellules cancéreuses de sein il agit sur la croissance et la migration/invasion (Reichardt, 2006). D'autre part, les cinétiques d'(in-)activation des phosphorylations des récepteurs tyrosine kinase déterminent l'ordre et la qualité du recrutement des adaptateurs sous les récepteurs entraînant la diversité des réponses biologiques (Santos *et al*, 2007; D'Alessio *et al*, 2007). Sur la base de cinétiques d'activation, nous étudierons, dans les différents modèles, l'association des adaptateurs à TrkA en fonction de sa phosphorylation. Les protéines seront co-immunoprécipitées à l'aide des anticorps dirigés contre le tag HA. Elles seront ensuite séparées sur SDS-PAGE puis identifiées par spectrométrie de masse (LC-MS/MS). Les associations seront confirmées par des techniques d'immunoprécipitation inverse et de colocalisation TrkA/protéine en microscopie confocale. Les sites de liaison de ces adaptateurs seront définis par des mutations ponctuelles, sur le récepteur, des résidus tyrosines en phénylalanines et des sérines/thréonines en alanines. Pour les résidus tyrosines, nous avons choisi d'effectuer la mutagénèse dirigée par PCR. Les couples d'amorces nécessaires à la mutagénèse dirigée sont indiqués dans la figure 15.



Site à muter	Amorces (de 5' à 3')			
	1	2	3	4
Tyr-490	TACAGCGCTGG GAGGAGGA	GCATCACTGAAGAA TTGTGGG	CCCACAATTCTTCAG TGATGC	ACCTGGCTAGCCAC GGCCA
Tyr-670/674/675	TGGCCGTGGCT AGCCAGGT	GGAAAAAGTCGGTG CTGAAGA	TCTTCAGCACCGACT TTTTCC	TCCGCGGCTAGCCC AGGACATCCA
Tyr-695	TGGCCGTGGCT AGCCAGGT	GAACTTACGGAACA GGATGCT	AGCATCCTGTTCCGT AAGTTC	AGGCACGTGGCCGC TCCAA
Tyr-751	TGGCCGTGGCT AGCCAGGT	CATGATGGCGAAGA CCTCTG	CAGAGGTCTTCGCC ATCATG	TCCGCGGCTAGCCC AGGACATCCA
Tyr-785	TTGGAGCGGC CACGTGCCT	ACATCCAGGAAGAC AGGAGG	CCTCCTGTCTTCCTG GATGT	CCTAACGTGGCTTCT TCTGC

AGCGCT : site AfeI GCTAGC : site NheI CACGTG: site PmlI

Figure 15 : Schématisation de la stratégie de mutagénèse dirigée et liste des amorces utilisées.

Outre les aspects qualitatifs, quantitatifs et cinétiques, nous rechercherons plus particulièrement les associations avec TrkA au niveau de la membrane plasmique. Pour cela, nous reprendrons les stratégies de marquage à la biotine des protéines exposées à la surface cellulaire (Zhang *et al*, 2009). Cette approche aura également l'intérêt de simplifier l'interactome de TrkA et permettre l'identification de partenaires minoritaires.

3- Implication des phosphorylations de TrkA dans le développement tumoral

Le développement tumoral est la résultante de nombreux paramètres qui concourent à l'accroissement de la taille de la tumeur mais aussi à la formation des métastases (Gatenby et Gillies, 2008). La phosphorylation de TrkA et le recrutement des adaptateurs que nous étudions participent à ce développement. Nous analyserons donc les phénomènes biologiques associés à l'(in-)activation des phosphorylations de TrkA et de ses adaptateurs.

Nous utiliserons pour cela les mutants du récepteur sur ses résidus phosphorylés que nous aurons développés et/ou l'invalidation par siRNA de quelques partenaires. Nous ciblerons nos études sur deux points en particulier qui semblent le mieux définir l'action du NGF dans les tumeurs : la croissance et la migration/invasion.

- la croissance : elle sera appréciée d'une façon générale par comptage cellulaire. Néanmoins les régulations induites par le NGF semblent plutôt influencer la croissance en absence d'ancrage et la résistance aux stress des cellules cancéreuses. Nous étudierons donc les modifications sur la survie induites par le NGF suite à une perte d'adhérence (anoïkis). Pour cela, les cellules seront cultivées en suspension dans des boites traitées au poly Hydroxy-éthyl-méthacrylique acide (poly Hema) pour prévenir tout ancrage. La résistance aux stress sera quant à elle évaluée avec des traitements connus pour induire l'apoptose comme les céramides C2 ou TRAIL. Mais de façon plus appliquée, nous étudierons les effets de l'activation de TrkA par le NGF sur la survie en présence de drogues utilisées en clinique pour le traitement des cancers du sein (Tamoxifène, doxorubicine),

- la migration et l'invasion : elles seront étudiées en utilisant des chambres de Boyden de type « transwell » à pores de 8 µm traitées avec différentes dilutions de matrigel. La mesure de la capacité migratoire et invasive des cellules sera également corrélée aux modifications d'expression et/ou d'activité de protéines dont le rôle dans les processus métastatiques est clairement établi (métalloprotéases, plasmine…) (Rydlova *et al*, 2008).

4- <u>Retombées attendues dans le domaine du cancer</u>

Sur le plan fondamental, notre projet de recherche permettra d'appréhender la signalisation induite par le NGF et TrkA dans le cancer du sein. La continuité de ces recherches, sur un plan plus appliqué, pourrait être assurée par des études translationnelles

sur modèles animaux en partenariat avec l'industrie pharmaceutique et la clinique. En effet, elles permettront d'établir des marqueurs d'activation de TrkA spécifiques du cancer du sein (phosphorylation des résidus ou découverte de nouveaux partenaires) dans un cadre diagnostique et/ou pronostique. D'autre part, notre étude pourrait être la base du développement d'inhibiteurs pharmacologiques plus spécifiques de TrkA et/ou de sa signalisation dans le cadre d'une utilisation thérapeutique.

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Stable 'portrait' of breast tumors during progression: data from biology, pathology and genetics

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Abstract

It is widely believed that ductal breast cancer dissemination involves a succession of clinical and pathological stages starting with carcinoma *in situ*, progressing into invasive lesion and culminating in metastatic disease. Such changes have frequently been attributed to the sequential acquisition of various alterations in a single cell followed by clonal selection and expansion, thus leading to intra-tumor diversity. According to this multi-step view, extensive genotype and phenotype (marker expression, grade) shift may occur in the same tumor during progression; this may lead to the co-existence of molecularly and/or pathologically different areas within the same lesion. An increasing amount of data of various natures now appear to challenge this concept: only a few distinct 'portraits', in relation to estrogen receptor (ER) status and grade, may be found among tumors. Moreover, although undergoing increasing genetic alteration, most individual lesions largely maintain their phenotype when they evolve from *in situ* to the metastatic state. While many of the data presented here are related to ductal tumors, lobular cancer is also discussed.

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Introduction

In Western countries, about 1 in 11 women will develop a breast carcinoma, generally of ductal origin. Despite considerable progress in the knowledge of tumor biology and treatment, more than two-thirds of patients still succumb to the disease. In most cases, death results from metastasis of breast cancer cells (BCCs). Elucidating the mechanisms that ultimately confer metastatic properties to BCCs remains, therefore, a major research challenge.

It has been widely believed that breast cancer dissemination involves a sequential progression through clinical and pathological stages starting with carcinoma *in situ*, progressing into invasive carcinoma and culminating in metastasis. To explain the transition from one stage to another, a multi-step hypothesis has been proposed. Based on the fact that BCCs are genetically unstable, the hypothesis postulates that tumor-stage changes are associated with the sequential acquisition of various genetic and, consequently, phenotypic alterations in a single cell followed by clonal selection and expansion. The successive clonal populations are thought to acquire an increasing aggressiveness, with numerous alterations in properties such as proliferation, adhesion, proteolysis, motility, angiogenic ability etc. and loss of estrogen receptor (ER). It is also believed that considerable intra-tumor diversity may result from the co-existence of these clonal populations. In particular, the characteristics of metastatic cells are expected to often be significantly different from those observed in BCCs *in situ*.

In fact, a number of recent data—of pathological, molecular and genetic nature—have revealed that despite increasing genetic alteration, the 'portrait' of breast tumors remains amazingly stable during progression, and that no major change appears to explain why a tumor may progress to the metastatic stage. A series of these data are reviewed here.

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Phenotype studies in breast tumors—molecular markers and grade

Before examining the occurrence of phenotype changes during tumor progression, it is necessary to specify which phenotype categories will be considered here. The categories are based on: (1) a series of molecular markers related to ER status; (2) grade. The rationale behind this choice is that both ER status and grade have long been used to classify tumors. As a consequence, there is abundant literature examining correlations between them and other parameters such as marker expression or proliferation. A classification based on an ER-positive/ER-negative or a low-grade/high-grade dichotomy hardly suffices to describe the complex spectrum of breast tumors; however, its simplicity will facilitate our main purpose: evaluating the stability of the breast tumor 'portrait'.

Molecular markers

ER appears as a major discriminator in the molecular classification of breast tumors. As a mediator of (anti)-estrogen action, its key role in the biology and treatment of breast cancer is well established. ER level has been evaluated in tumors for more than 30 years (Leclercq *et al*. 2002). The receptor, encoded by the *ESR1* gene, was long believed to be unique, until an isoform named ER-beta and encoded by a specific gene, *ESR2*, was identified. The 'older' isoform, subsequently named ER-alpha, seems to be functionally the most important in breast tumors (Speirs 2002). We have shown that the ER protein content, evaluated in breast tumors by a ligand-binding assay which measures both ER-alpha and -beta isoforms, was linearly correlated with the level of mRNA specific for *ESR1*, while the *ESR2* mRNA was undetectable in samples (Lacroix *et al*. 2001). Here, ER-beta will be considered as being of secondary importance in breast tumors, and the term ER will refer to the alpha isoform, unless otherwise indicated. ER is expressed by about 60–80% of breast tumors ('ER-positive'), while 20–40% are considered ER-negative.

During the last decade, many genes and/or proteins with an expression level positively or negatively correlated to that of ER have been identified in tumors. A series of these genes are listed in Table 1a (positive correlation to ER) and b (negative correlation to ER). From these studies, it appeared that two highly different phenotypes could be found in breast tumors according to their ER status. In addition, the co-existence in the same tumor of markers related to both ER-positive and ER-negative phenotypes was rarely observed. For instance, it is well known that ER and epidermal growth factor receptor (EGFR) levels are inversely correlated in most ductal tumors. Both receptors are, however, occasionally co-expressed in lesions, but are then, in the vast majority of cases, localized in distinct tumor cells, or in interspersed groups of cells ('mosaic expression', as described for instance in van Agthoven *et al*. 1994). Whether ER-poor/EGFR-rich BCCs were derived from ER-rich/EGFR-poor cells in these tumors is unknown. If this was the case, the observations suggest that such an event is infrequent and does not seem to be related to any significant advantage for progression. Rare co-expressions have also been observed with other pairs of markers related to distinct BCC phenotypes (not discussed here).

A series of proliferation/apoptosis-related markers has also been measured in tumors. In general, their expression levels reflected the fact that mitotic/apoptotic activity is higher in ER-negative than in ER-positive lesions (Keshgegian & Cnaan 1995, Gandhi *et al*. 1998, Lipponen 1999). For instance, the expression of the apoptosis inhibitor BCL2 was correlated to that of ER (Gee *et al*. 1994, Binder *et al*. 1995, Yang *et al*. 1999). P53 levels were repeatedly found to be higher in ER-negative lesions (see notably Rudolph *et al*. 1999a). Proliferation markers KI-67 and topoisomerase II alpha levels were also positively correlated to ER-negative status (Molino *et al*. 1997, Rudolph *et al*. 1999b). Regarding cyclins, while cyclin E was associated with the absence of ER, the inverse was observed for cyclin D1 (Nielsen *et al*. 1997, Barnes & Gillett 1998, Reed *et al*. 1999, Spyratos *et al*. 2000, Park *et al*. 2001, Loden *et al*. 2002). The level of the cyclin-dependent kinases inhibitors 1A ($P21^{WAF1/CIP1}$) and 1B ($P27^{KIP1}$) was found to be higher in ER-positive tumors (Barbareschi 1999, Reed *et al*. 1999, Barbareschi *et al*. 2000, Oh *et al*. 2001).

The evaluation of multiple markers in multiple tumor samples has recently been facilitated by the use of tissue micro-arrays (TMAs) (Kononen *et al*. 1998, Camp *et al*. 2000, Simon *et al*. 2004). In general, TMA-based data are in good agreement with previous observations. For instance, TMA analysis was applied to 107 breast carcinomas samples, to assess the pattern of expression of ER, PR, P53, ERBB2, MYC, $P27^{KIP1}$, cyclin D, cyclin E, BCL2, MIB1, MCM2 (minichromosome maintenance protein 2, see Gonzales *et al*. 2003), basal cytokeratins CK5/6, epithelial cytokeratins CK8/18. Cluster analysis of the data classified samples into two main groups: the first ('ER-related') included about two-thirds of the tumors and was characterized by a high expression level of ER, PR, BCL2, cyclin D, $P27^{KIP1}$, CK8/18, MYC; the second group expressed relatively high levels of P53, ERBB2, CK5/6, and of the proliferation markers MCM2, MIB, and cyclin E (Callagy *et al*. 2003). In another study, 15 markers (ER, PR, P53, ERBB2, EGFR, cyclin A, cyclin D1, cyclin E, BCL2, $P21^{WAF1/CIP1}$, $P27^{KIP1}$, CK5/6, CK8/

Table 1a Series of genes differentially expressed in breast tumors – genes expressed at higher levels in ER-positive and/or in low-grade tumors.

Gene name	Gene product name(s)	Higher expression in		References
		ER-positive tumors	Low-grade tumors	
CA12	Carbonic anhydrase XII	*	*	Watson *et al.* 2003
CDH1	Cadherin type 1, epithelial cadherin (E-cadherin)	*	*	Sommers *et al.* 1994, Parker *et al.* 2001
CLDN7	Claudin 7		*	Kominski *et al.* 2003
DSP	Desmoplakin (DPI, DPII)		*	Sommers *et al.* 1994, Davies *et al.* 1999
ERBB3	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	*	*	Bieche *et al.* 2003
ERBB4	V-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	*	*	Bieche *et al.* 2003
ESR1	Estrogen receptor alpha	*	*	Tong *et al.* 1999, Zafrani *et al.* 2000, Ringberg *et al.* 2001
GATA3	GATA sequence binding protein 3	*		Hoch *et al.* 1999
GREB1	Greb1 protein	*		Ghosh *et al.* 2000
MDM2	Mdm2, p53 binding protein	*	*	Gudas *et al.* 1995, Hori *et al.* 2002
NME1	Protein expressed in non-metastatic cells (nm23A)		*	Hartsough & Steeg 2000, 2001
PDZK1	PDZ domain containing 1	*		Ghosh *et al.* 2000
PGR	Progesterone receptor	*	*	Hall *et al.* 1990, Tong *et al.* 1999, Zafrani *et al.* 2000
PRDM2	PR domain containing 2 (RIZ1), transcript 1			Du *et al.* 2001
PRLR	Prolactin receptor	*		Peirce *et al.* 2001, Gill *et al.* 2001
PTPRA	Protein tyrosine phosphatase, receptor type, A	*	*	Ardini *et al.* 2000
RERG	Ras-like, estrogen-regulated, growth-inhibitor	*		Finlin *et al.* 2001
SLC9A3R1	Solute carrier family 9, isoform 3 regulatory factor 1	*		Stemmer-Rachamimov *et al.* 2001
STC2	Stanniocalcin 2	*		Bouras *et al.* 2002
TFAP2C	Transcription factor activator protein 2 gamma	*		Kuang *et al.* 1998
TFF1	Trefoil factor 1 (pS2, BCEI)	*	*	Tong *et al.* 1999
TFF3	Trefoil factor 3	*		Gillesby & Zacharewski 1999
TJP1	Tight junction protein 1 (ZO-1)		*	Sommers *et al.* 1994, Hoover *et al.* 1998

Table 1b Series of genes differentially expressed in breast tumors – genes expressed at higher levels in ER-negative and/or in high-grade tumors.

Gene name	Gene product name(s)	Higher expression in		References
		ER-negative tumors	High-grade tumors	
AKT3	V-akt murine thymoma viral oncogene homolog 3	*		Nakatani *et al.* 1999
CCNE1	Cyclin E1	*	*	Donnellan *et al.* 2001
CDH3	Cadherin 3, placental cadherin (P-cadherin)	*	*	Paredes *et al.* 2002
CDKN2A	Cyclin-dependent kinase inhibitor 2A (p16)	*	*	Hui *et al.* 2000
CTSB	Cathepsin B	*	*	Lah *et al.* 2000
CTSL	Cathepsin L	*	*	Lah *et al.* 2000
DFNA5	Deafness, autosomal dominant 5 (ICERE-1)	*		Thompson & Wiegel 1998
EGFR	Epidermal growth factor receptor	*	*	Hall *et al.* 1990, Klijn *et al.* 1992, Walker & Dearing 1999
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (avian)	*	*	Tsuda *et al.* 1990, Rilke *et al.* 1991, Tagliabue *et al.* 1999, Spizzo *et al.* 2002, Hoque *et al.* 2002, Hoff *et al.* 2002
GPX1	Glutathione peroxidase 1	*		Townsend *et al.* 1991, Esworthy *et al.* 1995
GSTP1	Glutathione S-transferase pi	*		Moscow *et al.* 1988
HGF	Hepatocyte growth factor (hepapoietin A; scatter factor)		*	Beviglia *et al.* 1997
HMGIY	High-mobility group protein isoforms I and Y		*	Liu *et al.* 1999
HXB	Hexabrachion (tenascin-C)	*	*	Dandachi *et al.* 2001
IL8	Interleukin-8	*		De Larco *et al.* 2001
MET	Met proto-oncogene (HGF receptor)		*	Beviglia *et al.* 1997
MSN	Moesin	*		Carmeci *et al.* 1998
MT1E	Metallothionein 1E	*		Friedline *et al.* 1998, Jin *et al.* 2000
PLAU	Plasminogen activator, urokinase	*	*	Grøndahl-Hansen *et al.* 1993, Tetu *et al.* 2001, Look *et al.* 2002
PTGS2	Prostaglandin-endoperoxide synthase 2	*	*	Ristimaki *et al.* 2002
RARB	Retinoic acid receptor, beta	*	*	Widschwendter *et al.* 2000
S100A4	S100 calcium binding protein A4 (metastasin)	*		Pedrocchi *et al.* 1994, Sherbet & Lakshmi 1998
SDC1	Syndecan 1	*	*	Barbareschi *et al.* 2003
SERPINB5	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 5	*	*	Umekita *et al.* 2002
SERPINE1	Plasminogen activator inhibitor type 1 (nexin)	*	*	Grøndahl-Hansen *et al.* 1993, Look *et al.* 2002
SNAI1	Snail homolog 1	*	*	Blanco *et al.* 2002
STMN1	Stathmin 1 (oncoprotein 18)	*	*	Curmi *et al.* 2000
STS	Steroid sulfatase	*	*	Miyoshi *et al.* 2003
TIMP1	Tissue inhibitor of metalloproteinase 1	*		Yoshiji *et al.* 1998, McCarthy *et al.* 1999, Remacle *et al.* 2000
VIM	Vimentin	*		Sommers *et al.* 1994, Dandachi *et al.* 2001

18 and SMA (smooth muscle actin)) were evaluated in 166 breast cancer samples. Tumors expressing the basal CK5/6 showed higher levels of cyclin A, KI-67, P53 and EGFR expression; tumors expressing CK8/18 but not CK5/6 were associated with a higher level of ER, PR, $P21^{WAF1/CIP1}$, $P27^{KIP1}$, ERBB2 and BCL2. When cluster analysis was applied to the data, three classes were found. Besides those defined by the presence or absence of CK5/6, a third class appeared which was characterized by ERBB2 over-expression and *ERBB2* amplification (Korsching *et al*. 2002). Thus, a specific 'portrait' may occasionally be associated with ERBB2-overexpressing tumors. This is also found in DNA-micro-array-based gene expression studies (see Micro-array studies in section on Genetic studies on breast tumors).

Grade

One of the most widely accepted classification systems for breast carcinomas is grading. The majority of grading systems, such as those based on the Scarff, Bloom and Richardson (SBR) method, combine histological assessment of nuclear pleomorphism, mitotic activity and tubule formation (Elston & Ellis 2002). Tumors classified as 'grade I' or 'low grade' have well-differentiated attributes, while 'grade III' or 'high-grade' tumors have poorly differentiated attributes. Grade II tumors fall into an intermediate category. High-grade ductal carcinoma have been associated with the highest rate of local recurrence (25–30%), low-grade tumors have very low recurrence (0–5%), while intermediate-grade tumors have a recurrence rate somewhere in between (10–15%) in 12 years of median follow-up (Polyak 2001). Moreover, high-grade tumors recur within a shorter time than the low-grade ones (for instance, in Cserni (2002), the median recurrence time is 88, 42 and 23 months for grades I, II and III respectively).

Grading is not directly based on molecular expression profiles. It may thus be asked whether or not grades are associated with the expression of specific sets of tumor markers, and more precisely if they are correlated to the distinct ER-status phenotypes described above for tumors. It has been repeatedly reported that most ER-positive tumors are of low grade. Conversely, high-grade tumors are mainly ER-negative (see for instance Tong *et al*. 1999, Zafrani *et al*. 2000, Ringberg 2001). Unsurprisingly, several markers whose expression is positively correlated to that of ER in tumors have also been associated with low-grade tumors; these are listed in Table 1a. Conversely, high-grade tumors are characterized by the expression of markers more related to the ER profiles in tumors; these are listed in Table 1b. None of the genes positively correlated to ER in tumors were found associated with high-grade/poorly differentiated carcinomas. None of the genes negatively correlated to ER in BCC in tumors was found associated with low-grade/well-differentiated lesions.

Proliferation/apoptosis-related markers appear to reflect the fact that mitotic/apoptotic activity is higher in high-grade/poorly differentiated tumors. For instance, a study examining mitotic index (MI, number of mitoses/1000 nuclei) and apoptotic index (AI, number of apoptosis/1000 nuclei) found MI and AI values to be up to 20-fold and 2.5-fold higher, respectively, in poorly differentiated ductal carcinoma *in situ* (DCIS), as compared with well-differentiated DCIS (Buerger *et al*. 2000*a*). This is in line with results from other groups (Keshgegian & Cnaan 1995, Gandhi *et al*. 1998). For instance, in an immunohistochemical (IHC) study of 46 DCIS, it was shown that BCL2 was expressed in 12/12, 14/20 and 1/14 well-, moderately and poorly differentiated tumors respectively. For P53, the respective values were 0/12, 6/20 and 12/14, while for KI-67 (Viacava *et al*. 1999) they were 0/12, 10/20 and 14/14. In another report, KI-67 expression was found in only 5% of low-grade but in 70% of high-grade DCIS. For P53, the respective percentages were 5% and 50% (Krishnamurthy & Sneige 2002). Regarding cyclins, while cyclin E has been associated with high-grade tumors, the inverse was observed for cyclin D1 (Loden *et al*. 2002). The level of $P21^{WAF1/CIP1}$ and $P27^{KIP1}$ was found to be higher in low-grade or well-differentiated tumors (Barbareschi 1999, Reed *et al*. 1999, Wu *et al*. 1999, Oh *et al*. 2001, Barbareschi *et al*. 2000).

The relationship between marker expression and grade has also been investigated by TMA analysis (see below). For instance, in a study of 107 breast cancer samples, the expression of 11 markers showed an association with grade: ER, PR, BCL2, $P27^{KIP1}$ and cyclin D levels were higher in low-grade samples; ERBB2, P53, cyclin E, CK5/6, MCM2 and MIB1 levels were higher in high-grade tumors (Callagy *et al*. 2003). These results are in agreement with previous data.

Concluding remarks on molecular markers and grade

A number of studies have shown that the phenotype categories based on ER status and on grade largely overlap. ER-positive and well-differentiated/low-grade tumors on the one hand, and ER-negative and poorly differentiated lesions on the other hand, share many features. But the two phenotypes defined in each category (ER-positive and -negative, well and poorly differentiated) appear highly different, so that a frequent transition from one to the other during progression seems rather unlikely. However, this does not exclude the possiblity that co-expression of the two phenotypes could occasionally occur in the same lesion, as suggested by the

existence of grade II tumors. However, it is largely unknown whether such cases result from phenotype changes during tumor growth, or are derived from two populations that appeared early in tumorigenesis.

Macroscopic homogeneity of breast tumors — stable 'portrait' during progression

According to the multi-step view, progression from primary to metastatic tumor should be accompanied by the sequential acquisition of phenotype changes, allowing BCCs to invade, disseminate and colonize distant sites. Notably, it has been proposed that BCCs *in vivo* might undergo a transition from the ER-positive- to the ER-negative-associated phenotype. Along the same lines, it has been repeatedly proposed that tumor progression is characterized by a shift from the well-differentiated/low-grade to the poorly differentiated/high-grade phenotype. Nevertheless, most investigations have revealed that progression is not accompanied by major changes in marker expression or grade.

Progression to invasiveness and markers/grade

If we hypothesize that progression from *in situ* to invasive carcinoma is often accompanied by extensive phenotype changes, it should then be easy to find in a significant part of invasive tumors both ER-positive- and ER-negative-associated, and both low- and high-grade, compartments. ER-negative-associated markers and high-grade areas should normally be observed more frequently in the invasive than in the *in situ* tumor compartment. In fact, most studies examining this point have revealed a striking similarity between both parts of breast carcinomas (Lampejo *et al*. 1994, Moriya & Silverberg. 1994, Iglehart *et al*. 1995, Douglas-Jones *et al*. 1996, Millis *et al*. 1998, Foster *et al*. 2000, Mommers *et al*. 2001 Warnberg *et al*. 2001,). For instance, grade and tumor marker (P53, ERBB2, KI-67, ER, PR, BCL2 and angiogenesis) expression were compared in 194 pure DCIS, 127 small invasive lesions and 305 lesions with both an invasive and *in situ* component. Grade concordance was high between *in situ* and invasive components of the same tumor. All markers were found to correlate with grade rather than with invasiveness. No marker was clearly associated with the progression from *in situ* to invasiveness. The expression of tumor markers was similar, at 80–90%, in the two components of mixed lesions (Warnberg *et al*. 2001). The DNA content and the expression of ERBB2 were examined simultaneously in both non-invasive and invasive phases of primary breast cancers, by image analysis. DNA content in the intraductal and invasive components was virtually identical. Expression of ERBB2 was similar in both growth phases, strongly suggesting identity of the ERBB2 genotype (Iglehart *et al*. 1995). An IHC study examined the expression of proteins involved in proliferation and apoptosis (KI-67, cyclin D1, ERBB2, $P21^{WAF1/CIP1}$, $P27^{KIP1}$, P53 and BCL2) in 61 DCIS and 53 invasive lesions. More proliferation tended to be observed in invasive cancers. However, well-differentiated DCIS and invasive lesions shared many aberrations in expression of the proliferation-associated proteins, as did poorly differentiated DCIS and invasive lesions. In contrast, many differences were observed between the well- and poorly differentiated lesions (Mommers *et al*. 2001). In a study of 102 patients, a 67% concordance in grade was found between *in situ* and infiltrating components (Millis *et al*. 1998). Another study of 64 cases indicated an 86% grade concordance between both components (Moriya & Silverberg. 1994). These studies and others (Lampejo *et al*. 1994, Douglas-Jones *et al*. 1996) indicated a strong correlation between the grade of DCIS and the grade of infiltrating carcinoma in which both components were present.

It is thus striking that patterns of grade or the other markers did not seem to change significantly during the transition from *in situ* to invasive carcinoma. Invasive cancer seems to occur independently of tumor grade. This is further supported by comparative genetic hybridization and micro-array data (see below).

Recurrence, metastasis

Metastatic and recurrent BCCs are often believed to have accumulated phenotypic alterations, as they are associated with late stages in tumor progression. In addition, metastatic cells may colonize various tissues that are highly different from the breast (bone, lung, brain, etc.) after having completed all steps of a complex process including local invasion, intravasation, resistance to blood pressure, adhesion to blood vessels, and extravasation. This suggests that they have sequentially acquired specific adaptive properties and it has thus been hypothesized that metastatic and recurrent cells could express a phenotype significantly different from that observed in the primary tumor.

Attempts have been made to compare the expression of various markers and/or the histological grade in primary tumors and their corresponding metastases and/or recurrences. It was shown that CK8 and CK19 expression were similar in both primary carcinomas and their lymph node (LN) metastases (Su *et al*. 1996). In an IHC study of 38 LN metastases and their corresponding primaries, very good concordance was found for KI-67 (85%), ER (96%), PR (82%), P53 (76%) and ERBB2

(84%) (Briffod *et al*. 2000). In another comparative IHC study involving 102 LN metastases, an 80% concordance was found for ER (Nedergaard *et al*. 1995). Investigations on a total of 31 LN, 35 lung, 25 skin, 1 liver and 2 contralateral breast metastases revealed good concordance with primaries for ER, PR, P53 and ERBB2 (Barnes *et al*. 1988, Kayser *et al*. 1998, Shimizu *et al*. 2000). This was also the case for ER, PR and EGFR evaluated in 26 LN and 2 distant metastases. In this latter study, expression of ER and EGFR was inverse regarding the individual tumor cells in both primary tumors and metastases (van Agthoven *et al*. 1995). By ligand-binding assay, it has been estimated that no more than 20% of the ER-positive primary tumors will produce ER-negative metastases. It has even been established that the expression of the frequent ER-alpha variant transcripts is conserved in primary tumors and their matched, concurrent LN metastases (Fuqua 2001). A good concordance was also found for grade; for instance, a study of 102 primaries and LN metastases revealed that both had the same grade (I, II or III) in 79% of cases (Millis *et al*. 1998). In a tissue micro-array analysis, it was found that 77% of ERBB2-positive primary tumors had entirely ERBB2-positive LN metastases, 6.5% had entirely ERBB2-negative metastases and 16.5% had a mixture of ERBB2-positive and ERBB2-negative metastases. For ERBB2-negative primary tumors, a 95% concordance was found for the LN metastases. Moreover, the ERBB2 status within individual tumors was fairly homogenous, as was the status of primary tumors and their metastases (Simon *et al*. 2001).

Along the same lines, several studies have examined grade and marker (ER-alpha, ER-beta, PR, P53, ERBB2 and TFF1 (formerly pS2)) expression in recurrent breast cancers (Millis *et al*. 1998, Horiguchi *et al*. 2000, Shimizu *et al*. 2000, Bijker *et al*. 2001, Jensen *et al*. 2001). Good concordance was found in most cases; for instance, in an analysis of 116 cases of recurrence, only four patients were found to have developed poorly differentiated DCIS or grade III invasive carcinoma after well-differentiated DCIS (Bijker *et al*. 2001). Regarding ER-alpha and ER-beta, their expression was even found to be higher in recurrences than in the corresponding primaries (Jensen *et al*. 2001). In a study of six cases of recurrence, histological type was the same as the initial one. There was concordance in ER, PR, TFF1, ERBB2 and P53 status between the recurrence and the primary cancer (Horiguchi *et al*. 2000). In a study of 49 primaries and recurrences, a 78% grade (I, II or III) concordance was found; in 36 patients who developed both metastasis and recurrence, grade concordance between them was also 78% (Millis *et al*. 1998). In an analysis of 84 patients for which axillary metastases and/or local and/or regional recurrence(s) were found, 78 and 81% concordance was demonstrated between primaries and their metastases and first recurrences respectively. In the cases where successive (up to six) recurrences were found, there was still a 74% concordance between the last recurrence and the initial tumor sample (Cserni 2002).

Conclusions

In conclusion, breast tumor phenotype does not appear, in most cases, to change extensively during tumor progression from *in situ* carcinoma to secondary site colonization. However, nearly all of the studies described here showed percentages of concordance in the 65–95% range. This indicates the existence of a substantial number of cases in which progression (to invasiveness, to metastasis or recurrence) is accompanied by qualitative changes in marker expression or grade. The fact that BCCs could occasionally undergo profound phenotype alterations has been suggested for years. For instance, it has been proposed that epithelial–mesenchymal transition (EMT) could occur in BCCs. This process would be reminiscent of the transition that is observed during embryonic development at precise times and locations (Boyer *et al*. 2000). EMT in BCCs would consist of the turning-off of genes encoding epithelial markers (ER-alpha, PR, E-cadherin, tight junction proteins etc.) and the increase of markers such as vimentin. While the reality of EMT has been little substantiated *in vitro*, the possibility that it could happen *in vivo* cannot be definitely excluded.

Genetic studies on breast tumors

Karyotype and cytogenetic studies

Breast cancer is characterized by multiple genetic alterations. They may include whole chromosome copy gain or loss (aneuploidy), gain and loss of parts of chromosomes (detected by comparative genetic hybridization and loss of heterozygosity analysis), amplifications or deletions of single genes, insertions and translocations, and mutations of a single or a few nucleotide(s).

Aneuploidy

Aneuploidy is frequent in breast carcinomas. In a study of 127 000 breast tumors, about one-half were found to be diploid or near diploid, the others exhibiting various types of aneuploidy (Wenger *et al*. 1993). In an analysis of 256 patients, 384 modal chromosome numbers were detected, ranging between 29 and 211; 74% of these modal numbers were between 41 and 50, 19% between 51 and 80. Only 3% were lower than 41 and 4% higher than 80 (Teixeira *et al*. 2002). Our cumulative study of breast tumor series (Ried *et al*. 1995, Schwendel *et al*. 1998, Adeyinka *et al*. 1999,

Roylance *et al*. 1999, Davidson *et al*. 2000, Dellas *et al*. 2002, Zudaire *et al*. 2002) emphasized that monosomy was observed mainly for chromosomes 7, 19, 20 and X, while trisomy most frequently concerned chromosomes 4, 18, 19 and X. By analyzing a set of relatively small and partly overlapping series of BCC lines (Forozan *et al*. 1999, 2000, Kytola *et al*. 2000, Davidson *et al*. 2000, Larramendy *et al*. 2000), the same variations were observed, except that loss of chromosome X was relatively less frequent in cell lines. As most available cultured BCC lines are of metastatic origin, this suggests that the qualitative pattern of whole chromosome losses and gains remains constant during tumor progression. But what about the quantitative pattern?

Fluorescence *in situ* hybridization (FISH) analysis of chromosomes 7, 8, 16 and 17 was applied to foci of residual DCIS and a representative area of co-existing invasive neoplasm. Most hybridization pairs (7/12, 58%) showed a gain in chromosomal copy number between the *in situ* and corresponding invasive area, whereas 29% showed no apparent change and 13% showed loss in copy number. Hybridizations from areas of invasive carcinoma, thus, were more frequently characterized by tumor cells with trisomy/polysomy (78%) than neoplastic cells from residual DCIS (50%) and less frequently characterized by cells with monosomy (10% versus 16%). Even when DCIS cells exhibited chromosome trisomy, 65% of hybridizations demonstrated a significantly greater proportion of trisomic cells in the corresponding invasive population (Mendelin *et al*. 1999). In another FISH study, a high proportion (54%) of 214 invasive breast carcinomas displayed aneusomy of chromosome 17. Aneusomy was not associated with survival, suggesting that it is not significantly related to the development of metastases. In contrast, an association was found with grade III carcinoma and ER negativity (Watters *et al*. 2003).

Indeed, although several FISH studies have attempted to identify chromosome gain or loss responsible for breast tumorigenesis and progression, no specific alterations can yet be repeatedly attached to certain histopathological stage. On the other hand, the patterns of aneuploidy may differ according to tumor grade. For instance, in a FISH study of numerical alterations of chromosomes 7, 8, 16 and 17 in 28 DCIS, grade I lesions were characterized by a complete lack of significant chromosome gain, but 29% showed partial (focal) monosomy. Grade III lesions, in contrast, showed partial or complete trisomy/polysomy in 88% hybridizations versus monosomy in only 4%. Grade II DCIS exhibited a mixed pattern of chromosome aneuploidy: 38% hybridizations were disomic, 36% trisomic/polysomic and 26% monosomic (8 out of 10 hybridizations showing complete monosomy occurred in grade II lesions). In morphologically heterogenous lesions, higher-grade foci were characterized by chromosome copy gain relative to corresponding lower-grade areas in 17 of 22 (77%) hybridizations (Visscher *et al*. 2000).

In conclusion, despite the fact that most tumors tend to gain chromosomes during progression, a higher proportion of high-grade carcinomas are believed to progress to near-triploidy (Pandis *et al*. 1996). However, no specific chromosome number change has been as yet clearly associated with progression.

An important criticism that is often addressed to karyotype analysis is that it may be biased, at least when it is applied to long-term cell cultures. The resulting karyotypes may represent minor malignant cell clones of the tumors expressing a growth advantage in culture (see for instance Truong *et al*. 1999). It is also possible that some of the simple abnormal karyotypes might be due to mitoses of non-malignant breast lesions (Lundin & Mertens 1998, Persson *et al*. 1999). This underlines the need for additional techniques to detect genetic alterations.

Comparative genomic hybridization (CGH) studies

In addition to changes in chromosome number, DNA losses or gains larger than 10 mb have been detected in tumors by CGH. Table 2 reports the frequency of DNA losses or gains affecting the ten most involved chromosome arms, as determined from a cumulative set of breast tumors (Ried *et al*. 1995, Schwendel *et al*. 1998, Adeyinka *et al*. 1999, Roylance *et al*. 1999, Davidson *et al*. 2000, Loveday *et al*. 2000, Guenther *et al*. 2001, Zudaire *et al*. 2002, Cingoz *et al*. 2003). These data have been confirmed by a recent study of 305 unselected primary invasive breast tumors, according to which the six most commonly observed gains were on 1q (55%), 8q (41%), 16p (40%), 17q (28%), 20q (19%) and 11q (16%) and the three most commonly observed losses were on 13q (27%), 16q (22%) and 8p (18%) (Rennstam *et al*. 2003).

Several studies using CGH analysis on DCIS have demonstrated a large number of chromosomal alterations

Table 2a Chromosome arms most frequently altered in a cumulative series of 542 breast tumors (CGH analysis), in order of decreasing frequency – DNA losses

16q	1p	8p	13q	11q	17p	22q	6q	Xp	Xq
25%	21%	20%	19%	19%	18%	17%	14%	11%	11%

Table 2b DNA gains

1q	8q	17q	20q	16p	11q	12q	7q	6q	3q
51%	46%	24%	24%	22%	21%	17%	15%	15%	15%

including gains on 1q, 6q, 8q, 17q, 19q, 20q and Xq, and losses on 13q, 16q, 17p and 22q. Most of these alterations resemble those identified in invasive ductal carcinoma (IDC), adding weight to the idea that DCIS is a direct precursor lesion of IDC (Aubele *et al*. 2002).

It has been shown that DNA loss at 16q is less frequent in high-grade ductal carcinoma (Roylance *et al*. 1999, 2002, Richard *et al*. 2000, Boecker *et al*. 2001, Cingoz *et al*. 2003) and in ER-negative tumors (Zudaire *et al*. 2002). This constitutes a strong argument against the theory supporting a frequent tumor progression from low to high grade and from ER-positive to ER-negative status. Indeed, it appears unlikely that grade III tumors could arise from grade I tumors through a process involving regain of 16q. Besides similar observations on 16q, Richard *et al*. (2000) also noted a higher frequency of 7q gains in ER-negative carcinomas and of 3q gains in ER-negative and high-grade carcinomas, as well as a lower frequency of 16p gains in ER-negative tumors and of 22q losses in ER-negative and high-grade carcinomas. In an analysis of 22 tumors (DCIS and IDC), ER positivity was significantly higher in cases displaying 16q losses and 20q gains (Cingoz *et al*. 2003). Other investigators pointed out that, contrasting with the higher global frequency of chromosome changes in high-grade carcinomas, 16q losses were more frequent in low-grade carcinomas, while the frequency of 20q gains was the same in both low- and high-grade lesions (Boecker *et al*. 2001). Thus, while several qualitative differences have been highlighted by CGH in ER-positive/low-grade tumors, as compared with ER-negative/high-grade lesions, only one change, loss at 16q, has been repeatedly observed. Whether it is crucially involved in phenotype definition is unlikely, as not all ER-positive/low-grade tumors are characterized by this loss, while it is occasionally observed in ER-negative/high-grade tumors.

Total DNA changes were found to be 1.7-fold more frequent in ER-negative than in ER-positive tumors (Richard *et al*. 2000), while between 1.5- and 3.2-fold more genetic alterations were observed in grade III/high-grade/poorly differentiated than in grade I/low-grade/well-differentiated samples (Schwendel *et al*. 1998, Roylance *et al*. 1999, 2002, Buerger *et al*. 2000*a*, Richard *et al*. 2000) . For instance, amplification of *ERBB2* (17q12), *TOP2A* (17q24), *MYC* (8q23), and *CCND1* (11q13) was more frequently found in high- than in low-grade tumors. However, major amplifications in pure *in situ* carcinoma and in intraductal carcinoma with an invasive component did not differ (Glockner *et al*. 2001). That no specific gross DNA alteration was associated with invasion was confirmed by analysis of a series of ductal (but also lobular) tumors submitted to CGH following micro-dissection (Buerger *et al*. 2000*b*).

Loss of heterozygosity (LOH) studies

LOH analysis detects allelic loss at specific loci by a PCR-based screening with polymorphic microsatellite markers spaced across the region of interest. This technique has been widely used to detect the loss of putative suppressor genes.

LOH in breast cancer has been observed in multiple chromosomal regions, notably 1p, 1q, 3p, 6q, 7q, 8p, 9p, 11q, 13q, 16q, 17p, 17q, 18q and 22q. The highest rate of LOH in DCIS approaches 50–80% and involves loci on chromosomes 16q, 17p and 17q, suggesting that altered genes in these regions may play a role in the development of DCIS. In general, LOH frequency has been found to be higher in IDC than in DCIS (see notably Ando *et al*. 2000). Eighty per cent of the DCIS shared their LOH patterns with invasive carcinomas from the same breast, strongly supporting a precursor relationship between these lesions and the cancers they accompany (Deng *et al*. 1996).

Differences in LOH frequencies according to grade have been repeatedly observed in breast tumors. Thus, Ando *et al*. (2000) observed higher frequency of LOH at 16q in low- and intermediate-grade DCIS, while LOH at 11p and 17p were less frequent for these grades.

The fact that regions showing the highest levels of LOH were different in tumors of different grades has notably been illustrated by Shen *et al*. (2000). In their genome-wide search using laser capture micro-dissected tissue of breast carcinoma, these authors found LOH frequencies as follows:

Well-differentiated lesions: 16q22.1 (47.6%), 16q22.3 (42.1%), 17p12 (37.5%), 11q22.1 (35.0%), 9q22.33 (35.0%), 1q24.2 (35.0%), 12p12.3 (33.3%), 3p22.1 (33.3%), 1q24.2 (32.0%), 1q44 (31.6%).

Moderately differentiated lesions: 11q22.2. (42.9%), 17p12 (42.1%), 14q31.1 (41.7%), 14q32.11 (41.2%), Xq13.3 (40.0%), 16q22.1 (40.0%), 14q24.1 (39.1%), 8p12 (39.1%), 17q21.31 (37.0%), 16q24.3 (37.0%).

Poorly differentiated lesions: 17p13.3 (90.9%), 17p12 (86.7%), 17p13.3 (77.7%), 17p13.2 (73.3%), 4p15.1 (73.3%), 1q32.1 (71.4%), 4q28.2 (70.0%), 17q21.31 (63.2%), 3q23 (62.5%), 1p36.12 (61.1%), 22q12.3 (60.0%), 8q24.3 (60.0%), 3p14.3 (60.0%).

Such results are in agreement with the observations of Ando *et al*. (2000) on 16q and 17p.

Instability at the nucleotide level

Genomic instability may also exist at the nucleotide level, resulting in base substitutions (nucleotide instability, or NIN) or in deletions or insertions of a few nucleotides (micro-satellite instability, or MIN). In breast tumors however, MIN has been observed only in a small subset (<10%) of tumors (Ingvarsson 1999), and there is little

evidence of mutation hotspots to support a significant etiological role of NIN in this type of cancer.

Concluding remarks on karyotype and cytogenetic studies

Breast cancer progression is accompanied by an increase in the number of genetic alterations. However, no specific gross alteration has been clearly and repeatedly associated with a specific tumor stage. In contrast, qualitative and quantitative differences exist between ER-positive and ER-negative tumors, as well as between low- and high-grade lesions. The lower occurrence of 16q loss in grade III cancers, repeatedly observed, appears to challenge the conception of tumor progression from ER-positive to ER-negative status and from low to high grade.

The data presented in this section concern major (high-frequency) DNA changes. Additional studies have demonstrated that a high genetic divergence characterizes BCC *in vivo*. A number of gains or losses of chromosomal material occur in tumors at a low frequency and at many different sites. This is in agreement with the concept of micro-heterogeneity in breast cancer. Multiple karyotypically related as well as unrelated clones (i.e. no single chromosomal abnormality is shared by them) have been found in a high proportion of carcinomas, suggesting that genetic mechanisms are crucially involved in the generation of small cell-to-cell and clone-to-clone variation in tumors (Aubele *et al*. 1999, Teixeira *et al*. 2002). Thus, invasive breast cancer may be viewed as a disease with multiple cytogenetic sub-clones and since no specific DNA alteration has been associated with invasion (see for instance Buerger *et al*. 2000*b*), it is concluded that complex patterns of non-specific changes are acquired during tumor progression. Accumulation of these minor (low-frequency) alterations distributed along the genome could ultimately overcome the mechanisms preventing cell aggressiveness. It has been found that the number of genomic aberrations is higher in tumors that give rise to recurrences (Dellas *et al*. 2002). Moreover, analysis of distant metastases (brain) showed that they were characterized by an accumulation of various genetic alterations and increased LOH frequency at all loci examined (Hampl *et al*. 1998-1999). In another study, the total number of aberrations detected by CGH exclusively in the lymph nodes or distant metastases was higher than in the primary breast tumors (2.5 vs 0.7) (Nishizaki *et al*. 1997). Analysis of single disseminated tumor cells has also revealed a high genetic heterogeneity, irrespective of whether they resided within the same compartment or within different homing sites, or whether they were isolated on repeated bone-marrow aspirations (Klein *et al*. 2002).

Micro-array studies

During recent years, gene expression studies have gained considerably from the introduction of massive and simultaneous analysis tools. Micro-array-mediated studies have allowed measuring the level of up to thousands of mRNAs in tumors (Sgroi *et al*. 1999, Bertucci *et al*. 2000, 2002, Perou *et al*. 2000, Ross *et al*. 2000, Finlin *et al*. 2001, Gruvberger *et al*. 2001, Ross & Perou 2001, Sørlie *et al*. 2001, 2003, West *et al*. 2001, Zajchowski *et al*. 2001, Lacroix *et al*. 2002, van de Rijn *et al*. 2002, van't Veer *et al*. 2002, Ma *et al*. 2003, Sotiriou *et al*. 2003, Lacroix & Leclercq 2004*a*). The existence of a few major phenotypes in breast cancer has been confirmed by such investigations.

Cluster analyses of micro-array data from series of breast tumors have repeatedly led to the identification of a major 'luminal epithelial-like/ER-positive' subtype, comprising 60–65% of tumors. It was characterized by the high expression of a gene set including ESR1 (the ER-alpha itself) and genes either regulated by estrogens (*LIV-1, TFF1, TFF3*), or previously identified as co-expressed with ER (*GATA3*, for instance). Other genes correlated to *ESR1* expression were *BCL2*, *COX6C*, *CRABP2*, *ERBB3*, *FBP1*, *HNF3A*, *HPN*, *IGFBP2*, *IGFBP5*, *MYB*, *NAT1*, *SELENBP1*, *VAV3* and *XBP1* (Perou *et al*. 2000, Gruvberger *et al*. 2001, Ross & Perou 2001, Sørlie *et al*. 2001, West *et al*. 2001, van't Veer *et al*. 2002, Lacroix & Leclercq, 2004*b*; see also Table 3a). Besides the 'luminal epithelial-like/ER-positive' subtype, three subtypes characterized by low or no *ESR1* expression were found: a 'normal breast-like', grouping some tumors with samples of normal breast tissue; a 'basal/myoepithelial-like', comprising about 15–20% of tumors, and notably expressing high levels of keratins 5 (*KRT5*) and 17 (*KRT17*); an 'ERBB2+' group, characterized by the high level of expression of several genes in the *ERBB2* amplicon at 17q22.24 including *ERBB2*, *GRB7*, *MLN64* and others. Most tumors expressing a strong 'luminal epithelial-like/ER-positive' signature were of low grade, while the majority of tumors expressing mainly the other signatures were of high grade. Tumors expressing high levels of *KRT5* and *KRT17* (van de Rijn *et al*. 2002), or *ERBB2* were associated with poor clinical outcome.

Tumors expressing a basal/myoepithelial gene signature, based on micro-array studies, are expected to include the fraction of ductal carcinomas that are not pure myoepithelial cell carcinomas but that are of high grade, and for which a basaloid/myoepithelial cell differentiation and steroid receptor negativity has been demonstrated by IHC (see for instance Jones *et al*. 2001). Myoepithelial differentiation, high grade and ER negativity are also found in certain meta-plastic carcinomas (spindle-cell carcinomas and matrix-producing carcinomas), and in

Table 3a A list of genes directly correlated to *ESR1* expression in tumors, as determined by micro-array studies

Gene name	Gene product name(s)
AR	Androgen receptor
BCL2	B-cell CLL/lymphoma 2
CCND1	Cyclin D1
CLDN7	Claudin 7
COX6C	Cytochrome c oxidase subunit VIc
CRABP2	Cellular retinoic acid-binding protein 2
CUTL1	Cut-like 1, CCAAT displacement protein
ERBB3	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)
ESR1	Estrogen receptor-alpha
FBP1	Fructose-1,6-bisphosphatase 1
GATA3	GATA sequence-binding protein 3
HNF3A/FOXA1	Hepatocyte nuclear factor 3A/Forkhead box A1
HPN	Hepsin (transmembrane protease, serine 1)
IGF2	Insulin-like growth factor 2 (somatomedin A)
IGFBP2	Insulin-like growth factor-binding protein 2
IGFBP5	Insulin-like growth factor-binding protein 5
LIV-1/SLC39A6	Protein LIV-1/Solute carrier family 39 (metal ion transporter), member 6
MYB	V-myb myeloblastosis viral oncogene homolog (avian)
NAT1	*N*-acetyltransferase 1 (arylamine *N*-acetyl-transferase)
NPY1R	Neuropeptide Y receptor Y1
SELENBP1	Selenium-binding protein 1
SLC9A3R1	Solute carrier family 9, isoform 3 regulatory factor 1
STC2	Stanniocalcin 2
TFF1	Trefoil factor 1 (pS2, BCEI)
TFF3	Trefoil factor 3
TIMP3	Tissue inhibitor of metalloproteinase 3
VAV3	Vav 3 oncogene
XBP1	X-box-binding protein 1

Table 3b A list of genes inversely correlated to *ESR1* expression in tumors, as determined by micro-array studies

Gene name	Gene product name(s)
CDH3	Cadherin 3, placental cadherin (P-cadherin)
CX3C1	Chemokine (C-X3-C motif) receptor 1
EGFR	Epidermal growth factor receptor
FABP7	Fatty acid-binding protein 7, brain
GALNT3	UDP-*N*-acetyl-alpha-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 3 (GalNAc-T3)
GSTP1	Glutathione *S*-transferase pi
HMGIY	High-mobility group protein isoforms I and Y
KRT7	Keratin 7
LAD1	Ladinin 1
LCN2	Lipocalin 2 (oncogene 24p3)
S100A8	S100 calcium-binding protein A8 (calgranulin A)
S100A9	S100 calcium-binding protein A9 (calgranulin B)
SERPINB5	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 5
SLPI	Secretory leukocyte protease inhibitor (antileuko-proteinase)
SOD3	Superoxide dismutase 3, extracellular

invasive ductal carcinomas with large central acellular zones (Tsuda *et al*. 1999).

That ER status reflects major differences in tumor gene expression patterns and phenotypes was notably illustrated by Gruvberger *et al*. (2001) and West *et al*. (2001) who showed that the number of genes that discriminated tumors according to their ER status was high. Moreover, only a small proportion of these discriminator genes were known to be regulated by estrogens, suggesting that mechanisms underlying *ESR1* gene expression are, indeed, common to many genes. Detailed analysis of the transcription of such genes could be valuable in understanding the molecular mechanisms underlying *ESR1* expression, which remain largely unclear. Among the genes positively correlated to ER were those correlated to *ESR1* expression in the 'luminal epithelial-like/ER-positive' phenotype (see above), but also *AR, CCND1, CUTL1, IGF2, NPY1R, SLC9A3R1, STC2* and *TIMP3* (see Table 3a). Among those negatively correlated to ER were *CX3CL1, CDH3, EGFR, FABP7, GALNT3, GSTP1, HMGIY, KRT7, LAD1, LCN2, S100A8, S100A9, SERPINB5, SLPI* and *SOD3* (see also Table 3b).

According to micro-array studies, only a few distinct breast tumor classes seem to exist. This suggests that phenotype transition from one class (for instance the 'luminal epithelial-like/ER-positive') to another is unlikely to occur in the same tumor during progression. Tumor phenotypes seem to be defined very early in the development of the lesions. This is further supported by micro-array-mediated analysis of invasion.

Micro-array investigations have also aimed to define the genes, if any, contributing to the invasive phenotype of breast tumors. However, in a study of 36 ductal tumors, extensive similarities at the transcriptome level were found among the distinct stages of progression (atypical hyperplasia, carcinoma *in situ*, invasive carcinoma), supporting the hypothesis that alterations that confer on tumors their potential for invasive growth are already present in the pre-invasive stages. Contrasting with stage, different tumor grades were associated with distinct gene expression signatures, suggesting that tumor grade is unlikely to change significantly during progression. A few genes were found to have an increased expression both in high tumor grade and in the carcinoma *in situ*/invasive carcinoma transition. They included genes involved in the cell cycle, centrosomal function and DNA repair (Ma *et al*. 2003).

In another study, comparison of gene expression changes between cancer cells at the periphery and in the center of breast cancers was performed using a combination of micro-dissection and micro-array analysis

(Zhu *et al*. 2003). Of 1176 genes analyzed, only 22 changed their expression levels in the periphery relative to the central region: 15 were up-regulated (including *VIM* and *AHRC*, encoding the small GTPase RhoC) and 7 were down-regulated (including *TSG101*) (arbitrary threshold of 1.5-fold or greater). RhoC has already been found to have increased expression in more motile, invasive and metastatic tumors, and in the most lethal form of the locally advanced breast cancer, inflammatory breast cancer (Kleer *et al*. 2002). *VIM* up-regulation might indicate the initiation of epithelial–mesenchymal conversion at the periphery. *TSG101* has been previously proposed as a tumor suppressor gene, but it seems that its expression could rather be needed for activities associated with aspects of tumor progression (Wagner *et al*. 2003, Zhu *et al*. 2004). Whether the expression of these genes was altered under the influence of normal surrounding tissue is presently unknown.

While a few changes in gene expression and, possibly, phenotype at the invasive front of tumors are suggested by the previous example, various micro-array studies indicate that the ability of BCC to metastasize to distant sites could indeed be an early and inherent genetic property. For instance, a 70-gene expression signature was found to be a strong independent factor in predicting a short interval to distant metastases. Those breast cancer patients presenting with a good prognostic fingerprint had a 95% chance of surviving the next decade, whereas those with a bad fingerprint had only a 55% chance of surviving (van de Vijver *et al*. 2002, van't Veer *et al*. 2002).

Other investigators identified aggregate patterns of gene expression (called 'meta-genes') allowing the classification of breast tumors by their likelihood of having associated LN metastases at diagnosis and by 3-year recurrence risk (Huang *et al*. 2003).

Bone marrow (BM) is a common homing organ for metastatic BCCs. Micro-array analysis of 83 breast tumor samples showed distinct profiles between BM-positive ($n = 23$) and BM-negative ($n = 60$) lesions. Nine genes were up-regulated while 77 were down-regulated in BM-positive tumors. In the same study, the expression profile associated with lymphatic metastasis was also studied. Forty-four genes were found to distinguish between LN-positive and LN-negative lesions. Again, the number of up-regulated genes in LN-positive tumors was smaller ($n = 9$) than the number of down-regulated genes ($n = 35$), suggesting that transcriptional repression of genes is important for metastasis. Of interest, the gene signature associated with LN metastasis was distinct from the signature associated with BM micro-metastasis, with only nine genes in common, suggesting that the two routes of dissemination could be governed by different molecular determinants (Woelfle *et al*. 2003)

There are studies suggesting that metastatic signatures could be common to cancers of various origins. Thus, a 17-gene expression signature that distinguished primary from metastatic adenocarcinomas was found from lung, breast, prostate, colorectal, uterus and ovary cancers. It was applied to 279 primary solid tumors (lung, breast, prostate, lymphoma and medulloblastoma). Those tumors carrying the gene expression signature were most likely to be associated with metastasis and poor clinical outcome ($P < 0.03$) (Ramaswamy *et al*. 2003).

All this suggests that the clinical outcome of individuals with cancer can be predicted using the gene expression profiles of primary tumors at diagnosis. It is proposed that some tumors could be pre-ordained to spread, while some would have a favorable combination of initiating events making them less likely to disseminate. The existence of early-expressed metastatic signatures is a further argument against the widely accepted idea that metastatic potential is acquired relatively late during multi-step tumorigenesis. It supposes that not just a few rare cells in the tumor acquire metastatic ability, but that all cells within such tumors have this ability to metastasize. It must be mentioned, however, that the metastatic signatures found by different groups have only a few, if any, genes in common, raising some questions about their potential use as clinical tools.

Concluding remarks on micro-array studies

Based on their pattern of gene expression, it appears that breast tumors may be grouped in a limited number of distinct classes largely correlated to ER status and grade. A transition of tumors from one to another of these classes seems unlikely, considering the number of differences in gene expression that discriminate them. Moreover, no 'mixed class' has been observed. Micro-array data also suggest that a (very) few genes could be susceptible to allowing distinction between *in situ* and invasive breast tumors. Other genes, such as ERBB2, KRT5 and KRT17, seem to be associated with higher aggressiveness.

Epigenetic alterations

Epigenetic alterations are heritable modifications of gene expression that do not involve mutation. They include hypermethylation of CpG island-rich promoters, which contributes to the transcriptional inactivation of a number of tumor-related genes in many types of cancer (Malik & Brown 2000, Widschwendter & Jones 2002). A series of genes frequently hypermethylated in breast cancers are listed in Table 4. The heritability of methylation states and the secondary nature of the decision to attract or exclude methylation suggest that DNA methylation is adapted for the cellular memory

Table 4 A list of genes for which CpG island promoter hypermethylation has been demonstrated in tumors

Gene name	Gene product name(s)
APC	Adenomatosis polyposis coli
ARHI	Ras homolog gene family, member I
ASC	Apoptosis-associated speck-like protein containing a CARD
BRCA1	Breast cancer 1, early onset
CCND2	Cyclin D2
CDH1	Cadherin 1, epithelial cadherin (E-cadherin)
CDH13	Cadherin 13, H-cadherin (heart)
CDKN2A	Cyclin-dependent kinase inhibitor 2A (p16)
DAPK1	Death-associated protein kinase 1
ESR1	Estrogen receptor-alpha
FABP3	Fatty acid-binding protein 3 (MDGI)
FHIT	Fragile histidine triad gene
GJB2	Gap junction protein, beta 2, 26kD (connexin 26)
GPC3	Glypican 3
GSN	Gelsolin (amyloidosis, Finnish type)
GSTP1	Glutathione *S*-transferase pi
HIC1	Hypermethylated in cancer 1
HOXA5	Homeo box A5
HSHIN1	High in normal-1
IL6	Interleukin-6
KLK10	Kallikrein 10
MGMT	Methylguanine-DNA methyltransferase
NME1	Protein expressed in non-metastatic cells 1 (nm23A)
PGR	Progesterone receptor
PLAU	Plasminogen activator, urokinase
PRDM2(1)	PR domain-containing protein 2 (RIZ1), transcript 1
PRKCDBP	Protein kinase C, delta-binding protein (SRBC)
PRSS8	Protease, serine, 8 (prostasin)
RARB(2)	Retinoic acid receptor, beta (transcript 2)
RASSF1(A)	Ras association (RalGDS/AF-6) domain family 1 (transcript A)
SERPINB5	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 5
SFN	Stratifin
SLC19A1	Solute carrier family 19 (folate transporter), member 1
SNCG	Synuclein, gamma (breast cancer-specific protein 1)
SYK	Spleen tyrosine kinase
TFF1	Trefoil factor 1 (pS2, BCEI)
TIMP3	Tissue inhibitor of metalloproteinase 3
WT1	Wilms tumor 1

For references, see Widschwendter *et al.* 2002, Paz *et al.* 2003.

(Bird 2002). Hypermethylation could participate in the development and the preservation of specific cell phenotypes, by definitely 'bolting' specific sets of genes.

Few studies have examined potential correlations between promoter hypermethylation and tumor stage or grade. However, in a laser capture micro-dissection-assisted analysis of 16 specimens with intraductal and invasively growing breast cancer, promoter hypermethylation of *CDKN2A* (p16), *SFN* (stratifin), *RASSF1A* and *CCND2* (cyclin D2) was found to be largely conserved between both compartments. This suggests that in most cases the epigenetic inactivation takes place before invasive growth develops (Lehmann *et al*. 2002). A number of hypermethylated genes are associated with the distinctive phenotypes observed in tumors. For instance, *ESR1, PGR, CDH1, TFF1* etc. are associated with the ER-positive/low-grade phenotype. In contrast, *CDKN2A, GSTPI, PLAU* etc. are preferentially found in ER-negative/high-grade tumors. The fact that the expression of many genes for which promoter methylation has been shown is correlated to the ER status was demonstrated by Yan *et al*. (1999). These authors performed a methylation profile analysis of 7776 CpG islands, which led to the identification of CpG island clusters that can significantly distinguish ER−/PR− from ER+/PR+ breast tumors. Thus, epigenetic events might significantly contribute to stabilize the phenotype of tumors. As we have concluded that the BCC phenotype is unlikely to change significantly during progression, we conclude that hypermethylation is not expected to play a key role in this progression.

Conclusion on genetic/epigenetic studies

(Epi)genetic studies have not revealed major changes in the gross DNA alterations or in the gene expression patterns of breast tumors during progression. Tumor progression to invasiveness and metastasis probably results from the accumulation by *in situ* carcinoma of various minor and localized genetic or epigenetic events. This would eventually alter the molecular balances controlling cell adhesion, migratory ability, proteolysis and/or angiogenesis. Such evolution is suggested by the known micro-heterogeneity of tumor tissues.

Tumor size and progression

Pre-invasive cells express almost all of the features associated with a full-blown cancer phenotype: sustained cell proliferation, disregard of growth and differentiation controlling signals, evasion of apoptosis, immortalization and induction of angiogenesis. However, they (apparently) lack the ability to invade surrounding tissue. How is this property acquired?

Tumor size may play a role in the acquisition of invasiveness. In fact, and with the possible exception of BRCA1-associated tumors (Foulkes 2004), a relation has been repeatedly found between tumor size, on the one hand, and LN status and reduced survival, on the other hand (see for instance Carter *et al*. 1989, Hayes *et al*. 2002).

When growing, a tumor accumulates genetic alterations (see for instance Sato *et al*. 1991). This may allow the emergence of different cell sub-populations sharing essentially the same 'portrait', but exhibiting minor phenotype differences. One may speculate that a local complex co-operation between these sub-populations might favor invasion. A growing *in situ* tumor is also believed to exert a mechanical stress on its neighboring basement membrane. Moreover, BCC accumulation in a confined space might lead to local concentrations of various secreted molecules (for instance metalloproteinases (MMPs)) high enough to overcome the mechanical and molecular resistance expressed (for instance through secretion of MMP inhibitors) by the surrounding normal cells.

Interactions between tumor cells and their cellular environment

It is now widely accepted that tumor evolution is highly dependent on interactions (by direct contact or through paracrine signaling) between BCCs and other cell types present in their vicinity. BCCs modulate stromal cell activity. In turn, the stromal micro-environment profoundly influences many steps of tumor progression. In various experimental tumor models, the micro-environment affects the efficiency of tumor formation, the rate of tumor growth, the onset of angiogenesis, the extent of invasiveness and the ability of tumor cells to metastasize (Elenbaas & Weinberg 2001). Among the cell types with which BCCs may interact are normal breast epithelial cells, blood cells, vascular endothelial cells and, at metastatic sites, specialized cells from brain, lung, liver, bone, bone marrow etc. (Lacroix *et al*. 1996, 2000, Sierra *et al*. 1997, Siwek *et al*. 1997, Dano *et al*. 1999, Yoneda 2000, Moore 2001, Deugnier *et al*. 2002, Toillon *et al*. 2002*a,b*, Ben-Hur *et al*. 2002, Blot *et al*. 2003). Among others, myoepithelial cells and stromal fibroblasts are thought to be implicated in the first steps of invasion.

Myoepithelial cells have been seen as 'natural tumor suppressors' (Deugnier *et al*. 2002, Barsky 2003). Surrounding the mammary ducts, they deposit extra-cellular matrix components, express high amounts of several proteinase inhibitors and appear responsive for limiting invasive behavior. The loss of this cell type, observed only in invasive tumors, should permit subsequent invasion and tumor progression (Sternlicht *et al*. 1997, Xiao *et al*. 1999, Barsky 2003). However, the mechanisms by which BCCs may reduce the amount of myoepithelial cells in their neighborhood remain largely unknown.

BCCs also have paracrine interactions with their surrounding stromal fibroblasts. In tumors, these latter are often phenotypically different from normal fibroblasts. For example, they may express smooth muscle differentiation with increased motility into collagen gel 'myofibroblasts' (Wang & Tetu 2002). Myofibroblasts, which comprise a predominant stromal cell type in breast tumors, are often seen in close association with the myoepithelium surrounding carcinoma *in situ*. Under the influence of BCCs, stromal (myo)fibroblasts can increase their production of various components of the urokinase (uPA) system (Schnack Nielsen *et al*. 2002) and of MMPs (Heppner *et al*. 1996). Since BCCs themselves are able to produce proteolysis-related molecules (uPA, uPAR, PAI-1, MMPs, matriptase/ST14) (Oberst *et al*. 2001), this is could lead to a considerable local matrix degradation and cancer progression (Dano *et al*. 1999).

Lobular breast cancer

Lobular tumors represent a minority (5–10%) of all breast carcinomas, but their occurrence appears to have increased steadily and disproportionately in recent years, possibly in association with increased use of combined hormone replacement therapy (Li *et al*. 2003*a*, Verkooijen *et al*. 2003). Compared with the ductal type, the characteristics of lobular tumors and the mechanisms of their progression have been less investigated. While additional studies will be necessary to draw firm conclusions, current data on lobular cancer will be summarized here.

Lobular tumors are usually composed of small monomorphic round cells, without significant nuclear atypia or abundant cytoplasm. Cells are most often arranged in single files. When present, invasion typically occurs in a manner that does not destroy anatomic structures or excite a substantial connective tissue response. Cells infiltrate alone or in files. Targetoid arrangements around non-neoplastic ducts may be observed. Besides the usual type, several variants have been described (signet-ring, alveolar, solid and others). The most studied of these, the pleomorphic variant, is characterized by a marked nuclear enlargement and pleomorphism, and small nuclei. It has moderate to high nuclear grade, contrasting with the low grade found in the usual type (Weidner & Semple 1992, Frykberg 1999, Soslow *et al*. 2000). In addition, lobular cancer may occasionally be observed in association with low-grade ductal cancer.

According to a series of data on proliferation, biology and genetics, lobular tumors in general appear to exhibit many similarities with low-grade tumors, but few with high-grade ductal tumors. Among lobular tumors, the usual type expresses a more well-differentiated and a less-proliferative phenotype than the pleomorphic type.

For instance, proliferation and apoptotic indexes were shown to be higher in ductal (in general) than in lobular cancers. These indexes were higher in pleomorphic than in

the usual lobular tumors (Kruger *et al*. 1999, Frolik *et al*. 2001, Arpino *et al*. 2004).

As also frequently observed in the low-grade ductal type: (1) the great majority of *in situ* and invasive lobular tumors express significant levels of ER, PR, BCL2, TFF1 and TFF3; (2) they are rarely ERBB2- and P53-positive, and their vimentin, VEGF and EGFR levels are low or null (Domagala *et al*. 1990, Poulsom *et al*. 1997, Lee *et al*. 1998, Frolik *et al*. 2001, Rosenthal *et al*. 2002, Arpino *et al*. 2004). The most noticeable feature distinguishing lobular and (low-grade) ductal tumors is the absence of E-cadherin expression in the former (Berx *et al*. 1996, Vos *et al*. 1997, Lehr *et al*. 2000, Goldstein *et al*. 2001, Wahed *et al*. 2002). In two independent gene expression studies comparing ductal and lobular carcinomas, the sole common discriminator identified was *CDH1*, which was significantly down-regulated in lobular samples (Korkola *et al*. 2003, Zhao *et al*. 2004). While marker expression is essentially similar in the usual lobular cancer and its variants (Soslow *et al*. 2000), the pleomorphic type has been found to have less ER and PR, and more P53 positivity than the usual type (Radhi 2000).

Using CGH, a higher number of genetic alterations has been observed in invasive, as compared with *in situ* lobular tumors. On the other hand, a frequent concomitant 1q gain and 16q loss appears to occur in both ductal and (*in situ* and invasive) lobular carcinomas. This has been associated with ER and PR presence and low proliferation (Etzell *et al*. 2001, Rennstam *et al*. 2003, Farabegoli *et al*. 2004). In lobular cancers, the most frequent losses are found at 16q followed by 17p, as also observed by LOH studies in well-differentiated ductal cancers (see above and in Shen *et al*. 2000).

Most studies, including many of those cited above, have indicated that when invasive and *in situ* components are present in lobular tumors, both are of the same type (usual, pleomorphic etc.) (see also Sneige *et al*. 2002) and are very similar, based on their expression of biological markers and their pattern of genetic alterations. For instance, a frequent hypermethylation of the five cancer-related genes *RASSF1A, HIN1, RARB, CCND2* and *TWIST*, has been observed in *in situ* as well as in invasive lobular cancers (Fackler *et al*. 2003).

A major difference between lobular and ductal tumors is their associated pattern of metastasis. Lobular carcinomas frequently metastasize to ovary, gastrointestinal tract, peritoneum and bone marrow; they less frequently colonize the lungs and the central nervous system (Borst & Ingold 1993, Arpino *et al*. 2004, Ferlicot *et al*. 2004). This difference could be essentially due to the lack of *CDH1* expression in lobular cancer, as compared with the ductal type (see above). In the absence of this adhesion molecule, the permeation of cancer cells through tissues could be facilitated (Goldstein 2002). It has been shown that *CDH1* expression may favor the appearance of intralymphatic tumor emboli, which are rarely observed in lobular cancer (Gupta *et al*. 2003). In addition to the specific metastatic pattern, the absence of CDH1 could also explain most of the peculiar cytological aspects of lobular cancer cells.

In summary, numerous features of lobular tumors, especially when they are of the usual type, are also observed in low-grade ductal tumors. No major difference seems to be associated with the progression of this type of cancer.

The origin of tumors — breast cancer stem cells

Breast tumors may exhibit different 'portraits' that are essentially maintained during progression from *in situ* to metastasis. On the other hand, it is now widely admitted that most tumors are clonal and represent the progeny of a single cell. Therefore, it may be asked whether the distinct tumor phenotypes are already present in the original tumor-initiating cells, or if phenotype divergence occurs at a later stage of tumor evolution from a common precursor.

Various studies on normal mammary tissue have identified a population of supra-basal cells that are able to generate both themselves and differentiated luminal epithelial and myoepithelial cells. These stem cells have a long life and a large replicating potential, making them good candidates for the cells of origin of cancer (Stingl *et al*. 2001, Boecker & Buerger 2003, Clarke *et al*. 2003, Dontu *et al*. 2003, Petersen *et al*. 2003, Smalley & Ashworth 2003).

Observations have long suggested that not all transformed cells composing a breast tumor are able to regenerate the tumor upon transplantation. The existence of 'breast cancer stem cells' (BCSCs) has recently gained more credibility through an elegant series of experiments by Al-Hajj *et al*. (2003). These authors identified in several tumors a sub-population of cluster of differentiation (CD)44+ CD24−/low epithelial-specific antigen (ESA)+ cells, of which as few as 200/1000 were consistently able to form tumors in mice. In contrast, 20 000 cells from other sub-populations were unable to do so. CD44 is a marker of basal/myoepithelial cells. CD24 and ESA are found in luminal epithelial cells. Thus, BCSCs express some (but not all) markers representative of both phenotypes, and it is conceivable that the progeny of these cells could evolve, probably rapidly, towards only one of these phenotypes. The final phenotype could result from specific events. For instance, tumors induced in transgenic mice by components of the WNT1 signaling pathway (wnt-1, beta-catenin, c-myc) were found to contain both luminal epithelial and

myoepithelial tumor cells; in contrast, no myoepithelial tumor cells were observed in tumors induced by ERBB2 (Rosner *et al*. 2002, Li *et al*. 2003*b*). Most BRCA1-associated breast tumors have a basal-like 'portrait'. To explain this, it has been hypothesized that an intact BRCA1 is needed to allow breast cells to acquire a luminal epithelial phenotype (Foulkes *et al*. 2003). Cells in which BRCA1 is truncated would be committed to a basal-like lineage.

The existence of 'monophenotypic' self-renewing BCSCs from which distinct tumor 'portraits' could be obtained may have important therapeutic implications. For instance, ER-positive tumors could indeed be composed of a bulk of ER-expressing cells associated with a very few BCSCs. In such a case, anti-estrogen-based therapy would be efficient only on ER-positive cells, allowing BCSCs to later reconstitute the tumor. New therapeutic targets should be identified that could be exploited to eliminate BCSCs from patients.

On the other hand, the increasing evidence that tumors maintain most of their 'portrait' during progression should also have consequences on their treatment. Different phenotypes mean different expression patterns of various drug targets, or modulators of drug action: components of estrogen metabolic pathways, proteinases, transporter proteins etc. The efficacy of strategies developed against such molecules is expected to be largely predictible from the most precise determination of the tumor phenotype.

General conclusions

Many attempts have been and are still being made to identify critical events responsible for the development and progression of breast cancer. In spite of this, the mechanisms underlying notably tumor invasion and BCC dissemination remain largely unclear. One of the current progression models for ductal tumors proposes that carcinoma *in situ* may evolve into invasive ductal carcinoma and subsequently produce metastases through an accumulation of molecular abnormalities possibly allowing extensive phenotype changes and gain of aggressiveness. To describe this progression, the 'clonal hypothesis' has generally been well received in the breast cancer community.

However, the data presented here indicate that most breast carcinomas cannot be viewed as a collection of a few successive clonal populations being associated with the major stages of progression. Rather unexpectedly, *in situ* and invasive components of carcinomas appear very similar, and this similarity has also been repeatedly observed in metastases, regardless of their localization, and in recurrences. In fact, at any step of their progression, breast tumors may be rather considered as collections of cell sub-populations exhibiting the same general pattern of gross recurrent genetic alterations and sharing the same major phenotypic features. Regarding phenotype, a few major 'portraits' may be identified. Tumors characterized by expression of ER and a series of correlated markers are generally associated with low proliferation/apoptosis indexes and a low grade (well-differentiated, luminal epithelial phenotype). Tumors characterized by the lack (or very low amounts) of ER are generally associated with high proliferation/apoptosis indexes and a basal-like, poorly differentiated aspect.

Although the tumor phenotype remains essentially stable, genetic alterations accumulate during progression. Micro-heterogeneity exists, due to minor (low-frequency) DNA changes, generally restricted to small sub-populations of BCCs. This could result in minor phenotype differences. Invasion could proceed from a local complex co-operation between different sub-populations. Moreover, dialogue between tumor cells and their surrounding normal cells could also play an important role in the establishment of biological conditions propitious to cell dissemination.

There are also data, obtained notably from micro-array studies, suggesting that breast tumors do not really progress, as they could possess very early the ability to invade and metastasize. According to such a view, the distinction between *in situ* and invasive carcinoma would not reflect a significant difference in the properties of BCCs. To reach definite conclusions on the most pertinent model, future investigations should exploit the most recent analysis techniques (including micro-dissection) to examine genotype and phenotype of individual BCCs close to the invasion front and in invaded tissues.

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Proteomics Demonstration That Normal Breast Epithelial Cells Can Induce Apoptosis of Breast Cancer Cells through Insulin-like Growth Factor-binding Protein-3 and Maspin*

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Normal breast epithelial cells are known to exert an apoptotic effect on breast cancer cells, resulting in a potential paracrine inhibition of breast tumor development. In this study we purified and characterized the apoptosis-inducing factors secreted by normal breast epithelial cells. Conditioned medium was concentrated by ultrafiltration and separated on reverse phase Sep-Pak C_{18} and HPLC. The proapoptotic activity of eluted fractions was tested on MCF-7 breast cancer cells, and nano-LC-nano-ESI-MS/MS allowed the identification of insulin-like growth factor-binding protein-3 (IGFBP-3) and maspin as the proapoptotic factors produced by normal breast epithelial cells. Western blot analysis of conditioned media confirmed the specific secretion of IGFBP-3 and maspin by normal cells but not by breast cancer cells. Immunodepletion of IGFBP-3 and maspin completely abolished the normal cell-induced apoptosis of cancer cells, and recombinant proteins reproduced the effect of normal cell-conditioned medium on apoptosis of breast cancer cells. Together our results indicated that normal breast epithelial cells can induce apoptosis of breast cancer cells through IGFBP-3 and maspin. These findings provide a molecular hypothesis for the long observed inhibitory effect of normal surrounding cells on breast cancer development. ***Molecular & Cellular Proteomics 6: 1239–1247, 2007.***

Breast cancer is the leading cause of cancer-related deaths in women of the western world, and despite significant improvements in cancer diagnosis and treatment, more than two-thirds of the patients still succumb to the disease (1). However, this pathology progresses slowly, and it has been estimated that the development of a clinically detectable tumor from one tumor cell may require 6–8 years. The normal human breast gland comprises a branching ductal-lobular system lined by an inner layer of luminal epithelial cells and an outer layer of myoepithelial cells separated from the interstitial stroma by an intact basement membrane. The luminal epithelial cells are polarized glandular cells with specialized apical and basolateral membrane domains expressing sialomucin and cell-cell adhesion molecules, respectively (2). The myoepithelial cells contribute significantly to the formation of basement membrane, and their myogenic differentiation is responsible for the contractile function. Breast cancer development involves defined clinical and pathological stages starting with atypical epithelial hyperplasia, progressing to *in situ* then invasive carcinomas, and culminating in metastatic disease (3). In *in situ* breast carcinomas, luminal epithelial cells lose their ability to maintain a single epithelial layer. At the same time, the number of myoepithelial cells decreases, and the number of stromal fibroblasts, lymphocytes, and endothelial cells increases. In invasive carcinoma, myoepithelial cells and the basement membrane are absent, and tumor cells are dispersed into the stroma.

It is now widely documented that tumor evolution is highly dependent on interactions between tumor cells and neighboring normal cells (4, 5). The paracrine interactions between neoplastic cells and adjacent normal cells may determine whether transformed cells undergo apoptosis, remain in a quiescent state, or advance to tumorigenesis. In various experimental tumor models, the microenvironment affects the efficiency of tumor formation, the rate of tumor growth, and the extent of invasiveness (6). Although fibroblasts and endothelial cells have been shown to favor tumor development, normal breast myoepithelial and epithelial cells are reported to have antitumor effects both *in vitro* and *in vivo* (7–13). We have demonstrated that normal breast epithelial

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cells (NBECs)[1] inhibit growth of cancer cells by inducing apoptosis (14, 15). The induction of apoptosis is mediated through a Fas-mediated pathway because conditioned medium from NBECs increases membrane-associated Fas and Fas ligand. More importantly both Fas-neutralizing antibody and dominant negative Fas completely abolish NBEC-induced apoptosis. The mechanisms of the apoptosis-inducing effect of NBECs on breast cancer cells, from the standpoint of both effector molecules and signal transduction, hold promise for the understanding of the natural paracrine tumor suppression as well as for cancer prevention. In the present study, we purified and characterized the apoptosis-inducing factors secreted by NBECs. Mass spectrometry analysis together with immunodepletion assay showed insulin-like growth factor-binding protein-3 (IGFBP-3) and maspin to be the major apoptosis-inducing factors produced by NBECs.

EXPERIMENTAL PROCEDURES

Chemicals—All cell culture reagents were obtained from BioWhittaker except insulin, which was obtained from Organon. Chemicals and anti-β-actin antibody were purchased from Sigma unless otherwise stated. Recombinant IGFBP-3 and anti-IGFBP-3 antibodies for Western blot and for immunodepletion assay were obtained from R&D Systems. Anti-maspin antibodies were produced by Biomerieux. Trypsin and soybean trypsin inhibitor were purchased from Roche Applied Science. GST-maspin was produced in *Escherichia coli* as described previously (16).

Cell Culture and Preparation of Conditioned Medium—NBEC cultures were established as described previously (11) from mammoplasty material (18–30-year-old women) obtained from the Department of Plastic Surgery (Prof. Pellerin) at the Medical University of Lille (Lille, France) in accordance with rules and regulations concerning ethical issues in France. In this study, NBECs from three primary cultures were used. Cells were cultured in DMEM/F-12 medium (1:1) containing 5% FCS, 10 μg/ml insulin, 5 μg/ml cortisol, 2 ng/ml EGF, 100 ng/ml cholera toxin, 100 IU/ml streptomycin, 100 μg/ml penicillin, and 45 μg/ml gentamicin. MCF-7, MDA-MB-231 and T-47D breast cancer cell lines were grown in Eagle's minimal essential medium supplemented with 10% FCS, 5 μg/ml insulin, 100 IU/ml streptomycin, 100 μg/ml penicillin, and 45 μg/ml gentamicin. For all experiments, cells were cultured in basal DMEM/F-12 medium without serum.

For preparation of conditioned medium, cells were plated in 75-cm^2 flasks (Nunc). When they reached preconfluence, they were washed two times with PBS and incubated in basal DMEM/F-12 medium (without serum, insulin, cortisol, EGF, or cholera toxin). Two hours later, the basal DMEM/F-12 medium was changed, and cells were further cultured for 24 h. The medium was then centrifuged at 200 $\times$ *g* for 10 min at 4 °C to remove cell debris and stored at −80 °C prior to use.

For immunodepletion of IGFBP-3 and maspin, conditioned medium was incubated with mouse anti-IGFBP-3 and/or anti-maspin antibodies overnight at 4 °C before incubation with protein A-agarose for 2 h on a roller system at 4 °C. Control was performed by incubating conditioned medium with an equal amount of irrelevant mouse immunoglobulin G. After centrifugation (10,000 $\times$ *g* for 10 min at 4 °C), the supernatants were used to determine apoptosis induction.

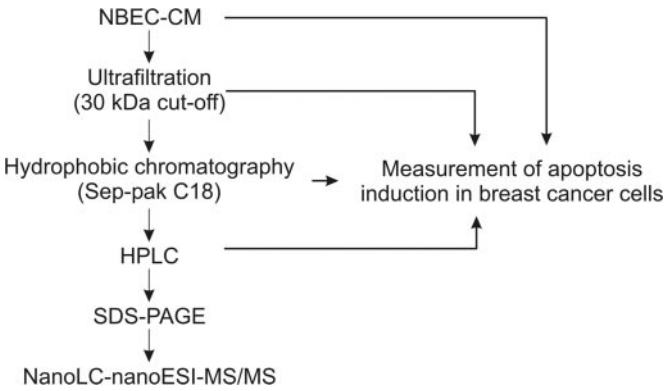

FIG. 1. **Protocol for purification and identification of apoptosis-inducing factors.**

Determination of Apoptotic Cells—Cells were seeded in 35-mm dishes (Nunc). After treatment with NBEC-conditioned medium (NBEC-CM), cells were fixed in cold methanol (−20 °C) for 10 min and washed twice in PBS before staining with 1 μg/ml Hoechst 33258 for 30 min at room temperature in the dark. Cells were then washed with PBS and mounted with coverslips using Glycergel (Dako). Apoptotic cells exhibiting condensed and fragmented nuclei were counted under an Olympus-BH2 fluorescence microscope as described previously (15). At least 500–1,000 cells in randomly selected fields were examined.

Purification Scheme of Apoptosis-inducing Factors—Apoptosis-inducing factors were purified following the protocol described in Fig. 1. To determine the approximate molecular size of apoptosis-inducing factors, size fractionation of NBEC-CM was performed by centrifugation in Centriplus tubes (Millipore) fitted with molecular sieve filters according to the manufacturer's instructions. For further purification, NBEC-CM (10 liters) was concentrated about 1,000 times using an Ultrasette type of ultrafiltration device (Filtron, Pall Gelman Science) (30-kDa cutoff). The concentrate was then loaded onto a reverse phase Sep-Pak C_{18} column and eluted with 50, 80, and 100% acetonitrile. The eluted fractions were freeze-dried and resuspended in DMSO before being loaded onto an HPLC column (Sephasil C_4, 250 $\times$ 10 mm, 5 μm; Vydac). Each eluted fraction was tested for its apoptosis-inducing activity in MCF-7 breast cancer cells as described above.

Protein Identification by Mass Spectrometry—The apoptosis-inducing fractions from HPLC were subjected to 12% SDS-PAGE followed by colloidal Coomassie Blue staining and trypsin digestion. The protein bands of interest were trypsin-digested and analyzed as described previously (17). Nano-LC-nano-ESI-MS/MS analysis of the trypsin digests was performed on an ion trap mass spectrometer (LCQ Deca XP$^+$, Thermo Electron) equipped with a nanoelectrospray ion source coupled with a nano-high pressure liquid chromatography system (LC Packings Dionex). Tryptic digests were resuspended in 10 μl of 0.1% HCOOH, and 1 μl was injected into the mass spectrometer using a Famos autosampler (LC Packings Dionex). The samples were first desalted and then concentrated on a reserve phase precolumn of 5 mm $\times$ 0.3-mm inner diameter (Dionex) by solvent A (H_2O/acetonitrile, 0.1% HCOOH (95:5)) delivered by the Switchos pumping device (LC Packings Dionex) at a flow rate of 10 μl/min for 3 min. Peptides were separated on a 15 cm $\times$ 75-μm-inner diameter C_{18} PepMap column (Dionex). The flow rate was set at 200 nl/min. Peptides were

[1] The abbreviations used are: NBEC, normal breast epithelial cell; Bax, Bcl-2-associated x protein; Bcl-2, B-cell/lymphoma 2; DMEM, Dulbecco's modified minimum essential medium; EGF, epidermal growth factor; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; NBEC-CM, NBEC-conditioned medium; GST-maspin, recombinant GST-maspin fusion protein; uPA, urokinase plasminogen activator.

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eluted using a 5–100% linear gradient of solvent B (H_2O/acetonitrile, 0.08% HCOOH (20:80)) in 45 min. Coated nanoelectrospray needles were obtained from New Objective (Woburn, MA). Spray voltage was set at 1.5 kV, and capillary temperature was set at 170 °C. The mass spectrometer was operated in positive ion mode. Data acquisition was performed in a data-dependent mode consisting of, alternatively in a single run, full-scan MS over the range *m/z* 500–2,000 and full MS/MS of the ion selected in an exclusion dynamic mode (the most intense ion is selected and excluded for further selection for a duration of 3 min). MS/MS data were acquired using a 2 *m/z* unit ion isolation window and a 35% relative collision energy. MS/MS raw data files were transformed to dta files with Bioworks 3.1 software (Thermo Electron). MS/MS spectra indicate primarily fragment ions originating from either the C terminus (y ion series) or N terminus (b ion series) of a peptide. Neutral mass of the precursor and sequence information were used to identify proteins in the Swiss-Prot database through MASCOT public interface using a mass tolerance of 0.8 Da for precursor, trypsin as the digestion enzyme, two possible missed cleavages, and oxidized methionine as a variable modification. Results were scored using probability-based Mowse score (protein score is $-10 \times \log(p)$ where p is the probability that the observed match is a random event. Scores greater than 42 are significant ($p < 0.05$). To ascertain unambiguous identification, searches were performed in parallel with Phenyx software using the same parameters.

Western Ligand Blotting and Western Blotting—Conditioned media (2 ml) of NBECs or MCF-7 cells were loaded onto a G-25 Sephadex gel filtration column. Fractions containing IGFBPs were eluted by 0.03 M ammonium acetate and lyophilized as described previously (18). Proteins from lyophilized samples were size-fractionated by 12.5% polyacrylamide gel electrophoresis under non-reducing conditions and electroblotted onto nitrocellulose membranes. The membranes were then incubated overnight in the presence of ^{125}I-labeled IGF-1 and ^{125}I-labeled IGF-2 (2×10^5 cpm for each ligand) (Amersham Biosciences) followed by washing with Tris-buffered saline (pH 7.2) and exposed to Eastman Kodak Co. X-Omat film for at least 48 h. For Western blotting, concentrated media and cell lysates were loaded onto a 10% SDS-polyacrylamide gel and then electrotransferred to nitrocellulose membrane (Hybond-C extra, Amersham Biosciences). After transfer, the blots were blocked with 3% BSA in TBS-T (20 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20) at room temperature and then incubated with anti-IGFBP-3 or anti-maspin antibodies (overnight at 4 °C). The detection was performed using a horseradish peroxidase-conjugated secondary antibody (1.5 h at room temperature) and the ECL detection system (Amersham Biosciences).

Statistical Analysis—Statistical significance was measured by Student's paired *t* test. The value of *p* for each data set is shown in the figures.

RESULTS

NBEC-conditioned Medium Induced Apoptosis of Breast Cancer Cells—MCF-7 breast cancer cells were cultured in serum-free medium in the presence of different dilutions of NBEC-conditioned medium. Apoptosis was determined following Hoechst staining (Fig. 2*A*). As shown in Fig. 2*B*, NBEC-conditioned medium induced apoptosis in a dose-dependent manner with a significant increase in apoptosis at a 1:40 dilution of NBEC-conditioned medium and a 4.5-fold increase in apoptosis at a 1:2 dilution. Similar results in apoptosis induction were obtained using a terminal deoxynucleotidyltransferase biotin-dUTP nick end labeling reaction (data not shown). Conditioned medium was then treated with heat and trypsin to determine whether the apoptosis-inducing factors were proteins. As shown in Fig. 2*C*, heating or trypsin treatment totally suppressed the apoptosis-inducing effect of NBEC-conditioned medium. This indicated that the apoptosis-inducing factors were temperature-sensitive proteins. We then evaluated the approximate molecular masses using a Centricon ultrafiltration system. Conditioned medium was concentrated with membrane filters of different cutoffs (10, 30, 50, and 100 kDa), and the apoptotic activity was determined in non-retained fractions. As shown in Fig. 2*C*, the non-retained fractions from 10- and 30-kDa-cutoff filters could not induce apoptosis, indicating that the molecular masses of apoptosis-inducing factors were greater than 30 kDa. In contrast, the total apoptosis-inducing activity was found in the non-retained fractions when filters of 50- and 100-kDa cutoff were used, indicating that the molecular masses of apoptosis-inducing factors were less than 50 kDa. Therefore the approximate molecular masses of apoptosis-inducing factors were estimated at between 30 and 50 kDa.

Purification of Apoptosis-inducing Factors—NBEC-conditioned medium was sequentially processed as described in Fig. 1. For each step, apoptosis induction of MCF-7 cells was determined. Conditioned medium (10 liters) was concentrated (1000-fold) and then subjected to a reverse phase Sep-Pak C_{18} column and eluted at 50, 80, and 100% acetonitrile. Apoptosis-inducing fractions were subsequently subjected to HPLC. The active fractions a and b were identified from HPLC (Fig. 3*A*). Fraction a was eluted at about 43% acetonitrile. After freeze-drying and dilution to a final concentration equivalent to 50% non-concentrated conditioned medium, this fraction induced about 28% of cancer cells into apoptosis. Fraction b was eluted at about 68% acetonitrile and induced about 17% of cells into apoptosis when cells were treated with a final concentration equivalent to 50% non-concentrated conditioned medium.

Identification of Apoptosis-inducing Factors by Mass Spectrometry—The two apoptosis-inducing fractions eluted by analytical HPLC were subjected to 12% SDS-PAGE followed by colloidal Coomassie Blue staining (Fig. 3*B*). Fraction a was revealed as an apparent double band at about 35–40 kDa. Fraction b was revealed as a single band at 42 kDa. These two individual protein bands were excised from a colloidal Coomassie Blue-stained gel and digested by trypsin, and the resulting peptides were processed for analysis by nano-LC-nano-ESI-MS/MS. The MS/MS spectra and the database search results are presented in Fig. 4. Two peptides were sequenced for each band. The 35-kDa band was identified as IGFBP-3 (score, 156; sequence coverage, 10.6%), and the 42-kDa band corresponded to maspin (score, 300; sequence coverage, 7.8%).

Western Blot Analysis of IGFBP-3 and Maspin—The presence of IGFBP-3 in conditioned media from normal and cancer cells was first verified by Western ligand blot (Fig. 5*A*). In NBEC-conditioned medium, three species of IGFBPs were visualized (IGFBP-2, IGFBP-3, and IGFBP-4) with IGFBP-3

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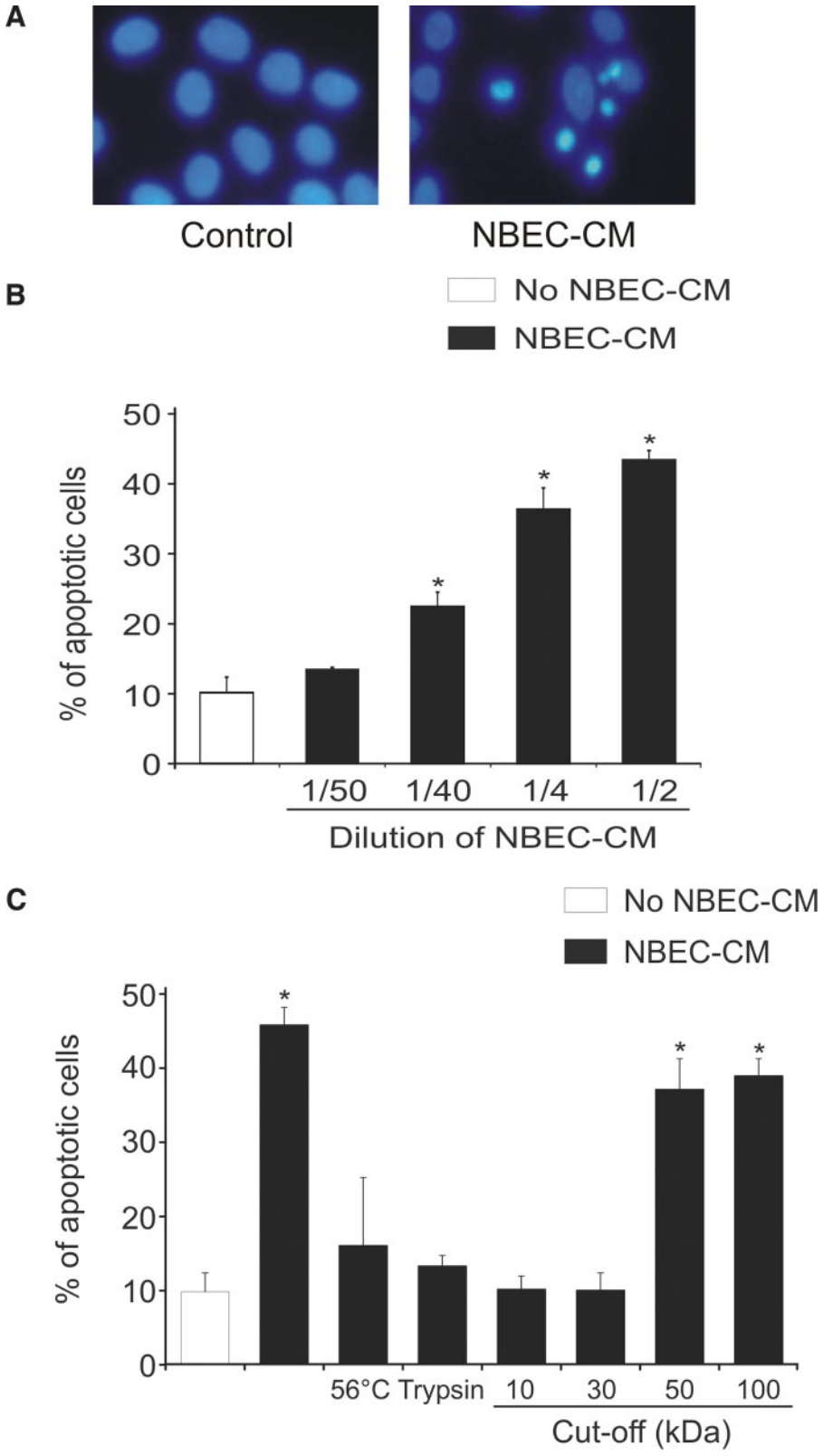

FIG. 2. **NBEC-CM induced apoptosis in MCF-7 breast cancer cells.** Cells were cultured in basal DMEM/F-12 medium for 24 h in the presence or absence of various dilutions of NBEC-CM. *A*, apoptosis detection after Hoechst staining. Apoptotic nuclei are condensed or fragmented. *B*, percentage of apoptotic cells. Data are the mean of three independent experiments. *C*, characterization of apoptosis-inducing activity in NBEC-CM. NBEC-CM was heated at 56 °C for 30 min or incubated with 2.5 μg/ml trypsin at 37 °C for 30 min. Molecular size fractionation of NBEC-CM was performed using 10-, 30-, 50-, and 100-kDa-cutoff sieve filters, and the non-retained fractions were used to determine apoptosis induction in MCF-7 cells as described. Apoptosis experiments were performed with a 1:2 dilution of conditioned medium. *Bars* show S.D. *, $p < 0.01$.

being the most abundant. In medium conditioned by MCF-7 cells, only IGFBP-2 and IGFBP-4 were detected.

The presence of IGFBP-3 and maspin in cell lysates and conditioned media from NBECs and MCF-7 cells was also verified by immunoblot analysis. As shown in Fig. 5, *B* and *C*, both IGFBP-3 and maspin were produced and secreted by

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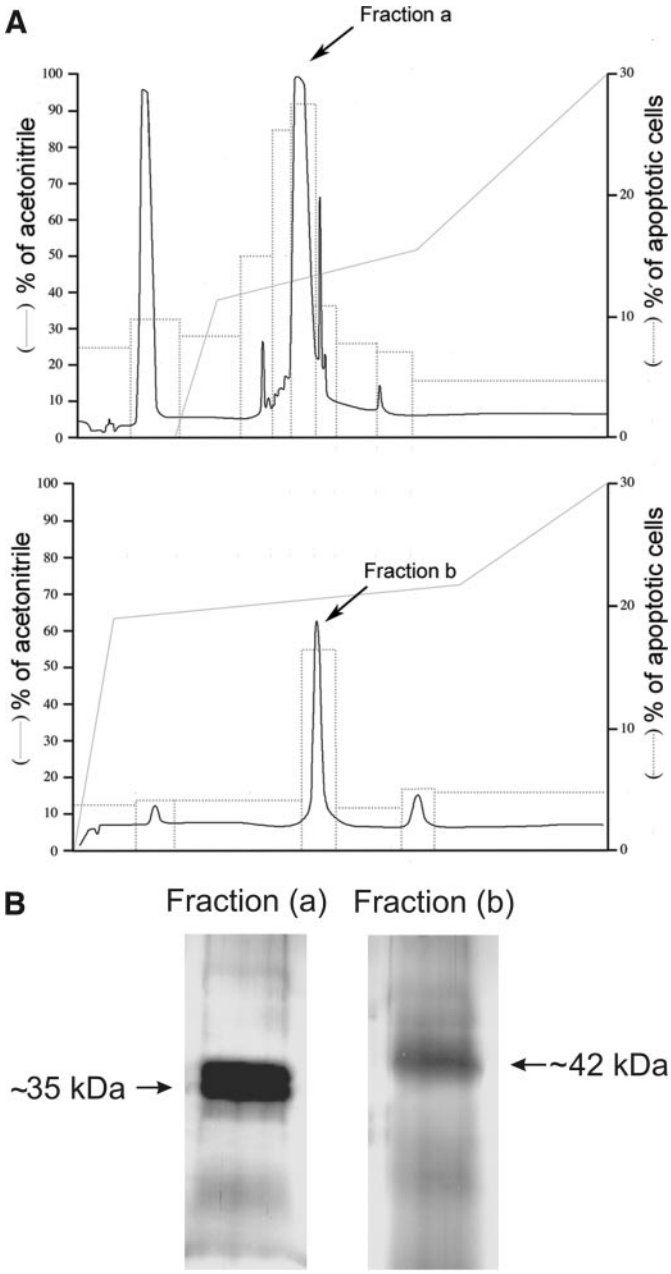

FIG. 3. **Elution profiles of HPLC and SDS-PAGE separation of active fractions.** *A*, HPLC elution profile reveals two active fractions, a and b. HPLC columns were eluted with a linear gradient of 0.1% TFA in acetonitrile. The flow rate was maintained at 1 ml/min, and the absorbance was monitored at 280 nm. Apoptotic activity of collected fractions was determined on MCF-7 cells after Hoechst staining. *B*, SDS-PAGE analysis of HPLC fractions. The active fractions a and b from HPLC were subjected to SDS-PAGE followed by colloidal Coomassie Blue staining.

NBECs. In contrast, neither IGFBP-3 nor maspin was detected in MCF-7 cell lysate and conditioned medium. Altogether these results suggested that IGFBP-3 and maspin were specifically secreted by NBECs but not by MCF-7 breast cancer cells.

Immunodepletion of IGFBP-3 and Maspin Abolished NBEC-CM-induced Apoptosis—To determine the extent to which secreted IGFBP-3 and maspin contributed to apoptosis induction, we immunodepleted IGFBP-3 and/or maspin by incubating NBEC-CM with anti-IGFBP-3 and/or anti-maspin antibodies. As shown in Fig. 6*A*, immunodepletion of NBEC-CM with the anti-IGFBP-3 antibody or the anti-maspin antibody diminished apoptosis induction in MCF-7 cells. Interestingly immunodepletion of both IGFBP-3 and maspin totally abolished apoptosis induction. This confirmed that IGFBP-3 and maspin secreted by NBECs did induce apoptosis of MCF-7 breast cancer cells.

IGFBP-3 and Maspin Synergistically Induced Apoptosis of Breast Cancer Cells—It has been described that both endogenous overexpressed maspin and IGFBP-3 can induce apoptosis or potentiate apoptosis induction by other agents (16, 19–22). To provide further evidence that extracellular IGFBP-3 and maspin could also induce apoptosis in breast cancer cells, recombinant proteins were used. As shown in Fig. 6*B*, IGFBP-3 and maspin alone significantly induced apoptosis. Interestingly the induction of apoptosis was further increased by cotreatment with IGFBP-3 and maspin.

DISCUSSION

Accumulating evidence suggests that dynamic cell-cell interaction may be as great a determinant of the behavior of a tumor cell as the specific oncogenetic or tumor suppressor alterations occurring within the malignant cells themselves (1–5). Normal breast myoepithelial and epithelial cells have been demonstrated to exert an inhibitory effect on breast cancer cells. We have reported previously that conditioned medium of normal breast epithelial cells strongly induces apoptosis of breast cancer cells (14, 15), but so far the apoptosis-inducing factors have yet to be identified. Here we used a proteomics-based approach to purify and identify apoptosis-inducing factors from normal breast epithelial cells. Proteomics offers the possibility of identifying proteins at very low concentrations, and this is of considerable interest in characterizing paracrine regulators as well as therapeutic targets in various pathologies such as breast cancers (23, 24). The use of sequential chromatography and mass spectrometry as well as immunodepletion assay has allowed us to identify IGFBP-3 and maspin as the two apoptogens secreted by normal breast epithelial cells.

IGFBP-3 is the most abundant of the circulating IGFBPs that bind IGFs with high affinity. IGFBP-3 inhibits cell proliferation and induces apoptosis by its ability to bind IGFs as well as through its IGF-independent effects. Hence IGFBP-3 can induce apoptosis by modulating the expression of Bcl-2 proteins in human breast cancer cells (25). More recently, Lee *et al.* (22) have demonstrated that in response to IGFBP-3 the retinoid X receptor-α-binding partner nuclear receptor Nur77 rapidly undergoes translocation from the nucleus to the mitochondria, resulting in rapid caspase activation. This nuclear

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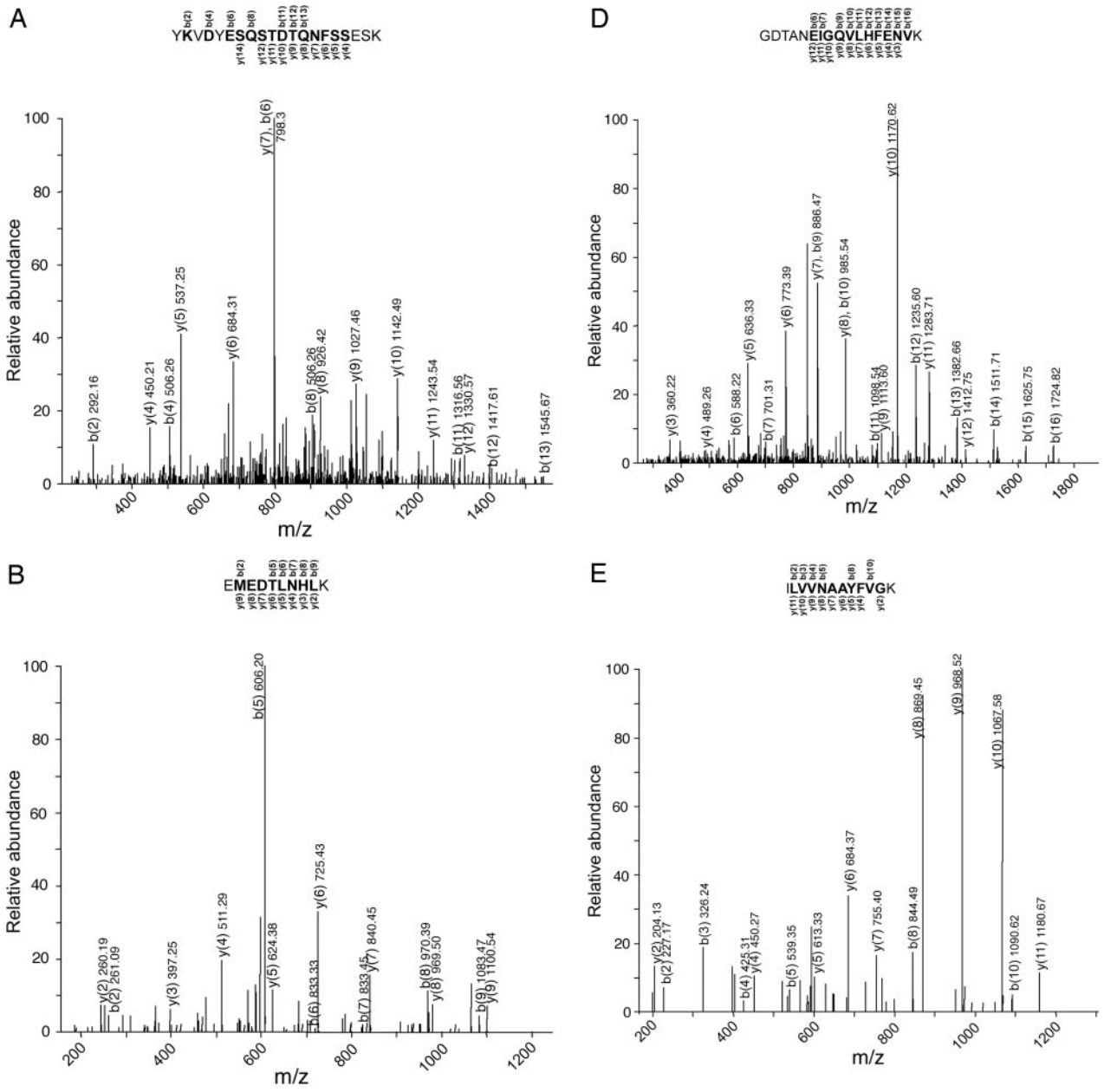

C Insulin-like growth factor binding protein-3
(P 17936)

MQRARPTLWA AALTLLVLLR GPPVARAGAS SGGLGPVVRC
EPCDARALAQ CAPPPAVCAE LVREPGCGCC LTCALSEGQP
CGIYTERCGS GLRCQPSPDE ARPLQALLDG RGLCVNASAV
SRLRAYLLPA PPAPGNASES EEDRSAGSVE SPSVSSTHRV
SDPKFHPLHS KIIIIKKGHA KDSQR**YKVDY** **ESQSTDTQNF**
SSESKRETEY GPCRR**EMEDT** **LNHLK**FLNVL SPRGVHIPNC
DKKGFYKKKQ CRPSKGRKRG FCWCVDKYGQ PLPGYTTKGK
EDVHCYSMQS K

F Maspin
(P 36952)

MDALQLANSA FAVDLFKQLC EKEPLGNVLF SPICLSTSLS
LAQVGAK**GDT** **ANEIGQVLHF** **ENVK**DIPFGF QTVTSDVNKL
SSFYSLKLIK RLYVDKSLNL STEFISSTKR PYAKELETVD
FKDKLEETKG QINNSIKDLT DGHFENILAD NSVNDQTK**IL**
VVNAAYFVGK WMKKFPESET KECPFRLNKT DTKPVQMMNM
EATFCMGNID SINCKIIELP FQNKHLSMFI LLPKDVEDES
TGLEKIEKQL NSESLSQWTN PSTMANAKVK LSIPKFKVEK
MIDPKACLEN LGLKHIFSED TSDFSGMSET KGVALSNVIH
KVCLEITEDG GDSIEVPGAR ILQHKDELNA DHPFIYIIRH
NKTRNIIFFG KFCSP

FIG. 4. **Mass spectrometry identification of IGFBP-3 and maspin.** The SDS-PAGE bands from fractions a and b were subjected to tryptic digestion. The tryptic digests were analyzed by nano-LC-nano-ESI-MS/MS. Spectra of the IGFBP-3 peptides are shown in *A* and *B*, and spectra of maspin are shown in *D* and *E*. The *bold letters* indicate the detected b and y ions matching the predicted ion mass in the database. The detected fragments (*bold letters*) are indicated in the sequence of full-length IGFBP-3 (*C*) and maspin (*F*).

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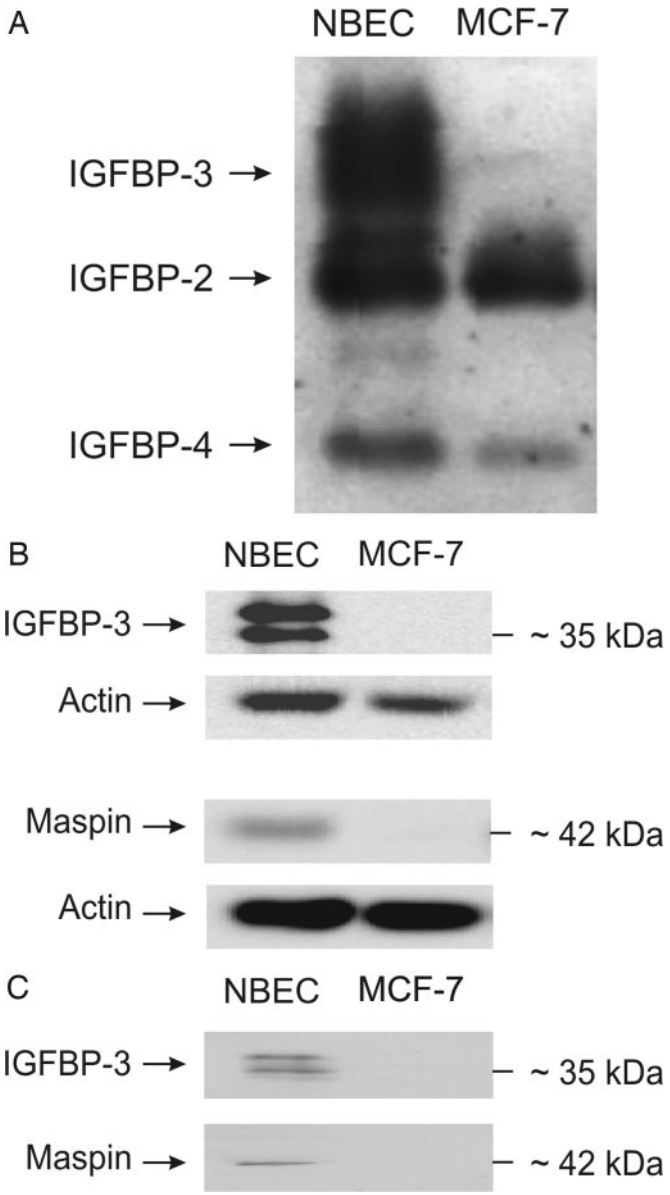

FIG. 5. **Western blot analysis of IGFBP-3 and maspin.** *A*, Western ligand blot analysis of IGFBPs. Conditioned media from NBECs and MCF-7 cells were collected, size-fractionated using 12% SDS-PAGE under non-reducing conditions, electroblotted onto nitrocellulose membranes, and treated with ^{125}I-labeled IGF-1 and -2 as indicated under "Experimental Procedures." *B* and *C*, detection of IGFBP-3 and maspin by Western blotting. Lysates (*B*) and concentrated conditioned media (*C*) from NBECs and MCF-7 cells were subjected to SDS-PAGE and immunoblotted as described under "Experimental Procedures." The loading and transfer of equal amounts of protein were confirmed by immunodetection of actin. The data are representative of three independent experiments.

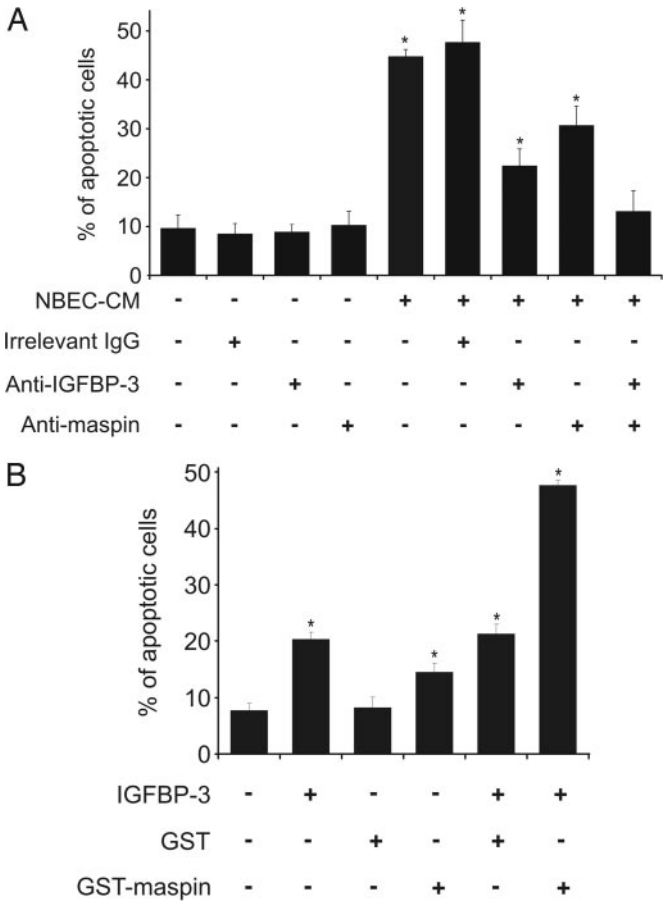

FIG. 6. **Effect of IGFBP-3 and maspin on apoptosis of MCF-7 breast cancer cells.** *A*, effect of IGFBP-3 and maspin immunodepletion from NBEC-conditioned medium. Conditioned medium was incubated with anti-IGFBP-3 and/or anti-maspin antibodies to immunodeplete IGFBP-3 and/or maspin. Irrelevant mouse immunoglobulin G (*IgG*) was used as control. Immunodepleted supernatants were added to MCF-7 cells with a final concentration equivalent to a 1:2 dilution of conditioned medium for 24 h. *B*, effect of recombinant IGFBP-3 and GST-maspin. MCF-7 cells were treated with IGFBP-3 (2.5 ng/ml) or GST-maspin (200 ng/ml) alone or in combination for 24 h. Apoptosis was determined after Hoechst staining. Results are the mean of three independent experiments. Bars show S.D. *, $p < 0.01$.

non-genotypic pathway requires the presence of retinoid X receptor-α. On the other hand, it has also been reported that inducible expression of IGFBP-3 leads to apoptosis induction of MCF-7 breast cancer cells by activating death receptor pathways (21). The level and activity of IGFBP-3 can be controlled by IGFBP-degrading proteases such as metalloproteinases (26, 27). Post-translational modifications including phosphorylation and glycosylation can also modify IGFBP-3 activity. Once phosphorylated, the binding of IGFBP-3 to IGF-I is enhanced (28). Phosphorylation of IGFBP-3 also increases the extracellular translocation of IGFBP-3 into the nucleus to exert IGF-independent effects (29, 30). Glycosylation, which influences bioavailability and cell surface association, may also enhance antiproliferative and proapoptotic effects (31). In our study, IGFBP-3 isolated from NBEC-conditioned medium seemed to be the full-length protein. However, two bands were obtained in the purified HPLC fraction as well as in Western blot analysis. Further studies are required to determine the nature of post-translational modifications of IGFBP-3.

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Maspin (mammary serpin) is a serine protease inhibitor with tumor suppression activities. Since its discovery in 1994, maspin has been consistently shown to suppress the aggressive tumor phenotypes, inhibiting cell invasion and mobility *in vitro* and inhibiting tumor growth and metastasis in experimental animal models (32–34). Maspin is localized both in cytoplasmic and nuclear compartments but may also be secreted. Maspin is the only proapoptotic serpin among all the serpins implicated in apoptosis regulation. However, the proapoptotic function of maspin has been so far observed by transfecting cancer cells with maspin cDNA (35). Transfected breast carcinoma cells are more sensitive to multiple apoptotic inducers. This apoptosis-sensitizing effect is mediated through the regulation of Bcl-2 family proteins (20, 35). The intracellular maspin can also translocate to the mitochondria to induce cytochrome *c* release and caspase activation (16). In this work, we showed that both normal breast epithelial cell-secreted maspin and recombinant maspin had the ability to induce apoptosis of breast cancer cells. The underlying mechanism is currently not known; however, maspin has been reported to dramatically decrease both cell-associated uPA/uPA receptor expression and uPA activity (36–38), and it is possible that maspin may induce apoptosis by reducing cell surface-associated prosurvival uPA·uPA receptor complex (34, 39). Alternatively because extracellular maspin is efficiently internalized (36, 40), it is also possible that internalized maspin induces apoptosis in a manner similar to overexpressed intracellular maspin by modulating expression of Bcl-2 family members or by targeting directly mitochondria (16, 20, 35). Consistent with this hypothesis, we have shown previously that conditioned medium from normal breast epithelial cells increases the level of Bax and decreases that of Bcl-2 (41).

Interestingly we showed that the release of IGFBP-3 and maspin is specifically observed for normal breast epithelial cells but not for breast cancer cells. This is consistent with findings that both IGFBP-3 and maspin are transcriptionally down-regulated or silenced by epigenetic changes such as methylation in cancer cells (42, 43). It is important to bear in mind that normal mammary epithelial cells that express high levels of maspin do not undergo detectable apoptosis either *in vitro* or *in vivo* (19). Furthermore maspin expression increases resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)- and staurosporine-induced apoptosis in normal breast epithelial cells (44). Recombinant IGFBP-3 does not modify growth kinetics of normal cells *per se* but can enhance proliferative effects of EGF in normal immortalized breast epithelial cells (MCF-10A) (45). In agreement with these data, we observed that recombinant IGFBP-3 and maspin alone or in combination did not affect growth of normal breast epithelial cells (data not shown). By contrast, MCF-7 breast cancer cells were dramatically induced into apoptosis when cotreated with IGFBP-3 and maspin. In the present study, apoptosis induction in MCF-7 breast cancer cells was used as a functional test to identify apoptosis-inducing factors produced by normal breast epithelial cells. However, we had shown previously that other breast cancer cell lines such as T-47D and MDA-MB-231 cells can also be induced into apoptosis by conditioned medium from normal breast epithelial cells (14, 15). Moreover recombinant IGFBP-3 and maspin synergistically induced apoptosis of these cells (data not shown). These findings indicate that IGFBP-3 and maspin exert a similar effect on distinct breast cell types.

In conclusion, using a proteomics-based approach we identified IGFBP-3 and maspin as the proapoptotic factors produced by normal breast epithelial cells. Although more physiological conditions, such as reconstituted three-dimensional culture systems and animal models, would be required to confirm *in vivo* the role of IGFBP-3 and maspin in breast epithelial cell homeostasis, our data provide a molecular basis for the long observed inhibitory effect of normal surrounding cells on breast cancer development.

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§ Both authors contributed equally to this work.

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REVIEW

Endocrine-Related Cancer (2006) **13** 293–325

p53 and breast cancer, an update

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Abstract

p53 plays a key role in mediating cell response to various stresses, mainly by inducing or repressing a number of genes involved in cell cycle arrest, senescence, apoptosis, DNA repair, and angiogenesis. According to this important function, p53 activity is controlled in a very complex manner, including several auto-regulatory loops, through the intervention of dozens of modulator proteins (the 'p53 interactome'). p53 mutations are observed in a significant minority of breast tumours. In the remaining cases, alterations of interactome components or target genes could contribute, to some extent, to reduce the ability of p53 to efficiently manage stress events. While the prognostic and predictive value of p53 is still debated, there is an increasing interest for p53-based therapies. The present paper aims to provide updated information on p53 regulation and function, with specific interest on its role in breast cancer.

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Introduction

While p53 seems to be dispensable for normal development (Donehower *et al*. 1992), it plays an important role in regulating cell fate in response to various stresses, either genotoxic (DNA alterations induced by irradiation, UV, carcinogens, cytotoxic drugs) or not (hypoxia, nucleotide depletion, oncogene activation, microtubule disruption, loss of normal cell contacts). The protein may be viewed as a node for the stress signals, which are then transduced, mainly through the ability of p53 to act as a transcription factor. p53 exerts its anti-proliferative action by inducing reversible or irreversible (senescence) cell cycle arrest, or apoptosis. It may also enhance DNA repair and inhibit angiogenesis.

Many types of stresses may be encountered during tumour development. The p53 function is often altered in cancer. It has been suggested that p53 could have evolved in higher organisms specifically to prevent tumour development (see notably in Vousden & Lu 2002). It is believed that this specific action is exerted mainly through the triggering of apoptosis (see notably in Haupt *et al*. 2003, Yu & Zhang 2005). Indeed, loss of p53 activity disrupts apoptosis and accelerates the appearance of tumours in transgenic mice (Attardi & Jacks 1999).

The qualitative and quantitative activity of p53 depends on its integrity (mutation status), its amount, and its specific posttranslational modifications induced by the activation of the different stress-induced signalling pathways. This leads to variable patterns of association between p53 and a number of other co-regulatory proteins, of which some may be tissue- or cell type-specific. Despite this complexity, p53 activity has been associated with prognosis and prediction of tumour response to various therapies and deserves further investigations with the perspective of developing more targeted treatments.

Structure of p53

p53 is encoded by the *Tp53* gene. Located at 17p13, this contains 11 exons spanning 20 kb. It belongs to a family of highly conserved genes that also includes *TP63* and *TP73*, encoding p63 and p73 respectively.

Three functionally distinct regions have been identified in p53.

1. An acidic N-terminal region (codons 1–101), itself containing two major domains. (i) A transactivation acidic domain (codons 1–42). Codons 17–28 may interact with the ubiquitin ligase mouse double minute-2 homologue

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(MDM2), which plays a major role in p53 degradation (see below). Codons 22–26 (LWKLL) constitute an LXXLL-type co-activator recognition motif (Savkur & Burris 2004) involved in histone acetyltransferase P300 binding. It is believed that codons 11–27 may function as a secondary nuclear export signal (NES) and that DNA damage-induced phosphorylation may inhibit this activity. (ii) A proline-rich domain (codons 63–97) required for interaction with various proteins involved in the induction of apoptosis. It contains five PXXP motifs (PRMP at 64–67; PVAP at 72–75; PAAP at 77–80; PAAP at 82–85; PSWP at 89–92) that are involved in p53 interaction with P300 (Dornan *et al*. 2003). Interestingly, a polymorphism has been demonstrated at codon 72, where the proline is frequently replaced by an arginine. Both forms are morphologically wild-type and do not differ in their ability to bind to DNA in a sequence-specific manner. However, there are a number of differences between these p53 variants in their abilities to bind components of the transcriptional machinery, to activate transcription, to induce apoptosis, and to repress the transformation of primary cells (Thomas *et al*. 1999).

2. A central DNA-binding core region (codons 102–292). It recognizes a promoter consensus motif made of two 10 bp segments (RRRCWWGYYY) separated by 0–13 bp. This region is highly conserved throughout evolution. It is also the most homologous region among p53 family members (P63, P73).
3. A basic C-terminal region (codons 293–393), involved in tetramerization and regulation of p53 activity. It notably contains: (i) three nuclear localization signals (codons 305–322, 369–375, 379–384) recognized by a heterodimeric complex composed of importin alpha and beta that allows the p53 nuclear import (Fabbro & Henderson 2003); (ii) a tetramerization domain (codons 323–356), itself containing a primary NES (codons 339–352) recognized by the export receptor CRM1/exportin (Fabbro & Henderson 2003). p53 is active as a transcription factor only in the homotetrameric form. Tetramerization of p53 masks the primary NES and prevents export from the nucleus; (iii) a negative regulatory region (codons 363–393). By binding short non-specific DNA sequences, this region may prevent specific DNA binding to the core region (Weinberg *et al*. 2004).

Genomic and non-genomic actions of p53

In normal cells not exposed to stress, the level and activity of p53 are very low. Upon stress, p53 is activated through a series of post-translational modifications and becomes able to bind to specific DNA sequences. The p53 recognition sequence is very loose and has been found in several hundred genes that are differentially modulated (induced or repressed) depending on the cell type, the nature of stress and the extent of damage. At low cellular levels, p53 modulates only a subset of the genes regulated at higher levels. The kinetics of target gene modulation may also vary.

In a study with a micro-array carrying 6000 capture sequences, 107 genes were found to be induced and 54 genes were repressed by p53 (Zhao *et al*. 2000). This result extrapolates to at least 500 up-regulated and 260 down-regulated p53 target genes.

Table 1, based on several papers (Yu *et al*. 1999, Vousden & Lu 2002, Liang & Pardee 2003, Nakamura 2004, Miled *et al*. 2005) lists a non-exhaustive series of p53-target genes that have been found to be altered by various stresses in many cell types.

Modulation of cell cycle-related genes by activated p53 may mediate arrest of cells at one of two major cell-cycle checkpoints, in G_1 near the border of S-phase (key role played by $P21^{WAF1/CIP1}$) or in G_2 before mitosis (important roles for GADD45 and 14-3-3σ). The transcriptional program responsible for p53-mediated apoptosis is much less clearly defined. However, the observation, for instance, that mice lacking the $P21^{WAF1/CIP1}$ gene (*CDKN1A*), unlike p53-null mice, do not develop tumours indicates that it is this apoptotic program that plays an essential role in p53 tumour suppression. p53 may modulate the expression of genes associated with either the extrinsic or the intrinsic apoptotic pathways. The extrinsic pathway (in which genes such as *TNFRSF10A*, *TNFRSF10B*, *FAS*, *PERP*, *LRDD* are implied) involves engagement of particular 'death' receptors. The intrinsic pathway is triggered in response to DNA damage and is associated with mitochondrial depolarization and release of cytochrome c from the mitochondrial inter-membrane space into the cytoplasm. Some genes associated with this pathway are *APAF1*, *BAK1*, *BAX*, *BCL2* (repressed), *FDXR*, *PMAIP1*,

Table 1 A non-exhaustive series of p53-target genes that have been found altered by various stresses in many cell types.

Function	Regulated genes
Cell cycle	14-3-3σ (*SFN*), ATF3 (*ATF3*), BTG2 (*BTG2*), CYCLIN A2 (*CCNA2*), CYCLIN B1 (*CCNB1*), CYCLIN G1 (*CCNG1*), DDA3 (*DDA3*), DSCP1 (*DSCP1*), GADD45 (*GADD45A*), $P21^{WAF1/CIP1}$ (*CDKN1A*), RAI3 (*GPCR5A*), REPRIMO (*RPRM*), TERT (*TERT*)
Apoptosis and survival	AMID/PRG3/AIF (*PRG3*), APAF-1 (*APAF1*), BAK1 (*BAK1*), BAX (*BAX*), BCL2 (*BCL2*), BCL2-like 14 (*BCL2L14*), BID (*BID*), BNIP3L/NIX (*BNIP3L*), BOK (*BOK*), DAPK1 (*DAPK1*), DR4 (*TNFRSF10A*), DR5/Killer (*TNFRSF10B*), FAS (*FAS*), FDXR (*FDXR*), IGFBP3 (*IGFBP3*), MAP4K4 (*MAP4K4*), MNSOD (*SOD2*), MYC (*MYC*), NDRG (*NDRG1*), NOXA (*PMAIP1*), P53AIP1 (*P53AIP1*), P53CSV (*HSPC132*), P53DINP1 (*TP53INP1*), P73 (*TP73*), PAC1 (*DUSP2*), PEG3/PW1 (*PEG3*), PERP (*PERP*), PIDD (*LRDD*), PIG3 (*TP53I3*), PIG6 (*PRODH*), PIG8 (*EI24*), PIG11 (*TP53I11*), PIR121 (*CYFIP2*), PTEN (*PTEN*), PUMA (*BBC3*), SURVIVIN (*BIRC5*), WIG-1/PAG608 (*WIG1*), WIP1 (*PPM1D*)
DNA repair	53BP2 (*TP53BP2*), DDB2 (*DDB2*), P53R2 (*RRM2B*), RECQ4 (*RECQL4*), XPC (*XPC*)
Angiogenesis	BAI1 (*BAI1*), ENDOSTATIN (*COL18A1*), KAI1/CD82 (*KAI1*), MASPIN (*SERPINB5*), MMP2 (*MMP2*), TSP1 (*THBS1*), VEGF (*VEGF*)
Others	BRCA1 (*BRCA1*), CHK1 (*CHEK1*), CHK2 (*CHEK2*), COP1 (*COP1*), CSPG2 (*CSPG2*), MDM2 (*MDM2*), PCAF (*PCAF*), PIRH2/ZNF363 (*RCHY1*), PML (*PML*)

and *BBC3*. Both pathways lead to a cascade of activation of caspases, ultimately causing apoptosis. p53 could promote the convergence of the extrinsic and intrinsic pathways through *BID* regulation.

Besides the regulation of apoptosis-related genes, p53 also appears to be able to act directly at the mitochondria. It can interact with BCL2 family members, such as the anti-apoptotic BCL2 itself and BCL-XL, and the pro-apoptotic BAK, thereby triggering mitochondrial outer membrane permeabilization and apoptosis (Schuler & Green 2005).

The quantitative, or even qualitative contribution of the direct, transcription-independent action to the global apoptotic activity of p53 has been debated. Observations such as the radio-resistant phenotype of the PUMA (*BBC3*)- and NOXA (*PMAIP1*)-knockout mice have been used as arguments against the general importance of transcription-independent mechanisms *in vivo* (Yu & Zhang 2005). It has also been observed that, in various cell lines, DNA damage induced by either ionizing radiation (IR) or topoisomerase inhibitors triggered a robust translocation of a fraction of p53 to mitochondria to a similar extent. Nevertheless, the cells succumbed to apoptosis only in response to topoisomerase inhibitors, but remained resistant to apoptosis induced by IR, suggesting that mitochondrial translocation of p53 does not *per se* lead to cell death (Essmann *et al*. 2005). Other investigators, by examining 179 mutant p53s, found no significant correlation between their apoptotic property and their ability to activate transcription of six p53-responsive genes (*CDKN1A*, *MDM2*, *SFN*, and the apoptosis-related *BAX*, *p53AIP1*, *BBC3*) (Kakudo *et al*. 2005). It is possible that rapid transactivation-independent events could modulate the extent of apoptosis, which would however depend on transactivation-dependent events. However, recent observations suggest that the inverse could be true. Indeed, it has been shown that after genotoxic stress, the major regulator of apoptosis, BCL-XL, sequestered cytoplasmic p53. Nuclear p53 caused expression of PUMA, which then displaced p53 from BCL-XL, allowing p53 to induce mitochondrial permeabilization. Mutant BCL-XL that bound p53, but not PUMA, rendered cells resistant to p53-induced apoptosis irrespective of PUMA expression. These observations thus identify PUMA as the protein coupling the nuclear and cytoplasmic pro-apoptotic functions of p53 (Chipuk *et al*. 2005).

The central core region of p53 is of key importance in regulating apoptotic function, either transcription-dependent or -independent, as supported by the number of mutations affecting this region in apoptosis-deficient p53 cells. In addition to inducing genes that drive apoptosis, p53 can also activate the expression of genes that inhibit survival signalling (such as *PTEN*) or inhibit inhibitors of apoptosis (such as *BIRC5*) (Vousden & Lu 2002, Haupt *et al*. 2003, Meek 2004, Nakamura 2004, Lu 2005, Yu & Zhang 2005). Besides the central core, the proline-rich domain has been specifically associated with the apoptotic activity of p53 (Walker & Levine 1996). Deletion of this region

Table 2 An overview of the p53 biochemical modifications that have been described to date.

Target residue or doublet	Type of modification	Modifying protein
Ser6	Phosphorylation	CHK1
Ser9	Phosphorylation	CHK1, CK1 (if Ser6 is phosphorylated)
Ser15	Phosphorylation	ATM, ATR, DNA-PK, CHK1, P38, RSK2
Thr18	Phosphorylation	CHK1, CK1 (if Ser15 is phosphorylated)
Ser20	Phosphorylation	CHK1, CHK2
Ser33	Phosphorylation	P38, CAK, JNK
Ser33-Pro34	*Cis-trans* isomerisation	PIN1
Ser37	Phosphorylation	ATR, DNA-PK
Ser46	Phosphorylation	P38, HIPK2
Ser46-Pro47	*Cis-trans* isomerisation	PIN1
Thr55	Phosphorylation	$TAF_{II}250$, ERK2
Thr81	Phosphorylation	JNK
Thr81-Pro82	*Cis-trans* isomerisation	PIN1
Ser127-Pro128	*Cis-trans* isomerisation (potential)	PIN1
Thr150-Pro151	*Cis-trans* isomerisation (potential)	PIN1
Thr155	Phosphorylation	CK2, CSN-associated kinases
Ser215	Phosphorylation	STK15
Lys305	Acetylation	P300
Ser313	Phosphorylation	CHK1, CHK2
Ser314	Phosphorylation	CHK1, CHK2
Ser315	Phosphorylation	STK15, CDKs, CDC2
Ser315-Pro316	*Cis-trans* isomerisation (potential)	PIN1
Lys320	Acetylation	PCAF
Ser366	Phosphorylation	CHK2
Lys370	Ubiquitination	MDM2 + other ubiquitin ligases?
	Acetylation	P300/CBP
	Neddylation	MDM2
Ser371	Phosphorylation	PKC
Lys372	Methylation	SET9
	Ubiquitination	MDM2 + other ubiquitin ligases?
	Acetylation	P300/CBP
	Neddylation	MDM2
Lys373	Ubiquitination	MDM2 + other ubiquitin ligases?
	Acetylation	P300/CBP
	Neddylation	MDM2
Ser376	Phosphorylation	PKC, CAK
Thr377	Phosphorylation	CHK1, CHK2
Ser378	Phosphorylation	PKC, CAK, CHK1, CHK2
Lys381	Ubiquitination	MDM2 + other ubiquitin ligases?
	Acetylation	P300/CBP
Lys382	Ubiquitination	MDM2 + other ubiquitin ligases?
	Acetylation	P300/CBP
Lys386	Ubiquitination	MDM2 + other ubiquitin ligases?
	Sumoylation	PIAS proteins
Thr387	Phosphorylation	CHK1
Ser392	Phosphorylation	PKR, FACT (complexed to CK2), P38

ATM, ataxia-telangiectasia mutated (gene *ATM*); ATR, ataxia-telangiectasia and Rad3-related (*ATR*); CAK, CDK activating kinase; CBP, CREB-binding protein (*CREBBP*); CDC2, cell division cycle 2 kinase (*CDC2*); CDKs, cyclin-dependent kinases (multiple members); CHK1, cell cycle checkpoint kinase 1 (*CHEK1*); CHK2, cell cycle checkpoint kinase 2 (*CHEK2*); CK1, casein kinase 1 (multiple isoforms); CK2, casein kinase 2 (multiple isoforms); CSN, COP9 signalosome (protein complex); DNA-PK, DNA-dependent protein kinase (*PRKDC*); ERK2, p42 mitogen activated protein kinase (*MAPK1*); FACT, facilitating chromatin-mediated transcription; HIPK2, homeodomain-interacting protein kinase 2 (*HIPK2*); JNK, Jun N-terminal kinase (*MAPK8*); MDM2, mouse double minute 2 homologue; P38, p38 mitogen activated protein kinase (*MAPK14*); P300, E1A-binding protein, 300-kDa (*EP300*); PCAF, P300/CBP-associated factor (*PCAF*); PKC, protein kinase C (multiple isoforms); PKR, double stranded RNA-dependent protein kinase (*PRKR*); PIAS, protein inhibitor of activated STAT (multiple isoforms); PIN1, peptidyl-prolyl-cis-trans isomerase 1 (*PIN1*); RSK2, ribosomal S6 kinase 2 (*RPS6KA3*); SET9, SET-domain containing protein 9 (*SET7*); STK15, serine/threonine protein kinase 15 (*STK15*); $TAF_{II}250$, TATA-binding protein associated factor 250-kD (*TAF1*).

leads to a complete loss of the apoptotic activity of p53. It could constitute an auxiliary protein-binding site and could be necessary for cellular cofactors specifically involved in the apoptotic activity of p53.

The p53-regulated genes that bring about senescence are less well characterized. However, *CSPG2* has been strongly associated with senescence in prostate cancer cells (Schwarze *et al*. 2005).

Biochemical modifications of p53

Posttranslational modification is a major mechanism regulating protein function. p53 may be phosphorylated, cis/trans isomerized, acetylated, ubiquitinated, methylated, sumoylated, neddylated, glycosylated at multiple sites, reflecting its biological importance. This multisite modification, which exhibits a cell and tissue specificity and depends on the position in the cell cycle, is a complex regulatory program that fluctuates in response to cellular signalling triggered by DNA damage, proliferation and senescence, and thus appears as a dynamic 'molecular barcodes' (Yang 2005).

An overview of the p53 modifications that have been described to date is provided in Table 2. It is based on papers used for Table 1 and additional reports (Appella & Anderson 2001, Meek 2002, Bode & Dong 2004, Ou *et al*. 2005).

Since it is impossible to give a detailed description of all p53 modifications, only the most widely observed and well-known alterations will be discussed briefly here.

Phosphorylation

p53 phosphorylation has been widely investigated. In most cases, it is associated with protein stabilization.

Three N-terminal sites, Ser15, Thr18, and Ser20, are particularly interesting because when phosphorylated, the interaction between p53 and its major negative regulator, MDM2, is diminished, while the binding of the acetyltransferase P300 is promoted, thereby increasing the level and stability of p53. Notably, Ser15 may be phosphorylated by IR (*via* ataxia-telangiectasia mutated; ATM) or UV (*via* ataxia-telangiectasia and Rad3-related; ATR). These stresses also lead to Ser20 phosphorylation, through the action of cell cycle checkpoint kinase 2 (CHK2) and CHK1 respectively. In fact, besides IR and UV, almost all stresses have been shown to induce Ser15 phosphorylation, which is thought to nucleate a series of subsequent p53 post-translational modifications (Meek 2004).

In some cases, p53 phosphorylation events are sequential. For instance, phosphorylation of Ser9 and Thr18 by CK1 is dependent of Ser6 and Ser15 phosphorylation respectively.

Another crucial N-terminal residue is Ser46. Its phosphorylation selectively promotes a p53 apoptotic response. Various kinases may be involved in this event, reflecting the activation of different stress pathways. For instance, HIPK2 mediates Ser46 phosphorylation in response to UV irradiation, although it seems that this alone is not sufficient to induce apoptosis. It has also been proposed that P38 can mediate the phosphorylation of Ser46 in response to UV. Neither P38 nor HIPK2 are involved in the Ser46 phosphorylation in response to IR, which requires both ATM and the p53-inducible gene, *Tp53INP1*, coding for p53DINP1. ATM does not directly phosphorylate p53, but it is likely to induce a kinase that might be co-activated by p53DINP1 to facilitate Ser46 phosphorylation (apoptosis-selective auto-regulatory loop) (Vousden & Lu 2002).

One important apoptosis-related protein, p53AIP1, is induced only when Ser46 is phosphorylated. Studies with the drug, etoposide, have confirmed that phosphorylation of p53 at Ser46 determines promoter selection and whether apoptosis is attenuated or amplified. High dose chemotherapy induced the phosphorylation of p53 on Ser46, whereas low dose chemotherapy did not. While Ser46-phosphorylated p53 targeted the promoter of the tumour suppressor *PTEN* in preference to *MDM2* (thus abrogating the auto-regulatory loop that contributes to keeping the p53 level low), the inverse was observed in the absence of Ser46 phosphorylation. Accordingly, only high dose chemotherapy led to *p53AIP1* induction, caspase 3 activation, and cell death (Mayo *et al*. 2005).

In addition to a common polymorphism at codon 72 (see below), p53 tumour also exhibits a rare single nucleotide polymorphism at residue 47. Wild-type p53 encodes proline at this residue, but in $<5\%$ of African Americans, this amino acid is serine. Notably, phosphorylation of the adjacent Ser46 by the proline-directed kinase P38 is known to greatly enhance the ability of p53 to induce apoptosis. The Ser47 polymorphic variant, which replaces the proline residue necessary for recognition by proline-directed kinases, is a markedly poorer substrate for phosphorylation on Ser46 by P38. Consistent with this finding, the Ser47 variant has an up to five-fold

decreased ability to induce apoptosis compared with wild-type p53. This variant has a decreased ability to transactivate two p53 apoptotic target genes, *p53AIP1* and *BBC3*, but not other p53 response genes; thus, the codon 47 polymorphism of p53 is functionally significant and may play a role in cancer risk, progression, and the efficacy of therapy (Li *et al*. 2005).

Experiments using p53 mutants with substitutions at Ser33, Ser46 or Thr81 have shown that phosphorylation of these sites (by P38 or Jun N-terminal kinase (JNK)) may independently lead to p53 stabilization, notably after exposure to UV (Appella & Anderson 2001).

In contrast to Ser315, Ser392 is phosphorylated only poorly after exposure of cells to IR, while it is strongly modified in response to UV (Appella & Anderson 2001).

In the C-terminal region of p53, phosphorylation of Ser315, Ser371, Ser376, Ser378, and Ser392 is well known. More recently, it has been shown that additional sites were also phosphorylated: Ser313, Ser314, Thr377, Ser378 (by both CHK1 and CHK2), Ser366 (by CHK2 only) and Thr387 (by CHK1 only). These events may alter the pattern of acetylation at Lys373 and Lys382, but not at Lys320, thus distinguishing between P300/CREB-binding protein (CBP) and P300/CBP-associated factor (PCAF) activity (see below) (Ou *et al*. 2005).

While most p53 phosphorylation events result in an increase in stability/activity of the protein, the phosphorylation of some sites (Thr55, Thr155, Ser215, Ser376) has been associated with enhanced p53 degradation. For instance, Thr55 can be phosphorylated by $TAF_{II}250$, the largest subunit of the general transcription factor TFIID, and this event enhances p53 degradation. Exposure of cells to UV decreases phosphorylation at Thr55 (Appella & Anderson 2001).

The COP9 signalosome (CSN) is an eight-subunit heteromeric complex that has homologies with the 26S proteasome bid complex. CSN has been reported to modulate ubiquitin ligase activity, as it directly interacts with cullin-domain ubiquitin ligases, catalyses deneddylation of these ligases, and is required for their proper function. Interestingly, CUL4A, a CSN-associated cullin-domain ubiquitin ligase has been shown to induce p53 degradation (see below). The CSN-associated kinases, CK2 and protein kinase D, are able to phosphorylate p53, and CK2 does so on Thr155. This dedicates p53 to rapid degradation by the ubiquitin–proteasome system. The importance of Thr155 is underlined by the fact that mutation of this residue is sufficient to stabilize p53 against human papilloma virus E6 oncoprotein-dependent degradation, which is mediated by E6AP, a ubiquitin ligase different from CUL4A. E6 is believed to play a major role in carcinoma of the cervix, where p53 mutations are rare.

Phosphorylation of Ser215 by the mitotic kinase serine/threonine protein kinase 15 (STK15) (also known as Aurora A) abrogates p53 DNA binding and transactivation activity (Liu *et al*. 2004*b*).

Ser376 (and Ser378) are constitutively phosphorylated by protein kinase C (PKC), which can contribute to p53 degradation (Chernov *et al*. 2001).

Not only the qualitative and quantitative pattern, but also the timing of p53 phosphorylation may vary depending on the stress. For instance, in response to IR increased phosphorylation of Ser6, Ser9, and Ser15 has been observed as early as 30 min after treatment, while exposure to UV induced a less-rapid, but more long-lived increase in the phosphorylation of these sites. This reflects the fact that ATR is more slowly activated than ATM (Appella & Anderson 2001).

Dephosphorylation

In vitro dephosphorylation of p53 by the phosphatases PP1, PP2A, PP5, PPM1D and CDC14 has been shown. These may have different specificities, as shown, for instance, by the fact that PP1, but not PP2A, can dephosphorylate phospho-Ser15 (Haneda *et al*. 2004). PPM1D is of high interest, as it is induced by p53 and may dephosphorylate both p53 (at Ser15) and CHK1 (which may phosphorylate p53 at various sites) (Lu *et al*. 2006). Amplification of the *PPM1D* gene has been observed in breast cancer and seems to be associated with high aggressiveness (Rauta *et al*. 2006). Dephosphorylation of Ser376 by an ATM-regulated phosphatase allows 14-3-3σ binding to phosphorylated Ser378, thereby contributing to p53 stabilization with consequent effects on site-specific DNA binding.

Cis/*trans* isomerization

p53 activation involves a conformational change, brought about by *cis*/*trans* isomerization of certain proline residues by peptidyl-prolyl-cis-trans isomerase 1 (PIN1). PIN1 binds protein sites consisting of a phosphorylated serine or threonine followed by a proline; it then catalyses the isomerization of proline residues, which changes the conformation

of p53. There are four Ser–Pro (Ser33–Pro34, Ser46–Pro47, Ser127–Pro128, Ser315–Pro316) and two Thr–Pro (Thr81–Pro82 and Thr150–Pro151) motifs on human p53 protein. Single mutations on these Ser–Pro or Thr–Pro sites do not lead to marked reduction of the p53-PIN1 interaction. However, a double point mutant (Ser33Ala, Ser315Ala) shows less binding to PIN1, and the triple point mutant (Ser33Ala, Ser315Ala, Thr81Ala) exhibits further reduced binding activity for PIN1, suggesting that these three sites are important for the p53-PIN1 interaction. It is possible that the Ser46–Pro47 site could also be involved in the process of *cis*/*trans* isomerization, considering the importance of Ser46 phosphorylation in p53 function. Whether the Ser127–Pro128 and Thr150–Pro151 motifs may be effectively targeted by PIN1 remains unknown at this time. The precise conformational changes induced by p53 due to different stress responses at different Ser–Pro or Thr–Pro sites are not yet clear. PIN1-induced conformational change in p53 inhibits the binding and/or stimulates the detachment of MDM2, leading to p53 stabilization. In addition, the conformational change may enhance the ability of P300 to acetylate p53 C-terminal lysines, and it may promote the binding of the p53 core domain to its specific promoter cognate sites, particularly those promoting apoptosis (Kohn & Pommier 2005).

Pro82 is essential for p53 interaction with CHK2 and consequent phosphorylation of Ser20 in response to DNA damage. These physical and functional interactions are regulated by PIN1. A sequence of events may thus be identified, in which phosphorylation of Thr81 allows PIN1 to isomerize p53, which further leads to p53-CHK2 interaction and phosphorylation of Ser20 (Berger *et al*. 2005).

Acetylation

Acetylation has been shown to augment p53 DNA binding and to stimulate p53-mediated transactivation of target genes through the recruitment of co-activators. Acetylation is also thought to contribute to p53 stabilization by impairing ubiquitination of the acetylated residues. Intriguingly, while all evidence so far indicates that acetylation positively regulates p53 function (Brooks & Gu 2003), this modification seems also to regulate p53 subcellular localization, at least in part by activating its nuclear export (Kawaguchi *et al*. 2006).

P300, CBP and PCAF are ubiquitous transcriptional co-activators. They act as histone acetyltransferases, but may also acetylate various transcription factors, including p53. According to current data, P300/CBP may compete with MDM2 for binding to N-terminus of p53, so that a decrease in MDM2-p53 interaction associated with phosphorylation of N-terminal (especially Ser15) sites may favour P300/CBP binding and acetylation of Lys373 and Lys382. On the other hand, Ser15 phosphorylation is not absolutely required for p53 acetylation, as shown, for instance, by actinomycin D, which does not induce Ser15 phosphorylation but is a powerful agent in triggering p53 acetylation (Appella & Anderson 2001). Other p53 residues acetylated by P300/CBP are Lys370, Lys372 and Lys381. PCAF may acetylate Lys320.

It has been shown that upon non-apoptotic DNA damage such as that induced by cytostatic doses of cisplatin, PCAF acting in cooperation with homeodomain-interacting protein kinase 2 (HIPK2) may acetylate p53. This HIPK2 action is independent of the Ser46 phosphorylation performed by the kinase upon severe genotoxic damage. Co-action of PCAF and HIPK2 selectively induce p53 transcriptional activity towards the *CDKN1A* promoter while depletion of either HIPK2 or PCAF abolishes this function. So, PCAF participates in the complex mechanisms allowing p53 to make a choice between growth arrest and apoptosis (Di Stefano *et al*. 2005). Interestingly, *PCAF* is a p53-induced gene (growth arrest-selective auto-regulatory loop) (Watts *et al*. 2004), while it is targeted for degradation (ubiquitinated) by MDM2 (Jin *et al*. 2004).

Experiments with histone deacetylase inhibitors on prostate cancer cells suggest that the acetylation of p53 at Lys373 is required for the p53-mediated induction of cell cycle arrest and apoptosis, while acetylation of p53 at Lys382 induces only cell cycle arrest (Roy *et al*. 2005).

The activation of p53 by P300/CBP can be achieved in a cooperative manner through the p53-binding proteins PRMT1 and CARM1 (co-activator-associated arginine methyltransferases). Whether p53 is a direct substrate for these two proteins is presently unknown.

Deacetylation

It is likely that deacetylation provides a quick acting mechanism to stop p53 function once transcriptional activation of target genes is no longer needed. Deacetylation of p53 may be performed by multiple histone deacetylases (HDACs), at least by HDAC 1-3. The deacetylase sirtuin 1 (SIRT1) shows an *in vitro* activity on p53 peptides and it

seems that cellular p53 is a major *in vivo* substrate of SIRT1 but not of the other six known SIRT proteins (SIRT 2-7) (Michishita *et al*. 2005). In fact, both HDAC1 and SIRT1 could be critical for p53-dependent stress response (Gu *et al*. 2004).

MTA2 (metastasis-associated protein 2)/PID (p53 target protein in the deacetylase complexes) specifically interacts with p53 both *in vitro* and *in vivo*, and its expression reduces significantly the steady-state levels of acetylated p53 by recruiting the HDAC1 complex. MTA2/PID expression strongly represses p53-dependent transcriptional activation, and, notably, it modulates p53-mediated cell growth arrest and apoptosis (Luo *et al*. 2000).

Numerous proteins modulating p53 activity have been shown to interfere with acetylation/deacetylation processes (not shown here).

Ubiquitination

In normal cells, degradation is the only mechanism that abrogates all functions of p53, and this appears to be accomplished, in part, by the ubiquitin-26S proteasome system (the other way is ubiquitin-independent). The highly conserved protein, ubiquitin, targets substrate proteins for degradation by the 26S proteasome to peptides. Ubiquitin ligases realise the last step of ubiquitination. These enzymes exhibit a high level of target specificity.

In normal cells, the RING domain MDM2 is considered as the main ubiquitin ligase regulating the amount of p53. MDM2 binds to the N-terminal region and represses p53 activity via two mechanisms: by promoting p53 export to the cytoplasm and its consequent degradation and by blocking p53 transcriptional activation. The export of p53 requires an intact p53 NES. Several lysine residues located at the C-terminus of p53 may be MDM2-ubiquitinated: Lys370, Lys372, Lys373, Lys381, Lys382, Lys386 (Rodriguez *et al*. 2000). The ubiquitination of these lysine residues in the p53 C-terminus, including Lys305, is required to expose the NES even when p53 is bundled as a tetramer.

MDM2 is up-regulated by activated p53 and this generates a p53-MDM2 auto regulatory loop.

According to a current view, DNA damage leads to destabilization and accelerated degradation of MDM2. This limits MDM2 binding to p53 during the stress response and enables p53 to accumulate and remain active, even as p53 transcriptionally activates more MDM2. Thus, the induction of MDM2 RNA by activated p53 may create a reserve of MDM2 that can inactivate p53 once the DNA damage stimulus has abated and MDM2 is re-stabilized.

The physiological relevance of the p53-MDM2 loop is supported by various observations: (1) MDM2-knockout mice have an embryonic lethal phenotype (which can be abolished by the simultaneous inactivation of p53; (2) disruption of the p53-MDM2 interaction with synthetic competitive inhibitors is sufficient to induce a p53 response in cultured cells; (3) blocking MDM2 degradation via proteasome inhibition prevents p53 transactivation in DNA-damaged cells; (4) the activity of MDM2 is controlled by numerous factors and the p53-MDM2 loop is the focal point of the many different stresses that activate the p53 pathway (see below)

As many tumours inactivate wild-type p53 through MDM2 over-expression, exploiting the pathways that trigger MDM2 auto-degradation may be an important new strategy for chemotherapeutic intervention (Stommel & Wahl 2005).

COP1 (constitutive photomorphogenesis protein 1) is a RING domain ubiquitin ligase that inhibits p53-dependent transcription. Depletion of COP1 by short interfering RNA (siRNA) stabilizes p53 and arrests cells in the G_1 phase of the cell cycle. Over-expression of COP1 correlates with a striking decrease in steady state p53 protein levels and attenuation of the downstream target gene, *CDKN1A*, in cancers that retain a wild-type p53 gene status. Moreover, like *MDM2*, *COP1* is a p53-inducible gene (Dornan *et al*. 2004).

The cytosolic chaperone-associated U-box domain ubiquitin ligase CHIP (C-terminus of hsc70-interacting protein) may induce the proteasomal degradation of p53. CHIP is thought to act in the quality control of protein folding, specifically ubiquitinating unfolded proteins associated with the molecular chaperones. CHIP-induced degradation has been observed for mutant p53, which was previously shown to associate with the chaperones Hsc70 and Hsp90, and for the wild-type form of the protein. Thus, mutant and wild-type p53 transiently associate with molecular chaperones and can be diverted onto a degradation pathway through this association (Esser *et al*. 2005).

The cullin-domain ubiquitin ligase CUL4A (cullin 4a) associates with MDM2 and p53, and ubiquitinates p53. Depletion of CUL4A leads to an accumulation of p53. CUL4A fails to increase the decay of p53 in mouse embryonic fibroblasts lacking MDM2. In addition, the CUL4A-mediated rapid decay of p53 is blocked by the MDM2 negative regulator $p19^{ARF}$(ARF for alternate reading frame).

The results provide evidence for a cooperative role of CUL4A in the MDM2-mediated proteolysis of p53 (Nag *et al*. 2004).

The E6 oncoprotein of human papilloma viruses (HPVs) that are associated with cervical cancer utilizes the HECT domain ubiquitin ligase E6AP (E6-associated protein) to target p53 for degradation. In normal cells (i.e. in the absence of E6), p53 degradation is mediated by MDM2 rather than by E6AP. In HPV-positive cancer cells, the E6-dependent pathway of p53 degradation is not only active but, moreover, is required for degradation of p53, whereas the MDM2-dependent pathway is inactive. As the p53 pathway was reported to be functional in HPV-positive cancer cells, this finding indicates clearly that the ability of the E6 oncoprotein to target p53 for degradation is required for the growth of HPV-positive cancer cells (Hengstermann *et al*. 2001).

Nuclear localization of p53 is essential for its tumour suppressor function. In contrast to most other ligases that act, or are believed to act in the nucleus, PARC (p53-associated parkin-like cytoplasmic protein), a RING domain ubiquitin ligase, directly interacts with p53 in the cytoplasm of unstressed cells. In the absence of stress, inactivation of PARC induces nuclear localization of endogenous p53 and activates p53-dependent apoptosis. Over-expression of PARC promotes cytoplasmic sequestration of ectopic p53. This suggests that PARC is a critical regulator in controlling p53 subcellular localization and subsequent function (Nikolaev *et al*. 2003).

PIRH2 (p53-induced protein, RING-H2 domain-containing) is a RING domain ubiquitin ligase that promotes p53 ubiquitination independently of MDM2. Expression of PIRH2 decreases the level of p53 protein and abrogation of endogenous PIRH2 expression increases the level of p53. Furthermore, PIRH2 represses p53 functions including p53-dependent trans-activation and growth inhibition. PIRH2, like MDM2 and COP1, participates in an auto-regulatory feedback loop that controls p53 function (Leng *et al*. 2003).

Using an osteosarcoma cell line, it was shown that TOPORS (topoisomerase I-binding arginine-serine-rich protein) could act on p53 as a RING finger-containing ubiquitin ligase. Over-expression of TOPORS was shown to result in a decrease in p53 protein expression (Rajendra *et al*. 2004). However, the exact role of TOPORS remains unclear, as it has also been shown to sumoylate p53, thereby abrogating its transcription activity. TOPORS was shown to associate with and stabilize p53, and to enhance the p53-dependent transcriptional activities of *CDKN1A*, *MDM2* and *BAX* promoters. Over-expression of TOPORS consequently resulted in the suppression of cell growth by cell cycle arrest and/or by the induction of apoptosis (Lin *et al*. 2005).

Although P300 is known as an acetyltransferase, it has been suggested that it could cooperate with MDM2 to induce p53 polyubiquitination. In the presence of MDM2, P300 could poly-ubiquitinate the p53 residues mono-ubiquitinated by MDM2, thus contributing to p53 degradation; in the absence of MDM2, P300 might only act as a p53 acetyltransferase and therefore stimulates the transcriptional activity of p53 (Kohn & Pommier 2005).

Apparently, multiple degradation pathways are employed to ensure proper destruction of p53. How can one explain the apparent redundancy of ubiquitin ligases? A possibility is that ubiquitin ligases are expressed or act optimally in different cell or tissue types. It is also possible that one or more of these ubiquitin ligases are involved in the maintenance of p53 levels in the non-stressed or basal state, while others act only after a stress-induced p53 is produced. It appears likely that each of these ubiquitin ligases form protein complexes in the cell and the associated proteins may well differ for each of these ligases, connecting them to different regulatory circuits.

Deubiquitination

USP7 (ubiquitin-specific protease 7, also known as HAUSP) has been shown to interact with p53, which can lead to p53 deubiquitination and stabilization. Its activity and global effect on p53 activity is, however, complex (see below).

Ubiquitin-independent p53 degradation

The proteasomal degradation of p53 is regulated by both (poly) ubiquitination, targeting p53 for degradation by the 26S proteasome and by a MDM2- and ubiquitin-independent process. This appears to be mediated by the core 20S catalytic chamber of the 26S proteasome and is regulated by NAD(P)H quinone oxidoreductase 1 (NQO1). NQO1 physically interacts with p53 in an NADH-dependent manner and protects it from 20S proteasomal degradation. Remarkably, the vast majority of NQO1 in cells is found in physical association with the 20S proteasomes, suggesting that NQO1

functions as a gatekeeper for these 20S proteasomes. By competing with NADH, NQO1 inhibitors including dicoumarol and various other coumarins and flavones induce ubiquitin-independent proteasomal p53 degradation and thus inhibit p53-induced apoptosis.

The NQO1 pathway plays a role in p53 accumulation in response to IR, as co-expression of NQO1-specific siRNA with p53 prevented the accumulation of the latter following IR. Escaping MDM2-mediated degradation is probably not sufficient for efficient p53 stabilization following IR, because p53 is still susceptible to 20S proteasomal degradation. In order to achieve efficient p53 accumulation following irradiation, NQO1-p53 interaction could be increased to eliminate p53 degradation by the 20S proteasomes. NQO1 might notably play a role in p53 accumulation under oxidative stress. Reactive oxygen species (ROS) are known to induce NQO1, which, in turn, reduces ROS. The ability of NQO1 to support p53 accumulation following oxidative stress may contribute to cellular defence mechanisms against ROS.

The core 20S proteasomes are abundant and ubiquitously present in the cells. They have been widely regarded as being incapable of degrading folded proteins and are therefore considered to be latent proteasomes. Degradation studies with natively unfolded proteins suggest that unstructured proteins might have an intrinsic capacity to enter the pore of the 20S proteasome. Furthermore, the unstructured protein even when flanked with well-structured regions is still susceptible to 20S proteasomal degradation. Therefore, a common feature of ubiquitin-independent and 20S proteasomal degraded proteins could be the presence of an unstructured protein region. Indeed, both the N- and the C-terminal regions of p53 have been identified as unstructured regions and could facilitate p53 degradation by the 20S proteasomes. p53 could be inherently unstable and degraded 'by default' by the 20S proteasome, unless stabilized by a molecule like NQO1. p53, when engaged in a large functional complex could be protected from 20S proteasomal degradation as a consequence of the masking of its unstructured regions. (For a review on NQO1 in p53 degradation, see Asher & Shaul 2005.)

The tumour suppressor $p19^{ARF}$, which inhibits the ability of MDM2 to target p53 for degradation (see below), also inhibits dicoumarol-induced p53 degradation. Therefore, $p19^{ARF}$ exhibits a double lock activity that inhibits p53 degradation by both the MDM2-dependent and the NQO1-regulated pathway, ensuring maximal p53 accumulation under certain physiological conditions.

Sumoylation

The p53 residue Lys386 may be sumoylated. SUMO (small ubiquitin-related modifier) is a ubiquitin-related protein that covalently binds to other proteins using a mechanism analogous to, but distinct from, ubiquitin. Protein inhibitor of activated STAT (PIAS)-1, PIASxα, PIASxβ, PIASy function as SUMO ligases for p53. In contrast to ubiquitination, sumoylation is not involved in protein degradation. Sumoylation affects target protein function by altering sub-cellular localization of the protein or by antagonizing other modifications (for example ubiquitination at the same acceptor site). Sumoylation most frequently correlates with decreased transcriptional activity and thus repression of target genes. PIAS proteins exert a strong repressive effect on p53-dependent transactivation (Schmidt & Muller 2002). It is thought that the physical association of MDM2 with p53 is important for the enhancement of SUMO conjugation to p53. However, mutant p53 that does not associate with MDM2 is still sumoylated, albeit at a reduced level.

Methylation

The p53 residue Lys372 may be methylated by the SET9 (SET domain-containing protein 9) methyltransferase. Methylated p53 is restricted to the nucleus and the modification positively affects its stability. SET9 regulates the expression of p53 target genes in a manner dependent on the p53-methylation site (Chuikov *et al.* 2004).

Neddylation

Unexpectedly, MDM2 was recently assigned a new role as neddylation ligase for p53. NEDD8 (neuronal precursor cell-expressed developmentally down-regulated protein 8) is a small ubiquitin-like protein. MDM2-dependent NEDD8 modification of p53 was shown to inhibit its transcriptional activity (Xirodimas *et al.* 2004).

ADP-ribosylation

Poly(ADP-ribosyl)ation is a reversible post-translational protein modification implicated in the regulation of a number of biological functions. It is

catalysed mainly by the enzyme poly(ADP-ribose) polymerase 1 (PARP-1). PARP-1 is rapidly activated by DNA strand breaks, which finally leads to the modulation of multiple protein activities in DNA replication, DNA repair and checkpoint control. PARP-1 may be involved in homologous recombination, and poly(ADP-ribosyl)ation of p53 represents one possible mechanism that activates p53 as a recombination surveillance factor (Wesierska-Gadek *et al*. 1996).

O-glycosylation

Addition of bulky residues such as sugar groups could disrupt p53 intramolecular interactions involving the basic region, thus activating DNA binding by p53 (Shaw *et al*. 1996).

Modulators of p53 activity

Besides those 'biochemical modificators' of p53 mentioned above, a considerable number of other proteins have been shown to interact with p53, thus underlining its crucial role in controlling cell fate. An extensive description of all these proteins (more than 100 have been identified) cannot be envisaged here, yet some of them will be discussed. Indeed, they illustrate how the activity of p53 may be quantitatively or qualitatively regulated, closely or remotely, by mechanisms allowing the finely tuned integration of various signals.

The key role of MDM2

As MDM2 is a major regulator of p53 level, it is not surprising that numerous proteins can modulate its own activity. This allows the integration of various stress signals.

Ribosomes, MDM2 and p53

The ribosomal proteins L5, L11 and L23 (RPL5, RPL11, and RPL23) lower MDM2 activity, thus preventing p53 ubiquitination and increasing its transcriptional activity. This suggests an important link between ribosomal biogenesis and p53 activity, perhaps highlighting a pathway that integrates the p53 response with protein synthesis (Coutts & La Thangue 2005). This link is also supported by the recent observation that another ribosomal protein, RPL26, is able preferentially to bind to the 5′ untranslated region of p53 mRNA after DNA damage and to enhance association of p53 mRNA with heavier polysomes, which increases the rate of p53 translation, induces G_1 cell-cycle arrest, and augments irradiation-induced apoptosis.

Growth factors, MDM2 and p53

AKT (v-akt murine thymoma viral oncogene homologue, also known as PKB/protein kinase B) is a serine/threonine kinase, which in mammals comprises three highly homologous members (AKT1-AKT3). AKT is activated in cells exposed to diverse stimuli such as hormones, growth factors (epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I)...), and extracellular matrix components. The activation mechanism occurs downstream of phosphoinositide 3-kinase (PI-3K), which is itself activated by phosphatidyl inositol triphosphate (PIP3). AKT signalling is believed to promote proliferation and increase cell survival by inhibiting apoptosis, thereby contributing to cancer progression. In agreement with this, phosphorylation of MDM2 at Ser166 and Ser188 by activated AKT results in inhibition of MDM2 self-ubiquitination and in its translocation into the nucleus where it reduces p53 activity (Milne *et al*. 2004). PTEN (phosphatase and tensin homologue), a dual specificity PIP3 phosphatase that antagonizes AKT signalling, is capable of blocking MDM2 nuclear translocation, thus preventing the negative effects of growth factors on p53 activity.

PTEN may be viewed as a tumour suppressor. In addition, PTEN appears to modulate *MDM2* transcription by negatively regulating its P1 promoter in a p53-independent manner (Chang *et al*. 2004). Indeed, the induction of MDM2 gene transcription by p53 requires the P2 promoter (Kohn & Pommier 2005). The induction of PTEN has been shown to be essential for p53-mediated apoptosis in mouse cells, underscoring the importance of the AKT survival signalling in determining the final outcome of the p53 response.

Oncogenes, MDM2 and p53

In the frequency of its disruption in human cancer, the *CDKN2A* (also known as *INK4A*/*ARF*) gene, located at 9p21, is second only to *Tp53* (Haber 1997). In fact, this locus encodes two proteins translated in alternate reading frames: $P16^{INK4A}$, a tumour suppressor, is a cyclin-dependent kinase inhibitor that acts upstream of retinoblastoma (RB) protein to promote cell-cycle arrest; $P19^{ARF}$ is more related to p53 activity. $P16^{INK4A}$ and

$P19^{ARF}$ are often co-deleted in tumour cells, as notably observed in the widely used, wild-type p53 MCF-7 breast cancer cell line (see Craig *et al*. 1998), but mice lacking $P19^{ARF}$ alone are highly susceptible to breast cancer (Haber 1997), thus underlining its importance.

$P19^{ARF}$ activates p53 by sequestering MDM2 into the nucleolus, thus preventing it degrading p53. The $P19^{ARF}$-p53 axis is critical for eliminating potential tumour cells containing deregulated oncogene expression. The adenoviral proteins E1A and MYC, when over-expressed, may promote apoptosis through p53 activation. By the same pathway, V-Ha-ras Harvey rat sarcoma viral oncogene homologue (HRAS) may induce cell senescence. It has been shown that $P19^{ARF}$ is strictly required to mediate these effects on p53 (Lowe 1999). $P19^{ARF}$ may also mediate the positive effects of beta-catenin on p53 activity (Harris & Levine 2005).

Interestingly, $P19^{ARF}$ also inhibits dicoumarol-induced p53 degradation. For instance, E1A, which stabilizes p53 by inducing $P19^{ARF}$, also inhibits dicoumarol-induced p53 degradation, which is mediated by NQO1. Therefore $P19^{ARF}$ exhibits a double lock activity that inhibits p53 degradation by both the MDM2-dependent and the NQO1-regulated pathway, ensuring maximal p53 accumulation under certain physiological conditions.

ABL, MDM2 and p53

ABL (v-abl Abelson murine leukemia viral oncogene homologue) is a ubiquitously expressed non-receptor tyrosine kinase and a critical factor that under physiological conditions is required for the maximal and efficient accumulation of active p53 in response to DNA damage. Mice that lack both p53 and ABL are not viable. ABL protects p53 by antagonizing the inhibitory effect of MDM2, an action that requires a direct MDM2 phosphorylation at Tyr394 by ABL, observed *in vivo* as well as *in vitro*. In addition, ABL has been shown to directly interact with p53 and could protect the latter from ubiquitination by other inhibitors of p53, such as the E6/E6AP complex that inhibits and degrades p53 in HPV-infected cells (Levav-Cohen *et al*. 2005).

MDM4 (MDMX), MDM2 and p53

MDM4 (mouse double minute 4, also known as MDMX) is a structural homologue of MDM2 that can bind to p53 and inhibit its transcription function. Knockout of MDM4 in mice results in embryonic lethality due to hyper-activation of p53. Thus, MDM4 is an essential regulator of p53 during embryonic development, which is not the case for MDM2. The current thought is that MDM4 inhibits p53 activity both directly and indirectly by facilitating the p53-MDM2 feedback loop. MDM4 alone does not promote p53 ubiquitination or degradation *in vivo*. However, formation of the MDM2-MDM4 heterodimer stimulates the ubiquitin ligase activity of MDM2 for itself and for p53, suggesting that MDM4 may serve as a regulator or cofactor of MDM2. Although the role of MDM4 in DNA damage-mediated control of p53 activity remains unclear, MDM2 is believed to target MDM4 for degradation after DNA damage, thereby increasing p53 activity (Coutts & La Thangue 2005, Pan & Chen 2005).

MDM4 also possesses the ability to inhibit 53-dependent transcription in an MDM2-independent manner. This could be a consequence of inhibition of P300/CBP-mediated acetylation of p53 (reviewed in Marine & Jochemsen 2005).

MDM4 over-expression can lead to transformation in cell culture; MDM4 gene amplification and over-expression have been observed in 5% of primary breast tumours, all of which retained wild-type p53. MDM4 is notably amplified and highly expressed in the widely used MCF-7, a breast cancer cell line harbouring wild-type p53, and siRNA-mediated reduction of MDM4 markedly inhibits the growth potential of these cells in a p53-dependent manner. Together, these results make MDM4 a putative drug target for cancer therapy (Danovi *et al*. 2004).

USP7 has been shown to interact with p53, which can lead to p53 deubiquitination and stabilization. However, it appears that total ablation of USP7 is indeed accompanied by an increase in p53 levels. In fact, USP7 may indirectly affect p53 activity and stability by associating with MDM2, leading to MDM2 stabilization. Furthermore, USP7 may also bind to MDM4, leading to its deubiquitination and stabilization. Of interest, the deubiquitination activity of USP7 towards MDM2 and MDM4 is impaired after DNA damage. Indeed, MDM2 and MDM4 phosphorylation by the DNA damage-activated ATM lowers their affinity for USP7, providing a possible mechanism for the instability of MDM2 and MDM4 after DNA damage. This example shows that USP7, MDM2, MDM4, and p53 entertain complex interactions (Meulmeester *et al*. 2005*a*,*b*).

The adenoviral protein E1A may stabilize p53 tumour suppressor through the activation of $P19^{ARF}$ (see above). E1A may also bind to MDM4 and form a complex with p53 in the presence of MDM4, resulting in the stabilization of p53 in a $P19^{ARF}$-independent manner. Although it has no effect on the p53-MDM2 interaction, E1A facilitates MDM4 binding to p53 and inhibits MDM2 binding to MDM4, resulting in decreased nuclear exportation of p53 (Li *et al*. 2004).

Gankyrin, MDM2 and p53

Gankyrin, also known as PSMD10 (proteasome 26S subunit, non-ATPase, 10), is an ankyrin repeat oncoprotein commonly over-expressed in certain carcinomas. Gankyrin has an anti-apoptotic activity in cells exposed to DNA damaging agents. Down-regulation of gankyrin induces apoptosis in cells with wild-type p53. Gankyrin binds to MDM2, facilitating p53-MDM2 binding, and increases ubiquitination and degradation of p53. Gankyrin also enhances MDM2 auto-ubiquitination in the absence of p53. Down-regulation of gankyrin reduced amounts of MDM2 and p53 associated with the 26S proteasome. Thus, gankyrin is a co-factor that increases the activities of MDM2 on p53 (Higashitsuji *et al*. 2005).

KAP1, MDM2 and p53

By interacting with MDM2, the nuclear co-repressor KAP1 (KRAB-associated protein 1, also known as TRIM28/tripartite motif-containing protein 28) inhibits p53 acetylation and promotes p53 ubiquitination and degradation. $P19^{ARF}$ competes with KAP1 in MDM2 binding and oncogene induction of $P19^{ARF}$ expression reduces MDM2-KAP1 interaction (Wang *et al*. 2005*a*).

RB1, MDM2 and p53

The RB1 (retinoblastoma 1) protein can be found in cells in a complex with MDM2 and p53, resulting in high p53 activity and enhanced apoptotic activity. RB1 is generally associated with the transcription factors E2Fs. By complexing to RB1, MDM2 allows the liberation of E2Fs. Both MDM2 and RB1 may be phosphorylated and inhibited by the cyclin E-cdk2 complex. Following DNA damage, activated p53 stimulates the synthesis of $P21^{WAF1/CIP1}$, the product of the *CDKN1A* gene. $P21^{WAF1/CIP1}$ inhibits the cyclin E-cdk2 complex, and this, in turn, acts positively upon the RB1-MDM2 complex that promotes p53 activity and apoptosis (apoptosis-selective auto-regulatory loop associated with RB1) (Yamasaki 2003, Harris & Levine 2005).

Of note, E2Fs not bound to RB1 contribute to p53 stabilization, notably by increasing transcription of $P19^{ARF}$, ATM and CHK2, and switches the p53 response from G_1 arrest to apoptosis, notably by up-regulating the expression of ASPP1, ASPP2, JMY and Tp53INP1, four pro-apoptotic cofactors of p53 (see below) (Hershko *et al*. 2005).

Interactions between p53 and p63/p73

p63 and p73 are highly related to p53. In contrast to p53, their genes are rarely affected by inactivating mutations. On the other hand, their targeted deletion causes severe developmental defects, in contrast to a deletion of *Tp53*. Hence, p63 and p73 appear responsible for biological effects that cannot be elicited by p53 alone. It has been speculated that, during the course of evolution, p63 and p73 have first pursued a broader range of activities, whereas p53 later specialized on genome maintenance (Blandino & Dobbelstein 2004).

A role of p73 in resistance to various drugs has been suggested (Melino *et al*. 2002). A complex network of interactions between p53, p63 and p73 has been demonstrated. p63 and p73 may exist as isoforms. Long isoforms (TAp63, TAp73) are able to transactivate the same target genes as p53, while short isoforms (DeltaN-p63, DeltaN-p73) have an opposite activity via dominant negative mechanisms. While common genes may be activated by p53 and p73, recent microarray analysis has, however, suggested that the cellular response induced by p73 during adriamycin treatment could involve specific genes, as suggested by microarray analysis (Vayssade *et al*. 2005)

Of interest, p53 has been shown to induce the expression of DeltaN-p73, at both the mRNA and protein levels, through a specific p53-responsive promoter element. This induction of DeltaN-p73 expression establishes an auto-regulatory feedback loop that keeps the trigger of cell death under tight control (Kartasheva *et al*. 2002).

Mechanisms of p53 apoptosis vs growth arrest – p53 apoptotic co-regulators

Apoptosis appears as the critical function of p53 in tumour suppression (Haupt *et al*. 2003, Yu &

Zhang 2005). The choice between growth arrest and apoptosis likely involves the complex interplay of numerous factors.

1. According to a quantitative model, genes involved in growth arrest contain high-affinity p53 binding sites in their promoter, while low-affinity sites are present in the promoter of apoptosis-related genes (Chen *et al.* 1996). This is in line with observations that increased levels or activity of p53 can lead to the onset of apoptosis, presumably by achieving a certain threshold level. Moreover, p53 mutants with marginally altered conformations retain sufficient activity to induce growth arrest but not apoptosis, presumably because they can still interact only with high-affinity sites. However, despite the degenerative nature of p53 binding sequences, the apoptotic targets of p53 do not necessarily contain low-affinity promoters. For example, chromatin immunoprecipitation experiments have revealed that the apoptotic gene *BBC3* contains high-affinity p53 binding sites (Kaeser & Iggo 2002). The quantitative model is thus not sufficient.
2. According to a qualitative model, the selective activation of the p53 apoptotic genes is mediated through the interaction of p53 with certain transcription co-activators. Several proteins may interact with p53 and specifically modulate apoptosis. For instance, ASPP1 (apoptosis stimulating protein of p53-1, also known as PPP1R13B/protein phosphatase 1, regulatory subunit 13B) and ASPP2 can both favour the interaction of p53 with the promoters of apoptotic genes *BAX* and *Tp53I3*/*PIG3*, but not that of *MDM2*, *CCNG1* or *CDKN1A* (Yu & Zhang 2005). The effects of ASPP1 and ASPP2 may be counteracted by iASPP (inhibitor of ASPP), the most conserved inhibitor of p53-mediated apoptosis. Both P63 and P73 are thought to favour selective binding of p53 to apoptotic promoters *BAX*, *PMAIP1*/*NOXA* and *PERP* (Yu & Zhang 2005), an effect that could be mediated through their interaction with ASPP1 and ASPP2 (Bergamaschi *et al.* 2004). DAXX (death-associated protein 6) is a transcriptional repressor of *CDKN1A* (involved in cell growth arrest), but it does not affect the activation of proapoptotic genes, and therefore acts by influencing the balance between cell cycle arrest and proapoptotic p53 targets (Gostissa *et al.* 2004). STAT1 (signal transducer and activator of transcription 1) can act as a co-activator of p53 to induce expression of *BAX*, *PMAIP1*/*NOXA*, and *FAS* (Yu & Zhang 2005).

As mentioned above, phosphorylation of the p53 residue Ser46 plays an important role in permitting the apoptotic function of the protein. The interaction between p53DINP1 and Ser46 may allow this phosphorylation.

Additional proteins do not interact directly with p53, but have been implied in its apoptotic function. JMY (junction-mediating and regulatory protein) interacts with P300 to enhance, selectively, the ability of p53 to induce expression of apoptotic genes such as *BAX* (Yu & Zhang 2005). STRAP (serine/threonine kinase receptor associated protein) was originally identified as a JMY-interacting protein. After DNA damage, its phosphorylation by activated ATM allows its localization to the nucleus. It is believed that this prompts p53 acetylation through recruitment of P300/JMY and the subsequent enhancement of p53 apoptosis (Coutts & La Thangue 2005).

E2F transcription factors may contribute to p53 stabilization by regulating genes such as $P19^{ARF}$, ATM and CHK2. In addition, E2F1 has been shown to up-regulate the expression of four pro-apoptotic cofactors of p53 – ASPP1, ASPP2, JMY and Tp53INP1 – through a direct transcriptional mechanism (Hershko *et al.* 2005).

Other interactors modulating the p53 transcriptional activity

Proteins that modulate p53 activity may exert their positive or negative effects through various ways that will not be discussed here.

Among positive regulators of p53 are 14-3-3σ (Yang *et al.* 2003), activating transcription factor 3 (ATF3, Yan *et al.* 2005), BRCA1-associated RING domain 1 (BARD1, Wu *et al.* 2006), breast cancer 1, early-onset (BRCA1, Fabbro *et al.* 2004), CCAAT-binding transcription factor 2 (CTF2, Uramoto *et al.* 2003), hypoxia-inducible factor 1 alpha (HIF1α, Fels & Koumenis 2005), high-mobility group box 1 (HMGB1, Banerjee & Kundu 2003), members of the ING (inhibitor of growth family) (Gong *et al.* 2005), nuclear factor Y (NF-Y, Imbriano *et al.* 2005), prohibitin (PHB, Fusaro *et al.* 2003), and STAT1 (Townsend *et al.* 2005).

Among negative regulators are bone marrow kinase, X-linked (BMX, Jiang *et al.* 2004), CCAAT/enhancer binding protein beta (C/EBPβ, Schneider-Merck *et al.* 2006), DNA methyltransferase-3a (DNMT3, Wang *et al.* 2005*c*), Kruppel-like factor 4 (KLF4, Rowland *et al.* 2005), SIN3 homologue A, transcription regulator (SIN3A, Zilfou *et al.* 2001), STAT3 (Niu *et al.* 2005), Y box-binding protein 1 (YB1, Homer *et al.* 2005), and YY1 transcription factor (YY1, Sui *et al.* 2004, Yakovleva *et al.* 2004).

Some of them may restrict p53 activity to specific promoters, for instance those of genes related to apoptosis.

The promyelocytic leukaemia (PML) protein is of specific interest. This tumour suppressor can selectively and dynamically recruit a number of proteins including p53 to form a sub-nuclear multi-protein chamber named PML-NBs, of which it is an essential component. After DNA damage, p53 is recruited into PML-NBs and modified by phosphorylations and acetylations, which in turn potentiate its transcriptional and pro-apoptotic activities. By sequestering p53, PML-NBs may regulate in a complex way its sub-nuclear distribution upon stress, thus allowing coordinate temporal patterns of p53-associated transcription (Bao-Lei *et al.* 2005, Coutts & La Thangue 2006).

Auto-regulatory loops in p53 action

The p53 pathway is intimately linked to other signal transduction pathways that may play a significant role in cancer. Most often, these pathways regulate entry of cells into the cell cycle. The coordination between p53 activity and these pathways may be ensured through a series of auto-regulatory loops. Here are some examples, notably based on the work of Harris & Levine (2005).

1. MDM2 is induced by p53. MDM2 promotes p53 degradation.
2. $P19^{ARF}$ down-regulates the MDM2 ubiquitin ligase activity, thus increasing p53 levels. Activated p53 down-regulates $P19^{ARF}$.
3. Activated P38 protein kinase increases p53. Activated p53 induces PPM1D which inactivates P38 by preventing its phosphorylation by the RAS pathway.
4. Activated p53 induces COP1 and PIRH2. These ubiquitin ligases contribute to p53 degradation.
5. Activated p53 induces DeltaN-P73. DeltaN-P73 represses p53 transcriptional activation.
6. MDM2 activity may be inhibited by phosphorylation on Thr216 (by the cyclin A/cdk2 complex). Activated p53 induces cyclin G; cyclin G makes a complex with PP2A phosphatase, which removes the phosphate at Thr216 and increases MDM2 activity, thus reducing p53 level (Ohtsuka *et al.* 2004).
7. Activated p53 induces the ubiquitin ligase, seven in absentia homologue (SIAH)-1. SIAH-1 degrades BETA-CATENIN, which is known to up-regulate $P19^{ARF}$ and, subsequently, to increase p53 levels.
8. Growth factors may activate AKT, which, in turn, phosphorylates and activates MDM2; it results in a decrease in p53 (survival pathway). p53 increases PTEN and PTEN decreases AKT activity.
9. Activated p53 induces 14-3-3σ. 14-3-3σ interacts with p53 and stabilizes it.
10. Activated p53 induces PML. PML helps to potentiate p53 activity.
11. PCAF is induced by p53. It contributes to p53 stabilization.
12. PPM1D is induced by p53. It may dephosphorylate both p53 and CHK1 (which may phosphorylate p53 at various sites), thus inactivating it (Lu *et al.* 2005).
13. BRCA1, CHK1 and CHK2 contribute to p53 activation upon stress. All three are down-regulated by activated p53 (Lohr *et al.* 2003, Matsui *et al.* 2004).

Mechanisms for loss of p53 activity in cancer

p53 is subject to tight regulation at multiple levels. In cancer cells, its function can be compromised by various mechanisms: mutations of Tp53, alteration of p53 regulators, alteration of p53 target genes.

p53 mutations

In humans, inheritance of a *Tp53* mutant allele results in a rare familial autosomal disorder, the Li–Fraumeni syndrome. It is characterized by a high incidence of multiple early cancers, including breast tumours.

However, most p53 mutations observed in breast cancer are of somatic origin. In fact, contrasting with the two p53 relatives p63 and p73 (Blandino & Dobbelstein 2004), p53 mutations are the most frequent genetic events in human cancer. They have been found in most types of tumours, with frequencies

ranging from 5% (cervix) to 50% (lung). Between 20 and 35% of breast tumours have been shown to express a mutant p53. However, most of the information on p53 mutations is derived from sequence analysis that included only exons 5–8 (residues 126–306) within *Tp53*, and examination of the whole p53 coding sequence is beginning to reveal an increasing number of mutations in the N- and C-termini of the protein (Vousden & Lu 2002). Nevertheless, the majority of p53 mutations appear to be localized in the DNA-binding domain, in the central part of p53. Notably, this domain is the binding site for ASPP1 and ASPP2, important cofactors in the transactivational activity of p53 in relation to apoptotic genes (see above). Most of the hot-spot p53 mutations render the protein unable to interact with ASPP1.

Since there is no evidence that *Tp53* lies in a hyper-mutable region of the genome, cells that have lost p53 function are likely to be selected during cancer development. In cells expressing a mutant p53, this protein is generally no longer able to control cell proliferation, which results in inefficient DNA repair and genetic instability. p53-deficient mice are developmentally normal but show a very high incidence of multiple early tumours and generally succumb before reaching the age of 1 year (Donehower *et al*. 1992). Moreover, when introduced into cells, a mutant p53 can transform and give to these cells a more aggressive phenotype.

The great majority of mutant p53s are defective in transactivation and may exert a dominant negative effect by preventing wild-type p53 from binding to the promoter of its target genes (Willis *et al*. 2004). However, it must be noted that not all p53 mutations are inactivating. For instance, some mutant p53s display only partial loss of their DNA binding activity, allowing the mutant to bind only to a subset of p53 response elements (Friedlander *et al*. 1996, Rowan *et al*. 1996). This has notably been observed with the mouse R172P mutation, equivalent to the human R175P alteration (Liu *et al*. 2004*a*), which is linked to differential transactivation ability. Biologically, such mutants have lost their apoptotic properties, but their cell cycle arrest activity remains similar to that of the wild-type protein. Along the same lines, it has been shown that p53 mutants can often trans-activate promoters containing a p53-responsive sequence like that found in *CDKN1A* (involved in growth arrest) but not like those present in *BAX* or *Tp53I3*/*PIG3* (involved in apoptosis) (Campomenosi *et al*. 2001). Regarding apoptosis, the hot-spot R175H p53 mutant was shown to strongly inhibit transcription of the *FAS* pro-apoptotic gene. This inhibition of transcription required binding of the mutant protein to a different promoter site from that recognized by wild-type p53. Other mutants (resulting from alterations of residues 248 and 273) have a similar, but less pronounced, property (Zalcenstein *et al*. 2003). Thus, several mutant p53s, in addition to preventing the apoptotic activity of a normal p53 (encoded by a non-mutated allele) could also exert anti-apoptotic actions.

In contrast, at least 18 mutant p53s expressing an apoptotic activity higher than that of wild-type p53 have been identified. The corresponding mutations tend to cluster at residues 121 (in the L1 loop – residues 115 to 135) or 290 to 292 (in the flanking region of the H2 helix). For instance, the S121F mutant is known as 'super' p53, due to its superior ability to induce apoptosis, as compared with wild-type p53 (Saller *et al*. 1999). The remaining 17 mutants are H214Q, K291E, K292T, Q144R, R290G, I162M, K291T, S121A, S121C, F212Y, E221Q, K291Q, S121Y, R156C, S215C, K292I and P153H. It has been shown that there was no significant correlation between their apoptotic property and their ability to activate transcription of six p53-responsive genes (*CDKN1A, MDM2, SFN,* and the apoptosis-related *BAX*, *p53AIP1*, *BBC3*). This suggests that transactivation-dependent mechanisms do not always play a major role in p53-dependent apoptosis (Kakudo *et al*. 2005). As expected, none of the super-apoptotic mutants described above is frequently observed in breast tumours.

Two alternative pathways that are either dependent or independent of the MDM2-ubiquitin-26S proteasome mediate proteasomal degradation of p53. The ubiquitin-independent pathway is regulated by NQO1 that prevents p53 degradation by the 20S proteasome. Compared with wild-type p53 and several mutants, the hot-spot p53 mutants R175H, R248H, and R273H were shown to exhibit increased binding to NQO1, and thus decreased degradation. However, they remained sensitive to MDM2-ubiquitin-mediated degradation. Thus, NQO1 has an important role in stabilizing some hot-spot p53 mutant proteins in human cancer. This could, at least in part, explain the relatively high steady state expression of these mutant proteins in cancer cells (Asher & Shaul 2005).

About 1400 p53 mutations observed in breast cancers are listed in the *Tp53* database maintained at the International Agency for Research on Cancer (IARC) (Olivier *et al*. 2004). The pattern

and codon distributions of p53 mutations in breast tumours show a very similar profile to all other cancers, including similar hot spots. Indeed, 34% of *Tp53* mutations affect only 10 residues – 175, 176, 179, 213, 220, 245, 248, 249, 273, and 282; three residues (175, 248 and 273) contribute 18% of mutations. More than 90% of all mutations affect the central core region (residues 103–292), which interacts with DNA. To date, only 2% and 5% of all mutations have been located to non-central regions 1–101 and 293–393 respectively. In breast cancer, there is an over-representation of TAC to TGC alteration at codon 163. This codon is rarely mutated in most cancers (less than 1%), but accounts for over 2% of all breast cancer mutations (Feki & Irminger-Finger 2004). The significance of this remains unknown.

Contrasting with other tumour-suppressor genes, p53 mutants are most frequently (~90%) missense ('point missense mutations'). Mutant p53 proteins generally have an increased stability and accumulate in the nucleus of neo-plastic cells. It is believed that this is a consequence of the inactive p53 mutant protein no longer driving the expression of the MDM2 protein required to target its own degradation. Immunohistochemical detection of the amount of nuclear p53 has long been used as an indicator of p53 alteration, but this parameter appears highly dependent on the type of mutation (see below).

The p53 status of numerous widely used breast cancer cell lines (Lacroix & Leclercq 2004*a*) has been determined. Some of these observations have been compiled at IARC (Olivier *et al*. 2004). Table 3 summarizes these data.

In agreement with the observations in tumours, the frequency of mutations in the central DNA-binding core is high in breast (see Table 3) and in non-breast cancer cell lines (O'Connor *et al*. 1997). Most mutations are missense. The percentage of cell lines with mutated p53 is higher than expected, based on the frequency of p53 mutations in breast tumours. It is possible that tumour cells expressing an altered p53 could be easier to establish in culture (for information about the bias in the process of cell lines isolation see Lacroix & Leclercq 2004*a*).

Alterations of p53 modulator and/or target proteins

The number of proteins able to interact with p53 or to be modulated by activated p53 is high. The qualitative and quantitative expression pattern of these proteins may vary from one tumour to another, thus composing a very complex picture that cannot be exhaustively detailed here.

The expression level of some proteins may reflect specific cell biology. It has been shown that cancer cells in oestrogen receptor (ER)-positive/low-grade/well differentiated breast tumours most often express a 'luminal-like' secretory phenotype, while those populating ER-negative/high-grade/poorly differentiated lesions express a 'basal/myoepithelial' portrait (Lacroix *et al*. 2004). Several effectors and targets of p53 have their expression restricted to one cell type and thus are found exclusively or at higher levels in the derived tumours. This is notably observed for P63, 14-3-3σ, IGF binding protein-3 (IGFBP3), and MASPIN (also known as SERPINB5), which are specific to the 'basal/myoepithelial', ER-negative phenotype and are expressed mainly in ER-negative/high-grade/poorly differentiated tumours (Shao *et al*. 1992, Lacroix & Leclercq 2004*a*, Lacroix *et al*. 2004, Simpson *et al*. 2004, Charafe-Jauffret *et al*. 2006). Other proteins related to the 'basal/myoepithelial' portrait and/or to high-grade steroid receptor-negative tumours are PERP, BARD1, and SURVIVIN (Singh *et al*. 2004, Span *et al*. 2004, Charafe-Jauffret *et al*. 2006, Wu *et al*. 2006).

On the other hand, the expression of BCL2, MDM2, MDM4, PTEN and USP7 has been associated with the 'luminal-like' phenotype of breast cancer cells (BCC) and/or tumours (Bozzetti *et al*. 1999, Kappes *et al*. 2001, Phelps *et al*. 2003, Garcia *et al*. 2004, Lacroix *et al*. 2004, Charafe-Jauffret *et al*. 2006).

The expression of other genes or proteins has been shown to be differentially regulated in breast tumours compared with normal breast tissue: *GADD45A*, *INK4A*/*ARF*, *PRG3* and *RPRM* are frequently down-regulated, while *RAI3* is often up-regulated in tumour tissues (Silva *et al*. 2003, Wu *et al*. 2004, Nagahata *et al*. 2005, Takahashi *et al*. 2005, Wang *et al*. 2005*b*).

In most cases, the absence of gene expression is associated with gene promoter methylation (Silva *et al*. 2003, Khan *et al*. 2004, Lacroix *et al*. 2004, Takahashi *et al*. 2005, Wang *et al*. 2005*b*).

Of interest, the expression of the pro-apoptotic factors, ASPP1 and ASPP2, is frequently down-regulated, while that of iASPP is frequently up-regulated in breast cancer. Thus, there could be a selective advantage for tumour cells to lose the expression of ASPP1 and ASPP2 and to gain iASPP (Liu *et al*. 2005).

Table 3 p53 mutations in breast cancer cell lines.

Cell line	Exon	Codon	Type	Nucleotide change	Residue change
BRC230	8	266	Nonsense	GGA → TGA	Gly (G) → Stop
BT-20	5	132	Missense	AAG → CAG	Lys (K) → Gln (Q)
BT-474	8	285	Missense	GAG → AAG	Glu (E) → Lys (K)
BT-483	7	246	Deletion (1 bp)		
BT-549	7	249	Missense	AGG → AGC	Arg (R) → Ser (S)
CAMA-1	8	280	Missense	AGA → ACA	Arg (R) → Thr (T)
EVSA-T	6	213	Nonsense	CGA → TGA	Arg (R) → Stop
HCC38	8	273	Missense	CGT → CTT	Arg (R) → Leu (L)
HCC70	7	248	Missense	CGG → CAG	Arg (R) → Gln (Q)
HCC1007	8	281	Missense	GAC → CAC	Asp (D) → His (H)
HCC1395	5	175	Missense	CGC → CAC	Arg (R) → His (H)
HCC1569	8	294 (*)	Nonsense	GAG → TAG	Glu (E) → Stop
HCC1806	7	256	Insertion (2 bp)		
HCC1937	8	306 (*)	Nonsense	CGA → TGA	Arg (R) → Stop
HCC2218	8	283	Missense	CGC → TGC	Arg (R) → Cys (C)
HDQ-P1	6	213	Nonsense	CGA → TGA	Arg (R) → Stop
HMT-3909	7	249	Missense	AGG → GGG	Arg (R) → Gly (G)
Hs578T	5	157	Missense	GTC → TTC	Val (V) → Phe (F)
MAST	7	244	Missense	GGC → AGC	Gly (G) → Ser (S)
MDA-MB-134-VI	8	285	Missense	GAG → AAG	Glu (E) → Lys (K)
MDA-MB-157	7	261	Deletion (26 bp)		
MDA-MB-231	8	280	Missense	AGA → AAA	Arg (R) → Lys (K)
MDA-MB-361	5	166	Nonsense	GAA → TAA	Glu (E) → Stop
MDA-MB-435	8	266	Missense	GGA → GAA	Gly (G) → Glu (E)
MDA-MB-436	6	205	Insertion (7 bp)		
MDA-MB-453	11	367 (*)	Deletion (30 bp)		
MDA-MB-468	8	273	Missense	CGT → CAT	Arg (R) → His (H)
SK-BR-3	5	175	Missense	CGC → CAC	Arg (R) → His (H)
T-47D	6	194	Missense	CTT → TTT	Leu (L) → Phe (F)
UACC893	10	342 (*)	Nonsense	CGA → TGA	Arg (R) → Stop
DU4475	Wild-type				
MCF-7	Wild-type				
MDA-MB-175-VII	Wild-type				
UACC-812	Wild-type				
ZR-75-1	Wild-type				
ZR-75-30	Wild-type				

(*) Mutation outside the central DNA-binding core.

Another source of differential gene expression in breast tumours is the occurrence of amplification/deletion events. For instance, *MYC* and *PPM1D* are frequently amplified in breast tumours, with a high-level copy number gain. *PPM1D* amplifications are found in aggressive primary lesions (Rauta *et al.* 2006). Interestingly, virtually none of the tumours with *PPM1D* amplification was shown to carry a p53 mutation, consistent with the idea that over-expressed PPM1D contributes to functional inactivation of p53, rendering its mutation unnecessary (Lu *et al.* 2005). *TERT* is another target of amplification at 5p12-p14. Other regulators of p53 activity may be amplified in breast cancer: MDM2, MDM4, COP1, CUL4A. MDM2 is amplified in ~5.7% of breast tumours (Al-Kuraya *et al.* 2004). MDM4 is amplified in ~5% of breast tumours, all of which have retained a wild-type p53 (Danovi *et al.* 2004). COP1 is over-expressed in a majority of breast tumours (~80%), of which most are negative for p53 (Dornan *et al.* 2004). CUL4A is amplified in 16% of primary breast cancers and over-expressed in 47% (Chen *et al.* 1998).

Other genes coding for important determinants of p53 activity may be deleted. This has notably been observed with *INK4A*/*ARF*, which is, for instance, deleted in the widely used MCF-7 BCC line (Craig

et al. 1998). *TNFRSF10A* is also frequently deleted in tumours (Naylor *et al*. 2005).

Hereditary breast cancer and p53

As mentioned above, *Tp53* mutations may be observed in the rare familial autosomal Li–Fraumeni syndrome. It is characterized by a high incidence of multiple early cancers, including breast tumours. Other hereditary breast cancers may be due to mutations in genes coding for p53 modulator proteins. A significant proportion of these cancers have been associated with mutations of *BRCA1*. BRCA1 may interact with p53 and has been viewed as a 'scaffold' for p53 response (Hohenstein & Giles 2003). Of interest, BRCA1 tumours often express *Tp53* mutations, but it remains to be established if this reflects the need for p53 inactivation for the development of BRCA1 tumours to occur, or rather if the loss of BRCA1-associated DNA repair properties may explain, at least partly, the high frequency of *Tp53* mutations (Lacroix & Leclercq 2005).

Other mutations leading to familial syndromes accompanied by a high occurrence of breast cancer may affect *BRCA2*, *ATM* (Ataxia-Telangiectasia), *CHEK2* (Li-Fraumeni-like syndrome), *STK11*/*LKB* (Peutz-Jeghers syndrome), or *PTEN* (Cowden syndrome) (Lacroix & Leclercq 2005). The products of two of these genes, *ATM* and *CHEK2*, are involved in p53 activation, while the product of *PTEN* increases p53 activity by antagonizing the cell survival effects mediated by the AKT-MDM2 pathway (see above).

p53 alterations, breast tumour characteristics, and prognosis

The potential relationships between p53 alterations and the expression of other tumour markers or pathological characteristics (grade) have been widely investigated. Many teams have also examined the value of p53 as a prognostic marker. The interpretation of data has, however, often been complicated by the fact that most initial studies used immunohistochemistry (IHC) to detect the amount of p53, while analysis of p53 mutations was performed by other investigators. The correlation between p53 accumulation measured by IHC and p53 mutation detected by sequencing has been estimated to be less than 75% in breast carcinomas (Norberg *et al*. 1998). Indeed, not all mutations yield a stable protein and some mutations lead to a truncated protein not detected by IHC. On the other hand, wild-type p53 may accumulate in some tumours as a result of a response to DNA damage or by binding to other cellular proteins, giving a positive IHC result.

Breast tumours expressing a high amount of p53 (as measured by IHC) are more frequently ER-negative and progesterone receptor (PgR)-negative. They are also associated with a high proliferation rate, high histological and nuclear grades, aneuploidy, and poorer survival. A high p53 level is frequently observed in tumours over-expressing ERBB2 (also known as Her-2/neu) (Feki & Irminger-Finger 2004).

The same relationships have been observed when p53 mutations were taken into account, instead of p53 accumulation. For instance, in a large (543 individuals) analysis of patients with node-negative breast cancer, p53 mutations were more frequent in breast carcinomas with amplification of the *ERBB2* gene (leading to ERBB2 over-expression). Patients with both p53 mutation and *ERBB2* amplification were associated with poor survival. The groups with p53 mutations (both with or without *ERBB2* amplification) were more likely to be ER- and PgR-negative, more likely to be grade 3 for both histological and nuclear grade, and less likely to have lobular subtype (Bull *et al*. 2004). The particularly bad prognosis associated with the coexistence of high ERBB2 and p53 alterations is supported by other studies (Rahko *et al*. 2003, Yamashita *et al*. 2004).

In a meta-analysis of more than 9000 patients, the prognostic and predictive value of high p53 expression in breast cancer, as evaluated by IHC, was found to be weak (Barbareschi 1996). On the other hand, more than 25 studies to date involving over 6000 patients have revealed the strong prognostic significance of p53 mutations (reviewed in Borresen-Dale 2003). A meta-analysis of 16 of these studies including over 3500 patients (Pharoah *et al*. 1999) confirmed that mutations in the *Tp53* confer a worse overall and disease-free survival in breast cancer cases, an effect that is independent of other risk factors. In several of the studies the presence of a *Tp53* mutation was the single most adverse prognostic indicator for both recurrence and death.

It seems that the prognostic significance of all types of mutations is not the same. Studies have shown that patients with mutations effecting or disrupting the zinc binding domains L2 and L3 (codons 163–195 and 236–251) or affecting amino acids directly involved in DNA binding, many of these

residing in the zinc binding domain, were related with the poorest prognosis (reviewed in Borresen-Dale 2003).

These findings indicate that not just p53 mutation *per se* but the full spectrum (i.e. different types, locations, and numbers) of p53 mutations needs to be examined when it is used as a prognostic marker of survival in breast cancer patients (Lai *et al*. 2004).

Recent technological advances have allowed the simultaneous evaluation of multiple RNAs (micro-arrays) or proteins (tissue arrays) in tumour samples or breast cancer cell lines. These studies have revealed that the breast tumours could be sorted into a very few classes characterized by the high level of expression of specific groups of genes/proteins. Moreover, these classes are 'stable', as most individual lesions largely maintain their 'portrait' when they evolve from *in situ* to the metastatic state (reviewed in Lacroix *et al*. 2004). The number of classes that have been defined in most micro-array-based or tissue array-based studies is three. About two-thirds of tumours express features characteristic of luminal cells. These lesions are often well differentiated, have a low grade and demonstrate relatively high levels of cytokeratins 8/18/19, ER, PgR, BCL2, CDH1 (E-cadherin), the three transcription factors GATA3, FOXA1, XBP1 (Lacroix & Leclercq 2004*b*), Treefoil factor (TFF)1 (pS2), TFF3, SLC39A6, $P21^{WAF1/CIP1}$, $P27^{KIP1}$, and cyclin D1. In contrast to the 'luminal-like' lesions, about 20% of tumours have a low level of the above cited markers, whereas they express relatively high levels of cytokeratins 5/6 and 17, CDH3 (P-cadherin), EGF receptor (EGFR), cyclin E, MIB1, MCM2, and other proliferation markers. Most of these 'basal/myoepithelial-like' tumours are poorly differentiated and have a high grade. Finally, tumours over-expressing ERBB2 as a consequence of gene amplification constitute a third class. It appears that p53 mutation is much more frequent in the 'basal/myoepithelial-like' and ERBB2 classes than in the 'luminal-like' one (82, 71 and 31% respectively, according to Sorlie *et al*. 2001). Moreover, the most well differentiated tumours have a very low level of p53 alteration (13% in Sorlie *et al*. 2001). Of note, up to 100% mutant p53 have been observed in medullary carcinoma, a specific subtype of breast cancer with a 'basal/myoepithelial-like' phenotype (de Cremoux *et al*. 1999).

The existence of breast tumour classes suggests that any tumour biology reflects to a large extent the biology of the cell of origin at the time of initiation. Tumours originating from more undifferentiated epithelial cells have a rapid growth pattern and more aggressive behaviour and outcome compared with those originating in more differentiated epithelial cells. Neoplastic progression might be p53-dependent in the tumours with a less-differentiated, 'basal/myoepithelial-like' phenotype and those over-expressing ERBB2, while it might be p53-independent in those tumours with a more differentiated, pure luminal form.

p53 alterations and response to therapy

Cell lines

Chemotherapy

O'Connor *et al*. (1997) correlated the endogenous p53 status of 58 cancer cell lines (lung, colon, breast, ovary, leukaemia, melanoma, kidney, prostate, CNS) of the National Cancer Institute (NCI) Anticancer Drug Screen with the growth-inhibitory potency of 123 anticancer agents, the majority of clinically approved cancer drugs at that time. These included: mitotic spindle poisons (microtubule inhibitors), such as paclitaxel and vincristine; anti-topoisomerase II, such as adriamycin (doxorubicin), morpholino-adriamycin and m-AMSA (amsacrine); anti-topoisomerase I, such as camptothecin; RNA antimetabolites, such as methotrexate and 5-fluorouracil; DNA antimetabolites, such as hydroxyurea and cytosine arabinoside; and alkylating agents such as carboplatin and cisplatin.

Of the 58 lines, 39 contained a mutant p53 sequence. The mutant protein was expressed at elevated basal levels in the majority of cases. In contrast to most of the wild-type p53-containing lines, cells containing a mutant p53 sequence were also deficient in γ-ray induction of $P21^{WAF1/CIP1}$, GADD45, and MDM2 mRNA and the ability to arrest in G_1 following γ-irradiation. This analysis revealed that lines with an endogenous mutant p53, while dramatically heterogeneous in their behaviour, still tended to be less sensitive than the wild-type p53 lines to most of the clinically used anticancer agents. Interestingly, however, mitotic spindle poisons were found to act independently from the p53 status.

Eight BCC lines were included in the NCI study. These were MCF-7, MCF-7/Adr (reported as MCF-7-derived cells, but their true origin remains questionable – see Lacroix & Leclercq 2004*a*), MDA-MB-231, Hs578T, MDA-MB-435 and its ERBB2-transfected derivative MDA-N, BT-549,

and T-47D. All cell lines except one, MCF-7, have a mutated p53. Two cell lines (MCF-7, T-47D) have a 'luminal-epithelial-like' phenotype, while the others have a more 'basal/myoepithelial-like' aspect (see Lacroix & Leclercq 2004*a*, de Longueville *et al*. 2005). Detailed examination of the sensitivity data (see http://www.broad.mit.edu/mpr/NCI60/GI50_RAW.txt) revealed that the most sensitive of all cell lines was MCF-7. All other BCC lines, except T-47D, were much less sensitive than MCF-7 cells. In fact, T-47D cells seemed to express an intermediary sensitivity pattern, suggesting that this feature may be only partially associated with the p53 status.

This is supported by a study in which two cell lines derived from basal epithelium (and immortalized) and two cell lines derived from luminal epithelium (MCF-7, ZR-75), all with wild-type p53, were treated with doxorubicin and 5-fluorouracil. Their transcriptional profile was thereafter analysed by microarray. While all cell lines expressed signatures of general stress response, distinct expression patterns were observed. Both luminal-like and basal-like types induced DNA damage response genes such as *CDKN1A*, but the response in the luminal cells showed higher fold changes. Luminal-like cell lines repressed a larger number of cell cycle regulated genes and other genes involved in cellular proliferation, whereas the basal-like cell lines did not. Instead, the basal-like cell lines repressed genes that were involved in differentiation. The two luminal-like cell lines showed similar response patterns to one another including the strong induction of DNA damage stress response genes, notably *CDKN1A* (Troester *et al*. 2004). Thus, despite expressing a similar p53 status, luminal epithelial-like cells seem to respond to at least two chemotherapeutic drugs to a higher qualitative and quantitative extent than basal/myoepithelial-like cells.

The role of p53 in modifying sensitivity to cytotoxic drugs has been commonly studied by creating transfection pairs of wild-type p53 parental cells and altered p53 daughter cells, or *vice versa*. Cimoli *et al*. (2004) performed a meta-analysis of 356 independent studies. Average changes of drug sensitivity after a change of p53 status were observed. These authors observed agreements between the data of O'Connor *et al*. (1997) and theirs, but the correspondence was only partial. The higher sensitivity of wild-type p53 versus mutant p53 lines to cytotoxic drugs (O'Connor *et al*. 1997) was in agreement with the finding that transfection with a wild-type p53 tends to increase sensitivity (Cimoli *et al*. 2004). However, unexpectedly, the reciprocal seemed not to be true, as transfection with a mutated p53 did little to change the drug sensitivity of most wild-type p53 cancer lines. Rather interestingly, cells transfected with a wild-type p53 and treated with mitotic spindle poisons did not follow the general trend of an increased sensitivity. In addition, in the opposite model (from a wild-type to an altered p53), mitotic spindle poisons tended to induce a modest (about 1.7 times) but statistically significant relative sensitization with respect to the remaining drugs. This is only in partial agreement with the NCI analysis, where mitotic spindle poisons seemed essentially p53-status insensitive. A crucial indication of these findings is that the role of p53 alone in determining sensitivity/resistance to cytotoxic drugs is limited: the individual molecular pathology and differentiation of a given cancer line prevail over any average trend, and are causal to a broad spreading of the data.

Radiotherapy

There are few studies examining and comparing the radio-sensitivity of breast cancer cell lines. We thus tested the viability of six of these cell lines after exposure to γ-rays. Table 4 describes the effect of a single 8 Gy dose on their viability, as assessed 96 h post-irradiation by the Crystal violet staining test.

Among these lines, the first three (MCF-7, ZR-75-1 and T-47D) are ER-positive and express a 'luminal-like' phenotype. The three others are ER-negative. The BT-20 cells have an amplified *EGFR* gene, while *ERBB2* is amplified in SK-BR-3 cells (Lacroix & Leclercq 2004*a*).

While the two wild-type p53 cell lines were sensitive to irradiation, as expected *a priori*, the T-47D cells also expressed a high sensitivity. This

Table 4 Effect of a single 8 Gy γ-rays dose on the viability of six breast cancer cell lines, as assessed 96 h post-irradiation by the Crystal violet staining test.

Cell lines	p53 status	% cells, as compared with control
MCF-7	Wild-type	<50%
ZR-75-1	Wild-type	<50%
T-47D	Mutant	<50%
MDA-MB-231	Mutant	~80%
BT-20	Mutant	~80%
SKBR-3	Mutant	~80%

suggests that p53-independent parameters, including cell origin, could be partly responsible for the observed effects.

It has been shown that activation of the PI-3K/AKT pathway ('growth factor pathway', see above) prevented radiation-induced apoptosis in breast cancer cells. BT-474 BCC, which over-express ERBB2 and have mutated p53 were resistant to G_1 arrest and apoptosis caused by irradiation. However, apoptosis following irradiation was significantly increased in these cells after treatment with the PI-3K inhibitor, wortmannin. On the other hand, pre-treatment of MCF-7, which have normal expression of ERBB2, with the ERBB2 ligand and PI-3K/AKT activator, heregulin-beta1, decreased apoptosis compared with the untreated controls. Furthermore, transfection of MCF-7 cells with constitutively active AKT made the cells more resistant against apoptosis. Thus, the PI-3K/AKT signalling pathway is involved in resistance to radiation-induced apoptosis in breast cancer cells in which this signalling pathway is over-stimulated (Soderlund *et al*. 2005). This seems to be, at least partly, p53-independent. Of note, EGFR over-expression may also induce the activation of the PI-3K/AKT signalling pathway. This activation could explain why we observed that BT-20 and SK-BR-3 cells were resistant to radiation.

Tumours

Chemotherapy

There is evidence from *in vitro* (Lowe *et al*. 1993) and animal studies (Lowe *et al*. 1994) that defective p53 is associated with resistance to chemotherapy. Furthermore, loss of p53 function correlates with multidrug resistance in many tumour types (Wallace-Brodeur & Lowe 1999).

It has been suggested in the past that p53 abnormalities could not be used as a predictor of a response to therapy (see, for instance, Elledge & Allred 1998). Indeed, the great majority of studies performed at this time were based on IHC detection of over-expressed p53. They either argued against a predictive role for p53 status or were not conclusive. For instance, several neoadjuvant studies have failed to detect a predictive value to p53 staining with regards to chemo responsiveness in breast cancers (MacGrogan *et al*. 1996, Niskanen *et al*. 1997, Bonetti *et al*. 1998, Rozan *et al*. 1998). However, the p53 over-expression detected by IHC does not necessarily correlate directly with p53 mutations. In fact, this lack of sensitivity and specificity account, in part, for the incongruity of these findings (Cleator *et al*. 2002, Feki & Irminger-Finger 2004).

Bergh *et al*. (1995) examined a series of 316 consecutively presented breast cancers. These authors found 69 internal mutations. Mutations in the conserved regions II (codons 117–142) and V (codons 270–286) were associated with worse prognosis. Adjuvant systemic therapy, especially with tamoxifen, together with radiotherapy, appeared of less value to tumours with a p53 mutation.

In a study of 243 patients with advanced breast cancer and receiving either tamoxifen or upfront chemotherapy, Berns *et al*. (2000) found that mutations in codons that directly affected DNA binding or within the zinc binding domain L3 showed the lowest response to tamoxifen (202 patients). p53 mutations were also associated with a poor, although not significant response to chemotherapy (cyclophosphamide/Methotrexate/5-fluorouracil (CMF) in 22 patients; cyclophosphamide/Adriamycin/5-Fluorouracil (CAF) in 16 patients; doxorubicin in 1 patient; platinum-containing chemotherapy in 2 patients).

p53 detected by IHC has recently been shown to be associated with worse clinical outcome, irrespective of ER status, in a study of 97 postmenopausal patients with axillary lymph node metastasis treated with an antioestrogen for a period of 3 years after primary surgery and radiotherapy. Thus, adjuvant therapy with antioestrogens appears insufficient in this patient population with p53-positive tumours (Rahko *et al*. 2006).

In a study of 63 patients with locally advanced breast cancers receiving doxorubicin in a neoadjuvant study, there was strong evidence that specific mutations disrupting the zinc binding domains correlate with primary resistance to the drug, and the presence of such mutations was predictive of an early relapse (Aas *et al*. 1996). These findings were further supported in an updated study from the same group including 90 patients (Geisler *et al*. 2001). Of note, a number of these mutations were not associated with enhanced staining for p53, which would explain why immunohistochemical studies have been inconclusive.

Geisler *et al*. (2003) also investigated 35 patients with locally advanced breast cancer for Tp53 mutations before receiving combination chemotherapy with 5-fluorouracil and mitomycin. Mutations in the *Tp53* gene, in particular those affecting loop domains L2 or L3 of the p53 protein, were associated with lack of response to chemotherapy. On the other

hand, no statistically significant correlation between *Tp53* loss of heterozygosity (LOH) and response to therapy was seen. Together with the previous finding that such mutations predict resistance to doxorubicin, these data suggest that mutations affecting this particular domain of the p53 protein may cause resistance to several different cytotoxic compounds applied in breast cancer treatment.

In another study, p53 staining and mutations were studied in relation to the response of 67 breast tumours to neoadjuvant 5-fluorouracil epirubicin cyclosphate (FEC) or paclitaxel chemotherapy. In the FEC group, treatment failure was related to both the presence of Tp53 gene mutations and a positive IHC. Apoptosis was almost exclusively found in tumours having normal p53 in both parameters. In the paclitaxel group, treatment response was neither related to apoptosis nor to normal p53. Combination of sequencing and IHC results revealed a significant association between abnormal p53 and response to paclitaxel. The efficiency of paclitaxel during mitosis might be supported by lack of G_1 arrest due to p53 deficiency. This suggests that patients with p53-deficient tumours may benefit from paclitaxel (Kandioler-Eckersberger *et al*. 2000).

Rahko *et al*. (2003) examined the predictive relevance of a mutated p53 in a series of 254 samples from primary breast cancer patients. The response rate to anthracycline-based chemotherapy in metastatic disease was low in the p53-positive cases.

It has recently been suggested that the status of codon 72 polymorphism (resulting in a Pro or an Arg) could affect the response of cancer cells to chemotherapy, notably through a different interaction between p53 and P73 (Bergamaschi *et al*. 2003). For instance, breast cancer patients with the Pro/Pro variant may be less sensitive to anthracycline-based treatment than those with the Pro/Arg or Arg/Arg variant (Xu *et al*. 2005). More generally, the response of cancer cells to chemotherapy could be influenced not only by p53, but also by the status of a network that contains p53, p73 and perhaps the closely related p63. However, interactions between these three proteins are expected to be cell type-dependent (i.e. p63 is expressed mainly in basal/myoepithelial breast cells (Matos *et al*. 2005) and its role remains to be clearly established in breast cancer).

In summary, p53 mutations, particularly those affecting the DNA binding core regions, are generally associated with tumour cell resistance to chemotherapeutic drugs, with the notable exception of mitotic spindle poisons. However, drug sensitivity of tumour cells might be related to additional parameters, as suggested by the higher qualitative and quantitative gene expression response of luminal cells to doxorubicin and 5-fluorouracil. The exact mechanisms underlying these different behaviours remain to be elucidated.

Radiotherapy

It is presently unclear whether the p53 status may provide an advantage in resistance to radiotherapy. Clinical studies examining the relationship between clinical radiosensitivity and tumour p53 status have largely failed to demonstrate a significant effect. Thus, many factors other than p53 status are expected to determine the sensitivity of tumour cells to irradiation (Ross 1999).

Additional comments on p53 and therapy

p53 isoforms

The difficulties in linking p53 status to the biological properties and drug sensitivity of cancer cells could be partly explained by the recently discovered differential expression of the p53 isoforms in cancer. Indeed, as previously observed for *Tp63* and *Tp73*, *Tp53* contains an alternative internal promoter in intron 4 and can transcribe 9 splice variants. p53 isoforms can bind differentially to promoters and can mediate p53 target gene expression and apoptosis. However, the pattern of isoform expression may vary from tumour to tumour (Bourdon *et al*. 2005), generating a complex landscape of possibilities.

p53 as a survival factor during therapy

According to a common view, p53 should sensitize tumour cells to therapy, as p53 is expected to trigger apoptotic events. However, it is likely that in some tumours the apoptotic function of p53, either mutated or not, could be lost, but not the ability of the protein to direct prolonged cell growth arrest and DNA repair. One may speculate that such a mechanism could explain why tumours expressing a mutant p53 are generally more sensitive to paclitaxel and other mitotic spindle poisons. In these cases, p53 could favour the recovery of cells damaged by therapy, thus acting as a survival factor preventing mitotic catastrophe, and p53 inhibitory therapies could be envisaged. Prediction

of tumour response to p53 inhibitors would require determination of the status of p53 in the tumour and, specifically, whether it can function to induce apoptosis (Gudkov & Komarova 2003, 2005).

p53-independent apoptosis

p53-independent apoptosis in response to IR and chemotherapy exists. It may be the consequence of mitotic catastrophe, which occurs after extended DNA damage. The mechanisms of mitotic catastrophe are unknown, but it likely results from a combination of deficient cell-cycle checkpoints (in particular the DNA structure checkpoints and the spindle assembly checkpoint) and cellular damage (Castedo *et al*. 2004). For instance, it has been shown that the primary mechanism of death in BCC lines exposed to the mitotic spindle poison docetaxel was mitotic catastrophe, as determined by scoring of micro nucleated cells and cells undergoing aberrant mitosis (Morse *et al*. 2005). More generally, there are indications that cells of epithelial tumours may often die by mitotic catastrophe during radiation therapy and chemotherapy (Hendry & West 1997).

Besides p53, another important determinant of breast cancer cell apoptosis is nuclear factor kappa B (NF-κB). It exerts strong anti-apoptotic functions in cancer cells. Many studies have demonstrated that inhibition of NF-κB activity by different means increased sensitivity of cancer cells to the apoptotic action of diverse effectors such as tumour necrosis factor-α (TNF-α) or chemo- or radiotherapies (Magné *et al*. 2006). Activation of NF-κB has been associated with ER negativity in tumours and cell lines. For instance, NF-κB was found to be constitutively active in the ER-negative MDA-MB-231 and MDA-MB-435 BCC, but not in the ER-positive MCF-7 and T-47D cell lines. This could partly explain the increased sensitivity of these latter two cell lines to most drugs and to IR (Nakshatri *et al*. 1997). Constitutive DNA binding of NF-κB was also observed with extracts from ER-negative, poorly differentiated primary breast tumours. As these tumours are frequently p53 mutated, some resistances attributed to p53 could, in fact, be due to NF-κB (Zhou *et al*. 2005).

The environment of tumour cells may also play a role in modulating the p53 response in these cells. For instance, activators of the PI-3K/AKT and NF-κB pathways might be produced by normal cells in the vicinity of tumour cells and contribute to the resistance of these cells to therapy.

p53 pathway-based therapies

The importance of p53 in cell death and the high frequency of mutations affecting this protein have generated a significant interest in exploiting the p53 pathway for novel cancer therapies. Various approaches have been exploited.

Small compounds have been used for the restoration of p53 function to lesions that carry full-length p53 protein with one amino acid change in the DNA-binding core domain. In theory, such compounds should only have an effect on cancer cells, because the core domain of wild-type p53 in normal cells is already structurally intact. Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole), the styrylquinazoline CP-31398, and PRIMA-1 (2,2-Bis(hydroxymethyl)-1-azabicyclo[2,2,2]octan-3-one) have been shown to restore function to a subset of p53 mutants (Foster *et al*. 1999, Bykov *et al*. 2002, Peng *et al*. 2003). PRIMA-1 (for p53 reactivation and induction of massive apoptosis-1) may synergize with chemotherapy (cisplatin) in inducing apoptosis in tumours, indicating the potential advantage of combined therapies (Bykov *et al*. 2005*a*). Screening of a chemical library identified another small molecule named RITA (for reactivation of p53 and induction of tumour cell apoptosis). It prevents p53-MDM2 interaction *in vitro* and *in vivo* and has anti-tumour activity (Issaeva *et al*. 2004). The maleimide-derived molecule, MIRA-1, can reactivate DNA binding and preserve the active conformation of mutant p53 protein *in vitro* and restore transcriptional transactivation to mutant p53 in living cells. The structural analogue MIRA-3 shows anti-tumour activity *in vivo* against human mutant p53-carrying tumour xenografts in SCID mice (Bykov *et al*. 2005*b*).

Attempts have been made to disrupt the p53-MDM2 interaction, thereby enhancing p53 activity. The first evidence that this approach could be successful came from peptide studies that culminated in the discovery of an optimized p53 octapeptide (Boettger *et al*. 1997). This, however, as well as the fungal metabolite cyclic peptide chlorofusin, was found to be poorly efficient *in vivo*. Small molecules have been designed to competitively inhibit the p53-MDM2 interaction. They include the following: synthetic chalcones (1,3-diphenyl-2-propen-1-ones), norbornane derivatives, cis-imidazoline derivatives (nutlins), a pyrazolidinedione sulphonamide, 1,4-benzodiazepine-2,5-diones, tryptophan derivatives, and the nine amino-acid peptide CDB3. The most promising drugs seem to

be the nutlins, as they have been shown to activate selectively the p53 pathway both *in vivo* and *in vitro* in human tumour cell lines that possess wild-type p53 and over-express MDM2, thus leading to growth inhibition and apoptosis. However, they appear to be significantly less cytotoxic to cancer cell lines harbouring mutant p53 (reviewed in Fischer & Lane 2004).

Chemosensitization of cancer cells has been obtained with anti sense oligonucleotides targeting the *MDM2* gene, which may inhibit the proliferation of tumour cells that possess wild-type as well as mutant p53 (reviewed in Bianco *et al.* 2005, Zhang *et al.* 2005). Radiosensitization has also been observed in cell lines from various cancers exposed to an anti-MDM2 oligonucleotide (Zhang *et al.* 2004). On the other hand, the use of an *MDM2* siRNA has also proved successful in inhibiting p53-dependent breast cancer (Liu *et al.* 2004*c*), suggesting that such molecules could be promising gene-specific drugs.

A 'global suppressor motif' involving codons 235, 239, and 240 has been identified in p53. With changes in these three amino acids, Baroni *et al.* (2004) were able to rescue 16 of 30 p53 cancer mutants. These rescued mutants are located within the beta-sandwich (codons 141, 157, 158, 163, 205, and 220), the L2 loop (codon 173), the L3 loop (codons 245 and 249), and the loop–sheet–helix motif (codons 272, 273, and 286), supporting the idea of a suppressor motif with a global rescue mechanism. Understanding the structural basis of this mechanism will allow the pursuit of small compounds able to achieve a similar stabilization of p53 cancer mutants.

ONYX-015 is a replication-conditional adenovirus. It induces wild-type p53 response, which halts viral replication and allows the cell to survive. However, in cells expressing a mutant p53, ONYX-015 replicates freely, causing cell death. A similar lethal effect is observed if wild-type p53 expression is abrogated by high MDM2 expression (an indirect way of p53 inactivation in some tumours). Although ONYX-015 as a single agent did not impress in initial clinical trials (in head and neck, ovarian, prostate, and lung cancers), it is being actively pursued in combination with chemo- and radiotherapy (Haupt & Haupt 2004, Stoklsa & Golab 2005).

Most p53-based therapeutic approaches aim to restore p53 function. However, in some tumours, p53 could have lost its apoptotic function but not its ability to direct prolonged cell growth arrest and DNA repair. In such cases, p53 could favour the recovery of cells damaged by therapy and prevent them inducing a mitotic catastrophe. Thus, p53 inhibitory therapies could be of interest in such cases. One molecule able to inhibit p53 activity is pifithrin-α. (Gudkov & Komarova 2003, 2005), but it seems to have limited solubility (Gary & Jensen 2005). There is thus a need for additional specific and stable p53 inhibitors.

Learning how p53 controls apoptosis through its targets might help devise better cancer therapeutics and prognostic tests. For example, the expression of p53 apoptotic targets might predict the prognosis in p53 gene therapy or other therapies designed to reactivate p53 in tumour cells. Unlike p53, most p53 apoptotic targets are relatively rarely mutated in human cancer. Therefore, small molecules that can activate these genes independent of p53 might afford new anticancer therapies. Some of the p53 apoptotic targets, such as *PUMA*, exhibit higher potency in apoptosis induction than p53. They can potentially be used as targets for identifying such small molecules, or as targets for gene therapy (Yu & Zhang 2005). Interestingly, in a recent study, core biopsies were taken from nine patients with locally advanced breast cancer, before and at 6 h after initiation of doxorubicin-based chemotherapy. Both samples were co-hybridized on the same microarray containing 18 000 cDNA spots. The analysis revealed marked differences in gene expression profile between treated and untreated samples. The gene that was most frequently found to be differentially expressed was *PUMA*. This gene was up-regulated in eight of nine patients with an average factor of 1.80 (range, 1.36–2.73). Another p53-regulated gene, *FXDR*, was also found to be induced. *In vitro* MCF-7 breast cancer cells exposed to clinically achievable doxorubicin concentrations for 6 h also revealed marked induction of *PUMA* mRNA, together with *Tp53INP1* (Middelburg *et al.* 2005). Another potential candidate for therapy (notably based on siRNA) is *RAI3* (Nagahata *et al.* 2005).

General conclusion

The crucial role of p53 as a mediator of stress in various cell types is demonstrated; however, its contribution to breast cancer has been difficult to evaluate. Indeed, the number of functions that it controls, the diversity of its mutations, the multiplicity of the proteins constituting its 'interactome', and the genetic variability inherent to cancer cell

progression may result in a tumour suppressor effect as well as an oncogenic action of p53. As an illustration of this complexity, the link between p53 and prognosis and prediction remains largely unclear, despite numerous studies.

Further investigations are needed to determine under which conditions a therapeutic approach targeting p53 could be of real benefit to breast cancer patients. The potential importance of this approach is, however, underlined by the number of compounds that are being developed to increase p53 level and/or to correct the mutant protein.

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Preclinical study

Interaction between estrogen receptor alpha, ionizing radiation and (anti-) estrogens in breast cancer cells

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Key words: breast cancer, estrogen receptor, ionizing radiation, *in vitro*

Summary

Purpose. Estrogen receptor alpha (ERα) plays a major role in breast cancer development. It acts as ligand-inducible transcription factor which determines growth, survival and differentiation of breast cancer cells. The aim of this study is to evaluate the potential interference between radiotherapy and estrogen receptor responsiveness.

Materials and methods. The effect of ionizing radiation was assessed on the estrogen receptor alpha status, growth (proliferation and apoptosis) and sensitivity of MCF-7 breast cancer cells to estrogenic (17β-estradiol (E_2)), selective estrogen receptor modulator (SERM) and anti-estrogenic compounds.

Results. We have observed a ligand-independent decrease in ERα expression after radiation, resulting from a specific reduction in mRNA level and protein synthesis. This ERα disappearance occurred 72 h post-irradiation at 8 Gy and decreased the transcriptional activity in ERα of these cells. On the other hand, E_2 impedes the growth inhibitory effects (essentially on proliferation) of ionizing radiation in MCF-7 cells, which potentially decreases radiosensitivity of these cells. This effect was totally blocked by SERM and anti-estrogenic treatments. Moreover, this growth effect of concurrent anti-estrogenic drugs and ionizing radiation appeared to be strongly synergistic.

Conclusions. This study may increase general comprehension of ERα modulation by radiotherapy and improve adjuvant therapeutic approaches based on co-administration of radiation and endocrine therapy.

Introduction

Breast cancer is the most common cancer affecting women in Europe and the USA: 12.5% of women will develop breast cancer during their life [1]. More than 300,000 new cases are diagnosed every year in Europe [2]. According to the American Cancer Society, 216,000 new cases should be observed in 2004 in the USA (www.cancer.org), with an incidence increasing steadily.

A majority (70%) of breast tumors express the alpha subtype of the estrogen receptor (ERα) [3] and ERα status is used as a predictive factor for hormone therapy. ERα-positive tumors have a 60–80% overall response rate to the SERMs (i.e. tamoxifen) [4]. The presence of ERα is also associated with increased survival and longer disease free intervals.

Primary treatment is normally breast-conserving surgery or mastectomy, in association with axillary's surgery and radiation therapy. Numerous prospective and retrospective trials demonstrated that a breast conserving surgery, consisting of segmental mastectomy with or without axillary's lymph node dissection, is equivalent to mastectomy in terms of overall and disease free survivals for patients with early stage breast carcinoma. Radiotherapy prevents local recurrence of breast cancer after breast-conserving surgery [5–7]. Both hormone therapy and adjuvant radiotherapy remain major treatments to manage early breast cancer [4]. Recently, the clinical advantage of concomitant hormonotherapy/radiotherapy in terms of efficacy has been suggested [8].

Until now, no rational clinical attitude has been proposed regarding the combination of hormone and adjuvant therapy. Biological explanation from *in vitro* studies does not provide any suggestion due to the large variety of investigated experimental conditions [9]. The potential impact of radiation on the ERα level and associated transcriptional activity is not established. In this regard, we reported in a previous study a decreased of estrogen binding ability in MCF-7 breast cancer cells irradiated at time of plating [10]. In the present paper, we further analyzed the impact of ionizing radiation on estrogen receptor expression and associated transcriptional activity under standard cell growth condition (i.e. exponential cell growth). The ligand-induced cell growth modulation was also assessed.

* These authors contributed equally to this work and should be considered as joint first authors.

Materials and methods

Chemicals

L-[^{35}S]-methionine (>1000 Ci/mmol), [^{3}H]-E_2 (88 Ci/mmol), [^{32}P]-dCTP were purchased from Amersham Biosciences (Buckinghamshire, UK). E_2, 4-hydroxytamoxifen (4-OH-TAM), PMSF (phenylmethylsulfonyl fluoride), TPCK (tosyl-L-phenylalanine-chloromethylketone) and agarose-bound anti-rabbit IgG antibody were from Sigma (St Louis, MO) whereas ICI 182, 780 was provided by Tocris (Illkirch, France). F-10 mouse monoclonal anti-human ERα antibody raised against F domain, D-12 mouse monoclonal anti-human ERα antibody raised against a recombinant protein corresponding to amino acids 2-185 mapping at the A/B domain of ERα, and HC-20 rabbit polyclonal anti-human ERα antibody raised against F domain were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). BCA protein assay kit, peroxidase-labeled goat anti-mouse IgG antibody was obtained from Pierce (Rockford, IL).

Cell culture

MCF-7 cells were routinely grown in basal EMEM medium supplemented with 10% inactivated FCS (fetal calf serum), 100 UI/ml streptomycin and 100 μg/ml penicillin. For experiments, cells were cultured in basal EMEM medium without phenol red and supplemented with 10% inactivated and charcoal-dextran stripped FCS.

Determination of the cytotoxic effects

MCF-7 cells were seeded in 96-well microtitration plates (1000 cells/well) to maintain cell growth during the whole duration of the experiment. Forty-eight hours later, cells were exposed to single agent (E_2; ICI 182,780; 4-OH-TAM); concentration ranges were as follows: $1\ nM < [E_2] < 100\ nM$, $1\ \mu M < [ICI\ 182{,}780] < 100\ \mu M$ and $1\ \mu M < [4\text{-OH-TAM}] < 100\ \mu M$.

Irradiation was performed, at room temperature, 48 h after plating using high energy photons from a linear accelerator 18 MV (Clinac, Varian Medical Systems) with 4 Gy/min. Medium was removed and replaced by fresh medium at time of irradiation. Dose effect curves were established using a total of 7 doses: 0.5, 1, 3, 5, 8, 10, 15 Gy. Cells were maintained in basal EMEM supplemented with 10% inactivated and charcoal-dextran stripped FCS during all radiation exposures.

At the end of the experiments, cells were gently washed once with PBS, fixed with 1% glutaraldehyde/PBS (15 min, 20 °C) and stained with 0.1% crystal violet (w/v in ddH_2O) (30 min, 20 °C). Excess of crystal violet dye was then removed by three washes of running tap water (15 min, 20 °C) and cells were lysed with 0.2% Triton X-100 (v/v in ddH_2O) (90 min, 20 °C, under agitation). The absorbance was measured at 550 nm using Microplate Autoreader EL309 (BIO-TEK Instruments).

Assessment of the effect of compounds combinations and/or radiation by isobolographic method for drug associations ICI 182,780 or 4-OH-TAM and/or E_2 in combination with γ-ray irradiation

Dose–response interactions between ICI 182,780 or 4-OH-TAM and/or E_2 in combination with ionizing radiations at 30, 50 and 75% cell growth inhibition ((IC30), (IC50) and (IC75)) were evaluated using crystal violet dye and results were analyzed by the classical isobolographic method described by Steel and Peckham [11]. The theoretical basis and procedure of the isobologram method have been described in details [12]. For a given level of efficacy (% survival) an 'envelope of additivity' curve was calculated from the dose effect curves of each compound (or drug combination) and from the dose effect curves of ionizing radiations (three doses). The coordinates of the experimental point are the drug concentration and the radiation dose which, when combined, give the level of efficacy. If the experimental point falls above, beyond or under the limits of the envelope of additivity, compounds and radiation combination give rise to antagonistic (Ant), additive (+) or synergistic (Syn) effects, respectively.

Cell cycle analysis

MCF-7 cells were maintained 48 h in estrogen-free medium before treatment. At the end of the experiment cells were trypsinized and washed twice with PBS. Cells were mixed thoroughly and stained using Coulter DNA-Prep reagent kit (Beckman Coulter, FL). Briefly, cells were resuspended in 50 μl of reagent A (15 s, 20 °C, under vortex agitation) and incubated in staining solution (950 μl of reagent B). After incubation (2 h, 4 °C, dark) cell cycle was analyzed with a Beckman FACS calibur analyzer and WinCycle software (Phoenix Flow Systems, San Diego, CA).

Determination of apoptosis

Apoptosis was determined by Annexin V staining (Biosource, Belgium). After treatment, cells were trypsinized and rinsed twice with PBS. Cell pellet was then incubated in Annexin V binding buffer in presence of 5 μl of Annexin V solution and 10 μl of Propidium iodide solution (15 min, 20 °C, in the dark). Staining solution was then discarded and replaced by Annexin V binding buffer. Annexin V positive, Propidium iodide negative cells (i.e. apoptotic cell) were detected by flow cytometry analysis using coulter xl cytometer.

ER binding determination

ER binding was measured by whole cell binding assays [13]. At the end of the treatment, cells were incubated

with increasing concentrations of [^{3}H]-E_2 (10^{-9} M to 5×10^{-11} M) with or without a 500-fold excess of unlabeled E_2 (1 h at 37 °C). Cells were then washed twice with PBS buffer, bound [^{3}H]-E_2 was extracted in 250 µl ethanol (20 min, 20 °C) and radioactivity was measured by scintillation counting. Results were analyzed by Scatchard plot and the binding capacity (B_{max}) was expressed in fmol/mg of protein (measured in cell extracts by BCA protein assay kit, Pierce).

EIA measurement of ERα

Total cellular extracts were used for determination of ERα content. MCF-7 cells were plated in 175 cm^2 Pétri dishes. At the end of the treatment, cells were washed twice in Hank's balance salt solution (HBSS), harvested by incubation with 1 mM EDTA, pelleted by centrifugation (800 × g, 10 min, 4 °C) and washed twice in phosphate buffer (10 mM K_2HPO_4, pH 7.4, 10 mM thioglycerol, 1.5 mM EDTA, 10% glycerol). Cells were then homogenized in phosphate buffer with a Teflon glass potter and extracts were clarified by ultracentrifugation (100,000 × g, 30 min, 4 °C). ERα amounts were measured by the Abbot Enzyme Immunoassay (ER-EIA), according to the manufacturer's instructions.

Western blots

Western blots were performed as previously described [14]. Briefly, cell cultures were washed with TBS and lysed for 30 min at 4 °C in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Igepal, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM NaF, 0.6 mM PMSF, 0.1 mM orthovanadate, 0.3 mM TPCK). Each sample (20 µg) was then loaded onto 5% stacking/12% running SDS polyacrylamide gel and transferred onto nitrocellulose membrane. Blots were incubated with primary antibody (1:1000 dilution, overnight, 4 °C). Detection was performed using a goat anti-mouse secondary antibody (1:2000 dilution, 1.5 h, room temperature) and an Western Pico Detection system.

Assessment of ER turnover

MCF-7 cells were plated in 100 mm Ø Pétri dishes (2×10^5 cells per dish). At the end of the experiment, cells were fed with MEM without L-methionine (2 h, 37 °C) and then exposed to 10 nM L-[^{35}S]-methionine (3 h, 37 °C). Five hundred micrograms of total protein extracts were submitted to immunoprecipitation [14]. Briefly, pre-cleared samples were incubated with a ER polyclonal antibody (HC-20, 2 µg, overnight, 4 °C) and the ER-antibody complexes were precipitated by incubation with an anti-rabbit agarose-bound IgG (45 µl, 2 h, 4 °C); Denatured samples were submitted to SDS–PAGE electrophoresis (4% stacking/12% running). Gels were then fixed (acetic acid 10%, methanol 40%, distilled water 50%) (30 min, 20 °C) and washed with distilled water (30 min, 20 °C). Radioactive signals were amplified by salicylic acid solution (1 M salicylic acid, 40% ethanol in distilled water) (90 min, 20 °C). Finally, gels were dried and submitted to fluorography to detect radio-labeled ER bands (67 kDa) (3 days, −80 °C) with hyperfilm MP (Amersham Biosciences, Buckinghamshire, UK).

ERα degradation. After labeling (as described above), cells were rinsed twice and allowed to grow in fresh medium containing unlabeled methionine for 0, 1, 2 or 3 h before harvesting (chase experiments). Remaining [^{35}S]-labeled ERα were quantified as describe above.

Northern blot analysis

Northern blot analysis was performed as previously described [15]. At the end of the experiment, total RNA was extracted with TriPure according to the instructions of the manufacturer (Roche). Total RNA (15 µg) was separated on a 1% agarose gel in 2.22% formaldehyde, 0.02 M 3-(*N*-morpholino) propane sulfonic acid (MOPS) and 1 mM EDTA before transfer onto a nylon membrane (*Hybond-N*, Amersham) and UV-cross linked. Pre-hybridization (4 h) and hybridization (18 h) were performed at 42 °C (at room temperature when using the 28S ribosomal oligonucleotide) in 50% formamide, 5 × SSPE (20 × SSPE = 0.2 M phosphate buffer (pH 7.4), 2.98 M NaCl, 0.02 M EDTA), 0.1% SDS, 10% 5 × Denhard, 5% dextran sulfate, and 100 µg/ml sheared salmon sperm DNA. Hybridized membranes with TFF1 cDNA probe (probe was obtained from American Type Culture Collection) were washed twice at room temperature in 2 × SSC (1 × SSC: 0.15 M NaCl and 15 mM sodium citrate, pH 7.0), 0.1% SDS, followed by three washes in 1 × SSC 0.1% SDS at 65 °C. The filters hybridized with the 28S ribosomal RNA oligonucleotide probe (Clontech) were washed only at room temperature in 2 × SSC 0.1% SDS. To account for variations in RNA loading, TFF1 values were normalized to 28S rRNA values. All membranes were exposed in autoradiography for various periods of time to ensure that only signals obtained in the linear range of film sensitivity were quantified. To rehybridize filters, former probes were first removed by incubating the membranes in pure water at 85 °C.

NASBA analysis

NASBA analyses were kindly performed by BioMérieux (Lyon, France). Briefly, 5 ng of RNA was added to 10 µl of NASBA buffer (final concentration in 20 µl reaction mixture: 40 mM Tris HCl, pH 8.5, 12 mM $MgCl_2$, 70 mM KCl, 5 mM dithiothreitol, 15% v/v DMSO, 1 mM of each dNTP, 2 mM of each NTP, 0.2 µM of ESR1 primers, 0.2 µM of PPIB primers and 0.1 µM of each gene specific molecular beacon), pre-incubated at 65 °C for 2 min, followed by 2 min at 41 °C. Five µl of enzyme mix (0.08 U RNase H, 32 U T7-RNA polymerase, 6.4 U RT) was then added to start the RNA amplification, and incubated at 41 °C for

90 min. ESR1 mRNA levels were normalized to PPIB mRNA levels. The GenBank accession numbers were X03635 for ESR1 and M60857 for PPIB.

Statistical analysis

Differences between the mean values were evaluated using either one-way ANOVA with Tukey's test or one-way ANOVA on ranks with Dunnetts' or Student–Newman–Keuls test, according to data distribution. $p = 0.05$ was considered as statistically significant. All analyses were carried out with the SPSS software (Paris, France).

Results

Impact of ionizing radiation on ERα content

Effect of radiation on [^{3}H]-E_2 binding parameters in MCF-7 cells (B_{max}, Kd) was analyzed by Scatchard plot (Figure 1a). A time-dependent decrease of binding capacity (B_{max}) was recorded in cells exposed to 8 Gy while B_{max} remained constant in non-irradiated cells. This inhibition became detectable 72 h after irradiation and reached 50% after 96 h. No statistically significant modification of B_{max} was observed for lower doses (3 and 5 Gy) (data not shown). Radiation did not affect the Kd value along the experiment, which remained around 1 nM indicating no change of the binding affinity of the remaining receptor for the hormone.

Influence of radiation on ERα content was then evaluated by EIA (Figure 1b) and Western blot (Figure 2) at the most efficient γ-rays dose of 8 Gy. EIA values indicated an ER loss (30% at 72 h and 50% at 96 h post-radiation, $p = 0.01$ and 0.00001, respectively) which was confirmed by Western blot analyses using F-10 antibody (raised against N-terminal domain of ERα). Indeed, densitometric quantification of the ERα (67 kDa) gave similar results as EIA quantification (significant decrease at 72 and 96 h with $p = 0.001$ and 0.001, respectively; Figure 2). Same results were obtained using antibody raised against C-terminal domain (D12 antibody) (data not shown), excluding the possibility of protein cleavage. Altogether our results indicated that 8 Gy radiation decreases ERα protein level in MCF-7 cells.

Ionizing radiation decreases ERα synthesis

In order to determine whether the 8 Gy radiation induced ERα loss was due to an inhibition of its synthesis or an accelerated degradation, receptor turn over rate was quantified by a [^{35}S]-methionine labeling

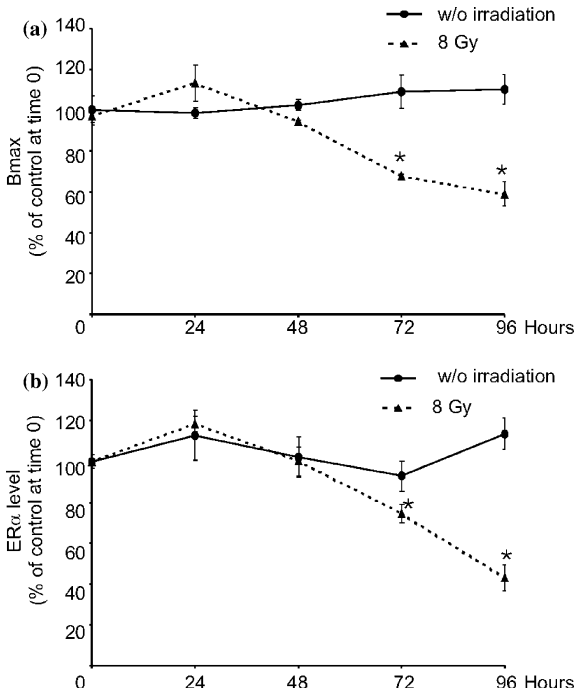

Figure 1. Effects of 8 Gy on the ER binding site content (B_{max}) and ERα expression. (a) Cells were irradiated 24, 48, 72, 96 h prior determination of ER binding site constant (B_{max}). B_{max} were obtained from Scatchard plot analysis and were expressed in % of control (= non-irradiated cells at time 0, $B_{max} = 526 \pm 37$ fmol/mg of proteins). Data refer to the mean value of three independent experiments performed in duplicate. Statistical significance of data was calculated by student *t*-test (irradiated versus non-irradiated sample for each experimental condition), $*p < 0.05$. (b) Cells were cultured up to 96 h after irradiation and whole cell ERα content was measured by EIA. Results were expressed in % of control (= non-irradiated cells at time 0, control = 575 ± 25.4 fmol/mg of proteins). Data refer to the mean value of three independent experiments performed in duplicate. Statistical significance of data was calculated by student *t*-test (irradiated versus non-irradiated sample for each experimental condition), $*p < 0.05$.

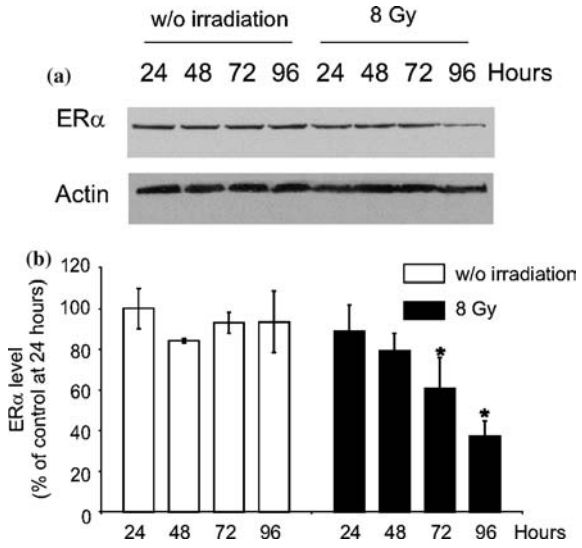

Figure 2. Western blot analysis of ionizing radiations on the ERα expression in MCF-7 cells. (a) ERα expression were detected 24–96 h post-irradiation by Western blot. Immunoblotting were performed using antibodies raised against N_T or C_T of ERα protein. Immunoblots were representative of three independent experiments. Equal loading was controlled by β-actin probe (see Materials and methods). (b) Immunoblots were submitted to densitometry analysis using Bio-Rad Quantityone Software. Results were representative of the three independent immunoblot analyses and expressed as percentage of control (non-irradiated cells at 24 h) corrected by actin value for each lane. Statistical significance of data was calculated by student *t*-test (irradiated versus non-irradiated sample for each experimental condition), $*p < 0.05$.

method (Figure 3). Radiation provoked a slight decrease of [^{35}S]-methionine ERα labeling (25%, 72 h post-irradiation, $p = 0.01$), which was more pronounced after 96 h (45%, $p = 0.001$). This ERα labeling decrease was not abolished by a proteasome inhibitor (10^{-6} M of MG-132, data not shown) suggesting that radiation affected ERα synthesis rather than its degradation by ubiquitin/proteasome pathway. In agreement with this view, pulse-chase experiment performed on [^{35}S]-methionine pre-labeled cells (Figure 4) failed to show any faster elimination of the remaining labeled ER after irradiation (maintenance of a 3 h period for the nearly total elimination of the receptor).

We next examined the effect of ionizing radiations on ERα mRNA expression by NASBA analysis (Figure 5). While no significant modification in ERα mRNA amount was observed at 72 h, 8 Gy induced a 50% decrease of ERα mRNA at 96 h as compared to non-irradiated cells (control), confirming that ionizing radiation affects ERα production.

Ionizing radiation fails to affect ERα transcriptional activity of the remaining receptor

Expression of TFF1 (pS2) (ERα reporter gene) was measured to evaluate the potential impact of ionizing radiation on its transcriptional activity. A slight increase of TFF1 mRNA levels was detected in basal conditions both at 72 and 96 h post-irradiation as already reported by Balcer-Kubiczek et al. [16]. A decrease of E_2-induced TFF1 level at 96 h closely related to the radiation induced ERα loss was also found, indicating that radiation did not modify transcriptional activity of the remaining receptors (Figure 6).

Ionizing radiation decreases E_2-induced cell growth

Potential impact of radiation on cell growth response to E2, 4-OH-TAM and ICI 182,780 was evaluated. As shown in Figure 7, 96 h post-irradiation, E_2-induced cell growth was significantly decreased by 33, 45 and 66% at 3, 5 and 8 Gy respectively. Moreover, growth rate of such E_2-exposed cells over passed the basal growth rate in irradiated cells, indicating that the hormone rescued cell growth. This growth rescuing action was abrogated by SERM or pure anti-estrogen (4-OH-TAM, ICI 182,780; at 100 nM) suggesting that it was relevant to a regulatory mechanism involving ER. The irradiation did not affect the growth inhibition by 4-OH-TAM or ICI 182,780 when tested alone (Figure 7). Interestingly, the protective effect of 17-β-estradiol seems to be

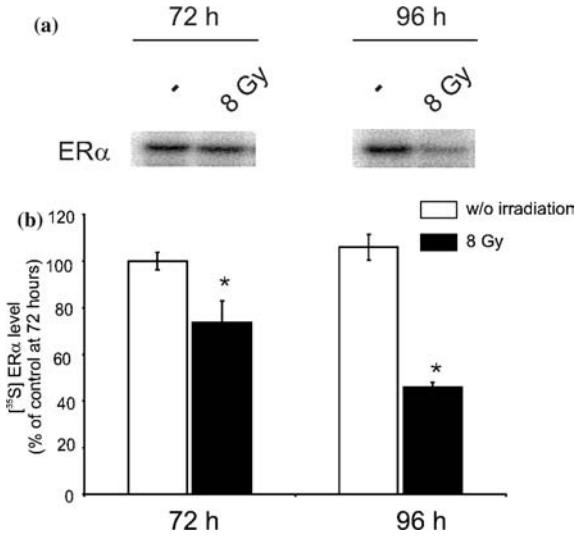

Figure 3. Effect of ionizing radiations on ERα protein synthesis in MCF-7 cells. 72 or 96 h prior irradiation, MCF-7 were incubated with 10 mM [^{35}S]-methionine for 3 h. At the end of the incubation, cells were lysed and immunoprecipitated ER was submitted to SDS–PAGE electrophoresis and fluorography. (a) Representative fluorography. (b) Densitometry analysis of three independent fluorography. Statistical significance of data was calculated by student *t*-test (irradiated versus non-irradiated sample for each experimental condition), $*p < 0.05$.

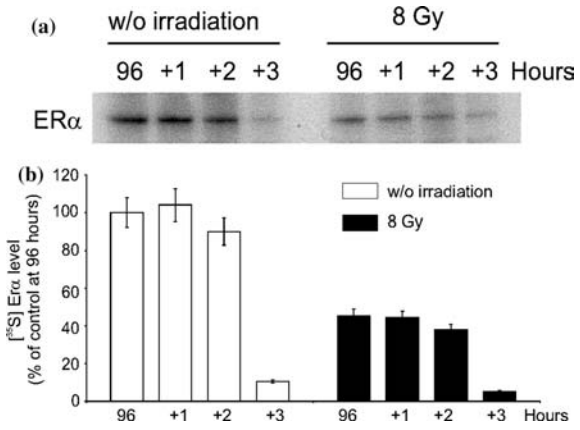

Figure 4. Effect of ionizing radiations on ERα protein degradation in MCF-7 cells. 72 or 96 h prior irradiation, MCF-7 were incubated with 10 mM [^{35}S]-methionine for 3 h. At the end of the incubation, cells were rinsed twice with non-radioactive medium and allowed to grow. Cells were then lysed and immunoprecipitated ER was submitted to SDS–PAGE electrophoresis and fluorography after 1, 2 or 3 h. (a) Representative fluorography. (b) Densitometry analysis of three independent fluorography. Statistical significance of data was calculated by ANOVA one way (irradiated and non-irradiated sample for each experimental condition), $*p < 0.05$.

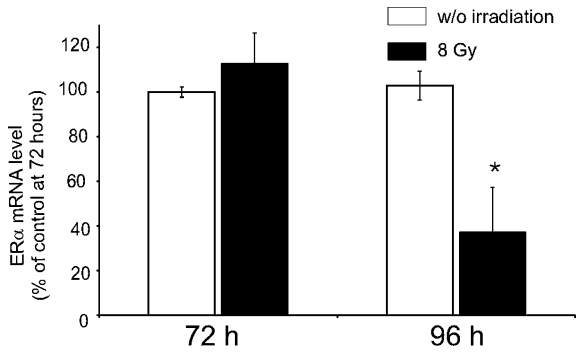

Figure 5. Effect of ionizing radiations on ERα mRNA amount. ERα mRNA levels were evaluated by NASBA after 72 or 96 h post-irradiation. Results were representative of the two independent analyses and expressed as percentage of control (non-irradiated cells) corrected by PPIB mRNA amount for each sample. Statistical significance of data was calculated by student *t*-test (irradiated versus non-irradiated sample for each experimental condition), $*p < 0.05$.

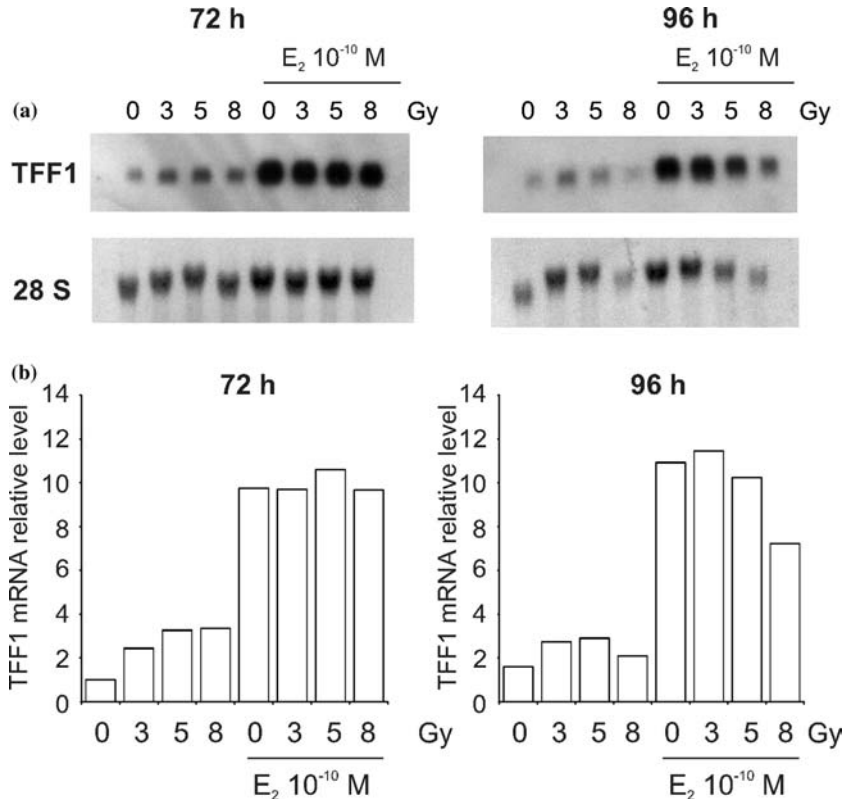

Figure 6. Effect of ionizing radiations on expression of TFF1. MCF-7 cells were cultured for 8 h without or with 10^{-10} M of E_2 prior to the end of experiments (72 or 96 h post-irradiation). (a) Basal rate and E_2-induced level were evaluated by Northern blot. The 28S levels are shown as internal control. Results representative of two independent experiments. (b) Results from densitometry analysis of the data shown in 'a' (normalized by the 28S RNA value).

maintained, even 10 days post-irradiation as measured in clonogenic assay. Indeed, at 8 Gy, MCF-7 cells were only able to grown in colonies in presence of 0.1 nM 17-β-estradiol; at lower irradiation doses, the hormone increased the number of colonies (data not shown).

The combination index (CI) at 30, 50 and 75% cell lethality are given in Table 1. Combination of ionizing radiations and E_2 conferred a strong antagonistic effect, while combinations with 4-OH-TAM and ICI 182,780 gave more often additive effects for 3 and 5 Gy and synergistic effects for 8 Gy.

17-β-estradiol impedes radiation cell cycle blockade

The effects of E_2 in cell cycle progression and apoptosis induction were determined after 8 Gy γ-rays exposure. As shown in Figure 8, radiation increased the percentage of cells in G0/G1 and G2/M after 48 h, indicating a cell cycle blockade in G0/G1 and G2/M phase as previously described [17]. 17-β-estradiol abrogated cell cycle blockade. Moreover, it still allowed cell cycle progression but to a lower extend than in non-irradiated cells. No significant modification of apoptosis induction under ionizing radiation was recorded both in the absence or presence of E_2 (Table 2); confirming the lack of potential correlation between radiosensitivity and apoptosis induction [17,18].

Discussion

The data reported here reveals two major findings: (1) 8 Gy-ionizing radiation induces a decrease of ERα synthesis but has no impact on receptor functions in MCF-7 breast cancer cells. Residual receptors seem to be unaffected, leading to the maintenance of a cell sensitivity to (anti)-estrogenic stimulation. (2) 17-β-estradiol may impede the cell growth inhibitory effect of 8 Gy-ionizing radiation.

Previous studies have revealed a loss of estrogen binding capacity without evidence of ER loss [9]. Difference in experimental protocols (i.e. radiation after plating (previous) versus during exponential growth phase (present)) may explain this partial discrepancy. Potential reasons for ER loss are multiple. Ionizing radiations are essentially characterized by DNA lesions which activate ATM/BRCA1 reparation pathways. Indeed, ATM and BRCA1 act as sensors of genetic alterations and activate downstream targets, like P53 and $P21^{waf1/cip1}$, responsible of the cell cycle arrest in G0/G1 or G2/M phases [17]. Changes in these transduction pathways interfere with ERα expression and/or activation providing possible explanation of our data. Thus, BRCA1 is a major co-repressor of ERα transcriptional activity. It may act by decreasing ERα binding to its target promoters and/or also inhibit transcriptional activity of ERE (estrogen response element) bound

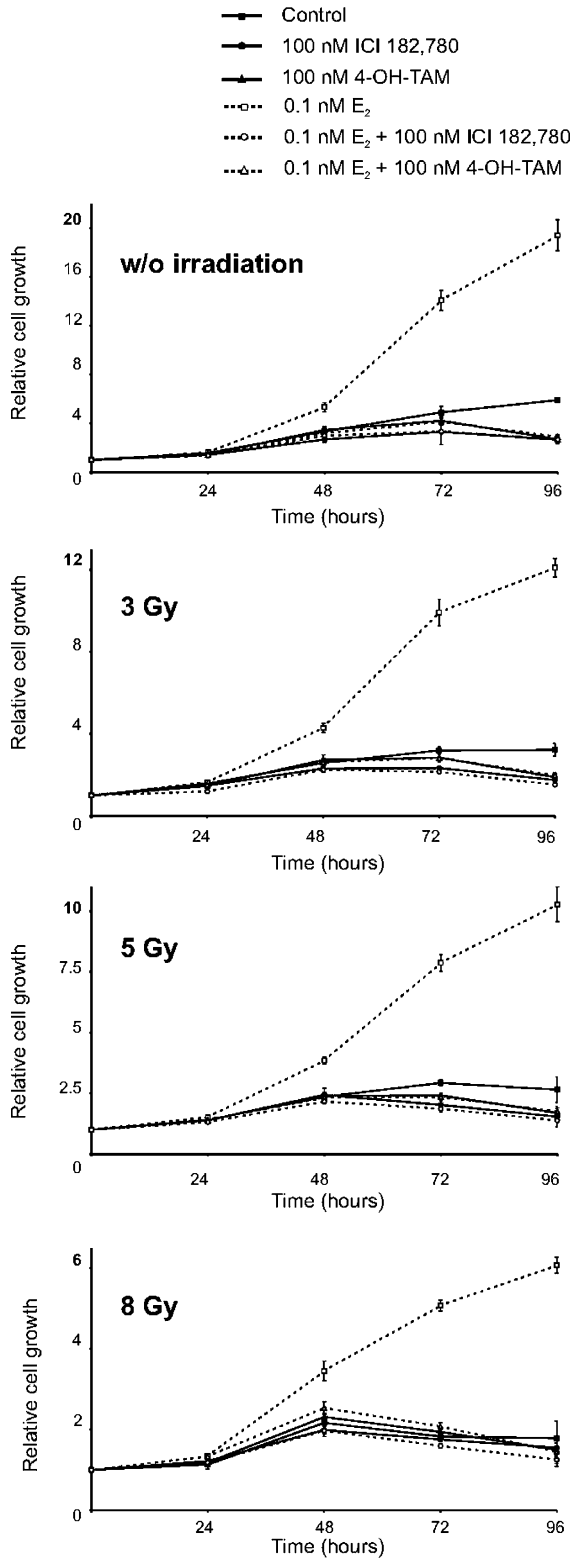

Figure 7. Effect of ionizing radiations on MCF-7 cell growth induced by E_2. Cells were irradiated (3, 5, 8 Gy) and subsequently maintained in culture for 96 h either in the absence or presence of estrogenic or/and anti-estrogenic ligands (E_2 at 0.1 nM, 4-OH-Tam at 100 nM, ICI 182,780 at 100 nM). Non-irradiated cells were cultured in parallel. Growth was estimated by crystal violet staining each day after irradiation. Results were expressed as a relative cell growth. Cell number at the time of irradiation was arbitrary defined as 1. Results are representative of three independent experiments performed at least six times.

Table 1. Results of isobolographic analyses of ionizing radiation estrogenic or anti-estrogenic drug interactions on MCF-7 cell line

Combined chemotherapy	Rγ Gy	30% surv	50% surv	75% surv	Mean
ICI 182780	3	Syn	Syn	+	Syn
	5	Syn	+	+	Syn
	8	+	Syn	Syn	Syn
4-OH-TAM	3	+	+	+	+
	5	Syn	Syn	+	Syn
	8	Syn	Syn	Syn	Syn
E_2	3	Ant	Ant	Ant	Ant
	5	Ant	Ant	Ant	Ant
	8	Ant	Ant	Ant	Ant
E_2 + ICI 182780	3	+	+	Syn	+
	5	Syn	+	Syn	Syn
	8	Syn	Syn	Syn	Syn
E_2 + 4-OH-TAM	3	+	+	Syn	+
	5	+	+	Syn	+
	8	Syn	Syn	Syn	Syn

Isobolographic interpretation at 30, 50 and 75% growth inhibition (results from three separate experiments). Mean were calculated from isobolographic values achieved at IC30, IC40, IC50, IC60 and IC75. Syn = synergistic effect; ant = antagonistic effect; + = additive effects; surv = survival.

receptor [19]. The decrease of ERα expression and activity are late events occurring after the cell cycle arrest exclude BRCA1-related effects. On the other hand, Angeloni et al. [20] recently demonstrated that p53 activation leads to a decrease of ERα expression in breast cancer cells, suggesting a potential implication of this protein.

ERα loss may also be a consequence of the cell cycle arrest. ERα expression in breast cancer cells MCF-7 is indeed dependent of cell cycle progression. Jakesz et al. [13] first revealed that G1 cell arrest decreases ERα content. Recently, de Graffenried et al. [21] demonstrated that ERα expression is dependent of S_P1, a transcription factor tightly regulated during cell cycle progression. On the other hand, cell cycle key proteins are under control of ubiquitin-like proteins which modulate their proteosomal degradation. Interestingly, level and activity of ERα and associated regulatory proteins are dependent of ubiquitin-like degradation pathways [22]. Thus, neddylation (a degradation pathway involved in cell cycle progression) also targets ERα and SRC-1 (ERα co-activator) to inhibit their activities. However, as shown in the present study, radiation-induced ERα decrease is due to a progressive arrest of its synthesis rather than to an enhancement of proteasomal degradation (MG-132 did not abrogate its loss) which seems to reject the implication of proteasome in radiation-induced ER loss.

It should be stressed that ionizing radiation inhibits E_2-induced cell growth independently of its ability to decrease ERα content since growth inhibition occurs

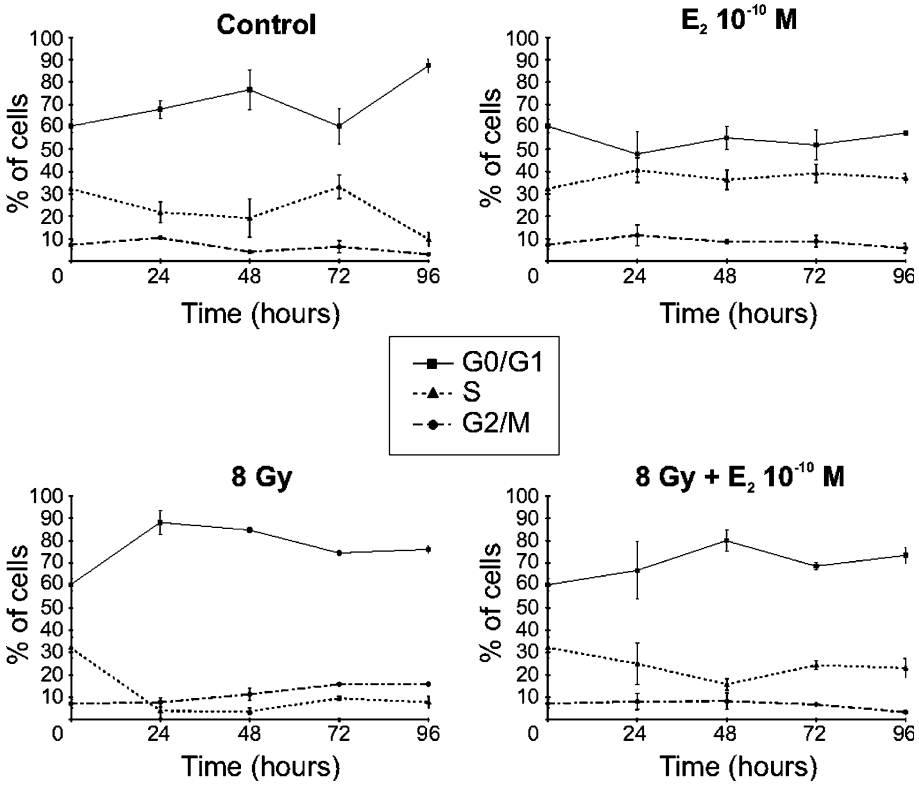

Figure 8. Effect of E_2 on cell cycle in MCF-7 cells. Cells were irradiated at 8 Gy and subsequently maintained in culture up to 96 h. Cell cycle was determined each day after radiation exposure by flow cytometry analysis. Results are representative of three independent experiments.

Table 2. Apoptosis determination in MCF-7 breast cancer cells

		0 h	24 h	48 h	72 h	96 h
Non-irradiated	–	2.12 ±1.15	2.51 ± 1.54	1.18 ± 0.76	0.70 ± 0.17	1.67 ± 0.14
	E_2	–	3.55 ± 2.79	0.81 ± 0.69	0.21 ± 0.03	2.42 ± 0.19
8 Gy	–	–	2.56 ± 0.65	0.68 ± 0.50	0.57 ± 0.19	2.16 ± 0.73
	E_2	–	4.23 ± 1.11	0.78 ± 0.72	0.33 ± 0.12	3.34 ± 2.56

Percent of annexin V positive, propidium iodide negative cells (i.e. % of apoptotic cells) were detected by flow cytometry analysis. E_2: 17β-estradiol (1×10^{-10} M). Results are mean value of three independent experiments.

largely before ER loss. Nevertheless, E_2 induced cell growth remains specific to ERα activation since they are totally blocked by SERM and anti-estrogens. Actually, ionizing radiation may interfere with E_2-induced cell growth by influencing E_2-dependent pathways such membrane signaling pathways or P53/P21$^{Waf\text{-}1/Cip1}$ axis [23,24]. Our results do not allow to conclude upon the potential implication of these cross-talks and further investigations are needed.

As compared to other experimental studies (reviewed in Schmidberger et al. [9]), our observations may be of clinical relevance. Association of SERM or anti-estrogen therapy before and concurrently to radiation may obviously block the effect of natural estrogen production; as a consequence, it may partially reduce the impact of a potential estrogen-associated radio-resistance. Clinical studies describing the sequence of endocrine therapy as tamoxifen or aromatase inhibitors and radiotherapy (concurrent versus sequential) are still rare, stressing the importance of our investigations. In retrospective studies with breast conservation in early stage breast cancer, Pierce et al. [25] and Christensen et al. [26] described that efficacy was not affected by the sequence of administration of tamoxifen and radiotherapy. In agreement with our experimental observations, the NASBP-B14 trial shows a significant decrease of breast relapse at 5 years with concomitant TAM treatment. On the other hand, results about toxicities are still closed up in debate but recent studies clearly demonstrated that toxic side effects are patient specific [8]. Furthermore concomitant treatment with pentoxifylline and alpha tocopherol treatment may avoid this toxic effects [27], raising the importance to pursue such investigations.

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BIOLOGY CONTRIBUTION

ESTROGENS DECREASE γ-RAY–INDUCED SENESCENCE AND MAINTAIN CELL CYCLE PROGRESSION IN BREAST CANCER CELLS INDEPENDENTLY OF p53

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Purpose: Sequential administration of radiotherapy and endocrine therapy is considered to be a standard adjuvant treatment of breast cancer. Recent clinical reports suggest that radiotherapy could be more efficient in association with endocrine therapy. The aim of this study was to evaluate the estrogen effects on irradiated breast cancer cells (IR-cells).

Methods and Materials: Using functional genomic analysis, we examined the effects of 17-β-estradiol (E_2, a natural estrogen) on MCF-7 breast cancer cells.

Results: Our results showed that E_2 sustained the growth of IR-cells. Specifically, estrogens prevented cell cycle blockade induced by γ-rays, and no modification of apoptotic rate was detected. In IR-cells we observed the induction of genes involved in premature senescence and cell cycle progression and investigated the effects of E_2 on the p53/p21$^{waf1/cip1}$/Rb pathways. We found that E_2 did not affect p53 activation but it decreased cyclin E binding to p21$^{waf1/cip1}$ and sustained downstream Rb hyperphosphorylation by functional inactivation of p21$^{waf1/cip1}$. We suggest that Rb inactivation could decrease senescence and allow cell cycle progression in IR-cells.

Conclusion: These results may help to elucidate the molecular mechanism underlying the maintenance of breast cancer cell growth by E_2 after irradiation-induced damage. They also offer clinicians a rational basis for the sequential administration of ionizing radiation and endocrine therapies. © 2007 Elsevier Inc.

Estrogen, Senescence, Breast cancer, Radiation, p53, p21$^{waf1/cip1}$, DNA damage, Rb, Cyclin.

INTRODUCTION

Estrogens are involved in the progression of most breast tumors (1). The biologic effects of estrogens are mediated by their binding to estrogen receptors (ERα or ERβ). Nevertheless, in breast cancer, ERα is predominant and the β-form is down-regulated (2, 3). The ERs are well known as ligand-dependent transcription factors. In addition, they increasingly appear to engage in cross-talk with growth factors signaling pathways independently of the transcriptional activity (4). Endocrine therapy is of major importance in the adjuvant treatment of breast cancer. However, endocrine therapy is generally associated with radiotherapy, and few experimental and/or clinical studies are available regarding the combined effects of both therapies (5).

Therefore, we designed *in vitro* experiments to determine the impact of estrogens on breast cancer cell (BCC) response to γ-ray irradiation. We have previously reported that estrogens decreased radiosensitivity of MCF-7 BCC (6). Direct and indirect actions of estrogens on target genes might explain this effect. For instance, the cross-talk between estrogen and growth factor transduction cascades, including the MAP-kinase and PI3-kinase pathways (7, 8), may potentially inhibit the effects of ionizing radiation (9). Direct estrogen effects through activated ERα activation with specific promoter sequences might also be involved in radioresistance. In particular, estrogens may induce cyclin D1 and c-myc expression, allowing cell cycle progression via cyclin/cdk activation and subsequent G_1/S and G_2/M transitions (10).

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Other hypotheses have been suggested to explain the effects of estrogen on radiosensitivity, including inactivation of p53 (11).

Based on these hypotheses and experimental data, we dissected the functional mechanisms underlying estrogen effects on BCC exposed to γ-rays, using MCF-7 cells as a model of hormone-dependent, p53 wild-type breast cancer cells (12).

METHODS AND MATERIALS

Cell culture

The MCF-7 BCC were routinely grown in basal Eagle's minimal essential medium (EMEM) supplemented with 10% inactivated fetal calf serum (FCS), 100 UI/ml streptomycin and 100 μg/ml penicillin. For experiments, cells were cultured in basal EMEM without phenol red and supplemented with 10% inactivated and charcoal-dextran–stripped FCS.

Reagents and antibodies

All cell culture reagents were from Life Technologies (Rockville, MD). Electrophoresis reagents and agarose bound antibodies were obtained from Sigma (Bornem, Belgium). Mouse monoclonal and rabbit polyclonal anti-$p21^{waf1/cip1}$, anti-p53, cyclin A, cyclin D1, cyclin E2, Rb antibodies were from Cell Signaling (Beverly, MA), mouse monoclonal anti-$p27^{kip1}$ antibody was provided by Novocastra (Zaventem, Belgium). Horseradish peroxidase–conjugated secondary antibodies were from Pierce (Erembodegem, Belgium). Pharmacologic inhibitors were from Calbiochem (Leuven, Belgium).

Ionizing radiation conditions

Irradiation was performed 48 h after plating using high-energy photons from a linear accelerator 18 MV (Clinac, Varian Medical Systems) with 4 Gy/min at room temperature. Medium was removed and replaced by fresh medium at the time of irradiation. Cells were maintained in basal EMEM supplemented with 10% inactivated and charcoal-dextran–stripped FCS during all radiation exposures.

Cell growth assays

Viable cell growth assay. Cells were gently washed once with phosphate-buffered saline (PBS), fixed with 1% glutaraldehyde/PBS (15 min, 20°C) and stained with 0.1% crystal violet (w/v in ddH_2O) (30 min, 20°C). Excess of crystal violet dye was then removed by three washes of running tap water (15 min, 20°C) and cells were lysed with 0.2% Triton X-100 (v/v in ddH_2O) (90 min, 20°C, under agitation). The absorbance was measured at 550 nm using a Microplate Autoreader EL309 (BIO-TEK Instruments, Winooski, VT).

Clonogenic assays. Clonogenic assays were performed as previously described (13). Briefly, MCF-7 cells were plated at a density of 25,000/dish in 35-mm dishes and cultured in basal EMEM without phenol red and supplemented with 10% inactivated and charcoal-dextran–stripped FCS. The bottom layer was prepared with 0.56% Bacto agar. Cells were seeded over the bottom layer in 0.37% Bacto agar containing medium. Cells were allowed to grow for 10 or 21 days before colonies of at least 20 or 50 cells were counted, respectively. Survival was fitted to the linear-quadratic model $S = e^{-(\alpha D + \beta D^2)}$.

Cell cycle analysis. MCF-7 cells were maintained 48 h in estrogen free medium before treatment. At the end of the experiment cells were trypsinized and washed twice with PBS. They were subsequently stained by Cell Cycle Coulter reagent (Beckman Coulter, Co., Fullerton, CA). Briefly, pelleted cells (1×10^6) were mixed thoroughly with 50 μl of reagent A (15 s, 20°C, under vortex agitation) and 950 μl of reagent B. After incubation (2 h, 4°C, dark), the cell cycle was analyzed with a Beckman Facs analyzer.

Senescence-associated β-galactosidase staining. Staining was performed as previously described (14). Four days after ionizing radiation, cells were washed twice and fixed with 3% paraformaldehyde, pH 7.2. Cells were then washed and stained with a solution of 1 mg/ml 5-bromo-4-chloro-indolyl-B-galactoside (X-Gal, Sigma) [pH 6], in 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium ferricyanide, 150 mmol/L NaCl, 40 mmol/L citric acid, and 2 mmol/L $MgCl_2$ (37°C, 24 h). After incubation, cells were washed twice with PBS and stained for SA–β-galactosidase assessment under light microscopy.

Microarray analysis

Total RNA was isolated from the cells (TRIzol reagent; Gibco BRL Life Technologies, Rockville, MD), and purified using RNeasy (Qiagen, Valencia, CA). cRNA was prepared exactly as described by the microarray manufacturer (Affymetrix, Santa Clara, CA). The concentration and the integrity/purity of each RNA sample were measured using RNA 6000 LabChip kit (Agilent Technologies, Palo Alto, CA) and an Agilent 2100 bioanalyzer. A 3-μg quantity of total RNA was used for the preparation of double-stranded cDNA using an oligo $(dT)_{24}$ primer with a T7 RNA polymerase promoter sequence at its 5′ end. After second strand synthesis, a labeled cRNA transcript was generated from the cDNA in an *in vitro* transcription reaction using Enzo BioArray high yield RNA transcript labeling (Enzo Diagnostics, Inc., Farmingdale, NY). The labeled anti-sense RNA was purified using RNeasy and each cRNA sample (20 μg) was fragmented (94°C, 35 min). The DNA microarrays used in this study were the U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA), containing almost 47,000 probe sets. Each probe set consisted of 22 different oligonucleotides (11 of which are a perfect match with the target transcript and 11 of which harbor a single-nucleotide mismatch in the middle). These 22 oligonucleotides were used to measure the level of a given transcript. Details of the RNA amplification, labeling, and hybridization steps are available at http://www.affymetrix.com. Chips were scanned, and background correction, normalization, and summarization of the data were done using the robust multi-array average procedure (15).

Cellular extract

Total extract. Cell cultures were washed with PBS and lysed for 30 min at 4°C in lysis buffer (50 mmol/L Tris [pH 7.5], 150 mmol/L NaCl, 1% Igepal, 0.1% SDS, 0.5% sodium deoxycholate, 50 mmol/L NaF, 0.6 mmol/L PMSF, 0.1 mmol/L orthovanadate, and 0.3 mmol/L TPCK). After centrifugation (15 min, 4°C, 16,000 *g*), the supernatants were collected as total extract.

Cytoplasmic and nuclear extracts. For the extracts, 2.5×10^6 cells were washed twice with ice-cold PBS and lysed in hypotonic buffer (10 mmol/L 4-(2-Hydroxyethyl) piperazin-1-ethanesulphonic acid [HEPES] [pH 7.9], 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 2 mmol/L Na_3VO_4, 0.2 mmol/L phenylmethylsulphonyl fluoride (PMSF), 3 μg/ml aprotinin, 25 μg/ml pepstatin, 25 μg/ml leupeptin, 25 μg/ml chymotrypsin, 5 mmol/L NaF, 0.5 mmol/L dithiothreitol (DTT), 0.1% Igepal). After centrifugation (5 min,

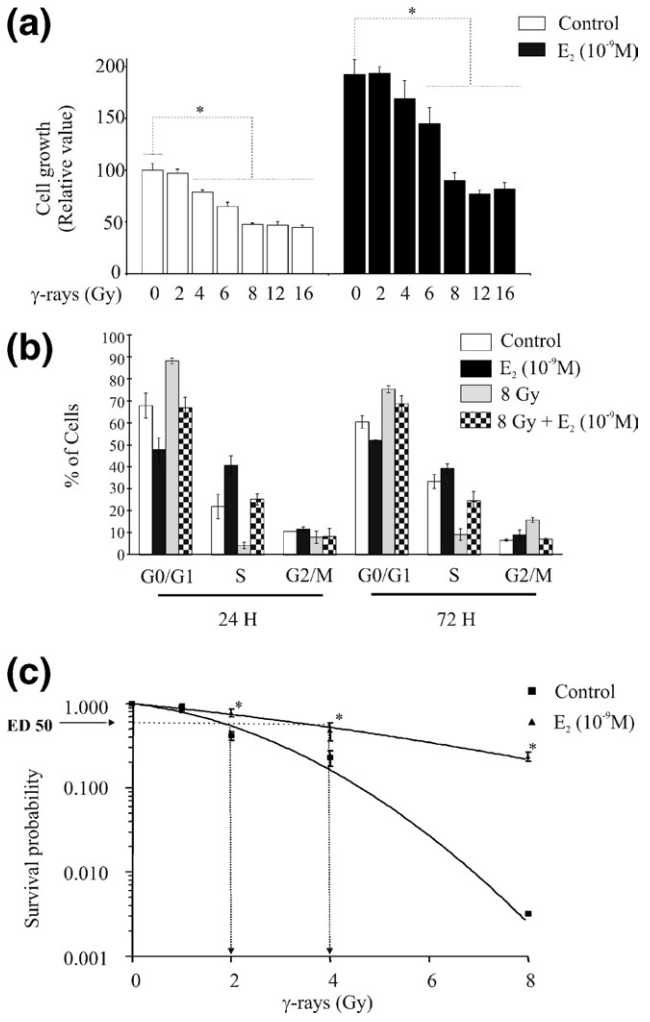

Fig. 1. Effects of E_2 on irradiated breast cancer cells (IR-cells) growth. (a) MCF-7 viable cell growth was assessed by crystal violet staining 96 h after γ-rays ionizing radiation exposure (0–16 Gy) in the absence or presence of 10^{-9} mol/L of 17-β-estradiol (E_2). (b) Flow-cytometric analysis of cell cycle distribution in IR-cells 24 and 72 h postirradiation at the 8-Gy γ-ray dose. (c) Clonogenic survival at Day 10 of MCF-7 cells upon γ-ray exposure (0–8 Gy), the experimental results are fitted to the linear-quadratic model. Results are representative of three independent experiments performed in triplicate assays. $*p < 0.05$.

4°C, 16, 000 g), the supernatants were collected as cytosol. Nuclear extracts were prepared by resuspension of the crude nuclei in high salt buffer (20 mmol/L HEPES [pH 7.9], 1.5 mmol/L $MgCl_2$, 420 mmol/L NaCl, 2 mmol/L Na_3VO_4, 0.2 mmol/L PMSF, 3 μg/ml aprotinin, 25 μg/ml pepstatin, 25 μg/ml leupeptin, 25 μg/ml chymotrypsin, 5 mmol/L NaF, 0.5 mmol/L DTT, 25% glycerol) (10 min, 4°C). The nucleoprotein-containing supernatants were collected after centrifugation (5 min, 4°C, 16,000 *g*) and conserved at −70°C until use.

Active p53 ELISA

At the end of experiment, 20 μg of nuclear extracts were used to measure p53 DNA-binding ability using a DuoSet IC active p53 ELISA kit as described by the manufacturer (R&D Systems, Abingdon, UK; www.RnDSystems.com).

Western blot

Western blots were performed as previously described (6). Blots were incubated with primary antibody (1:1,000 dilution, overnight at 4°C). Detection was performed using a goat anti-mouse secondary antibody or goat anti-rabbit secondary antibody (1:2,000 dilution, 1.5 h at room temperature) and Western Pico Detection system (Pierce, Erembodegem, Belgium).

Immunoprecipitation

Cells were washed with PBS and lysed for 30 min at 4°C in lysis buffer (50 mmol/L Tris [pH 7.5], 150 mmol/L NaCl, 1% Igepal, 1 mmol/L PMSF, 1 mmol/L orthovanadate, 1 mmol/L $Na_4P_2O_7$, 10 mg/ml aprotinin, and 10 mg/ml leupeptin). Lysates were cleared by centrifugation (12,000 *g,* 10 min). A 1-mg quantity of cellular extracts was pre-cleared with 5 μl protein A/G PLUS-Agarose (2 h, 4°C) and incubated with primary antibody (2 μg, overnight at

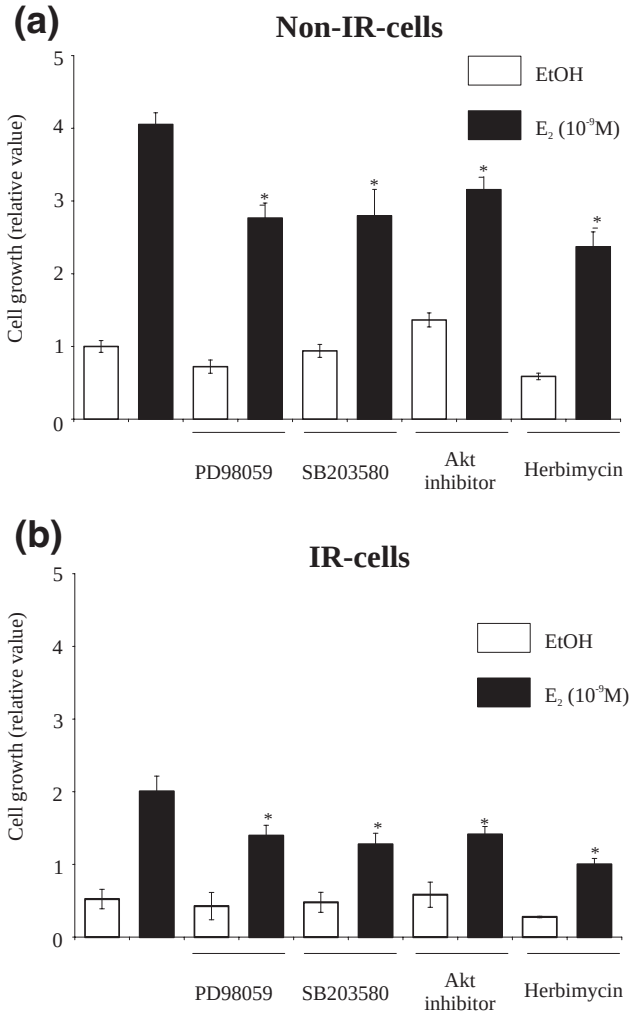

Fig. 2. Influence of pharmacologic inhibitors of growth factors transduction pathways on the estrogen receptor–α (ERα) rescue effects on cells growth. At 96 h postirradiation, viable cell growth was appreciated by crystal violet staining in presence of different combination of E_2 (10^{-9} mol/L) and pharmacologic inhibitors of MAP-kinase (PD98059, 10 μmol/L), P38 MAP-kinase (SB 203580, 5 μmol/L), Akt (Akt inhibitor, 5 μmol/L), and src (herbimycin, 100 nmol/L). Results are representative of three independent experiments performed in triplicate assays. $*p < 0.05$.

Table 1. Microarray analysis of effects of E_2 on γ-ray–modulated genes

Accession number	Gene name (Symbol)	Fold change (control = 1) 8 Gy	E_2	8 Gy + E_2	Pathways
NM_001107	ACYP1	0.46	1.94	1.68	
AI744123	ANKRD43	0.48	0.46	0.27	
NM_018685	ANLN	0.35	2.33	0.81	E2F
NM_030920	ANP32E	0.36	2.78	1.24	
NM_001159	AOX1	0.49	0.29	0.45	
NM_004900	APOBEC3B	0.36	0.50	0.32	
NM_018154	ASF1B	0.44	2.66	1.34	
NM_018123	ASPM	0.35	1.55	2.36	
AI925583	ATAD2	0.44	2.29	1.12	ER
AB011446	AURKB	0.41	2.75	1.08	E2F
BE672260	B3GNT5	0.46	0.51	0.25	
AA648913	BIRC5	0.46	2.15	1.07	p53/E2F
NM_000059	BRCA2	0.46	2.37	0.95	E2F
D38553	BRRN1	0.38	2.48	1.08	E2F
AF043294	BUB1	0.33	2.21	0.97	E2F
NM_001211	BUB1B	0.43	2.23	1.02	E2F
NM_018131	C10orf3	0.27	0.79	0.87	
BF792864	C15orf23	0.41	2.14	1.21	
BC001068	C20orf129	0.39	2.00	1.17	
NM_024053	C22orf18	0.44	1.89	0.95	
BG492359	C6orf173	0.48	3.00	1.35	
BF248364	CASC5	0.33	2.02	0.83	
R60224	CBLN2	0.41	0.34	0.22	
NM_001237	CCNA2	0.26	2.51	1.08	E2F
N90191	CCNB1	0.37	1.53	0.76	E2F
NM_004701	CCNB2	0.35	1.74	0.91	E2F
NM_001786	CDC2	0.41	1.53	0.83	E2F
NM_001255	CDC20	0.35	2.63	1.33	E2F
NM_001790	CDC25C	0.25	1.66	0.61	E2F
NM_003504	CDC45L	0.49	4.96	2.07	E2F
NM_001254	CDC6	0.39	4.23	1.82	E2F
AF326731	CDCA1	0.34	2.88	0.86	
T90295	CDCA2	0.40	1.98	1.20	
NM_031299	CDCA3	0.35	2.2	1.05	
BE614410	CDCA5	0.44	3.33	1.80	
AY029179	CDCA7	0.39	7.87	2.60	
BC001651	CDCA8	0.44	2.21	1.24	
NM_001262	CDKN2C	0.30	1.38	0.73	E2F
AF213040	CDKN3	0.31	1.99	1.06	E2F
AW075105	CDT1	0.43	3.29	1.43	E2F
NM_001809	CENPA	0.30	2.23	0.97	E2F
NM_001813	CENPE	0.40	2.19	3.70	E2F
NM_005196	CENPF	0.39	1.26	1.14	E2F
AL572471	CENPH	0.43	1.86	0.86	E2F
AI861788	CIT	0.48	1.45	2.17	
NM_001827	CKS2	0.46	1.98	1.18	E2F
AA406603	CLCC1	0.47	1.54	2.38	
NM_024094	DCC1	0.38	3.87	1.86	
NM_017779	DEPDC1	0.26	3.05	1.10	
AK001166	DEPDC1B	0.35	2.43	1.04	
NM_000791	DHFR	0.47	2.31	1.14	E2F
NM_014750	DLG7	0.31	1.91	0.82	
NM_016448	DTL	0.43	6.40	2.73	
AI341146	E2F7	0.47	5.66	2.07	E2F
NM_024680	E2F8	0.31	2.97	1.18	
NM_018098	ECT2	0.43	1.39	1.47	
BC040700	EP300	0.44	2.03	1.55	
NM_012291	ESPL1	0.37	1.66	0.78	E2F
NM_003686	EXO1	0.42	3.96	1.85	E2F
AI346350	EXOSC9	0.34	2.71	0.93	
AL512760	FADS1	0.50	1.39	1.01	
AL138828	FAM54A	0.46	3.15	1.66	

Continued

Table 1. Microarray analysis of effects of E_2 on γ-ray–modulated genes (*Continued*)

Accession number	Gene name (Symbol)	Fold change (control = 1)			Pathways
		8 Gy	E_2	8 Gy + E_2	
BC005004	FAM64A	0.46	2.30	1.18	
NM_012177	FBXO5	0.46	2.65	1.10	
NM_021953	FOXM1	0.41	1.97	3.88	
NM_006733	FSHPRH1	0.39	2.10	0.96	
AY028916	GAJ	0.36	6.04	1.88	
H37811	GAS2L3	0.41	1.67	0.91	
NM_016426	GTSE1	0.28	1.48	0.71	E2F
NM_022346	HCAP-G	0.28	3.82	1.43	
AI650364	HELLS	0.50	2.92	1.35	
BC000903	HMGB2	0.30	1.70	0.71	E2F
NM_012485	HMMR	0.26	2.00	0.73	
AW271106	IQGAP3	0.46	1.30	1.82	
NM_014736	KIAA0101	0.30	1.67	0.87	
NM_004523	KIF11	0.36	1.91	3.47	
NM_014875	KIF14	0.24	1.89	0.71	
NM_020242	KIF15	0.20	2.39	0.70	
NM_031217	KIF18A	0.44	1.91	3.34	
NM_005733	KIF20A	0.34	1.72	0.83	E2F
NM_004856	KIF23	0.29	1.47	0.68	E2F
U63743	KIF2C	0.30	2.10	1.04	E2F
NM_012310	KIF4A	0.32	1.67	0.79	E2F
BC000712	KIFC1	0.45	1.93	1.19	
NM_006101	KNTC2	0.31	2.10	0.96	E2F
NM_005573	LMNB1	0.29	2.33	0.83	
NM_017760	LUZP5	0.40	2.47	1.11	
NM_002358	MAD2L1	0.33	3.10	1.57	E2F
NM_018518	MCM10	0.35	5.87	2.51	E2F
NM_014791	MELK	0.41	2.32	1.12	E2F
AI871282	MGC70924	0.49	1.23	1.01	
AU147044	MKI67	0.30	1.91	0.64	E2F
NM_024629	MLF1IP	0.42	2.33	1.20	
NM_012329	MMD	0.41	2.09	0.99	
NM_002466	MYBL2	0.43	1.71	0.90	E2F
NM_018248	NEIL3	0.36	4.06	1.16	E2F
NM_002497	NEK2	0.31	1.71	0.77	E2F
NM_006681	NMU	0.42	5.15	2.11	
NM_002452	NUDT1	0.49	2.07	1.14	
NM_018454	NUSAP1	0.33	1.89	0.76	
BE045993	OIP5	0.44	2.70	1.19	
AI934557	PAQR5	0.39	0.65	0.68	
NM_018492	PBK	0.31	3.24	1.42	
NM_014264	PLK4	0.37	2.03	0.79	E2F/ER
NM_002692	POLE2	0.44	1.87	3.72	E2F
NM_003981	PRC1	0.34	2.19	0.97	E2F
NM_004219	PTTG1	0.34	1.84	0.91	E2F
AU153848	RACGAP1	0.41	1.47	0.82	E2F
BE966146	RAD51AP1	0.30	2.69	1.19	E2F
AB051846	RAP1A	0.17	0.24	0.22	E2F
BE966236	RRM2	0.30	3.78	2.00	E2F
AF116616	SCD	0.48	2.54	1.61	E2F
N31731	SGOL2	0.42	2.05	1.03	
NM_024745	SHCBP1	0.38	3.37	1.60	
NM_003035	SIL	0.48	2.01	1.05	
NM_005496	SMC4L1	0.49	1.73	3.00	E2F
NM_006461	SPAG5	0.40	2.40	1.10	
AF225416	SPBC25	0.37	2.23	0.82	
NM_003600	STK6	0.44	1.88	1.18	
NM_006342	TACC3	0.34	2.12	1.17	
BC002493	TCF19	0.43	2.19	1.17	E2F
NM_003258	TK1	0.47	1.73	1.03	E2F
BF338045	TNFAIP8L1	0.38	1.37	0.83	
AL561834	TOP2A	0.33	1.54	0.76	E2F

Continued

Table 1. Microarray analysis of effects of E_2 on γ-ray–modulated genes (*Continued*)

Accession number	Gene name (Symbol)	Fold change (control = 1) 8 Gy	E_2	8 Gy + E_2	Pathways
AF098158	TPX2	0.43	1.22	1.83	E2F
NM_004237	TRIP13	0.48	3.36	1.58	
NM_005480	TROAP	0.36	1.46	2.90	
NM_003318	TTK	0.27	2.19	0.97	E2F
NM_007019	UBE2C	0.32	1.73	0.91	E2F
AK025578	UHRF1	0.35	2.69	1.21	
AW772140	WDHD1	0.49	2.47	5.80	
NM_014950	ZBTB1	0.48	0.65	0.66	
N62196	ZNF367	0.38	4.30	2.15	
NM_007057	ZWINT	0.49	1.93	1.09	E2F
NM_001613	ACTA2	4.34	1.07	0.62	
NM_000700	ANXA1	2.23	0.94	1.19	
AW151108	ATRNL1	2.19	1.31	1.62	
NM_004324	BAX	2.22	1.22	0.84	p53
AI827789	BCMP11	2.04	1.24	1.25	
NM_006763	BTG2	2.71	0.58	0.90	p53/E2F
NM_001584	C11orf8	2.13	14.96	5.28	ER
NM_020375	C12orf5	2.19	1.19	1.24	p53
NM_020215	C14orf132	2.12	0.13	0.29	
NM_001753	CAV1	2.10	0.48	0.18	p53/senescence
BE903880	CD44	2.09	6.14	0.45	senescence
AW274756	CDK6	2.17	0.16	0.09	E2F
NM_000389	CDKN1A	3.65	0.66	1.06	p53/E2F
AF317887	Cep290	2.10	0.94	1.06	
AF101051	CLDN1	2.78	0.26	0.16	senescence
NM_001338	CXADR	2.06	2.58	0.99	
NM_000499	CYP1A1	2.48	0.42	0.24	
AU154504	CYP1B1	2.33	0.88	1.13	
NM_000107	DDB2	2.19	1.65	1.02	p53
U46745	DTNA	2.19	0.80	0.73	
NM_001394	DUSP4	2.75	2.51	2.32	
T15545	EPHA4	2.02	1.99	0.41	
NM_000043	FAS	6.15	2.08	1.04	p53
AK024690	FBXL20	2.15	0.89	0.50	
AA129444	FSTL5	2.88	0.63	0.26	
AF180519	GABARAPL3	2.21	0.46	0.33	
NM_001924	GADD45A	2.96	1.34	1.49	p53
AF003934	GDF15	6.12	1.76	2.16	p53
BM668595	GPATC2	2.01	0.30	0.24	
NM_002510	GPNMB	2.72	0.86	1.48	
BE675337	GSN	2.03	0.64	0.68	
AW731710	HINT3	2.13	0.59	0.57	
NM_003543	HIST1H4H	2.06	0.42	1.18	Senescence
M16276	HLA-DQB1	2.29	0.49	0.53	Myc/ER
NM_016545	IER5	2.18	2.00	1.36	
BM128432	IGFBP5	2.05	1.63	1.88	
AJ007557	KCNJ13	2.65	0.72	0.25	
U11058	KCNMA1	2.06	0.49	0.36	
NM_014732	KIAA0513	2.01	0.24	0.37	
NM_025081	KIAA1305	2.57	0.99	0.38	
NM_005780	LHFP	2.22	0.29	0.13	
AI375083	LRFN5	2.04	0.86	0.33	
NM_024548	LRRIQ2	2.26	2.65	0.85	
NM_005360	MAF	2.16	0.79	0.50	p53
NM_005757	MBNL2	2.09	0.87	0.48	
AJ276888	MDM2	2.22	1.73	1.53	p53
AF278532	NTN4	2.56	0.18	0.14	
NM_020190	OLFML3	2.18	0.52	1.30	
AI806586	OSBPL9	2.05	037	0.29	
AI524125	PCDH9	2.03	0.96	0.64	
AW968465	PGM5P1	2.01	0.63	0.33	
BF589462	PPM1L	2.18	0.96	0.57	

Continued

Table 1. Microarray analysis of effects of E_2 on γ-ray–modulated genes (*Continued*)

Accession number	Gene name (Symbol)	Fold change (control = 1)			Pathways
		8 Gy	E_2	8 Gy + E_2	
BG285881	PRICKLE2	2.15	0.66	0.60	
AW471145	PRSS23	2.42	4.87	1.11	ER/senescence
AW242315	PTGER3	2.46	0.37	0.24	
BC040303	PTP4A1	2.34	0.77	0.61	
BC003667	RPS27L	2.90	1.21	0.79	
NM_016656	RRAGB	2.09	0.59	0.51	
NM_002615	SERPIFN1	2.58	1.29	0.95	
AA488687	SLC7A11	2.71	32.11	4.31	
AI559300	SPATA18	5.08	1.07	0.70	
NM_001062	TCN1	3.25	72.41	63.46	
AV660825	TMEM49	2.31	0.32	0.22	
NM_014058	TMPRSS11E	2.83	0.53	0.32	
AF016266	TNFRSF10B	2.58	1.34	1.18	p53
NM_016629	TNFRSF21	2.01	2.33	1.18	
AW341649	Tp53INP1	2.83	0.08	0.30	p53
AW024437	TTC18	2.19	0.78	0.38	
L22431	VLDLR	3.04	2.95	0.69	
NM_022470	WIG1	3.85	0.48	0.55	p53

4°C, on rotating device). Immune complex was then collected after incubation with 25 μl of protein A/G PLUS-Agarose (2 h, 4°C, on rotating device) and centrifugation (12,000 *g*, 3 min). Immunoprecipitates were washed three times with ice-cold lysis buffer and analyzed by Western blot as previously described.

Statistical analysis

Statistical significance was measured by Student's paired *t* test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

E_2 prevented cell growth inhibitory effects of γ-rays on MCF-7 BCC

As shown in Fig. 1a, a dose-dependent decrease in MCF-7 cell growth was observed in irradiated breast cancer cells (IR-cells) up to 8 Gy, higher doses did not provoke further inhibition of cell growth. Our findings showed that E_2 sustained cell growth in IR-cells independently of the radiation doses. Therefore, cell cycle parameters were assessed at 8 Gy (Fig. 1b). In non-IR cells, E_2 rapidly induced cell proliferation (50% of cells in S and G2/M phases after 24 h) and maintained it at 72 h. In IR-cells, cell proliferation was stopped at 24 h (90% of cells in G0/G1-phase). A slight decrease in G0/G1 and an increase in G2/M were observed at 72 h, indicating that some cells bypassed the initial G0/G1 arrest but were then blocked in G2/M. Treatment with E_2 prevented cell cycle arrest in IR-cells. Confirming our previous report (6), no significant modification of apoptosis induction was associated with IR-cell cycle blockade either in the absence or in the presence of E_2 (data not shown).

The cell growth studies were substantiated by assessment of clonogenic survival at 10 and 21 days after γ-rays exposure. The γ-ray exposure reduced survival of MCF-7 cells after 10 days (Fig. 1c) and 21 days (data not shown). After 10 days the efficient dose to observe 50% of growth inhibition (ED-50) value was lower than in the monolayer culture condition and was evaluated to 2 Gy, corresponding to the relevant clinical dose. In these conditions, E_2 clearly decreased radio-sensitivity, increasing the ED-50 value to 4 Gy.

E_2-induced radio-resistance did not require cross-talk with growth factor pathways

To assess whether cross-talk with growth factor pathways was involved in E_2-induced cell growth rescue, various pharmacologic inhibitors of these pathways were used (Fig. 2). All inhibitors partly decreased E_2-induced cell growth in both non–IR-cells and IR-cells. However, none was able to abolish the rescue effect of E_2 on IR-cell growth.

Gene expression analysis of E_2-induced radio-resistance genes

Microarray analysis was performed to investigate gene modulation by E_2 in IR-cells (Tables 1 and 2). In all, 312 probe sets were found to be differentially modulated (at least 2-fold increase or decrease) upon γ-ray exposure corresponding to 204 genes (Table 1) Among these genes, 108 were relevant to cell growth. Comparing our data with those of previous studies, we determined that the majority of cell growth-associated genes modulated by 8 Gy γ-rays (79 genes) were related to p53 activation (15 genes) (16), induction of senescence (5 genes) (17–19), and/or inhibition of E2F (61 genes) (20) (Table 1). In IR-cells, E_2 treatment clearly down-regulated the induction of senescence-associated gene (*CDLN1, CAV1, HIST1H4H, PRSS23*, *CD44*) and up-regulated E2F-target genes (Table 1). We found that E_2 did not significantly modify the expression of the major p53-

Table 2. Microarray analysis of γ-ray modulation of E_2-dependent genes

Accession number	Gene name (Symbol)	Fold change (control = 1)			Pathways
		8 Gy	E_2	8 Gy + E_2	
AW069729	ACPL2	1.24	1.17	0.59	
N29801	ADAM22	0.98	1.52	0.65	EMT
AW203986	AFTIPHILIN	0.90	1.95	0.74	
AF279145	ANTXR1	0.89	1.56	0.59	
AB011132	AQR	1.03	1.21	0.52	
BG253884	BTBD15	0.87	1.10	0.40	
AI634652	C8orf44	0.99	1.75	0.81	
BF792631	CDC14B	0.83	1.52	0.58	p53
AF317887	Cep290	0.94	2.10	0.84	
AW514564	CHD2	0.98	1.00	0.48	
AF070621	DIXDC1	0.96	1.78	0.78	
NM_001406	EFNB3	0.76	1.30	0.49	EMT
AW138704	FAM76B	1.06	1.27	0.62	
D80480	FLJ14624	0.95	1.14	0.49	
AW975050	FLJ31568	0.88	1.27	0.47	
AI247824	FLJ36665	1.30	1.65	0.79	
AA129444	FSTL5	0.63	2.88	0.75	
AL041745	GPM6B	1.10	1.49	0.63	
BE544748	GSPT1	1.13	1.40	0.59	translation
AK001846	HNRPR	1.17	1.51	0.74	
N95466	HNRPU	1.49	1.66	0.78	
BE466675	IBSP	0.65	1.84	0.57	
M96843	ID2B	0.98	1.77	0.85	EMT
AI743396	KCMF1	1.05	1.19	0.56	
AJ007557	KCNJ13	0.72	2.65	0.69	
NM_025081	KIAA1305	0.99	2.57	1.00	
AA992480	KIDINS220	0.94	1.67	0.72	
NM_014398	LAMP3	1.04	1.47	0.50	p53
AW242720	LOC143381	1.08	1.61	0.79	
AA528080	LOC283070	0.56	1.18	0.32	
AA417117	LOC344595	0.94	3.12	1.25	
BE466160	LOC388526	1.03	2.12	0.89	
AI375083	LRFN5	0.87	2.04	0.67	
AA642143	M11S1	0.86	1.61	0.68	
BE674528	MAF	0.70	3.33	0.77	
NM_018298	MCOLN3	0.81	1.43	0.54	
AI382029	MYO9A	1.11	1.94	0.93	
BE670307	NA	0.68	1.74	0.45	
AU145501	NA	0.79	2.09	0.67	
BE464799	NA	0.84	1.33	0.55	
BF032500	NA	0.98	1.74	0.77	
BE552208	NA	0.95	1.03	0.45	
AW440490	NA	0.96	1.09	0.51	
BC026304	NA	1.92	2.15	1.01	
AU157716	NA	1.05	1.02	0.49	
AW083948	NFYB	0.68	1.23	0.39	senescence
AI743090	NPAS2	0.87	1.98	0.77	
AW003022	NRF1	1.15	1.15	0.52	EMT
AI149508	ORC4L	0.61	1.40	0.28	
AI818048	PHF10	0.93	1.25	0.59	
NM_005044	PRKX	0.95	1.54	0.68	
AL536268	RBMS2	0.91	2.60	1.04	
AI472310	RFP2	0.85	1.34	0.56	
NM_014746	RNF144	0.99	1.08	0.52	
AI694536	SNTB2	0.73	1.45	0.41	
NM_153039	TLOC1	1.00	1.72	0.76	
AK025872	TNRC8	1.00	1.70	0.70	
U31110	TRPC1	0.91	1.53	0.49	
AW024437	TTC18	0.78	1.16	0.44	
AW779859	UBE2Q1	1.27	1.53	0.73	
AC084239	ZNF228	0.85	1.39	0.57	
AF352582	ABCC11	1.51	1.10	9.85	IFN

Continued

Table 2. Microarray analysis of γ-ray modulation of E_2-dependent genes (*Continued*)

Accession number	Gene name (Symbol)	Fold change (control = 1)			Pathways
		8 Gy	E_2	8 Gy + E_2	
U05598	AKR1C2	0.90	0.91	2.08	PGR/IFN
NM_153042	AOF1	1.00	0.73	1.76	
D90427	AZGP1	0.66	1.05	4.18	ER
U37546	BIRC3	0.84	0.85	2.82	NF-κB
NM_004335	BST2	1.18	1.05	2.69	NF-κB /IFN
AI935123	C14orf78	1.01	0.56	1.33	
AI970144	CACNA2D2	0.97	0.63	1.27	stress
NM_004591	CCL20	1.09	1.20	4.69	NF-κB
NM_022467	CHST8	1.05	0.61	1.29	stress
NM_006536	CLCA2	0.92	1.05	4.97	
AV706254	CTSD	1.91	0.97	4.05	IFN/ER
NM_014314	DDX58	0.78	1.36	5.22	IFN
AI763378	EHF	0.86	0.81	3.03	
AF359241	FGFR4	0.97	0.77	1.64	
NM_001450	FHL2	0.82	0.70	1.82	NF-κB
NM_017631	FLJ20035	0.70	1.20	3.58	
AK023743	FLJ31033	0.56	0.92	1.97	
AI193973	FLJ42461	1.52	0.58	1.82	
NM_001453	FOXC1	1.72	0.75	3.91	
NM_005101	G1P2	0.51	1.14	2.94	IFN
NM_022873	G1P3	1.16	1.78	4.41	IFN/senescence
X61094	GM2A	0.81	0.64	1.56	NF-κB
NM_016295	GP2	1.15	0.74	2.13	
NM_018485	GPR77	1.36	0.61	2.22	
NM_000187	HGD	1.49	1.01	3.62	
NM_005532	IFI27	0.94	1.20	4.12	IFN
NM_001548	IFIT1	0.63	1.46	39.10	IFN
AA131041	IFIT2	0.71	1.88	6.05	IFN
AI075407	IFIT3	1.16	1.38	16.67	IFN
AA749101	IFITM1	0.97	1.07	4.27	IFN/senescence
NM_006084	ISGF3G	0.71	1.04	5.67	IFN
AI074145	KMO	1.88	0.94	6.55	
NM_014583	LMCD1	1.09	1.43	3.47	EMT
R49343	LOC200312	1.30	0.70	2.32	
BF691523	LOC283551	1.00	0.75	1.72	
AA570178	LOC392790	1.01	0.72	1.52	
AL021977	MAFF	1.05	0.75	2.18	
NM_005204	MAP3K8	1.01	0.96	2.18	
AA565509	MGC39606	0.92	0.93	2.38	
AF422798	MLL	1.30	0.75	2.35	
AF274945	NA	1.00	1.26	2.70	
AL049452	NA	1.00	0.33	0.93	
BF439063	NA	0.76	0.96	2.81	
AF228422	NMES1	1.03	0.99	4.10	
NM_002534	OAS1	0.72	1.06	4.46	NF-κB /IFN
NM_016817	OAS2	0.88	0.73	1.85	IFN/senescence
NM_003733	OASL	0.77	0.68	2.05	NF-κB
NM_024607	PPP1R3B	1.31	0.91	3.47	EMT
S78505	PRLR	1.23	0.83	2.08	EMT
AI440266	RDHE2	1.33	1.12	4.26	
AI337069	RSAD2	0.90	1.04	2.42	
NM_002963	S100A7	1.96	0.68	47.70	
NM_002964	S100A8	1.25	1.08	20.35	NF-κB
NM_002965	S100A9	0.68	0.80	3.58	NF-κB/senescence
NM_017654	SAMD9	0.94	1.04	6.96	
NM_002411	SCGB2A2	1.03	0.97	3.51	
NM_000295	SERPINA1	0.64	0.69	5.13	
NM_002639	SERPINB5	0.74	0.74	5.42	NF-κB
NM_020427	SLURP1	1.05	0.70	1.48	
AW015140	ST8SIA6	1.10	0.68	1.71	
BC002704	STAT1	0.89	1.03	2.24	IFN/NF-κB/senescence
AI141151	SYNPO2L	0.91	0.88	4.51	

Continued

Table 2. Microarray analysis of γ-ray modulation of E_2-dependent genes (*Continued*)

Accession number	Gene name (Symbol)	Fold change (control = 1) 8 Gy	E_2	8 Gy + E_2	Pathways
AV726673	THBS1	1.18	1.20	3.05	
AW272342	THRSP	1.51	1.18	7.66	
NM_018004	TMEM45A	1.37	1.20	3.73	
NM_006290	TNFAIP3	1.19	1.25	3.00	NF-κB
U06641	UGT2B15	2.00	1.72	15.53	
AF177272	UGT2B28	1.27	1.07	5.85	
NM_024626	VTCN1	0.62	0.70	1.43	

Abbreviations: EMT = epithelial mesenchyme transition; ER = estrogen-regulated gene; PGR = progesterone-regulated gene; IFN = interferon-associated gene.

induced cell cycle modulators (*CDKN1A* and *GADD45*) or pro-apoptotic gene (*FAS, BAX*) expression, but it did increase expression of p53 and of pro-survival genes (*BIRC5* and *GDF15*). In the absence of p53 regulation, we examined the expression of $p21^{waf1/cip1}$-dependent genes. As previously reported, $p21^{waf1/cip1}$ may support p53-dependent or -independent growth inhibition and senescence (21, 22). $p21^{waf1/cip1}$ modulates genes encode for growth- and senescence-related proteins (23). In the absence or presence of γ-ray treatment, E_2 impeded $p21^{waf1/cip1}$ dependent gene down-regulation (*e.g., TGFβ1, VAV3, MCM2, FEN1, RAD51A, TFF3, MKI67, CENPF, TOP2A*) and $p21^{waf1/cip1}$+-dependent gene expression (*CAV2*, *GRN*) (data not shown). These microarray data suggest that, in IR-cells, E_2 led to an increase in cell proliferation but to a decrease in senescence-modulating p21-dependent gene expression independently of p53 regulation.

We also analyzed the impact of ionizing radiation on E_2 response in BCC (Table 2). No significant modulation of E_2 response was observed for 1,325 probe sets (data not shown). As expected, E_2 increased expression of canonical E_2-targeted genes (*TFF1, PGR, STC2, CXCL12, NRIP1, MYC, TPD52L1*) (24) and modulated growth-associated gene in a sense favorable to cell growth. For instance, expressions of *CCNE2* and *MYC* were increased, whereas expressions of *SKP2* and *CCNG2* were decreased. By contrast, irradiation induced modulation of E_2 response for 132 genes (Table 2). The expression of 62 genes was significantly decreased (more than 2-fold) in IR-cells as compared with non–IR-cells in the presence of E_2. These genes did not appear to be related to any biologic process (as analyzed by Ingenuity Pathways software), but they did seem to be associated with an enhancement of differentiation (*PRLR* or epithelial mesenchyme transition (EMT)–related genes such as *NRF1, ID2B,* and *EFNB3*). On the other hand, simultaneous treatment by irradiation and E_2 specifically increased expression of 70 genes. They were clearly associated with cytokine (interferon and tumor necrosis factor) and/or nuclear factor–κB (NF-κB) responses (25–27). Altogether, these results indicated that irradiation modified E_2 effect on cell differentiation and enhanced E_2 response via NF-κB, which may increase resistance of breast cancer cells to ionizing radiation.

E_2 prevented premature senescence induced by γ-rays

As shown above, E_2 might prevent IR-cells from undergoing senescence. To confirm this hypothesis, premature senescence was assessed by SA–β-galactosidase staining. As shown in Fig. 3, The SA–β-galactosidase activity was increased in IR-cells (45% of cell stain) as compared with non-IR cells (10%). Increase of SA–β-galactosidase activity in MCF-7 cells was only shown 4 days after irradiation and remained constant up to 6 days (data not shown). The E_2 decreased the occurrence of SA–β-galactosidase–positive cells in both non-IR cells and IR-cell cultures, indicating that E_2 effectively precluded premature senescence in MCF-7 BCC.

E_2 prevented Rb dephosphorylation by γ-rays

Many E2F-target genes were down-regulated by γ-rays, whereas E_2 increased expression of these E2F targets in IR-cells (Table 1). It is possible that E2F activity is suppressed by binding to the dephosphorylated form of Rb protein. Phosphorylation of Rb allows dissociation of E2F, thereby permitting the induction of cell cycle genes (28). We hypothesized that the effects of γ-rays and E_2 on E2F were mediated by Rb; thus the level and phosphorylation status of Rb were evaluated by immuno-blotting (Fig. 4a).

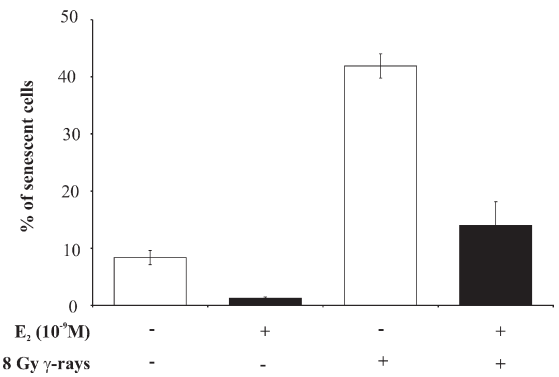

Fig. 3. Effects of E_2 on IR-cells senescence. Senescence induction was measured by SA–β-gal staining 96 h after 8-Gy γ-ray exposure. Results are representative of three independent experiments performed in triplicate.

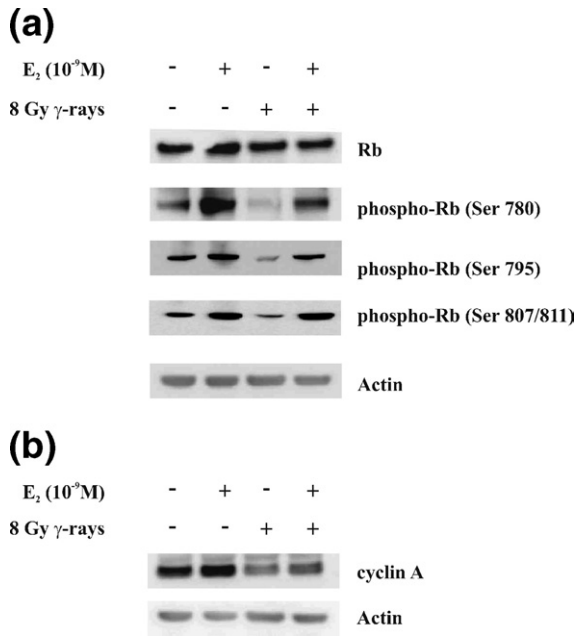

Fig. 4. Effects of E_2 on Rb regulation in irradiated breast cancer cells (IR-cells). (a) Total Rb expression, Rb protein phosphorylation status, and (b) levels of cyclin A, an E2F-dependent cell cycle protein, were assessed by western blot 24 h after 8-Gy γ-ray exposure. Proteins 25 μg for Rb and phospho-Rb; 50 μg for cyclin A were electrophoresed and western blot analysis performed as described in Methods and Materials. Loading and transfer of equal amounts of protein were confirmed by immunodetection of actin. Data shown are representative of three separate experiments.

No modification of Rb level was observed after γ-rays and/or E_2 treatments; Rb phosphorylation on several serine residues (780, 795 and 807/811) was then assessed. In non–IR-cells, E_2 increased Rb phosphorylation on Ser 780 and in a lesser extent on Ser 795 and Ser 807/811. On all serine residues 8-Gy γ-rays decreased Rb phosphorylation, an effect that was impeded by E_2. Because *CCNA2* is a well-known E2F target gene, we measured its product, cyclin A, by an immunoblotting technique (Fig. 4b). We found that E_2 slightly increased cyclin A level in non–IR-cells and sustained its expression in IR-cells, further supporting E2F activation.

E_2 induced dissociation of p21$^{waf1/cip1}$/cyclin E2 complexes independently of p53 regulation in IR cells

p53 activity was checked by immunoblotting (Fig. 5a), and treatment with γ-rays was found to increase p53 level. This increase followed a biphasic kinetic. Such complex kinetic was previously reported and attributed to the activation of the p53-associated MDM2 ubiquitin ligase (29). In IR-cells, E_2 did not prevent an increase in p53 level or its biphasic activation. The p53 activity, evaluated by its ability to bind its consensus DNA sequence, was then examined by DuoSet IC active p53 ELISA kit (Fig. 5b). Nuclear extracts from IR-cells exhibited an increase in p53 binding to the consensus sequence as compared with non–IR-cells. Treatment with E_2 did not modify the increase of p53 binding in IR-cells. p21$^{waf1/cip1}$ is known to play an essential role in p53-dependent and -independent cell cycle arrest and senescence (30). The p21$^{waf1/cip1}$ level was then determined by western blotting; as shown in Figure 5c, the p21$^{waf1/cip1}$ level was increased in IR-cells. We found that E_2 did not block p21$^{waf1/cip1}$ induction in IR-cells. In the absence of p21$^{waf1/cip1}$ modulation, we also analyzed other CDK inhibitors such as p16^{ink4a} and p27^{kip1}. As previously described, we did not observe p16^{ink4a} expression in MCF-7 cells (31) (data not shown). Expression of p27^{kip1} was also assessed

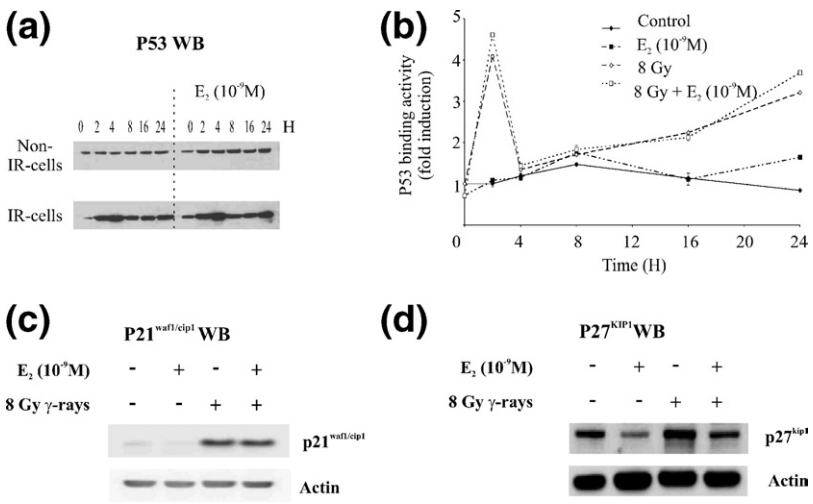

Fig. 5. Modulation of p21$^{waf1/cip1}$ by E_2 is independent of p53 in irradiated breast cancer cells (IR-cells). p53 levels were assessed by western blotting (a), and p53 activation was appreciated by its ability to bind consensus sequence (b) in 8-Gy γ-ray IR-cells up to 24 h. Western blot analysis of p21$^{waf1/cip1}$ (c) and p27^{kip1} in MCF-7 BCC (d). Proteins (25 μg) were electrophoresed and Western blots performed as described in Methods and Materials. Loading and transfer of equal amounts of protein were confirmed by immunodetection of actin. Results were representative of three independent experiments (a, c) and of two independent experiments (b, d) performed in triplicate.

by western blot (Fig. 5d). In non-IR cells, immunoblot analysis showed that E_2 decreased $p27^{kip1}$ basal expression. Moreover, E_2 prevented a γ-ray exposure–induced increase in $p27^{kip1}$ expression, which remained at the basal rate.

It has been shown previously that under E_2 stimulation, increased levels of cyclin D1/cdk4 associate to $p21^{waf1/cip1}$, which leads to the concomitant decrease in $p21^{waf1/cip1}$/cyclin E2/cdk2 complexes. Dissociated cyclin E2/cdk2 is activated and phosphorylates Rb (31, 32). To confirm such a hypothesis, $p21^{waf1/cip1}$ binding to cyclin D1 and E_2 were appreciated by immunoprecipitation. As shown in Fig. 6, E_2 did not significantly enhance cyclin D1 association with $p21^{waf/cip1}$ (Fig 6a), whereas it decreased $p21^{waf/cip1}$ binding to cyclin E_2 (Fig 6b). Altogether, these results suggest that E_2 decreased cyclin E2/$p21^{waf1/cip1}$ complexes in IR-cells.

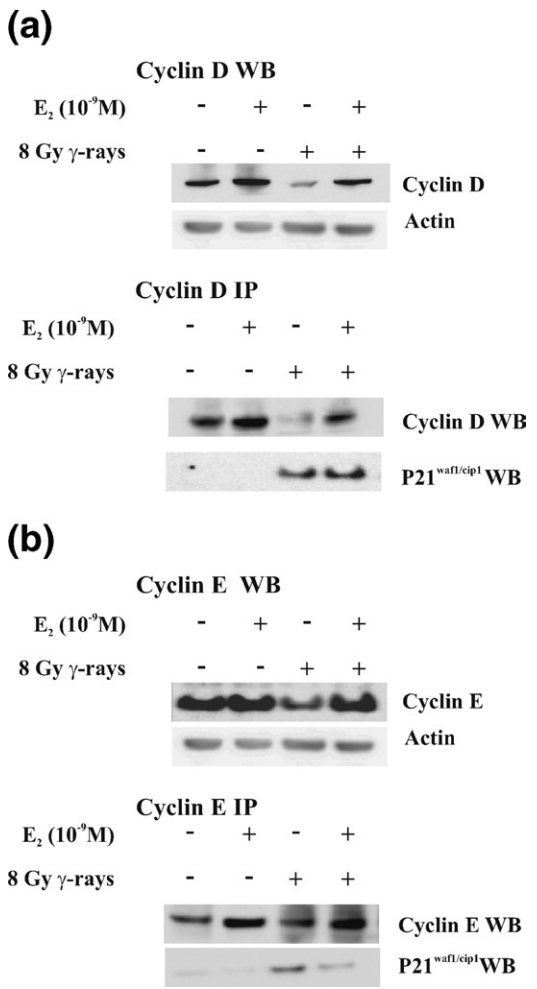

Fig. 6. E_2 induced redistribution of $p21^{waf1/cip1}$ from cyclin D1 to cyclin 2. At 24 h after γ-ray exposure, 1 mg of total cell extract was subjected to immunoprecipitation by a rabbit anti-cyclin D1 (a) or anti-cyclin E_2 (b) antibody and was followed by anti-cyclin D1 or anti-cyclin E_2 and anti-$p21^{waf1/cip1}$ immunoblots. As a control, 50 μg of total cell extract were also electrophoresed and Western blots for cyclin D1 and E_2 performed as described in Methods and Materials. Loading and transfer of equal amounts of protein were confirmed by immunodetection of actin. Results were representative of two (a) or three (b) independent experiments.

DISCUSSION

The present study shows that E_2 may partly prevent the BCC growth arrest induced by low γ-ray doses (<8 Gy), increasing clonogenic survival of BCC. At higher γ-ray doses, E_2 still sustained cell proliferation but no longer enhanced clonogenicity (data not shown). Moreover, we observed that E_2 prevented senescence in both non–IR-cells and IR-cells. Altogether, our data suggest that low γ-ray doses induce cell growth arrest through senescence that may be prevented by E_2. Higher γ-ray doses induce growth arrest through senescence and other mechanisms that may reflect harmful genetic lesions.

According to our microarray data, E_2 modulated IR-induced p53/$p21^{waf1/cip1}$/Rb pathways. Activation of p53 is known to result in a complex response in IR-cells. It seems insufficient to trigger apoptosis, but it induces a temporary (cell cycle arrest) or prolonged (senescence) cell growth arrest, and DNA repair (33). Interestingly, ERα expression in luminal breast cancer is rarely associated with mutation in *Tp53*, the p53 gene. Because most *Tp53* mutations lead to a p53 loss-of-function (34), this suggests that estrogen action on BCC mainly bypass p53 inhibitory effects. Accordingly, Molinari *et al.* (11) reported that E_2 stimulation may inactivate p53 through nuclear export. In agreement with a recent study (35), our data suggest that E_2 bypasses p53 growth inhibitory effects by acting on p53 downstream targets.

Under our conditions, E_2 did not inhibit p53 activation; but the hormone acted downstream p53, redistributing $p21^{waf1/cip1}$ from cyclin E_2 complexes. As a consequence of its effect on $p21^{waf1/cip1}$, E_2 prevented the dephosphorylation of Rb, an event that is known to mediate γ-ray–induced cell cycle arrest (36). Indeed, the hypophosphorylated Rb binds to members of the E2F transcription factor family and thereby antagonizes their ability to up-regulate genes involved in cell cycle progression and DNA repair (37). The hypophosphorylated Rb may also trigger senescence. Based on our data, it is thus likely that Rb inactivation and E2F activation may be responsible for the E_2 effects on cell growth, senescence, and radio-resistance.

Numerous reports indicate that DNA damage contributes to cellular senescence (21–23, 33, 38, 39). Senescence is associated with SA–β-galactosidase staining and is characterized by the expression of both growth inhibitor and paracrine tumor promoting factors. Two pathways have been implicated in the induction of cellular senescence involving p53 or $p16^{ink4a}$. In absence of $p16^{ink4a}$ expression in our MCF-7 BCC (data not shown), we have focused our investigation on p53-induced senescence. Nevertheless, we have shown that E_2 blocks senescence by disrupting $p21^{waf1/cip1}$ activation and not p53 in IR-cells. More precisely, E_2 treatment does not affect p53-dependent growth-regulated gene expression (*GADD45A, BAX, FAS*), whereas it increases p53-dependent growth-promoting factor (*GDF15, BIRC5*). These observations are consistent with a recent study demonstrating that p53 is involved in the cell growth arrest and

senescence just after γ-ray exposure, but that it acts as a protective factor that allows population recovery (33). Interestingly, our results indicated that E_2 may enhance this p53-protective effect. On the other hand, E_2 functionally targets $p21^{waf1/cip1}$, a downstream effector of p53. Based on our data, it appears that this effect of E_2 may be caused by the decrease in $p21^{waf1/cip1}$-dependent gene expression but also by $p21^{waf1/cip1}$/cyclin E complex dissociation. This effect of E_2 on $p21^{waf1/cip1}$ may explain the decrease in senescence. Nevertheless, we observed that E_2 also affects basal senescence induction in MCF-7 BCC in the absence of irradiation. These results emphasize the fact that $p21^{waf1/cip1}$ potentially enhanced senescence but was not necessary to trigger it. In these conditions, we suggest that senescence is probably linked to pRb phosphorylation status. Nevertheless, we cannot exclude E2F/pRb-independent effects of cyclin E on other substrates that may involve in DNA maintenance (40). Definitely, E_2 may both alter cdk-inhibitors expression ($p21^{waf1/cip1}$, $p15^{ink2b}$, $p27^{kip1}$) or activity ($p21^{waf1/cip1}$) and enhance cyclin expression (cyclin D, E, A) or activation (cyclin E). Altogether these effects of E_2 converge to increase pRb phosphorylation and its inhibition.

The effects of E_2 on radiosensitivity of BCC lines may also provide a rationale for the concomitant use of endocrine therapies and radiotherapy. Although endocrine therapies may affect the survival potential of IR-cells, clinical evidence of agonist vs. antagonist effects of tamoxifen endocrine therapy are still discussed (41). By contrast, concomitant anti-aromatase therapy seems to act synergistically (42). This discrepancy may reflect the complexity of the tamoxifen response in BCC. Indeed, tamoxifen is a selective estrogen receptor modulator which may act as an estrogen receptor antagonist but also as an agonist, depending of the cell context. This property may be the basis for the frequent appearance of tamoxifen resistance in BCC. Interestingly, irradiation induced a dramatic change in estrogen receptor-mediated response corresponding to an enhancement of NF-κB and/or interferon response (*BST2, IFI27, IFIT1, OAS1, OAS3, STAT1*); these genes are normally down-regulated by estrogen and up-regulated by inhibitors such as tamoxifen or aromatase inhibitors (43). Recently, Biswas *et al.* (44) have suggested that reactivation of NF-κB may contribute to hormone resistance of BCC. In addition, NF-κB induction is frequently associated with cancer cell resistance to radiation (45), chemotherapy (46), and apoptosis (47).

Our investigations have been performed *in vitro*. One cannot exclude the possibility that the BCC response *in vivo* could be different because of variations in factors relating to treatment (*e.g.,* dose, sequence, time lapse). Physiologic mechanisms such as repopulation and senescence induction might also be underestimated *in vitro*. Moreover, it is likely that the microenvironment of BCC *in vitro* may modulate the tumor responsiveness to radiotherapy and endocrine therapy. These modulations might result from bystander effects (48), direct ionizing radiation effects on neighboring normal cells such as fibroblasts (49), and complex integration of multiple autocrine, paracrine, or hormone-stimulated pathways (50, 51).

CONCLUSION

In conclusion, our studies showed that E_2 induced cell cycle regulators that sustained radioresistance of BCC and radiotherapy modified estrogen response in favor of cancer cell resistance. However, further studies are required to dissect physiologic mechanisms triggered by the hormone to provide a better understanding of the complex interactions between E_2 and $p53/p21^{waf1/cip1}$/Rb pathway and of the interference between hormone and adjuvant therapies (*i.e.,* endocrine, chemo-, and radiotherapies).

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Mini-Review

NF-κB modulation and ionizing radiation: mechanisms and future directions for cancer treatment

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Abstract

NF-κB transcription factor regulates important cellular processes ranging from establishment of the immune and inflammatory responses to regulation of cell proliferation or apoptosis, through the induction of a large array of target genes.

NF-κB is now considered as an important actor in the tumorigenic process mainly because it exerts strong anti-apoptotic functions in cancer cells. NF-κB is triggered by chimio- and radio-therapeutic strategies that are intended to eliminate cancerous cells through induction of apoptosis. Numerous studies have demonstrated that inhibition of NF-κB by different means increased sensitivity of cancer cells to the apoptotic action of diverses effectors such as TNFα or chemo- or radio-therapies. From these studies as emerged the concept that NF-κB blockade could be associated to conventional therapies in order to increase their efficiency.

This review focuses on the current knowledge on NF-κB regulation and discusses the therapeutic potential of targeting NF-κB in cancer in particular during radiotherapy.
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Keywords: NF-κB; Radiotherapy; Chemotherapy; Cell survival; Apoptosis

1. Introduction

NF-κB (Nuclear Factor-κB) was described for the first time in 1986 as a nuclear protein binding to the kappa immunoglobulin-light chain enhancer [1]. Since then, NF-κB has emerged as an ubiquitous factor involved in the regulation of numerous important processes as diverses as immune [2] and inflammatory responses [3], apoptosis [4] and cell proliferation [5]. These last two properties explain the implication of NF-κB in the tumorigenic process as well as the promise of a targeted therapeutic intervention [6]. In the present study, we described

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in the first part an overview of structure and functions of NF-κB and, in a second part, a focus on ionizing radiation, its impact on NF-κB pathway and the capacity of radiosensitization of NF-κB.

2. Structure of NF-κB /IKB complexes

Rel/NF-κB transcription factors share a highly conserved 300-amino-acid Rel Homology Domain (RH) containing sequences for dimerization, DNA binding, nuclear translocation and interaction with the inhibitory subunit IKB (Inhibitor of κB) [7,8]. Rel/NF-κB are organized in two classes. The p105 and p100 proteins of the NF-κB class are synthesized as precursors containing several C-terminal ankyrin repeats that are eliminated during maturation to respectively generate DNA-binding competent mature p50 and p52 proteins. RelA/p65, c-Rel and RelB of the Rel class are directely synthesized as mature proteins and possess, in contrast to the NF-κB class, a C-terminal transactivation domain (Fig. 1). Rel/NF-κB proteins associate as homo- or heterodimers, except for Rel-B that only forms heterodimers. Functional dimers bind specific κB sites on DNA (consensus sequence: 5′-GGGRNYYYCC-3′, where R is an unspecified purine, N is any nucleotide and Y is an unspecified pyrimidine). The most common and studied dimer is the combination p50/p65 RelA which is involved in regulating transcription of many κB-dependent genes. By contrast, p50/p50 or p52/p52 dimers are rather inhibitory due to the lack of a transactivator domain [2]. The 3D structure of a p50/p65 dimer bound to DNA showed that NF-κB proteins adopt a specific and unique conformation to recognize DNA using loops from both subunits and not alpha helixes like other transcription factors [9]. Each subunit contacts one half of the pseudo-symetric κB sites. The two halves (five bases) are separated by one base.

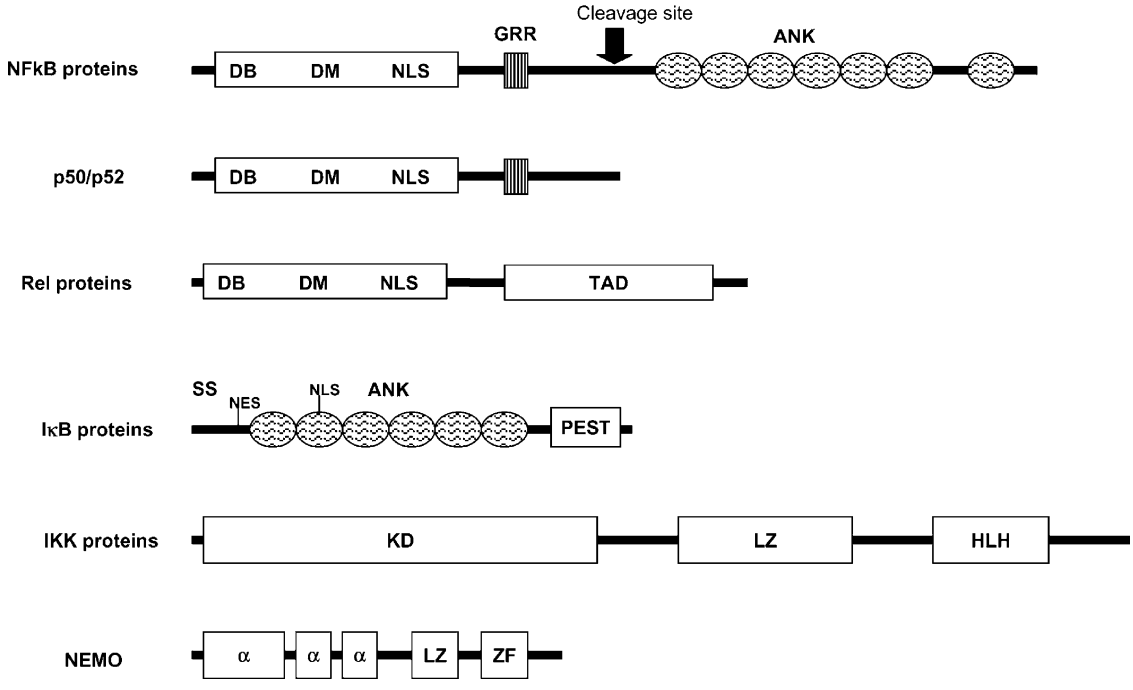

Fig. 1. Prototypal architecture of several actors of the NF-κB signalling pathway. DB, DNA binding; DM, dimerization domain; NLS, nuclear localisation sequence; GRR, glycin reach region; ANK, ANkyrin repeats; TAD, transactivation domain; SS, double serine phopshorylation sites; NES, nuclear export sequence; PEST, proline, glutamine acid, serine and threonine rich domain; KD, kinase domain; LZ, leucine zipper; HLH, helix loop helix; α, a helix; ZF, zinc finger.

3. Regulation of NF-κB activity

NF-κB activation is modulated by various different mechanisms involving protein/protein interactions, phosphorylation, and transcriptional modulation.

3.1. Inhibition of NF-κB by IKB proteins

NF-κB is sequestered inactive in the cytoplasm by interaction with an inhibitory subunit of the IKB (Inhibitor of κB) family. This family is composed of six proteins: IKB-alpha, IKB-beta, IKB-epsilon, IKB-gamma, IKB-zeta, Bcl-3 which possess 5–7 ankyrin repeats that mediate their inhibitory function [10] (Fig. 1). IKB masks nuclear localization signals (NLS) located on each NF-κB subunit to prevent their nuclear translocation. IKB-gamma corresponds to the ankyrin repeats-containing C-terminal part of p105 NF-κB1 that act by auto-inhibition and is eliminated during maturation. Bcl-3 is a peculiar member of the IKB family because it exerts a positive effect on gene transcription by displacing inactive p50/p50 or p52/p52 homodimers from DNA or through interaction with p52 [11,12]. IKBa/p50/p65 RelA are the most studied complexes. The IKBa protein can be divided in three parts: an N-terminal domain (SRD) that integrates activation signals, a central part bearing the ankyrin repeats involved in contact with and inhibition of NF-κB subunits, a C-terminal PEST region, rich in proline, serine, threonine and glutamic acid that regulate the constitutive half life of the molecule. Importantly, IKB not only interferes with nuclear translocation of NF-κB but can also displace NF-κB bound to DNA [13].

In 1999,150 different stimuly that can activate NF-κB have been listed [14]. In response to these diverse signals, the inhibitory subunit IKB is phosphorylated then degraded to free NF-κB. Most of NF-κB inducing stimuli such as TNFα, IL1, LPS and ionizing radiations [15] converge to the IKK (IkappaB Kinase) multi protein complex containing two IKB-specific kinases IKK1 and IKK2 and a scaffold protein IKKg/NEMO that is essential to signal transduction [16]. Activation of IKKs results in phosphorylation of IKB on two SRD serine residues (serines 32, 36 for IKBa). Doubly phosphorylated IKB is then recognized by E3RS, a specific ubiquitin ligase that catalyses ubiquitin transfert to the SRD (lysines 22, 26 for IKB). Ubiquitinated IKB is then recognized and degraded in situ by the 26S subunit of the proteasome, a multi-proteasic complex [17]. NF-κB is now free to translocate to the nucleus to activate transcription of target genes [14]. The gene coding for IKB is among the first to be transcribed by NF-κB. Newly synthesized IKB molecules migrate to the nucleus using an NLS to remove NF-κB dimers from DNA and turn off NF-κB -dependent transcription [18]. Then, using a nuclear export sequence (NES) NF-κB/IKB complexes return to the cytoplasm [19]. This regulation loop prevents overactivation of the NF-κB pathway. Besides this canonical/classical pathway, other means to activate NF-κB have been described. While IKK2 is mainly responsible for IKB phosphorylation to initiate the classical pathway, IKK1 phosphorylates NF-κB1/p100 to induce its maturation and to mobilize p52/RelB dimers [20]. UV irradiation induces NF-κB activation after phosphorylation and degradation of IKB apparently without participation of the IKK complex [15]. Oxydative stress and cell reoxygenation lead to NF-κB activation after phosphorylation of tyrosine 42 on IKB [21]. This pathway is independent of IKB degradation. Tyrosine phosphorylated IKB was shown to associate with the SH2 domain of the p85 PI3 kinase regulatory subunit that likely contributes to NF-κB/IKB dissociation [22].

3.2. Regulation of NF-κB transactivation by phosphorylation

NF-κB dimers are also succeptible to regulation by phosphorylation. For instance, hyperphosphorylation of p50 is required for its nuclear translocation following phorbol ester stimulation and increases its affinity for DNA in vitro [23]. By contrast, hypophosphorylated p50 cannot bind DNA in gel retardation assays [24]. The Akt kinase, downstream of PI3K can increase NF-κB transactivation potential via phosphorylation of the p65 transactivation domain [25]. In another example, the cyclic AMP-dependent protein kinase A (PKA) associates with NF-κB/IKB complexes in the cytosol and phosphorylate serine 276 on p65 once in the nucleus to increase its affinity for the transcriptional co-activators CBP/p300 [24].

3.3. Inhibition of NF-κB

Blockade of this pathway can be achieved at different levels in the cascade. Many natural or synthetic compounds have been ascribed with NF-κB inhibitory properties although most of them have a rather low specificity for the NF-κB pathway [26].

3.3.1. Anti-oxidants

On the front line are anti-oxidants that have been widely used for many years to inhibit NF-κB. NAC and PDTC were reported to block activation of NF-κB by various stimuli by interfering with IKK activation and IKB phosphorylation, suggesting at the time that ROS could be ubiquitous mediators of NF-κB activation [1]. Some anti-oxidants such as CAPE, were shown to directly prevent NF-κB binding to DNA [27]. Clearly NF-κB is sensitive to variations of the cellular redox potential, but most of the observed inhibitory actions were due to the numerous side effects of anti-oxidants on multiple cellular signalling pathways [28].

3.3.2. Proteasome inhibition

Degradation of the inhibitory subunit IKB to liberate NF-κB proceeds through the proteasome [29,30]. Proteasome inhibitors, in particular of the 26S catalytic activity such as lactacystine, MG-132, PS-341 [31], suppress NF-κB activation by stabilizing IKB despite phosphorylation by the IKK complex. It has to be kept in mind that apart from NF-κB inhibition, proteasome inhibitors also interfere with cell cycle progression since the half life of several cycle regulators is regulated by ubiquitination/degradation [32]. Although this has not yet been explored, NF-κB activation may be prevented via inhibition of the E3RS ubiquitin ligase [33].

3.3.3. Inhibition of IKB phosphorylation

Some inhibitors such as curcumin [34] or sanguinarine [35] come from plants or from fungi like gliotoxin [14] Others (BAY-11-7082; PS-1145) have been synthetized and selected for their ability to prevent IKB phosphorylation [36]. Similarly to other non steroidal anti-inflammatory drugs (NSAID), aspirin at the high doses used to treat chronic inflammation is an inhibitor of IKK2 [37]. Most of these agents have, however, a rather low specificity for the IKK which is likely to generate unwanted side effects and represent a major problem for a therapeutical use.

3.3.4. IKB super-repressor

Mutations of IKB-apha serines 32 and 36 prevents both phosphorylation and degradation of the inhibitor. Transfected mutated IKB competes with endogenous IKB molecules to block NF-κB activation. This mutant molecule is therefore referred as a super-repressor of NF-κB activation and has been used in numerous studies to evaluate NF-κB functions [38–40]. Although it is the most specific approach to date, it cannot be easily used in primary cells nor in clinics.

4. NF-κB functions

Since its discovery in 1986, NF-κB has primarily been known for its regulatory role for immune and inflammatory responses. Its functions have now been extended to the regulation of cell proliferation and survival and NF-κB is considered as both an important player in the tumorigenic process and a potential therapeutic target in cancer [5,41,42].

4.1. NF-κB in immune and inflammatory responses

Two main pathways are known to regulate immune responses: the NF-κB pathway and the glucocorticoid (GR) pathway. Both the GR receptor as well as NFKB are transcription factors but with opposite functions. While NF-κB is stimulatory for the immune system and has pro-inflammatory properties, GR are immunosuppresive and anti-inflammatory [43,44]. NF-κB induces expression of genes coding for antigen receptors on immune cells, adhesion molecules, pro-inflammatory cytokines (TNF, IL1, LPS) or chemoattractants (MCP-1) for inflammatory cells [14]. GR acts by suppressing NF-κB activation through several mechanisms such as up-regulation of IKB gene expression [26].

4.2. NF-κB and apoptosis

The implication of NF-κB in apoptosis was revealed in 1995 with the discovery that invalidation

of the RelA gene was embryonic lethal because of an increased sensitivity of hepatocytes to the apoptotic action of TNF [45].

Similar phenotypes were observed after inactivation of the genes coding for IKK2 or NEMO [46] demonstrating the anti-apoptotic role for NF-κB in liver functions. This role was further highlighted in many different cellular models using the IKB super-repressor to prevent NF-κB activation leading to increased apoptotic sensitivity of the cells to various stimuli or stress conditions [47–50].

4.2.1. NF-κB regulates anti-apoptotic genes

NF-κB can interfere with apoptotic signals at various levels. The best example is found in the TNF receptor I signalling pathway [51] (Fig. 2). This receptor is coupled via the FADD adaptor to a caspase cascade involving the initiator caspases 8 or 10. However, in many cell types TNF has no apoptotic effects. This is due to the parallel triggering by TNF of a signalling pathway that activates NF-κB via the TRADD and TRAF adaptors. Active NF-κB induces transcription of a set of genes coding for anti-apoptotic proteins. Among them are c-IAP-1 and c-IAP-2 that either directly block caspase functions or indirectly induce their ubiquitination and proteasome-dependent degradation [52]. NF-κB also regulates expression of several TRAF proteins that serve to amplify NF-κB activation and to interfere with the caspase cascade both at the TNFR1 level [53]. Another NF-κB target gene codes for A20 that also perturbs caspase activation at the TNF receptor [54]. A major anti-apoptotic protein whose gene is under NF-κB control is c-FLIP that has high homology with procaspase 8 but no catalytic activity. Upon induction, c-FLIP associates with TNFR to compete with and block caspase 8 activation [55]. A second apoptotic pathway proceeds via mitochondria. Changes in the mitochondrial transmembrane potential regulate cytochrome c release in the cytosol where, in

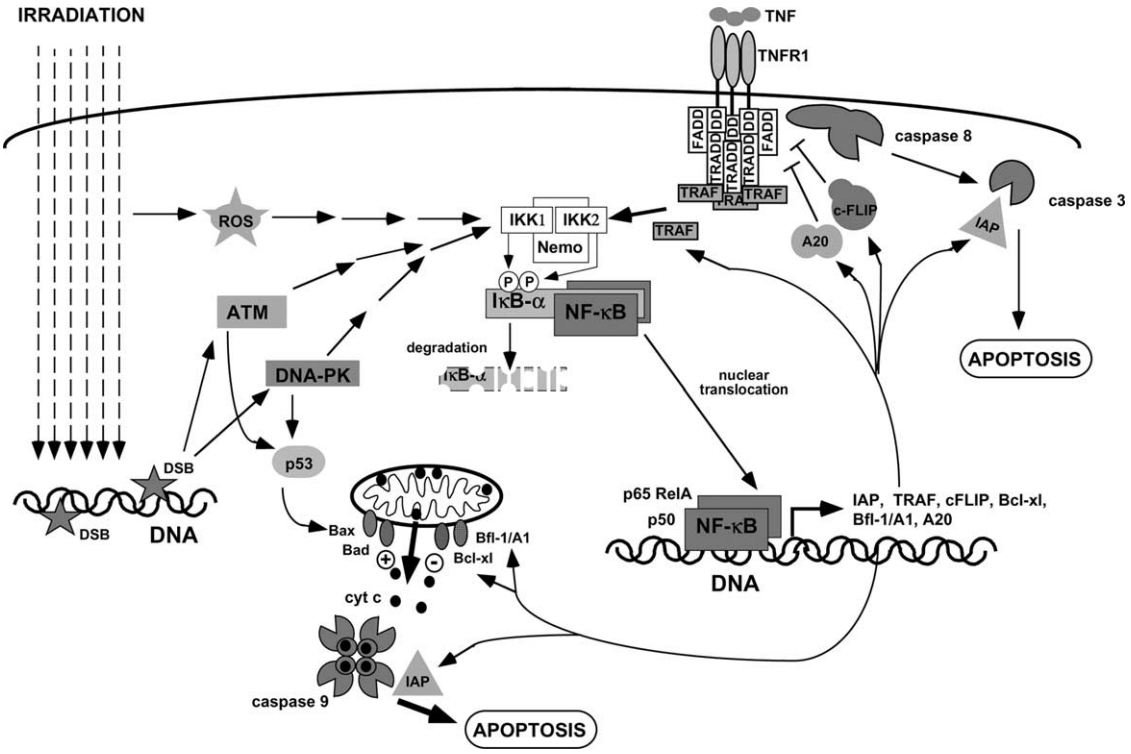

Fig. 2. Schematic activation of NF-κB by the TNF receptor 1 signalling pathway induced after irradiation. See text for details. DSB, double strand DNA breaks; ROS, reactive oxygen species; cyt *c*, cytochrome *c*; DD, death domain; TRAF, TNF-receptor associated factor; FADD, Fas-associated death domain; TRADD, TNFR-associated death domain; IAP, inhibitor of apoptosis.

coordination with Apaf-1 and d-ATP, it triggers activation of the initiator caspase 9 [56]. This pathway is controlled at the outer and inner mitochondrial membranes by proteins of the bcl-2 family that comprises both anti- (bcl-2, bclxl, bfl-1,…) or pro-apoptotic (bad, bax,…) members. NF-κB transcribes the genes coding for several anti-apoptotic proteins of this family such as bcl-xl, bfl-1/A1, Nr13 [57] all of which act to prevent cytochrome c release and the subsequent caspase 9 activation. NF-κB can interfere with induction by p53 of the pro-apoptotic protein Bax in colic carcinomas [39]. NF-κB and p53 have most of the time opposite functions although p53 can be induced by NF-κB [58,59]. The two transcription factors appear to compete for interaction with transcriptional co-activators [60,61]. In another cellular setting, NF-κB is required for the pro-apoptotic action of p53 [62] demonstrating that the interaction between the two pathways is complex.

4.2.2. NF-κB has pro-apoptotic functions

Under certain circumstances, NF-κB can promote or amplify cell death. Most of the effects are due to the induction of genes that code for the death receptor Fas or its ligand FasL. This is the case during elimination of activated T cells in the AICD response [63].

4.2.3. NF-κB and cell cycle regulation

Cell cycle and cell proliferation are interconnected in a delicate balance [64]. NF-κB mainly acts through the induction of the gene for cyclin D1 which is involved in the G1/S transition [65]. The cyclin A promoter that lacks a canonical KB site can be activated by NF-κB through an unknown mechanism. C-Rel has been shown to associate with the cyclinE/cdk2 complex involved in the G1/S transition, although the significance of this observation in terms of cdk2 or NF-κB functions is unknown [57].

4.2.4. NF-κB pathway:a target for caspases

Caspases are the executioners of the cell death responses and destroy cell structure and functions through the cleavage of strategic substrates. The NF-κB survival pathway is a substrate for caspases at different levels. The p50 and p65 proteins are cleaved by caspase 3 [60] and c-Rel has three cleavage sites for caspase 3 [66]. In all cases, proteolysis by caspase inactivate NF-κB proteins and transform them into dominant negative factors, still capable of DNA binding but devoid of transactivation potential. IKB is also a substrate for caspases that remove its most N-terminal region containing the two IKK specific phospho-acceptor serines, changing the inhibitor into a super-repressor protein [66]. Upon cell death, IKK2 is cleaved and crippled by caspase 3 therefore interrupting NF-κB-dependent survival signals emanating from the TNF receptor [67].

5. NF-κB and radioresistance

Radiotherapy acts through the induction of double strand breaks to DNA in order to induce elimination of cancerous cells via programmed cell death [68].

The efficiency of radiotherapy for cancer treatment is limited by toxic side effects impeding dose escalation. Moreover, cancer cells often develop radioresistance mechanisms that are related to the DNA repair response. The aim of combining chemo- to radio-therapy is to strengthen the efficiency of radiation by inhibition of DNA repair, and over-coming of apoptosis resistance.

NF-κB is activated by DNA damaging agents and could be involved in cell cycle arrest and prevention of apoptosis in order to allow DNA repair [69–71]. However, a sustained NF-κB activation could permit cells that have accumulated radiation-induced DNA damage to escape elimination by apoptosis [72]. Indeed, high constitutive NF-κB activity prevents cancerous cells from apoptosis [73] and results in a more aggressive potential for prostate [74] or ORL [75] cancer cell lines. Ionizing radiations and cytotoxic agents (daunorubicin, CPT-11) activate NF-κB via the protein kinases ATM or DNA-PK [76–80] (Fig. 2). ATM is a nuclear serine kinase that upon sensing DNA damage activates several signalling pathways to regulate cell cycle, stress and DNA repair [80]. Cells from Ataxia-Telangiectasia (AT) patients lack ATM and suffer from extreme radiosensitivity and cell cycle anomalies [77]. These cells also display a defect in NF-κB activation that could explain in part their enhanced apoptotic responses to DNA damaging agents [79]. Similarly, DNA-PK is important for NF-κB activation upon DNA damage [76]. Double strand DNA breaks are sensed by Ku proteins, the regulatory subunits of DNA-PK. It is not

clear yet how ATM and DNA-PK triggers the IKK complex although recent work by Panta et al. suggests that they could act via a kinase cascade involving MEK/ERK/p90 rsk [81].

Interestingly, ATM as well as DNA-PK induces pro-apoptotic responses via p53 stabilization and anti-apoptotic ones via NF-κB activation. The balance between these two opposing pathways determine the cell's fate [82].

In addition, reactive oxigen species (ROS) that are produced during irradiation could also be mediators of NF-κB activation (Fig. 2). It is possible that ROS could stimulate the IKK complex after inhibition of cellular protein phosphatases. It is interesting to note that in return NF-κB regulates the induction of the ROS detoxifying enzyme MnSOD [15].

Several studies have explored the impact of NF-κB inhibition on radiosensitivity in various models [83–86]. Expression of the IKB super-repressor resulted in increased apoptosis in irradiated fibroblasts [50] or ORL cancer cells [87,88]. Over-expression of wild type IKB sensitized human glioblastomas to radiations [89]. Moreover, the specific knock out of the IKK2 gene in intestinal epithelial cells demonstrated a radioprotective role of NF-κB in this cell type [90]. By contrast to these examples, the blockade of NF-κB activation with a super-repressor did not improve cell death of irradiated prostate carcinoma or Hodgkin lymphoma cell lines [91]. However, the interpretation of the results presented in that study was largely questioned [92].

Pharmacological inhibition of NF-κB would therefore be a very interesting approach to potentiate irradiation. Indeed, indomethacin, that suppresses NF-κB activation rendered HeLa cells more susceptible to apoptosis after irradiation [93]. Curcumin, a natural compound that interfere with IKK activation enhanced radiation-induced apoptosis of prostate PC-3 cancer cells [94]. Proteasome inhibitors are highly potent inhibitors of NF-κB activation through inhibition of IKB degradation (see above). When associated with irradiation, two of them, MG-132 and PS-341 augmented cell death of respectively Hodgkin [91,95] and glioblastoma [96] or colorectal cancer [31] cell lines. In the first study, however, no inhibition of NF-κB by MG-132 was observed.

Phase I studies with the proteasome inhibitor PS-341 (Bortezomib/Velcade) have demonstrated acceptable and manageable toxicities in patients with advanced cancers and phase II studies have been started on various hematologic cancers as well as on diverse solid tumors. Bortezomib is tested as a single molecule or in association with various chemo- or radio-therapies [97]. Given the promising results obtained in vitro on numerous and diverse cancer cell lines, therapeutical benefits are expected from these clinical trials.

Several recent studies have demonstrated that NF-κB is a molecular link between inflammation and cancer [98–100]. NF-κB plays a dual role by preventing apoptotic elimination of the cells submitted to the inflammatory insult and by mediating the release by macrophages of growth factors that sustain transformed cells. Ionizing radiations are inducers of inflammation [101]. Moreover, Hallahan et al. [102,103] have shown that radiations induce TNF-α production which mediates some lethal effects of radiations. Considering that TNF-α is both an inductor of and a target gene for NF-κB, a loop which amplifies the effects of radiations could settle, similarly to the situation of chronic inflammation. Inhibition of NF-κB will result in a complex situation with on one side an increased apoptosis of irradiated cells but on the other side lower TNF production and inflammation [104] decreasing the therapeutical effects of radiations. Altogether, these studies that used different approaches on different cancer models highlight the gain in apoptotic responses obtained by association of irradiation with NF-κB blockade. It is now crucial to confirm these results in an animal model.

6. Conclusion

NF-κB is now recognized as an important player in several critical steps of the tumorigenic process. Not only NF-κB promotes survival of cancer cells but it also contributes to abnormal proliferation and metastasis. As a stress factor, NF-κB is a crucial element of the cell's protective response to radiations and represents therefore an attractive target in new therapeuticals approaches to fight cancer. The pharmaceutical industry is producing several inhibitors of the NF-κB pathway that are going to be

used in association with conventional therapies and in particular with ionizing radiations. In vitro data have established this concept and clinical trials are now in sight. Inhibition of NF-κB is expected to increase the therapeutical efficiency of radiation.

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Concurrent hormone and radiation therapy in patients with breast cancer: what is the rationale?

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Endocrine therapy is often given together with postoperative radiotherapy in patients with breast cancer and positive hormone-receptor status. However, few experimental or clinical studies address the combined effects of hormone and radiation therapy. Preclinical models have shown changes in tumour cell kinetics with the addition of tamoxifen, and some show reduced tumour cell death with concurrent anti-oestrogen treatment and radiotherapy. Although data from in-vitro studies support the notion of antagonistic effects of concurrent tamoxifen and radiotherapy on tumour cells, in-vivo research suggests a synergistic effect that could be attributable to micro-environmental changes in tumour responsiveness to ionising radiation and hormone therapy. Retrospective studies suggest that in practical application, concurrent administration of tamoxifen with radiotherapy does not compromise local control but might increase toxicity. Preliminary results from simultaneous treatment with aromatase inhibitors and radiation indicate that this combination of endocrine and radiation therapy could enhance cytotoxicity and improve tumour response. Further studies are needed to clarify the physiological mechanisms activated by oestrogens, which will allow a more thorough understanding of the complex interactions between 17beta-oestradiol and P53/P21$^{WAF1/CIP1}$/Rb pathways and of the interaction between endocrine therapy and radiotherapy.

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Introduction

The identification of oestrogen's central role in mammary carcinogenesis has led to investigation of oestrogen pathways as major targets for breast-cancer therapy. The biological effects of oestrogen are mainly mediated through binding to oestrogen receptors alpha and beta; ligand-dependent transcription factors that determine growth, survival, and differentiation of breast-cancer cells.[1] Breast cancer cells (figure 1) are targeted by both endocrine therapy and radiotherapy, however, the mechanism underlying the mitogenic effect of 17beta-oestradiol has not been elucidated. For instance, adjuvant tamoxifen, an oestrogen antagonist, reduces the risk of distant metastases, local recurrence, and contralateral breast cancer incidence in women with tumours that express hormone receptors,[1–5] and therefore the combination of radiation and hormone therapy has become widespread clinical practice for receptor-positive breast cancer. Endocrine therapy is often combined with radiotherapy in patients with locally-advanced breast cancer, and then adjuvant chemotherapy can be administered. However, few studies in which the two modes of treatment are combined are available.[6] The sequencing of chemotherapy, radiation, and hormone therapy is a challenge for the oncologist when selecting the best treatment approach for breast cancer, and an important clinical question is whether to combine endocrine therapy and postoperative radiotherapy. Additionally, given the widespread application of adjuvant endocrine therapy, it is important to assess the safety and efficacy of cancer treatments relative to their sequence of administration.[7–9]

The aim of this review is to summarise in-vitro and clinical data that assess the sequencing of hormone and radiotherapy on outcomes in breast cancer, to improve understanding of concurrent endocrine and radiation treatment.

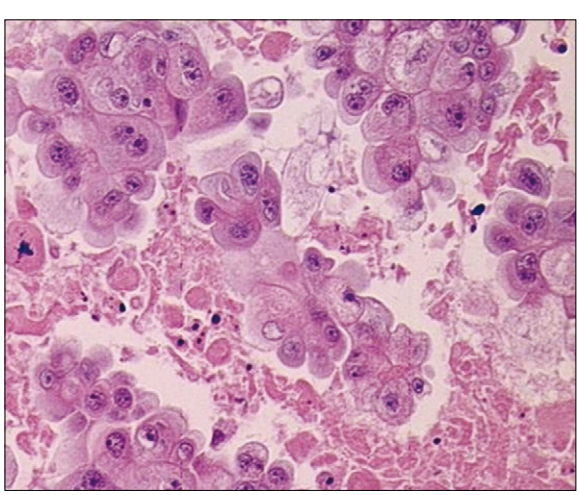

Figure 1: **Breast-cancer cells: a target for endocrine or radiation therapies**

Interaction between oestrogen and ionising radiation

The cellular effects of oestrogen are mediated by the secretion of hormone-regulated autocrine growth factors, which seem to engage in cross-talk with growth-factor signalling pathways independent of transcriptional effects.[10] The cross-talk between oestrogen and growth-factor signal cascades, including the MAP-kinase and PI3-kinase pathways,[11,12] might inhibit the effects of ionising radiation.[13] Other reports suggest that the effects of 17beta-oestradiol on radiosensitivity could be related to inactivation of P53, which maintains genomic integrity and protects cells against radiation-induced damage.[14,15] Molinari and colleagues[16] showed that oestrogen treatment of breast-cancer cells grown in serum-free

medium induced a modification of the intracellular distribution and functional inactivation of P53. Although a direct connection between P53 activation and radioresistance has not been shown, transcription factor NFκB is associated both with radioresistance and P53 activation.[17–19] Therefore, pharmacological inhibition of NFκB could be an approach to potentiate radiotherapy,[20] and treatment strategies designed to prevent or interrupt activation of NFκB could restore radiosensitivity in the setting of concurrent hormone therapy.[12]

Positive status for oestrogen receptor alpha is used as a predictor of the outcome of hormone therapy, with patients positive for oestrogen receptor alpha having a 60–80% overall response to selective oestrogen receptor modulators (SERMs).[21] Reduced oestrogen binding was reported in MCF-7 breast-cancer cells irradiated at the time of plating.[22] Toillon and colleagues[23] showed that one 8 Gy dose of radiation diminished synthesis of oestrogen-receptor-alpha but had no effect on receptor function; cell sensitivity to anti-oestrogenic agents was maintained. Radiation-induced apoptosis was maintained both in the presence or absence of 17beta-oestradiol. However, 17beta-oestradiol impeded the growth-inhibitory effects of ionising radiation in MCF-7 breast-cancer cells, indicating that the hormone lowered the radiosensitivity of these cells. The observed decrease in radiosensitivity and maintenance of apoptosis suggests the absence of an association between radiosensitivity and induction of apoptosis.[24] Moreover, 17beta-oestradiol might partly prevent cellular growth arrest induced by low doses of X-ray, enhancing clonogenic survival of breast-cancer cells and preventing senescence in both non-irradiated cells and irradiated cells by modulation of radiation-induced P53/P21$^{WAF1/CIP1}$/Rb pathways, leading to inhibition of Rb dephosphorylation, an event that is known to mediate radiation-induced cell-cycle arrest.[23] Work by Xia and colleagues[25] showed that P53 affected the transient radioresistance of MCF-7 breast-cancer cells after chronic exposure to ionising radiation. These results support the idea that 17beta-oestradiol-induced inactivation of P53 might have a role in the tumorigenesis of oestrogen-dependent cancers, thus making anti-oestrogen therapy a logical strategy. 17beta-oestradiol could bypass the growth inhibitory effects of P53, which induce temporary or prolonged arrest of cell growth and DNA repair, by acting on targets downstream of P53.[26] Other investigations have indicated that down-regulation of the anti-apoptotic genes *BCL2* and *PTGS2* and up-regulation of pro-apoptotic genes, such as *TP53* and *P21*, could enhance radiation-induced cytotoxicity and apoptosis in MCF-7 breast-cancer cells.[27]

17beta-oestradiol might induce *CCND1* and *MYC* expression, allowing cell-cycle-progression via cyclin-CDK activation and subsequent G_1/S and G_2/M transitions.[28] The MCF-7 breast-cancer cell line responds to stimulation by a physiological concentration of 17beta-oestradiol with increased proliferation.[29] Moreover, oestrogens can promote G_1 progression through direct activation of cell-cycle genes or through the Ras-mitogen-activated protein-kinase pathway. Oestrogen impedes the growth-inhibitory effects of ionising radiation in MCF-7 cells, which potentially decreases radiosensitivity of these cells. Oestrogen's obstruction of growth-inhibition can be blocked by SERMs and anti-oestrogen treatments.[30] The effects of 17beta-oestradiol on radiosensitivity of breast-cancer cell lines might also provide a rationale for the concomitant use of endocrine and radiation therapies.

Combining tamoxifen with radiotherapy

Tamoxifen is a SERM used for the treatment of both early and advanced breast cancers that are positive for oestrogen receptor in pre-menopausal and post-menopausal women. Although the effect of the drug on overall survival has been established in the adjuvant therapy of breast cancer, little data exist on the timing of tamoxifen therapy with or after radiotherapy.

Preclinical data

Although the mechanism of action of tamoxifen is not wholly understood, the drug has been shown to exert a number of hormonal and non-hormonal effects. Several studies have shown that tamoxifen inhibits proliferation of endocrine-receptor-positive human breast-cancer cells.[29,31,32] The fact that tamoxifen has no effect on the cell-cycle kinetics of the receptor-negative MDA-MB-231 cells suggests that the antioestrogen effect is mediated through the oestrogen receptor, and that regression of breast cancer in vivo is mostly due to blocking of oestrogen-dependent cells through the cell cycle rather than by a direct cytotoxic effect.

Using MCF-7 human mammary-epithelial cells, Sutherland and colleagues[31] demonstrated a dose-dependent decrease in the growth rate of tumour cells with the addition of tamoxifen, which was accompanied by a dose-dependent increase in the percentage of cells accumulating in G_0/G_1, and a decline in the proportion of cells in S phase. Several studies using [^{3}H]-thymidine incorporation techniques suggested that tamoxifen might block cells in a uniform phase of the cell cycle,[29] possibly in early G_1. Osborne and colleagues[32] investigated the effects of tamoxifen on the cell-cycle kinetics of the endocrine-responsive MCF-7 human breast-cancer cells and showed that tamoxifen inhibits proliferation. The ^{3}H-thymidine labelling-index was markedly reduced by tamoxifen, indicating a reduction in the fraction of cells in S phase. An associated depletion of G_2/M phase cells, as well as an accumulation of cells in G_1, was observed. These cell-cycle data indicate that tamoxifen inhibits proliferation of MCF-7 human breast-cancer cells by a reversible cell-cycle blockade in the early G_1 phase of the cell cycle.[32] Cells are most radiosensitive in the G_2/M phase, less sensitive in G_1, and least sensitive during the latter part of the S phase. Because cells are not as radiosensitive in early G_1 phase as in G_2, tamoxifen may

render cancer cells less responsive to radiotherapy, reducing cell death caused by ionising radiation. Tamoxifen-induced arrest in G_1 phase might also permit increased repair of DNA damage. Cell survival data indicate that non-homologous end joining is the predominant mechanism of double-strand break repair, and that this mechanism seems to be more important during the G_1 and early S phases.[32]

Wazer and colleagues[33] observed that cells in 1 µmol/L or 5 µmol/L tamoxifen were less radiosensitive than controls and showed a widened shoulder (increased survival) on the radiotherapy survival curve. Interpretation of the survival curves is complicated, however, by the fact that cell cultures in the two previous studies were grown in a medium containing phenol red and fetal bovine serum, two sources of exogenous oestrogenic compounds.[34] Data suggest that the antitumour effect of tamoxifen on MCF-7 cells is due to the expression of cyclin-dependent kinase inhibitors, especially $P21^{WAF1/CIP}$, that would normally be regulated by the activity of wild-type P53. This cytotoxic effect of kinase-inhibitor expression is a mechanism similar to that of ionising radiation and of other agents that cause DNA damage. Ichikawa and colleagues[35] found that the levels of P53 and $P21^{WAF1/CIP1}$ in tamoxifen-treated cells increased in a time-dependent and a dose-dependent manner, whereas those of $P27^{KIP1}$ and $P16^{INK4A}$ increased slightly or remained unchanged. A marked decrease in the S phase fraction and an increase in the number of cells in G_1 and G_2 accompanied the inhibitory effect of tamoxifen.[35]

Paulsen and colleagues[36] proposed that hormone therapy might alter radiation sensitivity, even in cells negative for oestrogen receptors, suggesting that hormonal agents might act both via receptor and non-receptor binding mechanisms. In-vitro exposure to tamoxifen increased radiation resistance in cell lines positive for oestrogen and progesterone receptors (eg, MCF-7 breast-cancer cells), while 17beta-oestradiol increased radiation sensitivity in cells negative for oestrogen and progesterone receptors (MDA-MB-231 cells). The expression of essential growth-controlling genes was studied in MCF-7 cells after exposure to cumulative radiation doses of 20 and 60 Gy.[37] A diminished response to tamoxifen-induced growth arrest and 17beta-oestradiol-induced growth stimulation was demonstrated by quantitative immune-peroxidase staining of single cells and by total cellular 17beta-oestradiol binding. Other results showed that single and repeated exposures to radiation might modulate the expression of both oestrogen receptors and epidermal growth factor-receptor (EGFR) in MCF-7 cells.[38] An inverse relationship in the expression of hormone receptors and EGFR was established for cells positive for oestrogen receptors, and maintained after single and repeated exposures. This suggests that radiotherapy acts through common regulatory circuits, modulating the cellular phenotype.[38]

However, other in-vitro assays indicated that growth-inhibitory concentrations of tamoxifen did not modify the radiation sensitivity of MCF-7 human breast-cancer cells. Sarkaria and colleagues[39] demonstrated that growth of MCF-7 cells was inhibited by 4-hydroxytamoxifen but found no substantial change in radiation sensitivity of 17beta-oestradiol-stimulated, or 4-hydroxytamoxifen-inhibited, cultures plated into growth-stimulating conditions after ionising radiation or an additional 24 h in oestrogen-free conditions. Several observations suggest a mechanism of action for antioestrogens that is independent of the presence of oestrogen receptors in tumour epithelial cells. Documented non-hormonal effects of tamoxifen include induction of cell secretion of the multifunctional cytokine transforming growth-factor β (TGF-β).[40] Although TGF-β was originally reported to be an oncogene, early studies also showed that it is a potent inhibitor of epithelial cell proliferation. TGF-β-mediated inhibition of growth could underlie the action of tamoxifen in tumour cells negative for oestrogen receptors.[41] Tamoxifen has been shown to induce the secretion of TGF-β from human breast-cancer lines in vitro, suggesting that some of the in-vivo effects of this class of drug might be mediated by the induction of such endogenous growth-inhibitors.[42] TGF-β, induced by anticancer therapy, has also been identified as a pro-metastatic signal in some tumour cells, providing a rationale for the use of TGF-β inhibitors in combination with radiotherapy.[43] However, recent results suggest that suppression of TGF-β signalling by activated Akt is correlated with the development of tamoxifen resistance in breast cancer.[44] TGF-β may also cause chemotaxis of fibroblasts,[45] explaining its importance in the pathogenesis of fibrosis in many animal models.[46]

Kantorowitz and colleagues[47] assessed in vivo the efficacy of irradiation given with concurrent tamoxifen. Female Sprague-Dawley rats with small tumours induced by 1-methyl-1-nitrosourea were treated with tamoxifen, radiation, or a combination of both. Radiation dose was 45 Gy delivered in 25 fractions, 1·80 Gy per fraction. Tamoxifen (500 mg/kg bodyweight) was administered subcutaneously each day during the irradiation interval. Combined tamoxifen and radiation resulted in significant reduction in tumour volumes and suppressed additional tumour growth, which was observed when radiation alone was administered. Sarkaria and colleagues[48] found a reduction in cell proliferation rate induced by 17beta-oestradiol deprivation in MCF-7 human breast xenografts during fractionated radiotherapy. A dimished cell growth rate resulted in a significantly decreased dependence on overall radiation treatment time in comparison with the more rapidly proliferating 17beta-oestradiol-stimulated tumours. In view of these results, and by contrast with in-vitro data, endocrine therapy can enhance radiosensitivity by inhibition of tumour regrowth, providing a rationale to give hormonal therapy along with post-operative radiotherapy (figure 2). However, endocrine

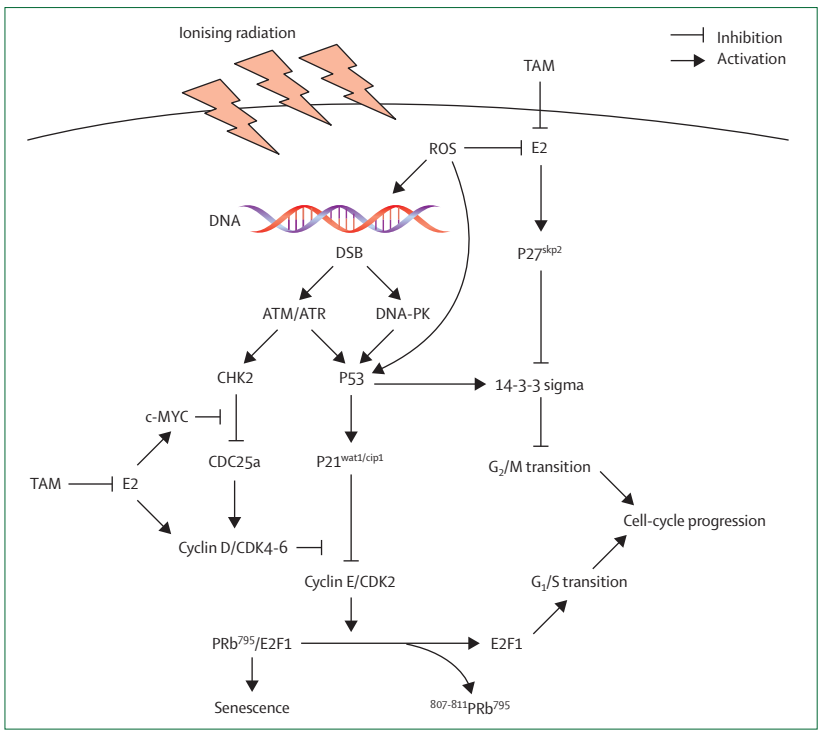

***Figure 2*: Potential direct genomic effect of estradiol, tamoxifen, and ionising radiation on inhibition of cell-cycle progression**

Ionising radiations induce direct or indirect (by ROS generation) DNA-strand breaks (DSB). DSB activate Ataxia telangiectasia-mutated gene (ATM) and Ataxia telangiectasia-mutated gene Rad3-related (ATR) and DNA-protein kinase (DNA-PK) signal transduction pathways. In turn, these kinases phosphorylate checkpoint kinase 2 (CHK2) or P53 and lead to subsequent cell growth arrest. Depending on downstream effectors (CDC25A, $P21^{waf1/cip1}$, 14-3-3 sigma), cell cycle is arrested at G_1/S or G_2/M transition. In irradiated cells, 17beta-estradiol (E2) might down-regulate these transduction pathways at several levels. Also, 17beta-oestradiol might decrease production of reactive oxygen species and subsequent cell response (by the inhibition of DNA damage induced by reactive oxygen species or by decreasing P53 activation). Moreover, 17beta-oestradiol increases expression of cell cycle regulators, such as *MYC* and *CCND1*, favouring G_1/S transition, while it acts on P27 and SKP2 to allow G_2/M transition. By contrast, tamoxifen impedes 17beta-oestradiol effects through its antioestrogen activity. This effect may reinforce growth inhibition induced by ionising radiation.

therapy could enhance the toxicity of radiation. Butta and colleagues[49] found that 3 months of tamoxifen treatment led to raised levels of extracellular TGF-β, implicated in the pathogenesis of radiation-induced fibrosis, around the stromal fibroblasts in breast-cancer biopsy specimens.

Clinical results

No randomised trials so far have investigated the clinical effect of the sequencing of tamoxifen and radiotherapy. Several studies have shown increased local control after breast-conserving surgery and radiotherapy in patients receiving tamoxifen compared with treatment without the drug; however, most reports do not specify the timing of the endocrine therapy relative to radiotherapy.[3,21,50] Few retrospective analyses have been published on the topic. A retrospective study by Pierce and colleagues[51] assessed 10-year tumour control according to the timing of radiotherapy relative to tamoxifen among patients treated with chemotherapy, followed by tamoxifen in addition to radiation. According to the cooperative group standard for this era of adjuvant trials, tamoxifen was prescribed for both receptor-positive and receptor-negative disease. Radiotherapy was given to patients who received breast-conserving surgery, either before adjuvant chemotherapy or after chemotherapy was completed. An exploratory analysis assessed the optimum sequencing of tamoxifen and radiotherapy after breast-conserving surgery. 107 patients received radiotherapy immediately after breast-conserving surgery but before tamoxifen, and 202 patients received radiotherapy and tamoxifen concurrently. No significant differences in grade 3 or 4 haematological toxicity were observed between the two groups, with 21% of concurrent radiotherapy patients having a grade 3 and 50% having a grade 4 granulocytopenia, compared with 18% and 47% for sequential radiotherapy (p=0·22). 10-year disease-free survival was similar for both concurrent and sequential treatment groups (93% in both groups) and 10-year overall survival was 88% and 90%, respectively (p=0·65). The report did not identify an effect on local or systemic control with concurrent versus sequential tamoxifen and radiotherapy in patients with node-negative breast cancer. This prospective trial, however, was not designed to study the sequencing of tamoxifen and radiotherapy. 45% of patients had hormone receptor–negative disease, which limits the extent to which the results can be generalised. Likewise, differences exist in patient characteristics between the groups, such as the proportion of patients who were receptor-positive, and no data are available with regard to the proportion who received a boost dose of radiotherapy. Additionally, chemotherapy was delayed further in patients who received radiation before tamoxifen, than in those who received concurrent treatment.

In a retrospective study, Ahn and colleagues[52] assessed the sequencing of tamoxifen relative to radiotherapy in 500 patients who underwent conservative surgery. Tamoxifen was administered at the same time as radiotherapy in 254 patients, and after completion of radiotherapy in 241 patients. The timing of tamoxifen was unclear in the five remaining patients. After a median follow-up of 10·0 years, there were no significant differences between the concurrent and sequential groups in overall survival (84% *vs* 82%, p=0·45), distant metastasis-free rate (82% *vs* 78%; p=0·12), ipsilateral breast-relapse-free rate (90% *vs* 86%; p=0·86), or contralateral breast-relapse-free rate (95% *vs* 93%; p=0·66). Limitations of the study include differences between treatment groups in prognostic factors, such as age and adjuvant chemotherapy. Patients who received sequential radiotherapy and tamoxifen were slightly younger and more likely than patients with concurrent radiotherapy and hormone therapy to have received chemotherapy. Although this study is the largest published investigation of the sequencing of radiotherapy and tamoxifen therapy, the small sample size limits the

ability to detect small difference in outcomes.[53] Also, it is a retrospective study and is subject to inherent biases and limitations therein.

Harris and colleagues[54] did a similar retrospective analysis of tamoxifen timing in 278 patients (sequential *vs* concurrent) and, consistent with Ahn and colleagues' study, found no effect on 10-year local recurrence (7% *vs* 3%; p=0·52), overall survival (86% *vs* 81%; p=0·64), or relapse-free survival (76% *vs* 85%; p=0·35). Again, important differences between the treatment groups were noted; patients in the sequential-treatment group were younger and more likely than patients with concurrent hormone and radiotherapy to have received chemotherapy (p=0·001). Although these three studies independently reached the same conclusion, they are limited by biases and caveats characteristic of retrospective reviews.[55,56] For example, in these studies, the timing and sequencing of tamoxifen administration relative to radiotherapy was either variable or not reported. In view of the small number of patients in each of these three studies, these results are consistent with either a beneficial or a detrimental effect of radiotherapy followed sequentially by tamoxifen, compared with concurrent tamoxifen and radiotherapy.

Of the three studies[51,52,54] only Harris and colleagues[54] investigated toxicity—reporting on breast and arm oedema, pneumonitis, and cosmetic outcomes. Assessment of toxicity was not blinded to treatment and no remarkable differences in complication rates between sequential and concurrent radiation therapy and tamoxifen-treatment groups were reported. Few data exist on the risk of complications when treatments are given concurrently. Bentzen and colleagues[57] reported an increased risk of lung fibrosis in postmastectomy patients receiving radiotherapy and tamoxifen. The authors postulated that tamoxifen enhances radiation-induced lung fibrosis through the induction of TGF-β secretion.[57] In this retrospective study, radiotherapy was started 2–4 weeks after surgery and tamoxifen was initiated simultaneously, administered orally at a dose of 10 mg three times daily for 48 weeks. There was a significant association between tamoxifen treatment and the incidence of lung fibrosis (relative risk 2·0, 95% CI 1·2–3·5). However, only 38 of 84 patients received tamoxifen plus radiotherapy, and lung fibrosis was assessed by changes on conventional radiographs. By use of computed tomography, Wennberg and colleagues[58] quantified radiological changes in the lung after radiotherapy for breast cancer, and investigated the association between tissue changes and treatment techniques, as well as symptomatic radiation pneumonitis. The researchers found no association between post-radiotherapy changes in lung density and concurrent tamoxifen or smoking habits. Koc and colleagues[59] did a prospective assessment of 74 patients to investigate whether tamoxifen induced the development of pulmonary fibrosis when given simultaneously with post-mastectomy cobalt-60 radiotherapy. Results from computed tomography showed a significant rise in the risk of the lung fibrosis among patients treated with tamoxifen. Pulmonary fibrosis developed in 26 of 74 patients who were treated with combined radiotherapy and tamoxifen, versus five of 37 patients with radiotherapy alone (p=0·01). The median time for the development of pulmonary fibrosis was 8 months in tamoxifen-treated patients compared with 10 months in patients who were not treated with tamoxifen. The authors suggested that the effect on vascular tissue of decreased oestrogen along with combined tamoxifen and radiotherapy might have enhanced the risk of development of pulmonary fibrosis. Huang and colleagues[60] investigated possible associations between regional lymphatics in patients with breast cancer and pulmonary fibrosis after postmastectomy electron-beam irradiation of the chest wall. They found that tamoxifen could significantly promote the development of lung fibrosis (p=0·03).

Wazer and colleagues[61] assessed cosmesis and factors related to disease presentation, surgical and radiotherapy technique, and adjuvant systemic therapy in conservative treatment for early-stage breast carcinoma. They demonstrated a trend toward worse cosmesis (p=0·062) in patients receiving tamoxifen, with a greater incidence of fibrosis, telangiectasia, and hyperpigmentation. Fowble and colleagues[62] found a prolongation of breast erythema after completion of radiotherapy, but no increased incidence of other complications, in women who received tamoxifen. This retrospective analysis was limited to 491 women with tumours positive for oestrogen receptors undergoing conservative surgery and radio therapy. 154 patients received tamoxifen; however, the timing of the endocrine therapy with initiation of radiotherapy was unknown for 111. Of the remainder, 23 patients received tamoxifen during radiotherapy and 20 began tamoxifen after the completion of radiotherapy.

Azria and colleagues[63] retrospectively analysed 147 women with breast cancer to assess whether concomitant use of tamoxifen and radiotherapy was associated with an increased risk of subcutaneous fibrosis after conservative or radical surgery. Simultaneous radiotherapy and tamoxifen (20 mg/day) was prescribed in 90 hormone receptor-positive patients. The researchers found significant differences in terms of complication-relapse-free survival at 3 years (48% [95% CI 37·2–57·6] in the tamoxifen group *vs* 66% [95% CI 49·9–78·6] in the non-tamoxifen group), and complication-free survival at 2 years, (51% [n=40–61] *vs* 80% [n=67–89]). In each of these groups, complication-relapse-free survival was significantly lower for women with low levels of CD8 radiotherapy-induced apoptosis, compared with patients with normal or increased levels of CD8 radiotherapy-induced apoptosis. From these results, Azria and colleagues concluded that concomitant tamoxifen and radiotherapy was associated with a significantly higher incidence of grade 2 or greater subcutaneous fibrosis. However, the study was a retrospective analysis of patients

in a prospective trial that was not designed to study the sequencing of tamoxifen and radiotherapy. Moreover, the results suggest that the predictive radiotherapy-induced lymphocyte apoptosis assay could be highly specific and sensitive to discriminate subgroups of patients as a function of their intrinsic radiosensitivity.

Preclinical models show changes in tumour cell kinetics after the addition of tamoxifen, with some studies suggesting reduced tumour cell death with concurrent antioestrogens and radiotherapy. In-vitro data also indicate antagonistic effects of concurrent tamoxifen and radiotherapy on tumour cells; however, in-vivo models suggest a synergistic effect that might be partially due to microenvironmental modulation of tumour responsiveness to radiation and endocrine therapy. Such discrepancies indicate the complexity of the response to tamoxifen in breast-cancer cells. From a clinical perspective, a tumour-protective effect of tamoxifen therapy given with radiation is not evident. Retrospective studies indicate that in practical application, concurrent administration of tamoxifen with radiotherapy does not compromise local control but might increase toxicity. Tamoxifen might act as an antagonist for oestrogen receptor but also as an agonist, depending on the cellular context—a probable explanation for the frequent contradictory results. Because tamoxifen acts on a number of oestrogen and growth factor receptors, a complex experimental system is needed to study the interaction of tamoxifen and radiotherapy, and remains to be designed.

Combining aromatase inhibitors with radiotherapy

An alternative to tamoxifen therapy is treatment with third-generation aromatase inhibitors, which have improved disease-free survival in a variety of adjuvant settings for early breast cancer.[64–68] Aromatase inhibitors block conversion of androgens to oestrogens, by inhibition of aromatase enzyme function, leading to suppressed oestrogen synthesis. Data show long-term safety and efficacy of the aromatase inhibitor anastrozole, compared with tamoxifen, as initial adjuvant treatment for postmenopausal women with hormone-sensitive, early breast-cancer.[69] Evidence shows a significantly larger carryover effect after 5 years of adjuvant treatment with anastrozole compared with tamoxifen. Recurrence remained significantly lower on anastrozole than with tamoxifen after treatment completion (hazard ratio 0·75 [95% CI 0·61–0·94], p=0·01). Due to lower cancer recurrence, aromatase inhibitors have become standard adjuvant therapy for postmenopausal women with (hormone) receptor-positive early breast cancer. Additionally, a randomised trial showed that after 2–3 years of tamoxifen therapy, switching to exemestane was more effective than continuing tamoxifen therapy for the remainder of the 5 years of treatment. Exemestane therapy significantly improved disease-free survival compared with the standard 5 years of tamoxifen treatment.[70] However, few data are available on the rationale for concomitant use of aromatase inhibitors in adjuvant radiotherapy settings.

Azria and colleagues[71] examined in vitro whether letrozole sensitises breast-cancer cells to radiation doses ranging from 0 to 4 Gy. MCF-7 human breast-cancer cells were incubated with androstenedione in the presence or absence of letrozole, and assessed using clonogenic assays, tetrazolium salt colorimetric assays, and cell number determinations. Results showed additive effects for the combined androstenedione-letrozole treatment. The survival fraction at 2 Gy was 0·66 for radiotherapy alone versus 0·44 for radiotherapy plus letrozole (p=0·02). Compared with radiotherapy alone, the combination of radiotherapy and letrozole produced a 50% decrease in the number of MCF-7 cells arrested in G_2 phase and a decrease of cells in the S phase, with proportional cell redistribution in the G_1 phase. Additionally, letrozole added 6 days before ionising radiation caused a greater fall in survival fractions than when letrozole was added 3 days before radiotherapy, or 3 days after radiotherapy, or when radiotherapy was delivered alone. These findings support concurrent use of aromatase inhibitors and radiotherapy in postsurgical settings.

Bollet and colleagues[72] retrospectively assessed responses and outcome of hormone therapy and radiotherapy given concurrently for large, positive hormone-receptor breast cancers in postmenopausal women. Treatment consisted of hormone therapy and radiotherapy followed by either surgery (n=29) or a radiotherapy boost without surgery (n=12). Hormone therapy consisted of tamoxifen (20 mg/day) for 38 tumours (90%) and anastrozole (1 mg/day) for four tumours (10%) and, for the preoperative group, respectively, 28 (93%) and two (7%). Concurrent radiotherapy and hormone therapy demonstrated high efficacy in terms of clinical and pathological complete responses (57% partial responses, 24% partial response, and 21% stable disease), allowing breast conservation with acceptable tolerance and good 5-year local control. However, severe mucocutaneous reactions associated with radiotherapy and concurrent use of anastrozole have been reported.[73]

Conclusion

Many issues are unresolved with regard to the optimum use of radiotherapy and endocrine therapy in premenopausal patients with breast cancer. At this time, mechanisms underlying the mitogenic effect of 17beta-oestradiol are unclear. Further studies are required to understand the physiological mechanisms triggered by oestrogens, the complex interactions between 17beta-oestradiol and P53/$P21^{WAF1/CIP1}$/Rb pathways, and the relationship between endocrine therapy and radiotherapy. No randomised clinical trials have investigated the sequencing of tamoxifen or other SERMs and radiotherapy. Results from retrospective clinical studies, which included treatment arms with and without tamoxifen, showed no reduction in local tumour recurrence. However, most of these studies were not

Search strategy and selection criteria

Data for this review were identified by searches of Medline and Cancerlit. Only papers published between January, 1976, and May, 2008, were included. Literature was reviewed with respect to experimental and clinical data on interaction of tamoxifen and radiation on tumour control and radiation side-effects. The search terms "tamoxifen", "aromatase inhibitor", "ovarian ablation", "endocrine therapy", "radiation or radiotherapy", and "breast cancer or carcinoma" were used. There was no language restriction. References identified from within retrieved articles were also used. Only published articles, and no abstracts, were taken into consideration.

designed to assess the sequencing of tamoxifen and radiotherapy, and so the timing was often unspecified. Although some reports have noted increased risk of lung and breast fibrosis with concurrent tamoxifen and radiotherapy, others showed no differences in local control or survival after sequential versus concurrent treatment. Although doubt remains with regard to the potential long-term toxicity of delivering hormonal treatment concurrently with radiotherapy, there is no conclusive data on the effect on recurrence of delaying endocrine therapy after radiotherapy (a 7-week delay *vs* a 5-year treatment time). Given the lack of prospective data and potential increase in long-term toxicity, physicians should consider a concurrent approach with caution. The clinical relevance of these data remains to be assessed in a prospective manner. However, as long as there is no proof-of-principle study that demonstrates any benefit to adding endocrine therapy concurrently with breast or chest radiotherapy, we recommend that hormonal therapy should be delayed until after completion of adjuvant radiation therapy.

Conflicts of interest

The authors declared no conflicts of interest.

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SHORT COMMUNICATION

Tamoxifen and TRAIL synergistically induce apoptosis in breast cancer cells

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Tamoxifen (TAM), is widely used as a single agent in adjuvant treatment of breast cancer. Here, we investigated the effects of TAM in combination with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in estrogen receptor-α (ER-α)-positive and -negative breast cancer cells. We showed that cotreatment with TAM and TRAIL synergistically induced apoptosis regardless of ER-α status. By contrast, cotreatment did not affect the viability of normal breast epithelial cells. Cotreatment with TAM and TRAIL in breast cancer cells decreased the levels of antiapoptotic proteins including FLIPs and Bcl-2, and enhanced the levels of proapoptotic proteins such as FADD, caspase 8, tBid, Bax and caspase 9. Furthermore, cotreatment-induced apoptosis was efficiently reduced by FADD- or Bid-siRNA, indicating the implication of both extrinsic and intrinsic pathways in synergistic apoptosis induction. Importantly, cotreatment totally arrested tumor growth in an ER-α-negative MDA-MB-231 tumor xenograft model. The abrogation of tumor growth correlated with enhanced apoptosis in tumor tissues. Our findings raise the possibility to use TAM in combination with TRAIL for breast cancers, regardless of ER-α status.
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Keywords: breast cancer; tamoxifen; TRAIL; apoptosis; tumor xenograft

Introduction

The antiestrogenic drug, tamoxifen (TAM), a nonsteroidal selective estrogen receptor modulator, has been used as a single agent in the treatment of estrogen receptor-α (ER-α)-positive breast cancer (Lover, 1989; Jordan, 1992). Clinical response to TAM is shown to be associated with both decreased proliferation and increased apoptosis (Gelman, 1997; McClay *et al*., 2000). Thus, TAM is thought to exert antitumor effect via ER-α-dependent inhibition of cell proliferation and induction of apoptosis. However, an important additional feature of TAM is its effectiveness in the treatment of ER-α-negative neoplasia including breast cancer, malignant gliomas, pancreatic carcinoma and melanoma (Gelman, 1997; McClay *et al*., 2000). Moreover, at clinical achievable concentrations (1–10 μM), TAM strongly induces apoptosis in both ER-α-positive and ER-α-negative breast cancer cells. The apoptosis-inducing effect is not reversible by addition of estrogens, suggesting that ER-α-independent induction of apoptosis could be a major mechanism of the observed antitumor effect of TAM (Hawkins *et al*., 2000; Mandlekar and Kong, 2001). TAM can induce apoptosis through several distinct pathways including production of oxidative stress and ceramide, as well as transcriptional regulation of expression of Bcl-2 protein family members (Mandlekar and Kong, 2001; Nazarewicz *et al*., 2007). Therefore, a critical question remaining is whether TAM, if given in combination with other agents, will be more efficient to eliminate both ER-α-positive and ER-α-negative breast cancer cells.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), has recently emerged as a novel biological agent for cancer therapy (LeBlanc and Ashkenazi, 2003). TRAIL exerts its tumoricidal effect by directly inducing apoptosis of cancer cells via a receptor-mediated process. TRAIL interacts with five different receptors: two functional receptors (DR4 and DR5), two decoy receptors (DcR1 and DcR2) that can bind TRAIL but lack the functional intracellular death domain, thus incapable of transducing a death signal, and one soluble protein (osteoprotegerin) that binds TRAIL at low affinity. Binding of TRAIL to DR4 or DR5 results in the recruitment of the adapter protein FADD, which in turn recruits and activates caspases 8 or 10. These active caspases initiate apoptosis either by direct cleavage of downstream effector caspases (extrinsic pathway) or by cleaving Bid. Truncated Bid (tBid) becomes inserted into mitochondrial membrane to favor the release of proapoptotic factors such as cytochrome *c*, leading to caspase 9 activation and subsequent apoptosis. This mitochondria-mediated signaling cascade is also called intrinsic pathway (LeBlanc and Ashkenazi, 2003).

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Although the majority of cultured cancer cells show a certain degree of sensitivity to the cytotoxic effect of TRAIL, it has been well documented that cotreatment with TRAIL and chemotherapeutic agents such as topoisomerase inhibitors, antimetabolites and antimicrotubule agents results in a synergistic induction of tumor cell apoptosis and a marked inhibition of tumor growth in murine xenograft models (Duiker *et al*., 2006).

Despite the existence of plenty of convincing data describing the ability of TRAIL to synergize chemotherapeutic agent-induced apoptosis of cancer cells, nothing is known about the effect of combined treatment with TAM and TRAIL on breast cancer cells, especially in ER-α-negative breast cancer cells. Here, we show that these two agents synergize to induce apoptosis in both ER-α-positive and ER-α-negative breast cancer cells. Moreover, TAM and TRAIL together totally abolish tumor growth in an ER-α-negative MDA-MB-231 tumor xenograft model. Our findings provide a proof of principle that the clinical efficacy of TAM could be enhanced by combined treatment with TRAIL not only for ER-α-positive but also for ER-α-negative breast cancers.

Results and discussion

Cotreatment with TAM and TRAIL enhanced apoptosis induction in breast cancer cells

We investigated whether the combination of TAM and TRAIL could enhance apoptosis more than either agent alone. As shown in Figure 1, TAM (2 μM) and TRAIL (1 ng ml^{-1}) alone only moderately induced apoptosis (10–20%) in breast cancer cells. In contrast, cotreatment with TAM and TRAIL synergistically induced apoptosis in all breast cancer cell lines tested regardless of ER-α status, since the combination index was calculated as being less than 1 (0.4–0.6). Synergistic induction of cell death was also confirmed by MTT assay (Supplementary Figure 1). Interestingly, neither agent alone nor in combination affected apoptosis in normal breast epithelial cells (Figure 1b and Supplementary Figure 1). Since TAM is known to induce cell cycle blockage in ER-α-positive breast cancer cells (Osborne, 1998), we then performed flow cytometric analysis to see if there were any modifications of cell cycle distribution upon cotreatment (Supplementary Table 1). TAM alone or in combination with TRAIL induced an accumulation of ER-α-positive MCF-7 cells in G_1 phase; no significant modifications of cell cycle profile were observed in ER-α-negative MDA-MB-231 cells. However, the percentages of cells in subG_1 were strongly increased by cotreatment in both MCF-7 and MDA-MB-231 cells. Collectively, these results indicated that the combination of TAM and TRAIL synergistically induced apoptosis in breast cancer cells regardless of ER-α status. Results shown were obtained in serum-free culture medium to avoid eventual protective effects of serum-derived survival factors. However, similar results were observed in the presence of 5% serum (data not shown). Of note, the apoptosis-inducing effects of TAM alone or in combination with TRAIL were somewhat stronger in ER-α-positive cells. Since TAM can acts through ER-α inhibition to induce apoptosis (Mandlekar and Kong, 2001), it is possible that the superiority of observed effects in ER-α-positive breast cancer cells may be partly mediated by signaling through ER-α. Nevertheless, at clinical achievable concentrations (1–10 μM), TAM has already been shown to induce apoptosis through mechanisms independent of ER-α including production of oxidative stress and ceramide, activation of JNK, as well as transcriptional regulation of expression of Bcl-2 protein family members (Mandlekar and Kong, 2001; Kallio *et al*., 2005; Nazarewicz *et al*., 2007). Accordingly, we observed that inhibition of JNK activity, using the specific JNK inhibitor SP600125 or a dominant-negative form of JNK, reduced cotreatment-induced apoptosis (data not shown).

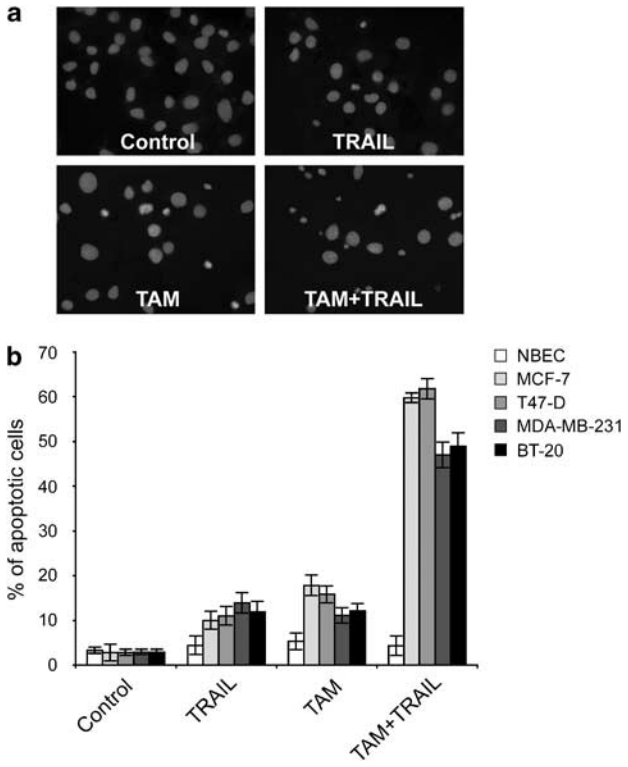

Figure 1 Effects of tamoxifen (TAM) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) on apoptosis in breast cancer and normal epithelial cells. ER-α-positive (MCF-7, T47-D) and ER-α-negative (MDA-MB-231, BT-20) human breast cancer cells as well as normal breast epithelial cells (NBEC) were treated in serum-free medium with 2 μM TAM and/or 1 ng ml^{-1} TRAIL for 24 h. (**a**) Hoechst staining of MCF-7 cells. Apoptotic nuclei are condensed or fragmented. (**b**) Percentage of apoptotic cells was determined after Hoechst staining as described (Chopin *et al*., 2004). Values represent mean ± s.d. of three experiments. Synergism was calculated as described (Supplementary Data).

Both extrinsic and intrinsic pathways were involved in cotreatment-induced apoptosis

TRAIL-induced apoptosis in breast cancer cells is known to involve both extrinsic (death receptor) and

intrinsic (mitochondrial) pathways (Suliman *et al*., 2001). We first determined the effects of cotreatment on the levels of molecular determinants of TRAIL signaling pathways (Figure 2a and Supplementary Table 2). Cotreatment increased the expression of the death receptor adapter FADD and reduced the expression of the short FLICE inhibitory protein (FLIP) isoform (FLIPs) in both MCF-7 and MDA-MB-231 cells. As FLIP competes with procaspase 8 to inhibit procaspase 8 recruitment by FADD, the observed modifications presumably lead to more recruitment of procaspase 8, and therefore increase caspase 8 activation and subsequent cell death. Accordingly, FADD-siRNA efficiently reduced the expression of FADD and the synergistic induction of apoptosis by cotreatment (Figure 3a), confirming the implication of extrinsic pathways in synergistic induction of apoptosis.

Cross talk between the death receptor and mitochondrial pathways can be mediated by caspase 8 cleavage of Bid. tBid translocates to mitochondria and interacts with pro- and antiapoptotic members to activate or antagonize their functions, leading to cytochrome *c* release (LeBlanc and Ashkenazi, 2003). We showed that combined treatment with TAM and TRAIL strongly increased the cleavage of Bid in breast cancer cells (Figure 2a and Supplementary Table 2). Bid-siRNA efficiently reduced the expression of Bid and the synergistic induction of apoptosis by cotreatment (Figure 3b). However, Bid inhibition could not totally abolish apoptosis induced by cotreatment, suggesting that other Bcl-2 family proteins may be also implicated. Indeed, cotreatment increased the levels of proapoptotic protein Bax and decreased the levels of antiapoptotic protein Bcl-2. Thus, downregulation of antiapoptotic protein Bcl-2 together with upregulation of proapoptotic proteins tBid and Bax may constitute a complementary positive amplification loop to promote mitochondrial dysfunctions, as confirmed by the release of mitochondrial cytochrome *c* (Figures 2b and c) and enhanced levels of active caspase 9 (Figure 2a and Supplementary Table 2). The mechanism by which cotreatment regulates the levels of Bcl-2 family members

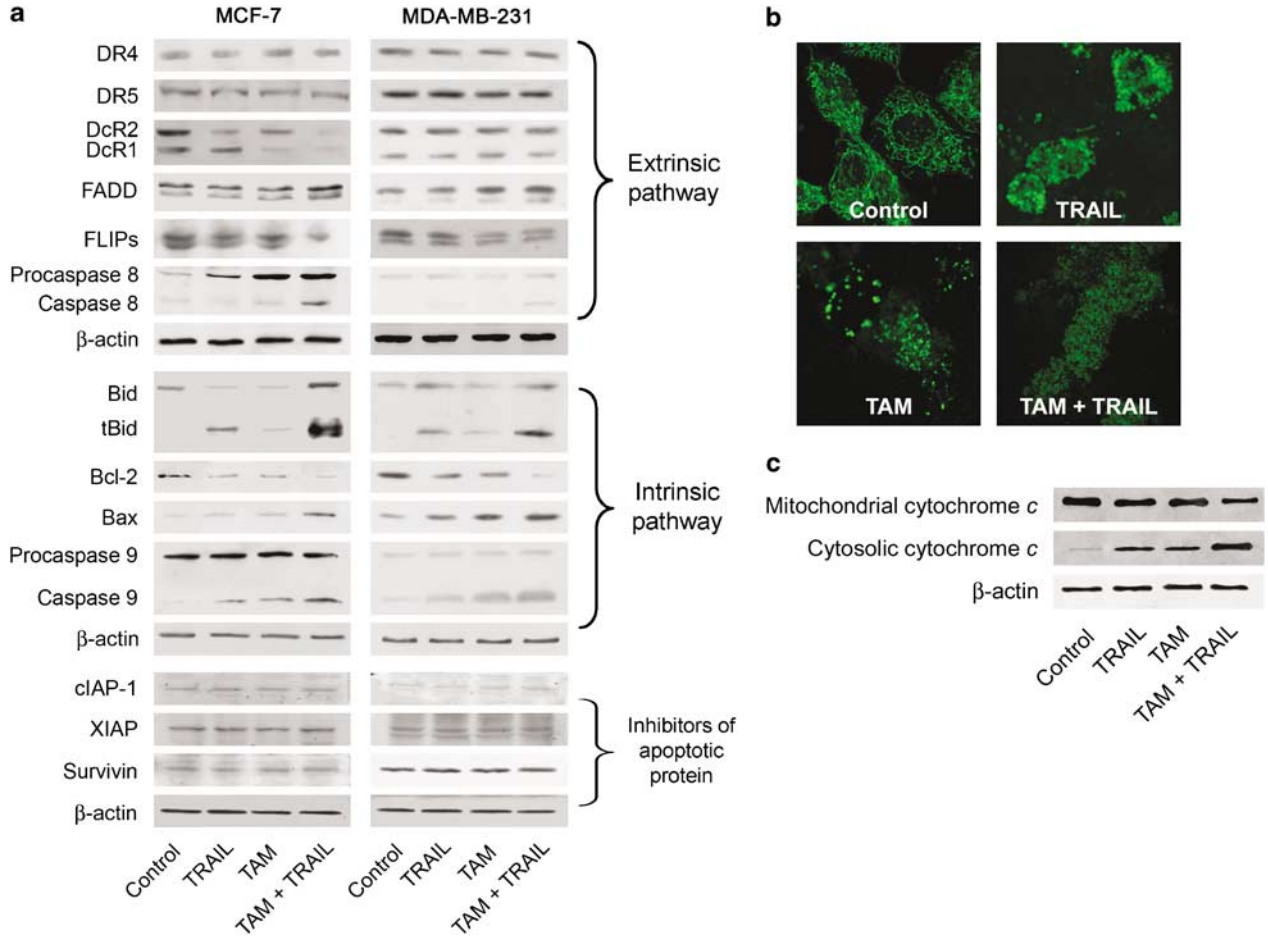

Figure 2 Modulations of apoptosis regulating proteins by cotreatment with tamoxifen (TAM) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). MCF-7 and MDA-MB-231 breast cancer cells were treated in serum-free medium with 2 μM TAM and/or 1 ng ml^{-1} TRAIL for 24 h. Western blot and confocal microscopy were then performed as described (Chopin *et al*., 2004). (**a**) Western blot analysis of whole cell lysates. (**b**) Confocal microscopy analysis of cytochrome *c* in MCF-7 cells. Typical punctuated mitochondrial staining was observed in control cells. A diffuse cytosolic signal was observed in cells treated with TRAIL and TAM alone, or in combination, suggesting the translocation of cytochrome *c* from mitochondria to cytosol. (**c**) Western blot analysis of mitochondrial and cytosolic cytochrome *c* in MCF-7 cells.

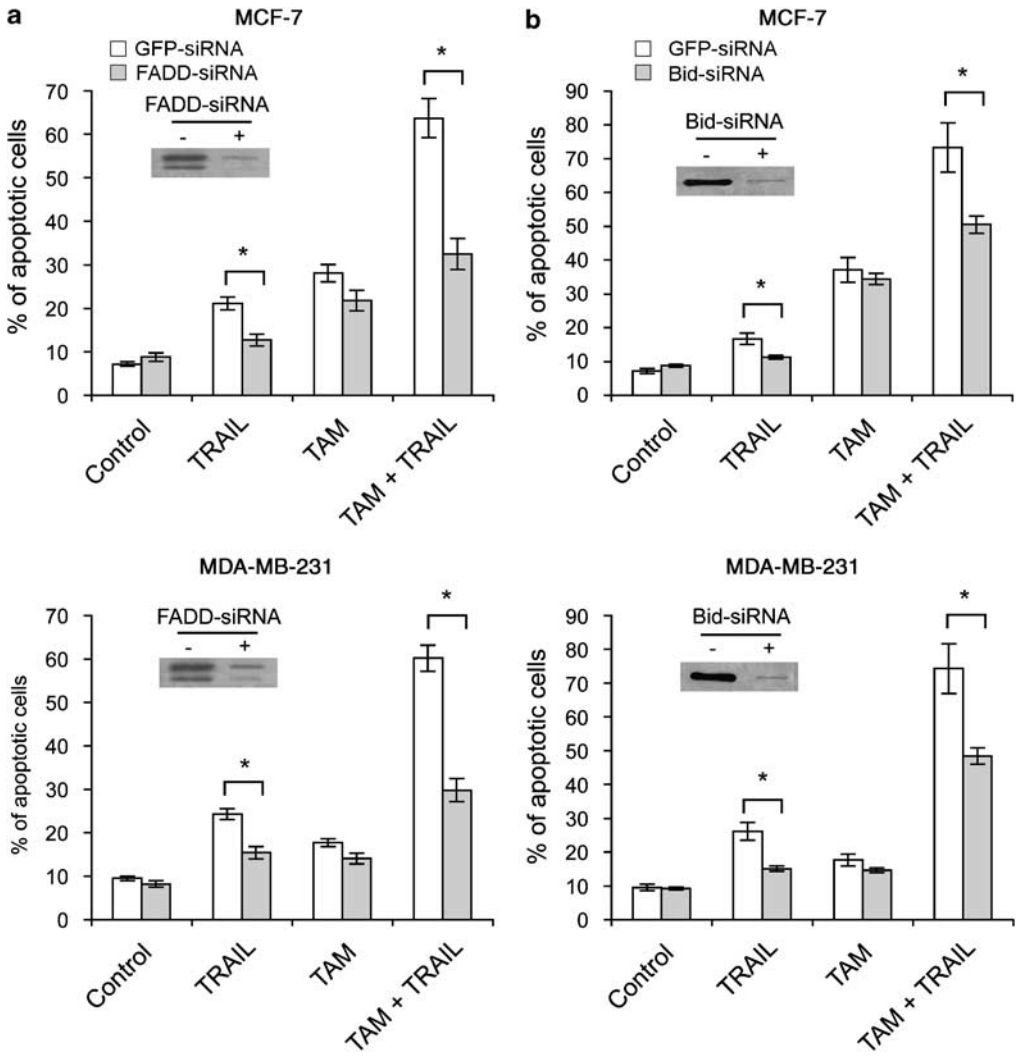

Figure 3 Effects of FADD-siRNA and Bid-siRNA on synergistic apoptosis induction. MCF-7 and MDA-MB-231 cells were subjected to one round of transfection with FADD-specific siRNA (**a**) or two sequential rounds of transfection with Bid-specific siRNA (**b**) as described (Grambihler *et al*., 2003; Broaddus *et al*., 2005). Twenty-four hours after transfection, cells were treated in serum-free medium with 2 μM tamoxifen (TAM) and 1 ng ml^{-1} tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) alone or both for 24 h. Results were from one experiment representative of three independent experiments. *$P<0.05$ (Student's *t*-test).

remains unknown. One explanation might be the involvement of JNK pathway. In this regard, activated JNK has been reported to enhance cleavage of Bid by enhanced activation of caspase 8 and to stimulate the expression of proapoptotic members such as Bid and Bax (Nakano *et al*., 2006). Moreover, Yanamadala *et al*. (2007) have recently reported that phosphorylation of Bcl-2 by JNK leads to degradation of Bcl-2 protein via the proteasome pathway.

Abrogation of tumor growth by cotreatment with TAM and TRAIL in SCID mice

Because TAM and TRAIL synergized to induce apoptosis of breast cancer cells *in vitro*, we examined the effectiveness of combined treatment in an ER-α-negative breast cancer xenograft model. As shown in Figure 4a, TRAIL slightly reduced tumor growth while TAM had no significant effect when compared to control mice. Interestingly, combined treatment completely arrested tumor growth, as tumor volume remained the same during the periods of treatment. Quantification of terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL)-positive cells in tumor sections showed that TAM had no significant effect on apoptosis induction, whereas TRAIL alone or in combination with TAM induced more than two- or sevenfold increase of apoptosis, respectively (Figures 4b and c). Interestingly, intensity of proliferating cell nuclear antigen (PCNA) expression index, an indicator of cell proliferation, was similar in tumors of different animal groups (Figure 4d). Thus, in the ER-α-negative breast cancer xenograft model, cotreatment induced apoptosis without modification of cell proliferation. Importantly, cotreatment did not affect survival in normal breast epithelial cells *in vitro* and was well tolerated by mice during the periods of treatment, as

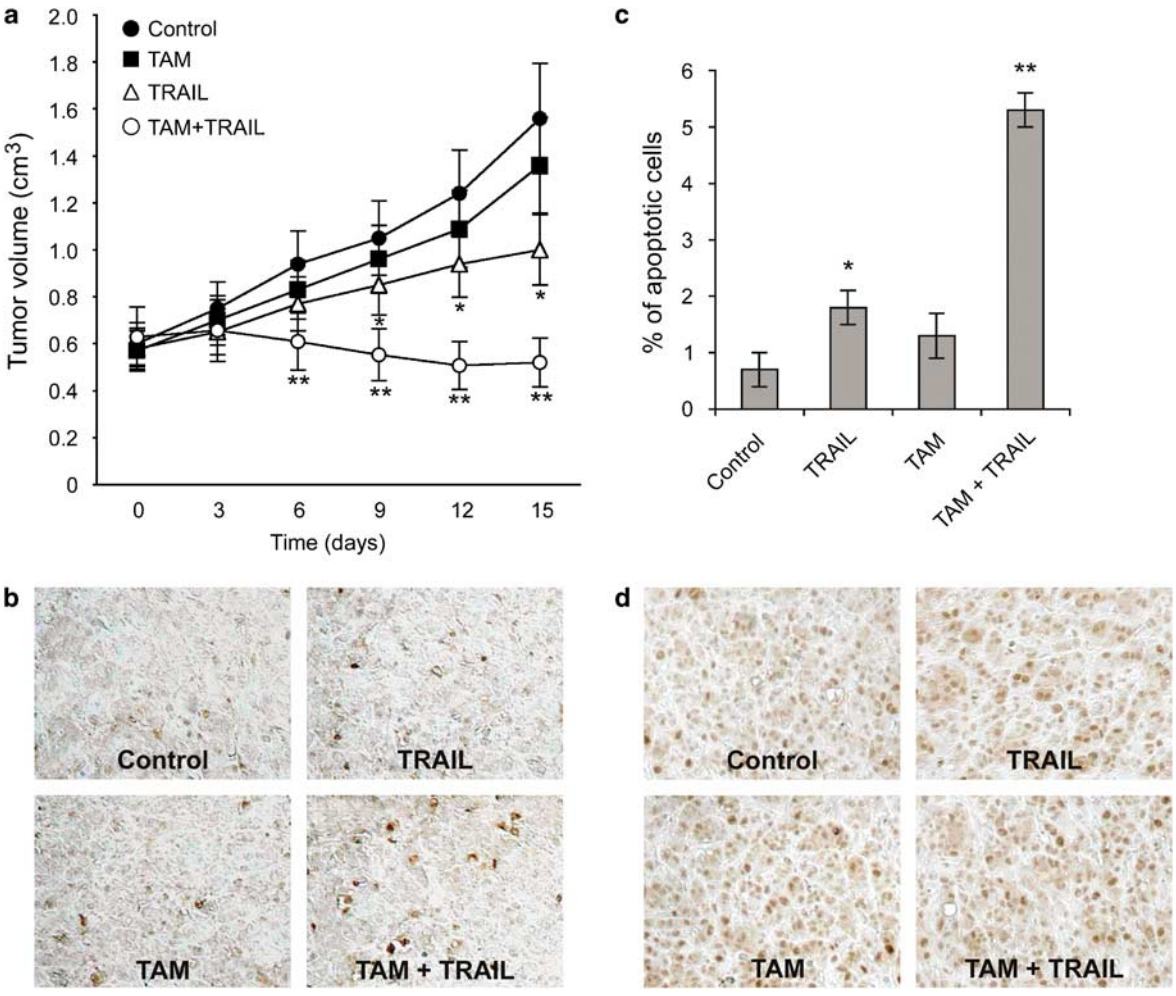

Figure 4 Effects of cotreatment with tamoxifen (TAM) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) on tumor growth in SCID mice. The ER-α-negative breast cancer cells MDA-MB-231 (2×10^6) were injected subcutaneously into the right flank of 6-week-old SCID mice (Charles River Laboratories. L'Arbresle, France). Tumor volumes were monitored every 3 days with calipers and calculated using the formula: $4/3\pi \times r1^2 \times r2$, where $r1$ and $r2$ are the minor and the major dimensions, respectively. When tumor volume attained 0.6 cm^3, the animals were grouped ($n=8$ for each group) and treated with an intraperitoneal injection of TRAIL (30 μg in 100 μl PBS) and/or TAM (250 μg in 100 μl corn oil) every other day for 15 days. (**a**) Tumor volumes at different times of treatment. Data represent mean ± s.d. ($n=8$). (**b** and **c**) Analysis of apoptosis after TUNEL staining (ApopTag Peroxidase *in situ* Apoptosis Detection Kit, R&D systems, Lille, France). (**d**) Analysis of cell proliferation after PCNA staining (Santa Cruz, Le Perray en Yvelines, France). Significant differences (Student's *t*-test) between treated and untreated mice are indicated with asterisks. $*P<0.05$; $**P<0.01$.

there were no abnormal appearance and behavior (ruffled fur/lethargy) in mice. Moreover, no modification in body weight was observed after 15 days of treatment (data not shown).

Up to the present, TAM has been used to treat ER-α-positive breast cancers. However, approximately 40–50% of primary breast cancers are either ER-α-negative or ER-α-positive but resistant to TAM (Osborne, 1998). Thus a substantial proportion of breast cancers are not suited to treatment with TAM. It is now admitted that resistance to apoptosis is a major cause of nonresponsiveness of cancers leading to treatment failure. An exciting outcome from our study is the observation that suboptimal doses of both TAM and TRAIL can synergize to induce cell death even in ER-α-negative breast cancer cells. This raises the possibility to use TAM in combination with TRAIL for breast cancers, regardless of ER-α status. Moreover, cotreatment with low doses may be advantageous since high concentrations found to be active *in vitro* may often not be achieved *in vivo*. This is also of importance in reducing potential toxic side effects of each agent. Altogether, our findings may open up a new therapeutic window in the fight against breast cancers by combined use of TAM and TRAIL.

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ORIGINAL ARTICLE

TrkA overexpression enhances growth and metastasis of breast cancer cells

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The Trk family of neurotrophin tyrosine kinase receptors is emerging as an important player in carcinogenic progression in non-neuronal tissues. Here, we show that breast tumors present high levels of TrkA and phospho-TrkA compared to normal breast tissues. To further evaluate the precise functions of TrkA overexpression in breast cancer development, we have performed a series of biological tests using breast cancer cells that stably overexpress TrkA. We show that (1) TrkA overexpression promoted cell growth, migration and invasion *in vitro*; (2) overexpression of TrkA *per se* conferred constitutive activation of its tyrosine kinase activity; (3) signal pathways including PI3K-Akt and ERK/p38 MAP kinases were activated by TrkA overexpression and were required for the maintenance of a more aggressive cellular phenotype; and (4) TrkA overexpression enhanced tumor growth, angiogenesis and metastasis of xenografted breast cancer cells in immunodeficient mice. Moreover, recovered metastatic cells from the lungs exhibited enhanced anoikis resistance that was abolished by the pharmacological inhibitor K252a, suggesting that TrkA-promoted breast tumor metastasis could be mediated at least in part by enhancing anoikis resistance. Together, these results provide the first direct evidence that TrkA overexpression enhances the tumorigenic properties of breast cancer cells and point to TrkA as a potential target in breast cancer therapy.
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Introduction

Breast cancer progression depends not only on primary tumor growth but also on the ability of tumor cells to metastasize to distant sites. Several sets of growth factors and their cognate receptors are known to be significantly involved in the regulation of these processes (Mercurio *et al*., 2005; Jechlinger *et al*., 2006; Ursini-Siegel *et al*., 2007). Thus, disruption of growth factors and receptor signaling is a current strategy for the development of anticancer drugs. So far, several drugs have shown a therapeutic efficiency, such as Herceptin (specific inhibitor of Erb-B2), but its use is limited because only 20–30% of breast cancers overexpress Erb-B2 and less than 30% of patients with Erb-B2 overexpressing metastatic breast cancer respond to Herceptin as a single agent in first-line treatment (Vogel *et al*., 2002; Sawaki *et al*., 2004). Identification of other growth factors and their receptors implicated in breast tumor development is therefore essential to improve therapeutic efficiency.

Neurotrophins consist of nerve growth factor (NGF), brain-derived neurotrophic factor, neurotrophin 3 (NT-3) and NT-4/5. Neurotrophins bind two classes of receptors, the p75 neurotrophin receptor ($p75^{NTR}$) and the tyrosine kinase receptors of Trk family (TrkA, TrkB, TrkC). $p75^{NTR}$ binds all neurotrophins with similar affinities (Chao, 1994) and regulates cellular processes through interactions between the cytoplasmic domain of $p75^{NTR}$ and effector molecules (Mukai *et al*., 2003). The Trk tyrosine kinase receptors activate several signaling pathways that regulate survival and differentiation in neuronal cells (Reichardt, 2006). Although neurotrophin-mediated signaling has been extensively studied in PC12 and neuronal cells, their effects on non-neuronal cells are not fully understood. Accumulating data have demonstrated that NGF and its tyrosine kinase receptor TrkA are involved in tumor growth and the progression of non-neuronal cancers, including medullary thyroid carcinoma (McGregor *et al*., 1999), lung (Ricci *et al*., 2001), pancreatic (Zhu *et al*., 1999), prostatic (Weeraratna *et al*., 2000) and ovarian carcinomas (Davidson *et al*., 2003).

Both $p75^{NTR}$ and TrkA are expressed in breast cancers (Aragona *et al*., 2001; Descamps *et al*., 2001a). Moreover, we have shown that NGF acts as an autocrine growth factor to stimulate cell proliferation and survival (Dolle *et al*., 2003). Interestingly, NGF cooperates with p185(HER2) to stimulate breast cancer cell growth (Tagliabue *et al*., 2000), and Tamoxifen, the commonly drug used in hormonotherapy, inhibits the proliferative effects of NGF (Chiarenza *et al*., 2001). More recently, we have shown that NGF is expressed in the majority of human breast tumors, especially in epithelial cancer cells. Moreover, anti-NGF antibodies and small-interfering RNA against NGF strongly inhibit the tumor growth and metastasis of breast cancer cells xenografted in immunodeficient mice (Adriaenssens *et al*., 2008).

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These *in vitro* and *in vivo* results suggest that NGF may actively be involved in mammary tumor development. In agreement with this hypothesis, high levels of activated TrkA (phospho-TrkA, pTrkA) are observed in breast cancer effusions compared to primary cancers (Davidson *et al*., 2004). Unfortunately, commonly used breast cancer cell lines express relatively low levels of TrkA compared to tumor biopsies (Descamps *et al*., 2001a). In this study, we first showed that breast tumor biopsies expressed high levels of TrkA and pTrkA compared to normal biopsies, and then stably transfected TrkA in MDA-MB-231 human breast cancer cells to determine the functional importance of TrkA. Our results show that TrkA overexpression promoted cell growth, migration and invasion *in vitro*. Moreover, TrkA overexpression enhanced tumor growth, angiogenesis and metastasis of xenografted breast cancer cells in immunodeficient mice.

Results

Breast tumor biopsies present high levels of TrkA and pTrkA

TrkA expression in breast biopsies was first quantified by real-time RT–PCR (Figure 1a). TrkA was expressed in normal biopsies with levels roughly equivalent to those detected in MDA-MB-231 breast cancer cell line. In contrast, the levels of TrkA mRNA in tumor biopsies were about 10- to 140-fold higher than those in normal samples. The high level of TrkA expression in breast tumor biopsies was further confirmed by western blot analysis (Figure 1b). Moreover, pTrkA was detected in the majority of cancer biopsies, whereas it was undetectable in normal ones; no strict correlation was observed between the levels of TrkA and pTrkA in these samples. We then extended our analysis by immunohistochemical staining of breast tissue microarrays (Figure 1c). The levels of these proteins were scored from 0 to 3 (Table 1). Normal tissues expressed no or low levels of TrkA (score $\leqslant 1$). In contrast, of 37 cancer biopsies, 28 were found to present high levels of TrkA and pTrkA (score $\geqslant 2$).

TrkA overexpression promotes cell growth, migration, invasion and survival

Commonly used breast cancer cell lines express relatively low levels of TrkA compared to tumor biopsies (Descamps *et al*., 2001a; Figures 1a and b). Moreover, PCR analysis showed that only the TrkAI isoform is expressed in breast cancer cell lines (data not shown), so to determine the functional importance of TrkA overexpression in breast cancer development, we established TrkAI overexpressing MDA-MB-231 breast cancer cells as pools or single clones (Figure 1d). Interestingly, an increase in pTrkA was also observed in TrkA overexpressing cells, indicating that TrkA was activated following its overexpression (Figure 1d). We observed similar results in terms of cell behavior in culture for both the pooled cells and the two selected clones (C1 and C2). To simplify the presentation, only results with the pooled cells are shown (Figure 2). In standard cell culture conditions, TrkA overexpression resulted in accelerated cell proliferation (Figure 2a). After 6 days of culture, the number of TrkA overexpressing cells is about twice that of mock cells. When cell migration was evaluated using Transwell Boyden chambers, more TrkA overexpressing cells were found to migrate to the bottom chamber than did the empty-vector-transfected cells (Figure 2b). Similar results were obtained using the wound-healing method (Figure 2c). The invasive capacities of the cells were assessed using Transwells with filters coated with Matrigel and were also found to be enhanced in TrkA overexpressing cells (Figure 2d). Such an increase in migration and invasion was not due to differences in cell proliferation as no difference in cell number was observed after 24 h of culture in the same conditions (data not shown).

We then determined the anoikis resistance of cells on poly-HEMA-coated wells that effectively inhibit cell attachment (Figures 2e and f). TrkA overexpressing cells could survive and proliferate as large spheroid aggregates in suspension, resulting in a twofold increase in cell numbers by 36 h of culture. Of note, during different culture times, the viability of TrkA overexpressing cells remained superior or similar to that at the beginning of the experiment whereas mock cells underwent rapidly cell death. Morphological analysis after Hoechst staining revealed that after 72 h of culture, more than 60% of mock cells were apoptotic whereas less than 5% of TrkA overexpressing cells were apoptotic (data not shown). Moreover, TrkA overexpressing cells formed more colonies than did mock cells in soft agar assay (Figure 2g). This reinforced the fact that TrkA overexpression bypasses the need for anchorage. Finally, TrkA overexpression enhanced the resistance of cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis (Figure 2h).

Surprisingly, although exogenous NGF further stimulated cell growth in soft agar, migration and invasion, it did not modify anoikis resistance either in mock or in TrkA overexpressing cells. Although we have previously shown that NGF can stimulate the growth of breast cancer cells both *in vitro* and *in vivo* by an autocrine loop (Dolle *et al*., 2003; Adriaenssens *et al*., 2008), we then determined whether the effects of TrkA overexpression were due to the activation of the autocrine loop. PCR analysis revealed no modification of NGF expression in TrkA overexpressing MDA-MB-231 cells compared to that of mock cells (data not shown). Neutralizing anti-NGF antibody partially inhibited the migration of TrkA overexpressing MDA-MB-231 cells, but had no effect on anoikis resistance; similar results were obtained in MCF-7 breast cancer cells (Supplementary Figure 1). These data suggest that NGF produced by breast cancer cells is efficient in stimulating TrkA-induced migration but not TrkA-enhanced anoikis resistance. Together, whatever the involvement of NGF, TrkA overexpression increased cell growth, invasion and survival in breast cancer cells.

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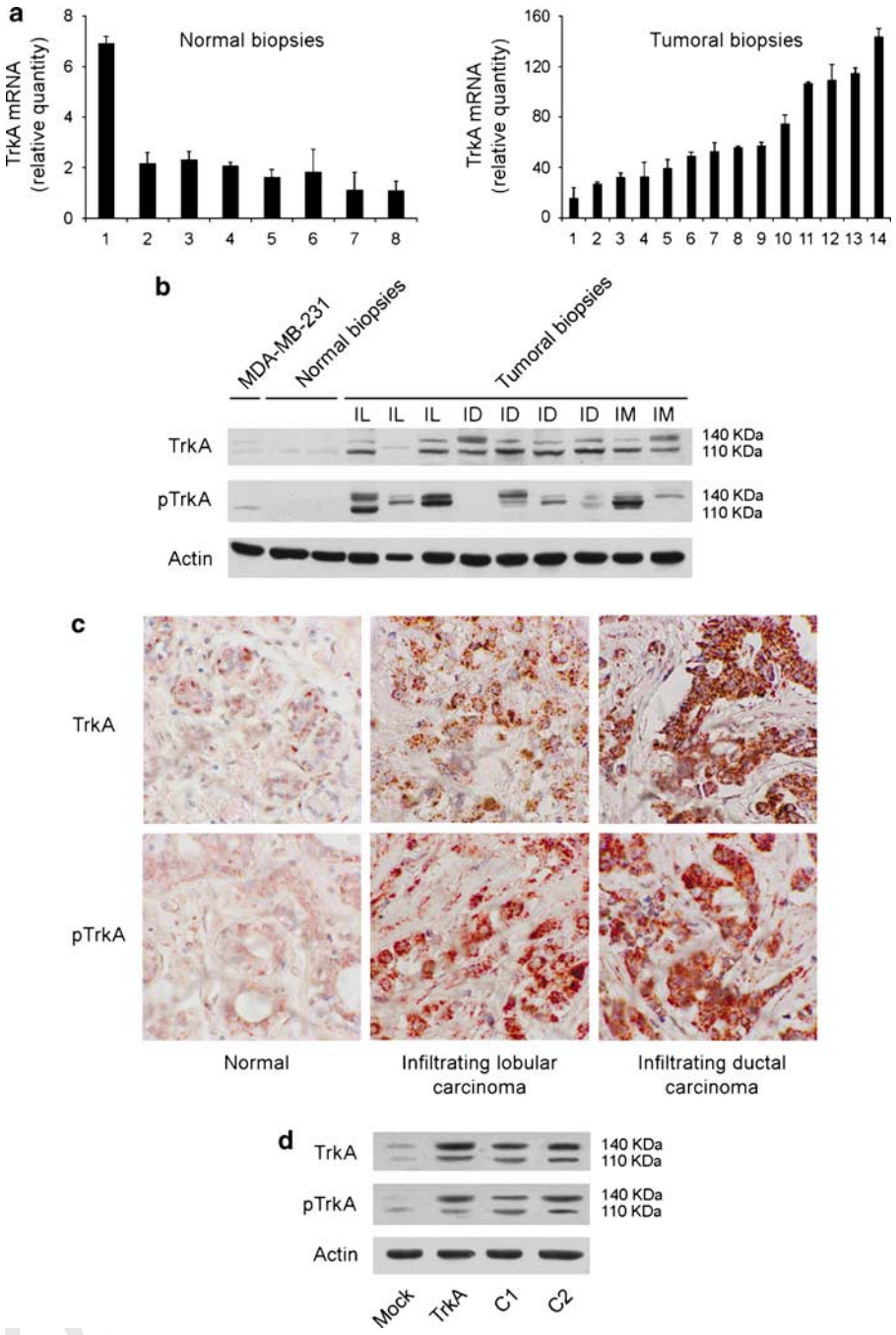

Figure 1 Detection of TrkA and phospho-TrkA (pTrkA) in breast biopsies and MDA-MB-231 breast cancer cells. (**a**) Quantitative PCR detection of TrkA mRNA in breast biopsies. Eight normal breast tissues and 14 tumor biopsies (mainly infiltrating ductal carcinomas) were used. Levels of TrkA mRNA in individual samples are expressed as relative quantity compared to those expressed by MDA-MB-231 breast cancer cells (considered as 1). (**b**) Western blot analysis of TrkA and pTrkA. Cancer biopsies used are infiltrating lobular carcinomas (IL), infiltrating ductal carcinomas (ID) and infiltrating mixed carcinomas (IM). (**c**) TrkA and pTrkA immunostaining of breast tissue microarrays. Representative staining in normal (scored as 1) and tumoral tissues (scored as 3) are shown. Immunostaining was essentially localized in epithelial cells. (**d**) Western analysis of TrkA and pTrkA in MDA-MB-231 cells. Cells were stably transfected with the full-length sequence of TrkAI cDNA and selected as pools and/or single clones. Mock, pools of empty vector-transfected cells; TrkA, pool of cells overexpressing TrkA; C1, TrkA overexpressing clone 1; C2, TrkA overexpressing clone 2.

PI3K-Akt and MEK MAP kinase are required for TrkA overexpression-promoted migration and anchorage-independent growth

Activation of signaling pathways such as PI3K-Akt and mitogen-activated protein (MAP) kinases in TrkA overexpressing cells was first determined by western blot analysis. Higher levels of phospho-Akt (pAkt), phospho-ERK (pERK) and phospho-p38 (pp38) MAP kinases were observed in TrkA overexpressing cells than in mock cells (Figure 3a; Supplementary Figure 2A–C).

Table 1 Relative quantification of immunostaining of TrkA and pTrkA in breast tissue arrays

Breast samples	*TrkA*							*pTrkA*						
	Score				*Mean*	*s.d.*	P-*value*	*Score*				*Mean*	*s.d.*	P-*value*
	0	1	2	3				0	1	2	3			
Normal tissues ($n=10$)	6	4	0	0	0.40	0.48		5	5	0	0	0.50	0.50	
Tumoral tissues ($n=37$)	1	8	14	14	2.11	0.67	1.9×10^{-8}	1	8	17	11	2.03	0.58	3.7×10^{-7}

Immunohistological staining of breast tissues was estimated from 0 (no staining) to 3 (intense staining) as described in Materials and methods section.

Inhibition of TrkA phosphorylation by K252a strongly reduced the levels of pAkt, pERK and pp38 MAP kinases without modifying the expression of corresponding proteins. Similar results were obtained when cells were cultured in suspension (Supplementary Figure 2D). This implies that overexpressed TrkA was functional in signal transduction by activating PI3K-Akt and ERK/p38 MAP kinases. Furthermore, pharmacological inhibitors of TrkA, PI3K-Akt and ERK MAP kinase more strongly reduced the migration and anoikis resistance of TrkA overexpressing cells than that of mock cells (Figures 3b and c; Supplementary Figure 3A–D). Colony formation of TrkA overexpressing cells was also diminished in the presence of inhibitors of TrkA and ERK/p38 MAP kinases (Figure 3d). However, cells did not survive in soft agar whatever the levels of TrkA in the presence of LY294002 or Akt inhibitor III (Figure 3d), confirming the crucial function of PI3K-Akt in colony formation in soft agar (Nakanishi *et al*., 2002).

TrkA overexpression accelerates tumor growth by enhancing cell proliferation and angiogenesis

MDA-MB-231 cells were subcutaneously injected into severe combined immunodeficiency (SCID) mice. All animals formed a tumor at the injection site, but the tumor growth rates were quite different: tumors formed by TrkA overexpressing cells were palpable 2 weeks after injection and attained a size of 2 cm^3 at about 5 weeks after injection. In contrast, empty-vector-transfected cells formed palpable tumors with a latency of 5 weeks and took 10 weeks to form tumors of similar size (about 2 cm^3) (Figure 4a). Primary tumors from MDA-MB-231 TrkA overexpressing cells maintained high levels of TrkA and pTrkA as revealed by immunohistochemical staining (Figure 4b). Similar results were obtained with another xenograft model T47-D human breast cancer cells that are less tumorigenic (Supplementary Figure 4). To determine whether apoptosis, we modified cell proliferation and angiogenesis in tumors formed by TrkA overexpressing cells, then performed terminal transferase dUTP nick-end labeling (TUNEL) assay, immunostaining of proliferating cell nuclear antigen (PCNA) and von Willebrand factor (vWF), which are markers of apoptosis, cell proliferation and angiogenesis, respectively. No significant differences in TUNEL-positive cells were observed between tumors formed by mock and TrkA overexpressing cells. In contrast, about twofold increases in the number of PCNA- and vWF-positive cells were found in TrkA overexpressing tumor sections (Figure 4c). These data indicated that accelerated tumor growth was due to the enhanced proliferation of TrkA overexpressing cells and increased angiogenesis.

TrkA overexpression enhances tumor metastasis

To evaluate metastasis *in vivo*, we generated MDA-MB-231 cells (overexpressing TrkA or not) stably expressing green fluorescent protein (GFP). The GFP-positive cells exhibited the same levels of TrkA and similar *in vitro* properties (proliferation, migration and survival) as their parental counterparts (data not shown). Metastatic potentials of the GFP-positive cells were then first evaluated after subcutaneous injection into SCID mice (Figures 5a and b; Supplementary Figure 5). More and bigger metastatic foci were found in the lungs, liver and brain of mice that received TrkA overexpressing cells (Figure 5a). Quantification of GFP-positive cells using fluorescence-activated cell sorting (FACS) showed that TrkA overexpression induced two- and threefold increase in the metastatic burden in lungs and liver, respectively (Figure 5b, Table 2). Interestingly, only three animals among eight bearing empty-vector-transfected cells developed brain metastases with a low tumor burden, as metastatic (GFP-positive) cells were found to be inferior to 0.2%. In contrast, nine out of nine animals receiving TrkA overexpressing cells developed brain metastases with an average tumor burden 25-fold higher than control animals. To evaluate the abilities of cells to metastasize independently of primary tumor formation and intravasation, we injected cells into the lateral tail veins of SCID mice. After 8 weeks, the mice were killed and the metastatic (GFP-positive) cells in the lungs were quantified. As shown in Figure 5c, a significant increase in metastatic cells was observed in the lungs of mice that received TrkA overexpressing cells, suggesting that TrkA overexpression also affects the later steps of metastasis.

The metastasis-promoting effect of TrkA overexpression was also observed by using another breast cancer cell line T47-D known to be tumorigenic but not metastatic (Supplementary Figure 4C).

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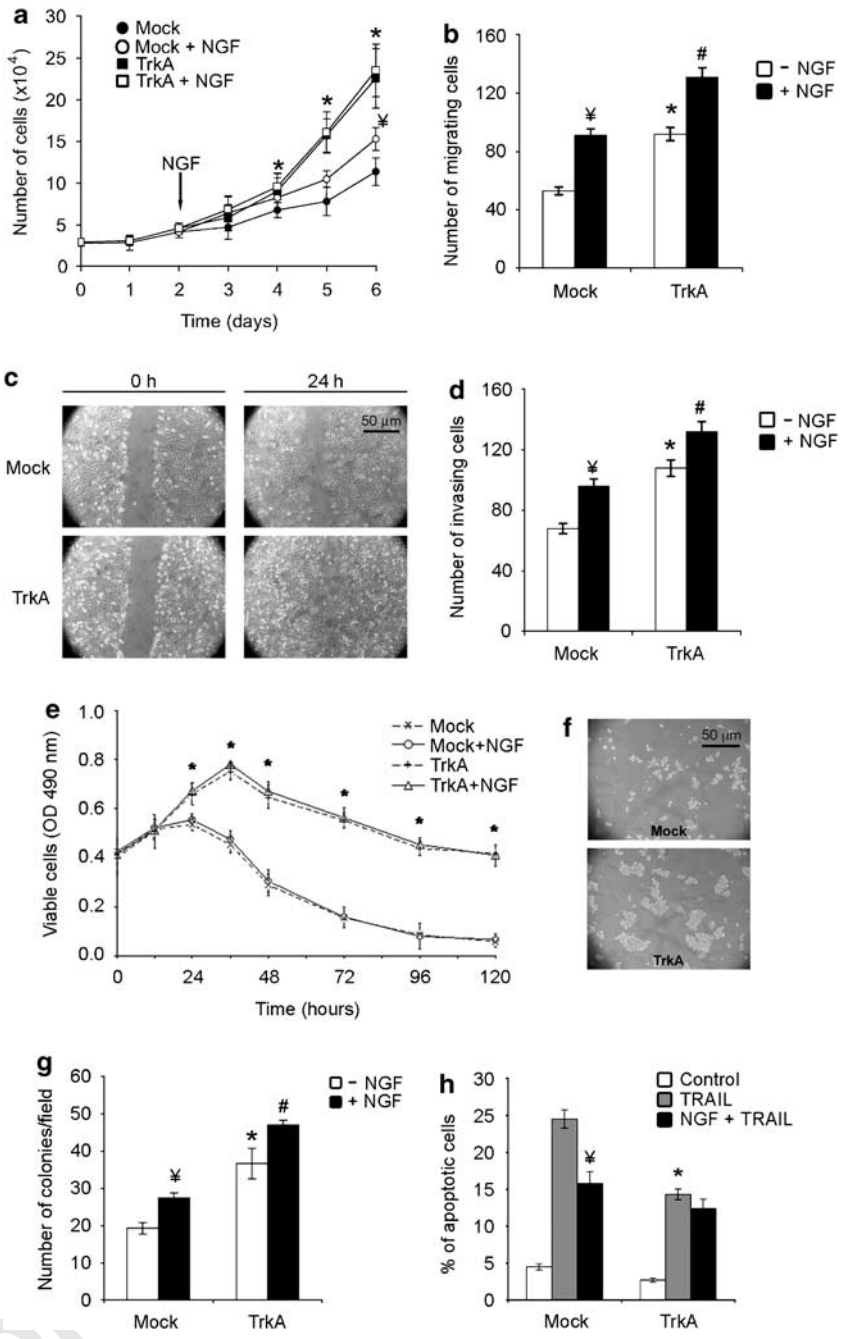

Figure 2 Pleiotropic *in vitro* effects of TrkA overexpression. (**a**) Growth assay on standard culture plastic was performed for various periods of time. (**b**) Migration assay (6 h) using Transwells. (**c**) Representative photographs of wound-healing assay in the presence of nerve growth factor (NGF). (**d**) Matrigel invasion (16 h) assay using Transwells. (**e**) Resistance to anoikis assay. Cells were cultured in poly-HEMA-coated 96 wells for various periods of time. Cell viability was determined by MTS. (**f**) Representative photographs of cells cultured in poly-HEMA-coated wells for 3 days in the absence of NGF. (**g**) Colony formation in soft agar. Colonies of more than 50 cells were counted after 3 weeks of culture. (**h**) Resistance to apoptosis induction by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). All results are the mean of at least three independent experiments each in triplicate. $^{*}P<0.01$, TrkA overexpressing versus mock cells; $^{¥}P<0.01$, NGF-treated versus NGF-nontreated mock cells; $^{\#}P<0.01$, NGF-treated versus NGF-nontreated TrkA overexpressing cells.

TrkA overexpressing cells isolated from lung metastases were more resistant to anoikis

To get insight into the mechanisms of TrkA-driven metastasis, we isolated and cultured TrkA overexpressing cells derived from the lungs of nine animals separately to evaluate their behavior in terms of growth, migration and resistance to TRAIL-induced apoptosis, as well as resistance to anoikis. As shown in Figure 6a, the levels of TrkA and pTrkA in recovered metastatic TrkA overexpressing cells were slightly higher than those of cells before injection. No modifications in cell growth, migration and resistance to TRAIL-induced

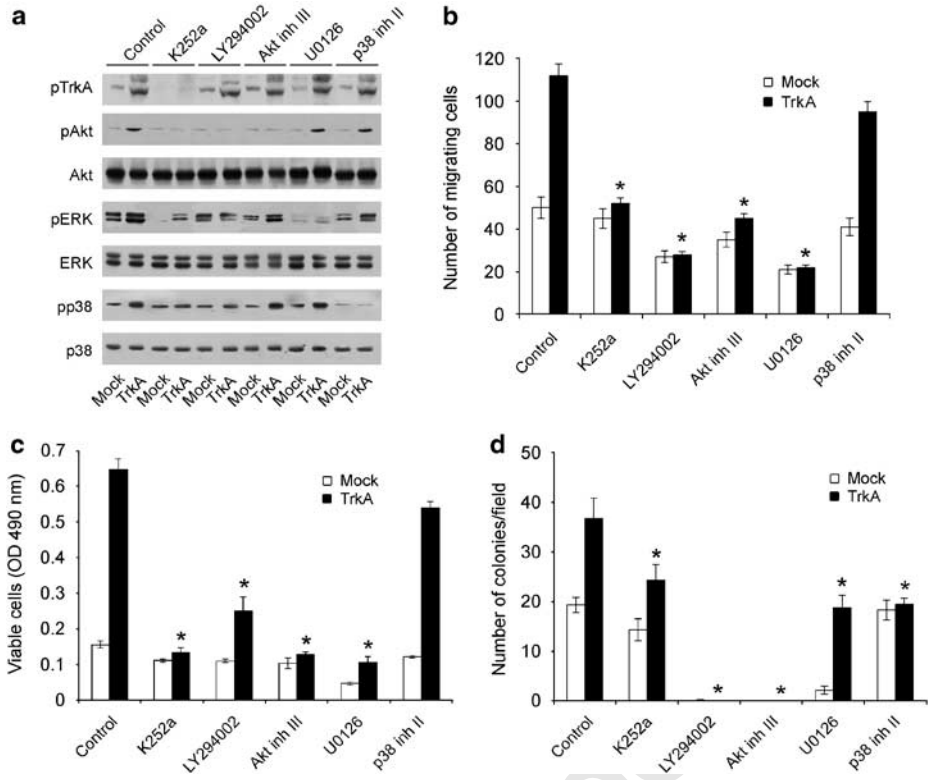

Figure 3 Involvement of PI3K-Akt and ERK/p38 mitogen-activated protein (MAP) kinases in TrkA-mediated biological effects. (**a**) Cells were treated in serum-free Eagle's minimal essential medium (EMEM) for 30 min with specific pharmacological inhibitors. Lysates were made and subjected to immunoblot using the antibodies indicated. Quantification of western blots from three experiments is shown in Supplementary Figure 2A–C. (**b**) Migration assay using Transwells in the presence of different inhibitors for 6 h. (**c**) Anoikis assay in the presence of different inhibitors for 72 h. Anoikis resistance was quantified by MTS. (**d**) Colony formation in soft agar in the presence of different inhibitors for 3 weeks. For b–d, results are the mean of three independent experiments. $*P<0.01$, inhibitors-treated versus -untreated TrkA overexpressing cells.

apoptosis were observed (Figures 6b–d). In contrast, the recovered metastatic TrkA overexpressing cells exhibited enhanced anoikis resistance compared to parental TrkA overexpressing cells. This enhanced anoikis resistance was specific to metastatic cells as no difference was observed between cells derived from primary tumors and their parental cells before injection (data not shown). More importantly, TrkA inhibition with K252a totally abolished the anoikis resistance of these metastatic cells (Figure 6e). Taken together, these results suggested that the enhanced anoikis resistance could be at least partially responsible for the increased metastatic capacity of TrkA overexpressing cells.

Discussion

We first showed that TrkA was overexpressed in human breast cancer biopsies compared to normal breast tissues with levels of pTrkA also being higher in breast cancer biopsies. TrkA can be activated following mutation, overexpression or binding to NGF. On one hand, no mutations have been found either in breast cancer biopsies or in breast cancer cell lines including MDA-MB-231 and MCF-7 (unpublished personal data). On the other hand, the majority of breast cancers express NGF (Adriaenssens *et al*., 2008) that may activate TrkA in an autocrine manner. To determine the incidence of TrkA overexpression in breast cancer biology, we stably transfected MDA-MB-231 breast cancer cells with the full-length TrkAI isoform cDNA (the only TrkA isoform found in these cells). We showed that TrkA overexpressing cells (pooled and separated clones) exhibited increased cell proliferation, migration, invasion as well as survival. These results were further confirmed using two other prototypic breast cancer cell lines MCF-7 and T47-D that are known to be less tumorigenic. However, we had previously shown that upon NGF stimulation, endogenous TrkA is only involved in cell proliferation but not in the survival of breast cancer cells (Descamps *et al*., 2001b), this may be explained by the fact that a relatively low level of endogenous TrkA is not sufficient to activate survival pathways. Accordingly, in TrkA overexpressing cells, tyrosine receptor signaling pathways were found to be constitutively activated, as revealed by the high levels of pAkt, pERK and pp38. Moreover, both PI3K-Akt pathway and ERK MAP kinase were necessary for TrkA-stimulated biological effects such as migration, anoikis resistance and colony formation in soft agar. p38 MAP kinase only seemed to be implicated in colony formation of TrkA overexpressing cells.

TrkA overexpression not only promoted cell growth *in vitro* but also accelerated primary tumor growth *in vivo* in SCID mice. The enhanced primary tumor

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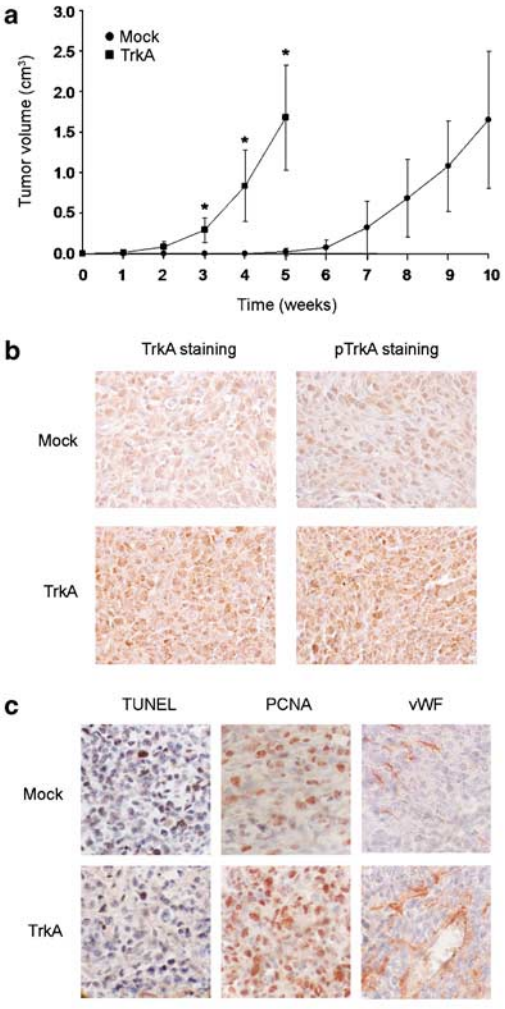

	Mock	TrkA
% of apoptotic cells	3.6 ± 1.4	3.1 ± 1.3
% of PCNA-positive cells	38.8 ± 4.3	72.3 ± 4.6*
% of vWF-positive cells	7.2 ± 1.5	18.0 ± 4.0*

Figure 4 Xenograft tumor growth in severe combined immunodeficiency (SCID) mice. Cells (2×10^6) were subcutaneously injected into mice (nine mice per group). Tumor growth was monitored weekly until mean tumor volume approached 2 cm^3 (10 weeks for mock cells, 5 weeks for TrkA overexpressing cells). Xenografted tumors were then sectioned for further analysis. (**a**) Growth curve of tumors. Data are representative of three independent experiments. $*P<0.01$, TrkA overexpressing versus mock cells. (**b**) Immunostaining of TrkA and pTrkA of xenografted tumor sections. (**c**) Terminal transferase dUTP nick-end labeling (TUNEL) reaction, immunostaining of proliferating cell nuclear antigen (PCNA) and von Willebrand factor (vWF) of xenografted tumor sections. Results are representative of two independent experiments. $*P<0.01$, TrkA overexpressing versus mock cells.

growth was found to be associated with increased cell proliferation and angiogenesis. These data together with our previous findings showing that inhibition of TrkA by the tyrosine inhibitor K252a efficiently inhibits tumor growth and angiogenesis (Adriaenssens *et al*., 2008) prompted us to suggest a model in which relatively low endogenous TrkA and NGF are sufficient to sustain tumor growth by an autocrine loop. However, this loop is probably suboptimal as TrkA overexpression enables stronger signaling, resulting in enhanced tumor growth and angiogenesis. NGF has already been shown to stimulate angiogenesis (Cantarella *et al*., 2002). In addition, a correlative analysis suggests a function for NGF in ovarian cancer angiogenesis (Davidson *et al*., 2003). It is not yet known if NGF can exert a direct and/or indirect action, as NGF can also induce the expression of pro-angiogenic factors such as VEGF (Campos *et al*., 2007). Clearly, it needs to be clarified how TrkA overexpressing tumor cells can stimulate tumor angiogenesis, but our present findings show that TrkA impact in breast carcinogenesis may also encompass angiogenesis.

Another important finding of our work is the increased metastatic ability of TrkA overexpressing cells. Metastasis is a complex process consisting of multiple steps. These steps include growth of the primary tumor, growth of vessels (blood vessels and lymphatics) in and around the tumor, intravasation, transport, arrest of tumor cells and formation of secondary tumors at distant sites. Acquisition of resistance to anoikis may allows the survival of cancer cells during systemic circulation and facilitate tumor dormancy or metastasis in distant organs (Frisch and Screaton, 2001; Rennebeck *et al*., 2005). In support of this concept, it has been reported that overexpression of TrkB renders normal intestinal epithelial cells anoikis resistant and highly tumorigenic (Douma *et al*., 2004; Geiger and Peeper, 2007). Here, we showed overexpression of TrkA rendered breast cancer cells more resistant to anoikis. In addition, TrkA overexpressing cells derived from lung metastases were more resistant to anoikis than their parental TrkA overexpressing cells. These correlations strongly suggested that the increased metastatic capacity of TrkA overexpressing cells could be at least in part due to the enhanced anoikis resistance. It is also reasonable to presume that the increase in lung metastasis due to TrkA overexpression after tail vein injection was relevant with increased capacity of anchorage-independent growth. To our knowledge, this is the first report of TrkA being involved in anoikis resistance, which extends our understanding of the function of TrkA in breast tumor development. Surprisingly, we found that anoikis resistance was independent of NGF action, as neither exogenous NGF nor neutralizing anti-NGF could modify anoikis resistance in both control and TrkA overexpressing cells. It is unclear whether overexpressed TrkA can act in an intracrine manner or through a direct interaction with other proteins such as adhesion molecules. Indeed, it has been reported that E-cadherin-mediated suppression of anoikis is associated with ligand-independent activation of Erb-B1 (Shen and Kramer, 2004). Studies to address these questions are now under way.

However, given the complexity of metastatic process, cancer cells must acquire a series of traits that enable them to overcome the multiple barriers erected by normal tissues. Apart from the enhanced anoikis

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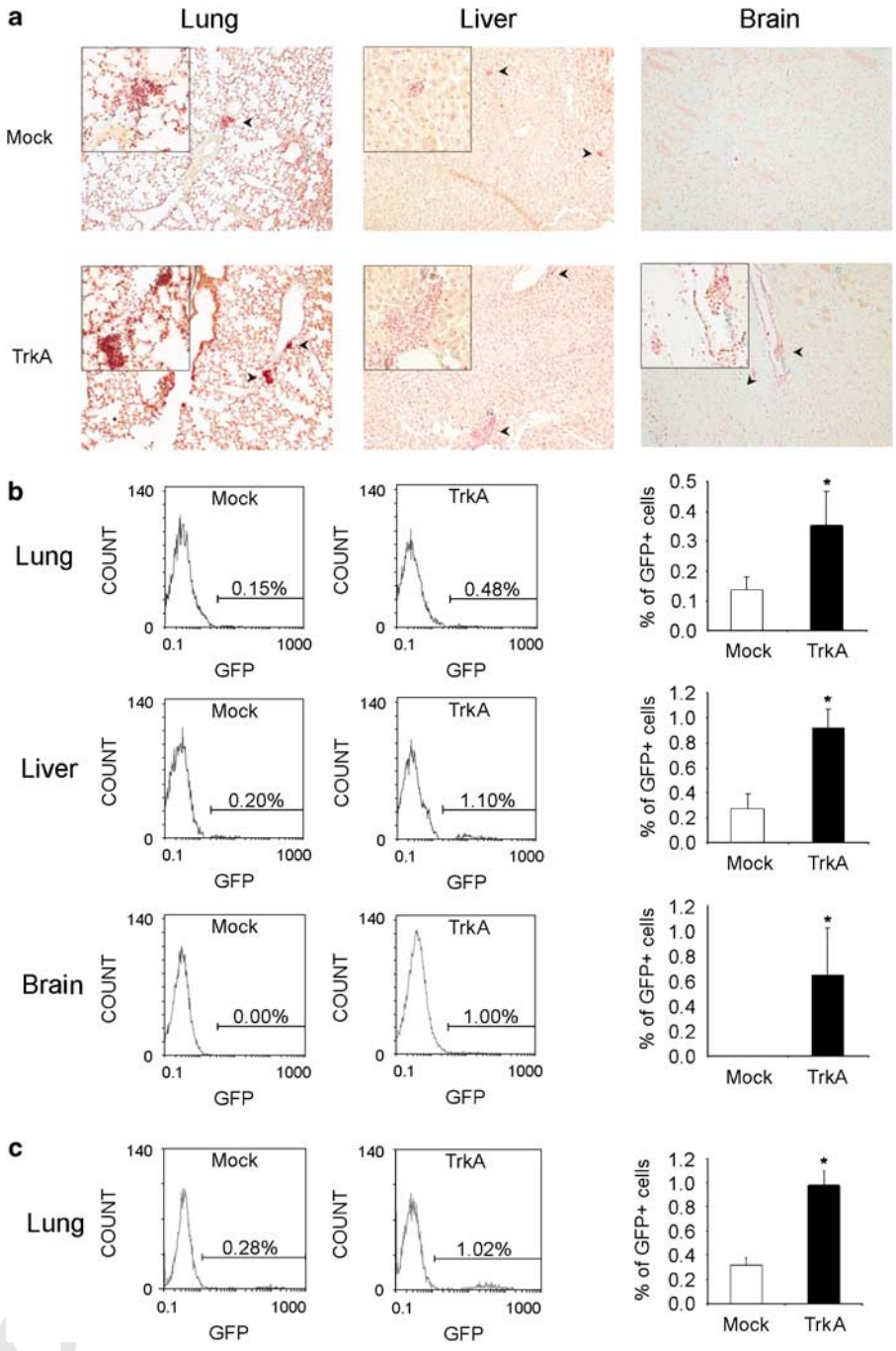

Figure 5 Metastasis assessment. Green fluorescent protein (GFP)-mock or GFP-TrkA overexpressing cells were subcutaneously injected into severe combined immunodeficiency (SCID) mice (**a** and **b**). Mice were killed when tumor volume approached 2 cm^3. Lungs, liver and brain were removed and processed for the detection of metastatic cells. (**a**) Paraffin sections of lungs, liver and brain were stained with nuclear red and picroindigocarmine. Arrows indicate metastatic foci (insets: higher magnification of the metastatic foci). (**b**) Quantification of GFP-positive cells by fluorescence-activated cell sorting (FACS) analysis. Histograms represent the mean of results from eight mice injected with mock cells and nine mice injected with TrkA overexpressing cells. $*P<0.01$, TrkA overexpressing versus mock cells. (**c**) GFP-mock or GFP-TrkA overexpressing cells were injected into the lateral tail veins of SCID mice (nine mice per group). After 8 weeks, the mice were killed and GFP-positive cells in their lungs were quantified by FACS. $*P<0.01$, TrkA overexpressing versus mock cells.

resistance, the increased abilities of proliferation, migration and invasion may also potentiate metastasis of TrkA overexpressing cells. Therefore, more detailed quantitative analysis *in vivo* as recently reported (Hedley *et al*., 2008) is required to see the exact impact of TrkA overexpression in the numerous steps of metastatic process. Nevertheless, our findings provide primary explanations for the observation that pleural effusions of breast cancers present high levels of pTrkA compared to corresponding primary tumors (Davidson *et al*.,

Table 2 Effect of TrkA overexpression on incidence and severity of metastasis

GFP-positive cells (%)	*Mock*				*TrkA*			
	<0.2	*0.2–0.5*	*0.5–1*	*>1*	*<0.2*	*0.2–0.5*	*0.5–1*	*>1*
Liver	2/8	4/8	1/8	1/8	0/9	3/9	5/9	1/9
Lung	6/8	2/8	0/8	0/8	4/9	4/9	1/9	0/9
Brain	3/8	0/8	0/8	0/8	1/9	5/9	2/9	1/9

Mice injected with mock cells (eight animals) or with TrkA overexpressing cells (nine animals) were killed when the average volume of primary tumors approached 2 cm^3. The liver, lungs and brain of each mouse were recovered and digested with trypsin, collagenase XI and hyaluronidase for 1 h at 37 °C to obtain individual cells. The percentage of GFP-positive cells was then analysed by FACS.

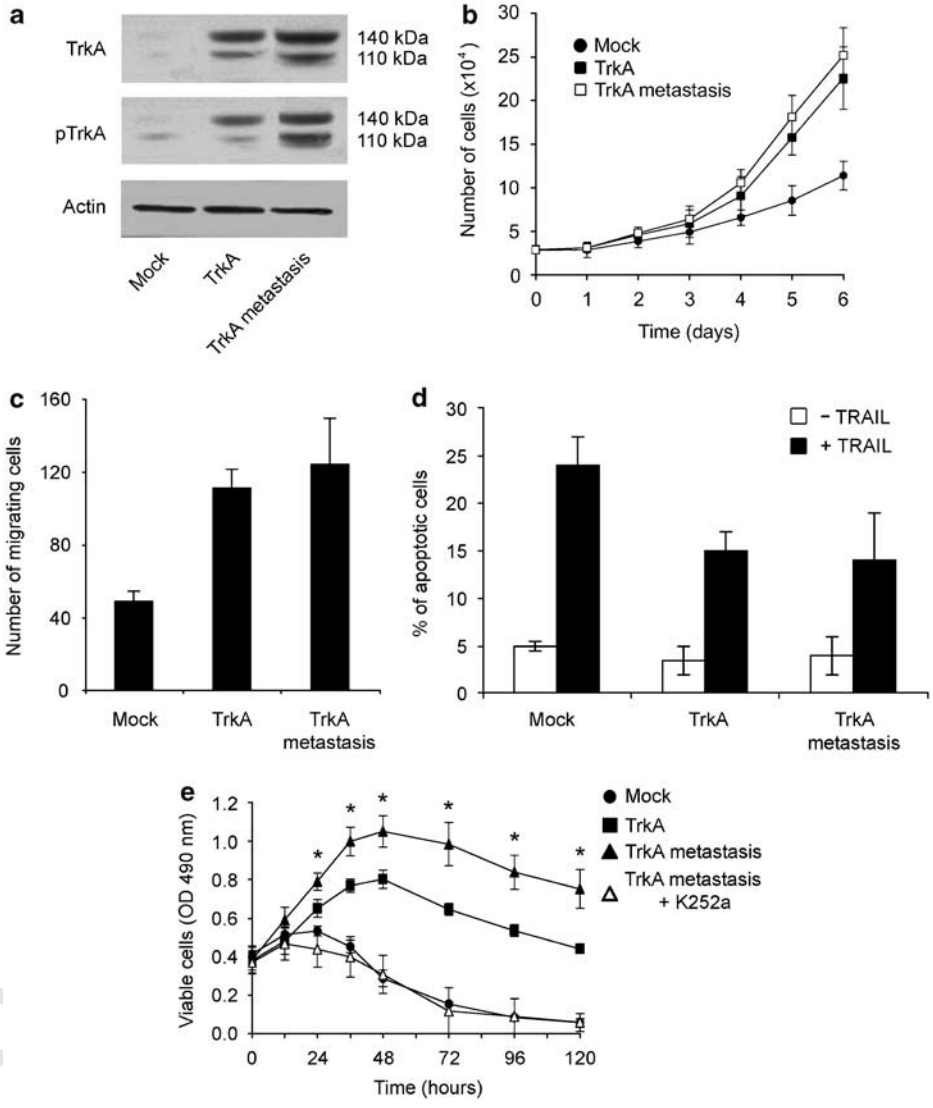

Figure 6 Properties of lung metastatic TrkA overexpressing cells. Lung metastatic TrkA overexpressing cells were recultivated (nine cultures) from the lungs of nine mice subcutaneously injected with TrkA overexpressing cells. (**a**) Immunoblots of TrkA and phospho-TrkA (pTrkA) from pooled metastatic cells. (**b**) Growth assay on standard culture plastic. (**c**) Migration assay using Transwells. (**d**) Resistance to terminal transferase dUTP nick-end labeling (TRAIL)-induced apoptosis. (**e**) Anoikis resistance of cells treated or not with K252a. *$P<0.01$, metastatic versus parental TrkA overexpressing cells.

2004). These results are not in agreement with our previous report in which TrkA mRNA expression predicted improved overall survival (Descamps *et al*., 2001a). This difference may be attributed to the fact that the levels of TrkA protein especially in its activated form cannot be compared with the levels of mRNA. Another

explanation may relate to the cohort studied. Indeed, we analysed tumors from patients who presented variable disease stages, with highly variable clinical outcomes, whereas the cohort used by Davidson *et al*. (2004) was uniformly composed of patients who developed stage IV disease at some point along the clinical course, and who, despite long disease-free periods in some cases, all suffered tumor-related death.

In conclusion, we show that TrkA is overexpressed in breast carcinoma. We also provide the first direct evidence that TrkA overexpression in breast cancer cells enhances their tumorigenic properties. Our findings imply that the targeting of TrkA signaling in breast cancers is of potential interest. Given the correlation of anoikis resistance and metastatic potential, suppression of anoikis resistance would contribute to limiting metastasis and could be useful in combination with treatments that directly target primary tumor growth.

Materials and methods

Human breast biopsies and animals

Normal breast tissues were obtained from individuals treated by mastectomy at the Hospital of Lille (France) and breast carcinoma specimens were obtained from the Clinique du Croisé Laroche (Lille, France). All these samples were collected with institutional safety review board approval. Q1 Breast cancer tissue microarrays (Cliniscience) included 10 normal breast samples and 37 tumoral breast samples (3 infiltrating lobular carcinomas, 2 *in situ* ductal carcinomas, 32 infiltrating ductal carcinomas). Animal studies were performed in accordance with the *Animal Care and Use Committee Procedures and Guidelines* of the Institut Pasteur de Lille (Lille, France).

Cell culture, transfection and generation of TrkA overexpressing cancer cells

The MDA-MB-231 breast cancer cell line was obtained from the American Type Culture Collection. Cells were maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum (FCS) and stably transfected with TrkA cDNA and/or GFP cDNA as described in the Supplementary data.

Quantitative real-time PCR and western blot analysis

The protocols were detailed in the Supplementary data. TrkA isoforms were determined by reverse-transcriptase (RT)–PCR using specific primers as described (Tacconelli *et al*., 2004).

Assays of cell behavior in culture

Protocols were detailed in the Supplementary data (cell growth, migration, matrigel chemoinvasion, wound-healing assay, anoikis assay, soft agar assay for colony formation, apoptosis analysis). Specific inhibition with pharmacological inhibitors was performed with 10 nM K252a, 15 μM LY294002, 15 μM Akt inhibitor III, 10 μM U0126 or 10 μM p38 inhibitor II, Q2 all from Calbiochem. Control cells were treated with DMSO at a 1:1000 dilution. The concentrations used were based upon the absence of toxicity in the MDA-MB-231 cells, which was determined by cell proliferation assay in serum-free medium for 48 h.

Tumor growth in SCID mice

Female SCID mice (6-week old) were purchased from the Charles River Laboratories (France) and acclimatized for at least 2 weeks. MDA-MB-231 mock and TrkA overexpressing cells were harvested and resuspended in phosphate-buffered solution before subcutaneous injection into the flanks (2×10^6 cells per flank) of 8-week-old SCID mice. The tumor volume was determined every week by measuring the length (l) and width (w) and then calculating the volume as $\frac{\pi}{6} lw \frac{(l+w)}{2}$.

Immunohistochemistry and TUNEL staining

TrkA and pTrkA staining was performed using tissue microarrays according to the manufacturer's instructions (Cliniscience). Apoptotic cells were detected by the TUNEL assay *in situ* cell death detection kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. Q3 For detailed protocols, see the Supplementary data.

Spontaneous and experimental metastasis assays

To measure spontaneous metastasis, we subcutaneously injected GFP-positive cells into the flanks (2×10^6 cells per flank) of 8-week-old SCID mice as described above. Once the average tumor size approached 2 cm^3, the mice were killed and their lungs, liver and brain were removed for further analysis. The organs were cut to two parts: one half for histological analysis, the other half was digested with enzymes (2.5 mg/ml trypsin, 0.5 mg/ml collagenase XI, 0.5 mg/ml hyaluronidase) for 1 h at 37 °C before quantification of the metastatic cells (GFP-positive) by FACS analysis. Cultured GFP-positive MDA-MB-231 cells were used as a positive control. Cells derived from the lungs, liver and brain of a mouse injected with GFP-negative MDA-MB-231 cells were used as a negative control. To recultivate cancer cells derived from primary tumors and lungs, we washed enzymatic digests once in 10 ml EMEM 10% FCS and seeded in cell culture dishes in EMEM 10% FCS with 800 μg/ml hygromycin for 2–3 weeks. Selected tumor cells were confirmed after examination of GFP expression under fluorescent microscopy.

To measure experimental metastasis, we injected 5×10^5 GFP-positive cells into the lateral tail veins of 8-week-old SCID mice. After 8 weeks of injection, the mice were killed and metastatic cells in their lungs were quantified as described above.

Statistical analysis

Statistical differences were determined with two-tailed Student's t-tests. All P-values were two sided. $P < 0.01$ was considered as statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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Reviews

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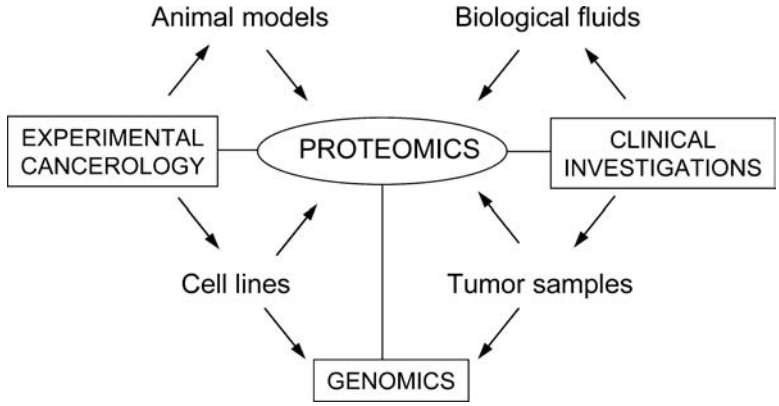

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Proteomics of Breast Cancer: The Quest for Markers and Therapeutic Targets

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Proteomics of breast cancer has already delivered significant data in terms of proteome profiling in addition to the identification of a few proteins of potential interest for diagnosis and treatment. With more pathological and experimental situations being studied, it now enters into a new phase dominated by the concepts of deep proteome analysis and the definition of protein–protein interaction networks leading to mammary cell deregulation and cancer progression. Together, what could be called "Systems Proteomics", integrating with information from the genomics and the physiopathology, is clearly emerging to become the frame for future investigations. However, difficulties ahead should not be underestimated. First, the proteome is complex, and current tools are still far from providing a definitive solution for its exploration. Second, breast cancer is a multifactorial disease which is so diverse that a great deal of time and efforts will be necessary to define its associated proteome modifications and translate it into practical applications for the clinic.

Keywords: Breast cancer • Biomarkers • Therapeutic targets • Proteomics

Introduction

During the past decade, with the completion of human genome sequencing and its limited number of direct outcomes in cancer, it gradually became clear that looking solely at genes and their expression would not provide a definitive way for the comprehension, detection, and treatment of cancer. Concomitantly, with progress made in proteomic analysis, more interest has been directed toward the proteome as a source of future markers and therapeutic targets.[1] In the field of cancer, as in many others, it now appears that one of the outcomes, if not the major outcome, of the human genome sequencing has finally been to open the way to the exploration of the proteome, transferring the goals and hopes in terms of biomedical applications. Here we are today, in the postgenomic era, trying to find new proteins, new post-translational modifications, or new protein networks that could be used to better understand, detect, or treat cancer. This will be illustrated with the case of breast cancer.

1. Diagnosis and Treatment of Breast Cancer: What We Have and What We Need

Current screening methods used to detect breast tumors, either benign or malignant, include clinical examination, ultrasound, and mammography. Although mammography is the most acute approach, there are intrinsic limitations to mammography. To be detected in mammography, a breast tumor should be at least a few millimeters in size. However, a tumor of this size already contains several hundred thousand cells, and given that a single cell can lead to the development of a whole tumor (clonal origin of cancer), it is already late when a breast tumor is detected by mammography. In clinical practice, after the surgical removal of a tumor, its characterization as a malignant or benign tumor is made by histology. Such parameters as tumor size and inflammation, histoprognostic grading, and node involvement are then used to decide treatment and prognosis. However, breast cancer is not a homogeneous disease, and there are different categories of breast tumors. Depending on the cellular and histological origin of the cancer cells and on the evolution of the disease, a broad range of breast tumor types has been described. The main biological markers recommended for routine use in breast cancer are the presence of estrogen (estradiol) and progesterone receptors, for the selection of patients potentially responding to treatment with antiestrogen such as tamoxifen.[2] Interestingly, practical consequences for treatment derived from a better understanding of the molecular basis of cancer cell growth are now emerging. This is evidenced by the development of targeted strategies based, for example, on the inhibition of tyrosine kinase receptors. This approach is well-illustrated with ErbB2, a tyrosine kinase receptor overexpressed in about 20% of breast tumors. Specific inhibition of ErbB2 using Herceptin, a truncated blocking-antibody directed against it, has been successfully developed and has now entered into clinical practice.[3] However, the growth of breast cancer cells can be regulated by other growth factors that either stimulate

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or inhibit their proliferation, migration, and differentiation, thus, acting in concert to promote tumor growth and metastasis. Probably related to this, the efficacy of Herceptin appears to be limited to a small proportion of breast cancers, and the identification of other targets, and corresponding drugs, remains a major interest for the development of targeted therapeutic strategies. Clearly, there is a critical need to find new molecular parameters not only for detection, but also for typing and treatment of breast cancer. As proteomics provides a global approach for the identification of protein regulation during pathological processes, the discovery of new markers and therapeutic targets is a highly anticipated outcome. Such data would provide the knowledge base for the development of new diagnosis and therapeutic strategies against breast cancer.

2. Differential Analysis of Cell Cultures and Tissue Biopsies: Should We Expect More?

We have now more than 30 years of investigations since the first analysis of proteome content of normal versus cancer biopsies was published. Hundreds of research manuscripts have been published. A set of few proteins of interest have been established and will be described below. Although the value of these studies for the molecular description and understanding of the pathology should not be underestimated, none of the proteins identified in more than 30 years of two-dimensional electrophoresis (2DE) and proteomics have found a practical use for the management of breast cancer at the clinical level. Why is this? A first way to deal with this question is to point that we have so far only studied the most abundant proteins and that the constant evolution of proteomic technologies will enable us to have increasing access to the less abundant proteins—the deep proteome—with more relevant markers to be identified. Although this hypothesis cannot be excluded, it obviously does not take under account the cellular and molecular complexity of the mammary gland that makes intrinsic limitations to the differential analysis of cell cultures and tissue biopsies.

Normal mammary gland tissues and breast tumors are dynamic and heterogeneous cellular structures, and therefore, a global differential analysis of their proteomes refers to a comparative study of cell mixtures with different proportions of epithelial and other cell types including myoepithelial, fibroblast, and endothelial cells, supplemented by adipocytes, nerve fibers, circulating cells, and macrophages. Therefore, analyzing breast tumor biopsies clearly means dealing with a mixture of different cell types with tumor cells representing only a fraction of the whole. From early times of 2DE, several studies have reported differential proteomic analyses of breast tumor biopsies. In 1974, the use of 2DE to resolve serum proteins was reported, with differences being noted in the protein patterns of individuals suffering cancer;[4] however, this study was essentially descriptive, and no protein identifications were made. In 1984, it was found that most polypeptides were consistently present in both malignant and nonmalignant breast tissues, as only 10 polypeptides differed out of 350 resolved.[5] Subsequently, using better technology, it was shown that of approximately 1000 silver-stained cytosolic polypeptides observed, the 2DE patterns from normal and malignant tissue differed in only 6 places qualitatively, and only 22 places quantitatively.[6,7] A more precise characterization of such polypeptide differences was published in the early 1990s with the demonstration of a defect in tropomyosin 1-2 and -3 expression in mammary carcinoma, suggesting that such abnormalities may play a role in breast neoplasia.[8,9] Differential distribution of heat shock protein (HSP) family members has also been described.[10,11] More recently, comparisons of subclasses of breast cancer, or during epithelial-mesenchyme transition, have been published with several dozens proteins found to be differentially expressed.[12–14] From all of these data, it appears that the set of differentially expressed proteins identified are different from one study to another, suggesting either a lack of experimental standardization or problems of heterogeneity between the biological materials used in each study. Considering the fact that such a global analysis is in fact describing the proteomes of different cell types, the lack of specificity found in cancer samples might not be regarded surprising. In addition, as assessed by the presence of serum albumin, breast tumor biopsies also contain blood compounds due to the vascular irrigation of breast tumors. As blood cannot be washed out of biopsies during their processing, proteomic analyses of breast tumors also measure proteins from circulating cells and from the plasma, further confounding an already complex situation. This emphasizes the importance of widespread validation—by complementary approaches such as Western blotting and immunohistochemistry—of proteins identified as a mean of excluding protein expression differentials due to blood compound, hormonal influence, or different proportion of cell types.

Given the cellular complexity of mammary tumors, microdissection can be regarded as a reasonable alternative for selectively isolating cell types to be analyzed. Techniques such as laser capture microdissection can be used for the isolation of malignant cells prior to sample preparation for proteomic analysis and might facilitate marker protein discovery. However, initial enthusiasm for microdissection has progressively been diminished by several limiting aspects. The first problem is quantitative: as no amplification technique can be applied to proteins (as PCR for DNA/RNA), it is necessary to start any proteomic analysis with a significant amount of material. In our hands, a minimum of 100 000 cells is necessary to perform analysis in a way that allows subsequent characterization of proteins by mass spectrometry; this has recently been illustrated. Despite being at the upper-limit of the theoretical capabilities of current microdissection techniques, the preparation of such a number of cells represents an important quantity of work with a constant problem in the choice of which cells to select. The second problem of using microdissection for proteomic analysis is related to the biochemical quality of the dissected material. The use of fixatives must be avoided as the requisite compounds create artificial boundaries between amino acid residues. In addition, due to the time necessary to perform microdissection, protein modifications such as degradation or dephosphorylation are likely to take place. This last point is related to a more general concern about working with tissue biopsies: the collecting procedure of the samples must be standardized to a maximum in order to avoid differential protein modification and/or degradation. This difficult situation is nevertheless being challenged,[15,16] and future progress in coupling microdissection and proteomics of breast cancer can eventually be expected.

When comparing cancer tissues to normal tissues or the corresponding dissected cells, a fundamental physiological question also arises: what is a normal breast tissue? Because of the changing hormone concentration (estrogens and progesterone) during the course of menstrual cycle, the status of

normal breast tissue is clearly also changing, and therefore, it is difficult to define a reference state. Hormonal stimulation of breast epithelial cells will be different depending on hormonal status of the women at the time the sampling is made, and the same concern applies to breast tumor biopsies. The stimulation by hormones is known to modify gene expression in both normal and cancer cells and consequently the proteome of breast epithelial cells. Thus, the hormonal environment of biopsied tissue should ideally be evaluated, but this is practically very difficult if not impossible to implement.

Given the limitations of analyzing tumor biopsies, cell cultures look like a reasonable alternative. As soon as 1980, a secreted 46 kDa glycoprotein, induced by estrogens in human breast cancer cell lines, was identified with specific antibodies as being the protease cathepsin D.[17] In 1989, a computer-based analysis of 2DE gels reported a total of 8 polypeptide differences between cancerous and normal breast epithelial cells in tissue culture.[18] More precise characterization of such polypeptide differences was published in the early 1990s with the demonstration that normal breast epithelial cells produce keratins K5, K6, K7, and K17, whereas tumor cells produce mainly keratins K8, K18, and K19.[19] This distribution was secondarily confirmed in tumor samples,[20] and cytokeratin immunodetection is now eventually used at the clinical level to help discriminate benign from malignant cells on histopathological slides. More sophisticated characterization of normal breast epithelial/myoepithelial cell proteins and breast cancer cells was more recently performed and revealed a limited number of differences[21] even when using technological innovations.[22] The study of cells in culture, both normal and cancerous, definitely offers the possibility to directly investigate proteins specifically produced, or not produced, by breast cancer cells. This is the case with proteinase inhibitors TIMP-1 and PAI-1, originally studied in breast cancer cells, which have been shown to be complementary in determining prognosis of breast tumors.[23] Cell fractionation prior to proteomic analysis has also been explored, as been illustrated for plasma membranes of breast cancer cells.[24,25] Importantly, proteomic analysis from cell cultures can be realized in standardized medium conditions, providing a comparable and controlable status of hormone and growth factor stimulation. In addition, phenotype and behavior of the cells, that is, proliferation/migration/differentiation/survival, as well as corresponding signaling, can be experimentally studied. A step toward the identification of signaling proteins regulated in cancer cells has been provided by the molecular chaperone 14-3-3 sigma, and interestingly, this is a good illustration of the complementarity of genomic- and proteomic-based approaches. 14-3-3 is a family of highly conserved protein forms (alpha, beta, delta, sigma, zeta) of 25−30 kDa, expressed in all eukaryotic cells, that plays a role in the regulation of signal transduction pathways implicated in the control of cell proliferation, differentiation, and survival.[26] 14-3-3 proteins are known to associate directly or indirectly with signaling proteins such as the IGF-1 receptor, Raf, MEK kinases, and PI3-kinase, modulating their activities.[26] The down-regulation of 14-3-3 sigma has been shown to be a major modification of the proteome associated with breast epithelial cell carcinogenesis, and subsequently, its distribution in breast cancer biopsies was investigated, and it was established that the level of 14-3-3 sigma is systematically down-regulated in tumor biopsies.[27] This demonstrates that starting with a cellular proteome, which exhibits a lower level of complexity, facilitates the identification of relevant proteins that can secondarily be found and quantified in tumor biospies. At the mRNA level, it was shown that gene expression of 14-3-3 sigma is decreased in breast cancer cells due to the high frequency of hypermethylation of the 14-3-3 sigma locus.[28] From the clinical point of view, it has been reported that down-regulation of 14-3-3 sigma gene expression is an early event in breast carcinogenesis[29] and a contributor to drug resistance.[30] Nevertheless the introduction of 14-3-3 sigma detection into the clinic looks unlikely because its down-regulation does not appear to be systematically observed in all tumor types[31] and relationship with other clinicopathological parameters has not been established so far. Interestingly, the case of 14-3-3 illustrates that existing cell lines are not representative of all types of breast cancer and that, although they are advantages of working with them, their pathological relevance do not reflect the entire spectrum of breast cancer diversity.

Recently, other studies dealing with differential analysis of cell cultures and/or tumor biopsies,[32–36] some with technological innovation such as shotgun approach[37] or FT-ICR MS[38] have been published, but it should be recognized that, despite their interest for better defining the proteome of breast cancer, none of them have really evidenced any marker that can be envisioned for future practical use. Thus, it seems reasonable to postulate that we have reached a plateau and that the molecular and cellular complexity and diversity of mammary gland and breast cancer remains the major limitation to differential proteomic investigation. Whether the exploration of the deep proteome will overcome these difficulties will have to be tested, but as described above, there are serious reasons to be pessimistic about differential analysis of cell cultures and tissue biopsies and, in this context, the exploration of alternative sources of biomarkers appears appropriate.

3. Biological Fluids and Tissue Slides: New Promising Sources of Biomarkers

Although it is difficult to imagine that a biomarker not found in cell cultures and tissue biopsies would be detectable in biological fluids or tissue slides, the exploration of biological fluids and tissue slides offer some theoretical advantages. Exploring biological fluids can be a way to look at secreted proteins avoiding the vast majority of housekeeping cellular components that will constitute the majority in cell or tissue extracts.[39] Several recent studies have shown the potential of looking at plasma membrane[40,41] or cell-conditioned media,[42] and in terms of biomedical applications, the detection of proteomic markers in the biological fluids such as plasma and urine is of promising interest. To be able to detect breast cancer, or any other cancer, from a simple blood analysis would represent a major breakthrough, and such an achievement provides impetus to the currently ongoing plasma project.[43,44] However, even though significant progress has already been made,[45–52] searching for tumor-released proteins in plasma or their fragments in urine will still require a lot of technological development before the deep proteome can fully be investigated. From this perspective, the current challenges are definitely on the technological side. On the other hand, the mammary gland offers the possibility to access local fluids. Not to be forgotten, the milk is a production of normal breast epithelial cells, and the analysis of its proteome has been performed with the same limitation as for plasma, that is, once again the presence of major proteins (casein, albumin) that represent a technological challenge for the detection of less abundant ones. Although milk is only produced during lacta-

tion and cannot be proposed as a diagnosis origin for the diagnosis of the vast majority of breast cancer, nipple aspiration liquid could represent a reasonable possibility. Even out of lactation period, breast ducts contain a physiological liquid that can be aspirated. Clinical protocols for using it on a clinical basis are presently being developed with the idea of aspirating and detecting cancer cells if they are present. The advantage of nipple aspirate is that it allows to specifically access the fluid surrounding ducts and breast epithelial cells, with no contamination with other interstitial fluids or cell types of the mammary gland. Although cells can easily be removed, the extracted fluids have been studied, and even if no real markers have emerged so far, it definitely appears very promising.[53–56] Serious difficulties remain in terms of low volume/quantity of material that can be extracted, but considering the specificity of this fluid, interesting outcomes can reasonably be anticipated.

Aside from biological fluids, tissue slides could also become an alternative source for identification of biomarkers of breast cancer. As for the majority of solid tumors, diagnosis, prognosis, and treatment choice of breast cancers are largely based on histological analyses. After resection, breast tumors are systematically fixed and/or frozen before slicing for histological analysis, and evaluation of cell differentiation and tissue invasion serve as a basis for tumor type determination and grading. The clinical routine analyses of breast tumors are based on histology since decades, and vast quantities of tissues and tissue slides have been stored, with corresponding crucial clinical data such as patient survival, and are potentially available for analysis. The problem consists of two aspects. First, tissues used for histology are often being fixed, inducing covalent chemical reaction inside individual protein between different proteins or between proteins and other cellular molecules such as lipids. This is definitely a problem for protein separation and for subsequent identification in mass spectrometry. Second, tissue slides contain very little material quantities, and analyzing proteins from 5 μm slides with classical protein extraction and separation procedures is clearly limited for access to low-abundance proteins. However, this challenging context seems to be progressively overcome by recent significant progress in mass spectrometry based analysis of tissue slides. MALDI-TOF imaging and now the possibility to envision MS/MS sequencing from tissue slides opens new perspectives.[57] The problems caused by fixatives are naturally minimized by direct MS analysis, and it should be noted that new protocols are progressively elaborated to increase their compatibility with proteomic analysis.[58] The exploration of biological fluids and tissue slides represent promising alternatives that will deserve attention during the coming years. Interestingly, and representative of the general situation of the field of proteomics, success will depend on both the technological developments and the increased knowledge and understanding of the pertinence of the biological materials used. Thus, large collaborative projects including protein analysis and mass spectrometry—medical and clinical management of breast cancer—as well as bioinformatics for data integration, now appear, more than ever before, as a necessary requirement.

4. Functional Proteomics of Breast Cancer: To Understand More than To Describe

In addition to providing a mean to look for markers, proteomics is also increasingly used to decipher the molecular mechanisms underlying breast cancer cell growth and metastasis. The objectives are double: understanding the basic mechanisms of cancer initiation and progression and identifying new therapeutic targets. In breast cancer, some information regarding the activity of oncogenic and tumor suppressor proteins have been obtained through the use of proteomics. The genetic of breast cancer is marked by the existence of the predisposition genes BRCA1 and BRCA2, and proteomics have led to substantial information on their mechanism of action. Notably, it allowed to demonstrate that BRCA1 ubiquitinates RPB8 in response to DNA damage,[59] providing a pathway of BRCA1 activity. Same type of approach was developed for studying the metastasis suppressor gene BRMS1[60] or for analyzing p53 to demonstrate that the heat shock protein 90 is a target for p53 mutation reactivation by PRIMA-1 in breast cancer cells.[61] Other studies have dealt with proteomic modifications induced by oncogenic or suppressor proteins. This was done for the mammary serpin maspin for which a wide range of proteome modifications was observed.[62] In case of H19, oncogenic for breast cancer cells, it was shown by proteomics that this noncoding mRNA acts through thioredoxin post-transcriptional regulation[63] and for CTCF for which overexpression is associated with resistance to apoptosis.[64]

Another area of considerable interest is intracellular signaling initiated by extracellular messengers such as growth factors or hormones. Estrogenic hormones and growth factors play an important role in survival, growth, and metastasis of breast cancer cells, and importantly, elucidating the associated signaling pathways could lead to the identification of therapeutic targets. This concept has been illustrated first with the elaboration of antiestrogenic drugs such as tamoxifen which block the estrogen receptor and has been used for antiestrogen therapy of breast cancer for decades. The action of estrogens and tamoxifen on breast cancer or the influence of the hormone-dependence status have been studied by proteomics.[65–67] However, more than 50% of breast tumors are estrogen-independent, rendering antiestrogen-based therapy inefficient. A more recent illustration concerns the tyrosine kinase membrane receptor ErB-B2/HER-2/Neu which is overexpressed in about a quarter of breast cancers, offering a possibility of a specific targeting. At the clinical level, this is already implemented, and ErB-B2 can be targeted with Herceptin, a truncated blocking-antibody, to treat ErB-B2 overexpressing breast tumors. Proteomic modifications associated to ErB-B2 activation have been reported[68,69] and particularly at the phosphoproteome level to highlight the downstream pathways involved.[70] However, ErB-B2 is overexpressed only in 20% of breast tumors, and the identification of further targets is badly needed. Many growth factors initiate intracellular signaling through tyrosine kinase-membrane receptors, which in turn induce intracellular protein–protein interaction and phosphorylation cascades involving a variety of signaling proteins such as the mitogen activated protein-kinases (MAP-kinases). These cascades of protein phosphorylation ultimately induce changes in gene expression, with consequent modifications in protein synthesis, leading to cell survival, proliferation, differentiation, or migration. In breast cancer, insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), fibroblast growth factors (FGFs), and transforming growth factor-β (TGF β) have long been recognized for their potent intervention in tumor cell growth and development, and their respective signaling has been investigated by proteomics. For EGF, which is a strong mitogen for breast cancer cells, it has been shown that the overexpression of its receptor enhances the expression of the epithelial

marker cytokeratin 19,[71] and the molecular mechanism of the inhibiting drug lapatinib has been delineated by proteomics.[72] Interestingly, EGF signaling in breast cancer cells has also been used as a model for the development of MRM (multiple reaction monitoring) as a quantitative method for the analysis of cellular signaling networks.[73] Very elegant proteomic approaches have also led to a better understanding of TGFβ signaling in breast cancer cells,[74,75] demonstrating the importance of the transcription factor TFII-I in the Samd3 mediated activation of transcription. Of particular interest is also FGF signaling in breast cancer cells. FGF not only stimulates proliferation of breast cancer cells, it also activates their survival and migration then favoring both tumor growth and metastasis. The effect of FGFs on the proteome and phosphoproteome profiles of breast cancer cells has been explored,[76,77] underlining several features such as the role of HSP90 in accompanying growth factor induced-phenotype or the importance of the serine-threonine kinase AKT[78] in the resistance to apoptosis which is a characteristic of most breast tumor cells. AKT is crucial to breast cancer development as its impairment inevitably results in apoptosis of breast cancer cells, placing AKT as a potential therapeutic target. However, AKT is a largely distributed kinase which also controls a variety of other cellular processes such as metabolism, and therefore, targeting AKT is likely to have a wide series of side effects limiting practical applications. A way to overcome this problem would be to define signaling proteins downstream to AKT and specifically involved in tumor cell survival. Proteomics exploration has revealed a list of 10 potential AKT interacting partners that are related to cell survival.[79] Among them, and of particular interest, was the ATPase valosin-containing protein (VCP) which appears to be required to AKT antiapoptotic signaling in breast cancer cells. VCP interacts and is a substrate of the kinase activity of AKT, the serine residues involved have been identified, and their mutation resulted in a potent induction of breast cancer cells.[80] Importantly, the same study reported that the impairment of AKT/VCP interaction in breast cancer cells potentiated the effect of several chemotherapeutic drugs, such as 5FU or etoposide, indicating the potential interest of targeting VCP in breast cancer therapy. To finish on proteomic exploration of growth factor signaling in breast cancer, the proto-oncogenic tyrosine kinase receptor TrkA should also be mentioned. TrkA is the membrane receptor of nerve growth factor, and its stimulation leads to cell proliferation in breast cancer cells.[81,82] Although TrkA cannot be regarded as a marker of breast cancers,[83] NGF itself is overexpressed in breast tumors and can be targeted to inhibit breast cancer cell growth and metastasis.[84] A very recent proteomics-based study has evidenced a series of TrkA signaling partners that are related to cell growth, and the DNA repair protein Ku70 was also identified as involved in TrkA signaling.[85] It was shown that during the course of stimulating cell proliferation, TrkA also induces the activation of Ku70 to prevent apoptosis, reinforcing the agressivity of breast cancer cells and their progression toward a more advanced cancer phenotype.

In conclusion, although it is too early to know if one of the hits defined so far will be of clinical value (a mean of about 10 years separate the identification of a target to clinical use), proteomics-based approaches have proven their efficacy and are increasingly used as a tool to decipher the basic mechanisms of carcinogenesis. Thus, it can reasonably be envisioned that the proteomics pipeline will soon allow to complement antiestrogens and anti-ErB-B2 strategies by other targeted therapies, permitting a wider coverage of the broad spectrum of the disease.

5. Systems Proteomics: Integrating Proteomics into a Bigger Picture

Now that more and more data are being collected about the proteome of breast cancer, a concomitant need to have a better integration appears. Just as information about the sole genome does not allow a comprehension of complex biological systems, proteomic view alone is also not sufficient. If proteins are the functional outcomes of genes, they are not the finality of gene transcription, and just as RNA are only messengers for the synthesis of proteins, proteins themselves are also intermediates. Ultimately, proteins only carry necessary information to drive cells toward appropriate phenotypes and behaviors, and therefore, further understanding of complex biological systems, like mammary gland and breast cancer, now requires more integrated approaches and strategies. The need for a better integration can be regarded at two different levels. First, from the molecular point of view, putting together genomic, transcriptomic, and proteomic data would give access to a global view of molecular changes occurring in cells during the initiation and progression of cancer as well as potential way of rational intervention to destroy or normalize them. Although the way is going to be long, and dependent on progresses in bioinformatics, attempts in that direction have recently been made for breast cancer.[86–88] The second possible level of integration is from a more functional perspective, taking under consideration the cellular interactions occurring in the mammary gland. The breast is organized around the mammary gland that has 15−20 lobes, and within each lobe, there are many smaller lobules ending in dozens of tiny bulbs that can eventually produce milk. The lobules are all linked by thin tubes called ducts, and all ducts lead to the nipple. The cells forming the ducts and lobules are epithelial cells whose main function is to produce and to secrete the various constituents of milk. In addition, epithelial cells are surrounded by a layer of myoepithelial cells, attached on a basal membrane, whose role is to maintain the tubular structure of ducts and lobules. Surrounding the lobules and ducts, connective and fat tissues are composed of fibroblasts, with their abundant extracellular matrix and adipocytes. In addition, both blood vessels and lymph vessels irrigate the mammary gland, and nerve fibers, mostly sensory and sympathetic, are also present. Few studies have dealt with cell−cell interaction in breast cancer using *in vitro* models. Then it was shown that normal breast epithelial cells do produce IGFBP3 and maspin, hence, contributing to contain the development of breast tumors by inducing apoptosis of tumor cells.[89] From analysis of human tissues, the molecular circuitry of epithelial−adipocyte stromal interaction was also studied,[90] but with naturally no experimental approach, investigation on human tissues fast reaches its limitations. An alternative solution can be provided by the use of animal models, and there have recently been intensive efforts to take advantage of animal models in breast cancer and their analysis by proteomics.[91] From the study of xenografted human cancer cells in immunodeficient mice to the use of isogenic models,[92–94] animal models also offer the possibility to integrate data with genomic analysis[95] for a better knowledge of global *in vivo* molecular modifications associated with breast cancer. Together, it seems clear that what could be called systems proteomics (Figure 1), with integration at both the

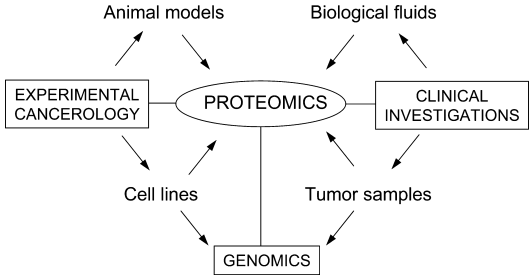

Figure 1. Systems proteomics for descriptive and functional analysis of breast cancer. Integration at both molecular and physiopathological levels is becoming the new framework for identification and validation of markers and therapeutic targets. It is to be noted that (1) all types of biological samples (cell lines, animal models, biological fluids, and tumor samples) can be analyzed by proteomics, placing the approach at a central and crossing level, and (2) this schematic representation applies not only to breast cancer but to most human pathologies.

molecular and physiological levels, is under way to open a new dimension for both differential and functional analyses of breast cancer.

6. Transition from Bench to Clinical Application: Still a Bridge Too Far

It has become common to hear that none of the biomarkers or targets identified so far by proteomics have actually entered into the clinical practice, and breast cancer makes no exception. However, this should not be a disappointment, not only because a mean of 10 years are usually necessary between discovery and clinical use of both biomarkers and therapeutic targets, but also because we have so far only explored the most obvious aspects of the human proteome. Despite much agitation and emphasis around proteomics and its potential in biomedicine, one should not forget that most of the complexity of the proteome, in terms of protein numbers and post-translational modifications, is still not accessible with our current tools. With about a million proteins and 200 types of post-translational modifications, what we have explored so far in breast cancer—as in other pathologies—is only the very surface, and we are going to need much more time and efforts, along with technological improvements, to have access to the deep proteome. Another area of current limitation is quantitation. One should not forget that we are almost blind when it comes to quantitation and stoichiometry in proteomics, and it has a too often forgotten impact when it comes to find proteins that are differentially expressed in cancer. Finally, post-translational modifications is another area where we are still mostly blind and there again our ability to explore the refinement of the situation will require more technological progress. The human proteome is much more complex than what we have explored so far, and we should realize the time and efforts that will be necessary first to simply describe it and then to understand how it works. This is why a worldwide organization such as HUPO (HUman Proteome Organization) is absolutely needed to bring together expertise and to provide the framework for a long run program.

More than 30 years after Nixon's declaration of war against cancer, humankind is still facing this problem of a multifactorial and diverse disease which is constantly progressing. It is a fact that no major breakthrough (like the discovery of antibiotics for infectious disease) has been made, and we are now entrenched in what looks like a long battle against cancer. Although it is too early to know if proteomics will change the course of the war, it is clearly a new weapon introduced into the battlefield. Just as tanks first used during World War I, proteomics shows potentials as well as limitations, and we now have to improve it to make it work along with other weapons in the complex battlefield against breast cancer.

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