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THESE

POUR L'OBTENTION DU GRADE DE DOCTEUR DE L'UNIVERSITE DE LILLE I
EN SCIENCES DE LA VIE ET DE LA SANTE

Présentée par

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PHENOTYPES DE CELLULES DE CANCER DU SEIN SUREXPRIMANT LE GENE *H19*: ATTRIBUTION DE PROPRIETES ONCOGENIQUES ET D'UN STATUT DE *TRANS*-RIBOREGULATEUR POSITIF.

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- **Lottin S.**, Adriaenssens E., Dupressoir T., Bertaux N., Coll J., Dugimont T. et Curgy JJ. Overexpression of an ectopic *H19* gene enhances the tumorigenic properties of breast cancer cells. (Soumis pour publication).

- Adriaenssens E., **Lottin S.**, Bertaux N., Hornez L., Fauquette W., Fafeur V., Peyrat JP., Le Bourhis X., Hondermarck H., Coll J., Dugimont T. et Curgy JJ. Cross-talk between mesenchyme and epithelium results in an increase of *H19* gene expression during scattering and morphogenesis of epithelial cells. (Soumis pour publication).

COMMUNICATIONS CONCERNANT MON TRAVAIL DE THESE

- Adriaenssens E., Dumont L., **Lottin S.,** Bolle D., Delobelle A., Dugimont T., Coll J., Curgy J.J. (1997). La surexpression du gène *H19* dans le sein signe-t-elle le mauvais pronostic d'un cancer et est-elle impliquée dans les relations épithélio-mésenchymateuses? *Eurocancer*, **97**, p94. Eds Boiron M. et Marty M., John Libbey Eurotext.

- Adriaenssens E., **Lottin S.**, Le Bourhis X., Dugimont T., Desbiens X., Coll J., Boilly B., Curgy J.J. (1998). Epithelium-mesenchyme relationships within breast cancers: *in vitro* experimental procedures which reproduce the rare up-regulation of *H19*, a putative suppressor gene, in tumor cells. *Eurocancer*, **98**, p178. Eds Boiron M. et Marty M., John Libbey Eurotext Limited.

- **Lottin S.**, Adriaenssens E., Dugimont T., Coll J., Curgy J.J. (2000). Le gène *H19*: expression et régulation dans des cellules normales ou cancéreuses. Forum de Cancérologie de Lille, Institut de Biologie de Lille, France.

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- Dumont L., Adriaenssens E., Bolle D., **Lottin S.**, Delobelle A., Bouali F., Dugimont T., Coll J., Curgy J.J. (1997). Phénoménologie et analyse statistique de l'expression du gène *H19* dans les carcinomes du sein. *Bull. Cancer*, **84**, p 483.

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Sommaire

Introduction

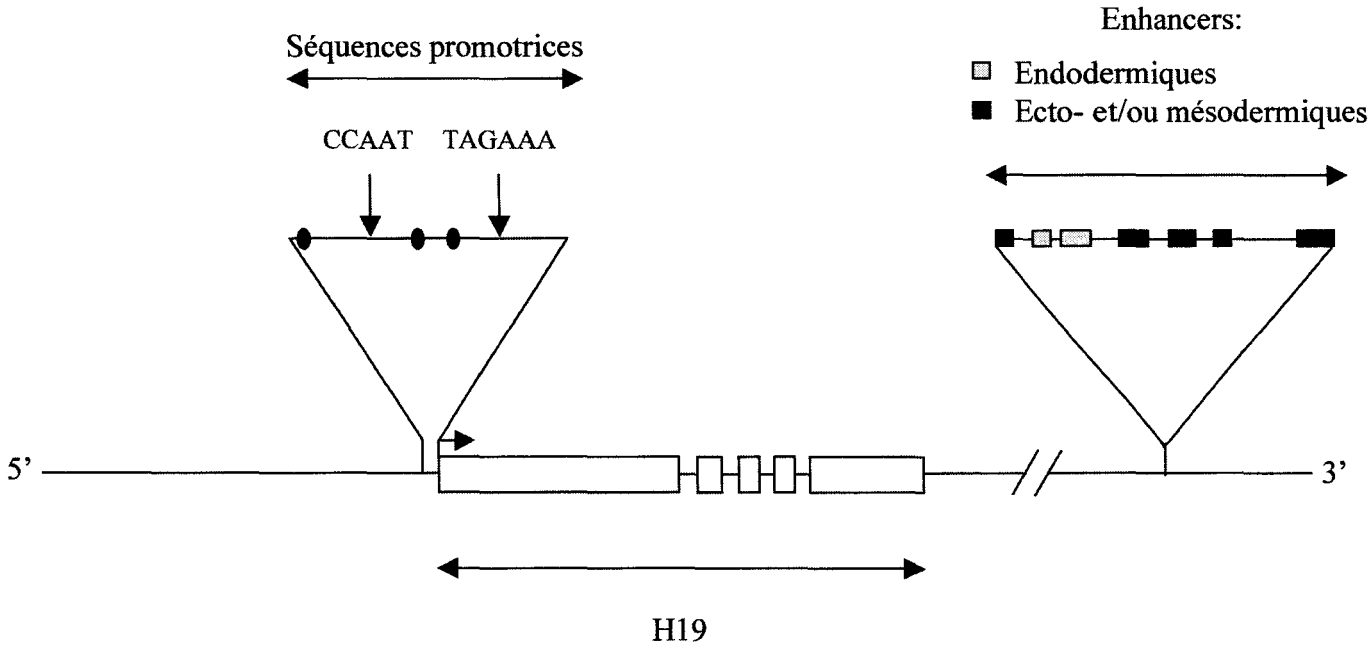
1) Découverte du gène *H19*

Afin d'identifier des gènes structuraux co-régulés en *trans* avec le gène de l'*alpha-foetoproteine* (*AFP*) par le locus *raf*, c'est à dire des gènes dont la forte expression fœtale est réprimée après la naissance, l'équipe de Tilghman (USA) a criblé une banque d'ADNc de foie fœtal de souris et a isolé un gène, qu'elle a nommé *H19*, seul gène répondant à ces critères. La co-régulation de ces deux gènes est également retrouvée dans l'endoderme viscéral et dans l'intestin où la répression périnatale est totale. Contrairement au gène de l'*AFP*, *H19* est exprimé dans le muscle cardiaque et les muscles squelettiques fœtaux et une expression réduite mais significative persiste chez la souris adulte. Toutefois, dans ces tissus, la régulation du gène *H19* n'est plus sous le control de *raf*. Chez la souris, le gène *H19* se situe sur le chromosome 7 et n'est génétiquement lié ni au gène de l'*AFP*, ni à celui de *raf* (Pachnis *et al.*, 1984). Six ans plus tard, la même équipe isole le gène *H19* humain, dont il existe, comme chez la souris, une copie par génome haploïde (Brannan *et al.*, 1990).

2) Structure du gène *H19*

La détermination de la séquence du gène *H19* murin et sa comparaison avec celle du gène de l'*AFP* indiquent que ces deux gènes ne dérivent pas d'un ancêtre commun et que leur co-régulation provient d'une évolution indépendante et convergente. Chez la souris le gène *H19* est constitué de 5 exons (de 5' en 3': 1307,135, 119, 127 et 560 nucléotides) séparés par 4 introns remarquables par leur brièveté (de 5' en 3': 81, 53, 76 et 60 nucléotides). Son promoteur ne présente pas une réelle boîte TATA, mais possède en -27 (par rapport au site d'initiation de la transcription) une séquence non canonique TAGAAA. Il est également pourvu d'une séquence CCAAT en -72 sur le brin non codant. Le reste du promoteur est très riche en GC et contient, à -42 et –55 pb, deux séquences consensus pour le facteur de transcription Sp1. Une autre séquence, plus longue, riche en GC et située à -100 pb, pourrait également être la cible de Sp1 (Pachnis *et al.*, 1988).

Le gène *H19* humain est localisé sur le bras court du chromosome 11 en p15.5 et sa structure, décrite figure 1, est analogue à celle de son homologue murin. Les gènes des deux espèces présentent 77 % d'identité de séquences, néanmoins ce pourcentage varie selon les régions géniques analysées. Le domaine le plus conservé (85 % d'homologie) recouvre la fin de l'exon 1 et l'exon 2. Les exons 3, 4 et 5 sont moins conservés: 65, 80 et 72 % d'homologie respectivement. Les introns ont une homologie de séquences proche de celle des exons les moins conservés soit 69 % environ. La séquence promotrice est également fortement conservée ; en particulier, les 175 premières paires de bases en amont du site d'initiation de la transcription présentent une identité de séquence de 80 % avec celles de la souris (Brannan *et al.*, 1990).



Site d'initiation de la transcription

Exons

Site de fixation pour Sp1

Figure 1: Structure du gène *H19* humain
Il est constitué de 5 exons de 1328, 135, 113 et 123 pb séparés par 4 petits introns de 96, 90, 81 et 80 pb.

Des séquences régulatrices ont été mises en évidence en 3' du gène et les premières à avoir été bien décrites sont deux séquences "enhancers" spécifiques des tissus d'origine endodermique situées à 5 et 6,5 kpb en aval du site de polyadénylation du gène murin (Yoo-Warren *et al.*, 1988). Plus récemment, le séquençage des 30 kpb situées en 3' du gène *H19* humain et la comparaison de cette séquence avec celle de son homologue murin a permis d'identifier dix séquences conservées (68 à 85 % d'identité). Sept d'entre elles, dont deux correspondent aux "enhancers" endodermiques décrits précédemment, se sont révélées être des séquences "enhancers" spécifiques de tissus d'origine ectodermique et/ou d'origine mésodermique lors de tests d'activité dans des souris transgéniques (Ishihara *et al.*, 2000). Grâce à des transfections transitoires d'un gène rapporteur sous la dépendance de différentes séquences de la région 3' du gène *H19* (+10 à +42 kpb) dans des cellules musculaires, les "enhancers" spécifiques des muscles squelettiques ont été localisés dans la région +22 à +28 kpb (Kaffer *et al.*, 2000). Bien que les "enhancers" contrôlant l'expression du gène *H19* dans de nombreux types cellulaires soient contenus dans les 35 kpb en 3' de ce dernier, il semblerait que les "enhancers" responsables de son expression dans les tissus d'origine mésodermique tels que le cœur, les poumons ou les reins se situent plus en aval (Ainscough *et al.*, 2000; Kaffer *et al.*, 2000).

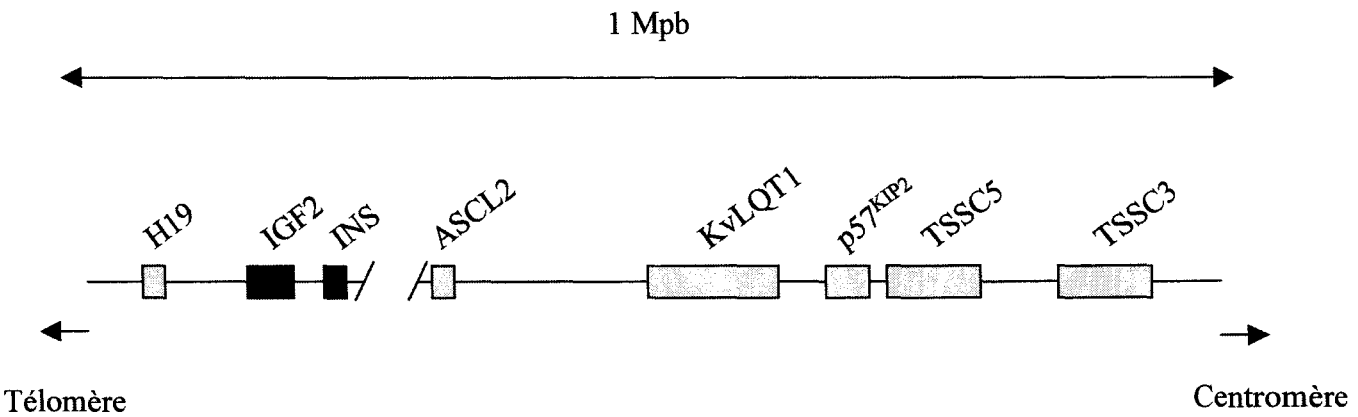
3) Empreinte génomique parentale du gène *H19*

Chez les Mammifères, le génome maternel et le génome paternel ne sont pas équivalents mais complémentaires, et par conséquent tous deux nécessaires au développement normal d'un embryon. En effet, des embryons ayant un génome uniquement maternel (gynogénotes) ou uniquement paternel (androgénotes) ne sont pas viables (Surani *et al.*, 1984). Cette non-équivalence fonctionnelle des deux génomes

parentaux est due à une empreinte génomique, dite parentale, mécanisme complexe conduisant à l'expression d'un seul allèle en fonction de l'origine parentale du gène. Elle est établie au cours de la gamétogenèse, transmise lors de la fécondation, maintenue dans toutes les cellules de l'embryon au cours du développement, et effacée pour laisser place à une nouvelle empreinte dans les cellules de la lignée germinale de la génération suivante. Le mécanisme de l'empreinte génomique repose sur des modifications épigénétiques différentielles permettant de distinguer les deux allèles parentaux, c'est à dire des modifications chromosomiques, héréditaires de génération cellulaire en génération cellulaire et réversibles, qui se surimpose à la séquence nucléotidique.

A l'heure actuelle, au moins 28 gènes ont été identifiés comme soumis à l'empreinte chez la souris (Beechey *et al.*, 1998) et la plupart le sont également chez l'homme (Reik *et al.*, 1998). Ces gènes ont la particularité d'être regroupés au niveau de larges domaines chromosomiques (Ainscough *et al.*, 1998; Feinberg, 1999) et cette régionalisation suggère que l'empreinte est régulée par des séquences influençant l'ensemble du domaine. Les domaines les plus étudiés sont le domaine humain localisé en 11p15 et son orthologue murin situé dans la partie distale du chromosome 7. Ce dernier, décrit figure 2, s'étend sur environ 1 Mpb et contient 8 gènes soumis à l'empreinte dont 6 sont exprimés maternellement et 2 paternellement (Feinberg, 1999 ; Onyango et al., 2000). Parmi ces gènes, nous nous intéresserons plus particulièrement au gène *H19*.

Chez l'humain, *H19* se situe à 200 kpb environ (90 kpb chez la souris) en 3' du gène *IGF2* qui code un facteur de croissance fœtal. Les deux gènes sont co-exprimés au cours du développement dans les tissus d'origine endodermique et d'origine mésodermique et subissent une empreinte génomique réciproque. Ainsi, seul l'allèle maternel du gène *H19* s'exprime (Bartolomei *et al.*, 1991 ; Zhang et Tycko, 1992), alors que seul l'allèle paternel d'*IGF2* est transcrit (DeChiara *et al.*, 1991, Giannoukakis *et al.*, 1993). Différentes



Gènes exprimés maternellement

Gènes exprimés paternellement

Figure 2: Représentation schématique du domaine 11p15 humain.
D'après Feinberg *et al.*, 1999 et Onyango *et al.*, 2000

expériences de délétions ont suggéré que l'expression et l'empreinte de ces deux gènes sont régulées par des régions communes. Une première délétion, qui a consisté à remplacer une séquence de 13 kpb, contenant le gène *H19* et sa région 5', par un gène de résistance à la néomycine a conduit à une perte de l'empreinte du gène *Igf2* lorsque cette délétion était transmise maternellement (Leighton *et al.*, 1995a). Une nouvelle délétion, consistant cette fois à remplacer une séquence de 3 kpb ne contenant que le gène *H19* et une petite partie de son promoteur, a eu pour conséquences une expression biallélique du gène *Igf2* et la mise en place d'une empreinte au niveau du gène de résistance à la néomycine remplaçant *H19* (Ripoche *et al.*, 1997). Cette seconde délétion, non seulement confirme l'implication du gène *H19* dans l'empreinte génomique d'*Igf2*, mais surtout suggère que la séquence de 10 kpb en amont du gène *H19*, seule différence entre les deux types de délétions, contient un élément contrôlant l'empreinte. Une dernière délétion en 3', concernant les "enhancers" endodermiques du gène *H19* entraînant une perte d'expression de *H19* mais également d'*Igf2*, a permis de montrer que les deux gènes partagent les mêmes "enhancers" dans les tissus d'origine endodermique (Leighton *et al.*, 1995b). Le partage des séquences "enhancers" du gène *H19* semble s'étendre aux tissus d'origine ectodermique et/ou d'origine mésodermique puisqu' *in vitro* les "enhancers" spécifiques de ces tissus ont des effets activateurs sur un gène rapporteur placé sous le contrôle des promoteurs P2 et P3 du gène *Igf2* (Ishihara *et al.*, 2000).

Le gène *H19*, son promoteur et les séquences situées en amont sont le siège de modifications épigénétiques différentielles qui comprennent la méthylation de l'ADN et une organisation particulière de la chromatine. La méthylation de l'ADN provient du transfert par l' ADN-méthyl transférase d'un groupement méthyl sur la cytidine au niveau de dinucléotides cytidine-guanine (CpG). L'importance de cette méthylation dans le mécanisme de l'empreinte génomique est fondée sur une étude de souris transgéniques

présentant un "knock-out" du gène de l'ADN-méthyl transférase, puisque des souris homozygotes pour cette mutation présentent une hypométhylation générale du génome, associée à une perte d'empreinte de plusieurs gènes dont *H19* et *Igf2* (Li *et al.*, 1993). L'étude de la méthylation du gène *H19* indique que l'allèle paternel (allèle silencieux) est hyperméthylé, alors que l'allèle maternel (allèle exprimé) est hypométhylé. Ces régions différentiellement méthylées (DMR) se répartissent à la fois dans les séquences en amont du gène, dans le promoteur et dans la partie 5'de la séquence transcrite (Bartolomei *et al.*, 1993 ; Ferguson-Smith *et al.*, 1993 ; Zhang *et al.*, 1993), mais n'ont pas le même statut. En effet, alors que la DMR située en amont du gène *H19* constitue une DMR primaire, c'est à dire établie dans la lignée germinale et maintenue au cours de l'implantation (Davis *et al.*, 2000 ; Ueda *et al.*, 2000), celle située dans le promoteur et dans la portion 5'du gène constitue une DMR secondaire, c'est à dire établie après la fécondation et/ou au moment de la phase de méthylation *de novo* qui a lieu au stade précoce de la post-implantation (Mann *et al.*, 2000). Néanmoins, la méthylation de *H19* au niveau de ses séquences transcrites est partielle. En effet, alors que le niveau de méthylation de *H19* est stable au cours du développement quel que soit le tissu considéré, celui des séquences internes du gène est plus variable selon les tissus et les stades du développement (Weber *et al.*, 2001).

La DMR primaire s'étend sur 2 kpb et est localisée dans la région comprise entre -2 et -4 kpb en amont du site d'initiation de la transcription (Tremblay *et al.*, 1997). Une délétion de 1,6 kpb de cette région conduit sur le chromosome maternel à une réactivation du gène *Igf2* et sur le chromosome paternel à une réactivation de *H19* (Thorvaldsen *et al.*, 1998). Ces résultats montrent le caractère primordial de cette région dans le mécanisme de l'empreinte, lui donnant ainsi le statut d'ICR (Imprinting Control Region). Lorsque l'ICR est insérée entre les "enhancers" endodermiques et mésodermiques, l'expression de *H19* dans le foie reste normale, mais son expression dans les muscles squelettiques est

considérablement réduite (Kaffer *et al.*, 2000). De ces expériences, les auteurs déduisent que l'ICR agit comme un isolateur, c'est à dire une séquence qui placée entre le promoteur du gène *Igf2* et ses séquences "enhancer" empêche leur interaction. Ces auteurs ont également démontré que cette région contient les informations suffisantes pour marquer son origine parentale et qu'elle isole promoteur et "enhancer" de façon non spécifique. En revanche, cette fonction d'isolateur est unidirectionnelle puisque la relocalisation des "enhancers" en amont de l'ICR n'empêche pas leur action sur le promoteur du gène *H19* (Webber *et al.*, 1998). Il a été également montré que l'ICR du chromosome maternel contient des sites d'hypersensibilité à la DNase I qui correspondent aux sites les plus méthylés de son homologue paternel (Hark et Tilghman, 1998 ; Khosla *et al.*, 1999). L'équipe de Feil démontre, de plus, que ces sites sont au cœur d'une organisation nucléosomale non canonique indiquant que cette région est la cible de protéines autres que les histones (Khosla *et al.*, 1999). Des expériences de "footprinting" *in vivo* ont permis de mettre en évidence quatre répétitions de 21 pb (Szabo et al., 2000), séquences qui ne fixent la protéine CTCF (CCCTC-binding factor) dans des expériences de retard sur gel que si elles ne sont pas méthylées (Bell et Felsenfeld, 2000; Hark *et al.*, 2000). Cette protéine en doigt de zinc, capable d'activer ou réprimer la transcription, est connue pour son implication dans le fonctionnement plus général d'isolateur de vertébrés (Bell et Felsenfeld, 1999). Des délétions ont permis de préciser le rôle de l'ICR en montrant qu'outre sa fonction d'isolateur vis-à vis du gène *Igf2*, l'ICR est également répresseur de la transcription de *H19* (Drewell *et al.*, 2000 ; Srivastava *et al.*, 2000). La délétion de l'ICR dans les cellules germinales ou dans le zygote conduit à une activation de l'allèle paternel de *H19* associée à une perte des méthylations des DMR secondaires, alors que la même délétion au moment de la différenciation des cellules musculaires est sans effet sur cet allèle (Srivastava *et al.*, 2000). Les auteurs proposent donc que l'ICR réprime l'expression

du gène *H19* sur l'allèle paternel en permettant la méthylation des DMR secondaires de celui-ci. Néanmoins, la méthylation ne semble pas être la seule modification épigénétique responsable de la répression de l'allèle paternel de *H19*, puisque la délétion de 1,2 kpb de l'ICR provoque la réactivation de *H19*, sans perte de la méthylation des DMR secondaires (Drewell *et al.*, 2000). Plus précisément, il a été montré que le promoteur de l'allèle paternel silencieux de *H19* est méthylé, mais aussi hypoacétylé, alors que l'allèle maternel actif est hypométhylé et hyperacétylé. En conséquence, une combinaison d'inhibiteurs de la méthylation et de la désacétylation est nécessaire à la réactivation de *H19* (Pedone et al., 1999 ; Grandjean et al., 2001).

Finalement, le modèle de compétition vis-à-vis des "enhancers", qui fut le premier mécanisme proposé pour expliquer l'empreinte réciproque des gènes *H19* et *IGF2* (Leighton *et al.*, 1996) laisse place à un nouveau modèle (figure 3), fondé sur la double fonction de l'ICR : isolateur pour le gène *IGF2*, répresseur pour le gène *H19*. Ainsi, sur le chromosome maternel, quatre protéines CTCF se fixent sur l'ICR non méthylée et isolent les promoteurs d'*IGF2* des "enhancers" situés en 3' du gène *H19* ; les "enhancers" activent alors la transcription de *H19*. Sur le chromosome paternel, la méthylation de l'ICR inhibe la fixation des protéines CTCF et en empêche donc la fonction d'isolateur, laissant ainsi les promoteurs d'*IGF2* accessibles aux "enhancers". L'inactivation de l'isolateur, associée à la méthylation et la structure chromatinienne répressive du promoteur du gène *H19*, permet aux "enhancers" d'activer la transcription d'*IGF2* (Bell et Felsenfeld, 2000 ; Hark et Tilghman, 2000 ; Reik et Murrell, 2000).

Chromosome 7 murin

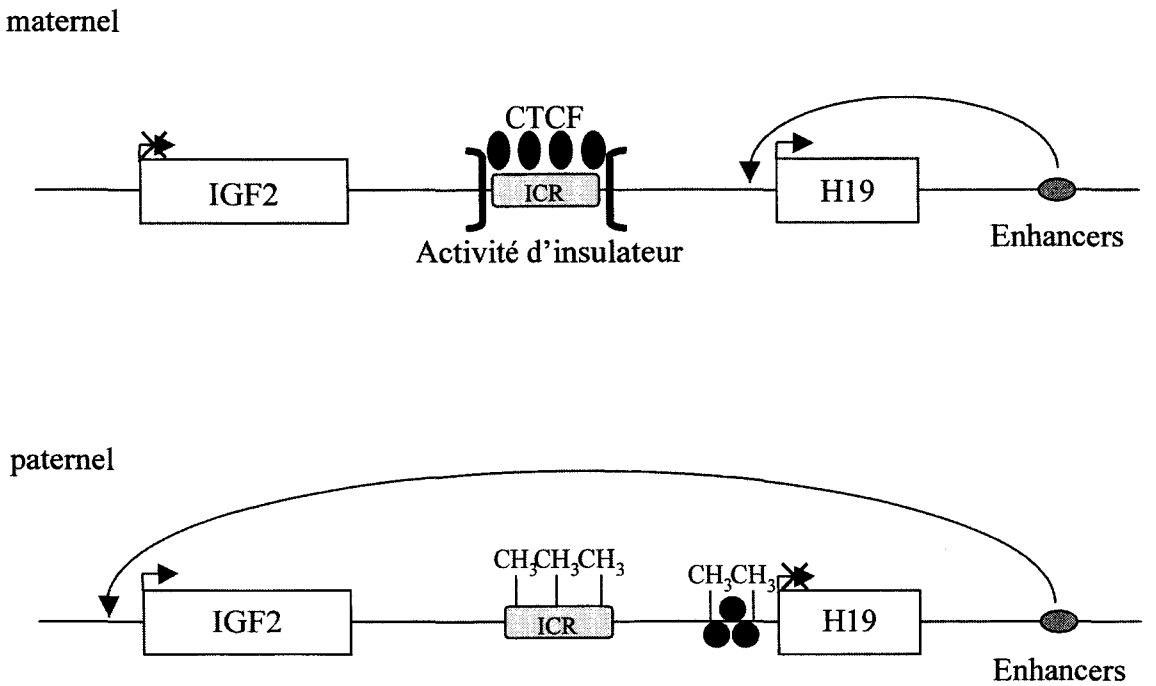


Figure3: Représentation schématique du mécanisme de régulation de l'empreinte génomique parentale réciproque des gènes *H19* et *IGF2* (d'après Mann, 2001). Sur le chromosome maternel, l'ICR (Imprinting Control Region) qui est non méthylée, fixe quatre protéines CTCF. Ce complexe fonctionne alors comme un isolateur empêchant l'interaction entre le promoteur d'*IGF2* et les enhancers. Les enhancers activent ainsi le promoteur *H19* sur cet allèle.
Sur le chromosome paternel, l'ICR méthylée ne fixe plus les protéines CTCF et ne fonctionne donc plus comme un isolateur. Les enhancers peuvent alors interagir avec le promoteur d'*IGF2* et l'activer. De plus, la méthylation et la conformation chromatinienne répressive du promoteur du gène *H19*, sur cet allèle, empêche sa transcription.

4) Expression et régulation du gène *H19*

Le gène *H19*, comme la plupart des gènes soumis à l'empreinte génomique parentale, est fortement exprimé au cours de l'embryogenèse dans les tissus extra-embryonnaires et dans la majorité des tissus fœtaux, en particulier dans les tissus d'origine endodermique et d'origine mésodermique (Poirier *et al.*, 1991; Lustig *et al.*, 1994), mais également dans les tissus d'origine ectodermique comme le système nerveux central ou la glande mammaire (Hemberger *et al.*, 1998; Adriaenssens *et al.*, 1999). A la naissance, il subit une répression générale qui restreint son expression à quelques tissus chez l'adulte. C'est alors dans les muscles squelettiques que son expression reste la plus élevée, bien que largement plus faible que dans les mêmes tissus fœtaux (Pachnis *et al.*, 1984 ; Leibovitch *et al.*, 1991 ; Han et Liau, 1992 ; Douc-Rasy *et al.* 1993 ; Milligan *et al.*, 2000). Une expression basale, mais significative, est également détectée dans les poumons, le cœur et le thymus (Poirier *et al.*, 1991), dans la glande mammaire (Douc-Rasy *et al.*, 1993 ; Dugimont *et al.*, 1995 ; Adriaenssens *et al.*, 1999), dans les glandes surrénales (Liu *et al.*, 1995) et dans l'utérus (Ariel *et al.*,1997).

Non ou modérément transcrit chez l'adulte, le gène *H19* est toutefois ré-exprimé ou surexprimé dans un grand nombre de cancers, tels que les cancers du sein (Douc-Rasy *et al.*, 1993 ; Dugimont *et al.*, 1995 ; Adriaenssens *et al.*, 1998), de la vessie (Ariel *et al.*, 1995 ; Elkin *et al.*, 1995), du poumon (Kondo *et al.*, 1995), des glandes surrénales (Liu *et al.*, 1995), du col de l'utérus (Douc-Rasy *et al.*, 1996), de l'œsophage et les tumeurs colorectales (Hibi *et al.*,1996), les gliomes (Uyeno *et al.*, 1996), les cancers du foie (Ariel *et al.*, 1998), de l'ovaire (Kim *et al* ., 1998 ; Tanos *et al.*, 1999 ; Chen *et al.*, 2000), de l'ensemble tête-cou (El-Naggar *et al.*, 1999) et dans les léiomyomes utérins (Rainho *et al.*, 1999). Dans un grand nombre de ces tumeurs (vessie, poumons, col de l'utérus, œsophage, ovaire et tête-cou) la surexpression de *H19* est associée à une perte de l'empreinte

génomique, néanmoins il a été montré qu'une empreinte génomique normale est maintenue dans les tumeurs mammaires (Yballe *et al.*, 1996 ; Van Roozendaal *et al.*, 1998), les tumeurs colorectales (Hibi *et al.*,1996), les gliomes (Uyeno *et al.*, 1996) et les léiomyomes utérins (Rainho *et al.*, 1999).

Peu d'équipes se sont intéressées à l'étude de la régulation du gène *H19* par des hormones, des facteurs de croissance ou autres cytokines. Dans des cellules fœtales de glandes surrénales, Voutilainen et ses collaborateurs ont montré que le gène *H19* est activé par l'IGFI et l'IGFII et réprimé par le TGFβ1, le TNFα et l'IFNγ; ces auteurs suggèrent également que ces modulations dépendraient d'une balance entre les voies de la protéine kinase A (PKA) et celles de la protéine kinase C (PKC), puisque des activateurs de la PKA augmentent l'expression du gène, alors que des activateurs de la PKC l'inhibent (Voutilainen *et al.*, 1994 ; Liu *et al.*, 1995, 1997). Chez le lapin, le gène *F31*, homologue du gène *H19*, est activé quand des cellules musculaires lisses fœtales prolifèrent, réprimé lors de la confluence des cellules, enfin réactivé par l'ajout de sérum ou d'insuline dans le milieu de culture. Dans les mêmes cellules, le gène *F31* est positivement régulé par les facteurs de croissance IGFI et IGFII, alors que l'EGF ou le FGF1 ont peu d'effets (Han et Liau, 1992 ; Han *et al.*,1996). Plus récemment, il a été démontré dans des cellules issues de tumeurs testiculaires germinales humaines que le gène *H19* est régulé positivement par l'acide rétinoïque et ces travaux suggèrent que cette activation, qui nécessite à la fois le promoteur et les séquences en 3' du gène, s'effectue *via* le facteur de transcription AP-2 (Kopf *et al.*, 1998). Dans des cellules de carcinomes de vessie, cette même équipe a constaté que contrairement aux cellules n'exprimant pas *H19*, les cellules qui le surexpriment cessent de proliférer lorsqu'elles sont cultivées dans un milieu appauvri en sérum (Ohana *et al.*, 1999). Dans notre groupe, les modulations d'expression du gène *H19* ont été étudiées au cours du développement de la glande mammaire de souris et nous avons

observé deux périodes de forte expression du gène: l'une lors de la puberté, l'autre au cours de la gestation. Il s'agit en fait de deux phases au cours desquelles le développement est particulièrement dépendant d'hormones stéroïdes. Nous avons confirmé cette régulation du gène *H19* par les hormones stéroïdes dans des cellules mammaires cancéreuses. Dans ces cellules, le 17-β-oestradiol augmente l'expression de *H19*, stimulation qui est inhibée par la progestérone ou par le tamoxifène, un antagoniste des œstrogènes (Adriaenssens *et al.*, 1999).

De l'ensemble de ces études, il ressort que selon les modèles *H19* peut être activé soit par des agents induisant une prolifération, soit par des agents responsables d'une différenciation. Enfin, cette régulation complexe est éventuellement liée à son statut de gènes soumis à l'empreinte, pour lesquels plusieurs travaux indiquent que leur expression est particulièrement sensible aux conditions de culture. En effet, dans des fibroblastes embryonnaires, une étude concernant huit gènes soumis à l'empreinte a montré que tous sont régulés positivement par un arrêt de croissance, alors que seul un tiers de gènes non-imprimés pris au hasard sont activé dans les mêmes conditions (Hayashida *et al.*, 1997). De même, la simple culture de blastocystes de souris ou de cellules souches embryonnaires (ES), soit dans un milieu de culture particulier (Doherty *et al.*, 2000), soit en présence de sérum (Dean *et al.*, 1998 ; Kohsla *et al.*, 2001), est capable de déréguler l'expression du gène *H19* en induisant des changements de méthylation de ses séquences situées en 5'. De plus, ces méthylations aberrantes persistent après l'implantation et conduisent à un développement anormal des fœtus.

Enfin, des études récentes précisent que la régulation du gène *H19* s'effectue aussi bien au niveau transcriptionnel qu'au niveau post-transcriptionnel. Un premier travail a suggéré une régulation post-transcriptionnelle du gène qui contribuerait au maintien de son expression monoallélique (Jouvenot *et al.*, 1999). En utilisant la technique du RNA FISH

(Fluorescent *In Situ* Hybridization), qui permet de détecter des foyers de transcription au niveau des chromosomes, les auteurs ont constaté que dans 17% des cellules de foie fœtal, les deux allèles sont transcrits. Alors que dans le cytoplasme seul le transcrit d'origine maternelle est détecté, les transcrits paternels, supposés très instables, sont dégradés avant d'atteindre le cytoplasme. Un second travail consacré à la régulation post-transcriptionnelle du gène *H19* a consisté à comparer l'activité transcriptionnelle du gène avec le taux d'ARN exprimé au cours de la différenciation myogénique de cellules C2C12. Lorsque ces cellules sont induites à se différencier, le taux d'ARN *H19* est fortement augmenté ; cependant, dans le même temps, le taux de transcription reste inchangé. Les auteurs de cette étude montrent que l'augmentation du taux de transcrits *H19* au cours de la différenciation des cellules musculaires est uniquement due à leur stabilisation qui dépend de la synthèse protéique, et suggèrent que cette stabilisation nécessite l'induction de la traduction d'une protéine stabilisante ayant une courte durée de vie (Milligan *et al.*, 2000).

5) Le produit fonctionnel du gène *H19* est un ARN non traductible

Le gène *H19* est transcrit par l'ARN polymérase II, et présente toutes les caractéristiques usuelles d'un ARN messager (mise en place d'une coiffe, épissage et polyadénylation, transport vers le cytoplasme), néanmoins il est maintenant bien admis que cet ARN n'est pas traductible *in vivo*. C'est l'équipe de Tilghman, découvreuse du gène *H19* chez la souris, qui après avoir caractérisé le gène *H19* humain et comparé sa séquence avec son homologue murin, propose que le produit fonctionnel de ce gène est un ARN régulateur qu'elle nomme riborégulateur (Brannan *et al.*, 1990). Les homologues murin et humain présentent chacun de multiples phases de lecture ouverte (ORF) dont la plus grande coderait une protéine putative de 26 kDa chez l'homme et de 14 kDa chez la souris.

Cependant, l'absence de conservation des ORF, l'absence d'association entre les ARN *H19* et la machinerie de traduction et l'absence de conservation des séquences protéiques putatives sont autant d'arguments en faveur d'une non-traduction des transcrits *H19*. Chez la souris, la production d'une protéine par traduction en milieu acellulaire n'est possible que si la région 5' non-traduite (UTR 5') est entièrement délétée (Pachnis *et al.*, 1988). Joubel et ses collaborateurs ont transfecté, dans des cellules de rein de singe dépourvues de gène *H19*, différents ADNc de *H19* contenant des délétions partielles plus ou moins conséquentes et/ou des mutations ponctuelles de l'UTR 5' et ont alors rendu traductibles ces différents construits. Après ces analyses en condition transitoire, les auteurs ont ainsi confirmé que l'UTR 5' empêche la synthèse d'une protéine H19 et que cette inhibition est essentiellement due à l'ORF juste en amont de l'ORF traduite. La possibilité d'une régulation de la traduction par de petites ORF de l'UTR 5' a déjà été décrite pour plusieurs protéines, parmi lesquelles le facteur de transcription GCN4 de levure (Hinnebush, 1990 ; Abastado *et al.*, 1991), les facteurs de croissance TGF β1 (Romeo *et al.*, 1993) et TGF β3 (Arrick et al., 1991) ou encore le récepteur à l'acide rétinoïque RAR β2 chez les Mammifères (Zimmer *et al.*, 1994), néanmoins dans tous ces exemples la protéine codée par chacun de ces gènes est à un moment ou un autre détectable *in vivo*, et ne subit pas une absence absolue de traduction comme pour *H19*.

Récemment, une équipe a cloné et séquencé le gène *H19* du chat, du lynx, de l'éléphant, du spermophile et de l'orang-outang. En menant une étude phylogénétique sur ces cinq nouvelles séquences et sur les quatre séquences déjà connues de l'humain, de la souris, du rat et du lapin, les auteurs confirment qu'aucune ORF n'est conservée, mais ils rapportent essentiellement que la structure secondaire, composée de 16 appariements de bases en épingle à cheveux, est parfaitement conservée (Juan *et al.*, 2000). Même si, contrairement à ce qui a été rapporté par l'équipe de Tilghman (Brannan *et al.*, 1990), on

sait aujourd'hui que le transcrit du gène *H19* peut s'associer à des polysomes dans de nombreux types cellulaires humains ou murins (Li *et al.*, 1998 ; Milligan *et al.*, 2000), cela ne remet pas en cause l'absence d'un produit protéique H19 *in vivo*.

Finalement, le produit fonctionnel du gène *H19* est un ARN mature, dont la fonction de riborégulateur requiert probablement la capacité d'adopter une structure secondaire bien définie et de s'associer à la machinerie de traduction de la cellule.

6) Rôle du gène *H19*

Dix-sept ans après sa découverte et en dépit de nombreux travaux publiés par différentes équipes, le rôle du gène *H19* reste encore largement méconnu. Les gènes *Igf2* et *H19* sont les premiers gènes imprimés découverts. Ainsi, la majorité des travaux concernant *H19* sont consacrés à la compréhension des mécanismes moléculaires qui régissent l'empreinte génomique des *loci H19* et *Igf2* et la contribution du gène *H19* dans cette régulation. Récemment, il a été démontré que l'ARN *H19* n'était pas nécessaire au maintien de l'empreinte d'*Igf2* (Jones *et al.*, 1998), néanmoins il semble que le gène *H19* régule en *trans* le taux d'ARNm *IGF2* (Wilkin *et al.*, 2000), ainsi que sa traductibilité (Li *et al.*, 1998).

Par ailleurs, le patron d'expression du gène *H19*, à savoir sa forte expression chez le fœtus, sa répression chez l'adulte et sa ré-expression dans des cancers, suggère qu'il est impliqué dans les processus de cancérogenèse. En 1991, Wiseman et ses collaborateurs travaillent sur des cellules SHE (Syrian Hamster Embryo cells) pré-néoplasiques et isolent deux clones de ces cellules qui diffèrent par leur capacité à supprimer la tumorigénicité de cellules SHE transformées lors de la formation d'hybrides somatiques. Afin d'identifier les gènes impliqués dans cet effet suppresseur de tumeurs, les auteurs ont réalisé un criblage

différentiel de banques d'ADNc issues de ces deux clones. Parmi les gènes surexprimés dans les cellules capables de supprimer la tumorigénicité, se trouve le gène *H19* (Wiseman *et al.*,1991). Cette hypothèse conférant au gène *H19* une fonction de suppresseur de tumeurs a été largement renforcée par les expériences de Hao et de ses collaborateurs, qui ont démontré que des cellules G401 (cellules issues d'une tumeur de Wilms) transfectées avec le gène *H19* manifestent un retard de croissance, une perte de capacité à croître en absence d'ancrage et une perte de tumorigénicité dans des souris *nude*. Cependant, il faut noter que lors de cette étude deux autres lignées ont également été transfectées ; l'une d'elle n'a montré qu'un retard de croissance, alors que l'autre n'a manifesté aucune correction du phénotype néoplasique (Hao *et al.*, 1993). Bien que la transfection de cette même construction dans des cellules SHE corrobore le rôle de suppresseur de tumeurs attribué à *H19* (Isfort *et al.*, 1997), d'autres travaux, au contraire, le réfutent. En effet, des souris transgéniques présentant une délétion du gène *H19* par recombinaison homologue (Leighton *et al.*, 1995a ; Ripoche *et al.*, 1997) ne développent pas de tumeurs. Le seul phénotype associé à cette délétion fut une augmentation de leur poids par rapport aux souriceaux normaux de la même portée. Par ailleurs, nous avons déjà rapporté que le gène *H19* est ré-exprimé ou sur-exprimé dans un grand nombre de cancers, mais plus particulièrement, dans les cancers de la vessie, son expression est associée à la progression tumorale et à la récurrence précoce de ces tumeurs (Ariel *et al.*, 1995 ; Cooper *et al.*, 1996 ; Ariel *et al.*, 2000). Dans les cancers urothéliaux, le taux d'expression du gène *H19* accompagne les capacités invasives des cellules (Biran *et al.*, 1994). De même, dans des tumeurs induites par l'injection dans des souris *nude* de cellules issues de cancer de vessie, *H19* est fortement exprimé alors que ces cellules ne l'exprimaient que très faiblement avant l'injection (Elkin *et al.*, 1995). Des résultats similaires ont été obtenus avec des cellules de choriocarcinome (JEG-3), mais contrairement aux cellules précédentes, les

JEG-3 issues des tumeurs induites conservent une forte expression de *H19*, même au-delà de 15 passages après leur remise en culture. De plus, ces cellules dont la croissance en présence et en absence d'ancrage n'est pas altérée par la surexpression du gène *H19*, sont devenues plus tumorigènes que les cellules originelles (Rachmilewitz *et al.*, 1995 ; Lustig-Yariv *et al.*, 1997). Des propriétés oncogéniques furent également attribuées au gène *H19* par Tsujimoto et ses collaborateurs après qu'ils aient constaté que ce gène est exprimé dans les hybrides de cellules HeLa et de fibroblastes de peau, seulement lorsque ces hybrides deviennent spontanément tumorigènes (Tsujimoto *et al.*, 1999). Cependant, la seule transfection des hybrides non-tumorigènes avec le gène *H19* ne confère pas de phénotype tumorigène à ces hybrides. Ces résultats suggèrent que *H19* est nécessaire lors de processus oncogéniques, mais n'est pas suffisant pour induire une tumorigenèse (Tsujimoto *et al.*, 2001).

Objectifs de notre recherche

Le groupe "H19" au sein duquel j'ai effectué mon stage doctoral s'intéresse depuis une dizaine d'années aux modulations de l'expression du gène *H19* dans des tissus normaux, tels que la glande mammaire en développement ou l'utérus soumis au cycle oestrien, mais également lors de situations pathologiques dans des tumeurs bénignes ou malignes du sein, de l'utérus ou de la prostate. Lors de mon intégration dans le groupe "H19", il m'a été confié, comme objectif majeur, de découvrir une ou plusieurs fonctions de ce gène, mais l'intérêt des modulations de l'expression du gène en situation normale ou pathologique n'a pas été abandonné pour autant. Lors de mon arrivée, des travaux consacrés à l'étude de l'expression du gène *H19* au cours du développement de la glande mammaire et dans des carcinomes primaires du sein étaient en cours. Je me suis donc immédiatement impliquée dans ces recherches au cours desquelles nous avons constaté, entre autres, une accumulation préférentielle des transcrits *H19* aux interfaces épithélium/mésenchyme, soit au niveau des deux types de tissus, soit seulement au niveau de l'un d'eux. Ces fréquentes observations suggèrent une implication de l'expression de *H19* dans les interactions épithélium/mésenchyme; ce gène étant éventuellement régulé par des facteurs paracrines libérés par l'un des types cellulaires et agissant sur l'autre. Ces observations, qui nous semblaient importantes, nous ont conduits à reproduire ces interactions en culture cellulaire et

à déterminer le ou les facteurs libérés par les cellules mésenchymateuses pouvant être responsables d'une activation du gène *H19* dans les cellules épithéliales. Le choix du sens mésenchyme vers épithélium a d'abord été retenu, car nous étions préoccupés par le rôle de *H19* dans les cellules cancéreuses et non par son rôle dans le stroma.

Ainsi, la première partie de mon travail de thèse comprend une étude de l'expression du gène *H19* de différentes lignées de cellules épithéliales de sein placées dans différentes conditions de culture. Ces différentes conditions comprennent la teneur en sérum de veau fœtal, la densité des cellules, leur capacité de croissance sur fond de boîte ou entre deux couches de collagène, l'influence de facteurs de croissance, parmi lesquels " l'Hepatocyte Growth Factor/Scatter Factor "(HGF/SF) bien connu pour être l'une des principales molécules libérées par les fibroblastes et capable d'avoir des effets mitogène, morphogène et/ou motogène sur les cellules épithéliales.

La deuxième partie de mon travail concerne la découverte des fonctions du gène *H19*. En effet, malgré de nombreux travaux menés par des équipes très actives de par le monde, le rôle du gène restait (et reste encore malgré des avancées significatives) mystérieux. La controverse se manifestait de façon la plus aiguë à propos de l'attribution d'une fonction oncogénique ou anti-oncogénique au gène *H19*, les conclusions des publications aboutissant aux deux statuts selon les modèles envisagés. Dès lors la stratégie envisagée par notre équipe était d'observer des phénotypes de cellules surexprimant le gène. L'ensemble des résultats obtenus concernant les modulations de l'expression du gène *H19* dans différentes lignées de cellules de cancer du sein, nous a convaincu de poursuivre notre étude sur l'une de ces lignées. Les cellules choisies furent les cellules malignes, MDA-MB-231, car ces cellules invasives et hormono-indépendantes avaient été précédemment analysées et jugées intéressantes pour leur faible taux d'expression de l'endogène. La question d'une surexpression d'un gène ectopique pouvait donc être aisément envisagée en transfectant

stablement une séquence génomique de *H19* dans ces cellules. Le choix de ce type de séquence (génomique *versus* ADNc) se justifie car il permet aux cellules de transcrire le ou les gène(s) étranger(s) comme leur propre gène et de maturer les transcrits. C'est ainsi que nous avons analysé les clones sélectionnés et les témoins en termes de croissance, de sensibilité à divers facteurs de croissance, de clonogénicité sur fond de boîte ou en absence d'ancrage (agar mou) et enfin dans des souris immunodéficientes (*scid*). Ces différentes analyses devaient nous permettre de proposer un rôle à *H19* en lui conférant une fonction soit d'oncogène, soit de suppresseur de tumeurs dans notre modèle.

Enfin, parmi les particularités peu ordinaires de notre gène d'intérêt, celle d'être transcrit en un ARN qui a toutes les qualités d'un messager, mais qui ne peut être traduit sans modifications majeures de l'UTR 5' a évidemment retenu notre attention. C'est pourquoi le rôle de riborégulateur, défini dans mon introduction, a été le centre d'intérêt de la troisième partie de ce mémoire. C'est ainsi que nous avons voulu mettre à profit la surexpression du transgène par nos clones, pour rechercher, grâce à une analyse protéomique, d'éventuelles protéines régulées par le gène *H19*.

Résultats

Chapitre I

Implication du gène H19 dans les interactions épithélium/mésenchyme

Lors de précédents travaux, nous avons démontré que l'expression du gène *H19* varie au cours du développement normal de la glande mammaire de souris et que ces variations concernent essentiellement les cellules épithéliales; le taux d'ARN *H19* restant relativement constant dans les cellules mésenchymateuses (Adriaenssens *et al.*, 1999). En effet, on constate un premier pic d'expression, au moment de la puberté, dans les tubules en croissance et plus particulièrement au niveau des bourgeons terminaux qui se ramifient et pénètrent activement le mésenchyme adjacent. Un second pic d'expression est observé au cours de la gestation, lorsque les bourgeons terminaux se différencient en *acini*. Ainsi, dans la glande mammaire en développement l'expression du gène *H19* est la plus élevée aux moments où les interactions épithélium/mésenchyme sont prépondérantes. Par ailleurs, dans les cancers du sein, nous avons observé une surexpression du gène *H19* dans plus de 70% des cas étudiés. Cette surexpression se localise soit dans les cellules stromales, soit dans les cellules épithéliales, soit simultanément dans les deux types de cellules. De plus, de la même manière que dans la glande mammaire normale nous avons remarqué une accumulation préférentielle des transcrits à l'interface épithélium/stroma (Adriaenssens *et al.*, 1998). L'ensemble de ces résultats suggère une implication du gène *H19* dans les interactions épithélium/mésenchyme, qui nous a incité à mimer ces interactions dans

différentes conditions de culture de cellules et à déterminer des facteurs responsables de l'activation du gène *H19*.

Article 1

Nous avons d'abord montré, par RT-PCR quantitative en temps réel, que l'expression du gène *H19* varie selon les lignées cellulaires étudiées; *H19* étant plus fortement exprimé dans la lignée MCF-7 (cellules mammaires cancéreuses ayant conservées des caractéristiques de cellules différenciées) que dans la lignée HBL-100 (cellules mammaires normales immortalisées par le virus SV40). Par ailleurs, quelle que soit la lignée, la densité cellulaire n'a que très peu d'influence sur cette expression. Enfin, l'expression du gène augmente graduellement avec le pourcentage de sérum dans la lignée MCF-7 alors qu'elle reste inchangée dans les cellules HBL-100.

Afin de placer les cellules dans une situation proche de celle qui existe *in vivo*, nous avons cultivé les cellules dans des conditions dites en "3 dimensions" (3D), c'est à dire entre deux couches de collagène. Par hybridation moléculaire *in situ* (HIS) et RT-PCR quantitative en temps réel, nous avons observé que le simple passage d'une culture en 2 dimensions (2D) sur fond de boîte à une culture en 3D active le gène *H19*, quelle que soit la lignée étudiée. Nous avons alors testé l'influence de cellules du mésenchyme sur l'expression du gène *H19*, en traitant les cellules avec du milieu conditionné par des fibroblastes humains normaux issus de poumons embryonnaires (lignée MRC-5), milieu connu pour induire une morphogenèse ou un essaimage des cellules épithéliales mammaires cultivées en 3D (Montesano *et al.*, 1991a ; Fauquette *et al.*, 1997). En présence de milieu conditionné, les cellules HBL-100 s'organisent en pseudo-tubules et les cellules

MDA-MB-231 (cellules mammaires cancéreuses invasives) se dispersent dans un gel de collagène. En revanche, les cellules MCF-7 restent insensibles à ce milieu. Parallèlement, l'expression du gène *H19* augmente dans les HBL-100 et les MDA-MB-231, alors qu'elle reste inchangée dans les MCF-7. Ces résultats indiquent que la migration, induite par le milieu conditionné, s'accompagne d'une surexpression du gène *H19*. Nous avons alors cherché à approfondir cette étude en déterminant quels facteurs contenus dans le milieu conditionnés pouvaient être responsables de cette activation.

L'Hepatocyte Growth Factor/Scatter Factor (HGF/SF) est un facteur de croissance mitogène, motogène et morphogène, synthétisé et sécrété par les cellules mésenchymateuses. L'HGF/SF a fait l'objet de nombreuses études et a été reconnu comme étant un acteur majeur des interactions épithélium/mésenchyme (Stoker *et al.*, 1987 ; Sonnenberg *et al.*, 1993). Nous avons donc évalué les effets de ce facteur sur les différentes lignées cultivées en 3D. Comme le milieu conditionné, l'HGF/SF provoque une tubulogenèse des HBL-100 et un essaimage des MDA-MB-231, effets respectivement accompagnés d'une surexpression du gène *H19*. En traitant les cellules avec du milieu conditionné, soit en présence d'un anticorps neutralisant anti-HGF/SF, soit en présence d'un anticorps non-relevant, nous avons confirmé que les effets du milieu conditionné implique l'action de l'HGF/SF. Les expériences mettant en œuvre l'HGF/SF et les cellules MCF-7 confirment les résultats obtenus avec le milieu conditionné à savoir les absences de déplacement et de surexpression du gène *H19*.

Enfin, grâce à des transfections transitoires du gène rapporteur de la *luciférase* sous le contrôle du promoteur du gène *H19*, nous avons montré que l'HGF/SF est capable d'activer le promoteur de *H19* et que cette activation dépend de la voie de transduction Ras-MAP kinases et de la phospholipase C-γ1. Ces voies de transduction sont connues pour être activées lors de processus de dispersion ou de tubulogenèse induits par l'HGF/SF

(Furge *et al*, 2000 ; Gual *et al.*, 2000). En parallèle, nous avons testé les effets d'autres facteurs de croissance et nous avons montré que le gène *H19* est également activé par l'EGF et le FGF-2, alors que l'IGF II, le TNF-α et le TGF-β sont sans effet.

De l'ensemble de ces résultats, nous pouvons conclure que des facteurs paracrines produits par des cellules mésenchymateuses activent le gène *H19* de cellules épithéliales, et que cette activation accompagne un phénotype de migration de ces cellules. Parmi ces facteurs, l'action de l'HGF/SF paraît prépondérante, néanmoins d'autres facteurs y participent très certainement. Il semble donc que le gène *H19* soit impliqué dans les interactions épithélio-mésenchymateuses conduisant soit à la formation des tubules au cours du développement normal de la glande mammaire, soit à l'envahissement du stroma par la tumeur lors de processus néoplasiques.

Article 1

Cross-talk between mesenchyme and epithelium results in an increase of H19 gene expression during scattering and morphogenesis of epithelial cells.

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Running title : Induction of *H19* gene expression by HGF/SF.

Keywords : *H19* gene; epithelium-mesenchyme interactions; scattering, morphogenesis, extra-cellular matrix; HGF/SF.

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SUMMARY

The *H19* gene is an imprinted gene that is expressed from the maternal allele and is known to function as an RNA molecule. We previously reported that in breast adenocarcinoma, *H19* is often overexpressed in stromal cells and preferentially located at the epithelium/stroma boundary, suggesting that epithelial/mesenchymal interactions can control *H19* RNA expression. In some cases of breast adenocarcinoma with poor prognosis value, *H19* is overexpressed in epithelial cells. So, we have now examined whether mesenchymal factors can induce *H19* expression in epithelial cells. Using quantitative RT-PCR and *in situ* hybridization, we found that culture of mammary epithelial cells in collagen gels strongly up-regulates *H19* expression compared to culture on plastic. These conditions of culture in collagen gels allow three-dimensional growth of epithelial cells and morphogenetic responses to soluble factors. Conditioned medium from MRC5 fibroblasts caused branching morphogenesis in HBL-100 cells, invasive growth of MDA-MB-231 cells, whereas MCF7 cells were unresponsive. Induction of *H19* expression is correlated with the morphological changes in HBL-100 and in MDA-MB-231 cells, whereas *H19* expression was not induced in MCF7 cells. Using a blocking antibody, HGF/SF was identified as the fibroblast derived-growth factor capable of inducing *H19* expression and cell morphogenesis. We further demonstrate that promoter activity of *H19* was stimulated by various growth factors using transient transfections in MDCK epithelial cells. HGF/SF was more efficient than EGF or FGF-2 to transactivate the *H19* promoter, whereas IGF-II, TGFβ1 and TNFα were ineffective. This activation by HGF/SF was prevented by pharmacological inhibition of MEK or of phospholipase C. Here we find that *H19* is a target gene of HGF/SF, a known regulator of epithelial/mesenchymal interactions, and data suggest that the up-regulation of *H19* could match morphogenesis and/or migration of epithelial cells.

INTRODUCTION

In mammary gland, formation of epithelial structures during embryonic or post-natal development involves sequential morphogenetic processes that finally result in elaboration of organized multicellular structures. Particularly, proper epithelial morphogenesis requires interactions with proximal mesenchymal cells [1]. These interactions are mediated both by extracellular matrix (ECM) and molecules synthesized by mesenchymal cells. Indeed, this was well demonstrated using three-dimensional culture that promotes the organization of epithelial cells in structures similar to those existing *in vivo*. For example, a three dimensional culture of thyroid follicular cells in collagen gels can lead to the formation of follicles [2]. Furthermore, addition of fibroblast-conditioned medium allows the formation of tubule-like structures by normal mammary epithelial cells cultured in three-dimension condition [3, 4]. The hepatocyte growth factor/scatter factor (HGF/SF) has been identified as one of the main paracrine mediators of the morphogenetic effects of epithelial-mesenchymal interactions [5]. This growth factor stimulates the proliferation of a large panel of epithelial cells and also increases their motility and morphogenesis [6]. HGF/SF provokes scattering of epithelial cells cultured on plastic dishes (2D-condition) and morphogenesis when these cells are embedded within a collagen gel (3D-condition) [7]. During this process, HGF/SF and other factors responsible for epithelial morphogenesis modulate numerous genes like those coding for the *urokinase-type plasminogen activator (uPA)* and its receptor *(uPAR)*. Their up-regulation by HGF/SF or fibroblast-conditioned medium allows proteolytic activity required for cell migration [8]. Finally, the knowledge of genes involved in and/or responsible for the molecular nature of signals given by the mesenchyme and received by epithelium , which responds by morphogenesis, differentiation and growth during organ development is of major importance in tumor progression.

The human *H19* gene maps to chromosome 11 p15.5 and lies within 200 kbp downstream of *IGF-2* gene; these two genes have been shown to be imprinted, leading to selective expression of the paternal *IGF-2* and the maternal *H19* alleles [9, 10]. Shared enhancers 3' to *H19* are necessary for co-ordinate regulated transcription of the two genes [11] and in cells of endodermal lineage, the reciprocal imprinting of the two genes has been rationalized in terms of an enhancer competition model [12]. However, strict enhancer competition is not sufficient, since an intergenic differentially methylated domain in the *H19* upstream region is crucial in achieving the proper monoallelic usage [13, 14]. Although the reciprocal imprinting of the *H19* and *IGF2* genes appears to be a general phenomenon, there are exceptions with monoallelic expression of one gene and biallelic expression of the other [15-17]. In parallel, Van Gurp *et al.* investigated the patterns of allelic expression of the *H19* and *IGF-2* genes in human testicular germ cell tumors and determined that in contrast to normally developing embryos, these tumors showed a consistent expression of both parental alleles of the two genes [18]. In contrast, *H19* showed retention of monoallelic expression in all *in vivo* samples of breast cancers, indicating that *IGF-2*, but not *H19*, is prone to loss of imprinting in these tumors [19]. Recently a chromatin boundary model of genomic imprinting has been proposed. It suggests that chromatin boundary elements (insulators) act in *cis* to insulate a gene blocking thereby its transcription when placed between the gene and its enhancer. Such an insulator located upstream of the *H19* gene isolates *IGF2* from its enhancers. When the imprinting-control region (ICR) is unmethylated on the maternally inherited chromosome, there are nuclease hypersensitive regions that overlap with several short CpGs rich repetitive elements. These repeats are the target for the DNA-binding zinc finger protein CTCF (CCCTC-binding factor), implicated in vertebrate boundary function [20]. The latter factor interacts with the core insulator, resulting in a blocked activation of the maternal allele of *IGF2* by their common enhancers. The methylated ICR contains no hypersensitive sites and this prevents

CTCF binding, thereby inactivating the insulator and allowing the *H19* enhancer to activate *IGF2* [21-23].

Despite full filling all the characteristics of a *bona fide* mRNA, no protein encoded by *H19* RNA has ever been detected in physiological conditions [24]. Thus, some authors proposed hypothetical translation of sequences from several (nine) mammals including human and showed an absence of conserved open reading frame (ORF) of any size. At the same time, the existence of several RNA helical pairings is suggested and Juan *et al.* concluded that the mature RNA is the functional product of the *H19* gene and that its function requires the ability to fold into a specific secondary structure [25]. Only after performing deletions and/or point mutations in the 5'-long untranslated region of the human *H19* RNA our group was able to show a protein corresponding to the longer ORF, nevertheless these data remain obtained in artificial context [26]. Finally, it has been proposed that *H19* expression modulates directly or indirectly the cytoplasmic levels of *IGF-2* mRNAs, as well as *IGF-2* mRNA translatability [27] and that *H19* participates in the repression of *IGF-2*, at least in part through effects on *IGF-2* transcription [28]. Collectively, these results imply that *H19* RNAs function as riboregulators and are involved in regulation of expression of a linked gene.

H19 RNA is expressed during fetal development, predominantly in tissues of mesodermal and endodermal origin, and down regulated at birth [24, 29]. Thus, in adulthood a basal *H19* gene expression has been detected only in mammary gland, uterus, cardiac and skeletal muscles and to a lesser extent in kidney, adrenal gland and lung [24, 30-34]. In most cancer tissues, the *H19* gene is overexpressed compared to healthy tissues [32, 35-38]. Frequently, this overexpression was accompanied with a loss of imprinting or a loss of heterozygosity [37, 39-42]. Taken together, these studies reported that *H19* is expressed in several tumors in tissues that normally express *H19* RNA during fetal development, thus exhibiting the characteristics of an "oncofetal" factor [34].

Although the role of the *H19* gene is to date still questioned, molecular evolutionary comparison of the rat and the mouse versions of the gene indicate that the RNA is under stabilizing selection and hence is most likely functional [43]. Interestingly, Wilms' tumor investigations provided data which underscore the possibility that chromosome 11 p 15.5 harbors a tumor repressor gene that is preferentially expressed from the maternal allele. The observation that *H19* is generally inactive in Wilms' [44-46] and that a *H19* expression vector rescues the normal phenotype of rhabdomyosarcoma cells [47] indicated that *H19* acts as a tumor suppressor gene. In parallel, a growth suppression as a biological activity of *H19* has been shown [48, 49]. On the contrary, this gene is considered as an oncogene because its expression in some cases correlated with the tumor progression and several groups reported data that argue against *H19* being a tumor suppressor gene [35, 36, 50-54].

Few studies are available on the modulation of *H19* expression by growth factors in cell cultures. It was reported that insulin, IGF-I or IGF-II up-regulate the *H19* gene in rabbit smooth muscle cells, whereas treatments with EGF or FGF-1 had minimal effects on its expression in these cells [55]. In human fetal adrenal cells, positive regulation of *H19* by IGF-I and IGF-II was also reported; by contrast, TGF-β-1, TNF-α and IFN-γ reduced significantly *H19* RNA level in these cells [56].

In previous studies, we have demonstrated that the *H19* gene is often overexpressed in breast tumors, either in stromal and/or epithelial cells, and that this overexpression was often observed at the epithelium/stroma boundary [32, 38]. This accumulation of *H19* RNA at mesenchyme/epithelium interface may be the result of a paracrine stimulation of one of the two compartments on the other. To test whether the *H19* RNA synthesis can be regulated by paracrine mesenchymal factors, we analysed the effect of conditioned medium and signaling molecules produced by fibroblasts on the *H19* gene activity. Thus, we report modulations of *H19* gene expression in mammary epithelial cells examined in various culture conditions and

/or under mesenchymal influences. We further define the effects of various growth factors on *H19* gene expression. As HGF/SF stimulates tubulogenesis of mammary gland and kidney epithelial cells, we focused our study on the influence of HGF/SF on the *H19* RNA synthesis, keeping in mind that this growth factor is involved in the morphogenesis and scattering phenotype of epithelial cells.

MATERIALS AND METHODS

Materials

Epidermal growth factor (EGF, recombinant, human), hepatocyte growth factor (HGF/SF, recombinant, human), basic fibroblast growth factor (FGF-2, recombinant, human), transforming growth factor-β-1 (TGF-β-1, recombinant, human) and tumor necrosis factor-α-1 (TNF-α-1, recombinant, human) were purchased from R & D System. Insulin-like growth factor (IGF, recombinant, human) was purchased from Becton Dickinson. The MEK-inhibitor UO126, specific inhibitor of ras-ERK pathway was purchased from Promega. U73122 was purchased from Calbiochem. Wortmannin and LY 294002 (specific inhibitors of PI3-kinase) were purchased from Sigma.

Cell line cultures

MRC-5 is a human embryonic lung fibroblast cell line (provided by ATCC), which secretes an inductive factor of morphogenesis [3]. MRC-5 were cultured in Dulbecco modified Eagle's minimal essential medium (DMEM, BioWhittaker) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (BioWhittaker) and 40 μg/ml gentamycin.

MDA-MB-231 and MCF-7 are two human cancerous mammary epithelial cell lines provided by ATCC. HBL-100 is an immortalized human mammary epithelial cell line generously provided by Pr. M. Crespin (Faculté de Médecine, Bobigny, France). All mammary cell lines were cultured in MEM supplemented with 10% FCS and 1% non-essential amino-acids (BioWhittaker) and 5 μg/ml insulin (Organon). All cell lines were incubated at 37°C in a humidified atmosphere with 5% CO_2 and 95% air.

Two-dimensional (2D) cultures

Mammary epithelial cells were seeded in chamber slides (Nunc) at 20,000 cells per well in the appropriate culture medium in the presence or absence of 50% fibroblast conditioned medium (FCM). FCM was obtained by using the supernatant of MRC-5 cells (2.10^6/100 mm Petri dishes) grown for 72 hours. This FCM was centrifuged to eliminate cell fragments and stored at –80°C in siliconized tube until use. After experiments, cells were analysed by contrast microscopy, fixed in 4% paraformaldehyde solution (10 minutes), washed in PBS, dehydrated through increasing ethanol concentrations and stored at 4°C until *in situ* hybridization experiments.

Three-dimensional (3D) cultures

Type 1 collagen gels were prepared as previously described [4, 5]. Mammary epithelial cells were cultured in 3D-collagen gels either with MRC-5 fibroblasts (co-culture) or with 50% FCM. A bottom gel was dispensed in each well of 24-well plates (300 µl collagen/well). After collagen solidification , 10 µl of the culture medium containing $2.5x10^4$ mammary epithelial cells were layered in the centre of the gel and cells were allowed to attach to the substrate for 1hour at 37°C. A cell-free gel (200 µl/well) was then applied to the adherent epithelial cell layer. After solidification, wells were filled up with 500 µl of complete medium or with 50% complete medium-50% FCM and cells were cultured for one week. For co-culture experiments, the bottom layer gel was containing $5x10^5$ MRC-5/ml.

At the end of experiment, collagen gels were fixed, embedded in paraffin, sectioned and submitted to ISH technique.

In situ hybridization (ISH)

ISH were performed as previously described [57]. Labeled probes were obtained as follows: antisense and sense ^{35}S labelled RNA probes were transcribed by SP6 polymerase from a 1.3 kb *Stu* I fragment of the human *H19* cDNA cloned at a *Sma* I site into the pSP64 vector (Promega). Before *in vitro* transcription, the vector was linearized by *Hin*d III.

After dipping in a nuclear track emulsion (Kodak NTB2), slides were exposed for 3 weeks at 4°C before development. Then, they were stained by the intercalating dye Hoechst 33258, mounted in glycergel (Dako) and were observed under a double illumination using an Olympus BH2 photo-microscope with epifluorescence for Hoechst staining and a dark-field condenser for silver grain detection.

Total RNAs isolation

Total RNAs from cells cultured as a monolayer were isolated using Rneasy Mini Kit (Quiagen). Total RNAs from cells cultured in collagen gels were prepared by guanidium isothiocyanate-CsCl gradient method.

Real-time RT-PCR

Production of the RNA standards

We constructed RNA standards for *H19* and *TBP (TATA Binding Protein)*. Each standard was obtained after in vitro transcription (RiboMAX Large scale RNA Production System T7, Promega) of cloned fragment in a plasmid (pGem-T Vector Systems, Promega), as previously described [58].

PCR primers and TaqMan fluorogenic probes

PCR primers and TaqMan fluorigenic probes were designed using Primer Express software program (Demo version 1.0, Perkin-Elmer). Their sequences are summarized below:

H19 sense	CAC TAT GGC TGC CCT CTG
H19 antisense	TTC AAA GGC CCA GGC TTG
H19 probe [a]	CTC CCA GAA CCC ACA ACA TGA AGG AA

[a] TaqMan probes carried a 5' FAM (6-carboxy-fluorescein) reporter dye, a 3' TAMRA (6-carboxy-tetramethyl-rhodamine) quencher dye and a 3' phosphate group to prevent extension during PCR.

RT-PCR conditions

RT and PCR were performed in a one-step methodology as previously described [58]. The reaction mixture (50 µl final volume) contained 100 ng total RNA, 10X (5 µl) TaqMan buffer, $MgCl_2$ (6 mM), 20 units RNase inhibitor, 12.5 units MulV reverse transcriptase, 1.25 units AmpliTaq Gold DNA polymerase, 300 µM dATP, dCTP, dGTP, 600 µM dUTP, 200 nM forward and reverse primers, and 200 nM probe. Reverse transcription was performed at 48°C for 30 minutes. Activation of AmpliTaq Gold DNA polymerase (10 minutes at 95°C) was followed by PCR (15 seconds at 95°C and 90 seconds at 60°C for 40 cycles).

To normalise expression level of the *H19* gene, we used *TBP* mRNA, whose primers and probe are previously reported [59].

Analysis and Expression of the Real-time RT-PCR Data

Quantification of the starting amount of a specific mRNA in an unknown sample was performed using a standard curve of known dilutions of corresponding standard RNA. For each dilution, the Abi-Prism 7700 software generated a real-time amplification curve

constructed by relating fluorescence signal intensity (*ΔRn*) to the cycle number. The *ΔRn* value corresponds to the variation in reporter fluorescence intensity before and after PCR, normalised to the fluorescence of an internal passive reference present in buffer solution (6-carboxy-x-rhodamine, a rhodamine derivative). Standard curve was then generated on basis of the linear relationship existing between the *Ct* value (cycle threshold; corresponding to the cycle number at which a significant increase in the fluorescence signal was first detected) and the logarithm of the starting quantity [60]. The level of RNA synthesis of *H19* gene was calculated as a ratio between *H19* and *TBP* expression (in copies per µg of total RNA) and was referred as relative expression.

Northern blot analysis

RNAs (20 µg per sample), prepared by guanidium isothiocyanate-CsCl gradient method, were separated in a 1.2% agarose/formaldehyde denaturing gel and transferred overnight onto a nitrocellulose membrane (Hybond-C-extra, Amersham). Membrane was hybridized at 42°C with α^{32}P-dCTP random primed cDNA probes (Megaprime labeling system, Amersham): a 1.3 kbp *Stu* I *H19* fragment and a 1.2 kbp *Pst* I *GAPDH* (GlycerAldehyde-3'-PhosphoDesHydrogenase) fragment. *H19* and *GAPDH* RNAs were quantified using a PhosphorImager 425 (Molecular Dynamics Inc., Sunnyvale, CA/USA).

Transfection experiments

MDCK were transiently tranfected with pGL2 plasmid containing the *H19* minimal promoter region (823 bp) associated with luciferase reporter gene [61].

Transfections were performed using LipofectAMINE™ reagent (Gibco BRL): 0.25 µg of *H19*-luciferase plasmid was used for 35,000 cells per well seeded the day before transfection.

Cells were incubated with transfection mixture for 6 hours at 37°C, then culture medium was changed. Sixteen hours later, cells were treated with fibroblast conditioned medium, growth factors or pharmacological inhibitors. 24 hours after treatment, cells were harvested with a reporter lysis buffer (Promega). Luciferase activity was assayed using a luciferase assay system (Promega) and measured using a lumat 9501 (Berthold). Luciferase activities were normalized with total protein concentrations determined by densitometry (DU^R64 spectrophotometer, Beckman) at 562 nm wavelength after bicinchoninic acid reactions.

RESULTS

H19 gene is differentially expressed in various mammary cell lines

For this study, we have chosen two mammary epithelial cell lines HBL-100 and MCF-7 which exhibit obvious different migratory phenotypes with respectively highly and weakly motile ability [4, 62], and lung fibroblasts (MRC-5) which are used below to yield conditioned medium (FCM). Real-time RT-PCR shows that MCF-7 cells contained more *H19* RNA than HBL-100 cells (Fig. 1A). Elsewhere, MRC-5 fibroblasts express more *H19* transcripts than mammary cells (25 to 100 fold, data not shown). The low *H19* RNA levels in mammary epithelial lines (MCF-7, HBL-100, MDA-MB-231) have been routinely observed by Northern-blotting analysis (data not shown). These observations are consistent with *H19* expression pattern in primary breast cancers, mainly contributed by the stromal component [32, 38].

We found that cell density in dishes (subconfluence, confluence, hyperconfluence) weakly modified the *H19* RNA quantities evaluated after real-time RT-PCR (Fig. 1A). To test the influence of serum on *H19* RNA synthesis, we varied the percentage of fetal calf serum (FCS) added to the culture medium. It appeared that the susceptibility of the *H19* expression was dependent on the cell line, for instance, the gene was up-regulated by serum in MCF-7, but not in HBL-100 (Fig. 1B).

Morphogenesis and growth in collagen gels (3D-condition) strikingly enhances *H19* gene expression

We test whether cellular anchorage and cell shape can modify the *H19* gene expression. Thus, the two cell lines growing in clusters (MCF-7) or not (HBL-100) were cultured between two layers of collagen gel, the so-called "3D-condition". For both cell types, the ISH technique

indicates an increasing synthesis of *H19* RNA, compared to the so-called "2D-condition" (Fig. 2). This up-regulation of *H19* expression was quantified by real-time RT-PCR, as illustrated by figure 2.

Conditioned medium and HGF/SF up-regulate the *H19* gene expression of cells cultured in collagen gel-condition

We have previously found that in breast adenocarcinomas, *H19* gene overexpression is mainly observed at the epithelium-mesenchyme boundary [32, 38]. This observation led us to investigate the possible modification of *H19* gene expression in human epithelial cells under stromal cells influence. We first investigated the sensitivity of mammary cells to MRC-5 fibroblast conditioned medium (FCM), which is known to promote cell scattering and tubulogenesis; [63]. FCM induced cell scattering and cell organization in strands for HBL-100 (Fig. 3A, B), and only a cell scattering for MDA-MB-231 (Fig. 4A, B). By contrast, no significant change was observed for MCF-7 grown in the presence of FCM; cells grew in cysts (Fig. 5A, B). Same results were obtained when co-cultures were performed with each epithelial cell type and MRC-5 fibroblasts (data not shown). In parallel, ISH technique indicated an obvious increase of *H19* RNA pool in HBL-100 and MDA-MB-231 cell types, when treated with FCM (Figs 3, 4; F and G respectively), but no increase was visible for MCF-7 line (Fig. 5F, G). These results indicate that *H19* RNA synthesis is related to the migratory phenotype of cultured cells.

Since HGF/SF has been identified as a potent morphogenetic factor released by mesenchymal cells, we studied its influence on cell phenotypes, and *H19* RNA synthesis. HGF/SF has potent motogenic, mitogenic and morphogenic activities on epithelial cells in culture [64, 65]. This factor exhibited effects (scattering and/or tubulogenesis) similarly to FCM on HBL-100 and MDA-MB-231 cells (Figs 3C, 4C), but seems to have no influence on the MCF-7 line

(Fig. 5C). In parallel, the ISH technique indicated that HGF/SF induced an increased synthesis of *H19* RNA in HBL-100 and MDA-MB-231 cells (Figs 3H, 4H respectively), but no significant change was observed in MCF-7 line (Fig. 5H). To determine to which extent HGF/SF is responsible for the scattering and for the *H19* overexpression in FCM, we used an HGF/SF antibody and an irrelevant antibody (Figs 3 to 5, D-E and I-J, respectively). When FCM was added to HBL-100 or MDA-MB-231 cell culture with a blocking anti-HGF/SF antibody, the same phenotype as that in control conditions was observed. FCM has no more effect, neither on cell migration (Figs 3, 4, compared D to A, respectively), nor on the *H19* RNA synthesis (Figs 3, 4, compare I to F, respectively). When an irrelevant antibody was added to FCM, results were similar to cells treated with FCM alone or HGF/SF (Figs 3, 4, compare E and J with B and G or C and H, respectively). In MCF-7 cells, relevant and irrelevant antibody treatments provided no significant change, neither in cell phenotypes, nor in *H19* expression (Fig. 5, compare D and E to A and B, respectively; compare I and J to F and G, respectively). These observations indicate that the positive effect of FCM on the *H19* RNA synthesis are likely due to HGF/SF.

The effect of FCM on the *H19* gene expression was questioned for HBL-100, MDA-MB-231 and MCF-7 lines cultured in 2D-condition. Northern-blotting technique (Fig. 6) indicated a conspicuous stimulation by FCM of *H19* RNA synthesis in HBL-100 cells (the lack of measurable *H19* signal in control does not allow the quantification of the induction by FCM), an increased expression in MDA-MB-231 cells (3.5 fold) but no effect in MCF-7 (1.3 fold).

_H19_ is a target gene for growth factors, in particular of HGF/SF

Cell phenotype considered together with ISH data, reported in the above paragraph, prompted us to study thoroughly the role of various growth factors on the control of the *H19* gene expression *via* their action on the *H19* promoter linked to the *luciferase* gene. Experiments

were performed in introducing the vector into MDCK cells. This model was chosen because it is a well recognized system for studying the HGF/SF effects.

Figure 7 (A and B) summaries transfection data obtained with cell cultured in a medium supplemented with 0.5% of FCS. HGF/SF, EGF, FGF-2 (Fig. 7A) and FCM (Fig. 7B), activated the *H19* promoter by 5, 3.3, 2.3 and 8.7 fold respectively, whereas IGF II, TGF-β-1 and TNF-α-1 (Fig. 7A) did not modify significantly luciferase activity. The anti-HGF/SF antibody reduced significantly FCM stimulating action on *H19* promoter (Fig. 7B). This latter range of results is in agreement with those obtained by ISH on epithelial mammary cells. Our study as a whole indicates the potent role of HGF/SF on *H19* gene expression which activation correlates to cell motility.

To determine the signalling pathway involved in this HGF/SF stimulation of *H19* gene expression, we performed experiments using different pharmacological inhibitors. UO126 and U73122, inhibitors of ERK/MAPK and phospholipase C pathways respectively, reduced significantly HGF/SF up-regulation of *H19* promoter (Fig. 7B). By contrast, Wortmannin and LY 294002 (specific inhibitors of PI3-kinase) have no effect on *H19* induction (data not shown). Experiments performed with cell culture medium containing 10% FCS gave similar results with attenuated differentials.

Figure 1: Real-time RT-PCR showing *H19* relative mRNA expression in MCF-7 and HBL-100 mammary epithelial cells.

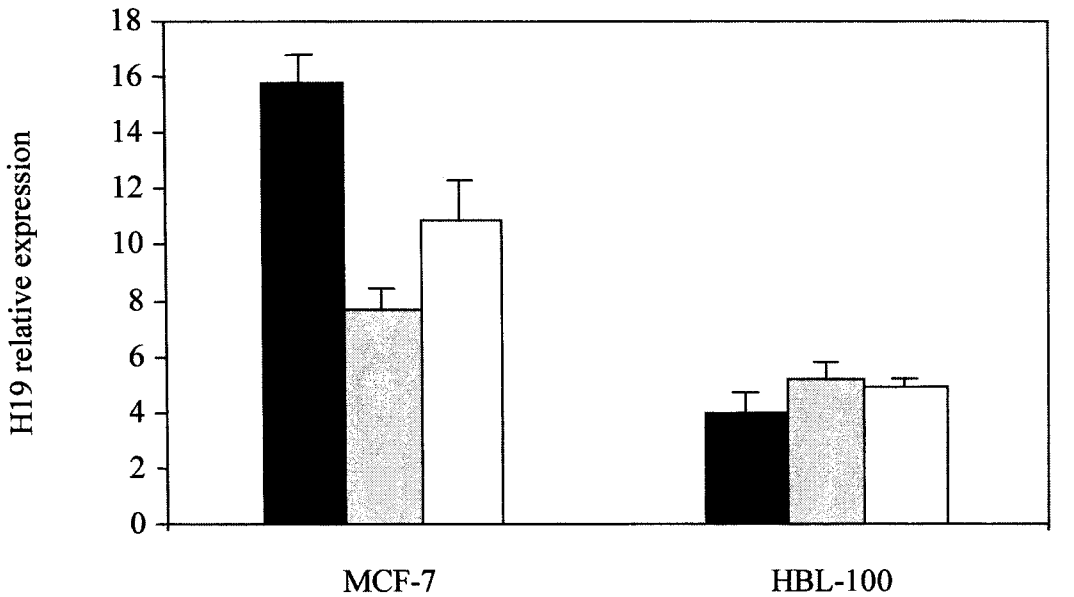
MCF-7 and HBL-100 cells were chosen for these experiments, because of their distinct migratory capacities, with HBL-100 being spontaneously more motile than MCF7.

A: Effect of cell density on *H19* mRNA relative expression. Cells were cultured until they were subconfluent (black bar), confluent (grey bar) and two days after confluence (white bar). The relative level of mRNA synthesis was calculated as a ratio between *H19* and *TBP* mRNA expression.

B: Effect of fetal calf serum on relative *H19* mRNA expression. The cells were grown for 3 days until they reach subconfluence in medium with serum percentage varying from 0 to 20%. The *H19* mRNA abundance in MCF-7 cells (grey columns) depends on the percentage of FCS, but not in HBL-100 cells (white columns).

Figure 1

A


H19 relative expression
18
16
14
12
10
8
6
4
2
0
MCF-7
HBL-100

B

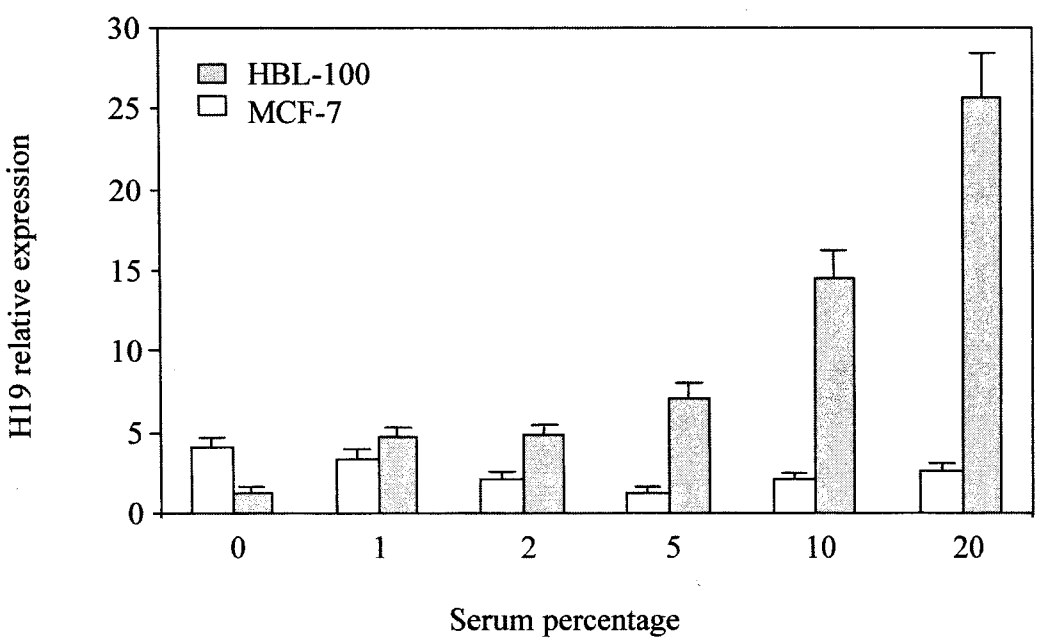

H19 relative expression
30
25
20
15
10
5
0
HBL-100
MCF-7
0
1
2
5
10
20
Serum percentage

Figure 2: Effect of plastic or collagen as a cell culture substrate on *H19* gene expression.

The left and right part of the figure corresponds to HBL-100 and MCF-7 cells, respectively. Cells were grown on plastic (two-dimensional conditions, 2D, top part) or in collagen gels (three-dimensional conditions, 3D, medium part) and were processed for observation by phase-contrast microscopy or for evaluation of *H19* mRNA expression using in situ hybridization. As depicted in the cartoon for 3D conditions, cells were grown in sandwich between two layers of collagen gel and developped as a micromass within 7 days. Photographs were taken above the well (arrow) and in situ hybridization was performed following the A-B section.

In 2D conditions, MCF-7 grew in cell clusters, with an epithelioid morphology, whereas HBL-100 cells grew as individual migrating epithelial cells, with a fibroblastoid morphology. In 3D conditions, both MCF-7 and HBL-100 grew in aggregates, but only HBL100 cells were able to invade the gel. For both MCF7 and HBL-100 cells, expression of *H19* was induced by culture in 3D- versus culture in 2D- conditions.

Bottom part: Real-time RT-PCR providing *H19* relative expression confirmed that *H19* expression was increased by cell culture in collagen gels. Similar results were obtained for MDA-MB-231 and normal mammary epithelial cells (data not shown).

Scale bars represent 30 μm in phase-contrast microscopy and 50 μm in hybridization photographs.

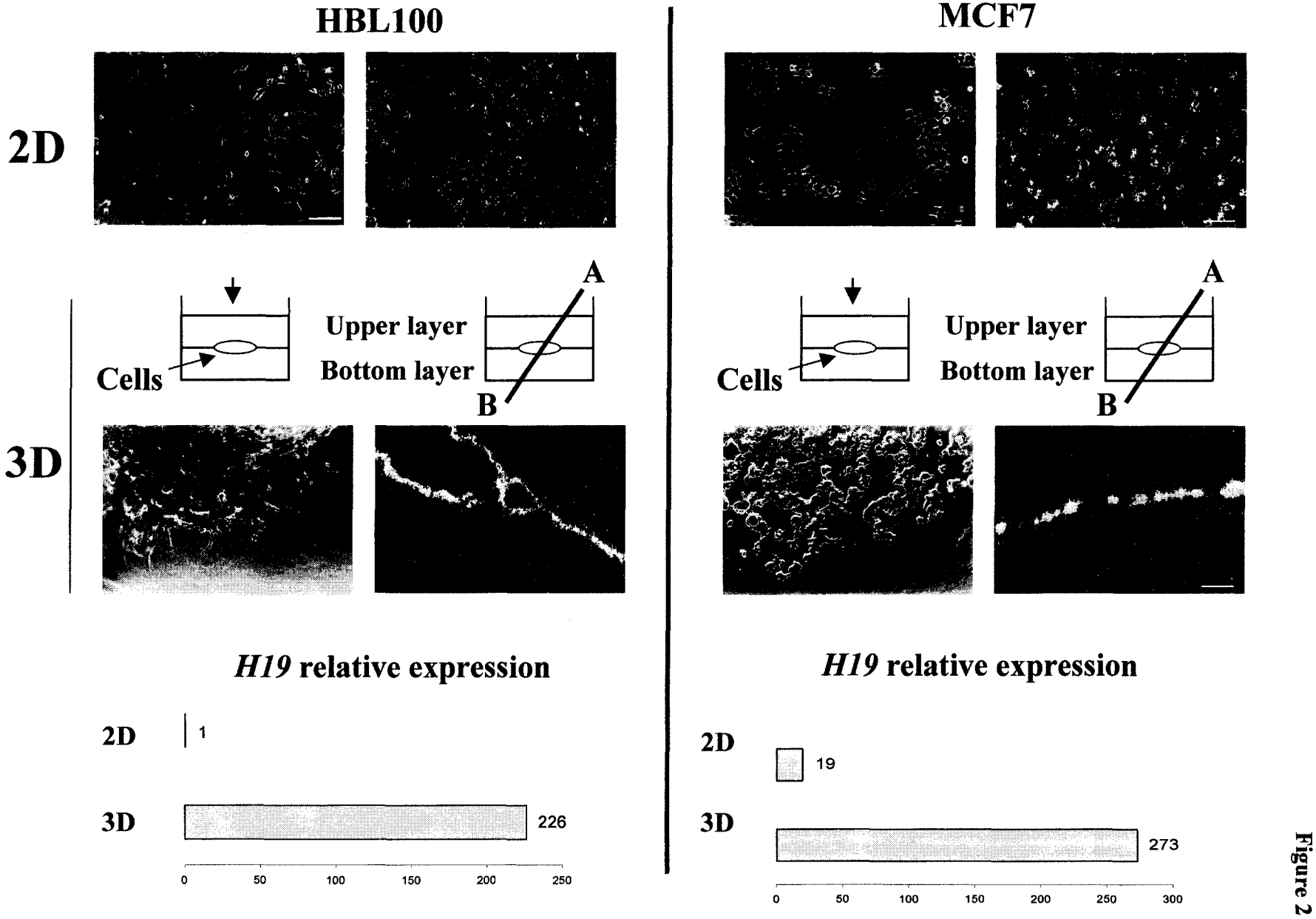

HBL100
MCF7
2D
3D
Cells
Upper layer
Bottom layer
A
B
H19 relative expression
2D
1
3D
226
0
50
100
150
200
250
H19 relative expression
2D
19
3D
273
0
50
100
150
200
250
300

Figure 2

Figure 3: Effect of fibroblast-conditioned medium and HGF/SF on *H19* expression from HBL-100 epithelial cells grown in collagen gels.

HBL-100 cells were grown in collagen gels for 7 days, in the presence of control medium (A-F), MRC5 fibroblast-conditioned medium (FCM) (B-G), HGF/SF (10 ng/ml) (C-H), FCM with anti-HGF/SF antibody (D-I), or FCM with irrelevant antibody (E-J). For all pictures, A to E shows cell morphology and F to J shows *H19* expression detected by in situ hybridization.

Scale bars represent 50 μm in phase-contrast microscopy and 75 μm in hybridization photographs.

Figure 3

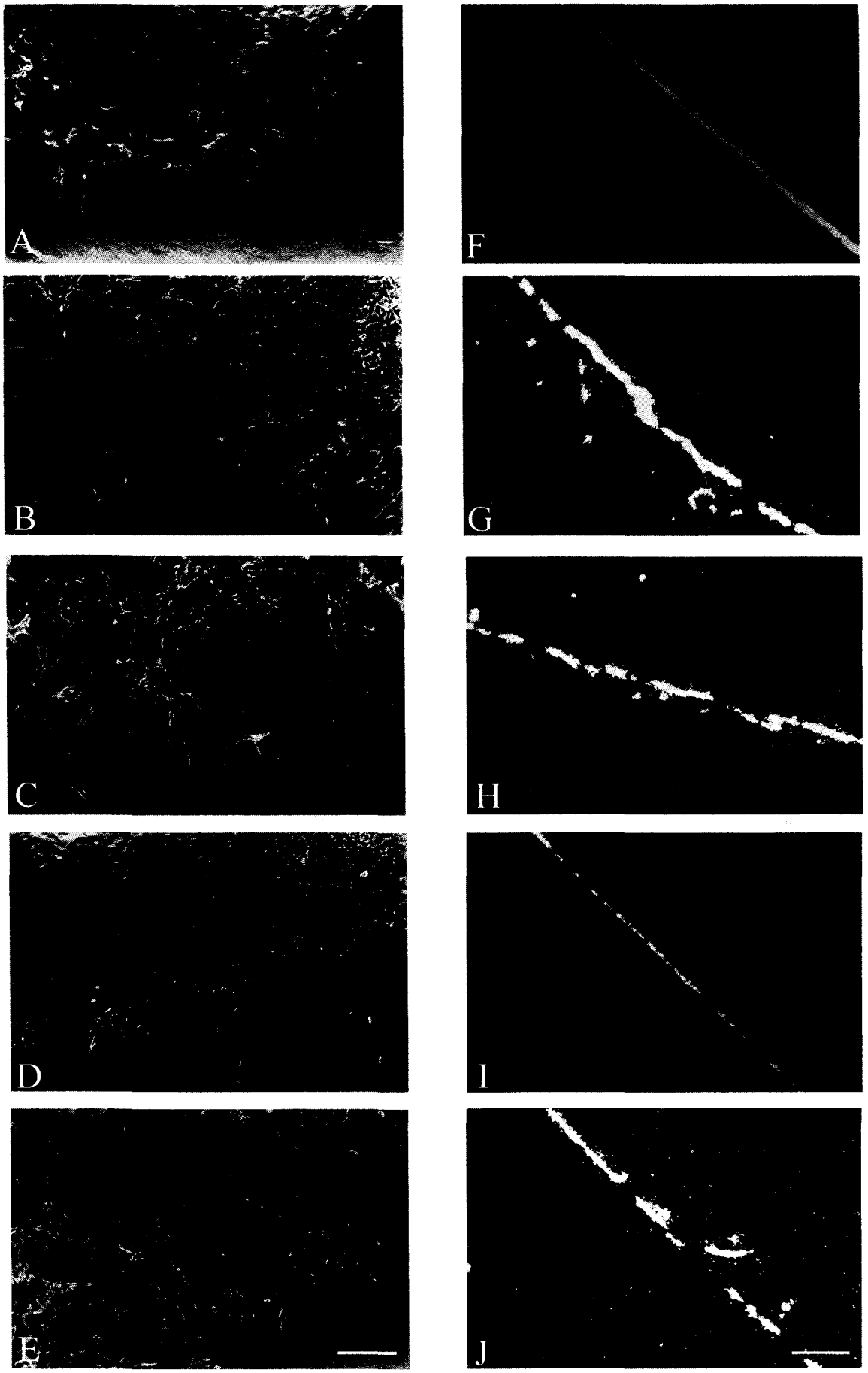


Figure 4: Effect of fibroblast-conditioned medium and HGF/SF on *H19* expression from MDA-MB-231 epithelial cells grown in collagen gels.

Protocols previously described in figure 3 were performed with MDA-MB-231 cell line.

Figure 4

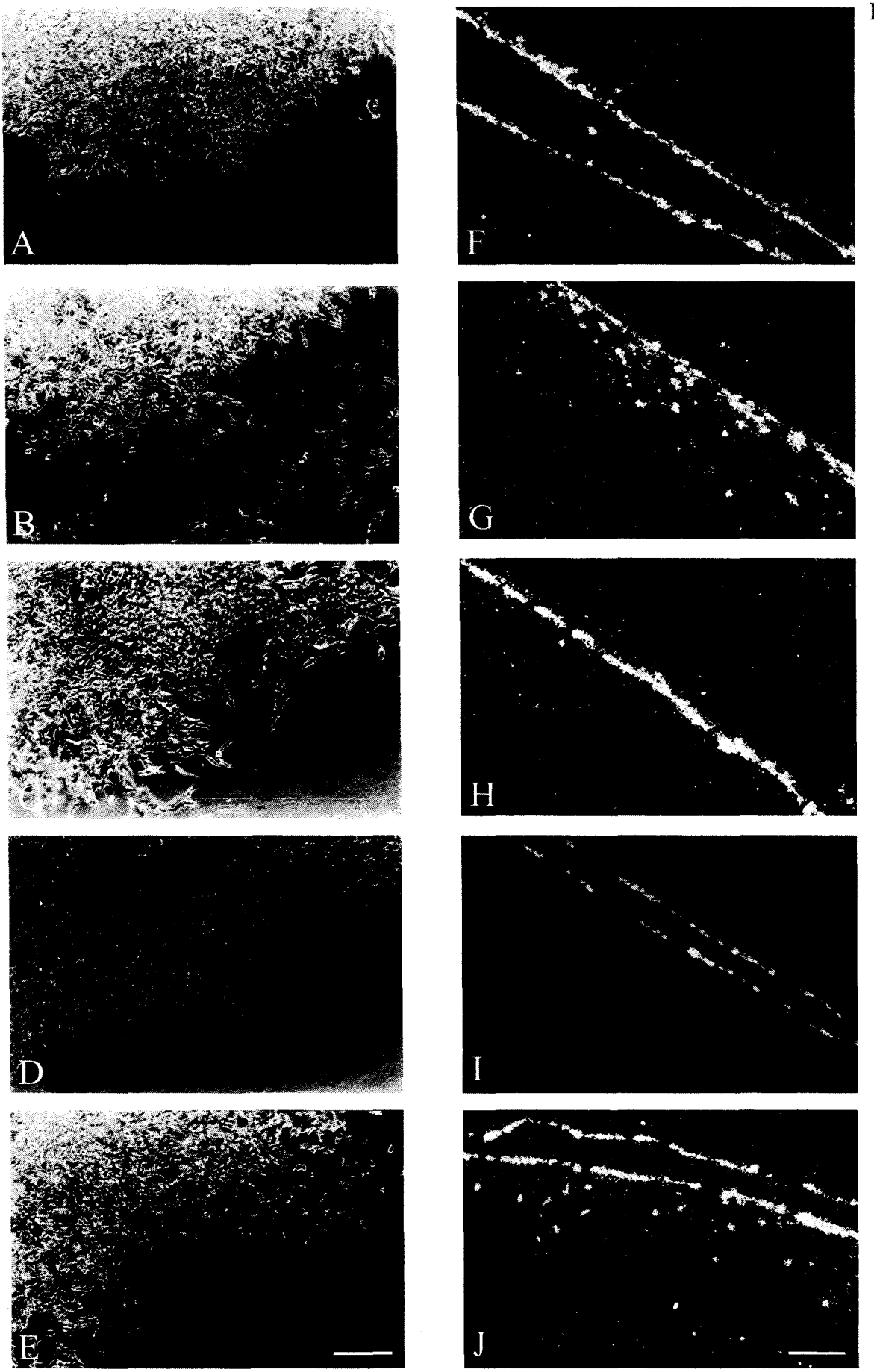


Figure 5: Effect of fibroblast-conditioned medium and HGF/SF on *H19* expression from MCF-7 epithelial cells grown in collagen gels.

Protocols previously described in figure 3 were performed with MCF-7 cell line.

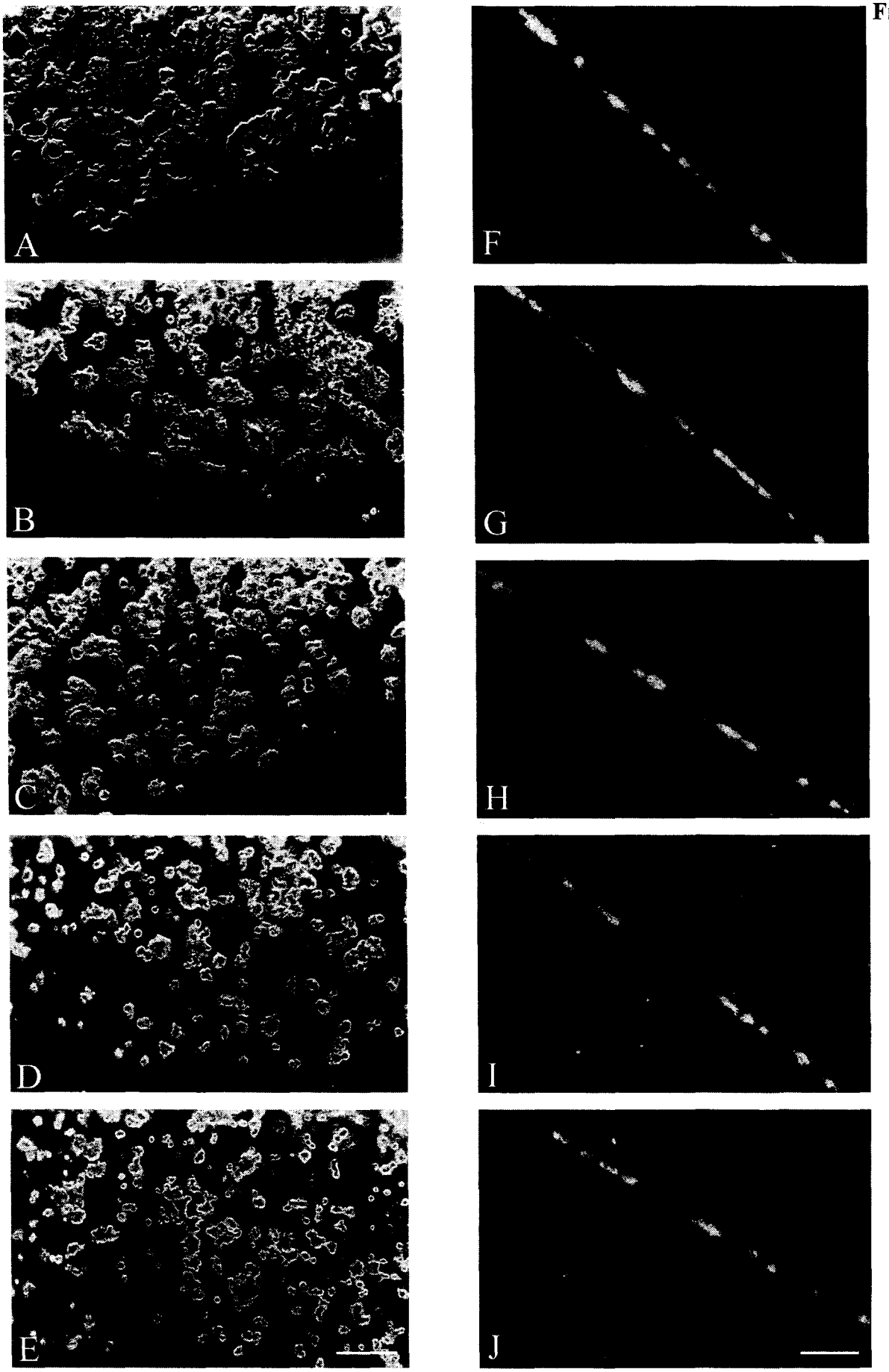


Figure 5

Figure 6: Effect of fibroblast-conditioned medium on *H19* expression from epithelial cells grown on plastic.

mRNAs were isolated from MCF-7, HBL-100 and MDA-MB-231 cells grown on plastic, with or without fibroblast conditioned medium. Results were analyzed by Northern blot. Blots were hybridized with human *H19* or *GAPDH* cDNA probes. *GAPDH* was used as the control for the amount of mRNA loaded onto the gel.

Figure 6

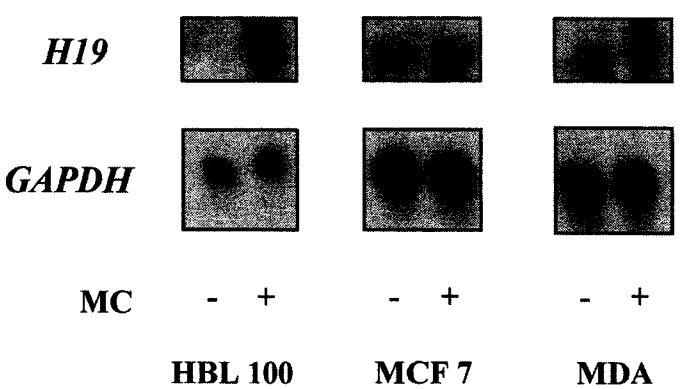


Figure 7: Effect of various factors on *H19* promoter activity in MDCK epithelial cells

MDCK cells were transiently transfected with 0.25 μg of the *H19* reporter vector. The following day, cells were incubated in DMEM-05 % FCS in the presence or absence of:

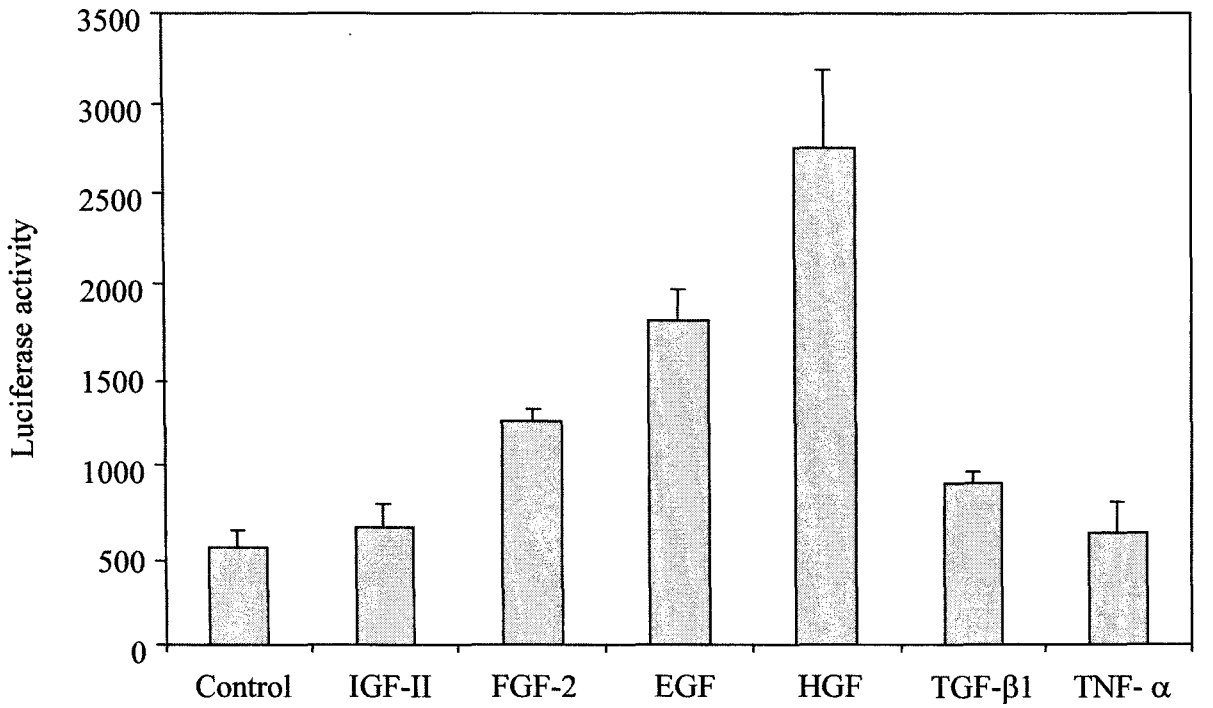
A: IGF II, FGF2, HGF/SF, TGF-β-1, TNF-α (10 ng/ml) or EGF (100 ng/ml).

B: fibroblast-conditioned media (FCM), HGF/SF (10 ng/ml), FCM + anti-HGF, HGF/SF with 25 μm of U0126 (HGF + U0126) or HGF/SF + 10 μm of U73122 (HGF + U73122).

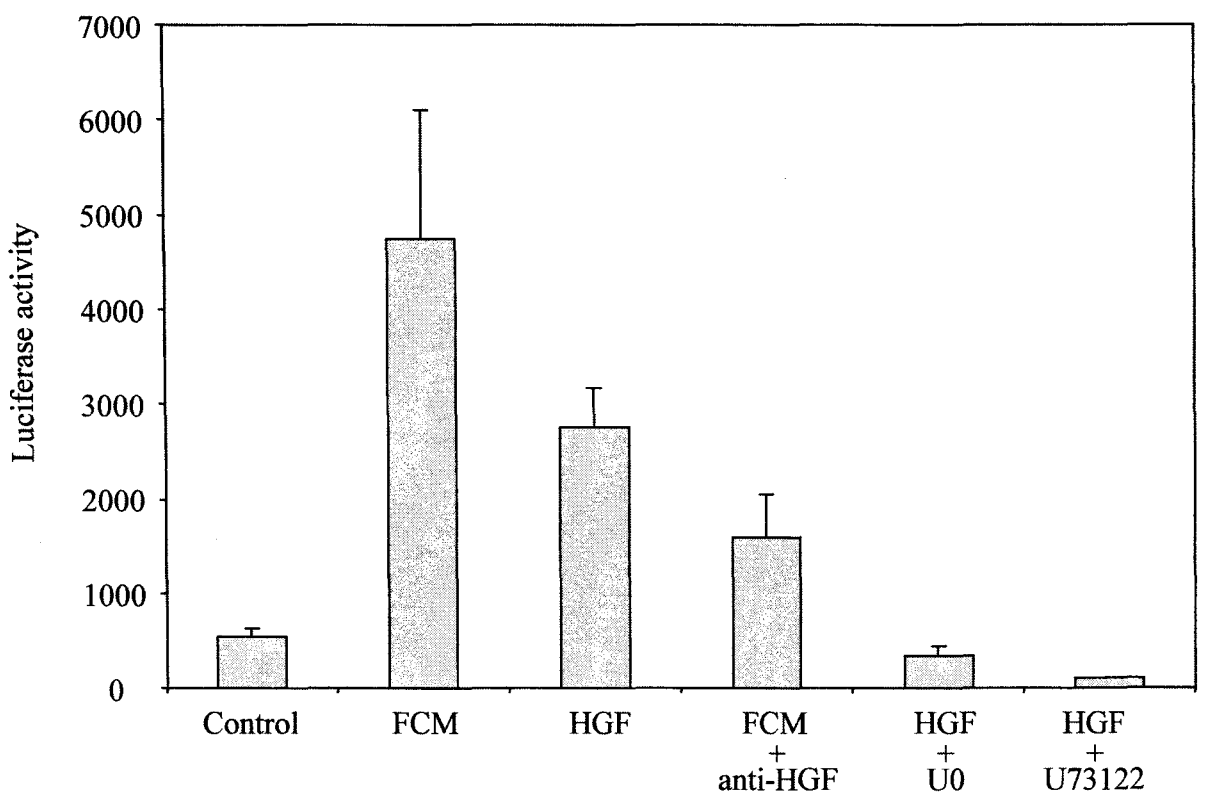
Cells were processed for luciferase assay 24 hours later. The values are means +/- s.d. of three independent experiments, performed in duplicates.

Figure 7

A


Luciferase activity
3500
3000
2500
2000
1500
1000
500
0
Control
IGF-II
FGF-2
EGF
HGF
TGF-β1
TNF- α

B


Luciferase activity
7000
6000
5000
4000
3000
2000
1000
0
Control
FCM
HGF
FCM + anti-HGF
HGF + U0
HGF + U73122

DISCUSSION

Epithelial-mesenchymal interactions are crucial for regular epithelial growth and differentiation during embryogenesis and in the adult organism. Epithelium homeostasis is controlled by multifactorial signalling events implicating the underlying mesenchymal cells. Moreover, mesenchymal stroma was also involved in epithelial carcinogenesis. Fibroblasts have been shown to promote [66, 67] or suppress [68, 69] the development of cancer. This dual role is probably mediated by cross-talk between epithelial cells and fibroblasts. Thus, the latter cells play a major role in tumor development and spread, affecting proliferation, differentiation, invasion or regression of cancer cells, keeping in mind that tumors contain various types of fibroblasts [70].

In this field of investigation, we observed, as previously reported [4], that the fibroblast conditioned medium (FCM) induced organization of epithelial tubules and/or epithelial scattering for HBL-100 and MDA-MB-231, but no significant change was observed for MCF-7. *In situ* hybridization data indicate that the migratory phenotype was accompanied with an increase of the *H19* RNA synthesis (ISH data, Figs 3 to 5). This up-regulation of the *H19* gene by FCM was confirmed by Northern-blotting and transfection assays (Figs 6, 7). Among the numerous growth factors produced by mesenchymal cells, HGF/SF is clearly identified. HGF/SF is synthesized by mesenchymal cells, whereas its receptor, the tyrosine-kinase c-Met, is located in epithelial cell cytoplasmic membrane [64, 65] mediating important mesenchymal-epithelial interactions *in vivo* [71-73]. One of the major post-natal morphogenetic periods of mammary gland development is dependent on sequential mesenchymal-epithelial interactions mediated by HGF/SF, during ductal branching in the virgin animal [74]. This key-role prompted us to study thoroughly activity of FCM, by testing directly the effect of HGF/SF on epithelial cells. The motogenic phenotype of cells (HBL-100, MDA-MB-231) induced by this factor, brought together with the up-regulation of the

H19 gene, lead to conclude that the *H19* expression is related with the morphogenesis and scattering of epithelial cells. This linkage is further emphasized by lack of *H19* up-regulation in HGF/SF-non-responsive MCF-7 cells. Specificity of HGF/SF stimulation was further confirmed by using a blocking antibody. Otherwise, it is noteworthy that in hepatoma cells, HGF/SF regulates negatively the α-fetoprotein gene [75], which is tightly co-regulated with *H19* during fetal life [76], indicating thus that the up-regulation of *H19* by the scatter factor is probably not a general process. Nevertheless, our experiments demonstrate that positive action of HGF/SF on *H19* expression was not restricted to mammary epithelial cells, and can be extended to the MDCK kidney epithelial cell, the latter being an attractive model for studying morphogenesis of branching tubules induced by HGF/SF [64, 65].

In epithelial cells, activation of HGF/SF receptor, c-Met, induces specific response during scattering or branching tubulogenesis phenotypes. Kinetic and mechanistic developments of this HGF/SF-induced response are complex, not yet fully elucidated, and probably due to an integration of several signalling pathways. c-Met activation leads in particular to PI3-kinase and Ras activation [77-79], Ras-MAP-kinase cascade stimulation [80], activation of STAT pathway [81] and recruitment of PLC-γ-1 and the docking protein Gab 1 (GRB 2 associated Binder-1) [82]. It is well established that Ras-MAP-kinase, PI3-kinase and PLC-γ-1 activation is necessary to cell scattering and tubulogenesis [79, 82]. A comprehensive view of the signalling pathways implicated by HGF/SF stimulation is available in the review of Furge *et al.* [83]. To determine the signalling pathway responsible for *H19* gene induction, we used pharmacological inhibitors specific for the Ras-MAP-kinase or PLC-γ-1 pathways. Our results indicate that inhibitions of Ras-MAP-kinase (UO126) and PLC-γ-1 (U73122) lead to an obvious decrease of the *H19* gene induction by HGF. By contrast, inhibition of PI3-kinase pathway by wortmannin or LY has no effect on *H19* (data not shown).

Regulation and general expression pattern of *H19* in mammary gland, during fetal or postnatal life, could argue in favour of a role of the *H19* gene in epithelial cell migration. Indeed, during puberty and pregnancy, *H19* is strongly expressed in mammary terminal buds invading the fat-pad and mesenchymal components [84]. Nevertheless, these statements must be considered with discretion, since homozygous mutant animals with a targeted deletion of the *H19* gene were viable and fertile. These mice only displayed an overgrowth phenotype of 8% compared with wild-type littermates [85].

Even though HGF/SF among FCM components is the main factor responsible for morphogenetic action on cells and activation of their *H19* gene, positive regulation of *H19* promoter by two other growth factors (EGF and FGF-2) seems noteworthy. Indeed, it has been documented that the FGF-induced cell migration can involve MAP kinase pathway via ERK [86, 62].

In other respects, cell shape changes (spreading) that commonly accompany cell binding to extracellular matrix appear to be required for the growth of different types of anchorage-dependent cells [87, 88]. These observations raised the possibility that some form of anchorage signalling may regulate cell growth [89-91]. Elsewhere, hepatocyte attachment to a high density of fibronectin or laminin led to cell-spreading accompanied with histone mRNA expression and replication [92]. In our study, we observed that the possibility for cells to anchor between two collagen layers induced an up-regulation of the *H19* gene.

Futhermore, it has been reported that *H19* RNA synthesis can be modified by culture conditions. Expression of *H19* in cultured neointimal cells and in medial vascular smooth muscle cells (VSCM) appears tightly coupled to the growth state of the cells. Exponentially growing cells contain few or no *H19* transcripts. On the contrary, as cells approach confluence, expression of *H19* increases, reaching a maximum at three days or more after confluence. This finding suggests that expression of *H19* can be used as a marker for VSCM

differentiation and/or growth arrest [93]. Moreover, it was found that the gene is silent in undifferentiated embryonic carcinoma cells and becomes expressed when these cells were induced to differentiate into endoderm [94]. Similarly, Pachnis *et al.* indicated that C3H10T1/2 cells presented a dramatic increase in *H19* expression upon differentiation of precursor stem cells in myoblasts or in growth-arrest conditions [24]. Hayashida *et al.* reported that in the embryonic fibroblast line, normal Cl1 cells, the *H19* gene was up-regulated by contact inhibition and by growth factor depletion after reducing the serum concentration in dishes [95]. But it must be pointed out that *H19* expression is not an universal characteristic of growth-arrest cells. For instance, Davis *et al.* showed that confluent and quiescent Swiss 3T3 cells do not express *H19* [96]. In accordance, our data do not indicate dramatic changes of *H19* expression in mammary cells cultured at subconfluence, confluence or hyperconfluence (Fig. 1A). The above puzzling data suggest that an unique answer to cell abundance does not exist and that the *H19* RNA synthesis depends mostly on the cell type (Fig. 1A). Furthermore, growth factors and nutriments included in the fetal calf serum are responsible for general growth activation pathway, but up-regulation of the *H19* transcription depends on the cell line too (Fig. 1B).

Interestingly, *H19* RNA synthesis in the MCF-7 cell line appears insensitive to HGF/SF and fibroblast conditioned medium. On the contrary, fetal calf serum and 3D-culture induced an increase of *H19* gene expression accounting for the presence of functional signalling pathways in this cell line. In order to explain this discrepancy, we performed some classical experiments showing that c-Met protein synthesis is abnormal in MCF-7 cell cultivated in our laboratory (data not shown) as previously described elsewhere [97]. Even if *H19* gene can be modulated in MCF-7 cell, the defect in c-Met receptor leads to absence of *H19* induction by HGF/SF or FCM. Thus, well-differentiated breast carcinoma cell line (MCF-7) does not express c-Met protein and shows neither motile or invasive phenotype nor *H19* induction by

FCM. In opposition, poorly differentiated cell lines (MDA-MB-231 and HBL-100), sensitive to FCM and HGF/SF, exhibit a high level of *H19* gene expression concordant with a fully developed invasive phenotype. Moreover, Tsujimoto *et al.* performed a differential display screening with tumorigenic and nontumorigenic HeLa x normal human fibroblast hybrid cells. This experiment revealed that several genes might regulate tumorigenic expression, including *H19* and signal transduction and extracellular matrix genes [98]. Recently, this group confirms the previously exclusive genetic linkage between *H19* expression and tumorigenic phenotype [99]. A differential display experiment performed in a model of metanephric mesenchyme to nephron epithelium conversion demonstrated that FGF2 and conditioned medium induced overexpression of several genes, including those of syndecan-4, integrin-β1 and *H19* [100]. These results provide evidence that an up-regulation of genes encoding cell adhesion proteins can be accompanied with the increase of *H19* RNA synthesis. Finally these data and those described in the present study demonstrate similar phenotypic modulations of the *H19* gene expression in non-malignant and malignant cells that may reflect a common response to and a possible role of the *H19* gene in cell differentiation and/or adhesion going with migration.

In conclusion, we report that in mammal cells *H19* gene expression is depending on the cell line and the factors present in the culture medium. Furthermore, the cell ability to anchor between collagen layers increased the *H19* RNA synthesis. The main information of this study comes from the evidence of an up-regulation of *H19* induced by fibroblast conditioned medium (FCM) or by presence of mesenchymal cells in the culture medium. We showed that HGF/SF is the major factor responsible for the FCM positive control exerted on the *H19* gene even if other factors, such as EGF and FGF-2, can also be involved. We further studied the key-role of HGF/SF and demonstrated that its stimulating effect involves either the MAP kinase *via* ERK and the phospholipase C signalling pathway. Consequently, *H19* appears to

be a target gene of motogenic growth factors, induced by the epithelial-mesenchymal interactions which are known to be fundamental during normal development or neoplastic genesis. Thus, the potent role exhibited by HGF/SF correlates the *H19* expression to morphogenesis and/or scattering, possibly to be functioning to promote cancer progression and tumor metastasis.

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Chapitre II

Analyse des phénotypes tumorigènes de cellules épithéliales mammaires cancéreuses surexprimant H19

Nous avons montré, dans une précédente étude, que le gène *H19* est surexprimé dans plus de 70% des cancers du sein et bien que cette surexpression soit le plus souvent stromale, dans environ 10% des cas l'accumulation des transcrits est détectée dans les cellules épithéliales. Bien que le faible nombre de cas pour lesquels le gène *H19* n'est surexprimé que dans les cellules épithéliales ne nous ait pas permis de réaliser une corrélation statistique, nous avons remarqué que ce patron d'expression est toujours associé à des tumeurs de grade III, constituées de cellules ne présentant plus de récepteurs aux hormones stéroïdes, et que le décès des patientes était survenu dans les cinq ans suivant la tumorectomie (Adriaenssens *et al.*, 1998). Or, le rôle du gène *H19* dans les processus de tumorigenèse est fortement controversé, étant oncogénique pour certains auteurs et suppresseur de tumeurs pour d'autres. Afin de statuer sur ce point dans les cancers du sein, nous avons transfecté de façon stable le gène *H19* dans des cellules épithéliales mammaires cancéreuses humaines, puis avons analysé les effets de la surexpression du gène sur les propriétés tumorigènes de ces cellules.

Article 2

Pour réaliser le projet exposé ci-dessus, nous avons choisi de travailler sur la lignée hormono-indépendante MDA-MB-231 qui représente un stade avancé de cancer du sein et qui exprime très faiblement le gène *H19*. Nous avons transfecté cette lignée avec le vecteur d'expression pRC/CMV contenant un gène de résistance à la néomycine et dans lequel l'expression du transgène est sous le contrôle du promoteur du cytomégalovirus. Le transgène, qui correspond à celui utilisé par Hao et ses collaborateurs en 1993, est une séquence de 6 kpb d'ADN génomique de *H19* contenant la séquence complète du gène, 52 pb de la séquence en amont du site d'initiation de la transcription et 3140 pb de la séquence en aval du site de polyadénylation. Toutes les analyses réalisées dans la suite de notre travail ont été menées à la fois sur la lignée parentale (MDA), sur 2 clones transfectés avec le vecteur non recombiné (MDA-Neo) et sur 2 clones transfectés avec le vecteur recombiné par la séquence *H19* et surexprimant le transgène (MDA-H19).

Nous avons étudié les effets de la surexpression du gène *H19* sur la croissance des MDA-MB-231. Ainsi, les différentes lignées ont été cultivées, soit en absence, soit en présence de 5 ou 10 % de sérum de veau fœtal et leur croissance a été mesurée par comptage cellulaire et par incorporation de thymidine tritiée. Dans toutes ces situations, les cellules MDA-H19 croissent à la même vitesse que les témoins. Nous avons confirmé ces résultats en montrant que la répartition des cellules dans les différentes phases du cycle est identique dans toutes les lignées, transfectées ou non. Nous avons ensuite évalué la clonogénicité d'abord sur fond de boîte, puis en absence d'ancrage (agar mou). Sur fond de boîte, nous avons obtenu le même nombre de colonies quelle que soit la lignée; en revanche en agar mou les cellules MDA-H19 forment trois fois plus de colonies que les témoins, colonies qui sont également plus volumineuses, mais sans différences

morphologiques apparentes. La capacité de croître en absence d'ancrage étant généralement liée à l'acquisition d'une plus grande tumorigénicité des cellules, nous avons poursuivi notre étude par des injections sous-cutanées des différentes lignées dans des souris *scid* et suivi l'évolution des tumeurs. Quel que soit le type de cellules inoculé, les souris développent des tumeurs, néanmoins l'apparition de tumeurs palpables est plus rapide lorsque les souris sont injectées avec les cellules MDA-H19. Ainsi, le jour du sacrifice les tumeurs MDA-H19 ont un diamètre deux fois plus grand que celui des tumeurs induites par les cellules témoin. Du point de vue histologique, toutes les tumeurs sont constituées de cellules indifférenciées, donc de haut grade. Enfin, par hybridation moléculaire *in situ*, nous avons observé que le gène *H19* est seulement, mais alors fortement, exprimé dans certaines cellules des tumeurs induites par les cellules MDA-H19. Dans les tumeurs témoin, aucune expression du gène n'est détectée, ce qui est en accord avec le très faible niveau de son expression dans ces cellules avant leur injection. En accord avec ces données, dans les cellules MDA et MDA-Neo les transcrits *H19* ne sont détectés que par RT-PCR.

En conclusion, l'ensemble de ces résultats indique qu'une surexpression du gène *H19* accroît le phénotype tumorigène des cellules transfectées, ce qui confère des propriétés oncogéniques au gène dans les cellules de cancer du sein. De plus, il semble qu'il exerce ce pouvoir délétère non pas en augmentant les capacités prolifératives des cellules, mais plutôt en augmentant leur capacité à survivre en absence d'ancrage.

Article 2

Overexpression of an ectopic H19 gene enhances the tumorigenic properties of breast cancer cells

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ABSTRACT

The maternally expressed *H19* gene is transcribed in an untranslated RNA functioning as a riboregulator. We have previously reported that in about ten percent of breast cancers this transcript accumulates in epithelial cells. To gain further insight into the consequence of an overexpression of the *H19* gene upon the phenotype of human breast epithelial cells, we investigated the oncogenic potential of abundantly expressed RNA from an ectopic genomic *H19* sequence, stably transfected. The abundance of *H19* RNA did not modify proliferation capacity, timing of cell cycle phases, apoptotic events and anchorage-dependent ability of *H19*-transfected clones. On the contrary, in anchorage-independent growth assay the *H19*-recombinated cells formed more numerous and larger colonies in soft-agar *versus* control cells. To explore this phenotypic change, we analyzed the tumor development after subcutaneous injection of *H19*-recombinated cells into *scid* mice. The results show that the *H19* gene overexpression promotes tumor progression. These data support the proposal that an overdose of *H19* transcript is associated with a more tumorigenic phenotype of cells, conferring oncogenic properties to the *H19* gene in breast epithelial cells.

INTRODUCTION

The mouse *H19* gene was discovered in 1984 by virtue of its coordinate regulation with the alpha-fetoprotein gene under the control of the two loci *raf* and *Rif* (1). Its human counterpart is located at 11p15.5 within a cluster of imprinted genes and in close proximity of the *IGF2* gene (2). These two genes are oppositely imprinted, *IGF2* being only paternally expressed (3) while *H19* is transcribed exclusively from the maternal allele (4). *H19* is highly expressed in the extraembryonic tissues (placenta), in the embryo proper and in most of fetal tissues but is repressed or dramatically reduced after birth (5, 6). Only few adult organs such as cardiac and skeletal muscles (1), lung (6), uterus (7, 8) and mammary gland (9, 10) retained a basal level of expression.

The *H19* gene is transcribed by the RNA polymerase II in a capped, spliced and polyadenylated RNA. This transcript, which is localised in the cytoplasm and has recently been demonstrated as being associated with polysomes (11) is untranslated. Although, a 26 kDa protein has been obtained after transient transfections of construct carrying deletions and/or point mutations, the 5' untranslated region prevent the *H19* RNA from being translated *in vivo* (12). Moreover, the comparison between sequences of the mouse and human gene did not show any conserved open reading frames (ORF) despite an overall 77 % sequence homology (13). Theoretical translation of seven supplementary *H19* mammals sequences confirmed this absence of conserved ORF of any size but essentially the alignment of these sequences established the conservation of secondary structure at the RNA level (14). Finally, it has been largely admitted that the *H19* transcript belongs to the growing family of mRNA-like non-coding mRNA (15).

At the present time, the role of the *H19* gene is still controversial. Firstly, by a differential screening between cDNA of Syrian Hamster embryo (SHE) cells that have either retained or

lost the ability to suppress tumorigenicity of a highly malignant cell line in cell hybrid experiments, the *H19* gene has been found to be associated with tumor suppression (16). This putative tumor suppressor role was further proposed when Hao *et al.* (1993) stably transfected the *H19* gene in two embryonic tumors cell lines and showed a growth retardation of the both cell lines as also an abrogation of anchorage-independent growth and of tumorigenicity of one of these cell lines (17). In SHE cells, the reexpression of *H19* by stable transfection of the same construct as previously had little effect on cellular growth *in vitro* but did retard tumor growth in nude mice (18). In parallel, in non-experimental models the inactivation of *H19* in Wilms' tumor is in agreement with a tumor suppressor role (19, 20).

On the contrary numerous other types of cancers such as breast (9, 10, 21), bladder (22, 23, 24), lung (9, 25), esophageal (26), cervical carcinomas (27) exhibit an *H19* overexpression. More importantly, in bladder carcinomas *H19* expression was statistically correlated with tumor grade and suggests that *H19* may have oncogenic properties in this cancer (22, 24). Likewise, expression of the *H19* gene was observed in tumors induced by bladder cancer cell lines without detectable *H19* mRNA before injection in *nude* mice (23). The same results were obtained with choriocarcinomas cell lines (JEG-3), but contrarily to the cells isolated from the tumors produced by bladder cancer cells which loosed *H19* gene expression after three passages of *in vitro* culturing, the cells isolated from the JEG-3-derived tumors retained high *H19* gene expression even after fifteen passages. Not only the high level of *H19* mRNA did not alter the growth rate and the independent-anchorage growth of these cells, but also these cells were more tumorigenic than the original cells (28, 29). In HeLa x normal human fibroblast hybrids, it has also been shown that *H19* was specifically expressed in the spontaneous tumorigenic segregants from non-tumorigenic hybrids. Nevertheless, stable transfections of *H19* in the non-tumorigenic hybrids did not allow *in vivo* tumor growth in *nude* mice indicating that *H19*, although necessary to the tumorigenic process, did not

induced it (30, 31). Finally, in numerous models data argue in favor of an implication of the *H19* gene during tumorigenesis and contradict a tumor suppressor role of this gene.

In a previous study, we have demonstrated that *H19* was overexpressed in more than 70 % of breast adenocarcinomas and that this overexpression was preferentially localised in the stromal cells and more rarely in epithelial cells. Although the few number of cases where *H19* was activated in the sole epithelial cells did not allow a statistically correlation, we noticed that this pattern was associated with the absence of hormone receptors and the death of patients within the 5 years after tumorectomy (21). The overexpression of the *H19* gene in epithelial cells of about 10 percent of breast carcinomas and the controversial data about an oncogenic or anti-oncogenic role prompted us to evaluate the *H19* gene status in breast cancer. Consequently, we have stably transfected MDA-MB-231 breast cancer cells with the genomic sequence of the *H19* gene and investigate the effects of this *H19* overexpression on the growth rate, the clonogenic ability and the tumorigenicity of these cells.

MATERIALS AND METHODS

Cell culture

MDA-MB-231 breast cancer cells were originally obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in Minimal Essential Medium (MEM) supplemented with 20 mM Hepes, 2g/l sodium bicarbonate, 2 mM L-glutamine, 100 U/ml of penicillin-streptomycin, 50 µg/ml gentamycin, 1 % of non-essential amino acids (Biowhitaker) and 5 % of fetal calf serum (GIBCO-BRL) in a humidified, 37 °C, 5 % CO_2 incubator.

Stable transfection

MDA-MB-231 cells overexpressing *H19* were obtained by stable transfection using the expression vector pRC/CMV (Invitrogen) made by ligating a 6kbp genomic sequence containing the entire *H19* gene under the constitutive control of the Cytomegalovirus promoter. MDA-MB-231 cells were transfected using *ExGen 500* (Euromedex) according to the manufacturer's instructions. Twenty-four hours before transfection, cells were plated into 100 mm dishes at a density of 1.5×10^5 cells per dish. Forty-eight hours after transfection, the cells were diluted and divided into new culture dishes. Cells were selected by growing for fifteen days in medium containing 1000 µg/ml G418 sulfate (GIBCO-BRL). Cell colonies were isolated by trypsinization onto small squares of sterile filter paper. Clones harboring desired foreign DNA were selected by PCR. All experiments were performed in the absence of G418 after culture without G418 for one week.

DNA isolation and PCR

Confluent cells in monolayer were trypsinized, washed with PBS and lysed in TNE solution (Tris-HCl 10 mM, NaCl 100 mM and EDTA 1 mM). Genomic DNA was extracted using

proteinase K treatment (200 µg/ml, 30 min at 37° C) and phenol/chloroform extraction. For PCR, 50 ng of genomic DNA were used as template with 1 unit of AmpliTaq Gold (Applied Biosystems), 25 pmol of *H19* primers and 1 pmol of *TBP* (TATA Binding Protein) primers. The *H19* primers used were P2F, P3R previously described (32) and the *TBP* primers were: gF1, 5'- AATGCCTGTCAGTCTTTTCTCC -3' and gB1, 5'- AGGAAACTTCACATCACAGC -3' (GIBCO-BRL). DNA was first denatured for 7 min at 95° C, and then amplified by 40 cycles of 95° C for 1 min, 58° C for 1 min, 72° C forl min and finally extended for 7 min at 72° C. PCR products were electrophoresed through a 3 % agarose gel and visualised with ethidium bromide under UV light.

Northern blot analysis

Total RNA was extracted using the guanidium isothiocyanate-CsCl gradient method (33). RNA (20 µg) was denatured, electrophoresed through a 1.2% agarose gel containing formaldehyde and transferred by capillarity onto a nitrocellulose membrane (hybond-C-extra, Amersham). After baking 2 hours at 80° C, the membrane was hybridize at 42° C with α^{32}P-dCTP-labeled random primed cDNA probes (Megaprime Labeling System, Amersham). The *H19* cDNA probe was a 1.3 kbp *Pst* I digested fragment containing the end of the first exon and the exons 2 to 5. A GlycerAldehyde-3'-Phosphate DeHydrogenase (GAPDH) cDNA probe consisting of a 1.2 kbp *Pst* I digested fragment was also used to normalize *H19* signal.

Semi-quantitative RT-PCR

RNA was isolated as mentioned above and treated for 1 h with an amplification grade Rnase free-Dnase I (GIBCO-BRL). cDNA was obtained by Reverse Transcription of total RNA (5 µg) using Murine Leukemia Virus Reverse Transcriptase (Applied Biosystems). Aliquots of cDNA (1/20^{e}) were used for PCR amplification using AmpliTaq Gold (Applied Biosystems).

For semi-quantitative analysis *β-Actin* was co-amplified as an internal control. cDNA was first denatured for 7 min at 95° C, then amplified using 37 cycles of 30 s at 95° C, 30 s at 58° C and 30 s at 72° C, with a final extension of 7 min at 72° C. The forward and reverse primers (GIBCO-BRL) and expected size of the PCR products were: for *IGF2*, (25 pmol each), 5'-TCGATGCTGGTGCTTCTCACCTT -3' and 5'- TGGACTGCTTCCAGGTGTCATAT -3'; 345 bp and for *β-Actin* (1.5 pmol each): 5'- CACAGCAAGAGAGGCATCCT -3' and 5'-GTTGAAGGTCTCAAACATGA -3'; 219 bp. PCR products were electrophoresed through a 2 % agarose gel and visualized with ethidium bromide under UV light.

To examine the growth rate on plastic surfaces, 35 mm diameter dishes were inoculated with 1.10^5 cells per dish in 2 ml of medium containing various percentage of Fetal Calf Serum (FCS). The medium was changed every 48 h. Daily over 5 days, cells were trypsinized and counted using an hemocytometer.

Cell cycle analysis

Cells grown in chamber slides were fixed for 10 min in cold methanol at -20° C and rinsed with PBS. After hydrolysis in 5 N HCl for 1 h at room temperature, cell DNA content was Feulgen stained for 1 h with Schiff reagent. Image analysis of 300 randomly selected nuclei from each sample in duplicate was performed using a SAMBA 200 (Alcatel- TITN-France). The proportion of cells in G0/G1, S, G2/M phases was determined on computerized integrated optical density histograms.

Clonogenicity

Clonogenic ability on plastic surfaces was evaluated by plating 5.10^2 cells in 60 mm diameter dishes. The medium was changed every 48 h and after one week, cells were fixed with a 4 % ParaFormAldehyde (PFA)/Phosphate Buffer Saline (PBS) solution, rinsed twice with PBS

and finally stained with hematoxilin. Colonies consisting of more than 20 cells were counted using a light microscope.

Clonogenic ability in semi-solid medium was evaluated by seeding 5.10^4 well-separated cells in 0.37 % bacto-agar/DMEM (Dulbecco's modified MEM) medium containing 10 % of FCS on a bottom layer consisting of the same medium containing 0.56 % of bacto-agar (DIFCO). After one week, medium (DMEM-10 % FCS) was added and colonies of more than 20 cells were counted on the 15^{th} day.

Tumorigenesis in *scid* mice

Exponentially growing cells were harvested by trypsinization, washed with serum and antibiotic-free medium and resuspended in the same medium at the rate of 2.10^6 cells per 100 µl. Female BALB/cJHanHsd-*scid* mice of 6-8 weeks old were pursached from HARLAN FRANCE and kept under pathogen-free conditions. For each cell line, 2.10^6 cells were subcutaneously injected into the flank of 6 mice. Tumor diameters were measured with a caliper once a week and each mouse was sacrificed two month after injection. Tumors were removed and mice were macroscopically and microscopically analysed for the presence of metastasis in lung and liver.

***In situ* hybridization**

Soft-agar colonies and tumor were fixed in 4 % PFA/PBS, washed with PBS, dehydrated through increasing ethanol concentrations, embedded in paraffin and serially cut. Seven µm sections were transferred onto SuperFrost coated slides (Polylabo), incubated one week at 37° C and stored at 4° C until use. Sections were deparaffined with toluene, post-fixed with a 4 % PFA/PBS solution and treated with proteinase K. Slides were acetylated to reduce non-specific binding, dehydrated through increasing concentration of ethanol and hybridized with

^{35}S-labeled riboprobes (34). To obtain the riboprobes, a pSP64 vector containing a sense or antisense *Stu* I *H19* cDNA fragment were linearized by *Hin*d III, submitted to *in vitro* transcription in the presence of ^{35}S-CTP and reduced to an average 150 bp length. After hybridization, slides were dipped in NTB2 nuclear track emulsion (Kodak) and exposed for 3 weeks at 4° C. Following development and staining with the intercalating agent Hoechst 33258, slides were analysed with epifluorescence for nuclei visualization and a dark-field condenser for silver grain detection using an Olympus BH2 photomicroscope.

In parallel, one section of each tumor sample was colored with hemalun-eosin.

RESULTS

Isolation and characterization of clones overexpressing the *H19* gene.

To test the role of *H19* gene in breast cancer, the MDA-MB-231 cell line was transfected with a vector containing the entire genomic *H19* gene sequence placed under the control of the constitutive Cytomegalovirus promoter (pRC/CMV-H19 vector). G418-resistant clones derived from either the control vector- or the *H19*-transfected cells were isolated and expanded for further analysis. The presence of the transfected DNA in their genome were verified by PCR (Figure 1A), and the expression of the *H19* transgene were examined by Northern Blot. Typical Northern blot data is shown in Figure 1B. The *H19*-transfected clones (H19 S14 3 and H19 S14 4) exhibited a high level of the expected 2.3 kb transcript which corresponds to the correctly spliced and polyadenylated *H19* mRNA. By contrast, no *H19* signal was detected in parental (MDA) and control vector-transfected cells (Neo 2 and Neo 3), suggesting that *H19* transcripts were poorly represented in these cells (Figure 1B). Nevertheless, *H19* mRNA was not completely absent in these cells since *H19* expression became detectable after the potent RT-PCR technique (data not shown). In other respects, it has been demonstrated that *H19* negatively regulate the transcription and the traductibility of the *IGF2* gene (11, 35). To validate this regulation in our model, we have evaluated the *IGF2* mRNA level in the transfectants. As it was expected, the overexpression in the *H19*-recombinated cells induced a decrease of the *IGF2* transcription level of MDA-MB-231 (Figure 1C).

Growth characteristics of the *H19*-transfected MDA-MB-231cells.

When MDA-MB-231 were transfected with the vector alone or the *H19*-vector, no morphological changes of the cells were observed. To evaluate the effect of an overexpression

of the *H19* gene on the growth of MDA-MB-231 cell line, we examined the proliferation capacity of the various cell lines (parental, control vector-transfected and *H19*-transfected). Figure 2 shows that, in 5% fetal calf serum (FCS) containing medium, the *H19*-transfected cell lines grew at the same rate as the both parental and mock-transfected cell lines, doubling time being of about 24h for each cell line. The same results were obtained when the cells were grown in medium containing either 10% or 0% of FCS (data not shown). These results were further confirmed by [^{3}H]-thymidine incorporation assays (data not shown). After stoechiometric Feulgen staining of total DNA content and measures of the integrated optical density by image analysis, we evaluated the distribution of the cells in the G1, S and G2/M phases. Table I shows that regardless of the cell line, the percentage of cells in each cell cycle phase is unchanged.

In parallel, we studied the apoptosis of these cells after Hoechst 33258 staining which allow to visualize typical apoptic features of the nucleus and we determined that the percentage of apoptotic cells for *H19*-clones was that one of control cell lines (parental and mock-transfected). Likewise, the response of MDA-MB-231 cells to growth factors (Basic Fibroblast Growth Factor and Nerve Growth Factor) and to growth inhibitors (Sodium Butyrate and Normal Breast Epithelial Cells conditioned medium) was not changed when the cells overexpressed the *H19* gene (data not shown). All these data indicate that an overexpression of the *H19* gene does not modify the growth characteristics of MDA-MB-231 cell line.

Clonogenic ability of *H19*-transfected cell lines.

One week after plating the cells at a very low density, we evaluated the percentage of colonies formed for each cell line. Figure 3A shows that both the vector alone- and the *H19*-transfected cell lines exhibited the same clonogenic ability as the parental cell line. To get thoroughly in

the clonogenic capacity of the *H19*-transfected cell lines, we performed anchorage-independent growth assays. When the cells were grown in soft-agar, the *H19*-transfected cells showed a significantly 3-fold increased in colony formation compared with the parental and the vector alone-transfected cell lines (Figure 3B). In Figure 4, A to F illustrate the more rapid growth of the *H19*-transfected cells as the days progressed. On the fifteenth day, the colonies were counted and the *H19*-transfected cells exhibited much more (Figure 4G-H *versus* I) and larger colonies (Figure 4J-K *versus* L) that still overexpressed the *H19* gene, as seen by *in situ* hybridization (Figure 4M to O). No obvious difference in the morphology of individual colonies was observed.

Tumorigenicity analysis of the *H19*-transfected cells.

The anchorage-independent growth results prompted us to further investigate the effects of an *H19* gene expression change in the neoplastic process. To address this issue, we inoculated exponentially growing parental, control- or *H19*-transfected cells in *scid* mice and followed the progression of tumor genesis. Regardless of the cell line injected almost all the mice developed tumors (Table II). Mice injected with the parental and control-transfected cell lines showed a palpable tumor at a median time of 52 and 48 days after inoculation respectively, whereas only 27 days were necessary to make the same observations in the mice injected with the *H19*-transfected cells. Sixty days after injection, mice were sacrificed and the average diameter of *H19*-transfected cell derived tumors was twice that one of the control cell lines derived tumors (Table 2 and Figure 5A and B).Regardless to the cell line that was injected, no metastasis were observed in lung and liver. Histological study of the various cell-derived tumors gave, regardless of the cell lines inoculated, undifferentiated carcinoma of high grade with atypical cells having very chromatic nucleus (Figure 5C and D). Finally, by *in situ* hybridization we observed the *H19* gene expression in the tumors derived from the various

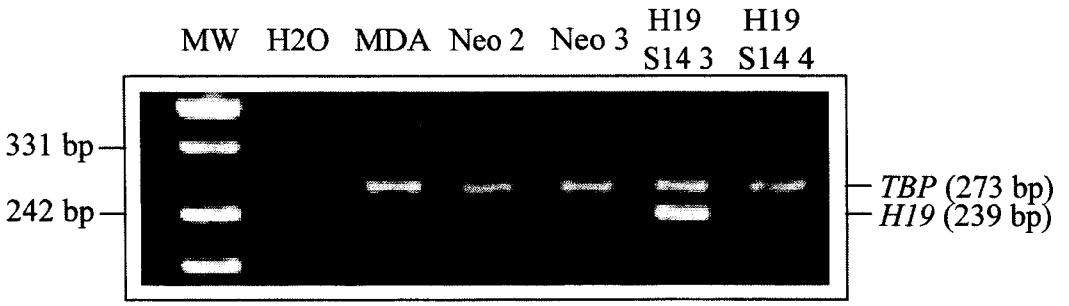
cell lines. In the parental and mock-transfected cell derived tumors, *H19* gene expression was never observed, what is in agreement with the difficulty to detect *H19* gene expression in these cells before injection (Figure 5E). By contrast, *H19*-transfected cell derived tumors exhibited a high *H19* gene expression level. In these tumors, this expression was not uniformly spread being in some cases very localized to certain parts of the tumor (Figure 5F) and more dispersed in others (Figure 5G). However, in both cases, the *H19* signal appears in epithélial cell clusters indicating that some cells only overexpressed *H19* gene whereas numerous cells have lost this expression.

Figure 1.

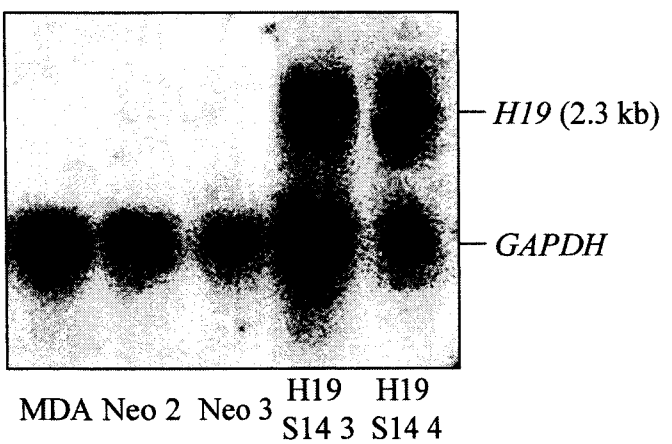
Isolation and characterization of the *H19*-transfected cell lines. A, PCR selection of *H19*-transfected cell lines harboring *H19* ectopic DNA. PCR experiments were performed using 50 ng of genomic DNA extracted from parental cell line (MDA) and from control-transfected (Neo 2, Neo 3) and *H19*-transfected (H19 S14 3, H19 S14 4) G418-resitant clones. TATA-binding protein (TBP) gene was co-amplified as an internal control. H2O lane corresponds to the same experiment performed without DNA; MW lane contains Puc19/M*sp*I molecular weight marker. B, Northen blot analysis of *H19* gene expression. Membrane was hybridized with a *H19* cDNA probe and a Glyceraldehyde-3'-phosphodehydrogenase (GAPDH) cDNA probe as an internal standard. C, Expression of *IGF2* gene by semi-quantitative RT-PCR. *β-actin* cDNA was co-amplified as an internal control. For each sample, a RT-PCR experiment was performed without Reverse transcription (-RT).

Figure 1

A


MW
H2O
MDA
Neo 2
Neo 3
H19 S14 3
H19 S14 4
331 bp
242 bp
TBP (273 bp)
H19 (239 bp)

B


H19 (2.3 kb)
GAPDH
MDA
Neo 2
Neo 3
H19 S14 3
H19 S14 4

C

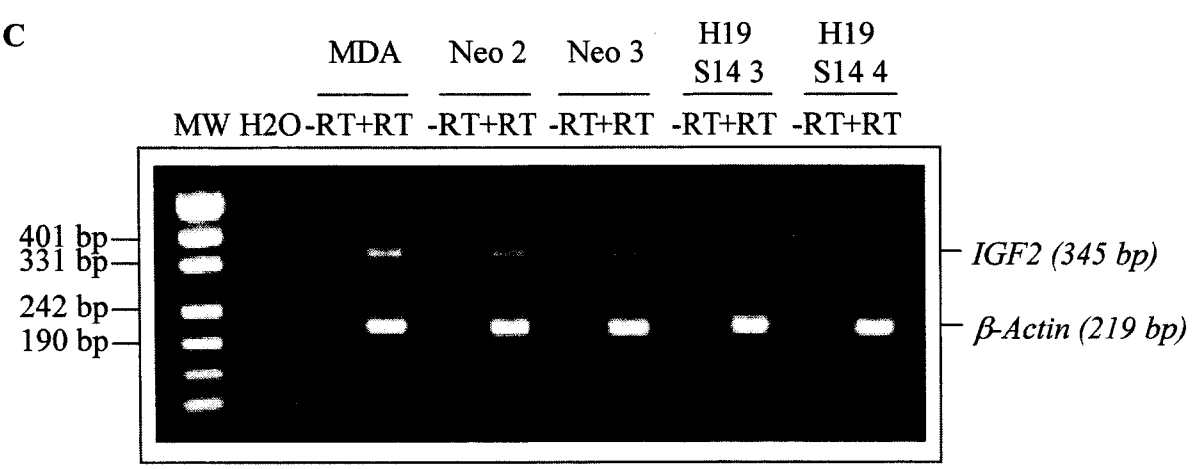

MDA
Neo 2
Neo 3
H19 S14 3
H19 S14 4
MW H2O -RT +RT -RT +RT -RT +RT -RT +RT -RT +RT
401 bp
331 bp
242 bp
190 bp
IGF2 (345 bp)
β-Actin (219 bp)

Figure 2

Effects of transfection with *H19* gene on the growth of the MDA-MB-231 cell line. Cells were plated at 10^5 cells per dish in MEM supplemented with 5% of FCS Cell number was measured daily and counted daily. ◊, parental cell line; □ and Δ, control transfected cell lines; ○ and * *H19*-transfected cell lines. Results represent the mean ± standard deviation of three independent experiments.

Figure 2

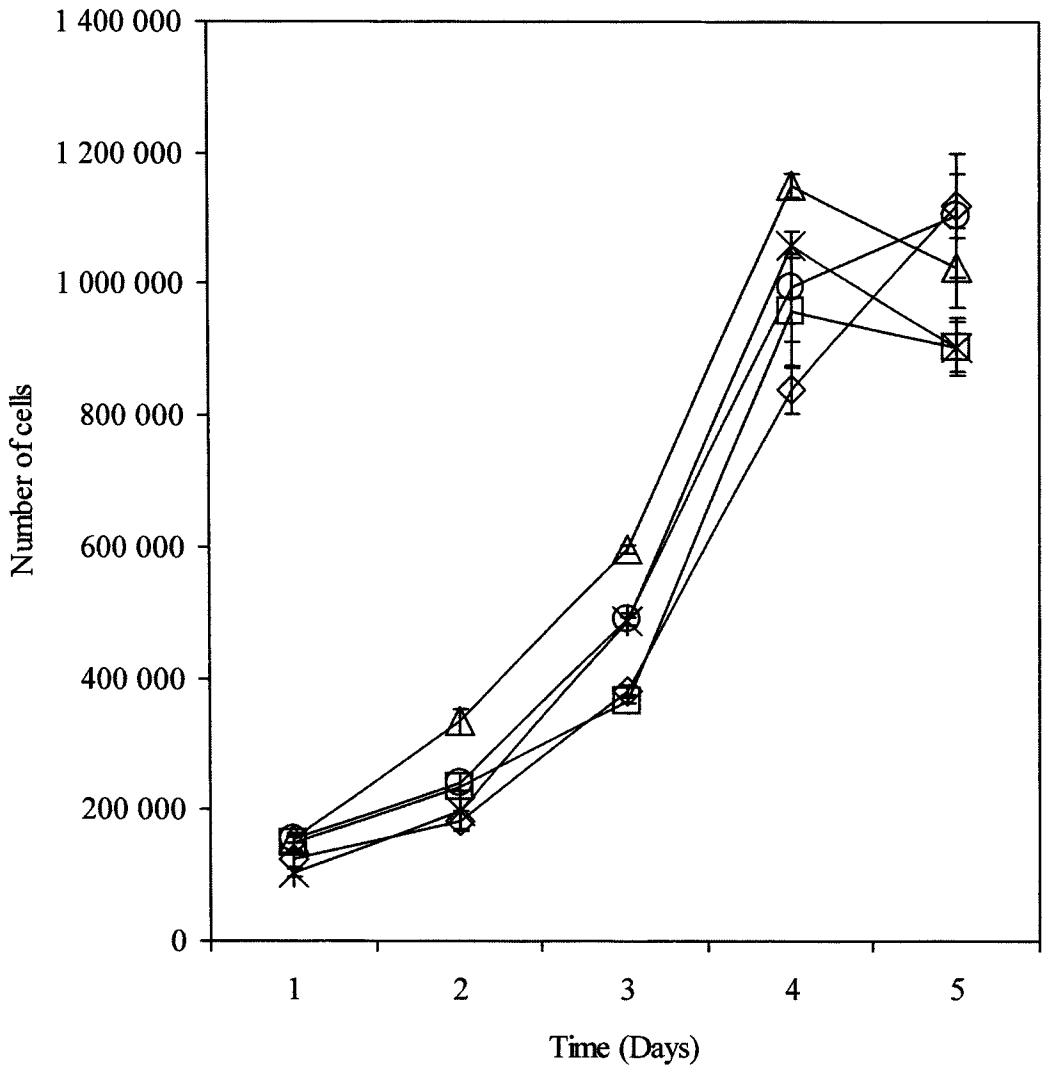

1 400 000
1 200 000
1 000 000
800 000
600 000
400 000
200 000
0
Number of cells
1
2
3
4
5
Time (Days)

Table I

Effects of transfection with the *H19* gene on cell cycle phases of MDA-MB-231 cell line.

Cell cycle phases	Controls	H19 S14 3	H19 S14 4
G1	36.4 ± 7.7	36.9 ± 5.5	42.1 ± 1.0
S	31.9 ± 0.1	29.2 ± 4.0	31.9 ± 1.2
G2/M	15.3 ± 6.8	17.0 ± 2.3	18.5 ± 0.3

The percentage of cells in each cycle phase is evaluated after Feulgen staining of total DNA and image analysis. Results represent the mean value ± standard deviation of thre experiments made in duplicate.

Figure 3

Effects of *H19* transfection on anchorage-dependent and anchorage-independent clonogenic ability of MDA-MB-231 cells. A, Cells were plated at very low density (5.10^2 cells/60-mm dishes) in MEM supplemented with 5% of FCS, so that colonies appeared one week later. Colonies consisting of more than 20 cells were counted. B, Cells (5.10^4) were seeded in soft agar and colonies of more than 20 cells were counted 15 days later. MDA, parental cell line; Neo 2 and Neo 3, control vector-transfected cell lines; H19 S14 3 and H19 S14 4, H19-recombinated cell lines. Values are the mean of four determinations performed in triplicate; bars, standard deviation.

Figure 3

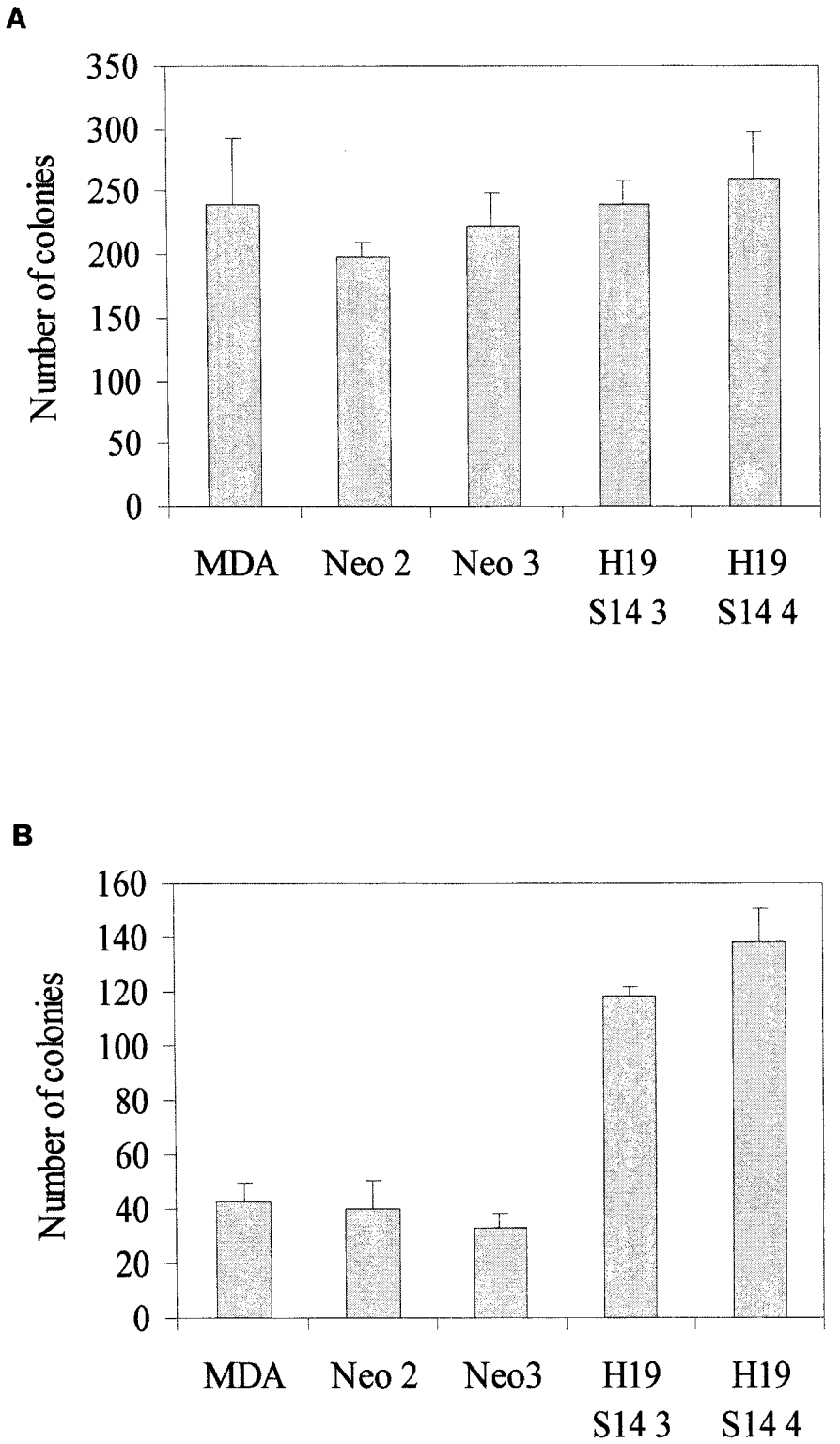

A
Number of colonies
350
300
250
200
150
100
50
0
MDA
Neo 2
Neo 3
H19 S14 3
H19 S14 4
B
Number of colonies
160
140
120
100
80
60
40
20
0
MDA
Neo 2
Neo3
H19 S14 3
H19 S14 4

Figure 4

H19-transfected cells rapidly formed numerous and larger colonies in soft agar. Light microscopy photographs on the 5th, 7th and 12th day of colonies in soft agar of control-vector transfected cells (A, B, C respectively) and *H19*-transfected cells (D, E, F respectively). G, H and I, light microscopy photographs on the 15th,of parental, control vector-transfected and *H19*-transfected cell colonies, respectively. J, K and L, light microscopy photographs, on the 15^{th,} of parental, control vector-transfected and *H19*-transfected individual colony, respectively. M, N and O, *in situ* hybridization with an antisense *H19* riboprobe on parental, control vector-transfected and *H19*-transfected colony, respectively. A to F and J to L, magnification × 100; G to I, magnification × 40; M to O, magnification × 200.

Figure 4

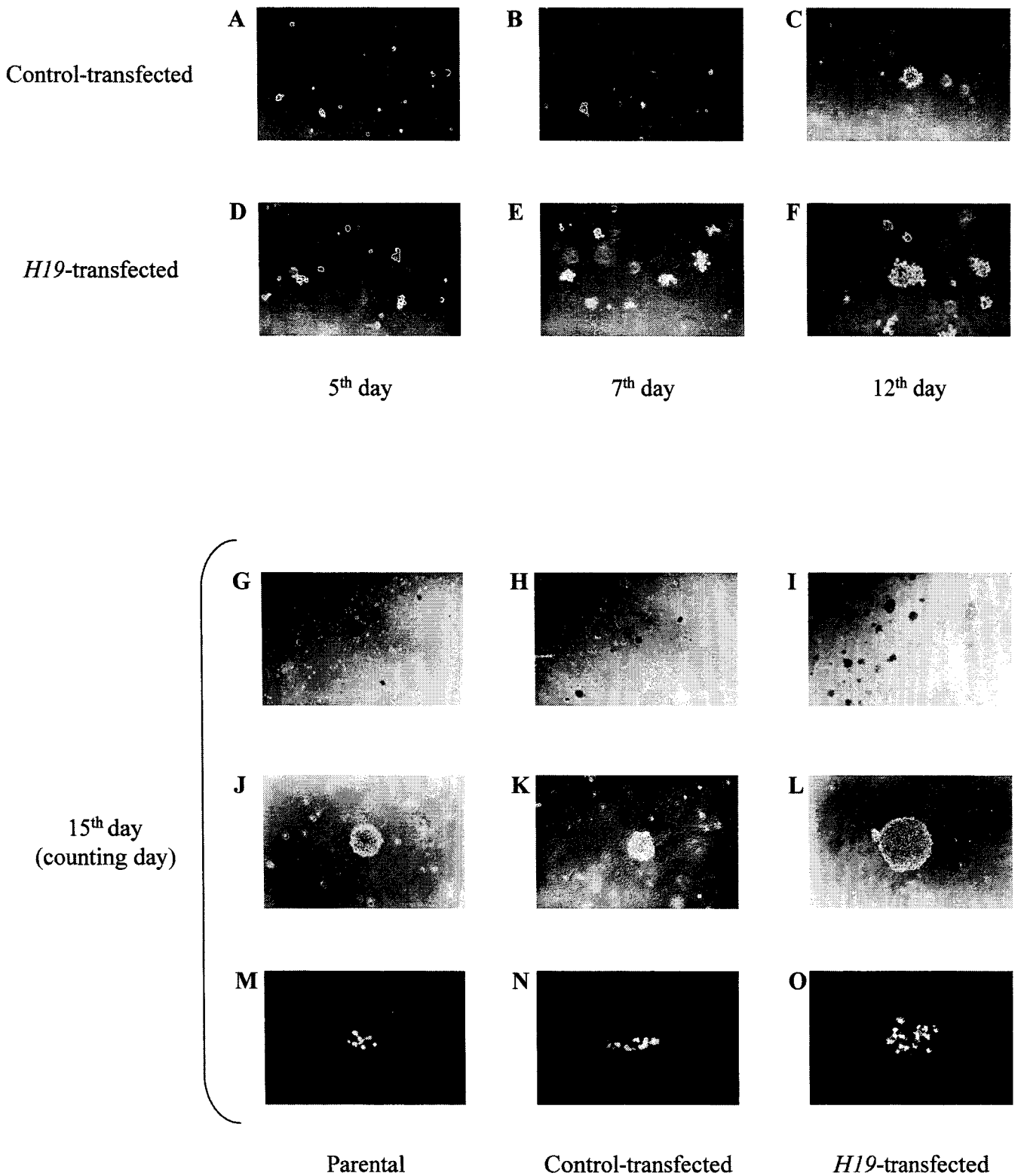

A
B
C
Control-transfected
D
E
F
H19-transfected
5th day
7th day
12th day
G
H
I
J
K
L
15th day
(counting day)
M
N
O
Parental
Control-transfected
H19-transfected

Table II

Effects of transfection with the *H19* gene on the tumorigenicity of MDA-MB-231 cell line.

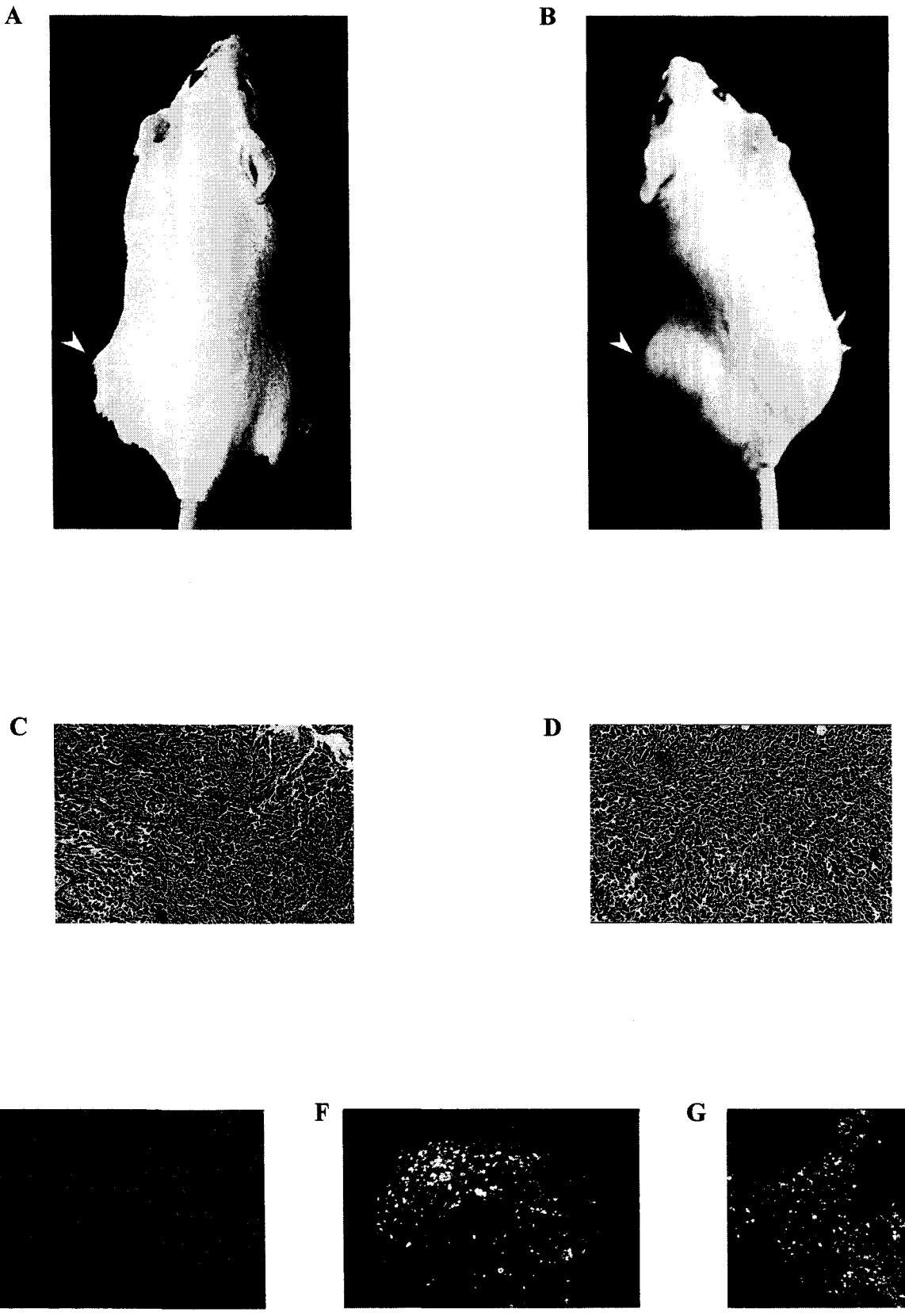
Cell lines	Tumor incidence (nb tumor/nb mice)	Median latency (Days)	Tumor diameter (mm)
Parental	5/6	52	6.0 ± 0.5
Control-transfected	5/6	48	6.2 ± 0.6
H19-transfected	6/6	27	13.4 ± 0.9

Exponentially growing cells (2.10^6) were injected subcutaneously in the flank of 6 *scid* mice for each cell line. Tumor incidence represents the number of tumor formed per number of injected mice. Median latency indicates the median day of apparition of a palpable tumor. Tumor diameter represents the mean value ± standard deviation of two diameter measures of each tumor of each one of the 6 mice.

Figure 5

Injection of *H19*-transfected cells into *scid* mice rapidly formed larger tumor. A, mice injected with control vector-transfected cells; B, mice injected with *H19*-transfected cells. Arrow head s indicate the tumor position. C and D, histological coloration of control- and *H19*-transfected cells derived tumors, respectively. E to G, *in situ* hybridization with an antisense *H19* riboprobe of control- (E) and *H19*-transfected cells (F,G) derived tumors. C and D, magnification × 140; E and G, magnification × 60.

Figure 5



DISCUSSION

The *H19* gene, which is highly expressed during embryogenesis and in subsequent fetal development, is repressed postnatally in most organs. Thus, *H19* is only marginally transcribed in adult tissues but is reexpressed in a wild range of tumors derived from tissues that expressed it during fetal life. This pattern of expression, typical of an oncofetal RNA (36), suggests that this gene would be implicated in normal development and tumorigenesis. Despite numerous years of research, the function of *H19* is not yet clearly established. Since Hao *et al.* (1993) have demonstrated that the transfection of *H19* in G401 cells was responsible for a growth retardation and a suppression of clonogenicity in soft agar and of tumorigenicity in *nude* mice (17), *H19* was considered as a tumor suppressor gene. However, in other models such as bladder carcinomas (22,24) and choriocarcinomas cells (28, 29) *H19* expression was associated with the tumorigenesis process.

In breast cancers, we have previously demonstrated that *H19* is overexpressed in about 72 % of breast adenocarcinoma. This overexpression is preferentially located in the stromal cells, however in some cases *H19* is overexpressed either in both the epithelial cells and the stromal cells or in the sole epithelial cells (21). Breast is of particular interest since it belongs to the few organs in which *H19* is not completely repressed after birth and in order to study the role of *H19* in breast cancer, we have stably transfected human cancerous mammary epithelial cells with the genomic DNA of the entire *H19* gene. The MDA-MB-231 cell line was chosen because it exhibited a very weak *H19* gene expression so that its transcript is hardly detectable in this cell line. Here, we report that when transfected with *H19*, MDA-MB-231 cells did not show any difference in growth on plastic surfaces regardless of the percentage of fetal calf serum, no more that in the distribution of cells in the various phases of cell cycle. These results are in agreement with our observations in breast adenocarcinoma where the *H19*

overexpression was not correlated with the presence of Ki-67, a marker specific of cells in cycle (21) and suggest that the *H19* gene does not play a major role in cell cycle regulation.

The *H19*-transfected cells showed unchanged anchorage-dependent clonogenic ability measured by colony formation on plastic surface but exhibited significantly increased anchorage-independent growth measured by colony formation in soft agar. As the anchorage-independent growth in semi-solid medium is a characteristic of cancerous cells, we assessed the effects of an *H19* overexpression on tumorigenicity of MDA-MB-231 cells in immune-deficient animals. Although incidence of tumors was identical when the *H19*-transfected cells were inoculated to *scid* mice, the time of appearance of these tumors was shorter and the tumors grew more rapidly. Thus, in MDA-MB-231 cells the *H19* gene behaves as an oncogene, rather than an anti-oncogene, by increasing their growth without anchorage and as xenografts in mice.

It has been reported that tumors derived from *H19*-transfected Syrian Hamster Embryo (SHE) cells lacked *H19* expression even though they retained the exogenous gene (18), consequently we analyse *H19* expression, by *in situ* hybridization, in our MDA-transfected induced tumors. We also observed that although some cells had conserved a high *H19* gene overexpression, some others had lost this expression. By comparing *in situ* hybridization with histological colorations, we established that necrosis cannot explain this absence of *H19* in certain cells. In transfection experiments, it is not rare that the expression of the transgene declines with the time and this extinction is not always solely the result of a lost of transcription. Indeed, it has been recently demonstrated that the human genome contains stable integration sites responsible for long-term expression of the transgene but also contains some unstable integration sites responsible for an elimination of the transgene from the host genome (37). Elsewhere, the *H19* gene is submitted to genomic imprinting and one of the epigenetic feature that has been found to be consistently implicated in this allele-specific expression is DNA

methylation of CpG dinucleotides. Both the upstream sequence of *H19* and the structural gene itself are differentially methylated, being hypermethylated on the silent paternal chromosome (38). Thus, the non-expression of *H19* in the *H19*-transfected derived tumors could be the result of a methylation of the ectopic sequence as it was shown in *H19*-transfected SHE cells (18). Recently, Milligan et al. (2000) have demonstrated that the modulations of expression were not only regulated transcriptionally but also post-transcriptionally. Indeed, during muscle cell differentiation the *H19* gene accumulates solely by a stabilization of its transcript (35). Consequently, the extinction of the *H19* signal in the *H19*-transfected derived tumor could be explained by a destabilization of *H19* mRNAs subsequent to the proliferation process that accompanied tumor development.

On the contrary, choriocarcinoma derived cell lines (JEG-3) have been injected in *nude* mice and the resulting tumors exhibited an increased *H19* expression compared with the cells before injection (28, 29). These results raised the possibility that *H19* expression promotes tumorigenesis or that *H19* expression is a consequence of the carcinogenic process. In HeLa x normal human fibroblast hybrid cells, it has been recently demonstrated by differential display screening that *H19* was specifically expressed in tumorigenic hybrids, however the reexpression of *H19* in these hybrids was not sufficient to restore a tumorigenic phenotype (30, 31). In our experiments the *H19*-transfected cells enhanced the tumorigenic capacity of MDA-MB-231 cells, but in parallel the formation of tumors did not induce *H19* gene expression in the parental cells. Therefore, we can conclude that in breast cancer cells *H19* expression is not necessarily concomitant to the tumorigenic process but is responsible for an increase of tumorigenesis of these cells.

Furthermore, *H19* and *IGF2* have been found to show coordinate reciprocal regulation in numerous situations. Especially, the *H19* flanking sequences are implicated in the regulation of *IGF2* imprinting in *cis* (39). Knockout experiments have shown that mice with a targeted

deletion of the *H19* normally active maternal allele have displayed only an overgrowth phenotype, a typical phenotype of an IGF2 overproduction (40, 41). A *trans*-function of *H19* in *IGF2* regulation has also been suggested since in a Wilms' tumor *IGF2* mRNA level and translatability has been found to be correlated with the *H19* mRNA level (11). This *trans*-function was further confirmed in hepatocellular carcinoma cells where *H19* sense and antisense transgenes modulate the *IGF2* mRNA levels (42). All these data raised the expectation that the role of *H19* in the tumorigenesis would be the consequence of its effect on *IGF2* expression. In this report, we show that the overexpression of *H19* in MDA-MB-231 cells caused a decrease of the *IGF2* mRNA level and confirmed, in breast cancer cells, the negative *trans*-riboregulator function of *H19* on *IGF2* expression. Moreover, this down-regulation of *IGF2* expression associated with the increased tumorigenicity of *H19*-transfected MDA-MB-231 cells in *scid* mice argued against an oncogenic activity of *H19* in breast cancer cells via an upregulation of *IGF2*.

Elsewhere, since the *H19* transcript is an mRNA-like non-coding RNA acting as a riboregulator, we have performed a proteomic study to investigate protein pattern changes in *H19*-overexpressing cells. After two-dimensional electrophoresis and mass spectrometry analysis, we have identified the thioredoxin as being positively regulated and shown that this regulation occurred at the post-transcriptional level (43). The thioredoxin, is a key protein of the reduction-oxidation metabolism (44) and it has been demonstrated that thioredoxin-transfected breast cancer cells showed unaltered growth on plastic surfaces, but exhibited severalfold increased colony formation in soft agar. Moreover, these cells transfected with a redox-inactive mutant of thioredoxin inhibited almost completely the tumor formation when they are inoculated in immune-deficient mice (45). Collectively, these statements lead us to propose that the increased level of thioredoxin in *H19*-transfected MDA-MB-231 cells could be involved in the neoplastic phenotypes exhibited by our *H19*-recombinated clones.

In conclusion, although it has been shown a tumor suppressor role in some models, this report established that the *H19* gene has an oncogenic status in breast cancer cells. As we and others have demonstrated that the *H19* gene regulate positively or negatively the expression of other genes at the transcriptional or post-transcriptional levels, we can speculate that the observed net effect of *H19* overexpression depends upon the status of these target genes and therefore is dependent on the cell types and of the developmental stage of the cells under study.

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Chapitre III

Etude de la fonction riborégulatrice de l'ARN H19

Bien que le transcrit du gène *H19* ait toutes les caractéristiques d'un ARN messager, aucune protéine H19 n'a pu être détectée *in vivo*. Ainsi, la forte conservation de la séquence et celle de structures secondaires de l'ARN *H19* au cours de l'évolution, associées à l'absence de conservation des ORF suggèrent que le produit fonctionnel du gène *H19* est son transcrit maturé, de conformation bien définie, agissant donc en tant que riborégulateur. Dans le but de préciser cette fonction, nous avons recherché d'éventuelles protéines dont le taux pouvait varier, donc être régulé par l'abondance des ARN *H19*. Pour mener à bien cette étude, nous avons réalisé une analyse protéomique des cellules MDA, MDA-Neo et MDA-H19, décrites dans la deuxième partie de ce mémoire.

Article 3

Après un marquage métabolique par la ^{35}S-méthionine et la ^{35}S-cystéine, les cellules sont lysées et les protéines sont séparées par électrophorèse bidimensionnelle. Une analyse informatisée des cartes protéiques ainsi obtenues, nous a permis de mettre en évidence un spot protéique, significativement augmenté, dans les cellules MDA-H19. Grâce à la

comparaison du patron des protéines de nos gels avec des banques de données de cartes protéiques, le point isoélectrique de cette protéine a été estimé à environ 5 et sa masse moléculaire à environ 12 kDa. Afin de caractériser la protéine régulée positivement par le gène *H19*, le spot est excisé de gels bidimensionnels colorés au nitrate d'argent, et les peptides issus de la digestion trypsique du spot sont analysés par spectrométrie de masse. Les données accumulées concernant la protéine (point isoélectrique, masse moléculaire, empreinte peptidique obtenus par spectrométrie de masse de type MALDI-TOF et micro-séquences obtenues par spectrométrie de masse en tandem) ont alors été comparées avec les banques de données. Nous avons pu ainsi identifier, sans ambiguïté, que la protéine, dont le taux est nettement augmenté dans les cellules MDA-H19, est la thiorédoxine (Trx).

Par Western blot, nous avons confirmé que le taux de Trx est bien accru dans les cellules MDA-H19. De plus, étant clairement établi que la Trx augmente la fixation du facteur de transcription NF-κB sur l'ADN (Hirota *et al.*, 1999), nous avons réalisé des transfections transitoires du gène rapporteur de la *luciférase*, dont l'expression est sous le contrôle de sites consensus NF-κB. Nous avons ainsi observé que l'augmentation de la Trx dans les cellules MDA-H19 est corrélée avec une augmentation de l'activité de NF-κB. Enfin, par RT-PCR semi-quantitative, nous avons montré que contrairement au taux de protéine Trx, le taux des ARNm *Trx* est inchangé dans les cellules surexprimant *H19*.

En conclusion, ce travail nous a permis de confirmer que la fonction du gène *H19* peut être remplie *via* son transcrit, qui agit donc comme un facteur *trans*, d'où le qualificatif de *trans*-riborégulateur qui lui a été attribué. De plus, nous montrons, pour la première fois à notre connaissance, que cette régulation est positive et se réalise au niveau post-transcriptionnel.

Article 3

Thioredoxin post-transcriptional regulation by H19 provides a new function to mRNA-like non-coding RNA

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Key words: *H19* gene; non-coding mRNA; riboregulator; proteomic; thioredoxin; oncogene.

Running title: The *H19* gene up-regulates thioredoxin.

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ABSTRACT

Classically, the functional product of coding genes is a protein whose synthesis is directed by an mRNA-template. However, in the last few years several genes yielding an mRNA-like non-coding RNA as a functional product have been identified (Erdmann *et al.*, 2000). In most cases these transcripts are synthesized by the RNA polymerase II, capped, spliced and polyadenylated, like classical mRNA. These latter have non-conserved open reading frames and seem to be untranslated. Consequently, it has been proposed and admitted that these genes act at the RNA level, and are so-called "riboregulators". *H19* belongs to this class of gene and its role remains a matter of debate: for some authors it is an oncogene, for others a tumour suppressor. Here, we demonstrate, using a proteomic approach, that an *H19* overexpression in human cancerous mammary epithelial cells stably transfected with genomic DNA containing the entire *H19* gene, is responsible for positively regulating at the post-transcriptional level the thioredoxin, a key protein of the cellular redox metabolism. Interestingly, this protein accumulates in many cancerous tissues, such as breast carcinomas in which we have also demonstrated an overexpression of the *H19* gene.

The *H19* gene is a paternally imprinted, maternally expressed gene (Bartolomei *et al.*, 1991; Zhang and Tycko, 1992). It is transcribed by RNA polymerase II and its transcript is spliced, polyadenylated and transported to the cytoplasm, but not translated (Brannan *et al.*, 1990). However, in transient transfections with different constructs, a 26 kDa H19 protein synthesized from the largest putative open reading frame (ORF) has been characterized after using extensive deletions and/or point mutations of the nucleotide sequence upstream of this ORF (Joubel *et al.*, 1996). Nevertheless, as the structure at the RNA level is evolutionary conserved and all the attempts to detect an H19 protein translated from the endogenous mRNA have failed, it has been proposed and admitted that the functional product of the *H19* gene is a structured RNA acting as a riboregulator (Brannan *et al.*, 1990, Juan *et al.*, 2000). *H19* gene is abundantly expressed in both extraembryonic and fetal tissues and its transcripts accumulate in tissues of endodermal and mesodermal origin (Pachnis *et al.*, 1988; Poirier *et al.*, 1991; Rachmilewitz *et al.*, 1992). After birth the gene is repressed in almost all tissues, but its transcripts remain detectable in a few adult organs, in particular mammary gland (Adriaenssens *et al*, 1999). Furthermore, *H19* gene activation has been reported in various cancer tissues (Douc-Rasy *et al.*, 1993; Dugimont *et al.*, 1995; Elkin *et al.*, 1995; Kondo *et al.*, 1995; Ariel *et al.*, 1997). Elsewhere, Cathala's group showed that in myoblastic cells, stabilization of the *H19* RNA is solely responsible for its accumulation during *in vitro* muscle cell differentiation (Milligan *et al.*, 2000). Consequently, *H19* expression is the result of a complex regulation at both the transcriptional and post-transcriptional levels. This statement should be considered when *H19* gene overexpression is observed in tumours. To date, accumulating evidence suggest that *H19* plays a role in oncogenesis, whereas other studies indicate that it possesses a tumour-suppressor activity. Collectively, these results indicate that the *H19* gene function is yet a matter of debate. Most recently, it has been proposed that *H19* participates in the repression of *IGF2*, at least in part through effects on *IGF2* transcription

(Wilkin *et al.*, 2000), alternatively, Li *et al.* (1998) reported that the *H19* RNA is associated with polysomes and may be an antagonist of *IGF2* translation.

To test if the *H19* transcript has a riboregulatory function, we investigated changes in protein synthesis in human cancerous mammary epithelial cells stably transfected with *H19* gene using high-resolution two-dimensional electrophoresis (2DE). MDA-MB-231 cells were chosen because they exhibit a very low level of endogenous *H19* mRNA, level which is undetectable by Northern blot analysis (Figure 1a, lane MDA) but can be detected by the potent semiquantitative RT-PCR technique (Figure 1b, lane MDA). This cell line was transfected with an expression vector, where 6 kbp of genomic DNA containing the entire *H19* gene was placed under the control of the cytomegalovirus promoter. In this vector, the presence of the neomycin resistance gene allowed the outgrowth of stably-transfected clones by G418 selection. By Northern blotting, we observed in *H19*-transfectants (Figure 1a, lanes H19 S14 3 and H19 S14 4) a 2.3 kb band which was absent in both the parental cell line (Figure 1a, lane MDA) and the control vector-transfectants (Figure 1a, lanes Neo 2 and Neo 3). This 2.3 kb transcript corresponds to the accurately transcribed and spliced *H19* mRNA. To quantify the rate of *H19* overexpression in these transfectants, we performed semiquantitative RT-PCR (Figure 1b) and showed that *H19*-transfected cell lines express 8- to 10-fold more *H19* transcripts than control cell lines (parental and mock-transfected).

A proteomic approach was used to investigate if the overexpression of the *H19* gene in the *H19*-transfected cells induced changes in protein synthesis. These variations in protein patterns were analysed by 2DE after incorporation of ^{35}S-labelled amino acids for 12h, and for each cell type an equal quantity of cpm was submitted to 2DE. A representative autoradiogram is shown in figure 2a. About 1,000 polypeptides were sufficiently intense and accurately defined to be analysed and localized in pH 4-8 and in a molecular mass range of 10-150 kDa. Computer analysis using the Melanie II software package allowed both the

detection and the quantification of variations in spot intensity between MDA controls and *H19*-transfected cells. As shown in figure 2, one spot was found to be significantly up-regulated in the *H19*-transfected cells (Figure 2c and 2d) compared to MDA control cells (Figure 2b). After comparison with our breast epithelial cell protein database and with reference 2DE gels of the SWISS-2D-PAGE database we have estimated the pI of this protein at about 5 and its molecular mass (MM) at about 12 kDa.

In order to identify this protein, we performed a mass spectrometry analysis of the spot. The spot excised from 2D silver stained gel was submitted to trypsin digestion and the resulting peptides were analysed by MALDI-TOF and nanoelectrospray tandem mass spectrometry (MS-MS). MALDI-TOF mass spectrometry provided a mass fingerprint of the protein shown in figure 2e whereas MS-MS was used to characterize a partial amino acid sequence of the selected peptides. One of the two MS-MS spectra obtained for the protein is shown in figure 2f. All the collected data about the protein namely isoelectric point, molecular mass, mass fingerprint and sequence tags were compiled and used for database searching. More than 65% of the peptidic fragments measured by MALDI-TOF matched the theoretical fragments with a difference between measured and calculated masses below 0.2 Da. The pI and the MM estimated are in agreement with the theoretical pI (4.82) and the theoretical average mass (11.6 kDa). The results of the database search, shown in Table 1, allowed us to unequivocally identify the protein which is up-regulated in *H19*-transfected cells as being the thioredoxin (Trx), one of the major proteins regulating the intracellular redox metabolism (Holmgren *et al.*, 1985).

The up-regulation of Trx in the *H19*-transfected cell lines was further confirmed by Western blot using a polyclonal anti-thioredoxin antibody (Figure 3a). The results established that the level of Trx is highly increased in the *H19*-transfected cells (Figure 3a, lanes H19 S14 3 and

H19 S14 4) compared to the non-transfected (Figure 3a, lanes MDA) and the mock-transfected cells (Figure 3a, lanes Neo 2 and Neo 3).

The thioredoxin system is a general protein disulphide reducing system, which includes the NADPH-dependent flavoprotein thioredoxin reductase. Trx is activated in response to oxidative stress caused by UV or X-ray irradiation or inflammatory cytokines. Depending on its degree, the oxidative stress can cause either a positive cellular response such as proliferation, or a negative response such as apoptosis (Nakamura *et al.*, 1997). Following an oxidative stress, Trx scavenges damages caused by the reactive oxygen species (Spector *et al.*, 1988; Bjornstedt *et al.*, 1994) but there is growing evidence that Trx modulates expression of various kinds of genes in regulating the DNA binding of several transcription factors (Hayashi *et al.*, 1997; Hirota *et al.*, 1997; Makino *et al.*, 1999; Ueno *et al.*, 1999). More particularly, it is well demonstrated that Trx increases transcription activity of NF-κB in enhancing its DNA binding in the nucleus (Hirota *et al.*, 1999). Consequently, to establish if the increase of Trx in *H19*-transfected is associated with an increase of its activity, we analysed NF-κB status in the control and *H19*-transfected cell lines. We performed transient transfections with a NF-κB-responsive vector containing the luciferase reporter gene under the control of thymidine-kinase minimal promoter and five NF-κB binding sites. As shown in figure 3b, while the NF-κB-dependent luciferase gene activity was identical in wild type and in vector alone-transfected MDA-MB-231 cells (MDA and Neo 2, Neo 3 respectively), this activity is significantly increased in *H19*-transfected cells (*H19* S14 3 and *H19* S14 4). This NF-κB activation can be correlated with the up-regulation of Trx.

Given that the mechanism of action of *H19* riboregulator is largely unknown, we wondered if this regulation of the Trx by *H19* acted at the transcriptional level. To address this question we determined the Trx mRNA level in the various cell lines using semiquantitative RT-PCR (Figure 3c). The relative amount of Trx mRNA was expressed at the bottom of figure 3c as a

density ratio between Trx and β-actin amplified bands. Because we clearly observed no variation of Trx mRNA synthesis in the *H19*-transfected cells, we conclude that the up-regulation of the level of Trx in *H19* overexpressing cells occurs post-transcriptionally.

The *H19* transcript is a classical mRNA transcribed by RNA polymerase II, capped, spliced and polyadenylated. However, given the evolutionary conservation of structure at the RNA level and the absence of conserved open reading frames, it is presumed that the functional product of the *H19* gene is a structured RNA (Juan *et al.*, 2000) that acts as a genetic regulator termed riboregulator (Brannan *et al.*, 1990). Both in the mouse and human, *H19* gene is submitted to imprinting and is located within a cluster of genes, which are either paternally imprinted like $p57^{kip2}$, *H19* and *KvlqtI* (Bartolomei *et al.*, 1991; Lee *et al.*, 1997; Matsuoka *et al.*, 1996) or maternally imprinted like *IGF-II* and *INS* (De Chiara *et al.*, 1991; Giddings *et al.*, 1994). Imprinting of *H19* and imprinting of *IGF-II*, its close physical neighbour, are co-ordinated and until today, the research devoted to the function of the *H19* transcript was essentially interested in its possible *cis*-implication in the establishment and/or maintenance of *IGF-II* imprinting. However, little is known about the *trans*-function of the *H19* transcript. Indeed, Li *et al.* (1998) have documented that in a Wilms' tumour the cytoplasmic level of the *H19* transcript is inversely correlated with the cytoplasmic level of *IGF-II* mRNA from one allele as well as its translatability from the other allele without being genetically linked. Except the *IGF-II* gene, which seems to be negatively regulated (Wilkin *et al.*, 2000), no other target genes of *H19* have been identified. Thus, in this report we provide evidence that *H19* acts as a *trans*-riboregulator to positively regulate at the post-transcriptional level the thioredoxin, an unexpected target considering the knowledge about *H19* gene. Genes, having as a functional product a riboregulator non-coding mRNA, have been identified in bacteria, plants and mammals, and this growing family is collected in a database (Erdmann *et al.*, 2000) (http://biobases.ibch.poznan.pl/ncRNA/). These non-coding RNAs are divided in four

categories (Erdmann *et al.*, 2001), which are gene regulators, abiotic and biotic stress signal response RNA and other RNA. Until now, the *H19* transcript has been classified within the gene regulator category. In this report, we demonstrate that *H19* regulates the thioredoxin, which is a key protein of the oxidative stress response caused not only by radiation but also by inflammatory cytokines. Thus, *H19* transcript could be included in the abiotic and biotic stress response RNA categories providing a new overview of the *H19* gene function. Moreover, published data indicate the possible involvement of Trx in the process of oncogenesis and it has been reported that this protein is expressed in tumours of several kinds (Fujii *et al.*, 1991; Kawahara *et al.*, 1996; Ueno *et al.*, 2000; Bini *et al.*, 1997; Grogan *et al.*, 2000). It is of particular interest to note that, like Trx, the *H19* gene is overexpressed in breast cancers (Dugimont *et al.*, 1995; Adriaenssens *et al.*, 1998).

In most cases, the function and/or the mechanism of action of these non coding-mRNAs are not well understood. Some of these genes are included in an imprinted domain such as *Xist*, *Tsix*, *IPW*, *KvDMR1* (Wevrick and Francke, 1997; Lee *et al.*, 1999; Smilinich *et al.*, 1999) and among them, some are transcribed as antisense mRNAs. Whereas the role of *Xist/Tsix* in chromosome X inactivation is well documented (Kelley and Kuroda, 2000), all are supposed to be implicated in imprinting processes. However, *H19* is not an antisense mRNA and its participation in *IGF-II* imprinting has been ruled out (Jones *et al.*, 1998). In Prokaryotes and in the case of antisense RNA, the common feature is that these riboregulators are involved in the recognition of nucleic acid targets *via* complementary base pairing, and this association is not always responsible for a negative regulation, but can also activate the translation by removing an inhibitory secondary structure (Lease *et al.*, 1998). This mechanism does not seem to be implicated in the *H19* riboregulation, as we did not detect primary structure complementarity between the *H19* transcript and Trx mRNA by dotplot (data not shown). In contrast to what has been claimed for many years, the *H19* transcript is associated with

polysomes in a variety of cells (Li *et al.*, 1998), and *H19* could therefore increase Trx translation in favouring the association of Trx mRNA with polysomes. Recently, *H19* has been shown to belong to a group of localized untranslated RNA (Runge *et al.*, 2000), we can also speculate that the *H19* transcript influences the translatability of Trx mRNA by controlling its subcytoplasmic localization.

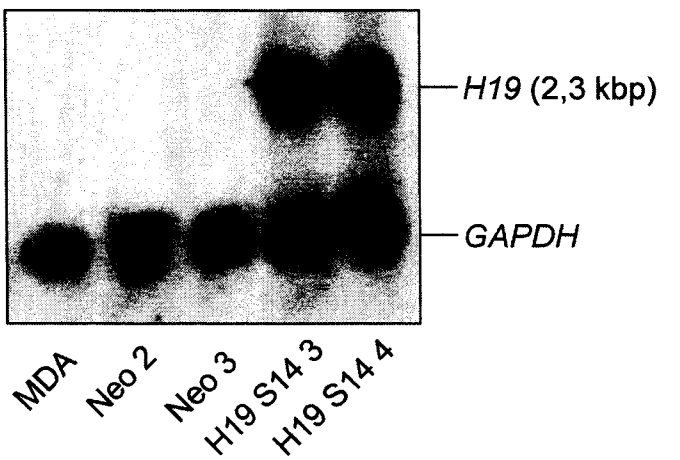
Finally, the identification of Trx as a target molecule for the *H19* gene, moreover positively regulated, allows a better understanding of the function of this riboregulator.

Figure 1

H19 gene overexpression in *H19*-transfected cells. **a**, Northern blot analysis of RNA extracted from parental (MDA), control vector-transfected (Neo 2, Neo 3) and from *H19*-transfected cell lines (H19 S14 3, H19 S14 4). Total RNA was extracted using the RNeasy Mini Kit (QIAGEN SA). RNA (20 μg) was denaturated, electrophoresed through a 1.2% agarose gel containing formaldehyde, transferred and hybridized as previously described (Adriaenssens *et al.*, 1999). **b**, Semiquantitative RT-PCR: *H19* gene was 8- to 10-fold overexpressed in *H19*-transfected cell lines compared to control cell lines. Total RNA (5 μg) was treated for 1 h at 25°C with amplification grade Rnase free-Dnase I (Life Technologies) and was reverse transcribed with 60 pmol of random hexamers, 50 mM KCl, 10 mM Tris-HCl, 3 mM $MgCl_2$, 0.5 mM dNTP, 20 U of Rnase inhibitor and with (+RT) or without (-RT) Murine Leukemia Virus Reverse Transcriptase for 45 min at 42°C. One twentieth of the cDNA reactions was amplified with 1 U of Ampli Taq Gold, 25 pmol of each primer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 50 mM KCl and 10 mM Tris-HCl (all PCR products were provided by Applied Biosystems). The primers used were as follows: for H19 amplification, 5'-CTGTTTCTTTACTTCCTCCACGG-3' and 5'-ATGAAGATGGAGTCGCCGGT-3'; and for β-actin amplification, 5'-CACAGCAAGAGAGGCATCCT-3' and 5'-GTTGAAGGTCTCAAACATGA -3'. The PCR conditions were as follows: 95 °C for 7 min, 36 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s followed by a final extension for 7 min at 72 °C. PCR products were run through a 2 % agarose gel and visualized with ethidium bromide. The molecular weight (MW) marker used was the PUC19 DNA/MspI marker (MBI Fermentas). H_2O represents the same PCR performed without cDNA.

Figure 1

a


H19 (2,3 kbp)
GAPDH
MDA
Neo 2
Neo 3
H19 S14 3
H19 S14 4

b

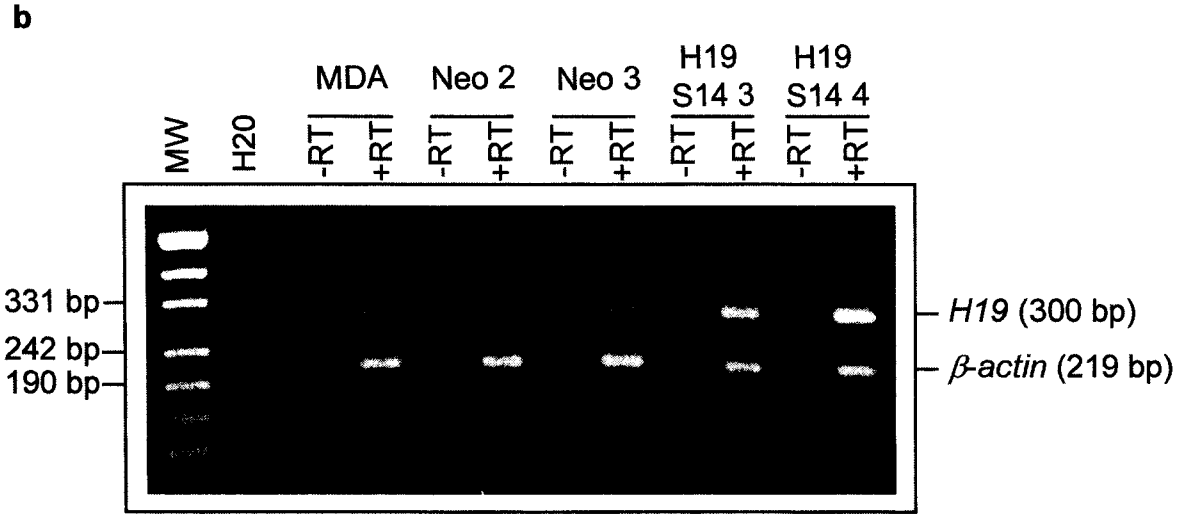

MW
H20
MDA
Neo 2
Neo 3
H19 S14 3
H19 S14 4
-RT
+RT
331 bp
242 bp
190 bp
H19 (300 bp)
β-actin (219 bp)

Figure 2

Thioredoxin is up-regulated in *H19*-transfected MDA-MB-231 breast cancer cells. **a**, 2D electrophoresis profil of ^{35}S-labelled proteins from control MDA cells. Starved cells were cultured for 12 h in the presence of ^{35}S-labelled amino acids at 50 μCi/ml and then lysed in a buffer containing 0.3 % SDS and 1 % β-mercaptoethanol. ^{35}S-labelled proteins (10^6 cpm) were separated on 2D gels with the Investigator 2D Electrophoresis System™ (Millipore) as described (Vercoutter-Edouart *et al.*, 2001). After electrophoresis, the gels were dried and exposed to autoradiography for 6 weeks. Molecular masses (in kilodaltons) and isoelectric point (pI) values are indicated. Details of 2D autoradiograms of **b**, control cells, and of H19-transfected cells: **c**, clone S14 3 and **d**, clone S14 4. Arrow-heads indicate the position of the regulated spot. Both gels and autoradiograms were scanned (scanner SM3, Pharmacia) using the Diversity One program (Pharmacia) and analysed using MELANIE II program (Bio-Rad) on a SUN-SPARC station. Molecular masses and pI were determinated after comparison with our own reference gels and with reference gels in the SWISS-2D-PAGE (Expasy) database (http://expasy.ch/). The regulated spot was excised from the silver-stained 2D gels and subjected to in-gel trypsin digestion and desalting prior to mass spectrometry analysis. Peptide fragments were analysed by MALDI-TOF (**e**) and nanoelectrospray tandem (MS-MS) mass spectrometry analyses of peaks m/z 1337.61 (**f**) and 1166.46 (data not shown) were performed as described (Vercoutter-Edouart *et al.*, 2001). T represents autolysis trypsin fragments. The sequence deduced from the MS-MS spectrum is indicated with the single letter code for the amino acids (**f**). Database searching performed using Peptident (http://www.expasy.ch/tools/peptident.html) allowed the identification of thioredoxin, as reported in Table 1.

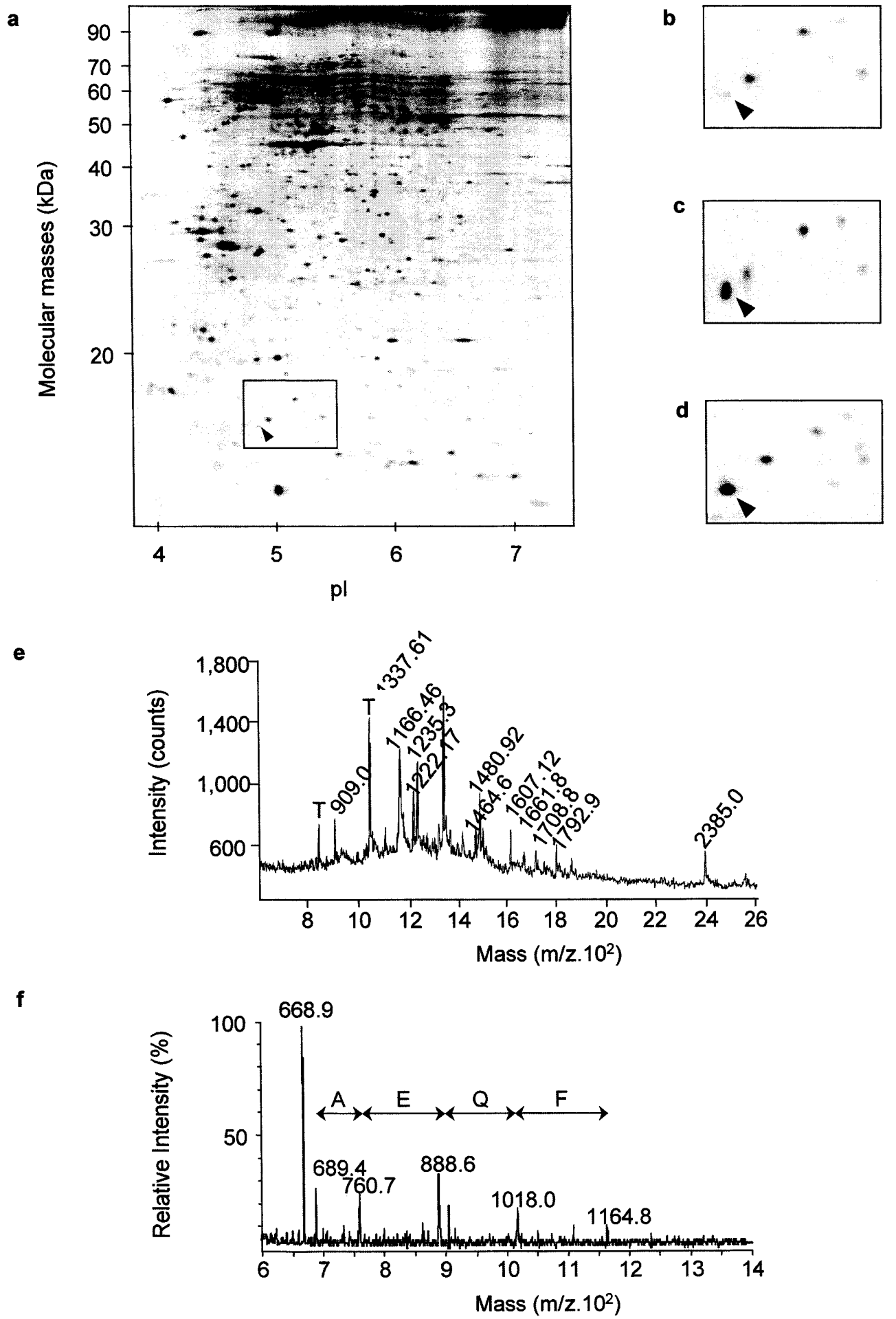

a
b
c
d
Molecular masses (kDa)
90
70
60
50
40
30
20
4
5
6
7
pI
e
Intensity (counts)
1,800
1,400
1,000
600
T
T
T
909.0
1337.61
1166.46
1235.3
1222.17
1480.92
1464.6
1607.12
1661.8
1708.8
1792.9
2385.0
8
10
12
14
16
18
20
22
24
26
Mass (m/z.10²)
f
Relative Intensity (%)
100
50
668.9
A
E
Q
F
689.4
760.7
888.6
1018.0
1164.8
6
7
8
9
10
11
12
13
14
Mass (m/z.10²)

Table 1 Mass spectrometry identification of Thioredoxin

m/z measured	m/z theoretical	Start-End amino acids	Peptide sequences
909.00	908.98	85-93	VGEFSGANK
1166.46	1166.27	85-95	V**GEFS**GANKEK
1222.17	1222.34	82-93	GQKVGEFSGANK
1337.61	1337.42	8-20	TA**FQEA**LDAAGDK
1464.60	1464.76	36-47	MIKPFFHSLSEK
1480.92	1480.76	36-47	MIKPFFHSLSEK [a]
1607.12	1606.94	72-84	CMPTFQFFKKGQK [a]
1661.80	1662.02	72-84	CMPTFQFFKKGQK [b]

a : Methionine oxydation

b : Cysteine alkylation by acrylamide

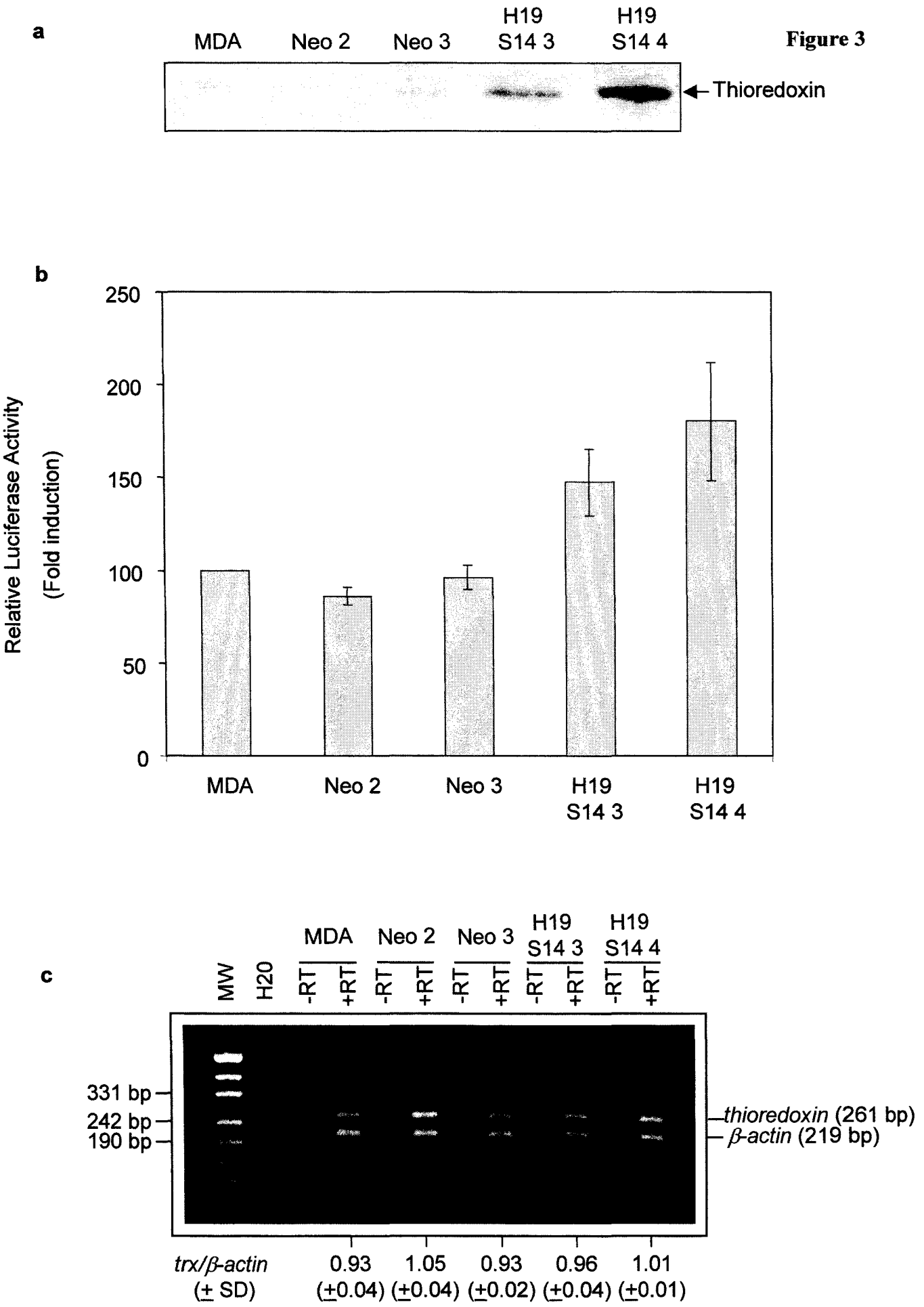
The tryptic fragment masses (in Daltons) that were obtained and their corresponding match to theoretical masses are listed. The amino acid sequences were deduced from the tryptic peptides after database searching. The sequence tags obtained by nanoelectrospray MS-MS are indicated in bold characters.

Figure 3

H19 regulates the thioredoxin at the post-transcriptional level. **a**, Thioredoxin levels which are weak in wild-type MDA-MB-231 (MDA) and in control vector-transfected cells (Neo 2, Neo 3), are up-regulated in *H19*-transfected cells (H19 S14 3, H19 S14 4). Cells were seeded in 100 mm dishes at 500,000 cells/dish and were starved for 24 h at 60 % of confluence. Proteins were extracted into ice-cold buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1 % Nonidet P-40 and protease inhibitors). After SDS-PAGE and transfer onto nitrocellulose membranes, Western-blot analysis was performed using a polyclonal anti-thioredoxin antibody (1/1,000 overnight at 4 °C, Sigma). The membrane was then incubated for 1 h with a Horseradish peroxidase-conjugated secondary antibody (Santa Cruz) and detection was performed by enhanced chemiluminescence (ECL) detection system (Pierce). **b**, Thioredoxin could be active in *H19*-transfected cells (H19 S14 3, H19 S14 4) since the up-regulation of the thioredoxin was correlated with an increase of NF-κB activity *versus* control cells (MDA, Neo 2 and Neo 3). NF-κB transactivation was measured using a five κB sites repeat coupled with a luciferase reporter gene in pTK transfection vector (NF-κB luc). Cells were seeded in six-well plates at 150,000 cells/well. The next day, they were transfected for 6 h at 37 °C by LIPOFECTIN® Reagent in OPTI-MEM I (Life Technologies) mixed with NF-κB luc (1.5 μg/well). After 36 h in MEM containing 5 % of fetal calf serum, cells were harvested and Luciferase activity was evaluated in a Lumat LB9501 luminometer (Berthold) using the Luciferase Assay Kit (Applied Biosystems). **c**, Thioredoxin mRNA was unchanged regardless of the cell line. Semiquantitative RT-PCR protocol was as previously detailed in Fig.1b. The thioredoxin primers were those previously described (Ferret *et al.*, 2000). One fortieth of cDNA reaction was subjected to the following PCR conditions: 7 min at 95 °C, then 35 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s and followed by 7 min of final

extension at 72 °C. PCR products were visualized as in Fig.1b. Values represent the ratio of the densitometry measures, obtained with the Diversity One program (Pharmacia), for thioredoxin and β-actin mRNA. SD corresponds to the standard deviation.

Figure 3


a
MDA
Neo 2
Neo 3
H19 S14 3
H19 S14 4
Thioredoxin
b
Relative Luciferase Activity (Fold induction)
250
200
150
100
50
0
MDA
Neo 2
Neo 3
H19 S14 3
H19 S14 4
c
MW
H20
-RT
+RT
331 bp
242 bp
190 bp
thioredoxin (261 bp)
β-actin (219 bp)
trx/β-actin (± SD)
0.93 (±0.04)
1.05 (±0.04)
0.93 (±0.02)
0.96 (±0.04)
1.01 (±0.01)

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Discussion générale

Le mode d'expression du gène *H19*, à savoir une forte expression dans le fœtus, peu ou pas d'expression chez l'adulte et une ré-expression dans de nombreux cancers, suggère que ce gène est impliqué dans le développement normal et dans des processus néoplasiques. Dans la glande mammaire normale, notre groupe a montré que l'expression basale du gène, qui persiste après la naissance, est fortement augmentée dans les bourgeons terminaux au moment de la puberté et dans les *acini* au cours d'une gestation. Les transcrits *H19* s'accumulent donc à l'interface épithélium/mésenchyme au moment où les interactions entre ces deux compartiments cellulaires sont prépondérantes et essentielles pour assurer la morphogenèse de la glande (Adriaenssens *et al.*, 1999). Parallèlement, dans les cancers du sein nous avons observé une surexpression du gène *H19* dans plus de 70% des adénocarcinomes étudiés et dans de nombreux cas cette surexpression se localise à la frontière cellules épithéliales/cellules stromales (Adriaenssens *et al.*, 1998). Une accumulation préférentielle des transcrits *H19* dans les cellules tumorales au contact ou proches du stroma a également été observée dans les cancers de la vessie (Cooper *et al.*, 1996).

Au cours de notre travail de thèse, nous avons recherché quelles pouvaient être les causes de l'activation du gène *H19* à l'interface épithélium/mésenchyme. Dans ce but,

nous avons analysé l'effet de facteurs paracrines mésenchymateux, en particulier celui de l'HGF/SF, sur l'expression du gène dans un système de culture dit en "3 dimensions" (3D). Ce système offre l'avantage d'être plus proche des situations existant *in vivo* que de celles qui prévalent en simple culture sur fond de boîte (2D). Ainsi, nous avons cultivé les cellules entre deux couches de collagène et nous avons montré que le simple changement d'une culture en 2D où la cellule établit des contacts restreints à une partie de sa surface, à une culture en 3D où la cellule peut établir des contacts au niveau de toute la surface de la membrane plasmique, augmente fortement l'expression de *H19*. Néanmoins, lorsque ces cellules sont cultivées en 2D sur différents substrats, tels que la fibronectine, la laminine, le collagène ou la poly-L-lysine, l'expression de *H19* reste inchangée (Adriaenssens, communication personnelle). Ce résultat suggère que l'expression du gène *H19* est sensible au changement de morphologie des cellules induit par le mode d'ancrage, et non par la nature du substrat sur lequel l'ancrage de la cellule s'établit. Dès la fin des années 70, des travaux sur les cellules épithéliales de cornée ont souligné combien la biologie des cellules dépendait de leur morphologie. En effet, ces cellules cultivées en 2D sont sensibles au FGF mais non à l'EGF, malgré la présence du récepteur spécifique de ce facteur. Au contraire, cultivées en 3D ces mêmes cellules ne répondent plus à une stimulation par le FGF, mais sont devenues sensibles à l'EGF (Gospodarowicz *et al.*, 1978).

Lorsque les cellules sont cultivées en 3D, le milieu conditionné par des fibroblastes (FCM) induit l'organisation des cellules HBL-100 en pseudo-tubules et un essaimage des cellules MDA-MB-231, mais n'a aucun effet sur les cellules MCF-7 (Fauquette *et al.*, 1997). Parallèlement, l'expression du gène *H19* est augmentée dans les cellules HBL-100 ou MDA-MB-231, mais non dans les cellules MCF-7. Parmi les facteurs synthétisés par les fibroblastes et impliqués dans les interactions épithélium/mésenchyme, l'HGF/SF a été identifié comme étant le facteur responsable de la tubulogenèse des cellules épithéliales en

culture (Montesano *et al.*, 1991a, 1991b, 1997), et nécessaire au cours de la morphogenèse de la glande mammaire (Yang *et al.*, 1995 ; Nieman *et al.*, 2000). Ces indications nous ont conduits à tester l'effet de l'HGF/SF sur les différentes lignées mammaires cultivées en 3D et à rechercher en parallèle une éventuelle modification de l'expression de *H19*. Comme le FCM, l'HGF/SF induit une augmentation de l'expression du gène *H19* concomitante d'une tubulogenèse des HBL-100 et d'un essaimage des MDA-MB-231, mais n'a aucun effet sur les cellules MCF-7 qui ne migrent pas. Un anticorps bloquant anti-HGF nous a permis de confirmer que les effets du FCM sur le phénotype des cellules et sur l'expression de *H19* sont principalement dus à l'action de l'HGF/SF. L'ensemble de ces résultats indique que la morphogenèse et/ou la migration induite par le FCM *via* l'HGF/SF s'accompagne d'une stimulation de l'expression du gène *H19* et suggère une implication du gène dans ces mécanismes.

L'HGF/SF sécrété par les cellules mésenchymateuses induit la dispersion et la morphogenèse des cellules épithéliales par l'intermédiaire de son récepteur Tyrosine-kinase, c-Met. Beviglia et ses collaborateurs (1997) ont rapporté que les cellules MCF-7 se différencient des cellules HBL-100 et MDA-MB-231 par leur incapacité à synthétiser le récepteur c-Met. Des expériences de Western blot nous ont permis de confirmer la présence du récepteur c-Met dans les HBL-100 et dans les MDA-MB-231, mais indiquent une synthèse anormale de ce récepteur dans les MCF-7 cultivées dans notre laboratoire. Il semble donc que l'absence de migration associée à l'absence d'activation de l'expression de *H19* par le FCM et l'HGF/SF dans les cellules MCF-7 soit due à un défaut de récepteurs c-Met fonctionnels.

Le récepteur c-Met est capable d'interagir avec de nombreux partenaires parmi lesquels GRB2 (Growth factor Receptor-Bound protein 2), la PI3K (Phosphatidyl-Inositol-3 Kinase), la PLCγ (PhosphoLipase Cγ), Src, SHC (SH2-Containing sequence), GAB1

(Grb-2 Associated Binder-1) ou encore STAT3 (Signal Transducer and Activator of Transcription) (Ponzetto *et al.*, 1994 ; Pelicci *et al.*, 1995 ; Fournier *et al.*, 1996 ; Weidner *et al.*, 1996 ; Boccaccio *et al.*, 1998). L'enjeu actuel est de comprendre le rôle de chacun de ces partenaires dans l'induction des voies de signalisation et les effets biologiques transduits par c-Met. En effet, l'activation d'une seule voie de signalisation ne rend pas compte de tous les effets induits par l'HGF/SF, effets qui nécessitent donc un croisement entre les différentes voies. Notamment, il a été démontré que l'induction de la dispersion et de la tubulogenèse nécessite l'activation de la voie Ras-MAP-kinase, de la PI3-kinase et de la PLCγ1 (Royal et Park, 1995; Potempa et Ridley, 1998; Gual *et al.*, 2000). A la suite de transfections transitoires d'un gène rapporteur sous le contrôle du promoteur du gène *H19* dans des cellules de rein de chien (MDCK), nous avons montré que l'HGF/SF est capable d'activer le promoteur du gène *H19* et que cette activation dépend de la voie Ras-MAP-kinase et de la PLCγ, mais ne dépend pas de la PI3-kinase. De plus, ces transfections indiquent que la régulation de *H19* par l'HGF/SF n'est pas restreinte aux seules cellules mammaires, mais peut être étendue à la lignée MDCK, qui est un modèle de choix pour l'étude de la tubulogenèse induite par l'HGF/SF (Stoker *et al.*, 1987 ; Sonnenberg *et al.*, 1993). Bien que l'activité de ce dernier semble primordiale dans les effets induits par le FCM, l'activation du promoteur de *H19* par l'EGF et le FGF2 suggère que ces facteurs puissent potentialiser ces mécanismes.

Finalement, il ressort de cette étude que le gène *H19* est activé dans les cellules épithéliales lors du processus de migration induit par les facteurs sécrétés par le mésenchyme. Le gène *H19* pourrait donc être impliqué dans la motilité des cellules, qui au cours du développement normal est associée au processus de morphogenèse, mais qui lors de processus néoplasiques est associée à la progression tumorale. Bien qu'ils soient en faveur d'une fonction oncogénique du gène *H19*, ces résultats ne nous permettent pas de

conclure. Or, eu égard à la forte controverse qui règne sur le rôle du gène *H19* dans les processus de cancérogenèse, il nous paraissait capital de déterminer le statut de ce gène dans les cancers du sein.

En conséquence, nous avons recombiné stablement le génome de cellules MDA-MB-231 avec une séquence d'ADN génomique de *H19* et nous avons analysé les effets de la surexpression de ce dernier sur les capacités tumorigènes des cellules. Nous avons ainsi montré que le gène *H19* se comporte comme un oncogène dans les cellules de cancer du sein. En effet, des souris *scid* auxquelles sont injectées les cellules MDA-MB-231 possédant le transgène *H19* (MDA-H19) développent plus rapidement des tumeurs que les souris ayant reçu les cellules témoin. Notre étude sur les interactions épithélium/mésenchyme suggère que le gène *H19* serait impliqué dans des mécanismes de migration, nous n'avons cependant pas détecté de métastases lors de nos expériences de tumorigenèse en souris immunodéficientes. Ainsi, bien que le gène *H19* semble nécessaire, lorsque des cellules capables de migrer sont induites à le faire, sa surexpression à elle seule ne suffit pas à provoquer une migration cellulaire.

Par ailleurs, des travaux menés sur des cellules de cancer de la vessie et sur des cellules issues d'un choriocarcinome ont montré que le gène *H19* est activé lorsque ces cellules, exprimant peu ou pas *H19*, sont injectées dans des souris *nude* (Elkin *et al.* 1995 ; Rachmilewitz *et al.*, 1995). Cependant, lors de nos expériences nous n'avons pas observé d'augmentation de l'expression de *H19* dans les tumeurs induites par les cellules MDA-MB-231 parentales. Ainsi, alors que le gène *H19* augmente la tumorigénicité des cellules de cancer du sein, la tumorigenèse au contraire n'active pas *H19* dans ces cellules. Dans les tumeurs induites par les cellules MDA-H19, nous avons observé que la surexpression de *H19* est conservée dans certaines cellules, mais est absente dans d'autres. Or l'analyse des colorations histologiques indique que cette perte d'expression n'est pas due à la

nécrose des cellules. Isfort et ses collaborateurs ont montré que la ré-expression du gène *H19* dans des cellules SHE ralentit la croissance tumorale dans des souris *nude* et ont observé que les tumeurs induites par ces cellules n'expriment plus le transgène à la suite de la méthylation des séquences exogènes (Isfort *et al.*, 1997). La séquence *H19* que nous avons transfectée étant identique à celle utilisée par ces auteurs, il est possible que la perte d'expression du transgène dans les tumeurs induites par les cellules MDA-H19 soit également la conséquence de méthylations. Par ailleurs, il a été récemment démontré que le génome contient deux types de sites d'intégration des transgènes: des sites dits stables et d'autres dits instables. Alors qu'une intégration au niveau d'un site stable permet une expression à long terme du transgène, une intégration au niveau d'un site instable conduit à l'élimination de la séquence exogène (Migliaccio *et al.*, 2000). Ainsi, l'absence d'expression de *H19* dans certaines cellules des tumeurs n'est peut être pas due à une extinction de la transcription, mais correspondrait éventuellement à une perte des transgènes par le génome hôte. Enfin, une dernière explication fondée sur la régulation post-transcriptionnelle du gène *H19* est envisageable. En effet, Milligan et ses collaborateurs (2000) ont démontré que l'accumulation des transcrits *H19* au cours de la différenciation des cellules musculaires ne dépend pas d'une activation transcriptionnelle, mais dépend uniquement d'une stabilisation des ARN. En conséquence, la prolifération à l'origine de la formation de la tumeur pourrait entraîner une déstabilisation des transcrits *H19* et donc une extinction du signal en hybridation moléculaire *in situ*.

Parallèlement aux études de tumorigenèse, nous avons évalué la croissance et la clonogénicité des cellules MDA-H19. Quel que soit le pourcentage de sérum dans le milieu de culture, la surexpression de *H19* dans les cellules MDA-MB-231 ne modifie ni leur prolifération, ni leur taux d'apoptose basal et ni leur répartition dans les différentes phases du cycle. Cette absence de lien entre le niveau d'expression du gène *H19* et la prolifération

cellulaire s'accorde avec ce que nous avons déjà constaté dans les adénocarcinomes mammaires, où la surexpression du gène n'est pas corrélée avec la présence d'un marqueur spécifique des cellules en cycle (Adriaenssens *et al.*, 1998). Les résultats obtenus en terme de clonogénicité confirment que les manifestations oncogéniques du gène *H19* ne sont pas dues à une augmentation des capacités prolifératives des cellules, mais semblent plutôt liées à une augmentation de leur capacité à survivre en absence d'ancrage. En effet, la surexpression de *H19* favorise la croissance en absence d'ancrage, alors que sur fond de boîte la clonogénicité des cellules est inchangée. Il est intéressant de noter qu'une fois de plus l'expression du gène *H19* se révèle très importante lorsque les cellules sont dans un contexte adéquat, permettant des contacts au niveau de toute la surface de la membrane plasmique (culture en agar-mou ou tumeur).

En 1998, Li et ses collaborateurs ont observé, d'une part qu'un taux élevé de transcrits *H19* est associé à un faible taux de transcrits *IGF2*, et d'autre part qu'une absence de transcrits *H19* est associée à un taux élevé d'ARN *IGF2*, et à une augmentation de leur traductibilité. Les auteurs ont alors suggéré que le gène *H19* régule négativement l'expression du gène *IGF2* en *trans*. Plus récemment, Wilkin et ses collaborateurs (2000) ont confirmé que *H19* réprime la transcription d'*IGF2*. Suite à des transfections transitoires de séquences sens et antisens du gène *H19*, cette équipe a montré qu'une séquence sens diminue l'activité du promoteur P3 du gène *IGF2*, alors que la séquence antisens est sans effet. Parallèlement, les auteurs ont introduit stablement le gène *H19* dans le génome de cellules de cancer du foie et ont observé qu'une séquence antisens augmente l'expression du gène *IGF2*; néanmoins, transfectée stablement, la séquence *H19* sens n'a pas entraîné de diminution de l'expression d'*IGF2* (Wilkin et al., 2000). Afin de vérifier la validité de cette régulation dans notre modèle, nous avons évalué le taux d'ARNm *IGF2*. Nous avons ainsi montré, contrairement aux travaux de Wilkin, que la transfection stable de la

séquence sens *H19* dans les cellules MDA-MB-231 provoque une diminution du niveau de transcrits *IGF2*, confirmant ainsi la fonction de *trans*-riborégulateur négatif du gène *H19* sur la transcription du gène *IGF2*. De plus, plusieurs travaux ont suggéré que le seul rôle du gène *H19* serait de réguler l'expression du gène *IGF2*. En effet, deux types de "knock-out" du gène *H19*, l'un concernant une région de 13 kpb, l'autre concernant une région de 3 kpb, ont abouti à un unique phénotype, qui est une augmentation du poids des souriceaux de 27 % dans le premier cas et de 8 % dans le second par rapport aux souriceaux sauvages de la même portée (Leighton *et al.*, 1995a ; Ripoche *et al.*, 1997). De même, la fréquente inactivation de *H19* dans les tumeurs de Wilms, généralement associée à une activation de l'allèle normalement silencieux d'*IGF2* (Steenman *et al.*, 1994 ; Tanigushi *et al.*, 1995), a conduit à proposer que le rôle du gène *H19* dans la tumorigenèse se réduirait à sa capacité à réguler l'expression d'*IGF2* (Moulton *et al.*, 1996 ; Reeve, 1996) et que la corrélation de son expression avec la progression tumorale ne serait que le reflet de la fréquente perte d'empreinte au niveau du domaine *H19/IGF2*. Ainsi, en observant que la surexpression du gène *H19* dans les cellules MDA-MB-231 augmente la tumorigénicité de ces cellules et parallèlement diminue l'expression de *IGF2*, nous démontrons non seulement que le gène *H19* a un statut d'oncogène dans les cellules de cancer du sein, mais surtout que son activité oncogénique est indépendante d'*IGF2*.

Comme nous venons de l'évoquer dans le paragraphe précédent, il a été observé et confirmé dans notre modèle, que le gène *H19* régule la transcription du gène *IGF2* en *trans* (Li *et al.*, 1998 ; Wilkin *et al.*, 2000). Curieusement, bien que cette fonction de riborégulateur ait été attribuée au gène *H19* très tôt après sa découverte, peu de travaux ont été consacrés à cette propriété du gène. Ainsi, dans le but de caractériser d'autres protéines régulées par l'ARN *H19* et d'identifier des cibles qui nous permettraient de préciser le rôle de ce gène, nous avons réalisé une étude protéomique des lignées MDA-MB-231

transfectées stablement avec le gène *H19*. Nous avons ainsi montré et confirmé par Western blot que le gène *H19* régule positivement la thiorédoxine (Trx) qui est une protéine clé du métabolisme redox de la cellule. La Trx est une protéine qui contient dans son site actif deux cystéines. Cette protéine peut exister sous une forme réduite dithiol ou sous une forme oxydée dans laquelle les deux résidus cystéine forment un pont disulfure intramoléculaire. La Trx catalyse des réactions d'échange dithiol en disulfure entre son centre actif et différentes protéines. Elle est activée en réponse à un stress oxydatif pouvant être causé par les ultra-violets, par des radiations ionisantes ou des cytokines inflammatoires, *stimuli* qui pour la plupart génèrent des radicaux oxygénés libres très réactifs (ROS pour Reactive oxygen species). La Trx protège les cellules vis-à-vis de ces ROS en régulant des enzymes de détoxification qui permettent de maintenir la balance redox intracellulaire (Das *et al.*,. 1997; Kang *et al.*, 1998), mais également en régulant l'état redox, et donc l'activité, de protéines contrôlant la croissance cellulaire. Notamment, plusieurs travaux ont rapporté que la Trx augmente la fixation sur l'ADN de divers facteurs de transcription tels qu'AP1 (Hirota *et al.*, 1997), le récepteur aux oestrogènes (Hayashi *et al.*, 1997), p53 (Ueno *et al.*, 1999), le récepteur aux glucocorticoïdes (Makino *et al.*, 1999) et NF-κB (Hirota *et al.*, 1999). Ainsi, des niveaux élevés de Trx réduite augmentent la croissance cellulaire, inhibent l'apoptose et favorisent la résistance des cellules aux drogues anticancéreuses (Powis *et al.*, 2000).

La Trx est surexprimée dans différents types de cancers tels que les cancers du col de l'utérus (Fujii *et al.*, 1991), de la peau (Wakita *et al.*, 1992), du poumon (Gasdaska *et al.*, 1994), du foie (Kawahara *et al.*, 1996), du colon (Berggren *et al.*, 1996), du sein (Bini *et al.*, 1997 ; Ueno *et al.*, 2000) et dans les cancers gastriques (Grogan *et al.*, 2000). Il est intéressant de noter que la plupart de ces tumeurs qui surexpriment la Trx sont des tumeurs pour lesquelles une surexpression du gène *H19* a été observée (Kondo *et al.*, 1995; Douc-

Rasy *et al.*, 1996; Hibi *et al.*, 1996; Ariel *et al.*, 1998; Adriaenssens *et al.*, 1998). La surexpression de la Trx dans les cancers associée à ses effets positifs sur la croissance cellulaire suggère qu'elle favorise la croissance tumorale. En accord avec cette proposition, il a été rapporté que dans les cellules mammaires cancéreuses MCF-7 la transfection stable de la Trx ne modifie pas la croissance de ces cellules sur fond de boîte, mais augmente de 3 à 4 fois leur croissance en absence d'ancrage. De plus, dans des souris immunodéficientes, la formation de tumeurs par des cellules MCF-7 transfectées avec un mutant de la Trx est presque totalement inhibée (Gallegos *et al.*, 1996). Les phénotypes décrits par Gallegos et ses collaborateurs nous permettent de proposer que l'augmentation des propriétés oncogéniques de nos cellules MDA-MB-231, après transfection stable du gène *H19*, pourrait être le résultat d'une élévation du taux de Trx. En conséquence, il serait intéressant d'éprouver cette hypothèse en observant les phénotypes des cellules MDA-H19 après inhibition de la Trx par des inhibiteurs pharmacologiques ou des oligonucléotides antisens. Dans une seconde étude, la même équipe a montré que l'activité NF-κB est amplifiée dans les cellules MCF-7 transfectées avec la Trx, mais n'est pas responsable de l'augmentation de la croissance de ces cellules en absence d'ancrage (Freemerman *et al.*, 1999). Ainsi pour notre part, bien que nous ayons constaté une augmentation de l'activité NF-κB dans les cellules MDA-H19, il est raisonnable de penser que si le gain d'agressivité de ces cellules est lié à la Trx, il est probablement indépendant de NF-κB. Enfin, Hirota et ses collaborateurs (1999) démontrent que la Trx favorise la fixation de NF-κB sur l'ADN uniquement lorsqu'elle est transloquée dans le noyau ; on peut donc supposer qu'au moins une partie de la Trx exprimée dans les cellules MDA-H19 est nucléaire.

Les résultats présentés dans la troisième partie de notre mémoire indiquent clairement que le transcrit *H19* est un *trans*-riborégulateur positif responsable d'un taux élévé de Trx dans les cellules. Au cours de notre travail, nous avons également observé que *H19* régule

négativement en *trans* la transcription du gène *IGF2*. Or, les travaux de Li et ses collaborateurs (1998) suggéraient déjà ce contrôle transcriptionnel d'*IGF2* et évoquait de plus la possibilité d'une régulation de la traductibilité des ARNm *IGF2*. Nous avons aussi cherché à préciser le niveau auquel la Trx est régulée par les transcrits *H19*. L'analyse du taux d'ARNm Trx, par RT-PCR semi-quantitative, indique que ce taux est stable ; en conséquence la régulation de la Trx par les ARN *H19* s'effectue au niveau post-transcriptionnel.

A l'heure actuelle, une trentaine de gènes dont le produit fonctionnel est un ARN non codant, ont été identifiés chez les Procaryotes et chez les Eucaryotes (Erdmann *et al.*, 2000; 2001), et sont répertoriés dans une base de données (http://biobases.ibch.poznan.pl/ncRNA/). Bien que dans la plupart des cas, la fonction et/ou le mécanisme d'action de cette catégorie d'ARN reste à découvrir, un mécanisme fondé sur des interactions ARN/ADN ou ARN/ARN semble se dégager. Pour notre part, des recherches par dotplot n'ont pas permis de mettre en évidence des complémentarités de séquences entre les transcrits *Trx* et *H19*. Par ailleurs, contrairement à ce qui avait été décrit par l'équipe de Tilghman (Brannan *et al.*, 1990), il a été depuis démontré que, bien que non traduits, les transcrits *H19* sont associés aux polysomes dans différents types cellulaires murins et humains (Li *et al.*, 1998). De même Milligan rapporte dans sa thèse (2000) que l'ARN *H19* est capable de s'associer à l'ARN ribosomique 18S. Il serait ainsi possible que le transcrit *H19* régule positivement le taux de Trx en favorisant son recrutement au niveau de la machinerie de traduction. Enfin, récemment Runge et ses collaborateurs (2000) ont montré que l'ARN *H19* contient quatre sites de fixation pour des "IGF2 m RNA binding protein" (IMP) au niveau de ses exons 4 et 5. La fixation de ces dernières permet la localisation subcytoplasmique des ARN *H19* dans une région périnucléaire ainsi qu'à l'extrêmité des lamellipodes de cellules de cancer du foie. Les

auteurs suggèrent que ces protéines constituent un lien mécanistique qui permettrait la régulation post-transcriptionnelle d'*IGF2* par le transcrit *H19*. Selon le même mode, on peut supposer que les ARN *H19* régulerait le taux de traduction des transcrits *Trx* en contrôlant leur localisation subcytoplasmique. Eu égard à la première partie de notre mémoire, dans laquelle nous suggérons une implication du gène *H19* dans les mécanismes de migration, il est intéressant de noter la localisation préférentielle des ARN *H19* au niveau des lamellipodes puisqu'ils sont en effet le siège de modulations de l'adhésion indispensables aux mécanismes de migration.

Conclusion

En résumé, nos travaux montrent que lors des interactions épithélium/mésenchyme le gène *H19* est activé dans les cellules épithéliales en réponse à des facteurs paracrines issus du mésenchyme. De plus, cette activation du gène est concomitante du mécanisme de migration accompagnant soit l'organogenèse lors du développement normal de la glande mammaire, soit la progression tumorale lors de processus néoplasiques. Nous avons également observé que la transfection stable du gène *H19* dans des cellules épithéliales mammaires cancéreuses augmente les capacités tumorigènes de ces cellules en favorisant leur croissance en absence d'ancrage (clonage en agar mou, développement de tumeurs chez des souris immunodéficientes). Enfin, nous rapportons que le gène *H19* régule positivement au niveau post-transcriptionnel la Trx, protéine ayant des effets positifs sur la croissance cellulaire et dont la surexpression dans des cellules mammaires cancéreuses potentialise leur comportement tumorigène. L'ensemble de nos résultats suggère donc que les transcrits *H19* jouent un rôle dans les processus évoqués en favorisant probablement une adhésion (cellules-cellules) et/ou un ancrage (cellules-substrat) des cellules, mais également un ancrage qui devient transitoire lors de l'induction d'une migration. Ce rôle n'est sans doute pas direct, mais serait assumé par des gènes cibles de *H19* qui restent à déterminer, et dont l'activité serait modulée par les ARN *H19*. Nous n'imaginons pas le

rôle de *H19* comme une clé de voûte des processus mentionnés, mais plutôt comme une action entrant en synergie avec celle d'autres partenaires. Pour conclure, il ressort que nos résultats confèrent au gène *H19* une fonction favorisant le développement de tumeurs, soit un statut d'oncogène dans les cellules de cancer du sein. Nos résultats permettent également d'établir pour la première fois que *H19* possède la propriété d'agir en tant que *trans*-riborégulateur positif.

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