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**Caractérisation du sécrétome des fibroblastes sénescents de derme
humains et de son impact sur les étapes initiales
de la carcinogenèse de la peau.**

Thèse de Doctorat

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Abstract

Cellular senescence is essentially characterized by an irreversible growth arrest, suggesting that mechanism as a potent barrier to tumorigenesis. Epidemiologic data however, correlate the risk to develop a carcinoma with an advancing age. In our laboratory, we investigated the molecular mechanisms underlying this correlation in a model of normal human primary epidermal keratinocytes (NHEK). In culture, NHEK accumulate oxydative stress and rapidly reach the senescence growth plateau. Nevertheless, contrarily to fibroblasts, a fraction of the cell population spontaneously and systemically escapes from the senescence plateau and grows as neoplastic cells (named Post-Senescence Emergence Keratinocytes). These PSE keratinocytes provide a suitable model of the earliest phases of age-related non-melanoma skin carcinogenesis. Emerging evidences connect the micro-environment to carcinoma initiation and progression. Particularly, senescent fibroblasts could enhance transformation, growth and invasion of cancerous cells by a specific pro-inflammatory secretome. The aim of my thesis was to characterize the secretome of senescent dermal human fibroblasts (NHDF) and then to determine whether it could influence early step of neoplastic PSE-keratinocytes.

We have confirmed that secretome of senescent NHDF is highly pro-inflammatory with secretion of cytokines, growth factors and metalloproteinases (MMPs). Conditioning of keratinocytes by senescent NHDF secretome enhanced the early steps of PSE-keratinocytes transformation by promoting Epithelial-to-Mesenchymal Transition and migration ability. In particular, MMP-1; -2 specifically secreted and activated by senescent NHDF, promote the early migratory capacity of cancer-initiated PSE-NHEKs, by activating the protease-activated receptor-1 (thrombin receptor, PAR-1), up to now associated to more aggressive cancers. The MMP-PAR-1 axis could thus be crucial for age-related cooperative switches in the early step of carcinoma.

By a comparative proteomic analysis of young and senescent NHDF conditioned media, we could observe a loss of several extracellular matrix glycoproteins secretion by senescent NHDF. Interestingly, these glycoproteins, Secreted Protein Acidic and Rich in Cystein (SPARC), Decorin and Pigment Epithelium-Derived Factor (PEDF) are strong inhibitors of epithelial cells activation and growth. Thus, the loss of SPARC, Decorin and PEDF could contribute to early step of keratinocytes transformation. Finally, we demonstrated for the first time that the defective secretion of these glycoproteins by senescent NHDF is the result of a constitutive endoplasmic reticulum stress and activation of the Unfolded Response Pathway (UPR) (in particular ATF-6 pathway).

Résumé

La sénescence ou vieillissement cellulaire est considéré comme un mécanisme suppresseur de tumeur de par sa capacité à bloquer le cycle cellulaire. Cependant le risque de développer un carcinome augmente fortement avec le vieillissement. Nous étudions les mécanismes des phases initiales de la carcinogenèse de la peau dans un modèle de culture de kératinocytes primaires humains de peau (NHEK). Contrairement aux fibroblastes, une fraction de NHEK est capable d'émerger du plateau de sénescence pour générer spontanément de nouvelles cellules proliférantes et néoplasiques (cellules appelées post-sénescence émergentes (PSE)). Le micro-environnement cellulaire a récemment été associé à la promotion du développement tumoral. En particulier, les fibroblastes sénescents peuvent stimuler la progression tumorale par l'intermédiaire d'un sécrétome pro-inflammatoire. Mon projet de thèse avait pour but de caractériser le sécrétome des fibroblastes sénescents de derme humains et d'en étudier l'impact sur l'émergence néoplasiques des kératinocytes.

Nous avons tout d'abord établi le caractère pro-inflammatoire du sécrétome des fibroblastes sénescents de derme par la sécrétion de cytokines (MCP-1 ; IL-6...), de facteurs de croissance (HGF/SF...) et de métalloprotéases (MMP-1 ; -2...). Le conditionnement des cultures primaires de kératinocytes par ce sécrétome des fibroblastes sénescents stimule la transition épithélium-mésenchyme et la migration des kératinocytes PSE néoplasiques. Nous démontrons que ce sont les MMP-1 et -2, sécrétées et activées par les fibroblastes sénescents qui sont à l'origine des capacités migratoires de ces kératinocytes PSE néoplasiques conditionnés par l'environnement sénescents. Pour cela les MMP-1 et -2 activent le récepteur à la thrombine PAR-1 (Proteases Activated Receptor) à la membrane des NHEK, processus jusque là associé aux processus métastatiques tardifs.

Par une analyse protéomique comparée de milieux conditionnés de fibroblastes jeunes et sénescents, nous avons pu aussi montrer un défaut de sécrétion de glycoprotéines de la matrice extracellulaire (SPARC (Secreted Protein Acidic and Rich in Cystein), Décorine et PEDF (Pigment Epithelium Derived Factor)) par les fibroblastes sénescents de derme. Ces glycoprotéines sont dotées de fortes capacités inhibitrices de l'activation et de la croissance cancéreuse. Les pertes de SPARC, décorine et PEDF pourraient donc contribuer à l'initiation cancéreuse des kératinocytes. Nous montrons également pour la première fois un lien entre le stress constitutif du réticulum endoplasmique et la sénescence via la description du rôle de la voie ATF-6 de l'UPR (Unfolded Protein Response) dans les défauts de sécrétion des glycoprotéines SPARC et décorine.

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Liste des abréviations

ADN	Acide désoxyribonucléique
AP-1	Activator protein-1
ARN	Acide ribonucléique
ATF-6	Activating transcription factor 6
ATM	Ataxia telangiectasia mutated
ATP	Adénosine monophosphate
BCC	Basal-cells carcinoma
BiP	Binding protein (protéine chaperone du reticulum endoplasmique)
CAF	Cancer associated fibroblast
CGH	Comparative genomic hybridization
CHK2	Checkpoint kinase 2
DDR	DNA damage response
DSB	Cassure à l'ADN double brin (Double-strand break)
DTC	Disseminated tumoral cell
EGF	Epidermal growth factor
ERM	Ezrin, radixin, moesin
ETS	E twenty-six specific
FAK	Focal adhesion kinase
FC	Facteur de croissance
FGF	Fibroblast growth factor
GAG	Glucosaminoglycannes
GFP	Green fluorescence protein
GPCR	G protein-coupled receptor
HGF/SF	Hepatocyte growth factor / Scatter factor
HMEC	Human mammary epithelial cell
HMLE	Human mammary cell line epithelial
hTERT	Human telomerase reverse transcriptase
ICAM	Intercellular adhesion molecule
IGF	Insulin-like growth factor
IGF-BP	IGF binding protein
IL	Interleukine
IMK	Immortal keratinocyte
IRE-1	Inositol-requiring protein 1
KGF	Keratinocyte growth factor
MAPK	Mitogen-activated protein kinase
MCF-7	Lignée cellulaire cancéreuse du sein
MCP-1	Monocyte chemoattractant protein 1 (CCL2)
MDA-MB-231	Lignée cellulaire cancéreuse du sein
MEC	Matrice extra-cellulaire
MEF	Mouse embryonic fibroblasts
MMP	Matrix metalloproteinase
MnSOD	Manganese superoxide dismutase
NF-κB	Nuclear factor kappa B

NHEK	Normal human epidermal keratinocyte
NHDF	Normal human dermal fibroblast
OIS	Oncogene-induced senescence
P16INK4a	Cyclin-dependent kinase inhibitor 2A/ Cyclin-dependent kinase 4 inhibitor A
P21cip/waf1	Cyclin-dependent kinase inhibitor 1A/CDK-interaction protein/wild-type p53-activated fragment 1
PAI-1	Plasminogen activator inhibitor-1
PAR-1	Protease activated receptor (Récepteur de la thrombine)
PARP-1	Poly ADP-ribose polymerase-1
PC3	Lignée cellulaire cancéreuse de prostate
PEA-3	Polynoma enhancer activator
PEDF	Pigment epithelium-derived factor
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PI3K	Phosphatidylinositol 3-kinases
PSE	Kératinocytes post-sénescence émergents
RE	Réticulum endoplasmique
Rb	Retinoblastoma
ROS	Reactive oxygen species
SA beta-gal	Senescence associated beta-galactosidase
SAHF	Senescence associated heterochromatin foci
SASP	Senescence associated secretory phenotype
SCC	Squamous-cells carcinoma
SDF-1	Stromal derived factor-1
SF-CM	Senescent fibroblasts conditioned medium
SIPS	Stress-induced premature senescence
SMS	Senescence messaging secretome
SPARC	Secreted protein, acidic and rich in cystein
SP-1	Specificity Protein 1
SSB	Cassure à l'ADN simple brin (single-strand breaks)
TEM	Transition épithélium-mesenchymateuse
TERT	Telomerase reverse transcriptase
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumor necrosis factor
TRF	Terminal Restriction Fragment
UPR	Unfolded protein response
UV	Ultra-violet
VEGF	Vascular endothelial growth factor
WI-38	Fibroblastes primaires de poumon embryonnaire
YF-CM	Young fibroblasts-conditioned medium

Introduction

A. Interactions de l'épithélium avec son micro-environnement

1. Définition et description du micro-environnement

Il est maintenant bien admis que les activités biologiques des cellules (ex : survie, mort, prolifération, différenciation, migration...) sont principalement contrôlées par des signaux extracellulaires. Ces signaux proviennent souvent de partenaires cellulaires ou structurels situés à proximité, c'est-à-dire dans leur micro-environnement. Dans la littérature, ce sont les communications entre les cellules épithéliales et leur micro-environnement (stroma) qui sont principalement décrites car ces interactions régulent l'homéostasie tissulaire dans des conditions normales et participent à l'évolution de certaines pathologies (cancers). Le tissu de la peau est un modèle type pour l'étude des interactions entre l'épithélium et son micro-environnement.

1.1. Structure et organisation d'un tissu : exemple de la peau

La peau est l'organe du corps dont la surface est la plus importante (environ 2m² chez l'homme adulte). Elle a pour principale fonction de protéger l'organisme des agressions mécaniques (les chocs et pressions), chimiques, des rayons UV (grâce à la mélanine) et des agents pathogènes. La peau est également essentielle pour la régulation thermique et la perception de l'environnement (toucher). Elle est constituée de 3 compartiments : l'épiderme, le derme et l'hypoderme (figure 1A).

L'épiderme est une succession de couches composées en majorité de cellules épithéliales appelées kératinocytes, responsables de la production de kératine associée aux propriétés de résistance et de protection (figure 1B) :

- la *couche cornée*, la plus superficielle, est composée de cellules mortes formant un revêtement imperméable.
- la *couche granuleuse* est composée de kératinocytes aplatis et très riches en kératine.
- la *couche de Malpighi* contient des kératinocytes, des mélanocytes, des terminaisons nerveuses et des cellules de Langerhans (cellules dendritiques présentatrices d'antigènes).
- la *couche basale* est constituée d'une assise de kératinocytes qui se divisent afin d'assurer le renouvellement des couches superficielles. Des cellules souches sont également présentes en faible quantité pour permettre le renouvellement des cellules épithéliales (Blanpain *et al*, 2007).

L'épiderme repose sur une lame ou membrane basale, fine couche de matrice extracellulaire très dense qui le sépare du derme. Elle est enrichie en collagène IV et laminine formant un réseau entrecroisé dense. Cette lame basale est primordiale pour l'adhésion et la différenciation des cellules épithéliales et sert de filtre pour les échanges de nutriments et de facteurs entre les deux compartiments (Kalluri, 2003).

Le derme ou stroma est un tissu conjonctif de soutien. Il supporte l'épiderme grâce à une matrice extra-cellulaire (ECM) dans laquelle cohabitent plusieurs types cellulaires (figure 1C). Le derme se divise en 2 principales zones : le derme papillaire et le réticulaire. Le derme papillaire, le plus superficiel, a une structure fibreuse très fine et est très riche en cellules (fibroblastes, capillaires sanguins, fibres nerveuses...) alors que le derme réticulaire, beaucoup plus dense et élastique, est pauvre en cellules (Mine *et al*, 2008).

Enfin, l'hypoderme, couche la plus profonde du derme, est constitué de tissus adipeux et est fortement vascularisé.

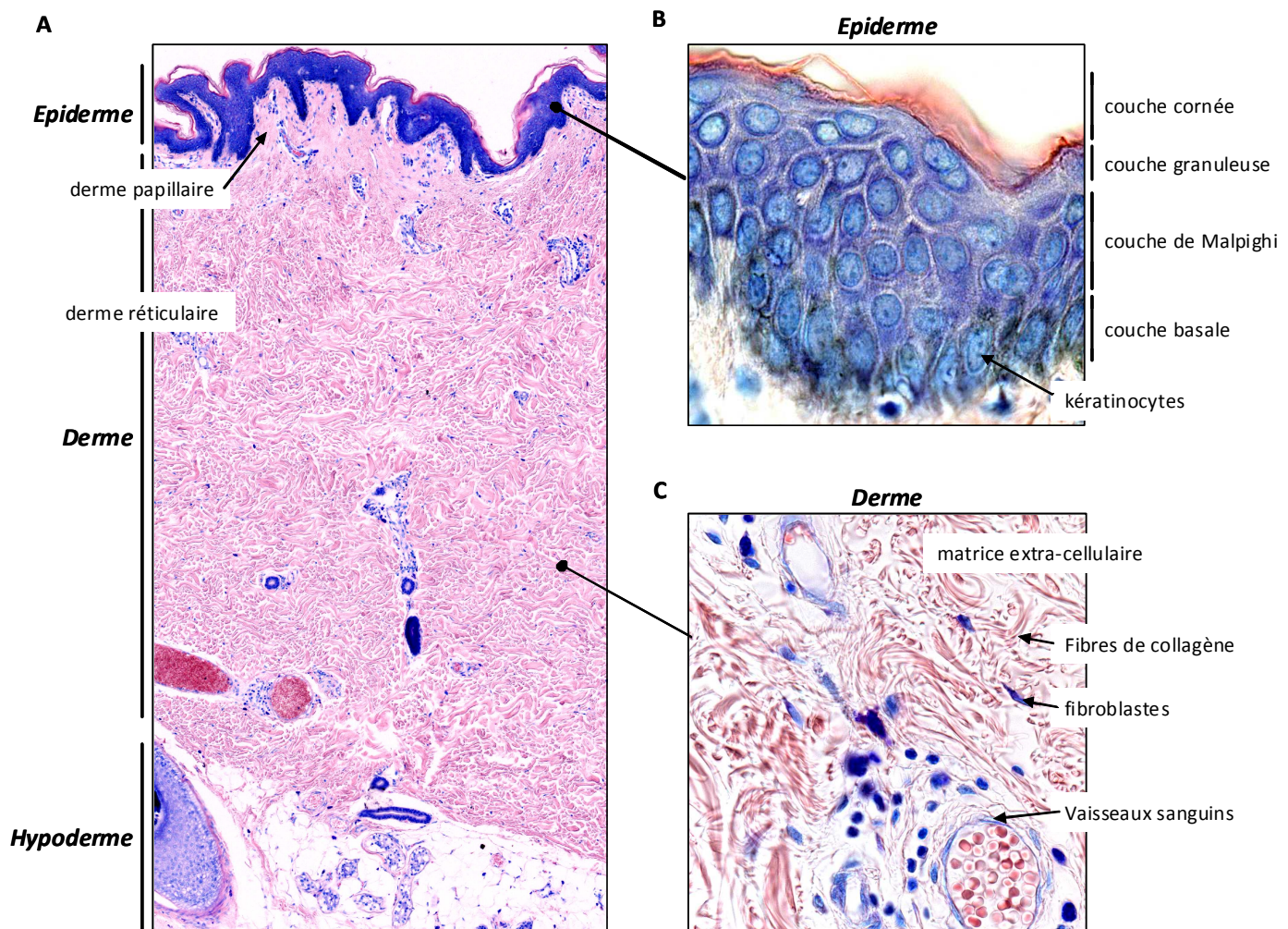


Figure 1 : Coloration histologique de May-Grünwald-Giemsa –MGG) d'une coupe de peau humaine

De par sa composition associant des couches structurées l'une par rapport à l'autre, la peau est un lieu d'interaction qui concourt à son homéostasie. Le derme constitue le micro-environnement qui agit sur le comportement des cellules épithéliales (kératinocytes) soit par l'intermédiaire des constituants de la matrice extracellulaire, soit par des facteurs solubles sécrétés par les cellules stromales (fibroblastes, vaisseaux sanguins, cellules immunitaires, etc....) qui y résident.

1.2. Les cellules du micro-environnement

1.2.1 Les fibroblastes

Les fibroblastes sont des cellules fusiformes allongées ou en étoile, peu différenciées et majoritaires dans le stroma. Chez l'adulte les fibroblastes peuvent dériver des cellules souches mésenchymateuses. Ce sont les cellules qui sécrètent majoritairement les constituants de la matrice extracellulaire (protéines formant les fibres du tissu conjonctif) propre à chaque organe et les protéases capables de remodeler cette même matrice. Les fibroblastes sécrètent également divers facteurs solubles (facteurs de croissance, cytokines...) nécessaires aux interactions avec les cellules épithéliales et les autres cellules du stroma.

1.2.2 Vaisseaux sanguins et lymphatiques

L'épiderme n'étant pas vascularisé, tous les apports nutritifs se font à partir du stroma qui contient un important réseau de vaisseaux sanguins et lymphatiques. Les cellules endothéliales (cellules aplaties et polarisées) forment la paroi intérieure des vaisseaux. A proximité de l'épithélium, le réseau est essentiellement composé de capillaires (les plus fins et plus petits vaisseaux) qui permettent la diffusion des nutriments, de l'oxygène, etc... nécessaires au métabolisme cellulaire. Les réseaux sanguins et lymphatiques permettent également d'apporter rapidement les cellules immunitaires et inflammatoires en cas d'agression au niveau des blessures.

1.2.3 Cellules immunitaires et inflammatoires

Le système immunitaire est une composante importante du micro-environnement qui permet de protéger l'organisme contre les éléments du non-soi comme les pathogènes extérieurs (virus, bactéries...) et de lutter contre les cellules cancéreuses. Sans rentrer dans les détails, le système immunitaire est divisé en 2 catégories : inné et adaptatif. Le système immunitaire inné composé

de macrophages, mastocytes, cellules natural killer, cellules dendritiques, leucocytes... permet une défense très rapide et non spécifique contre les agressions extérieures. Ces cellules, très présentes au niveau de la peau et dans les muqueuses, induisent une réaction inflammatoire mais aussi l'activation du système immunitaire adaptatif. L'immunité adaptative est une défense spécifique effectuée par les lymphocytes B (production d'anticorps spécifiques) et les lymphocytes T qui reconnaissent spécifiquement les antigènes étrangers et détruisent directement les cellules ou pathogènes.

1.3. Les molécules du micro-environnement

1.3.1 La matrice extra-cellulaire

La matrice extra-cellulaire (MEC) constitue la majeure partie du tissu conjonctif de soutien sur lequel repose l'épithélium. Bien que la composition de la MEC varie selon les organes, elle est constituée principalement de fibres et protéines fortement glycosylées.

Les protéines fibrillaires forment un réseau complexe responsable du soutien mécanique et de la plasticité du tissu. Les protéines fibrillaires les plus abondantes dans le derme sont les collagènes. Ils sont constitués de 3 chaînes α glycosylées et repliées en hélices les rendant très résistants aux contraintes mécaniques. Il existe différentes sous-unités et leur combinaison sont à l'origine de la diversité de la famille des collagènes (plus de 20 collagènes identifiés). Le collagène de type I, prototype de la famille des collagènes, est le plus abondant dans les tissus conjonctifs en particulier dans le derme et dans les tendons. Les fibres d'élastines sont également des constituants importants de la MEC apportant les propriétés de souplesse aux tissus.

La MEC contient également diverses glycoprotéines matricielles qui sont responsables de l'assemblage et de la réticulation des protéines fibrillaires mais aussi de l'ancrage des cellules à la matrice. Au sein des MEC, on peut observer : *(i)* Des glycoprotéines d'adhérence des constituants de la MEC entre eux et/ou avec les cellules. Par exemple la fibronectine, dimère de grande taille en forme de V, permet d'ancrer les cellules (par interaction avec les intégrines) à la MEC (par interaction avec les collagènes). Aussi SPARC (Secreted Protein, Acidic and Rich in cystein), glycoprotéine présente dans le derme, participe à l'assemblage, l'organisation et le renouvellement des fibres de collagène (Brekken & Sage, 2001). *(ii)* Des glucosaminoglycannes (GAG) qui sont des longues chaînes polysaccharidiques qui forment un gel hydraté de remplissage du tissu conjonctif (ex : acide hyluronique). *(iii)* Les protéoglycannes sont constitués d'un cœur protéique sur lequel se fixent de nombreux GAG.

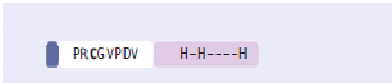
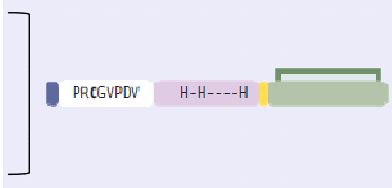
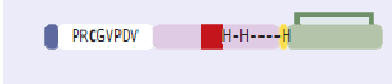
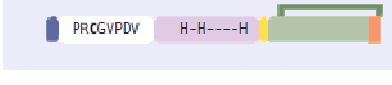
Ces molécules sont également importantes dans l'organisation de la MEC. Par exemple la décorine, petit protéoglycane riche en leucine, est fortement exprimée dans le derme dans lequel elle joue un rôle majeur dans l'assemblage des fibres de collagène (Reed & Iozzo, 2002). La plupart des glycoprotéines matricielles peuvent contrôler directement les activités biologiques des cellules stromales et épithéliales en interagissant avec les récepteurs membranaires ou en séquestrant les facteurs solubles (ex : facteurs de croissance). La MEC constitue donc un environnement complexe qui n'est pas simplement un tissu conjonctif de soutien inerte et qui participe activement aux interactions entre l'épithélium et son micro-environnement dans les processus physiologiques et pathologiques.

1.3.2 Les métalloprotéases

La MEC peut être remodelée par des protéases matricielles dont les métalloprotéases (MMPs). Les MMPs constituent une grande famille de protéases essentiellement matricielles recrutables au niveau des membranes dont l'activité nécessite la présence d'ions métalliques (Zn^{2+}). Elles sont classées en plusieurs groupes en fonction de leurs structures et de leurs substrats.

i Structure et classification des MMPs

Jusqu'à présent, au moins 23 MMPs ont été décrites chez l'homme et regroupées en 4 classes de MMPs sécrétées (matrilysines, collagénase, stromélysines et gélatinases) et 1 classe de MMPs membranaires (figure 2). La structure minimum des MMPs comprend 3 domaines : un **pré-domaine** (séquence signal en N-terminal) qui permet l'acheminement intra-cellulaire des MMPs et qui est éliminé dans le réticulum endoplasmique ; un **pro-domaine** qui maintient les MMPs sous forme latente ; et le **domaine catalytique** responsable du clivage. La plupart des MMPs (sauf les matrilysines) possèdent également des domaines de reconnaissance et de liaison aux substrats : domaine homologue de l'hémopexine et séquence analogue à la fibronectine pour les gélatinases (MMP-2 et -9). Enfin, les MMPs membranaires possèdent un site hydrophobe nécessaire pour la localisation à la surface des membranes cellulaires (Van Wart & Birkedal-Hansen, 1990).

Classe	MMP n°	Structure	Principaux substrats	Principales sources de production
Matrilysines	-7; -26		Gélatine, fibronectine	carcinomes
Collagénases	-1; -8; -13		Collagène fibrillaire	Carcinomes, fibroblastes
Stromélysines	-3; -10; -11		Glycoprotéines, protéoglycanes	Carcinomes, fibroblastes, cell. épithéliales
Gélatinases	-2; -9		Gélatine, collagène IV	Carcinomes, fibroblastes, cell. de l'inflammation, endothéliales
MMPs membranaires	-14; -15 ...		MMPs, collagène, etc...	Carcinomes, fibroblastes, cell. épithéliales








 Pré-domaine	 Pro-domaine avec son résidu cystéine (C)	 Domaine catalytique avec 3 résidus histidine (H)	 Site hydrophobe transmembranaire
 Région charnière riche en proline	 Séquence analogue à la fibronectine	 Domaine homologue de l'hémopexine	

Figure 2 : Structure et classification des MMPs adapté de (Chantrain & DeClerck, 2002)

ii Régulation des MMPs

Les MMPs sont tout d'abord régulées au niveau de leur expression qui peut être déclenchée par différents facteurs solubles du micro-environnement. Les gènes des MMPs, regroupés sur le chromosome 11 (q22-q23) (Formstone *et al*, 1993) présentent une structure relativement bien conservée au niveau de leur promoteur : une boîte TATA et des sites de recrutement de facteurs de transcription comme PEA-3, AP-1, Sp-1 ou les membres de la famille des facteurs de transcription ETS (Yan & Boyd, 2007).

L'activité des MMPs est tout d'abord régulée au niveau de leur activation. Les MMPs sont sécrétées sous une forme latente (pro-MMP) maintenue par le pro-domaine qui contient un résidu cystéine interagissant avec l'atome de Zn^{2+} du domaine catalytique. L'ion Zn^{2+} , relié par 3 résidus Histidine du domaine catalytique, doit être libéré de la liaison avec la cystéine pour que la MMP soit active. Cette activation appelée « cystein switch » consiste en un clivage du pro-domaine réalisé par des sérines protéases (plasmine, furine...), par d'autres MMPs actives ou même par une auto-activation (Van Wart & Birkedal-Hansen, 1990).

Par exemple la MMP membranaire de type 1 (MMP-14 ou MT1-MMP) active la pro-MMP-2 à la surface des cellules migrantes (Cao *et al*, 1995).

L'activité des MMPs est aussi contrôlée par deux familles d'inhibiteurs endogènes : l' α 2-macroglobuline et les TIMPs (Tissue Inhibitors of Metalloproteinases). L' α 2-macroglobuline est le principal inhibiteur de protéases dans le plasma. Après fixation aux MMPs pour les séquestrer, les complexes formés sont dégradés par endocytose (Sottrup-Jensen & Birkedal-Hansen, 1989). Les TIMPs constituent une famille de 4 inhibiteurs (TIMP-1 à -4) des MMPs dans la matrice extra-cellulaire. Les TIMPs se fixent aux MMPs de manière stoechiométrique (1 :1) de manière réversible.

Dans des conditions physiologiques (développement embryonnaire, morphogénèse...) l'activité des MMPs est maintenue par un équilibre strict entre les facteurs impliqués dans leur activation et dans leur inhibition. La dérégulation de cet équilibre est critique pour le développement de certaines pathologies (cancers).

iii Rôles biologiques des MMPs

A l'origine les MMPs ont été décrites pour leur capacité à dégrader les composants structurels de la MEC : les collagénases dégradent les collagènes fibrillaires ; les gélatinases clivent le collagène dénaturé (gélatine) et le collagène de type IV (lame basale) ; les stromélysines clivent de nombreux protéoglycanes.... De ce fait, elles ont surtout été associées à la migration et à l'invasion cellulaire dans des processus physiologiques (développement embryonnaire, cicatrisation) comme pathologiques (métastases des cancers).

Mais ces dernières années de nombreuses études ont montré que les MMPs clivent des substrats très variés (facteurs de croissance, récepteurs membranaires, etc...) et par conséquent leurs rôles biologiques se sont diversifiés (Kessenbrock *et al*, 2010) (figure 3). Ainsi les MMPs sont impliqués dans la régulation de la prolifération/ différenciation, survie/ apoptose, angiogenèse, inflammation, transition épithélium-mésenchyme... (Egeblad & Werb, 2002). Ainsi le clivage de constituants de la MEC par les MMPs peut générer des fragments aux propriétés biologiques indépendantes. Les exemples les plus décrits sont liés à la production de facteurs anti-angiogéniques. Ainsi le clivage du collagène XVIII (MMP-3 ; -9 ; 12...) génère l'endostatine (O'Reilly *et al*, 1997) et le clivage du plasminogène (MMP-2 ; -3 ; -7...) génère l'angiostatine (Cornelius *et al*, 1998), deux inhibiteurs de la prolifération et de l'invasion des cellules endothéliales.

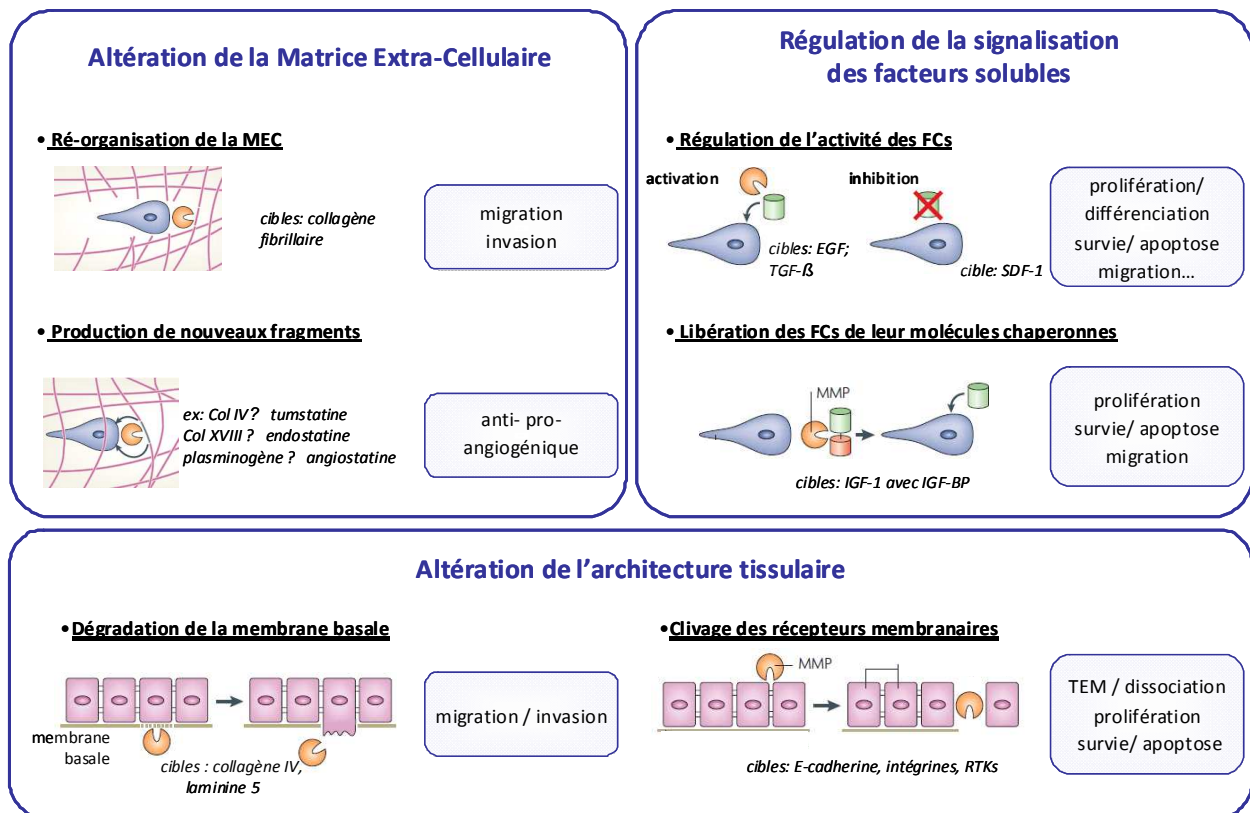


Figure 3 : Rôles biologiques des MMPs, d'après (Page-McCaw *et al*, 2007)

Les MMPs peuvent également contrôler les activités biologiques des cellules via la libération et/ou l'activation de certains facteurs solubles séquestrés dans la MEC. Ainsi, la forme latente du TGF-β1 est activée par les MMP-2 ; -9 et -14 (MT1-MMP) (Yu & Stamenkovic, 2000) ; (Mu *et al*, 2002), et la MMP-9 régule l'activité du VEGF (Bergers *et al*, 2000). Les MMPs peuvent aussi libérer ces facteurs solubles de leur molécule chaperonne. Ainsi le clivage de l'IGF-BP (Insulin Growth Factor-Binding Protein) par la MMP-11 (stromelysine-3) permet de libérer et d'activer l'IGF-1 (Mañes *et al*, 1997). Enfin, le clivage des récepteurs membranaires par les MMPs peut modifier directement l'ancrage des cellules à la MEC (ex : clivage des intégrines) et/ou la capacité des cellules à détecter des signaux extra-cellulaires (ex : clivage des récepteurs à tyrosine kinase).

1.3.3 Les facteurs de croissance

Les facteurs de croissance (FC) constituent une très large famille de molécules solubles qui régulent les activités biologiques des cellules (prolifération/différenciation ; survie/mort ; migration...). Les FC sont sécrétés dans le milieu extra-cellulaire spécifiquement par chaque type cellulaire et sont les principaux messagers des communications entre les cellules épithéliales et leur micro-environnement. Voici quelques exemples de FC qui régulent l'homéostasie du tissu de la peau (table 1) :

	Nom des facteurs	Cellules sécrétrices	Récepteur sur les kératinocytes
Facteurs stimulant la prolifération cellulaire			
KGF (FGF-7)	Keratinocyte Growth Factor	Fibroblastes	FGFR2IIIb
FGF-2 (FGF-b)	Fibroblasts Growth Factor basic	Fibroblastes	FGF-R
HGF / SF	Hepatocyte Growth Factor / Scatter Factor	Fibroblastes	c-Met
IGF-1	Insulin Growth Factor - I	Fibroblastes	IGF-RI
EGF	Epidermal Growth Factor	kératinocytes	EGFR- ErbB (1-4)
Facteurs inhibant la prolifération cellulaire			
TGF-β1	Transforming Growth Fractor β 1	Fibroblastes / kératinocytes	TGR RII
IFN-γ	Interferon - γ	Lymphocytes T/ kératinocytes	

Table 1 : Exemple des facteurs de croissance contrôlant la prolifération et la différenciation des cellules épithéliales de la peau

Les FCs se fixent spécifiquement sur leur récepteur membranaire généralement de type tyrosine kinase. Dans ce cas, cela va induire la dimérisation du récepteur, son autophosphorylation, le recrutement des différentes protéines de signalisation et l'activation de différentes voies de transduction. Le couple HGF/SF (Hepatocyte Growth Factor / scatter factor) et son récepteur c-Met est un exemple-type des interactions entre l'épithélium et son micro-environnement. En effet, l'HGF/SF est spécifiquement sécrété par les fibroblastes dans le micro-environnement sous forme d'un précurseur inactif formé d'une chaîne unique qui doit être clivé par des protéases matricielles. L'HGF/SF sera alors composé d'une sous-unité α (69 kDa) et d'une sous-unité β (34kDa) reliés par des ponts disulfures (Nakamura, 1991). Le récepteur Met est quant à lui exprimé principalement sur les cellules épithéliales (Bottaro *et al*, 1991; Naldini *et al*, 1991 ; Gherardi & Stoker, 1990). Donc, par l'HGF/SF les fibroblastes contrôlent la prolifération, la survie, la migration ou encore la morphogenèse des cellules épithéliales (Stoker *et al*, 1987 ; Weidner *et al*, 1993).

1.3.4 Les cytokines et le système immunitaire

En cas de perturbations dans l'homéostasie tissulaire, les cellules de l'inflammation (mais aussi les cellules épithéliales et les fibroblastes) vont produire une grosse quantité de facteurs solubles appelés cytokines qui vont contribuer à l'activation cellulaire et amplifier la réponse immunitaire par le recrutement des effecteurs. Il existe de nombreuses cytokines réparties en plusieurs

familles dont les Interférons, les interleukines (au moins 35 IL- différentes), les chimiokines ou chémokines (plus de 40), la famille du facteur de nécrose tumorale (TNF), des colony stimulating factors (CSF), etc....

Ces facteurs activent leurs récepteurs spécifiques exprimés sur les cellules cibles pour induire des activités biologiques (expression, prolifération, migration...). Les cytokines sont principalement impliquées dans la régulation des réponses inflammatoires et immunitaires (innées et adaptatives) en favorisant l'activation et le recrutement des cellules impliquées (par chemoattraction). Néanmoins, les cytokines peuvent également stimuler directement la croissance et la migration des cellules épithéliales et des fibroblastes.

1.3.5 Mécanismes de sécrétion des molécules du micro-environnement

Les constituants de la MEC sont principalement sécrétés par les fibroblastes. Ces derniers synthétisent aussi des facteurs de croissance, protéases et cytokines qui peuvent être également sécrétés par les cellules immunitaires, les cellules endothéliales voir même par les cellules épithéliales. Toutes ces protéines, une fois insérées de façon co-traductionnelle dans le réticulum endoplasmique, vont subir une série de modifications pour être correctement maturées au cours de leur parcours dans les différents compartiments endomembranaires des cellules (réticulum endoplasmique, appareil de Golgi, vésicules de sécrétion...) avant d'être sécrétées. Ainsi la maturation post-traductionnelle des protéines débute dans la lumière du réticulum endoplasmique (RE) par l'acquisition de leur conformation correcte. Ce sont des protéines chaperonnes (dont BiP (Binding protein) qui est la plus abondante) du RE qui sont responsables de ces repliements. Aussi, les modifications post-traductionnelles des protéines, comme la glycosylation (N-glycosylation) et la formation de ponts disulfures, sont initiées dans le RE. Enfin un contrôle qualité est mis en place dans le RE afin de vérifier que le repliement des protéines ainsi que leur glycosylation ont été correctement réalisés. A noter que les protéines mal conformées vont être dirigées vers les voies de dégradation du protéasome après qu'elles eussent été ubiquitinées (Ellgaard *et al*, 1999). Les protéines sont ensuite adressées vers l'appareil de Golgi pour poursuivre leurs modifications post-traductionnelles (N- et O-glycosylation) avant d'être emmenées jusqu'à la surface des cellules par des vésicules de transport puis sécrétées par exocytose. Il existe deux voies de sécrétion : la première, dite constitutive, permet de sécréter de manière non spécifique la plupart des protéines matricielles ; la seconde, dite contrôlée, nécessite un signal extérieur pour initier l'exocytose. Cette sécrétion contrôlée est principalement observée dans les cellules spécialisées dans la libération rapide sur demande (ex : hormones,

neurotransmetteurs, enzymes digestives...). Le transport des protéines dans les vésicules vers la surface membranaire (transport antérograde) doit être compensé par un transport dans le sens inverse (transport rétrograde).

Dans certaines situations physiologiques anormales (carence en glucose, hypoxie, statut redox et taux de calcium anormaux, inflammation, ...) ou pathologiques (diabète, cancers), la sécrétion des protéines peut être perturbée. Ainsi, ces événements peuvent perturber la maturation protéique au niveau du réticulum endoplasmique (RE) et plus particulièrement le processus de repliement des protéines (Yoshida, 2007). L'accumulation des protéines mal conformées au niveau de la lumière du RE va provoquer un état de stress du RE, déclenchant l'activation d'une réponse adaptative physiologique de la cellule appelée « Unfolded Protein Response » (UPR) (Schröder & Kaufman, 2005). Chez les mammifères, l'UPR est constituée de 3 voies de signalisation initiées par trois protéines transmembranaires senseurs du stress du RE : PERK (PKR-related Endoplasmic Reticulum Kinase) ; IRE-1 (Inositol Requiring Enzyme 1) et ATF6 (Activating Transcription Factor 6). Dans des conditions normales, PERK, IRE-1 et ATF-6 sont inactivées par la fixation de la protéine chaperonne BiP. La dissociation de BiP (au profit des protéines mal conformées présentes dans la lumière du RE) active PERK et IRE-1 par dimérisation puis auto-phosphorylation, et ATF-6 par clivage après sa re-localisation au niveau de l'appareil de Golgi (Okamura *et al*, 2000). L'activation de ces protéines entraîne l'expression et/ou l'activation spécifique de facteurs de transcription. Ainsi l'activation de PERK, inhibe EIF2 α (eucaryotic initiation factor 2) par phosphorylation, participant à une atténuation globale de la traduction des ARNm hormis celle de l'ARNm du facteur de transcription ATF4. IRE-1 induit l'épissage alternatif de l'ARNm du facteur de transcription XBP-1 nécessaire à son activation (Yoshida *et al*, 2001). Le clivage d'ATF-6 au niveau de l'appareil de Golgi produit un fragment (ATF6 p50) présentant une fonction de facteur de transcription (Wang *et al*, 2000). Ces facteurs de transcription sont à l'origine de la mise en place d'un programme transcriptionnel permettant (i) de rétablir l'homéostasie (calcique) du RE, (ii) de diminuer la synthèse des protéines afin de réduire leur accumulation dans la lumière du RE, (iii) d'augmenter la synthèse des protéines chaperonnes et (iv) d'augmenter la synthèse des protéines impliquées dans les voies de dégradation protéasomale et autophagique. Généralement une activation trop importante et prolongée des voies de l'UPR va induire la mort des cellules par apoptose (Xu *et al*, 2005)

2. Rôles physiologiques du micro-environnement

En conditions normales, les interactions entre l'épithélium et son micro-environnement sont responsables du maintien de l'état de quiescence des organes fonctionnels et de leur homéostasie. Néanmoins certains processus physiologiques (développement embryonnaire, morphogenèse de branchement, cicatrisation...) nécessitent l'activation du stroma de manière fortement contrôlée.

2.1. Embryogenèse et morphogenèse

Des interactions actives entre les cellules épithéliales et leur micro-environnement sont tout d'abord nécessaires pour la mise en place des feuillettes et des tissus épithéliaux lors du développement embryonnaire. Ainsi des MMPs sont nécessaires pour remodeler les MEC et des facteurs solubles vont stimuler la prolifération et la migration des cellules épithéliales. Un exemple bien caractérisé est celui du couple HGF/SF et de son récepteur Met dont les rôles lors du développement embryonnaire ont été mis en évidence grâce à des modèles de souris Knock-out. L'extinction de l'HGF/SF ou de Met entraîne la mort de l'embryon (environ après 15 jours de développement) avec des défauts d'organisation du placenta, une réduction de la taille du foie et de muscles squelettiques (Bladt *et al*, 1995 ; Schmidt *et al*, 1995; Uehara *et al*, 1995). L'HGF exprimé dans le mésenchyme va avoir un effet paracrine sur la survie, la prolifération et la migration des cellules épithéliales et endothéliales qui expriment Met.

Chez l'adulte, le micro-environnement peut aussi contrôler la formation de certains organes (glande mammaire, poumons, prostate...) par morphogenèse de branchement. Ainsi, lors du développement de la glande mammaire, l'HGF/SF exprimé par les cellules du stroma va stimuler la ramification des cellules épithéliales mammaires (Silberstein, 2001). Aussi l'interaction des cellules épithéliales avec leur MEC via les intégrines (ex : beta-1) est nécessaire pour la morphogenèse des glandes mammaires et de la prostate (Howlett *et al*, 1995 ; Klinowska *et al*, 1999).

2.2. Homéostasie et quiescence des tissus

Dans un organe fonctionnel, le stroma interagit avec l'épithélium essentiellement par des communications paracrines (sécrétion de FC, cytokines...) afin de maintenir un état de quiescence et l'homéostasie nécessaire au renouvellement des tissus (Donjacour & Cunha, 1991 ; Howlett & Bissell, 1993). Par exemple au niveau de la peau, les couches superficielles de l'épiderme étant en constante desquamation, les kératinocytes de la couche basale doivent se diviser afin de remplacer ces cellules. Les états de différenciation et de prolifération sont régulés essentiellement par des FC sécrétés soit par les kératinocytes (contrôle de la prolifération

autocrine), soit par les cellules du derme en particulier les fibroblastes (stimulation paracrine) (Shirakata, 2010) (figure 4).

Les kératinocytes stimulent leur propre prolifération principalement par la sécrétion de FC de la famille de l'EGF (Epidermal Growth Factor) (Hashimoto, 2000). Les kératinocytes contrôlent également indirectement leur prolifération en régulant la production de FC dans les autres types cellulaires du derme (Werner & Smola, 2001). Par exemple les interleukines IL-1 α et IL-1 β sécrétés par les kératinocytes vont stimuler la sécrétion du KGF (Keratinocyte Growth Factor) par les fibroblastes (Maas-Szabowski *et al*, 1999). Le KGF ou FGF-7, membre de la famille des Fibroblast Growth Factor, est le principal facteur mitogène des kératinocytes. La prolifération des cellules épithéliales peut être inhibée par des facteurs sécrétés par les kératinocytes et/ou les fibroblastes comme le TGF- β 1. Ainsi l'ajout de TGF- β 1 dans des cultures de kératinocytes inhibe leur prolifération (Matsumoto *et al*, 1990). Dans ces conditions de quiescence, il y a un équilibre entre les stimuli pro- et anti- prolifératifs pour assurer une homéostasie correcte.

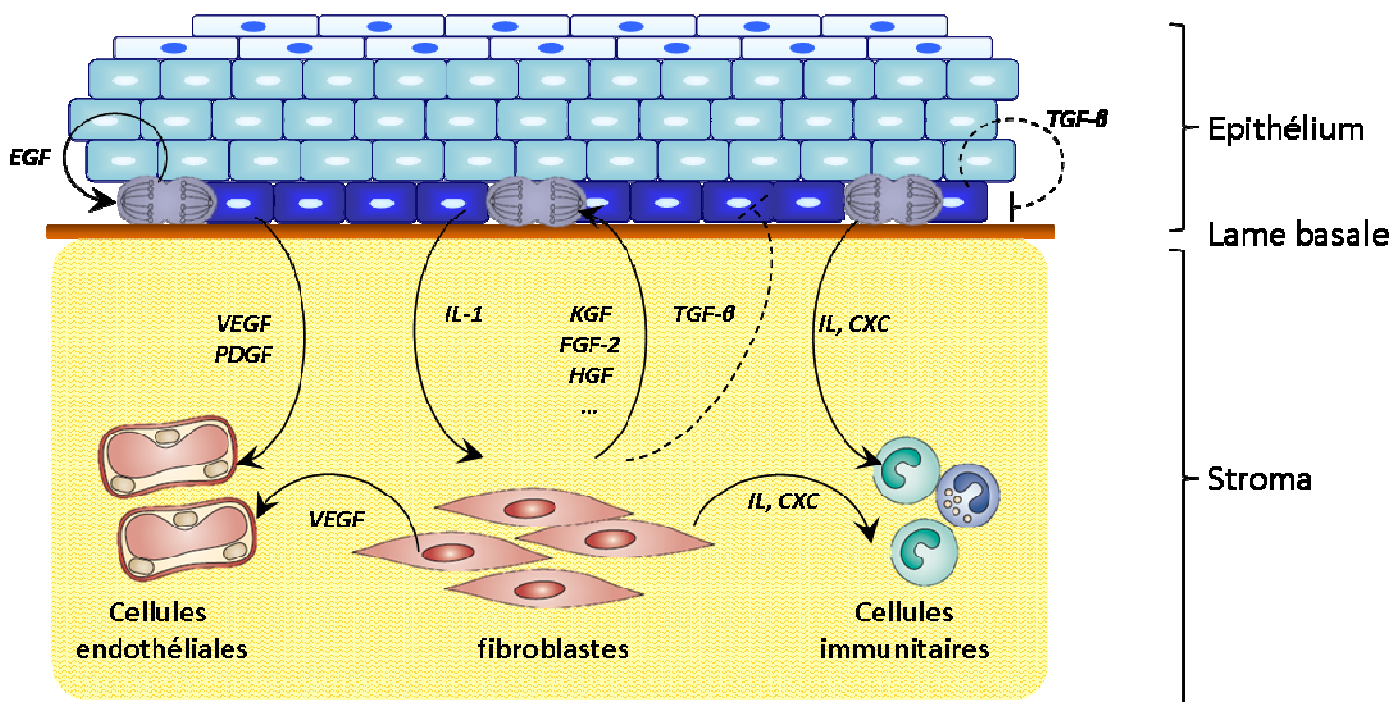


Figure 4 : Interactions équilibrées et contrôlées entre les cellules épithéliales et leur micro-environnement dans des conditions normales de quiescence.

Le comportement des cellules épithéliales est aussi contrôlé par leur contact physique avec la MEC. Ainsi des récepteurs d'adhésion comme les intégrines transmettent les signaux extérieurs de la MEC induisant la survie et la différenciation des kératinocytes (Müller *et al*, 2008). Les constituants de la MEC (fibres de collagène, glycoprotéines...) régulent également ces interactions en séquestrant les FC (FGF-2, HGF/SF...). La libération et l'activation de ces FC sont effectuées par les protéases matricielles (serines protéases, MMPs...) (Taipale & Keski-Oja, 1997).

2.3. Cicatrisation

Lorsqu'un tissu subit une blessure, des interactions activatrices entre les cellules épithéliales et celles du stroma sont nécessaires pour permettre, dans un contexte inflammatoire, une cicatrisation efficace (el-Ghalbzouri *et al*, 2002; Werner *et al*, 2007). La cicatrisation (wound healing) est une succession d'étapes visant à éliminer les pathogènes et à réparer le tissu endommagé :

- La *phase inflammatoire* débute très rapidement après la blessure, par la sécrétion de signaux pro-inflammatoires (ex : IL-1) des kératinocytes et/ou des fibroblastes. Ces signaux permettent le recrutement des cellules de l'inflammation.
- La *phase de prolifération* consiste en une accumulation de fibroblastes et kératinocytes autour de la blessure et une néo-vascularisation. La prolifération et la migration des cellules sont stimulées par des FCs. Aussi une nouvelle MEC temporaire est produite (riche en collagène III et fibronectine) par les fibroblastes.
- La *phase de ré-épithélisation* a pour but de recouvrir la blessure. Pour cela la migration et la prolifération des kératinocytes sont fortement stimulées par les facteurs de croissances et protéases sécrétés par les fibroblastes.
- La *phase de contraction*, essentielle pour la fermeture de la blessure, nécessite la différenciation des fibroblastes en myofibroblastes aux caractéristiques de cellules contractiles (expression de l'alpha-actine). La différenciation des myofibroblastes est induite par la sécrétion de TGF- β par les kératinocytes.

Au final, bien qu'un microenvironnement très pro-inflammatoire soit rapidement mis en place par les interactions kératinocytes/fibroblastes lors de la cicatrisation, celui-ci est assez vite régulé par une production de facteurs inhibiteurs tels que le TGF- β et le TNF- α . Mais une sur-activation de ces interactions peut être à l'origine de fibrose (desmoplasie).

3. Rôle du micro-environnement sur le développement des carcinomes

3.1. Généralités sur le développement des carcinomes

Dans les pays industrialisés le cancer est la principale cause de mortalité (environ 150 000 personnes par an en France). La grande majorité des cancers chez l'humain se développent à partir des cellules épithéliales, ce sont les carcinomes. Du fait de leur incidence élevée et de leur mortalité importante les cancers de la prostate, du sein, des poumons et du colon sont très étudiés. Néanmoins les cancers les plus fréquents sont les carcinomes non-mélanocytaires de la peau, répartis en deux catégories. Les *carcinomes basocellulaires* (basal-cell carcinoma-BCC) sont les plus fréquents. Ils se forment à partir des cellules épithéliales les plus basales de l'épiderme, se développent in situ et métastasent peu. Les *carcinomes spino-cellulaires* (squamous-cell carcinoma-SCC), second cancer de la peau le plus fréquent, se développent à partir des cellules épithéliales des couches superficielles de l'épiderme. Bien que plus rares, les SCC sont plus agressifs que les BCC et présentent un risque plus important de métastases. Le principal facteur de risque des carcinomes de la peau est l'exposition aux U.V et aux radiations. Les carcinomes non-mélanocytaires de la peau sont les plus communs des populations âgées, à la morbidité élevée (surtout les carcinomes squameux), et au cinquième rang des cancers les plus coûteux à traiter représentant un enjeu de santé publique (Boyle *et al*, 2004). De plus ces carcinomes sont associés à un risque plus important de développer un autre carcinome (Wheless *et al*, 2010).

Les principales caractéristiques des cellules cancéreuses sont (i) une augmentation des capacités prolifératives (qui peut être indépendante des signaux extérieurs), (ii) une prolifération illimitée, (iii) un échappement aux systèmes de contrôle du cycle cellulaire, (iv) une résistance aux processus de mort (apoptose), (v) l'acquisition des capacités invasives et (vi) la capacité à induire l'angiogenèse (Weinberg, cell, 2011). Ces caractéristiques sont acquises au fur et à mesure de la transformation cellulaire notamment par une succession d'altérations génétiques. Ainsi l'activation et/ou la sur-expression d'oncogènes apporte un gain de fonction à la cellule. Parmi les oncogènes les plus souvent activés dans les carcinomes, on peut citer les membres de la famille Ras, myc, Raf.... Aussi l'altération de fonction des gènes suppresseurs de tumeur va favoriser la croissance des cellules cancéreuses. L'altération de TP53, gardien du génome est retrouvée dans environ 50% des cancers humains. De même des mutations de BRCA1 constituent des facteurs héréditaires de prédisposition aux cancers du sein. Dans le cas des carcinomes non-mélanocytaires de la peau induit par les UV et les radiations, l'activation de Ras

et l'altération de p53 sont les plus couramment observées (van der Schroeff *et al.*, 1990; Harris, 1996; Boukamp, 2005).

Bien que les causes et les mécanismes moléculaires des cancers soient multiples, le schéma classique de l'évolution des carcinomes reste commun (figure 5). La transformation des cellules normales débute par l'acquisition de capacités prolifératives anormales provoquée par les premières altérations génétiques ce qui conduit à la formation de masses bénignes (hyperplasie, néoplasie). Ces masses peuvent ensuite évoluer vers des stades dysplasiques, définis par une altération de l'architecture et de la fonction de l'épithélium en lien avec la présence de cellules peu différenciées, au potentiel prolifératif important. Les dysplasies les plus agressives vont évoluer vers les stades de carcinomes *in situ*, c'est-à-dire le développement d'une masse de cellules néoplasiques sous forme compacte et sans destruction de la membrane basale. Bien qu'ils altèrent le fonctionnement du tissu épithélial, ces carcinomes détectés à temps sont souvent de bon pronostic. Dans le cas contraire ces carcinomes *in situ* évoluent vers les stades cancéreux métastatiques. A ce stade la tumeur maligne, plus grosse, doit être vascularisée pour poursuivre sa croissance et échapper à une hypoxie sévère. Les cellules cancéreuses les plus agressives vont dégrader la membrane basale, envahir le micro-environnement et pénétrer dans le réseau vasculaire. Cela résulte dans la formation de métastases dans les organes secondaires souvent à l'origine du décès. Cependant il apparait que les étapes de métastase puissent être aussi très précoces.

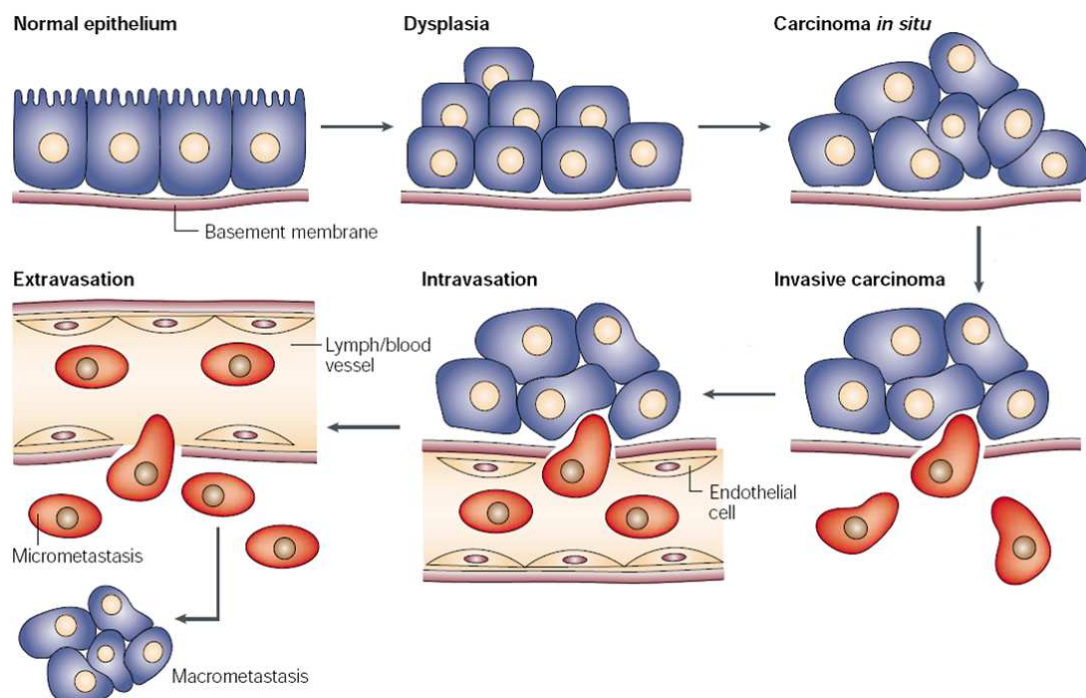


Figure 5 : Principales étapes associées à l'initiation et à la progression des carcinomes (adapté de Thiery, 2002).

Après s'être longtemps focalisées sur l'aspect génétique des cancers, de plus en plus d'études s'intéressent à l'importance du rôle des interactions épithélium/stroma dans l'initiation et le développement tumoral. Selon les conditions, le stroma peut jouer à la fois un rôle de suppresseur de tumeur ou pro-tumoral (Mueller & Fusenig, 2004). Le microenvironnement offre de nombreuses possibilités de cibles pour la prévention, les diagnostics, et les traitements des cancers.

3.2. Un micro-environnement jeune et sain inhibe l'initiation et la progression tumorale

Bien que la majorité des carcinomes soit diagnostiquée après 50 ans, des études ont montré que de nombreuses lésions pré-tumorales aux stades précoces (hyperplasie, néoplasie) sont présentes chez le « jeune adulte ». Dès 1935, Arnold Rich observe des lésions cancéreuses prostatiques microscopiques non diagnostiquées dans 14% d'échantillons prélevés post-mortem sur des hommes d'environ 50 ans dont le décès n'est pas lié aux cancers (Rich, 1979). Puis une autre étude a montré que dès la trentaine, un quart des hommes présentent des néoplasies intra-épithéliales au niveau de la prostate (table 2; (Sakr *et al*, 1993)) alors que l'âge moyen de diagnostic d'un cancer de la prostate est de 65 ans.

Diagnostic de néoplasies intra-épithélial prostatiques (Sakr <i>et al</i>, J Urol, 1993)		Observation de lésions tumorales au niveau des glandes mammaires (Nielsen <i>et al</i>, Br J Cancer, 1987)	
Age des patients	Pourcentage de positifs	Age des patients	Pourcentage de positifs
10 – 19 ans	0%	20 – 29 ans	0%
20 – 29 ans	9%	30 – 39 ans	8%
30 – 39 ans	27%	40 – 49 ans	39%
40 – 49 ans	34%	50-54 ans	33%
Age moyen de diagnostic des cancers de la prostate : 65 ans		Age moyen de diagnostic des cancers du sein : 60 ans	

Table 2 : Données expérimentales d'observation de lésions (pré)-tumorales au niveau de la prostate et des glandes mammaires.

De même, d'après une étude réalisée sur des glandes mammaires prélevées post-mortem, près de 40% de femmes présenteraient des hyperplasies et néoplasies à l'âge de 40 ans alors que l'âge moyen de diagnostic d'un cancer du sein est de 60 ans (table 2 ; (Nielsen *et al*, 1987)). Depuis, des observations identiques ont été faites dans différents organes (thyroïde, poumon, pancréas...) (Bissell & Hines, 2011). Ces données suggèrent donc que des cellules transformées et des lésions néoplasiques apparaissent très tôt au sein de différents organes.

Dans des cultures d'équivalent de peau, des kératinocytes normaux sont capables d'empêcher la croissance de cellules néoplasiques (IL-4) (Javaherian *et al*, 1998) mais pas celle de cellules de carcinomes de peau plus agressives (SCC13) (Zhang *et al*, 2005). Cela signifie donc que les cellules néoplasiques doivent échapper aux contrôles des cellules voisines encore normales pour progresser dans leur transformation. En effet l'intégrité de l'architecture des tissus épithéliaux normaux est essentielle pour empêcher les étapes précoces de la carcinogenèse. La structure du tissu épithélial est assurée par des jonctions intercellulaires très fortes (E-cadhérine, desmosomes) et par un ancrage des cellules à la membrane basale par les intégrines. Ces molécules d'adhérence contribuent à l'état de quiescence des cellules épithéliales non seulement par une cohésion mécanique, mais aussi par la transmission des signaux antiprolifératif et différenciateur du micro-environnement. Ainsi la E-cadherine, peut s'associer au récepteur de l'EGF (EGF-R) pour activer les voies PI3-K/Akt et induire un arrêt de la prolifération (Calautti *et al*, 2005 ; Müller *et al*, 2008; Perrais *et al*, 2007).

Un micro-environnement normalisé peut également inhiber la croissance tumorale voire même atténuer le phénotype tumoral. Plusieurs études montrent une atténuation des caractères de transformation par un environnement normal dans des conditions expérimentales physiologiques (culture en 3-dimensions dans un gel, équivalence de peau, *in vivo*...). La première démonstration a été réalisée en injectant des cellules de tératocarcinomes dans des blastocystes de souris. Les progénitures obtenues étaient parfaitement normales et sans tumeur démontrant que les cellules cancéreuses ont été reprogrammées lors du développement embryonnaire (Mintz & Illmensee, 1975 ; Illmensee & Mintz, 1976). Aussi, des cellules cancéreuses humaines transplantées dans des glandes mammaires saines de souris, sont reprogrammées par le microenvironnement et participent à la formation des structures normales (Bussard *et al*, 2010). Une reprogrammation des cellules cancéreuses mammaires (MDA-MB-231) vers un phénotype normal est également observée lorsqu'elles sont cultivées dans un gel riche en laminine (mimant la membrane basale) (Howlett *et al*, 1994 ; Bissell *et al*, 1999). La transplantation de MDA-MB-231 dans un organe sain peut induire la ré-expression de la E-Cadherine ce qui démontre une réversion de la transition epithelium-mésenchyme (TEM) qui est un phénotype de l'agressivité cancéreuse (Chao *et al*, 2010).

La composition de la matrice extra-cellulaire (MEC) est également un élément important pour l'inhibition de la progression tumorale. Ainsi, la présence de certaines glycoprotéines telle que la décorine inhibe la croissance tumorale en séquestrant dans la MEC les facteurs de croissance ou

en inhibant directement l'activité de certains récepteurs (ex : EGFR) (Seidler *et al*, 2006 ; Mohan *et al*, 2011).

Enfin le système immunitaire permet également de lutter contre le développement des tumeurs, essentiellement par l'intermédiaire de la réponse adaptative. En effet les antigènes des cellules cancéreuses peuvent être reconnus par des cellules de la réponse innée (ex : cellules dendritiques) qui vont activer les lymphocytes T CD8⁺ par la présentation d'antigène. Ces lymphocytes T CD8⁺ peuvent alors lyser spécifiquement les cellules tumorales. Le système immunitaire participe également au maintien des cellules cancéreuses en état de dormance (Quesnel, 2008; Morecki *et al*, 1996).

Il existe donc de nombreux arguments démontrant l'importance du contrôle négatif du micro-environnement sain et quiescent sur l'initiation et la progression tumorale.

3.3. Le stroma péri-tumoral contribue à l'évolution cancéreuse

Il est maintenant bien admis que le stroma péri-tumoral contribue activement aux différentes étapes du développement des cancers (Liotta & Kohn, 2001). Pendant longtemps seule la zone tumorale a été considérée comme étant altérée avec un tissu adjacent phénotypiquement normal. Pourtant l'observation des biopsies révèle qu'une zone stromale, à l'aspect de fibrose anormale (desmoplasie), se forme autour des tumeurs (Gao *et al*, 2010b). Ce stroma tumoral dit réactif se caractérise par la présence d'une MEC dense ainsi qu'une forte augmentation des populations cellulaires : infiltrations des cellules inflammatoires et immunitaires, forte vascularisation de vaisseaux sanguins et lymphatiques nouvellement formés et accumulations de fibroblastes dits activés (figure 6) (Rønnov-Jessen *et al*, 1996).

Les fibroblastes activés ou CAFs (Carcinoma Associated Fibroblasts) sont génétiquement normaux et sont caractérisés principalement par l'expression de marqueurs des myofibroblastes (fibroblastes responsables de la contraction tissulaire lors des phénomènes de cicatrisation) comme l' α -actine des muscles lisses (Tsukada *et al*, 1987). Ces propriétés contractiles augmentent les capacités migratoires des CAFs au sein de la MEC ce qui leur permet de s'agréger autour de la tumeur (Commandeur *et al*, 2011). Les CAFs sont aussi caractérisés par la production d'un sécrétome spécifique et fortement pro-tumoral : augmentation de l'expression de constituants de la MEC (collagène, fibronectine, tenascine...), de facteurs de croissance (HGF/SF, IGF-1, VEGF...), de cytokines (IL-1 ; MCP-1, SDF-1) et de MMPs (Kalluri & Zeisberg, 2006).

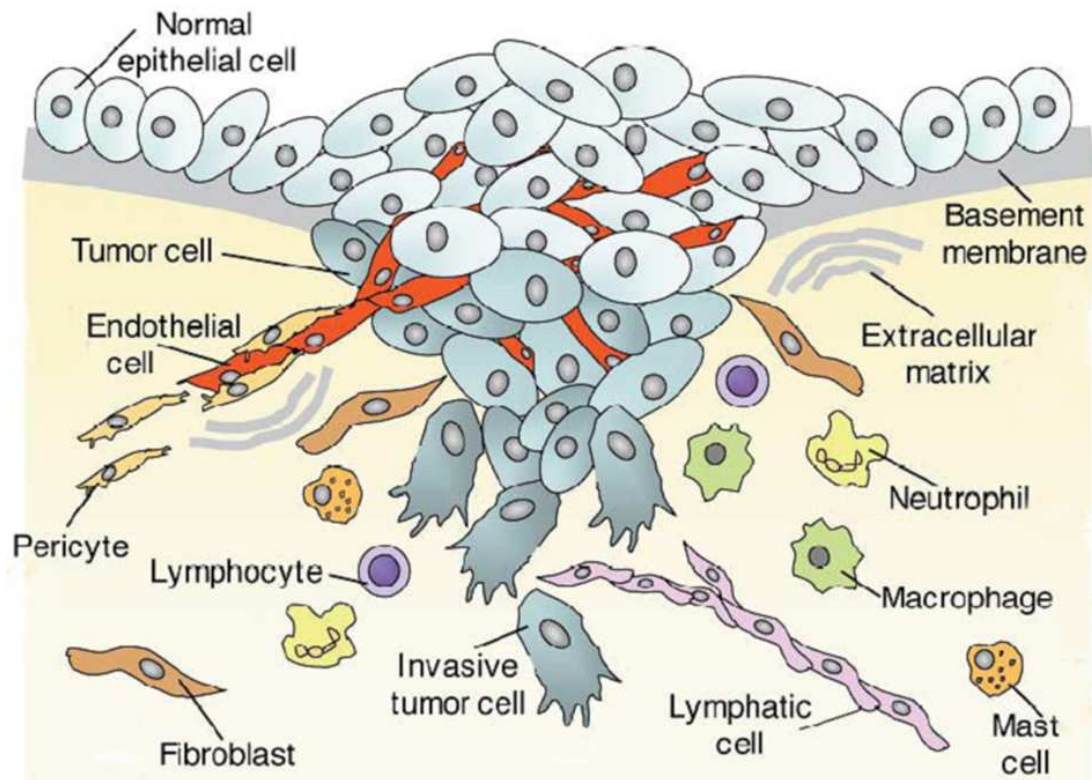


Figure 6 : Schéma d'un microenvironnement tumoral (d'après Joyce, 2005)

Ainsi, les CAFs, identifiés dans le stroma réactif de plusieurs types de carcinomes (ex : prostate, sein, peau...), peuvent stimuler la croissance, la transformation (par renforcement de la transition épithélium-mésenchyme) et l'invasion des cellules cancéreuses *in vitro* et *in vivo* (Olumi *et al*, 1999; Sternlicht *et al*, 1999; Kuperwasser *et al*, 2004; Orimo *et al*, 2005; Gao *et al*, 2010; Commandeur *et al*, 2011). L'activation des CAFs se fait principalement par les cellules cancéreuses elles-mêmes (Fromigué *et al*, 2003 ; Mishra *et al*, 2008) de manière paracrine (sécrétion de facteurs de croissance (TGF-beta, PDGF, FGF-2...)). Des contacts directs avec les cellules de l'inflammation (via les molécules d'adhésion ICAM), l'altération de la composition de la MEC ou des stress environnementaux (ROS, hypoxie...) peuvent également activer les CAFs (Zeisberg *et al*, 2000). Les cellules cancéreuses elles-mêmes peuvent être également une source de CAFs suite à une transition épithélium-mésenchyme (TEM) (Petersen *et al*, 2003). Enfin, cellules souches mésenchymateuses peuvent être reconverties en CAFs par une stimulation avec du milieu conditionné des cellules cancéreuses (Karnoub *et al*, 2007 ; Mishra *et al*, 2008).

Le développement des tumeurs est également accompagné par une importante infiltration de cellules immunitaires dans le stroma péri-tumoral. Alors que le système immunitaire permet dans

un premier de contenir le développement tumoral, une inflammation récurrente va au contraire le favoriser en produisant une grande quantité de facteurs de croissance, cytokines et MMPs (Coussens & Werb, 2002).

Les activations observées dans le stroma péri-tumoral sont donc proches de celles observées lors du processus de cicatrisation. Néanmoins, les régulations inhibitrices qui permettent de revenir à l'état quiescent du tissu à la fin du processus de cicatrisation ne sont pas activées (ou pas suffisamment) lors du développement tumoral.

4. Le micro-environnement : une bonne cible thérapeutique ?

Pendant longtemps les thérapies, en particulier les chimiothérapies, étaient uniquement dirigées contre les cellules cancéreuses. Mais l'instabilité génétique de ces cellules leur confère souvent une résistance aux traitements et provoque un fort risque de récurrence. De plus la distribution des drogues anti-tumorales peut être difficile au cœur des masses tumorales. C'est pourquoi de plus en plus d'approches thérapeutiques veulent cibler le micro-environnement tumoral. En effet les cellules du stroma péri-tumoral sont beaucoup plus stables génétiquement que les cellules cancéreuses et avec un accès plus simple pour les drogues. Le micro-environnement des cellules cancéreuses constitue donc un large répertoire de cibles thérapeutiques potentielles (Joyce, 2005). Les principales approches thérapeutiques ciblant le micro-environnement tumoral sont dirigées contre l'angiogenèse, et le système immunitaire et les MMPs. Jusqu'à présent les agents anti-angiogéniques, dont le bevacizumab/Avastin® ou le sunitinib/Sutent® sont les principaux inhibiteurs utilisés en thérapie. Des thérapies pour stimuler le système immunitaire de l'organisme contre ces cellules cancéreuses sont également développées dans les cancers du sein, de la prostate et du rein (Shiao *et al*, 2011). Enfin plusieurs inhibiteurs de l'activité des MMPs (Neovastat®, marimastat®, etc...) sont également en essais bien que les premiers résultats aient été décevants (Overall & Kleinfeld, 2006). En effet des difficultés sont associées aux approches thérapeutiques par le micro-environnement. Les facteurs du stroma forment généralement un réseau complexe d'interactions et modifier cet équilibre risque d'inhiber des capacités anti-tumorales et/ou activer des signaux promoteurs de tumeurs. C'est le cas notamment des MMPs qui, comme nous l'avons vu, possèdent des propriétés pro- et anti-tumorales. Inhiber leur activité pourrait donc avoir des effets néfastes sur le développement cancéreux (Overall & Kleinfeld, 2006).

B. Migration et invasion cellulaire

Les capacités migratoires et invasives des cellules sont essentielles pour le déroulement de certains processus physiologiques (développement embryonnaire, morphogenèse de branchement, cicatrisation ...) et pathologiques (métastases cancéreuses...). Alors que la migration est définie par le déplacement des cellules sur leur support, l'invasion se caractérise en plus par la capacité à traverser les barrières biologiques (lame basale, MEC). Comme les métastases restent la cause majeure de la mortalité liée aux cancers, les mécanismes associés aux processus invasifs sont donc particulièrement bien étudiés dans le but d'améliorer les traitements et la survie des patients.

1. Déroulement des processus migratoire et invasif

1.1. Acquisition des capacités migratoires par la transition épithélium-mésenchyme

1.1.1 Définition et mécanismes de la transition épithélium-mésenchyme

La Transition Epithélium-Mésenchyme (TEM) est considérée comme le mécanisme initial de l'acquisition des capacités migratoires des cellules. La TEM est définie par la perte progressive de marqueurs des cellules épithéliales pour acquérir des propriétés de cellules mésenchymateuses (Thiery, 2002) (figure 7). En effet, les cellules épithéliales sont très spécialisées avec une forte polarisation apicale/basolatérale fonctionnelle. Elles s'organisent en structure cohésive (épithélium) grâce à leurs jonctions inter-cellulaires : jonctions serrées (claudines, occludines...), jonctions d'adhérence (E-cadhérine, desmosomes), et jonctions de communication (connexines). Les cellules épithéliales, avec une organisation des fibres d'actine très polarisée, sont très peu capables de migrer et restent solidement attachées à la lame basale (Gumbiner, 1996). Les cellules mésenchymateuses (ex : les fibroblastes) sont des cellules beaucoup moins spécialisées, sans polarisation fonctionnelle et avec très peu de contacts inter-cellulaires. Situées généralement dans des tissus conjonctifs, ces cellules sont capables d'interagir avec la matrice extra-cellulaire (MEC) environnante par l'intermédiaire des intégrines et de la remodeler par la sécrétion de MMPs. Une fois activées, ces cellules présentent une mobilité importante au sein de cette matrice notamment grâce aux fibres d'actine de contraction (α -actine des muscles lisses).

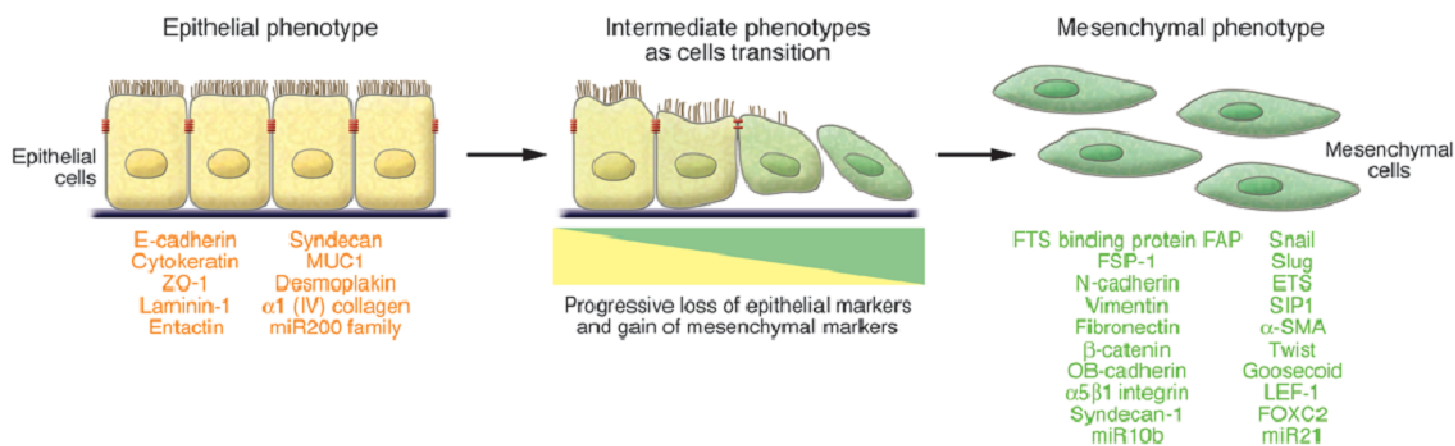


Figure 7: Marqueurs moléculaires associés à la transition épithélium-mésenchyme (extrait de (Kalluri & Weinberg, 2009))

Le déclenchement de la TEM est réalisé par des signaux extracellulaires, démontrant encore l'importance du microenvironnement (Thiery & Sleeman, 2006; Moustakas & Heldin, 2007). Les signaux qui peuvent initier une TEM sont des facteurs de croissance (ex : TGF- β , HGF/SF, FGFs, EGFs) (Li *et al*, 1994; Bladt *et al*, 1995, Nawshad *et al*, 2004), des cytokines (IL-6 ; IL-8...)(Fernando *et al*, 2011; Yadav *et al*, 2011), des changements dans les composants de la MEC, des MMPs (ex :MMP-3) (Radisky *et al*, 2005), l'hypoxie (Luo *et al*, 2006).

Ces signaux vont activer un réseau complexe de voies moléculaires initiées par l'activation des différents récepteurs membranaires (récepteurs à tyrosines kinases (Met, FGFR, ErbB), récepteurs au TGF- β ; récepteurs couplés aux protéines G, intégrines...). Ainsi la voie de signalisation Ras-MAPK est l'une des principales voies mises en œuvre lors des mécanismes de la TEM. On peut également citer l'implication des voies PI3K/Akt, GSK-3 (glycogen-synthase kinase-3), β -caténine, FAK (focal adhesion kinase)... L'ensemble de ces événements conduit à l'activation de facteurs de transcription, responsables de la sur-expression des marqueurs de cellules mésenchymateuses (ex : Vimentine, N-cadherine, Fibronectine, MMPs...) et/ou de l'inhibition d'expression de marqueurs des cellules épithéliales. (E-cadhérine, cytokératines...). Par exemple les facteurs de transcription de la famille SNAIL (SNAI1 (SNAIL), SNAI2 (slug)) sont les principaux responsables la répression de l'expression de l'E-cadhérine nécessaire à la dissociation des cellules épithéliales (Batlle *et al*, 2000 ; Cano *et al*, 2000). D'autres facteurs de transcription comme Twist-1 et Zeb1 contribuent également à la mise en place du programme transcriptionnel de la TEM (Yang *et al*, 2004 ; Peinado *et al*, 2007).

1.1.2 Rôles biologiques de la transition épithélium-mésenchyme

Il a récemment été proposé de distinguer 3 catégories de TEM en fonction de leurs rôles biologiques (Kalluri & Weinberg, 2009). La TEM de type 1 intervient sur des cellules épithéliales primitives lors de certaines étapes du développement embryonnaire dont la gastrulation (mouvements associés à la mise en place des feuillets) et l'émergence puis l'essaimage des cellules de la crête neurale. Lors de ces situations la réversion de la TEM par la transition mésenchyme-épithélium (TME) est nécessaire. Dans ces contextes, la TEM est nécessaire non seulement pour l'acquisition des capacités migratoires des cellules mais aussi pour les protéger contre les processus de mort par apoptose liés entre autre aux modulations des ancrages cellulaires (Vega *et al*, 2004).

La TEM de type 2 est associée aux processus de cicatrisation et de fibrose. Généralement causée par un état inflammatoire persistant, cette TEM permet la création de nouveaux fibroblastes à partir de cellules épithéliales ou de cellules endothéliales nécessaires à la fermeture de la blessure. Enfin la TEM de type 3 est associée à la transformation des cellules de carcinome. Etape cruciale dans la progression tumorale, elle permet aux cellules cancéreuses d'envahir leur stroma et de former des métastases (la croissance des métastases nécessitera une réversion de la TEM). Les marqueurs de la TEM, généralement absents dans les cellules normales différenciées, sont souvent exprimés dans les cellules cancéreuses aux propriétés métastatiques (Peinado *et al*, 2007; Ansieau *et al*, 2008a). En plus d'apporter des capacités invasives aux cellules cancéreuses, la TEM leur permet également de résister aux traitements pro-apoptotiques des chimiothérapies, radiothérapies... Ainsi, le traitement à la gemcitabine permet de distinguer deux catégories de lignées cancéreuses du pancréas : celles qui sont résistantes ou sensibles à l'effet pro-apoptotique. Il se trouve que contrairement aux lignées sensibles, les lignées cancéreuses résistantes expriment des marqueurs de la TEM (Arumugam *et al*, 2009). Cette TEM est un processus par lequel des cellules normales ou cancéreuses peuvent échapper aux principaux mécanismes suppresseurs de tumeurs induits par des stress : l'apoptose et la sénescence. Ainsi, le traitement des cellules cancéreuse (MCF-7) par l'adriamycine (drogue pro-apoptotique) peut induire une TEM sous le contrôle de Twist-1 protégeant les cellules du processus apoptotique et favorisant leurs capacités invasives et tumorigènes *in vivo* (Li *et al*, 2009). Comme nous le verrons dans le chapitre C, la sénescence permet de bloquer le cycle cellulaire afin d'empêcher la progression tumorale. L'induction de la TEM dans des cellules normales ou cancéreuses par l'expression des facteurs de transcription twist peut permettre aux cellules d'échapper à ce mécanisme de sénescence et de favoriser la progression tumorale (Ansieau *et al*, 2008a).

1.2. Attraction des cellules par le micro-environnement

Une fois que les cellules ont acquis les capacités migratoires par la TEM, leur invasion au sein de la MEC est stimulée par des facteurs chemoattractants émis par le micro-environnement : facteurs de croissance, cytokines inflammatoires et hypoxie.... Pour migrer, les cellules doivent donc également exprimer des récepteurs spécifiques capables de transmettre les signaux induits par ces facteurs.

1.2.1 Exemples classiques de récepteurs des chemoattractants : les Récepteurs à tyrosine kinase.

Comme nous l'avons déjà vu les facteurs de croissance et leurs récepteurs spécifiques sont des acteurs essentiels dans le comportement cellulaire notamment dans les processus migratoires et invasifs. Les facteurs de croissance activent principalement des récepteurs à tyrosines kinases qui eux-mêmes activent de nombreuses voies de signalisation. Par exemple, l'HGF/SF est un attractant très puissant par l'activation de son récepteur Met lors de la morphogenèse ou de la cicatrisation. Une dérégulation de l'activité du couple HGF/SF-Met (sur-expression de l'HGF ou de Met, activation indépendante du ligand) est souvent observée dans les cancers invasifs. Un gradient de facteurs chemoattractants est mis en place depuis la source de sécrétion afin d'orienter la migration. Ce gradient des facteurs chemoattractants va permettre aux cellules d'acquérir une polarisation morphologique (orientation des fibres d'actine) et moléculaire (répartition asymétriques des récepteurs sensibles aux chemoattractants, des intégrines...) ce qui va définir le front et l'arrière de la migration (Sullivan *et al*, 1984; Coates *et al*, 1992).

1.2.2 Exemple de récepteurs nouvellement impliqués dans les processus migratoires : les récepteurs PARs

i Description, expression et fonction de la famille des Récepteurs Activés par des Protéases (PAR).

La famille des récepteurs PARs (Protease Activated Receptor) est constituée de 4 membres (PAR-1 à -4). Ce sont des récepteurs membranaires couplés aux protéines G (GPCR) constitués d'une partie extra-cellulaire N-terminale glycosylée (N-glycosylation) qui est impliquée dans l'activation du récepteur, de sept domaines trans-membranaires et d'une partie cytosolique C-terminale impliquée dans le recrutement des protéines G (figure 8).

Historiquement, PAR-1, membre prototype de la famille, et la thrombine, son ligand principal ont été étudiés pour leur implication dans la coagulation du sang. Outre son rôle dans la conversion du fibrinogène en fibrine, la thrombine active PAR-1 fortement exprimé sur les plaquettes pour induire leur agrégation au niveau des blessures et favoriser la formation de caillots (KOWARZYK, 1952; Eagle, 1935 ; Ganguly, 1977). Néanmoins l'expression de PAR-1 est maintenant bien décrite dans de nombreux types cellulaires de différents organes et il est impliqué dans des processus biologiques variés (Macfarlane *et al*, 2001). Par exemple PAR-1 et la thrombine peuvent participer à l'angiogenèse et à l'inflammation (agrégation des neutrophiles). Aussi, sur des coupes de peau, il a été montré que PAR-1 est exprimé dans les kératinocytes et que sa stimulation induit la prolifération de ces cellules (Algermissen *et al*, 2000). Ces données suggèrent que PAR-1 et la thrombine pourrait avoir un rôle important lors des processus de cicatrisation (Xue *et al*, 2005; Gao *et al*, 2010a)

ii Mécanisme d'activation du récepteur PAR-1

L'activation des récepteurs PARs est particulière puisqu'elle est induite par un clivage protéasique réalisé au niveau de la partie extra-cellulaire. La thrombine, qui est une sérine protéase, est le ligand principal de PAR-1, PAR-3 et PAR-4. PAR-2 quant à lui ne peut pas être activé par la thrombine mais par d'autres sérines protéases telle que la trypsine. Depuis quelques années, il est montré que des protéases autres que la thrombine peuvent activer les récepteurs PARs avec plus ou moins d'affinité : trypsine, facteur de coagulation VII et FXa, plasmine, MMP-1.... (Coughlin, 2000 ; Arora *et al*, 2007). La thrombine est capable de se fixer physiquement sur la partie extra-cellulaire de PAR-1 au niveau de 2 séquences : le centre catalytique de la thrombine se fixe sur la séquence peptidique LDPR/S, le domaine de fixation au fibrinogène de la thrombine se fixe à une séquence analogue à l'hyrudine (inhibiteur de la thrombine) : DKYEPF (Vu *et al*, 1991b) ; (Liu *et al*, 1991) (figure 8). Seule la thrombine semble être capable de se fixer à PAR-1, les autres protéases doivent certainement être présentées par un co-facteur.

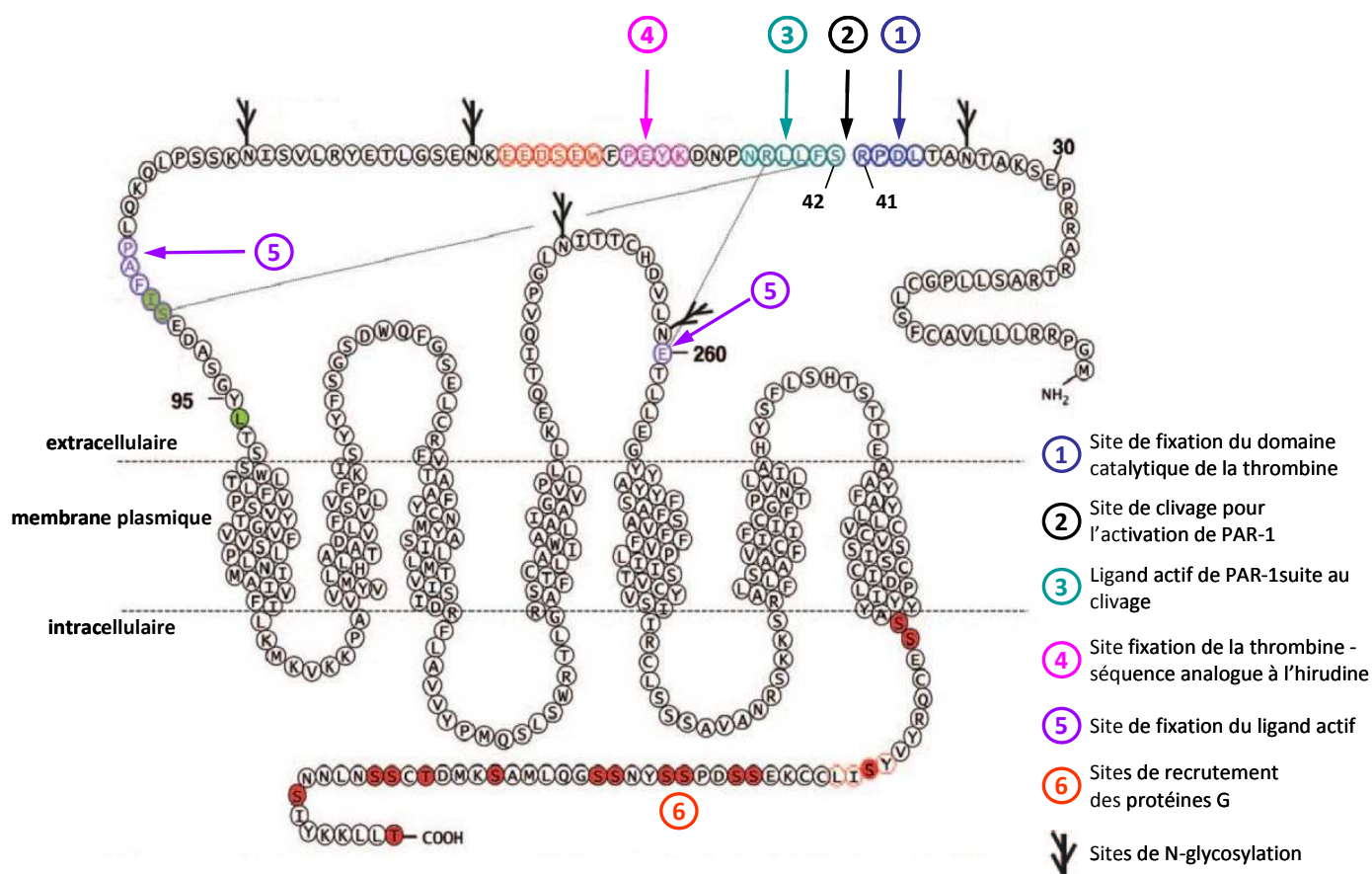


Figure 8: Structure et séquence peptidique du récepteur PAR-1 indiquant les sites de fixation pour la thrombine, le site de clivage (R^{41} - S^{42}) des protéases et le ligand actif nécessaire pour l'activation du récepteur (adapté de Steinberg, 2005)

Le clivage responsable de l'activation de PAR-1, réalisé de manière irréversible au niveau de la liaison peptidique R^{41} - S^{42} de la séquence LDPR/S, crée une nouvelle séquence N-terminale commençant par les acides aminés SFLLRN (Vu *et al*, 1991a). Cette séquence de 6 peptides devient alors le ligand minimum actif nécessaire à l'activation du récepteur (Scarborough *et al*, 1992). L'ajout de peptides synthétiques SFLLRN peut induire l'activation de PAR-1 indépendamment d'un clivage du récepteur (Vassallo *et al*, 1992). En revanche, le clivage sur des liaisons peptidiques autre que R^{41} - S^{42} par certaines protéases (Elastase, Cathepsine G, et Proteinase 3) empêche l'activation du récepteur (Renesto *et al*, 1997).

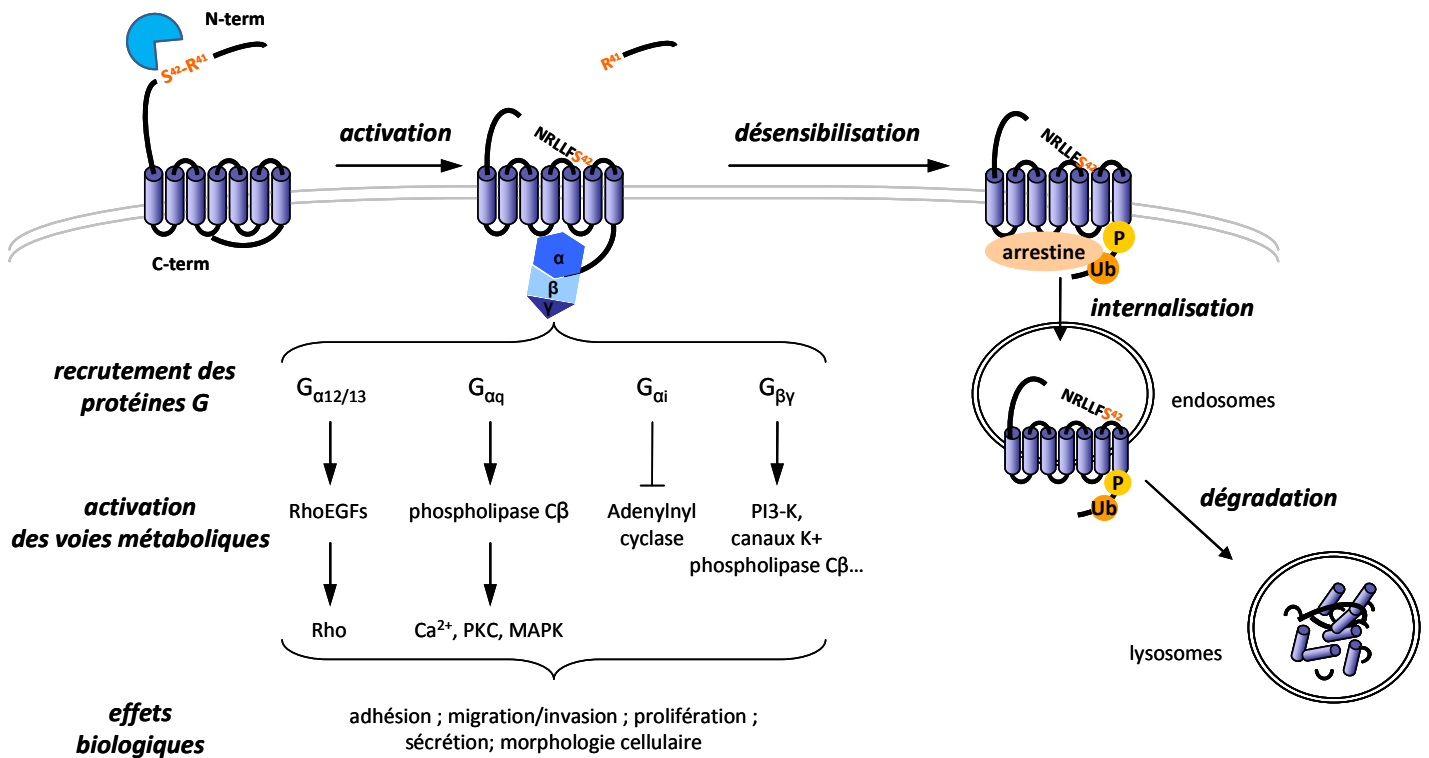


Figure 9: Activation, signalisation et régulation de l'activité du récepteur PAR-1 par internalisation et dégradation dans les lysosomes (adapté de(Arora et al, 2007).

Une fois démasqué, le ligand interne se lie au reste du récepteur, induit un changement de conformation favorisant le recrutement de complexes hétérotrimériques de protéines G de la famille $G_{\alpha12/13}$, G_q et G_i . Ces recrutements peuvent induire l'activation de nombreuses voies de signalisation (la voie des Rho-kinases, des MAPK, de la phospholipase C, la mobilisation du calcium intracellulaire) et l'inhibition de l'adénylate cyclase. Ces activations conduisent à des effets biologiques variés : morphologie cellulaire, adhésion, croissance, sécrétion et migration/invasion... (figure 9).

iii Mécanisme de régulation de l'activité de PAR-1

La régulation de l'activité de PAR-1 se fait essentiellement par des mécanismes de désensibilisation, d'internalisation et de dégradation des récepteurs (figure 9) (Marchese *et al*, 2008). Comme la plupart des GPCRs, PAR-1 est très rapidement désensibilisé par phosphorylation sur ses différents résidus Sérine et Thréonine situés dans la queue cytoplasmique du récepteur (figure 9, marqué en rouge) par des protéines kinases spécifiques des GPCR (GRK)

(Tiruppathi *et al*, 2000) ; (Ishii *et al*, 1994). Ces phosphorylations permettent le recrutement des arrestines qui empêchent l'interaction des protéines G avec le récepteur (Paing *et al*, 2002).

Les mécanismes d'internalisation de PAR-1 sont ensuite différents par rapport aux autres GPCRs. En effet, l'internalisation rapide de PAR-1 vers les endosomes est dépendante des molécules de clathrines et de dynamines mais indépendante de la présence des arrestines. Puis, au lieu d'être recyclé, PAR-1 est directement dégradé dans les lysosomes (Hoxie *et al*, 1993) ; (Brass *et al*, 1994 ; Trejo *et al*, 1998). Les mécanismes associés aux voies de dégradation de PAR-1 sont encore peu détaillés. Jusqu'à présent, il a été montré que l'ubiquitination de PAR-1 (dé-ubiquitinylé suite à son activation) n'est pas impliquée dans sa dégradation (Wolfe *et al*, 2007). Ces mécanismes de dégradation de PAR-1 sont essentiels pour le contrôle de la signalisation de PAR-1 (figure 9).

iv Implication de PAR-1 dans l'invasion tumorale

Au cours de ces 10 dernières années, l'expression des récepteurs PARs a été décrite dans de nombreux types de cancers, que soit ce soit dans les lignées cellulaires ou directement sur des biopsies tumorales. La compilation des résultats de plusieurs études a permis de montrer que PAR-1 est exprimé dans 77,3% des échantillons tumoraux analysés : 71% des tumeurs primaires et 63% des métastases sont positives (Elste & Petersen, 2010) (table 3).

Organe	Type de cancer	Echantillon d'identification
Prostate	Adénocarcinome	biopsie, lignée cellulaire
Sein	Carcinome	lignée cellulaire
ovaire	Carcinome	lignée cellulaire
endomètre	Adénocarcinome	biopsie
Peau	Mélanome	biopsie, lignée cellulaire
foie	Hépatocarcinome	biopsie, lignée cellulaire
rein	Adénocarcinome	biopsie, lignée cellulaire
poumon	Adénocarcinome et carcinome squameux	biopsie, lignée cellulaire
Tête et cou	Carcinome squameux	biopsie, lignée cellulaire
colon	Adénocarcinome	biopsie, lignée cellulaire

Table 3: Compilation des analyses d'expression du récepteur PAR-1 dans les biopsies tumorales et les lignées cellulaires (d'après Elste & Petersen, 2010).

Jusqu'à présent PAR-1 est essentiellement associé aux capacités invasives et métastatiques des cellules cancéreuses, ce qui en fait un marqueur de mauvais pronostic. Ainsi, PAR-1 est fortement exprimé dans les cellules cancéreuses de sein invasives (MDA-MB-231) alors qu'il n'est pas exprimé dans les cellules non-invasives (MCF-7). La transfection de PAR-1 dans les MCF-7 suffit à leur apporter ces capacités invasives (Boire *et al*, 2005).

Les mécanismes moléculaires par lesquels PAR-1 stimule l'invasion des cellules cancéreuses sont encore assez peu décrits. Néanmoins, quelques études montrent que PAR-1 favorise l'activation, l'expression ou le recrutement de protéines impliquées dans les processus migratoires des cellules comme les intégrines et les métalloprotéases. L'activation de PAR-1 dans des cellules cancéreuses d'ostéosarcomes induit l'expression de la MMP-9 et de l'intégrine $\beta 1$ nécessaire à l'invasion de ces cellules (Radjabi *et al*, 2008). Aussi l'activation de PAR-1 dans des cellules cancéreuses invasives peut permettre la ré-organisation à la membrane des intégrines ($\alpha V\beta 5$) en point focaux et l'activation de leur voie de signalisation par la phosphorylation des FAK (focal adhesion kinase) (Even-Ram *et al*, 2001). L'activation de PAR-1 peut également stimuler l'invasion en induisant une trans-activation persistante des récepteurs à l'EGF, EGFR et ErbB2 sur les cellules cancéreuses de sein MDA-MB-231 (Arora *et al*, 2008) ou des cellules de carcinomes de rein (Bergmann *et al*, 2006).

Pour la première fois en 2005, il a été montré que la MMP-1 sécrétée par des fibroblastes est capable d'activer le récepteur PAR-1 sur des cellules cancéreuses invasives de sein (MDA-MB-231) (Boire *et al*, 2005). Ils ont montré que la MMP-1, comme la thrombine, clive PAR-1 au niveau de la liaison peptidique R⁴¹-S⁴² entraînant la mobilisation du calcium intracellulaire et induisant les capacités invasives des cellules. Depuis, l'implication de l'axe MMP-1/PAR-1 dans les processus invasifs de différentes lignées cellulaires cancéreuses a été démontrée *in vitro* et *in vivo*. Par exemple MMP-1 et PAR-1 exprimés fortement dans les cellules métastatiques de mélanome sont nécessaires pour l'invasion de ces cellules (Blackburn *et al*, 2009). Aussi l'axe MMP-1/PAR-1 est impliqué dans l'invasion de cellules cancéreuses de l'ovaire (Wang *et al*, 2011).

1.3. Interaction des cellules avec la matrice extra-cellulaire

Le processus de migration et d'invasion cellulaire nécessite également un ancrage spécifique des cellules à la MEC ainsi que d'une dégradation localisée de cette MEC par les MMPs. Ces mécanismes sont principalement contrôlés par les récepteurs membranaires d'ancrage comme les intégrines et CD44.

1.3.1 Les intégrines

i Description et expression des intégrines

Les intégrines constituent une famille de glycoprotéines transmembranaires responsables de l'ancrage des cellules à la MEC (Hynes, 1987). Les intégrines sont des hétérodimères composés d'une sous-unité α (18 sous-unités α différentes) et d'une sous-unité β (8) formant au moins 24 couples (dimères) $\alpha\beta$ fonctionnels. Chaque intégrine peut se fixer spécifiquement à un ou plusieurs substrats matriciels (collagènes, laminines, fibronectines...) (figure 10).

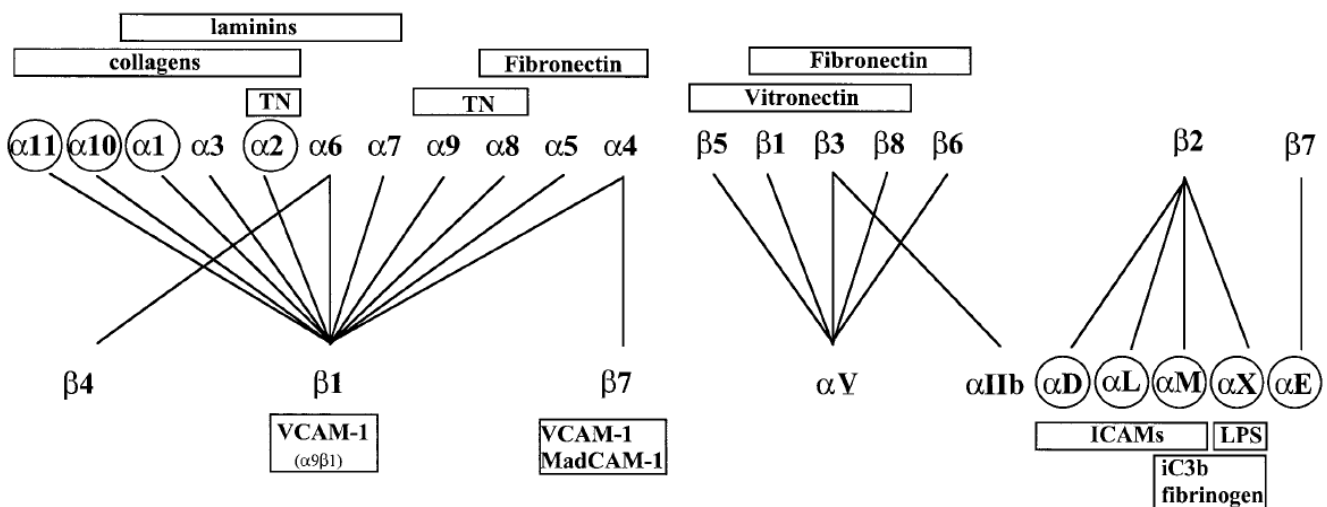


Figure 10: Combinaisons des différentes sous-unités des intégrines et les ligands connus (Ivaska & Heino, 2000)

Le domaine extracellulaire N-terminal des intégrines, qui contient des régions riches en cystéines, et sensibles aux cations (Mg^{2+} , Ca^{2+} ...), fixe les constituants de la MEC au niveau de sites de reconnaissance RGD (arginine-glycine-acide aspartique) (Tamkun *et al*, 1986). Ces interactions, nécessaires à l'ancrage des cellules contribuent également à l'assemblage et à l'organisation de la MEC (Barker *et al*, 2005). Le domaine cytoplasmique des intégrines est généralement très court, mais il est important pour l'interaction avec les constituants du cytosquelette (filaments

intermédiaires, filament d'actines...) et des protéines responsables de son organisation (α -actinine, vinculine, paxiline...) (Burrige *et al*, 1988). La nature de l'environnement matriciel influence la morphologie cellulaire par la distribution des intégrines sur leur membrane plasmique. Par exemple, dans les cellules épithéliales les intégrines sont polarisées pour que les cellules puissent adhérer à la lame basale alors que les fibroblastes s'ancrent à la MEC par l'intermédiaire de points de contact focalisés d'intégrines (Chen *et al*, 1985 ; Fath *et al*, 1989 De Luca *et al*, 1990).

Les intégrines participent également à la régulation des activités biologiques des cellules (survie, prolifération, différenciation...) en activant des voies de signalisation. Pour cela le domaine cytoplasmique des intégrines recrute des protéines kinases, comme la FAK (Focal Adhesion Kinase) (Schaller & Parsons, 1993) qui vont initier les cascades de transduction (ex : PI3K/Akt ; MAPK...). Les intégrines peuvent également moduler les activités des récepteurs à tyrosine kinases et des voies de signalisation associées (Comoglio *et al*, 2003). La présence d'une MEC va fournir des signaux de survie à la cellule (Giancotti, 1997 ; Manohar *et al*, 2004). La perte de ces signaux issus des intégrines peut entraîner une apoptose appelée anoikis (Meredith *et al*, 1993 ; Frisch & Ruoslahti, 1997).

L'expression des intégrines dépend du type cellulaire et de l'environnement matriciel dans lequel se trouvent les cellules. Par exemple pour les cellules normales de peau, les kératinocytes adhèrent à la membrane basale principalement par les intégrines $\alpha 6\beta 4$ (Nguyen *et al*, 2000) alors que les fibroblastes situés dans le derme adhèrent aux collagènes via $\alpha 1\beta 1$ et $\alpha 2\beta 1$ et à la fibronectine via $\alpha 4\beta 1$ (Gailit *et al*, 1993 ; Lange *et al*, 1994). Une dérégulation de l'expression des intégrines est souvent observée dans les cellules cancéreuses que ce soit des sur-expressions (ex : $\alpha V\beta 3$ dans le mélanome, $\alpha 6\beta 4$ dans des carcinomes) (Felding-Habermann *et al*, 1992) ou des sous-expressions (ex : $\alpha 5\beta 1$; $\alpha 2\beta 1$) (Ramirez *et al*, 2011). Expérimentalement la modulation d'expression des intégrines peut renforcer la transformation cellulaire ou au contraire rétablir un phénotype normal. (Weaver *et al*, 1997 ; Howlett *et al*, 1995 ; Ramos *et al*, 2002 ; Zhang *et al*, 2009).

ii Rôles des intégrines dans les processus migratoires et invasifs

Les rôles des intégrines dans les processus migratoires et invasifs des cellules sont multiples (Hood & Cheresch, 2002; Friedl & Wolf, 2003). En effet les intégrines peuvent participer aux différentes étapes nécessaires pour que la cellule puisse envahir son micro-environnement (Lauffenburger & Horwitz, 1996) :

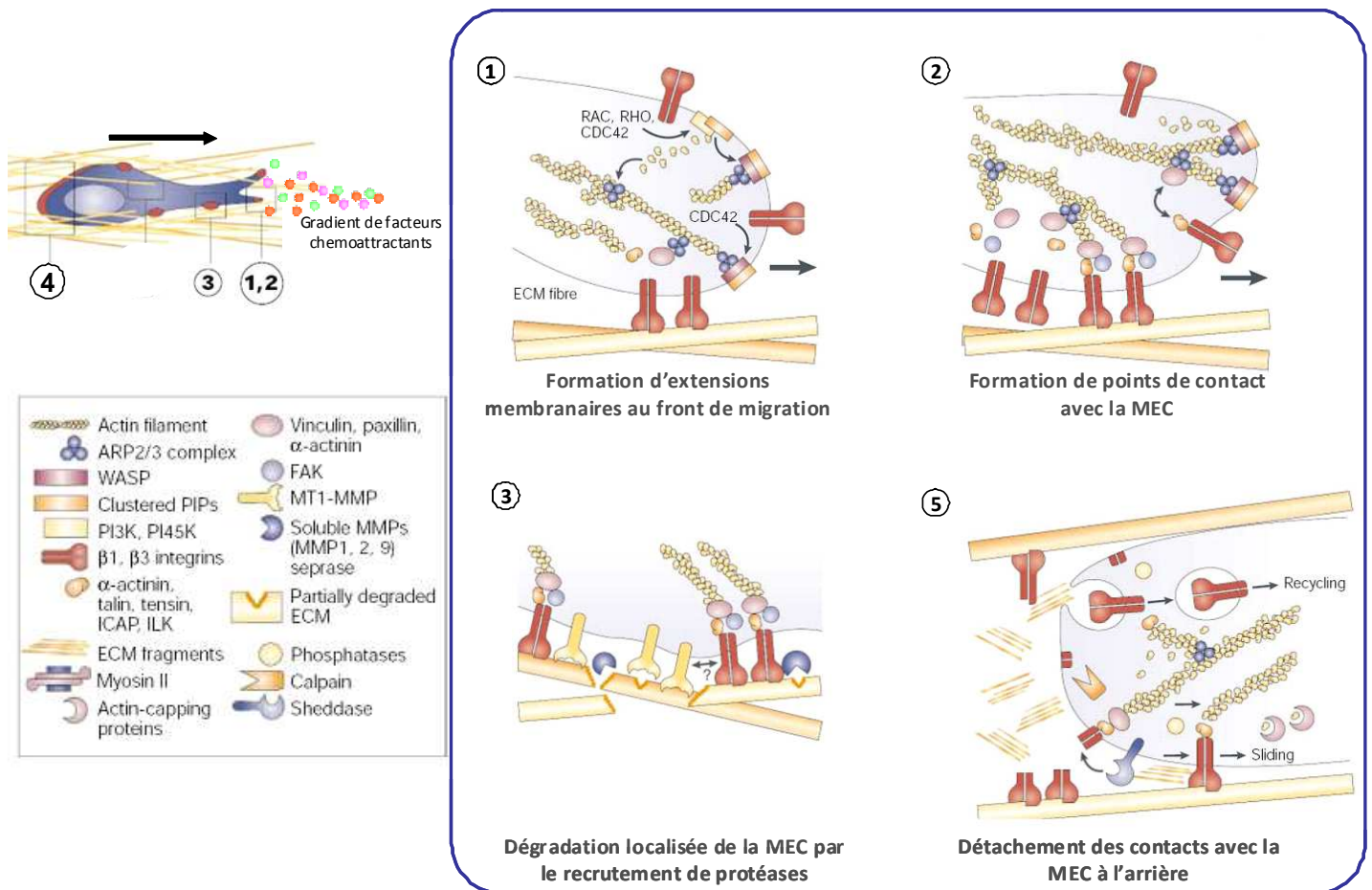


Figure 11 : Rôles des intégrines dans les processus migratoires/invasifs cellulaires (adapté de Friedl & Wolf, 2003)

- Contribution des intégrines à l'initiation des processus invasifs en favorisant la TEM des cellules épithéliales activée par le TGF- β (Munger *et al*, 1999; Galliher & Schiemann, 2006).
- Participation à la formation des extensions membranaires (filopodes, lamellipodes...) au niveau du front de migration par la polymérisation et la réorganisation des filaments d'actine (Schmidt *et al*, 1993; Huttenlocher *et al*, 1996 ; Cramer, 1999b) (figure 11, n°1)
- Ancrage des cellules aux constituants de la MEC (collagènes, fibronectine...) par l'intermédiaire des intégrines regroupées en points focaux (figure 11, n°2).

- Les intégrines favorisent la dégradation et le remodelage de la MEC au front d'invasion par le recrutement de MMPs comme par exemple la MMP-1 (Dumin *et al*, 2001) ou les gélatinases (MMP-2 et -9) (Brooks *et al*, 1996 ;Morini *et al*, 2000). Ce recrutement participe également à l'activation de ces MMPs. Ainsi pour que la MMP-2 soit activée par le complexe MT1-MMP et TIMP-2 elle doit être fixée par l'intégrine $\alpha V\beta 3$ (Hofmann *et al*, 2000) (figure 11, n°3)
- Détachement des intégrines de la MEC à l'arrière des cellules par clivage (effectué par la calpaine) ou par variation du calcium puis recyclage de ces intégrines vers le front de migration (Lawson & Maxfield, 1995) (figure 11, n°4).
- Les voies des transductions initiées par les points focaux d'intégrines stimulent l'expression de facteurs impliqués dans les processus migratoires dont des facteurs de croissance ou des MMPs (Lochter *et al*, 1999; Sugiura & Berditchevski, 1999; Ivaska & Heino, 2000).
- Les intégrines peuvent également interagir avec les récepteurs tyrosine kinase (RTK) pour participer à l'activation des voies de transduction passant par FAK (Plopper *et al*, 1995; Sieg *et al*, 1999; Sieg *et al*, 2000) (Bertotti *et al*, 2005; Guo *et al*, 2006).

1.3.2 Le récepteur à l'acide hyaluronique : CD44

i Structure et expression de CD44

CD44, récepteur principal de l'acide hyaluronique (composant majeur des MEC), est une glycoprotéine transmembranaire exprimée par la plupart des cellules. Bien qu'il existe de nombreuses isoformes, CD44 est issu d'un seul gène (locus 11p13). L'ARN messager de CD44 contient 20 exons dont 10 constants (exons n°1 à 5 et 16 à 20) et 10 variables (exons n°6 à 16, nommés v1 à v10) (figure 12). Les isoformes de CD44 sont obtenues par épissage alternatif de l'ARNm suite à l'activation des voies de transduction (ex :voie des MAP-kinases (Ras-MEK-ERK)) par des signaux extra-cellulaires aboutissant à l'activation de facteurs d'épissage (ex :sam68, ESRP1,SRm160...) (Weg-Remers *et al*, 2001 ; Matter *et al*, 2002 ; Warzecha *et al*, 2009).

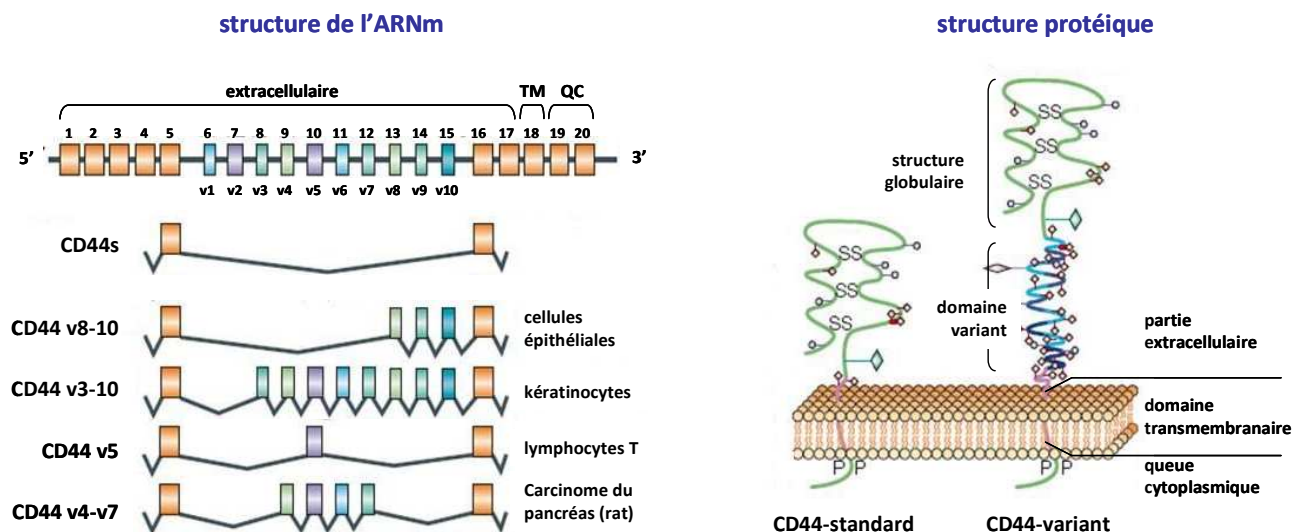


Figure 12: Structure de l'ARN messager et protéique de CD44 (adapté de Ponta et al, 2003)

La forme minimale (CD44-standard ou CD44s) est composée uniquement des 10 exons constants. Les dix autres exons variants peuvent être sélectionnés en différentes combinaisons pour former de nombreuses isoformes appelées CD44-variant (une vingtaine d'isoformes sont décrites jusqu'à présent) (figure 12). Alors que CD44-standard est exprimé sur la plupart des cellules, les CD44-variant sont exprimés spécifiquement dans certaines cellules et lors de certains processus physiologiques (développement embryonnaire, maturation et activation de lymphocytes...). Citons par exemple CD44v8-v10 exprimé par les cellules épithéliales et CD44v3-v10 par les kératinocytes (cellules épithéliales de la peau), CD44v5 par les lymphocytes (Mackay *et al*, 1994). L'expression aberrante d'isoformes variant de CD44 est souvent observée dans les cellules cancéreuses (CD44v4-v7, CD44v3, CD44v6, CD44v10...)(Bennett *et al*, 1995, 44; Mackay *et al*, 1994, 44; Bloor *et al*, 2001, 44).

Les exons 1 à 17 correspondent à la partie extracellulaire de CD44, l'exon 18 au domaine transmembranaire (TM) et les exons 19 et 20 à la queue cytoplasmique (QC). Les exons 1 à 5 en N-terminale codent pour une structure globulaire extra-cellulaire fortement glycosylée (N- et O-glycosylation) favorisant la fixation aux constituants de la matrice extra-cellulaire (acide hyaluronique, collagène, fibronectine, laminine...). En dessous de cette structure globulaire se trouve le domaine d'insertion des exons variants. La présence de certains exons variants influence alors directement l'interaction des cellules avec leur MEC et apporte également de nouvelles propriétés à CD44. Ensuite une courte séquence de 46 acides aminés, s'intercale entre le domaine variant et la région trans-membranaire. Elle présente un site de clivage pour les MMPs permettant de relarguer un fragment de CD44 dans le milieu extra-cellulaire qui peut promouvoir l'invasion cancéreuse (Okamoto *et al*, 1999)(Chetty *et al*, 2012). Enfin la queue

cytoplasmique de CD44 a pour fonction de recruter des protéines intra-cellulaires telles que l'ankyrin et les protéines ERM (erzin, radixin, moesin...). Ces protéines assurent les interactions de CD44 avec certaines protéines cytosquelette (actine, spectrine...).

ii Rôles de CD44 dans les processus migratoires et invasifs

Il est maintenant bien établi que les différentes isoformes de CD44 jouent un rôle important lors de la progression tumorale. CD44 est à la fois utilisé comme un marqueur de cellules souches (Zöller, 2011), mais aussi comme un marqueur de mauvais pronostic et de métastase (Lokeshwar *et al*, 1995). Bien que CD44 puisse favoriser la croissance et la survie des cellules tumorales, ce sont essentiellement ses rôles dans les processus d'adhésion, migratoires et invasifs qui sont principalement étudiés.

De nombreuses études ont pu démontrer que les expressions aberrantes de certaines isoformes de CD44 sont responsables des capacités invasives de lignées métastatiques (CD44v4-7 ; CD44v3, CD44v6...) (Günthert *et al*, 1991). La sur-expression de ces isoformes de CD44 dans des lignées non-métastatiques est suffisante pour induire des capacités invasives (Hofmann *et al*, 1991 ; Rudy *et al*, 1993). Ainsi la sur-expression de CD44v10 dans des lignées cellulaires de glandes mammaires non tumorigéniques est suffisante pour stimuler l'invasion en collagène de ces cellules (Iida & Bourguignon, 1997). A l'inverse, l'inhibition des CD44-variant (par anticorps bloquant ou siRNA) peut diminuer les capacités invasives (Seiter *et al*, 1993) ; (Reeder *et al*, 1998). L'expression aberrante des isoformes de CD44-variant est généralement causée par une dérégulation des systèmes d'épissage alternatif (activation de facteurs d'épissage tels que SRm160 ou TRA2- β 1) dans les cellules cancéreuses (Cheng & Sharp, 2006) ; (Watermann *et al*, 2006). A noter que le CD44-standard peut également être impliqué dans la progression tumorale et dans les capacités invasives des cellules cancéreuses (Afify *et al*, 2009).

La diversité des isoformes de CD44 permet à cette molécule d'être impliquée dans de nombreux mécanismes associés aux processus migratoires et invasifs des cellules cancéreuses (figure 13). Premièrement, la structure globulaire du CD44 permet l'ancrage des cellules à l'acide hyaluronique (composant principal de la MEC), ce qui participe aux processus de migration/invasion. (Bartolazzi *et al*, 1994). Cette interaction peut être modulée par différents mécanismes (*i*) l'expression d'exons variants (ex : CD44v3-10 ou v8-10) qui réduisent la fixation des cellules à l'acide hyaluronique (van der Voort *et al*, 1995) ; (*ii*) la glycosylation de CD44 au niveau du domaine globulaire ou des domaines variants peut également favoriser (Bartolazzi *et al*, 1996) ou inhiber (Kato *et al*, 1995) la fixation de cellules à l'acide hyaluronique ; (*iii*) le clivage

de CD44, par des protéases à sérines ou des MMPs, est aussi essentiel pour réguler son interaction avec l'acide hyaluronique et permettre l'invasion des cellules cancéreuses (Okamoto *et al*, 1999).

CD44 peut également recruter et activer des MMPs exprimées par les cellules cancéreuses ou présentes dans le milieu extra-cellulaire. Cela permet de concentrer les MMPs, (ex : MMP-2 et -9) au front d'invasion afin de renforcer la dégradation de la membrane basale et des fibres de la MEC (Yu & Stamenkovic, 1999). Le recrutement des MMPs par CD44 contribue également à l'activation du TGF- β stimulant lui-même l'invasion (Yu & Stamenkovic, 2000). De plus, la stimulation de CD44 (par l'interaction avec l'acide hyaluronique ou par des anticorps) dans des cellules cancéreuses peut induire une sur-expression et l'activation des MMP-2 et -9 (Takahashi *et al*, 1999 ; Murray *et al*, 2004).

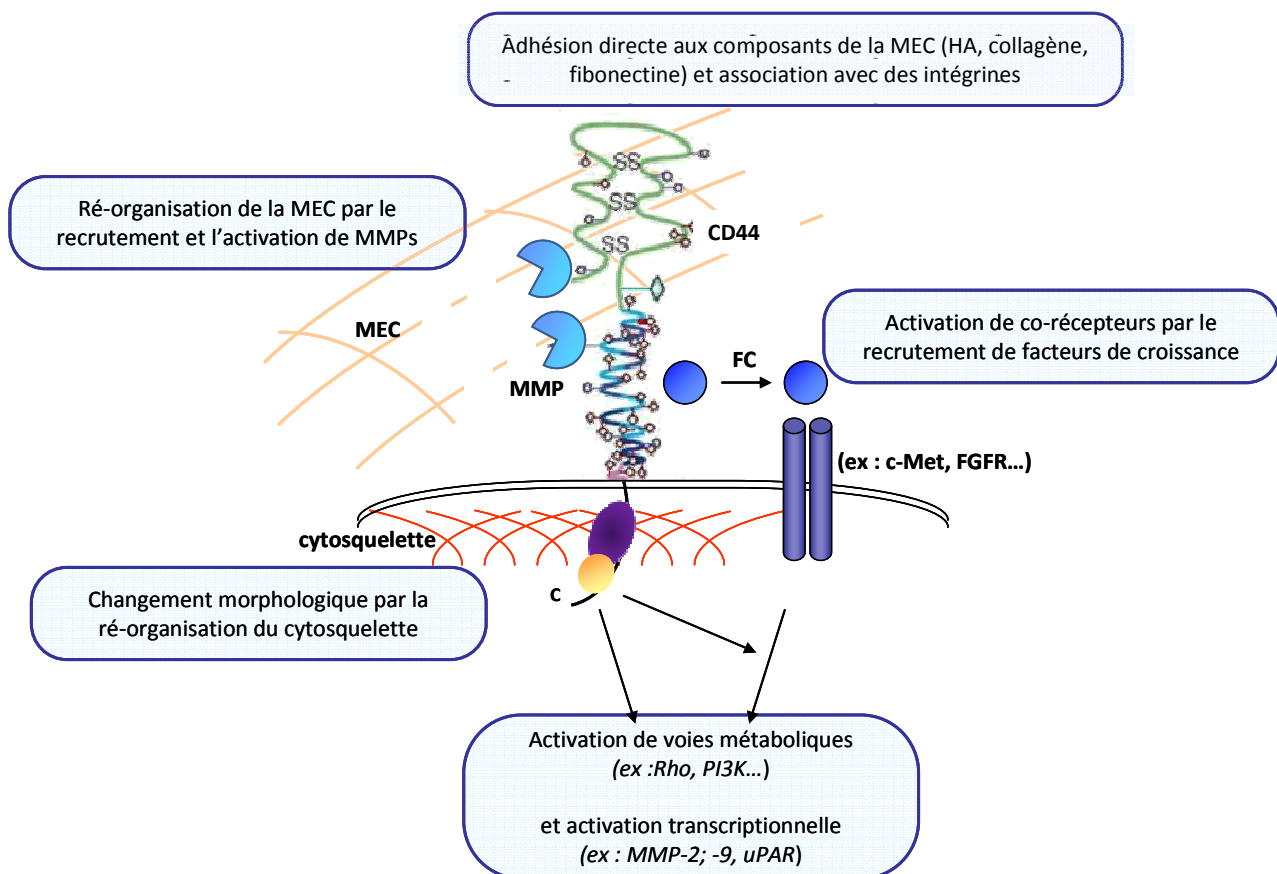


Figure 13: Fonctions moléculaires de CD44 lors des processus migratoires et invasifs

CD44 peut également servir de co-récepteur aux facteurs de croissance. Ainsi l'exon v3 comporte un site de fixation aux héparanes-sulfates favorisant le recrutement du FGF-2 et l'activation de son récepteur (FGF-R) (Bennett *et al*, 1995 ; Nedvetzki *et al*, 2003). De même CD44 est un co-récepteur bien établi du récepteur à tyrosine kinase c-Met en recrutant son ligand, l'HGF/SF, au niveau des exons v3 ou v6 (van der Voort *et al*, 1999 ; Orian-Rousseau *et al*, 2002). Récemment, certaines données suggèrent que CD44 pourrait être impliqué dans les mécanismes de transition épithélium-mésenchyme (TEM) (Zöller, 2011). Ainsi le complexe acide hyaluronique-CD44-ERM est nécessaire à la TEM induite par le TNF- α et le TGF- β (Takahashi *et al*, 2010).

Bien que généralement les CD44-variant soient généralement décrits pour favoriser la migration et l'invasion des cellules cancéreuses, dans certains cas, des isoformes peuvent au contraire inhiber ces processus. Ainsi la forme longue CD44v3-10 (isoforme spécifique aux kératinocytes normaux), réduit les capacités d'adhérence et d'invasion des cellules cancéreuses de prostate (PC3) (Harrison *et al*, 2006). De plus, il a été montré que la perte d'isoformes longues de CD44-variant au profit du CD44-standard est nécessaire pour induire la TEM dans des cellules mammaires immortalisées (HMLE) et permettre leur progression tumorale (Brown *et al*, 2011).

2. Invasion tumorale : évènement précoce ou tardif du développement tumoral ?

Les processus invasifs et métastatiques ont longtemps été associés exclusivement aux stades tardifs de la progression tumorale des carcinomes et à un mauvais pronostic (Thiery, 2002). En effet, le modèle classique du développement tumoral suggère que les cellules cancéreuses acquièrent progressivement leur agressivité suite aux mutations oncogéniques successives. Lors des stades tumoraux tardifs, les cellules cancéreuses les plus agressives envahissent leur micro-environnement suite à une transition épithélium-mésenchyme (TEM), pénètrent dans les vaisseaux sanguins et lymphatiques et établissent des macro-métastases préférentiellement dans des organes fortement vascularisés (poumon, foie, ganglions...) (figure 14). Une fois que les cellules métastatiques ont envahi les organes distants, la transition mésenchyme-épithélium est nécessaire pour permettre la croissance des foyers tumoraux secondaires.

Cependant certaines données cliniques ne corrèlent pas avec ce modèle classique de métastases lors des stades tardifs:

- Des cellules tumorales disséminées (DTC = Disseminated Tumoral Cells) isolées ou des micro-métastases peuvent être détectées en même temps que les stades initiaux de la tumeur primaire principalement au niveau des ganglions lymphatiques et dans la moelle osseuse (Engel *et al*, 2003).
- On peut observer le développement de métastases après l'élimination de petites tumeurs sans évidence de stade métastatique lors du diagnostic (Pantel *et al*, 2009)
- Certaines métastases sont génétiquement distinctes de la tumeur primaire suggérant une évolution de manière indépendante (Vecchi *et al*, 2008).
- Des métastases peuvent se développer chez des patients qui n'ont jamais été diagnostiqués pour des tumeurs primaires (CUP : cancer of unknown primary) (Pavlidis & Fizazi, 2005).

Ces observations cliniques peuvent être expliquées par un modèle de dissémination précoce de cellules transformées dès les premiers stades du développement tumoral. Mais il est très difficile de le démontrer expérimentalement pour plusieurs raisons : diagnostic trop tardif (cancers bien établis) chez les patients, peu de biopsies disponibles, utilisation de techniques pas suffisamment sensibles pour identifier une cellule DTC parmi des millions. Néanmoins, grâce à l'utilisation de modèles de souris transgéniques, plusieurs études récentes ont pu démontrer expérimentalement une dissémination précoce de cellules transformées :

- Des souris transgéniques (HER-2/Neu) développent, à la suite d'une stimulation par les hormones de la puberté, des hyperplasies mammaires à 7-9 semaines, des carcinomes in situ à 14-18 semaines et des cancers invasifs à 23-30 semaines. Pourtant, des cellules transformées (HER⁺ et cytokératines⁺) isolées provenant des glandes mammaires, sont présentes dans les poumons et la moelle osseuse des souris dès 4-9 semaines (stade hyperplasique), et les premières micro-métastases sont visibles dès 20 semaines. Cette dissémination précoce des cellules néoplasiques est associée à une sur-expression des MMPs et des cathepsines, ainsi que du facteur de transcription Twist-1 (Hüsemann *et al*, 2008)
- Dans le modèle de souris transgénique RET-AAD (sur-expression de l'oncogène RET uniquement dans les mélanocytes) des mélanomes se développent spontanément et très rapidement au niveau de l'œil. Des cellules tumorales disséminées (DTC) isolées sont

détectées rapidement dans la plupart des organes profonds (3 semaines) alors que l'apparition de métastases se fait plus tardivement (3 mois). Il s'avère que la réponse immunitaire par les lymphocytes T CD8 maintient les DTC isolées en dormance. La déplétion des souris en CD8 augmente le nombre de micro-métastases et accélère leur croissance (Eyles *et al*, 2010).

- Dans un modèle de souris transgéniques (PKYC), induisant des mutations Kras et p53 spécifiquement dans le pancréas, des cellules isolées avec des marqueurs de TEM sont identifiées dès les premiers stades de lésions précancéreuses. Ces cellules quittent très rapidement les structures épithéliales néoplasiques et disséminent dans le stroma, la circulation sanguine et peuvent se relocaliser dans un organe secondaire (foie, poumon). L'inflammation semble être un facteur critique de la TEM et la dissémination précoce (Rhim *et al*, 2012).

Ces données cliniques et expérimentales ont permis de proposer un modèle alternatif pour l'évolution du développement de carcinomes et en particulier pour les phénomènes invasifs (figure 14) (Klein, 2008 ; Ansieau *et al*, 2008a; Weinberg, 2008; Sabe, 2011). Dès les stades de lésions pré-néoplasiques, certaines cellules peuvent acquérir des caractéristiques de TEM avec notamment la sur-expression de Twist-1. Sous l'influence du micro-environnement (inflammation, MMPs), ces cellules quittent les structures épithéliales néoplasiques et disséminent à travers le stroma et dans les circulations sanguines et lymphatiques. Certaines de ces cellules peuvent se re-localiser dans les organes les plus fortement vascularisés (ganglions, poumons, foie...) dans lesquels le système immunitaire les empêchera de se développer (dormance tumorale). Plus tard, suite à l'altération du micro-environnement (vieillesse?, stress et agressions variées ?...), ces cellules disséminées et transformées pourront développer des macro-métastases indépendamment de la tumeur primaire (avec des mutations génétiques différentes).

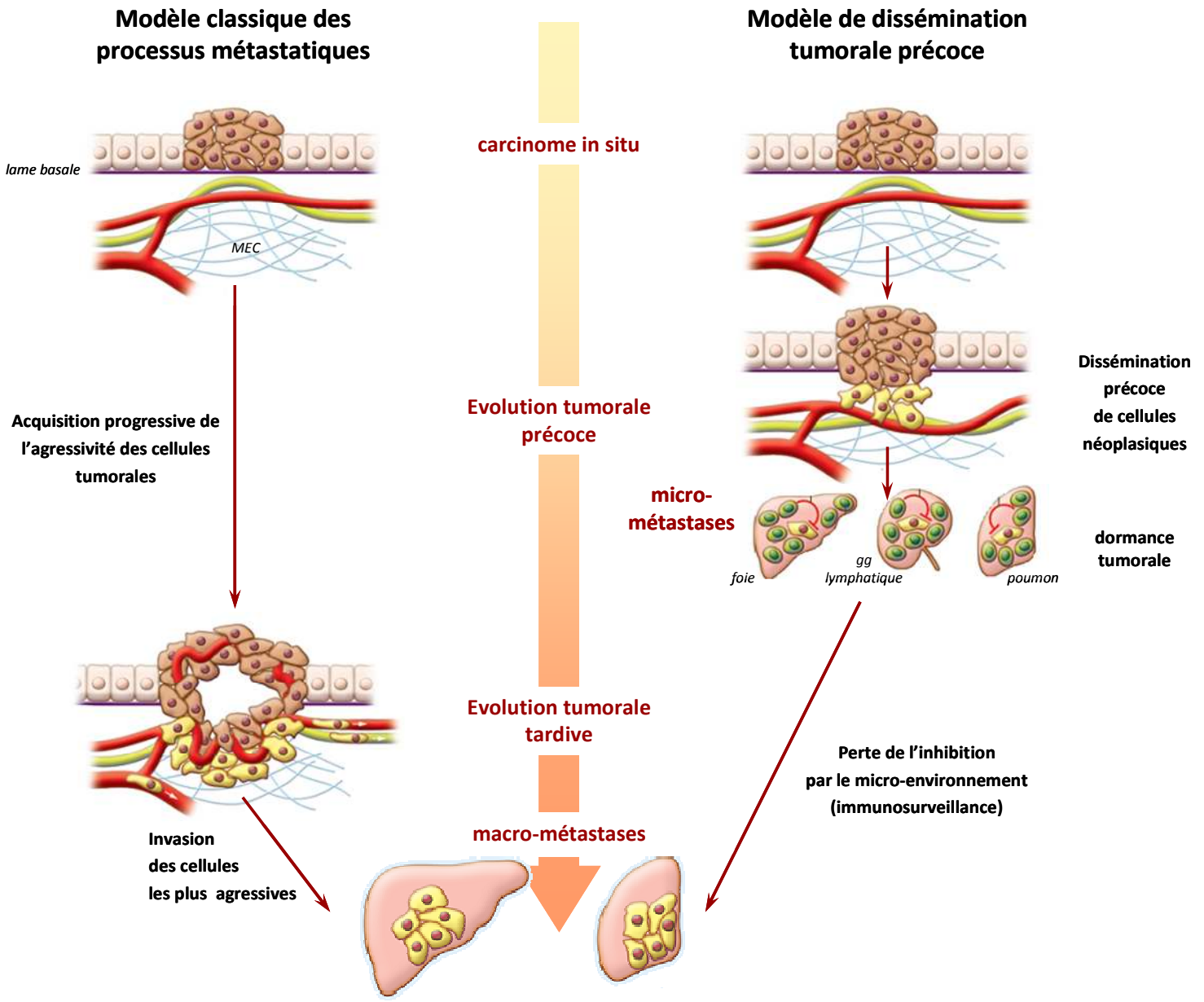


Figure 14: Modèles des processus métastatiques lors de l'évolution tumorale des carcinomes (adapté de Röcken, 2010).

C. La sénescence

1. Description de la sénescence cellulaire

1.1. Caractéristiques des cellules sénescentes

La sénescence cellulaire a été décrite pour la première fois *in vitro* par Leonard Hayflick en étudiant des fibroblastes primaires issus de poumons embryonnaires humains. Il a observé qu'après un nombre défini de doublements de population, ces fibroblastes cessent de proliférer et demeurent bloqués irréversiblement dans un plateau d'arrêt de croissance (Hayflick & Moorhead, 1961; Hayflick, 1965). Ce plateau nommé limite de Hayflick ou plateau de sénescence répllicative est observé pour la plupart des cellules normales (ex : fibroblastes, cellules endothéliales...). Les cellules en état de sénescence présentent de nombreuses caractéristiques phénotypiques :

- Augmentation de la taille des cellules et étalement sur leur support (Cristofalo & Kritchevsky, 1969; Greenberg *et al*, 1977).
- Augmentation de la taille des noyaux, parfois accompagnée d'une polynucléation (Matsumura, 1980).
- Changements dans la structure de la chromatine (foyers d'hétérochromatine, Senescence Associated Hetero-chromatine Foci (SAHF)) en lien avec des modifications des régulations épigénétiques (Narita *et al*, 2003).
- Augmentation de l'activité lysosomiale pouvant être associée à une augmentation de l'activité autophagique (Maciera-Coelho *et al*, 1971; Gosselin *et al*, 2009c).
- Accumulation d'agrégats lipido-protéiques (lipofuscine) (Terman *et al*, 2006).
- Désensibilisations des cellules aux stimuli de facteurs de croissance (Shiraha *et al*, 2000) et d'apoptose (Wang, 1995).
- Métabolisme actif permettant l'expression de transcriptomes (Bavik *et al*, 2006; Ohanna *et al*, 2011), protéomes (Benvenuti *et al*, 2002) et sécrétomes spécifiques (Coppé *et al*, 2008).

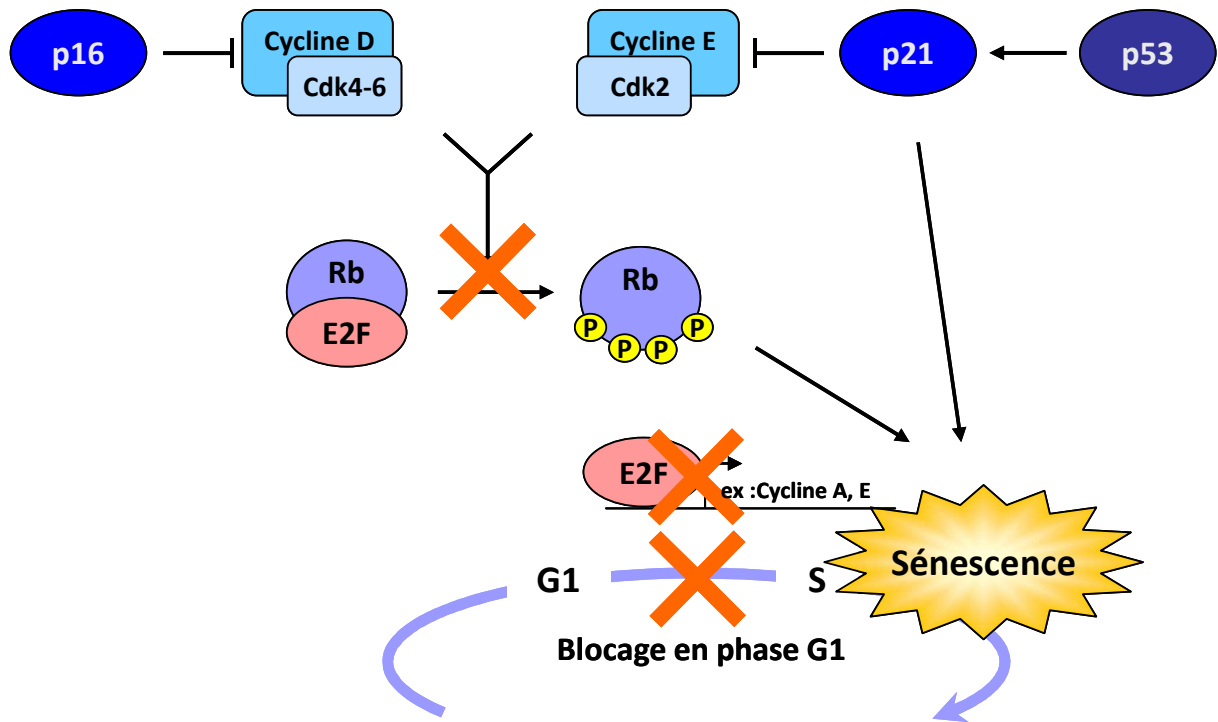


Figure 15 : Schéma simplifié des voies d'activation de l'arrêt du cycle cellulaire associé à la sénescence

Lors de la sénescence, le cycle cellulaire est généralement bloqué entre en phase G1 et l'entrée en phase S (phase de réplication de l'ADN) (Di Leonardo *et al*, 1994). La transition des phases G1/S est contrôlée par des complexes Cycline/Cdk (Cycline Dependante Kinase) : Cycline D/Cdk-4 ou 6 ; Cycline E/Cdk-2. Ces complexes hyper-phosphorylent et inactivent la protéine Rb (Rétinoblastoma protein), libérant ainsi les facteurs de transcription E2F qui contrôlent l'expression de gènes nécessaires à la transition G1/S (ex : cyclines A ; E...). L'arrêt de croissance au plateau de sénescence peut faire intervenir 2 voies moléculaires : **p53** et **p16^{INK4a}/Rb** (figure 15). L'activation de p53, suite aux signaux de sénescence, induit l'expression de p21^{CIP1/WAF1}, inhibiteur de l'activité des Cdk dont la Cycline E/Cdk-2 (Harper *et al*, 1995), participant ainsi à la desphosphorylation de Rb. De même l'activation de p16^{INK4a}, inhibiteur de la Cycline D/Cdk-4 ou 6, permet le maintien de Rb dans un état hypo-phosphorylé lui permettant de séquestrer E2F et d'empêcher ainsi la transition G1/S (figure 15). La surexpression de p53 ou p21^{CIP1/WAF1} suffit à induire un arrêt de croissance de sénescence (Sugrue *et al*, 1997; Fang *et al*, 1999). A l'inverse l'inactivation des voies p53-p21^{CIP1/WAF1} et/ou p16^{INK4a}-Rb repousse la survenue du plateau de sénescence démontrant leur importance dans ce mécanisme (Brown *et al*, 1997 ; Kiyono *et al*, 1998; Dickson *et al*, 2000; Beauséjour *et al*, 2003). Ces deux

voies peuvent agir de manière indépendante ou en collaboration et leur activation dépendant de la nature des signaux de sénescence et des types cellulaires.

Les dommages à l'ADN (cassures simple (SSB) et/ou double brins (DSB)) sont généralement l'évènement précurseur du blocage du cycle cellulaire lors de la sénescence par l'activation de voies de signalisation appelés DDR (DNA Damage response) (d' Adda di Fagagna, 2008). La DDR majoritairement décrite dans les mécanismes de sénescence est celle induite par les DSB. La détection des DSB par des complexes protéiques (MRN) induit la formation de foyers nucléaires composés de la kinase ATM (Ataxia-telangiectasia Mutated), de la forme phosphorylée de H2AX (γ H2AX) et des protéines médiatrices 53BP1 et MDC1 (Mediator of DNA-Damage Checkpoint 1). Ces foyers nucléaires, détectés dans les cellules sénescents, déclenchent ensuite l'activation de CHK2 (Checkpoint Kinase 2) puis de la voie p53 (d' Adda di Fagagna *et al*, 2003).

Ces caractéristiques sont cependant communes à d'autres mécanismes (différenciation, quiescence), et il existe jusqu'à présent peu de marqueurs spécifiques et exclusifs de la sénescence. Le marquage de l'activité de la Senescence-Associated β -galactosidase (SA- β gal) est le plus couramment utilisé pour identifier des cellules sénescents (Dimri *et al*, 1995 ; Debacq-Chainiaux *et al*, 2009). La β -galactosidase normalement active à pH 4,5 dans les lysosomes est capable de dégrader le composé X-gal dans des conditions non optimales (pH 6) dans les seules cellules sénescents du fait de leur forte activité lysosomiale (Lee *et al*, 2006). Bien que certaines études montrent que l'on puisse obtenir un marquage positif avec des cellules en forte confluence (Severino *et al*, 2000), le SA- β gal reste le marqueur le plus souvent utilisé pour identifier des cellules sénescents *in vitro* comme *in vivo*.

1.2. Mécanismes inducteurs de la sénescence

Il existe 2 principales catégories de sénescence cellulaire : la sénescence réplivative et la sénescence prématurée induite par des stress. Ces stress peuvent être de natures génétiques (activation d'oncogène), métaboliques (stress oxydant) ou être environnementaux (drogues cytotoxiques) (figure 16) (Collado & Serrano, 2006). Bien qu'il y ait des similarités phénotypiques et moléculaires, les sénescents réplivative et prématurée présentent des différences importantes.

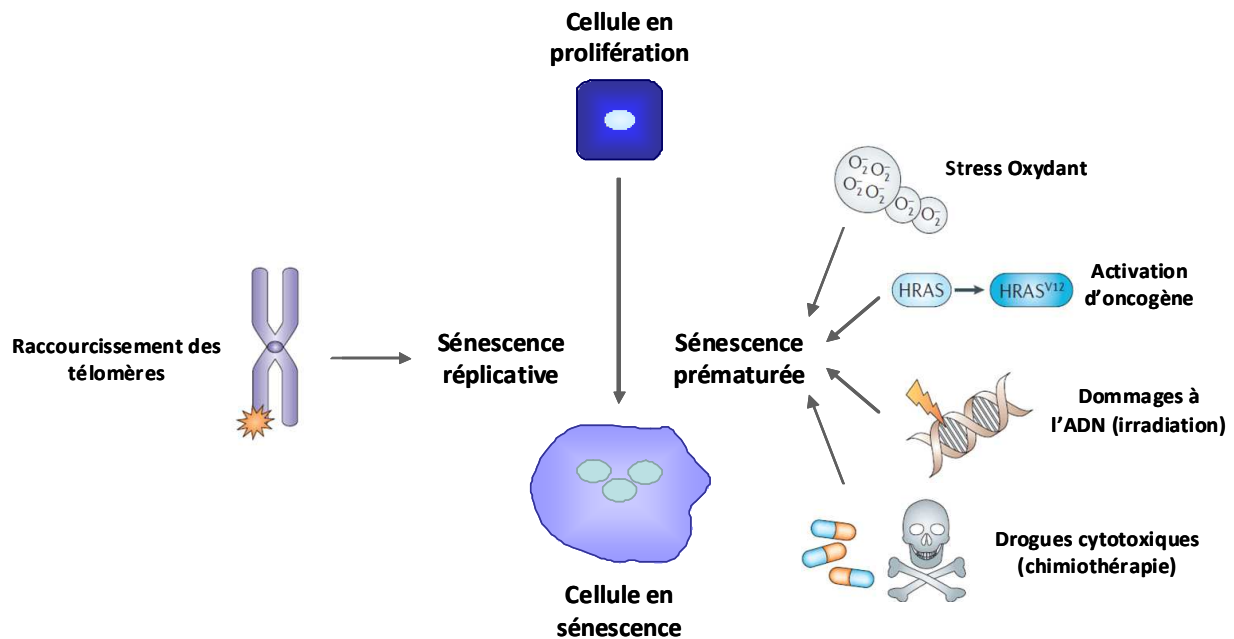


Figure 16 : Principales causes de la sénescence cellulaire (adapté de Collado et al, 2010)

1.2.1 La sénescence répliquative est causée par le raccourcissement des télomères

Les télomères sont les régions de séquences répétées (5'-TTAGGG-3' chez les vertébrés), d'environ 10-15kb chez l'homme, situés aux extrémités des chromosomes linéaires des cellules eucaryotes, qui ont pour but de protéger l'ADN de l'instabilité génomique (fusion chromosomique) pouvant résulter des divisions successives (Blackburn, 2001). Pour cela, l'extrémité 3' de l'ADN, dont une portion est sous forme simple brin, se replie au niveau des séquences répétées pour former une boucle T qui est stabilisée par un complexe protéique (composé principalement des protéines TRF-1 et -2 (Telomeric Repeat-binding Factors)...). Ce repliement empêche l'action des exonucléases et des systèmes de signalisation/réparation des cassures double-brin (Griffith *et al*, 1999).

Du fait de son incapacité à répliquer entièrement le brin d'ADN retardé, l'ADN polymérase provoque un raccourcissement des télomères à chaque division cellulaire (jusqu'à 200pb) jusqu'à atteindre une taille limite critique à laquelle la boucle T n'est plus capable de se former (Harley *et al*, 1990). Les extrémités des chromosomes sont alors détectées comme des cassures double-brin ce qui entraîne l'activation des voies de réponse aux dommages à l'ADN (DDR). En effet il a été montré que les marqueurs de DDR (foyers nucléaires de γ H2AX, TP53BP1,...) sont présents dans les fibroblastes sénescents au niveau de leurs télomères (d' Adda di Fagagna *et al*,

2003). Les DDR conduisent à l'activation de la voie p53-p21^{CIP1/WAF1} et par conséquent de l'arrêt du cycle cellulaire (Wright & Shay, 1992; Herbig *et al*, 2004 ;d' Adda di Fagagna, 2008). A noter que la voie p16^{INK4a} peut également être activée pour renforcer et maintenir l'arrêt dans le cycle (Alcorta *et al*, 1996). De plus un raccourcissement des télomères en lien avec le vieillissement a pu être mis en évidence *in vivo* par exemple au niveau de la peau (Lindsey *et al*, 1991). C'est pourquoi l'érosion des télomères jusqu'à leur taille minimale est considérée comme la principale cause de la sénescence répliquative irréversible (Allsopp *et al*, 1992; Allsopp & Harley, 1995; Deng *et al*, 2008).

L'implication de l'érosion des télomères dans la sénescence a été également appuyée par la découverte de la télomérase (Greider & Blackburn, 1985; Blackburn *et al*, 1989). La télomérase est une ribonucléoprotéine qui stabilise les télomères en ajoutant les séquences répétées aux extrémités 3' des chromosomes, compensant le raccourcissement télomérique. Elle est composée d'une sous-unité catalytique (TERT) et d'un ARN complémentaire (TERC) servant de matrice. Mise à part dans les cellules souches, la télomérase est peu ou pas dans exprimée dans les cellules normales différenciées expliquant leur durée de vie limitée. La plupart des cellules cancéreuses quant à elles sur-expriment la télomérase rendant leur prolifération illimitée. Il a été démontré que la ré-expression de la hTERT dans des fibroblastes normaux humains est suffisante pour empêcher la survenue du plateau de sénescence et à rendre les cellules immortalisées sans les transformer (Bodnar *et al*, 1998; Jiang *et al*, 1999; Morales *et al*, 1999).

Les cellules normales telles que les fibroblastes ont donc une horloge mitotique interne avec un nombre maximum de divisions qu'elles peuvent effectuer avant d'entrer en état de sénescence (Olovnikov, 1973; Harley, 1991).

1.2.2 La sénescence prématurée est induite par des stress

Il se peut qu'une cellule entre dans un état de sénescence avant qu'elle n'ait atteint la fin de son horloge mitotique. En effet, certains types cellulaires, en particulier les cellules épithéliales, subissent de nombreux stress *in vitro* comme *in vivo* provoquant un arrêt dans le cycle cellulaire et la survenue d'une sénescence prématurée (SIPS : Stress-induced premature senescence) (figure 16). A noter que ces différents stress peuvent provoquer la sénescence en induisant un stress oxydatif dans la cellule et/ou en induisant des dommages à l'ADN. Bien que l'origine des stress soit très variée, nous présenterons ici aux 2 principales causes décrites dans la littérature : le stress oxydant et l'activation oncogénique.

i Sénescence induite par un stress oxydant

Les espèces réactives de l'oxygène (ROS) sont des espèces chimiques oxygénées possédant des électrons non appariés les rendant très réactifs. Il existe plusieurs types de ROS dont l'anion superoxide ($O_2^{\circ-}$), le radical hydroxyle ($^{\circ}OH$) et le peroxyde d'oxygène (H_2O_2). En condition normale, les ROS sont formés principalement dans la mitochondrie, à la suite du transfert d'électrons entre les complexes enzymatiques de la chaîne respiratoire pour la synthèse de l'ATP (Chen *et al*, 2003). Au cours de ces réactions, le dioxygène (O_2) est réduit en eau (H_2O) par les complexes protéiques pour créer un gradient de proton nécessaire à la production d'ATP. Mais la réduction du dioxygène en eau n'est pas totalement efficace ce qui contribue à la formation d'anion superoxide ($O_2^{\circ-}$) dans la matrice mitochondriale. En condition normale, les cellules sont capables d'éliminer les ROS pour éviter leur accumulation grâce à l'expression d'enzymes anti-oxydantes (Djordjević, 2004).

La détoxification des ROS est un processus complexe dont voici le processus décrit de manière simplifiée (figure 17) :

- La famille des superoxydes dismutase (SOD), MnSOD (mitochondrie) et CuZnSOD (cytoplasme/noyau) exprimées chez l'homme, permettent la réduction de l'anion superoxide ($O_2^{\circ-}$) en peroxyde d'oxygène (H_2O_2).
- Le H_2O_2 peut à son tour être réduit en eau (H_2O) par d'autres enzymes telles que la catalase et le glutathion peroxydase.
- Contrairement à l'anion superoxide ($O_2^{\circ-}$), le peroxyde d'oxygène (H_2O_2) n'est pas chargé. Il peut donc diffuser à travers les membranes et se relocaliser dans les autres compartiments cellulaires et hors de la cellule (Bienert *et al*, 2007).
- En présence d'ions métalliques (ex : Fe^{2+} particulièrement présent dans le noyau) le peroxyde d'oxygène (H_2O_2) va générer le radical hydroxyle ($^{\circ}OH$) qui est extrêmement réactif. De plus il n'existe pas d'enzyme capable de réduire le radical hydroxyle (Valko *et al*, 2007).
- A noter que des molécules non-enzymatiques aux propriétés anti-oxydantes (acide ascorbique (Vitamine C) ; l' α -tocophérol (Vitamine E), les caroténoïdes, le glutathion, etc....) contribuent également à l'élimination des ROS dans les cellules (Balaban *et al*, 2005).

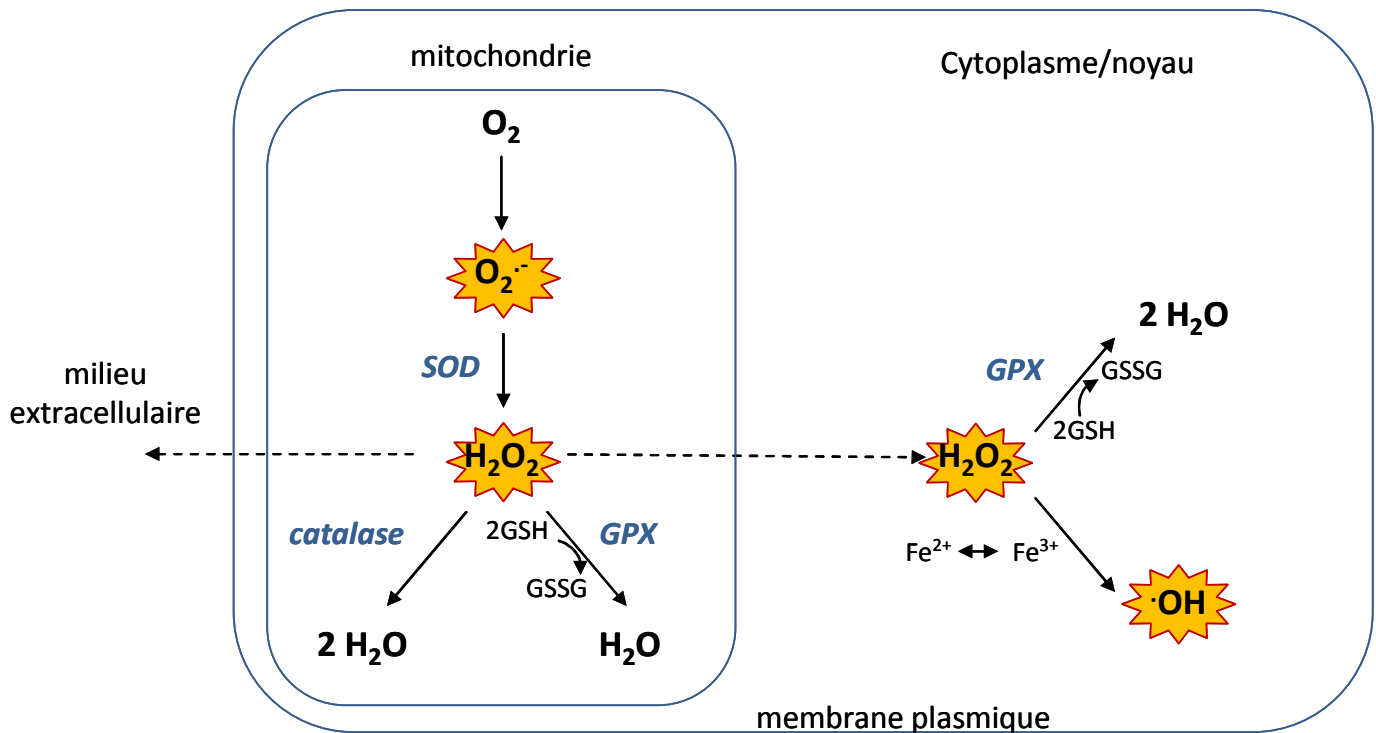


Figure 17: Schéma simplifié des mécanismes de la formation des ROS et des systèmes antioxydants.

Un stress oxydant est provoqué lorsqu'il y a une trop forte accumulation de ROS par la dérégulation de ces systèmes (sur-/sous-expression, mutations d'enzymes anti-oxydantes) et/ou par une surproduction des ROS (suite à un stress extérieur). Les ROS peuvent réagir avec les autres macromolécules de la cellule pour en modifier les propriétés physico-chimiques et donc altérer leur fonction.

- **Oxydation des protéines** : Les cystéines et les méthionines sont les 2 acides aminés les plus sensibles à l'oxydation. Ainsi l'oxydation des cystéines conduit à la formation de ponts disulfures intrachaînes (changement de conformation de la protéine) et/ou interchaînes (formation d'agrégats protéiques). L'oxydation de méthionines modifie quant à elle les propriétés physico-chimiques de la protéine. Les protéines oxydées sont dégradées par les voies du protéasome ou autophagique (Dunlop *et al*, 2009).
- **Oxydation des lipides** : la peroxydation des lipides par l'anion superoxide (O₂^{•-}) et le radical hydroxyle (•OH) conduit à la formation hydroperoxyde lipidique (LOOH) qui va (i) endommager les propriétés des membranes cellulaires, (ii) contribuer à oxyder et à altérer les autres macromolécules (exemple : production d'agrégats lipido-protéiques (lipofuscine) difficilement dégradables) (Terman & Brunk, 2004).

- **Oxydation de l'ADN** : l'oxydation de l'ADN par l'anion superoxide ($O_2^{\circ-}$) et le radical hydroxyle ($^{\circ}OH$) peut générer des cassures simple et double-brin ou des modifications de bases pouvant induire mutations ponctuelles. Le dommage à l'ADN le plus abondant est l'oxydation de la guanine qui génère la 8-oxo-hydroguanine (8-OH-G). Cette 8-OH-G peut s'apparier avec la cytosine mais aussi l'adénine augmentant ainsi le risque de mutations (par tranversion G : C vers A : T) (Lu *et al*, 2001).

Le stress oxydant a un impact très important sur le dysfonctionnement des cellules et le vieillissement d'un organisme notamment par les dérégulations des systèmes de défenses anti-oxydantes (Rhie *et al*, 2001; Harman, 1992). L'accumulation de ROS peut être aussi la conséquence d'autres stress (ex : UV, oncogènes, agents chimiques...). La sur-production chronique (ex : hyperoxie) ou ponctuelle (ex : traitement au H_2O_2) d'espèces réactives de l'oxygène, peut induire une sénescence prématurée en provoquant des dommages à l'ADN (cassures simple et double brin) et/ou en altérant les propriétés physico-chimiques des autres macromolécules. L'induction de la sénescence peut être indépendante du raccourcissement télomérique. En effet, les fibroblastes embryonnaires de souris (MEF), qui expriment la télomérase de manière endogène, atteignent rapidement un plateau de sénescence dont la survenue peut être repoussée en diminuant la concentration en dioxygène (O_2) (Prowse & Greider, 1995; Parrinello *et al*, 2003). De plus des traitements au peroxyde d'oxygène (H_2O_2) peuvent précipiter la survenue de la sénescence alors que l'ajout d'antioxydants la repousse. Les mécanismes moléculaire précis par lesquels les ROS induisent l'arrêt du cycle cellulaire sont encore peu connus mais il semble probable que les dommages à l'ADN par l'activation des voies DDR aient un rôle majeur. (Chen *et al*, 1998).

Outre sa capacité à induire une sénescence prématurée, le stress oxydant participe également à la survenue de la sénescence répllicative en accélérant le raccourcissement télomérique. En effet les télomères, riches en guanine, sont des cibles privilégiées pour les ROS. Les protéines télomériques TRF-1 et-2 sont très sensibles à la séquence consensus pour leur fixation. De cette façon les tranversions (G : C vers A : T) causé par la 8-OH-G participent donc à la déstabilisation de la structure des télomères (von Zglinicki *et al*, 1995).

ii Sénescence induite par des oncogènes (OIS – Oncogene Induced Senescence).

Les oncogènes constituent une catégorie de gènes dont les mutations et/ou sur-expression contribuent à la transformation cellulaire en apportant un gain de fonction. Dans des cellules normales, l'activation d'un seul oncogène n'est pas suffisante pour induire la transformation des cellules. Une combinaison d'activations d'oncogènes associées aux pertes de régulation par les gènes suppresseurs de tumeur est nécessaire pour obtenir une cellule cancéreuse.

Dans une cellule normale, la sur-expression ou l'activation d'un oncogène va avoir pour conséquence de déclencher un état de sénescence prématurée (OIS – Oncogene Induced Senescence). La première mise en évidence d'une OIS a été réalisée par la sur-expression de l'oncogène Ras actif (*HrasV12*) dans des fibroblastes primaires humains et murins induisant une accumulation de p53, p21^{CIP1/WAF1} et p16^{INK4a} (Serrano *et al*, 1997), alors qu'une sur-expression de Ras dans des cellules sans p53 ou p16^{INK4a} induit leur transformation (Donehower *et al*, 1992; Serrano *et al*, 1996). La sur-expression des effecteurs en aval de Ras (Raf, PML, C/EBPbeta1) aboutit également à une OIS (Zhu *et al*, 1998; Pearson *et al*, 2000; Atwood & Sealy, 2010). Depuis, d'autres oncogènes ont été décrits pour induire une OIS comme E2F (Lazzerini Denchi *et al*, 2005); BRAF (Michaloglou *et al*, 2005) ; MEK (Lin *et al*, 1998) ; NF-κB (Bernard *et al*, 2004)), STAT5A (Malette *et al*, 2007) (Malette, GenDev 2007) ; SnoN (Pan *et al*, 2009)...

L'activation de voies de transduction des oncogènes va favoriser l'accumulation des dommages à l'ADN et donc activer une DDR. Comme précédemment, la DDR va alors activer les voies p53-p21^{CIP1/WAF1} et/ou p16^{INK4a} bloquant les cellules dans la phase G1 de leur cycle cellulaire. L'inactivation des principaux acteurs de la DDR lors d'une OIS permet aux cellules d'échapper à cette sénescence et permettre leur transformation (Di Micco *et al*, 2006). Aussi, l'OIS est associée à la formation de SAHF (Senescence-associated heterochromatin foci) contribuant au maintien de l'état sénéscent (Di Micco *et al*, 2011).

Les mécanismes d'induction des dommages à l'ADN et des SAHF par l'activation d'oncogènes sont encore peu connus. Un mécanisme possible est que la sur-expression d'un oncogène (*HrasV12*) conduit à une augmentation du nombre de fourches de réplication ce qui causerait un stress répliatif (Di Micco *et al*, 2006). D'autre part, les dommages à l'ADN lors de la survenue d'une OIS pourraient être en lien avec le stress oxydant. En effet la sur-expression de l'oncogène NF-κB dans des kératinocytes primaires provoque un stress oxydant important (suite à la sur-expression de la MnSOD) dans ces cellules conduisant à la sénescence (Bernard *et al*, 2001b, 2001a, 2004).

2. Sénescence cellulaire et vieillissement de l'organisme

Le vieillissement est généralement défini par la perte progressive des fonctions biologiques d'un organisme aboutissant aux pathologies liées à l'âge (maladie d'Alzheimer, cancers...) et à la mort de l'individu (Austad, 1998. ; Kirkwood & Austad, 2000). L'altération de l'homéostasie tissulaire (par le non-renouvellement des cellules) et du fonctionnement des cellules sont responsables du vieillissement. C'est pourquoi la sénescence cellulaire a été rapidement associée au vieillissement de l'organisme. La corrélation entre la sénescence cellulaire *in vitro* et le vieillissement *in vivo* a été montrée pour la première fois en comparant le comportement en culture de fibroblastes de derme humains provenant de donneurs jeunes (21-36 ans) ou âgés (63-92 ans). *In vitro*, la durée de la phase de prolifération des fibroblastes issus de donneurs âgés est plus courte que celle des fibroblastes des donneurs jeunes (Schneider & Mitsui, 1976). De plus grâce au marquage de l'activité de la senescence-associated β -galactosidase (SA- β gal) à pH6, il a été montré que les cellules sénescents s'accumulent dans les tissus avec le vieillissement notamment au niveau de l'épiderme et du derme dans des biopsies de peau humaines âgées (Dimri & Campisi, 1994; Dimri *et al*, 1995). Récemment l'accumulation de cellules sénescents *in vivo* avec l'âge a été montrée également par le marquage de foyers nucléaires de réponse aux dommages à l'ADN (ex : foyers γ -H2AX) (Wang *et al*, 2009).

La structure de la peau est fortement altérée lors du vieillissement (comme la plupart des tissus) diminuant ses fonctions protectrices contre les infections et les pathologies. L'épiderme, plus fin, est fragilisé par la baisse de la cohésion entre les cellules épithéliales (notamment dans les couches les plus superficielles) (Fenske & Lober, 1986) ; (Kurban & Bhawan, 1990). La lame basale s'épaissit bien que la quantité de collagène IV diminue (Vázquez *et al*, 1996). La zone de jonction entre l'épiderme et le derme est aplatie dans les tissus âgés. Le derme perd de son élasticité du fait d'une diminution des composés de la matrice extra-cellulaire du derme (collagène, fibronectine, elastine...) et une augmentation des enzymes de dégradation de la matrice (collagénases, gélatines...). Des changements importants dans la nature et la composition en glycoprotéines sont également observés (Carrino *et al*, 2011, 2003). Récemment une étude a montré que les altérations dans la composition du derme sont surtout dues aux fibroblastes situés dans la zone superficielle du derme (papillaire) et peu à ceux du derme profond (réticulaire) (Mine *et al*, 2008). Le nombre de cellules, la vascularisation et la surveillance du système immunitaire sont considérablement réduits. La diminution du nombre de lymphocytes T et B (provoquée par une atrophie du thymus), favorise les infections cutanées, les pathologies auto-immunes, et le développement de néoplasies.

3. Sénescence et cancer

Les données épidémiologiques (issues du NCI-National Cancer Institute) montrent que le risque de développer un cancer augmente fortement avec l'âge (DePinho, 2000). De plus la grande majorité (8 sur 10) des cancers sont d'origine épithéliale (carcinomes) chez les individus âgés au contraire des cancers développés chez l'enfant (1 sur 10) (figure 18).

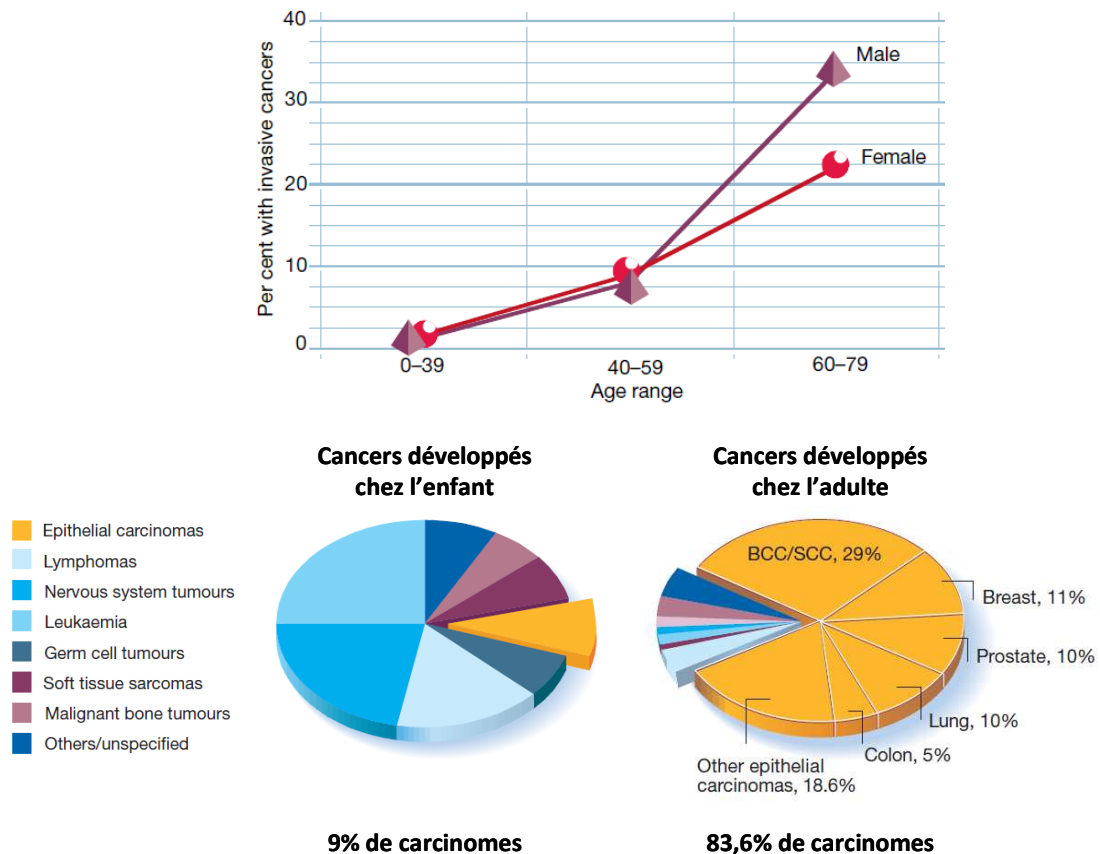


Figure 18 : Incidence et types des cancers développés chez l'homme en fonction de l'âge (adapté de DePinho, 2000).

Les carcinomes font donc partie des pathologies liées au vieillissement (comme les maladies de Parkinson ou d'Alzheimer). Pourtant la sénescence cellulaire est généralement considérée comme un mécanisme suppresseur de tumeur. Cette apparente contradiction suggère donc que l'effet anti-tumoral bénéfique de la sénescence deviendrait néfaste dans les organismes vieillissants (théorie de la pléiotropie antagoniste) (Campisi, 2003); (Campisi, 2005) ; (Ungewitter & Scoble, 2009).

3.1. L'échappement aux mécanismes de sénescence est une étape de la progression tumorale

De par sa capacité à bloquer le cycle cellulaire, la sénescence est considérée comme un important mécanisme suppresseur de tumeur, mécanisme qui semble être efficace dans les organismes « jeunes adultes » vu le faible nombre de cas de cancers. Le marquage de l'activité SA- β gal a permis de montrer que des cellules sénescents sont présentes au niveau de lésions tumorales précoces (ex : adénomes du poumon, nævi de mélanomes, néoplasies hépatiques, pancréatiques, prostatiques...) mais absentes dans leurs stades tumoraux tardifs respectifs (Braig *et al*, 2005; Chen *et al*, 2005 ; Collado *et al*, 2005; Collado & Serrano, 2010). Ces observations soutiennent donc que la sénescence serait une barrière contre le développement tumoral que les cellules néoplasiques doivent franchir pour poursuivre leur progression (Halazonetis *et al*, 2008). La sénescence des cellules retrouvées au sein des néoplasies est généralement prématurée en réponses à des stress (ex : ROS, activation d'oncogènes...) (Bartkova *et al*, 2006; Gray-Schopfer *et al*, 2006; Michaloglou *et al*, 2008). Par exemple, la mutation activatrice de l'oncogène BRAF (V600E) qui induit une sénescence prématurée est retrouvée au sein de lésions pré-cancéreuses du mélanome, les *nevi*, dans des cellules qui sont positives au marquage de l'activité SA- β gal (Michaloglou *et al*, 2005).

Contrairement à la sénescence répliquative, la sénescence prématurée permet aux cellules épithéliales de conserver des télomères suffisamment longs pour que les cellules puissent proliférer de nouveau en cas d'échappement à la sénescence (Rheinwald *et al*, 2002; Jacobs & de Lange, 2004). Expérimentalement l'inactivation des voies p53 et p16^{INK4a}/Rb ou des voies de la DDR dans des cellules épithéliales normales (prostate, mammaire...) permet d'échapper à la sénescence et peut contribuer à la transformation cellulaire (Jarrard *et al*, 1999; Brenner *et al*, 1998 ; Di Micco *et al*, 2006).

De plus des études ont pu démontrer que certains types de cellules épithéliales primaires sont capables de s'échapper spontanément du plateau de sénescence. C'est le cas notamment pour les HMEC (Human Mammary Epithelial Cell) (Romanov *et al*, 2001) ; (Tlsty *et al*, 2001) et pour les NHEK (Normal Human Epithelial Keratinocytes) (Gosselin *et al*, 2009c). Ces cellules qui émergent de la sénescence présentent des instabilités génétiques et acquièrent des caractéristiques de cellules néoplasiques (Yaswen & Stampfer, 2002; Martien & Abbadie, 2007). La sénescence n'est donc pas un phénomène forcément irréversible et peut même être à l'origine de la génération de cellules néoplasiques.

3.2. Le sécrétome des cellules sénescences contribue au développement tumoral.

Outre ces dérégulations intrinsèques, la sénescence peut également contribuer au développement tumoral par une action paracrine. Comme nous l'avons vu précédemment, le développement des tumeurs est stimulé par leur le stroma réactif adjacent et la présence de fibroblastes activés. Les fibroblastes sénescents, tout comme les fibroblastes activés, instaurent un micro-environnement très réactif qui va contribuer au développement tumoral (Rinehart & Torti, 1997). En effet bien que bloquées dans le cycle cellulaire, les cellules sénescences demeurent métaboliquement actives avec un profil d'expression spécifique (Bavik *et al*, 2006 ; Ohanna *et al*, 2011). Les fibroblastes sénescents produisent un sécrétome très pro-inflammatoire dont les principales caractéristiques sont : la sur-expression de nombreuses cytokines (IL-6, Gro-1...), facteurs de croissance (bFGF, HGF/SF...) et protéases (MMPs), un relargage de fragments de récepteurs membranaires et des changements dans la sécrétion de facteurs insolubles (collagènes, laminines...). Les termes de SASP (Senescent Associated Secretory Phenotype) ou SMS (senescence messaging secretome) ont été proposé (Coppé *et al*, 2008 ; Kuilman & Peeper, 2009). Les principaux facteurs composant les sécrétomes de cellules sénescences décrits jusqu'à présent dans la littérature sont listés ci-dessous.

Cytokines et facteurs de l'inflammation	Interleukines (IL)-1a ; -1b ; -6 ; -8, Gro -1 ; -2 ; -3 ; MCP -1 à -4 ; MIF ; GM-CSF ...
Facteurs de croissance	Amphireguline ; épiréguline ; EGF ; bFGF ; HGF/SF ; KGF ; VEGF ; SDF-1 ; IGFBP
Protéases et régulateurs	MMP-1 ; -2 ; -3 ; -10 ; MT1-MMP ; PAI-1 ; TIMP-1 (↓)
Fragments de récepteurs	ICAM-1 à -3 ; TFNR-1 ; EGFR
Composants de la matrice extra-cellulaire	Fibronectine ; collagène (↓) ; laminine (↓)
Autres	PGE2

Table 4 : Liste des principaux facteurs de sécrétome des cellules sénescences décrits dans la littérature (les diminutions de sécrétion sont précisées (↓)) (d'après Coppé *et al*, 2010).

Les mécanismes de régulation des sécrétomes des cellules sénescents sont encore peu connus. Néanmoins il a été montré récemment que la production du sécrétome pro-inflammatoire des fibroblastes sénescents humains (WI-38) nécessite un stress génotoxique, et une persistance dans l'activation des voies de réponse aux dommages à l'ADN (DDR) (Rodier *et al*, 2009, 2011; Rodier & Campisi, 2011). Les activations de la voie p38 MAPK et du facteur de transcription nucléaire factor- κ B (NF- κ B) semblent également essentielles (Freund *et al*, 2011) (Chien *et al*, 2011). Autre exemple dans des cellules de mélanomes sénescents, où l'activation des voies de dommage à l'ADN par la poly(ADP-ribose) polymérase-1 (PARP-1) et NF- κ B induit la production d'un sécrétome pro-inflammatoire (Ohanna *et al*, 2011). Par contre les voies p53 et p16^{INK4a}/Rb ne semblent pas être responsables de la production de ces sécrétomes, et même au contraire les inhibent (Coppé *et al*, 2008, 2011).

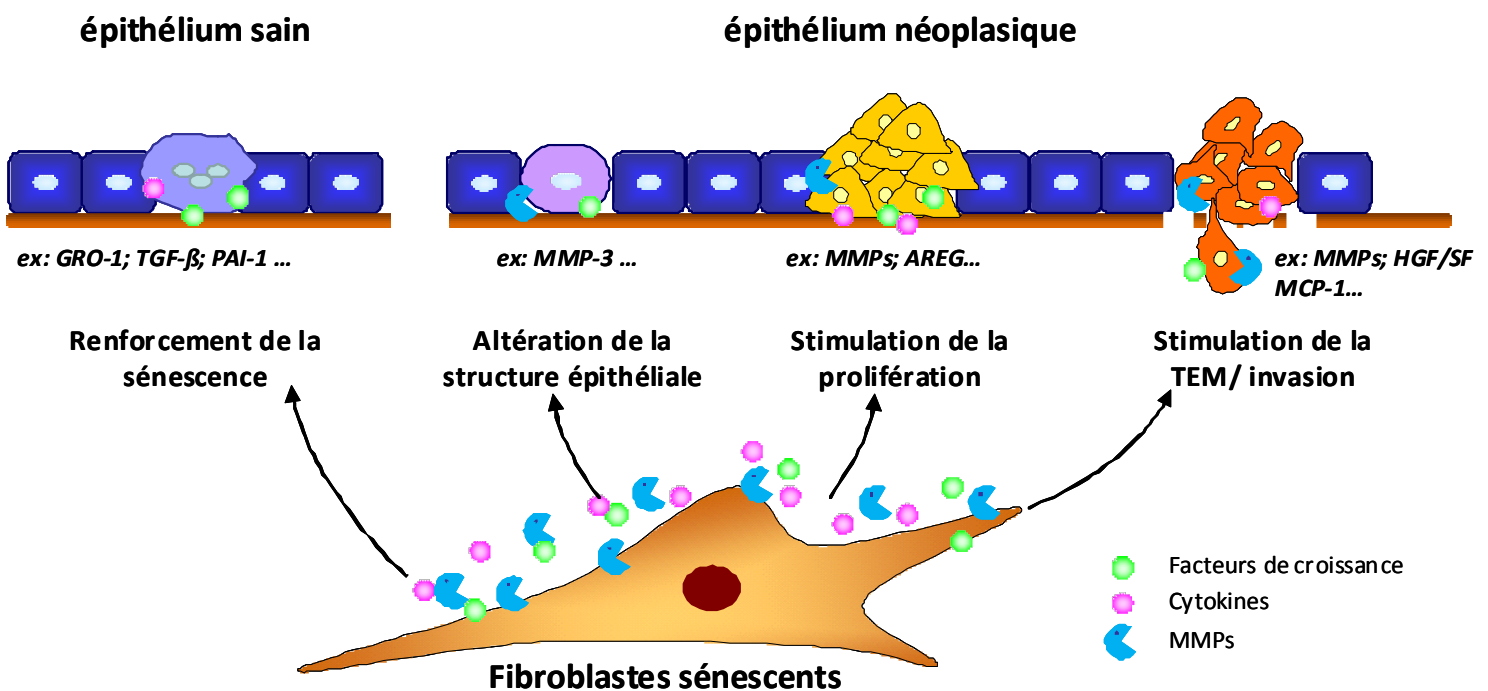


Figure 19: Effets du sécrétome des fibroblastes sénescents sur les cellules épithéliales normales et néoplasiques

Le sécrétome des fibroblastes sénescents a des effets multiples et divers sur le micro-environnement et les cellules adjacentes (figure 19). Outre l'altération de la composition de la matrice extra-cellulaire du stroma, ce sécrétome contribue dans un premier temps à renforcer l'état de sénescence des cellules voisines normales par l'intermédiaire de cytokines (ex : Gro-1, IL-8), de FC (ex : TGF- β 1) ou de PAI-1 (figure 19) (Yang *et al*, 2006; Acosta *et al*, 2008; Kortlever *et al*, 2006; Kuilman & Peeper, 2009).

Des fibroblastes sénescents ont été identifiés à proximité de carcinomes bien établis dans des biopsies humaines (Yang *et al*, 2006). Par leur sécrétome ils contribuent alors à l'évolution tumorale de cellules néoplasiques de différentes manières (figure 19): stimulation de la transformation et de la croissance de cellules pré-tumorales ou tumorales *in vitro* et *in vivo* (Krtolica *et al*, 2001; Krtolica & Campisi, 2002; Liu & Hornsby, 2007; Lawrenson *et al*, 2010), désorganisation des épithéliums (Parrinello *et al*, 2005) et induction de la transition épithélium-mésenchyme (TEM) pour stimuler les capacités invasives (Coppé *et al*, 2008; Ohanna *et al*, 2011; Laberge *et al*, 2011).

Ces données indiquent donc que les fibroblastes sénescents, tout comme des CAFs, contribuent activement aux différentes étapes du développement tumoral par l'intermédiaire d'un sécrétome fortement pro-inflammatoire.

D. Objectifs et plan de travail de la thèse

La thématique générale de l'équipe Initiation des Cancers Epithéliaux (ICE) est d'étudier les phases initiales de la carcinogenèse de la peau en lien avec le vieillissement. Pour cela, nous utilisons un modèle de culture des cellules primaires épithéliales de la peau issues de donneurs humains sains. *In vitro*, ces kératinocytes sont capables d'échapper spontanément de l'état de sénescence en acquérant des caractères de transformation. Nous avons également développé au sein du laboratoire un axe d'étude du lien entre le vieillissement du micro-environnement et ces étapes précoces de la carcinogenèse de la peau. L'objectif global de mon projet de thèse était de déterminer l'impact du sécrétome des fibroblastes sénescents de derme sur les étapes initiatrices de la transformation des kératinocytes. Pour cela nous avons initialement décidé d'aborder ce projet par deux axes étudiés en parallèle.

Le premier axe de ce travail de thèse consistait à caractériser *in vitro* les effets des milieux conditionnés de fibroblastes jeunes et sénescents sur les étapes d'émergence tumorale post-sénescence des kératinocytes par une approche de co-culture. Nos travaux ont permis de mettre en évidence que le sécrétome de fibroblastes sénescents renforce la transition épithélium-mésenchyme et les capacités migratoires des kératinocytes néoplasiques. Aussi, par des manipulations moléculaires et pharmacologiques, nous avons pu mettre en évidence que la stimulation de la migration de ces kératinocytes nécessite l'activation de leur récepteur à la thrombine (PAR-1) par les MMPs sécrétées par les fibroblastes sénescents.

Dans un second axe de recherche, nous avons cherché à décrire le sécrétome des fibroblastes sénescents de derme car peu de données bibliographiques existaient au début de ma thèse. Pour cela, j'ai comparé les milieux conditionnés (MC) des fibroblastes jeunes et sénescents de derme par des gels mono- et bi-dimensionnels et identifié les facteurs différenciellement sécrétés par protéomique (MALDI-TOF). La difficulté liée à la préparation des échantillons de MC pour réaliser ces expériences, nous a permis d'identifier seulement les facteurs les plus sécrétés. Néanmoins les résultats montrent de manière originale une perte de sécrétion de plusieurs glycoprotéines aux propriétés anti-tumorales dans le MC de fibroblastes sénescents. Nous avons donc dans un dernier temps cherché à décrire les mécanismes responsables de ces défauts de sécrétion dans les fibroblastes sénescents.

Résultats

A. Etude de l'impact du sécrétome des fibroblastes sénescents sur l'initiation cancéreuse des kératinocytes

1. La culture des kératinocytes primaires de peau est un modèle d'initiation cancéreuse

La thématique générale du laboratoire est d'étudier les mécanismes de l'initiation cancéreuse des kératinocytes primaires de peau issus de donneurs humains et sains en lien avec le vieillissement (NHEK = Normal Human Epithelial Keratinocytes).

Comme pour toutes les cellules primaires, la prolifération des NHEK en culture n'est pas illimitée. En effet les NHEK atteignent un plateau de sénescence après 15 à 20 doublements de population qui n'est pas lié à un raccourcissement télomérique. Les travaux antérieurs de l'équipe ont mis en évidence que la sénescence des NHEK est accompagnée d'une sur-expression du facteur de transcription Rel/nuclear factor (NF)- κ B qui lui-même induit une sur-expression de la MnSOD (Superoxyde Dismutase). Le principal rôle de la MnSOD est de réduire l'anion superoxyde ($O_2^{\cdot -}$) en peroxyde d'oxygène (H_2O_2) qui est une espèce réactive de l'oxygène (ROS) capable de diffuser à travers les membranes plasmiques. L'accumulation d' H_2O_2 par la sur-expression de la MnSOD induit des dommages oxydants aux différents constituants cellulaires notamment à l'ADN, directement responsables de l'arrêt de croissance des NHEK. (Bernard *et al*, 2001b, 2001a, 2002, 2004). La sénescence induite par le stress oxydant s'accompagne d'une augmentation de la taille des kératinocytes et de leur étalement sur le support, de l'accumulation de vésicules cytoplasmiques, d'une augmentation de l'activité beta-galactosidase et d'une augmentation de l'expression de p21^{CIP1/WAF1} responsable du blocage du cycle cellulaire. Ce stress oxydant peut avoir deux effets opposés sur le devenir des NHEK sénescents.

Premièrement notre équipe a pu démontrer que la grande majorité des cellules sénescents (plus de 99%) vont rapidement mourir par autophagie (et non pas par apoptose) (Gosselin *et al*, 2009a). En effet, les NHEK sénescents présentent des caractères phénotypiques de la macro-autophagie (vésicules autophagiques) et une sur-expression des marqueurs d'autophagie comme beclin-1 et -2. Contrairement à l'apoptose, la modulation des activités autophagiques impacte sur la survenue de la mort des NHEK sénescents. De plus la surexpression de la MnSOD ou des traitements au H_2O_2 suffisent à induire la mort des NHEK par autophagie après avoir induit une sénescence prématurée (Deruy *et al*, 2010).

Ce stress oxydant est cependant également responsable de l'apparition des cellules épithéliales néoplasiques. En effet une faible proportion des NHEK sénescents (moins de 1%) échappe au blocage du cycle cellulaire et au processus de mort par autophagie pour générer spontanément et systématiquement des clones de nouvelles cellules proliférantes. Ces cellules, appelées post-sénescence émergentes (PSE), présentent des caractères de transformation dont l'expression de plusieurs marqueurs de transition épithélium-mésenchyme et la formation d'hyperplasies et de carcinomes cutanés en souris *nude*. Les NHEK-PSE atteignent un second plateau de sénescence et pour certains donneurs, on observe par la suite une seconde émergence de cellules appelées Imk qui présentent des caractères plus transformés (caryotypes aberrants, morphologie plus fibroblastoïde...).

Ces travaux sont l'objet de l'article n°1 : ***Senescence-associated oxidative DNA damage promotes the generation of neoplastic cells.***

Un des axes développés actuellement au laboratoire est d'étudier l'impact du niveau du stress oxydant et de l'autophagie dans l'orientation des kératinocytes sénescents vers les processus de mort ou vers l'émergence néoplasiques de ces cellules. De manière intéressante, la fréquence d'émergence post-sénescence peut être changée en modulant les activités autophagiques dans les kératinocytes sénescents. En effet une inhibition partielle de l'autophagie par un inhibiteur pharmacologique (3-méthyladénine) ou par si-RNA (inhibition de l'expression d'Atg-5) augmente la fréquence d'émergence post-sénescence alors qu'une inhibition complète (par une augmentation de la concentration en drogue ou en siRNA) la diminue. Cela suggère que pour générer des cellules PSE, les kératinocytes sénescents doivent réduire leur activité autophagique pour échapper à la mort tout en conservant un niveau d'activité minimum afin d'éliminer les composés altérés. De plus, le niveau de stress oxydant présent dans les kératinocytes sénescents est directement responsable de l'intensité de l'activité autophagique et dommages mutagènes à l'ADN dans les cellules. Le niveau de stress oxydant nécessaire pour l'émergence post-sénescence de cellules transformées ne doit donc pas être trop intense afin de ne pas déclencher la mort par autophagie. Les kératinocytes PSE survivent donc au processus de macro-autophagie mais sont criblés de dommages oxydant à l'ADN responsables de leur transformation.

Ces résultats font l'objet d'un article actuellement en préparation. Article complémentaire n°1 : ***Levels of macroautophagy drive senescent keratinocytes into cell death or neoplastic transformation.***

Article n°1

*Senescence-associated oxidative DNA damage promotes
the generation of neoplastic cells.*

Senescence-Associated Oxidative DNA Damage Promotes the Generation of Neoplastic Cells

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Abstract

Studies on human fibroblasts have led to viewing senescence as a barrier against tumorigenesis. Using keratinocytes, we show here that partially transformed and tumorigenic cells systematically and spontaneously emerge from senescent cultures. We show that these emerging cells are generated from senescent cells, which are still competent for replication, by an unusual budding-mitosis mechanism. We further present data implicating reactive oxygen species that accumulate during senescence as a potential mutagenic motor of this post-senescence emergence. We conclude that senescence and its associated oxidative stress could be a tumor-promoting state for epithelial cells, potentially explaining why the incidence of carcinogenesis dramatically increases with advanced age. [Cancer Res 2009;69(20):7917–25]

Introduction

Both *in vitro* and *in vivo*, as a result of time and cumulative divisions, normal cells enter senescence, characterized by an enlarged morphology, lipofuscin accumulation, increased autophagic activity, cell cycle arrest, and frequent polynucleation (1–5). It is accepted that senescence results from cumulative oxidative damage and telomere shortening, each probably acting to a different degree according to cell type or environmental conditions. Oxidative damage is due mainly to enhanced production of reactive oxygen species and concerns all macromolecules. Oxidation of proteins and lipids may explain accumulation of lipofuscin and other damaged components (6, 7), and oxidative DNA damage may be a signal for cell cycle arrest (8). Telomere shortening is due primarily to the end-replication problem. It leads to deprotected chromosome ends that behave like DNA breaks and signal for cell cycle arrest (9, 10). Because of its associated cohort of damage and irreversible cell cycle arrest, senescence has been viewed as a tumor-suppressing mechanism that stops proliferation

of genetically altered cells (11). Consequently, it has been assumed that, to become tumoral, a cell has to bypass senescence. Yet, this assumption is questionable regarding *in vivo* data: the incidence of carcinomas in humans is 2- to 3-fold higher in the 60 to 79 age bracket than in the 40 to 59 age bracket; cancer is frequent in patients suffering from progeroid syndromes (12); and when ageing is delayed by caloric restriction, the incidence of cancer decreases (13). Hence, aging and tumorigenesis are positively linked, suggesting that senescence might precede and sustain tumorigenesis.

Here, after monitoring long-term cultures of human primary keratinocytes, we report the systematic and spontaneous emergence from senescence of cells displaying some transformed and tumorigenic characteristics, suggesting that senescence could indeed be a tumor-promoting state per se. We show that post-senescent-emerging cells potentially originate from all initial cells and not from a special subpopulation and that they have not bypassed senescence but have been formed, on the contrary, through division of cells with already senescent characteristics. We present evidence that the molecular switches necessary for emergence are set during senescence by reactive oxygen species accumulated with senescence. This supports the view that senescence-associated reactive oxygen species might be both a cause of senescence through their deleterious effects and a cause of emergence of pretumoral cells through their mutagenicity.

Materials and Methods

Cell culture and senescence-associated β -galactosidase assays. Normal human epidermal keratinocytes (NHEK) purchased from Clonetics were obtained from eight female donors: five Caucasians (ages 60, 31, 18, 37, and 19 years), one Black (age 33 years), and one Asian (age 40 years). They were grown in KGM-2 BulletKit medium consisting of modified MCBBD153 with 0.15 mmol/L calcium, supplemented with bovine pituitary extract, epidermal growth factor, insulin, hydrocortisone, transferrin, and epinephrin (Clonetics). Such a serum-free low-calcium medium has been shown to minimize keratinocyte terminal differentiation (14). The number of population doublings was calculated as follows at each passage: population doubling = $\ln(\text{number of collected cells} / \text{number of plated cells}) / \ln 2$. Senescence-associated β -galactosidase assays were done as initially described (15).

Western blotting. Cells were lysed in 27.5 mmol/L HEPES (pH 7.6), 1.1 mol/L urea, 0.33 mol/L NaCl, 0.1 mol/L EGTA, 2 mmol/L EDTA, 60 mmol/L KCl, 1 mmol/L DTT, and 1.1% NP-40. The total protein concentration was measured with the Bio-Rad protein assay. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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(Hybond-C extra; Amersham). Equal loading was checked by Ponceau red staining. Primary antibodies were mouse anti-rat proliferating cell nuclear antigen (DAKO), anti-human involucrin, anti-human cytokeratin 14 (Chemicon), anti-human E-cadherin (Transduction Labs), and anti-human actin (Santa Cruz). The secondary antibody was a peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories).

Karyotype analyses. Metaphase spreads were obtained using a standard method. Briefly, cells were incubated 1 h in Karyomax Colcemid (Invitrogen), trypsinized, and incubated in a 60 mmol/L KCl hypotonic buffer. Cells were fixed with methanol/acetic acid solution (3:1, v/v), spread onto frozen slides, and air-dried overnight. For MGG coloration, slides were incubated in 0.035% trypsin diluted in PBS 1 min 40 at 4°C, washed, and stained by 0.024% (w/v) Giemsa (Sigma) in Gurr's buffer 3 min 25 at room temperature. Metaphases were analyzed by the Cytovision software for G-banding. For multiplex fluorescence *in situ* hybridization, slides were fixed in 4% formaldehyde in PBS for 2 min, washed, and treated with pepsin (Sigma) at 1 mg/mL for 10 min at 37°C at pH 2.0. After a wash in PBS, formaldehyde fixation and washes were repeated and the slides were dehydrated with ethanol and air-dried. They were then hybridized with multiplex fluorescence *in situ* hybridization probes (MetaSystems) according to the manufacturer's recommendations.

Anatomopathologic analysis of tissue samples and *in situ* hybridization with human *Alu* sequence probes. Tissue samples were formalin-fixed, paraffin-embedded, sectioned, and processed for May-Grunwald-Giemsa stainings according to standard procedures. Images were recorded using an Axioplan2 Zeiss microscope using Axiovision Software. For *Alu in situ* hybridization, sections were treated with proteinase K and post-fixed. FITC-labeled *Alu* probe (BioGenex) was added and slides covered with sealed coverslips were heated to 90°C for 5 min and then to 37°C overnight. Post-hybridization washes were carried out at 40°C with 2× SSC/0.1% SDS for 2 × 5 min, 0.1× SSC for 10 min, and 2× SSC/0.1% SDS for 5 min. Unspecific binding sites were blocked with 3% bovine serum albumin in PBS + 0.1% Tween 20 for 1 h at room temperature followed by an avidin-biotin-blocker (Vector Laboratories). Probe detection was achieved by incubation with a biotinylated anti-FITC antibody (Vector Laboratories) followed by rhodamine RedX-conjugated streptavidin (Jackson ImmunoResearch) and nuclei counterstaining with Hoechst 33258 (40 ng/mL). Slides were examined under a Zeiss confocal microscope LSM70. Images were recorded using the software Zen.

Adenoviral vector encoding MnSOD. The human MnSOD cDNA, obtained after retrotranscription, was amplified by PCR and propagated in pcDNA3.1. The cDNA was then digested with *EcoRI* and inserted into the pAdCMV2 vector at the *XbaI* sites after filling with Klenow polymerase. Recombinant adenovirus vectors were obtained by homologous recombination in *Escherichia coli* BJ5183 as described previously (16). Viral stocks were amplified after infection of N52.E6 cells (17). Recombinant adenoviruses were purified with the ViraBind Adenovirus purification kit (Cell Biolabs) and titrated with the Adeno-X rapid titer kit (BD Biosciences Clontech). Cells were infected by adding virus stocks directly to the culture medium at an input multiplicity of 200 viral particles per cell.

Comet assays. Ten thousand cells were embedded in 80 μL of 0.5% low melting point agarose at 37°C, and the suspension was immediately laid onto a Trevigen comet slide. Agarose was allowed to solidify at 4°C for 30 min. The slides were then immersed in prechilled Lysis Solution [2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Tris, 1% Triton (pH 10)] at 4°C for 60 min and equilibrated in the electrophoresis buffer for 20 min at room temperature. The electrophoresis buffer was either 89 mmol/L Tris, 89 mmol/L boric acid, 2 mmol/L EDTA (pH 8) or 300 mmol/L NaOH, 1 mmol/L EDTA (pH 13). Migration was carried out at 1 V/cm for 20 min. After migration, the slides were neutralized with 0.4 mol/L Tris (pH 7.5) and stained with either SYBR Green I (Trevigen) according to the manufacturer's recommendations or propidium iodide (2.5 μg/mL). Tail moments were analyzed with the Tritex Comet Score freeware.

Immunofluorescence staining of 8-oxoguanines. Cells were fixed in 4% paraformaldehyde for 15 min at 4°C, dehydrated at -20°C in 70% and 95% methanol for 3 min each followed by 99% methanol for 30 min, and rehydrated for 3 min in 95% and 70% methanol at -20°C and three times in

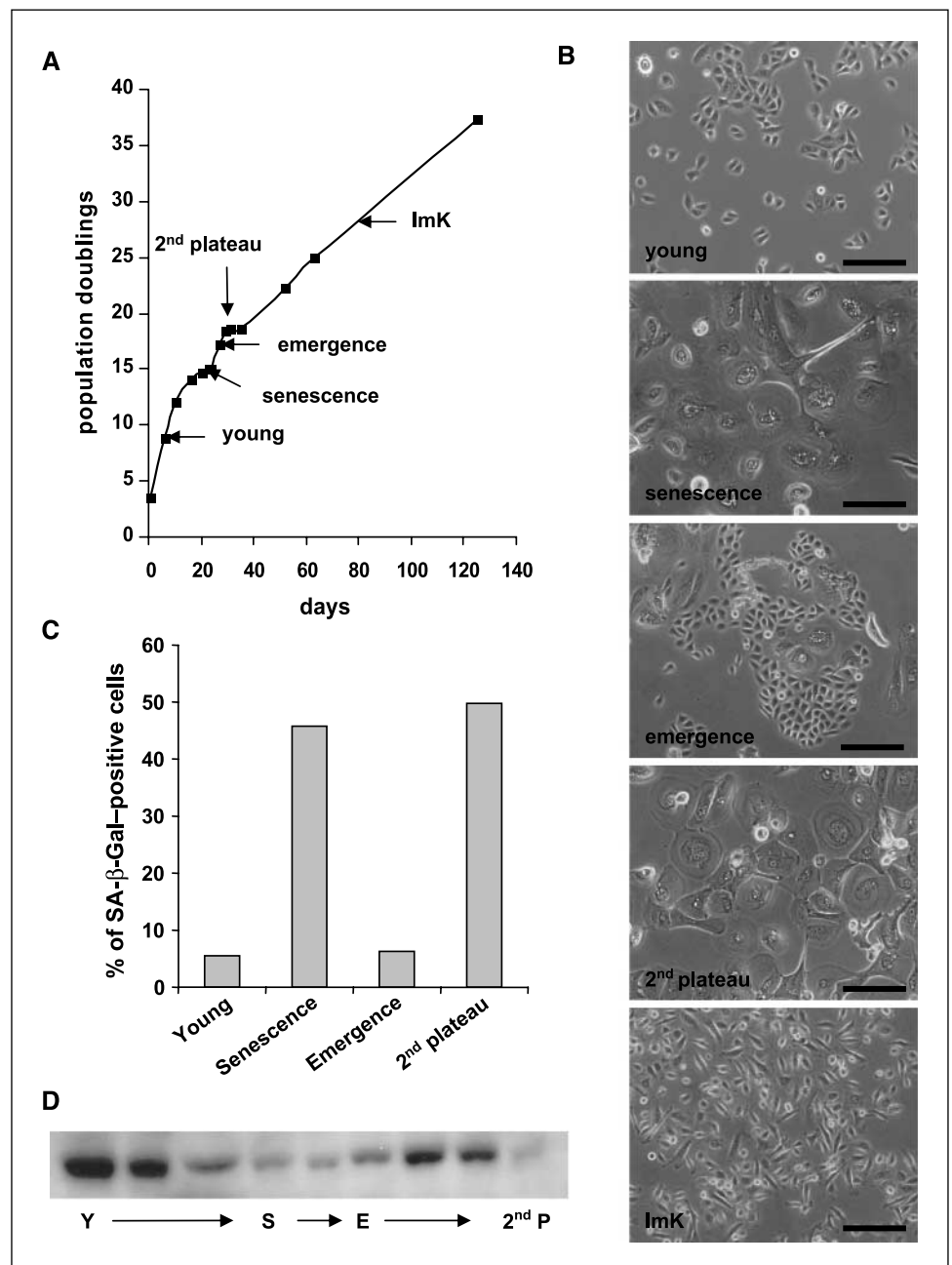
PBS. The anti-8-oxo-7,8-dihydroguanine (8-oxoG) antibody is from Trevigen. Because it recognizes 8-oxoG in both ribonucleotides and deoxyribonucleotides, we performed a RNase A treatment, which did not affect the percentage of positive cells or the intracellular localization of the signal (data not shown).

Results

Keratinocytes spontaneously give rise to transformed and tumorigenic cells. We monitored the behavior in culture of NHEK from eight female adult donors of different ages and races. Such cells first divided over ~3 weeks, making 15 to 20 population doublings, and then reached a plateau at which they display the characteristics of senescence, including increased cell size, polynucleation, accumulation of vacuoles and various damaged components, senescence-associated β-galactosidase activity, and decreased proliferating cell nuclear antigen expression (Fig. 1). After a few days to 3 weeks at this plateau, several clones of small cells appeared spontaneously and systematically in all cultures, whatever the donor, while most senescent cells died. These cells (henceforth called "post-senescent-emerging cells") were found to have resumed expression of proliferating cell nuclear antigen and to grow again for 5 to 15 population doublings, after which they reached a second plateau from which we observed a second emergence. The second emerging cells appeared more transformed than the initial ones (Fig. 1B). We first believed these cells were immortalized and named them ImKs for immortal keratinocytes (numbered IMK, IMK2, IMK3, ..., to identify the donor). ImKs, however, underwent up to 60 population doublings but then stopped and died (data not shown). Neither ImKs nor post-senescent-emerging cells showed any resumption of telomerase activity (Supplementary Fig. S1). We estimated the emergence frequency, that is, the number of emerging clones generated per cell at the plateau, by plating plateau cells at low density and counting the emerging clones. Depending on the experiment, the frequency of the first wave of emergence ranged from 10⁻⁵ to 10⁻² and that of second wave was 10⁻⁵ to 10⁻⁴. These frequencies are considerably higher than 10⁻⁷, the frequency of immortalization of SV40-transformed human fibroblasts (18, 19).

Post-senescent-emerging cells looked partly transformed, with some clones displaying a fibroblastoid morphology associated with a tendency to scatter (Supplementary Fig. S2A). The expression of involucrin and keratin 14, two markers of keratinocyte differentiation, increased with senescence and decreased again with emergence (Supplementary Fig. S2B). That of E-cadherin, involved in cell-cell interaction, also slightly decreased in post-senescent-emerging cells (Supplementary Fig. S2C). A transcriptomic analysis on DNA microarrays revealed that, on the 50 most up-regulated and the 50 most down-regulated genes in post-senescent-emerging cells, 15 turned out to be linked to adhesion or migration, 6 to cytoskeleton structure or dynamics, 9 to senescence, oxidative stress, or DNA damage, 9 to cell cycle progression or cell death, and 10 to diverse cancer-related pathways (Supplementary Table 1). Hence, ~50% of the genes whose expression changes in emerging keratinocytes are relevant to transformation. We also investigated the karyotypic of post-senescent-emerging cells and ImKs by analyzing metaphases by G-banding and multiplex fluorescence *in situ* hybridization. Post-senescent-emerging cells displayed no karyotypic aberrations. In contrast, 100% of ImK metaphases displayed various aberrations, mainly translocations (Fig. 2).

Figure 1. Senescence and emergence of NHEKs. *A*, growth curve. *B*, cell morphologies observed by phase-contrast microscopy. *Bar*, 80 μ m. *C*, percentages of senescence-associated β -galactosidase-positive cells counted among 300 total cells. *D*, Western blot analysis of proliferating cell nuclear antigen level in total cell extracts. Representative of several experiments done with cells from seven different donors.



Finally, we investigated the tumorigenic potential of emerging cells. Pre-senescent, post-senescent-emerging cells or ImKs were injected in the flank of *nude* mice. MDA-MB-231 and NIH-3T3 were used as controls. As expected, MDA-MB-231-injected mice showed significant tumors after 4 weeks. Tumors were also recorded in NIH-3T3-injected mice but only after 17 weeks. In mice injected with pre-senescent, post-senescent-emerging cells and ImKs, the xenograft poorly developed. However, from the 19th week onward, disseminated skin lesions appeared away from the injection site in 4 of 5 and 6 of 6 mice injected with post-senescent-emerging cells and ImKs, respectively (Fig. 3A). Macroscopically, these lesions resembled early nonmelanoma skin carcinomas (Fig. 3B). Anatomopathologic analyses indicated hyperplasia, hyperkeratotic plaques, and actinic keratosis as most

frequent precancerous phenotypes. Important mastocytosis was recorded facing hyperplasia and hyperkeratotic plaques, particularly at sites of basal lamina ruptures (Fig. 3B). Because these lesions developed very lately and away from the injection site, it was necessary to prove they really derive from the injected cells. We therefore performed the detection of primate-specific *Alu* sequences by fluorescence *in situ* hybridization. The results showed the presence of human cells within the epidermis, at the lesion sites (Fig. 3C).

Taken together, these results suggest that the cell populations present at both growth plateaus are able to generate partially transformed and moderately tumorigenic cells able to disseminate, with more and more marked phenotypes from the first emergence wave to the second.

Post-senescent-emerging cells are formed from a few senescent cells by an unusual budding mitosis mechanism.

One of our concerns was to elucidate the origin of post-senescent-emerging cells. Our first hypothesis was they might come from an initial subpopulation of already transformed cells present in the explants despite the healthy status of the donors. To test this hypothesis, we performed monoclonal cultures of young NHEKs, which we conducted to senescence and then monitored for emergence. Emerging clones appeared in ~75% of the cultures (Supplementary Table 2), indicating that emerging clones are not the progeny of a restricted initial subpopulation but rather that almost all initial young cells have the potential to yield emerging cells. This invalidated our initial hypothesis. We then reasoned that emerging cells might be generated during senescence. To test this hypothesis, we sorted senescent cells by fluorescence-activated cell sorting from a pre-senescent population as cells with the highest forward and scatter factors, that is, the largest and most granular. Sorted cells were plated and stained with fluorogenic filiation tracers (Vybrant diI or Vybrant CFDA SE). After ~1 week, emerging cells arose around some senescent cells and were stained by the fluorogenic tracers (Supplementary Fig. S3), proving that they are directly generated through division of fully senescent cells.

The fact that senescent cells can divide was surprising, because numerous studies have shown that proliferation of senescent cells is irreversibly impaired by their short telomeres. However, in the

case of keratinocytes, it has been shown that telomerase reexpression alone is insufficient to bypass senescence (20), suggesting that telomere length is not a limiting factor in this cell type. To confirm this point, we examined NHEK telomeres at senescence. Southern blot analysis showed that the telomere length continuously decreased from ~9 kb in young NHEKs to 6 kb at senescence and then 5 kb in emerging cells (Supplementary Fig. S4A). However, teloFISH analysis revealed that most cells at both plateaus still had substantial telomeres on all their chromosomes and that only a minority displayed some chromosomes with very short to undetectable telomeres likely to cause irreversible cell cycle arrest (Supplementary Fig. S4B and C). Senescent cells, including keratinocytes, have also been described as irreversibly arrested through induction of cyclin-dependent kinase inhibitors p16 and p21 (20–22). A quantitative reverse transcription-PCR analysis showed, as expected, that both cyclin-dependent kinase inhibitor mRNAs increased at the first plateau but decreased again in post-senescent-emerging cells (Supplementary Fig. S5), indicating that their up-regulation is only transient. Bromodeoxyuridine incorporation assays indicated, as expected, that most typical polynucleated senescent cells were bromodeoxyuridine-negative but revealed that some were bromodeoxyuridine-positive (Supplementary Fig. S6A). Moreover, we observed some typical large polynucleated senescent cells with one nucleus in metaphase after a colcemid treatment (Supplementary Fig. S6B). Taken together,

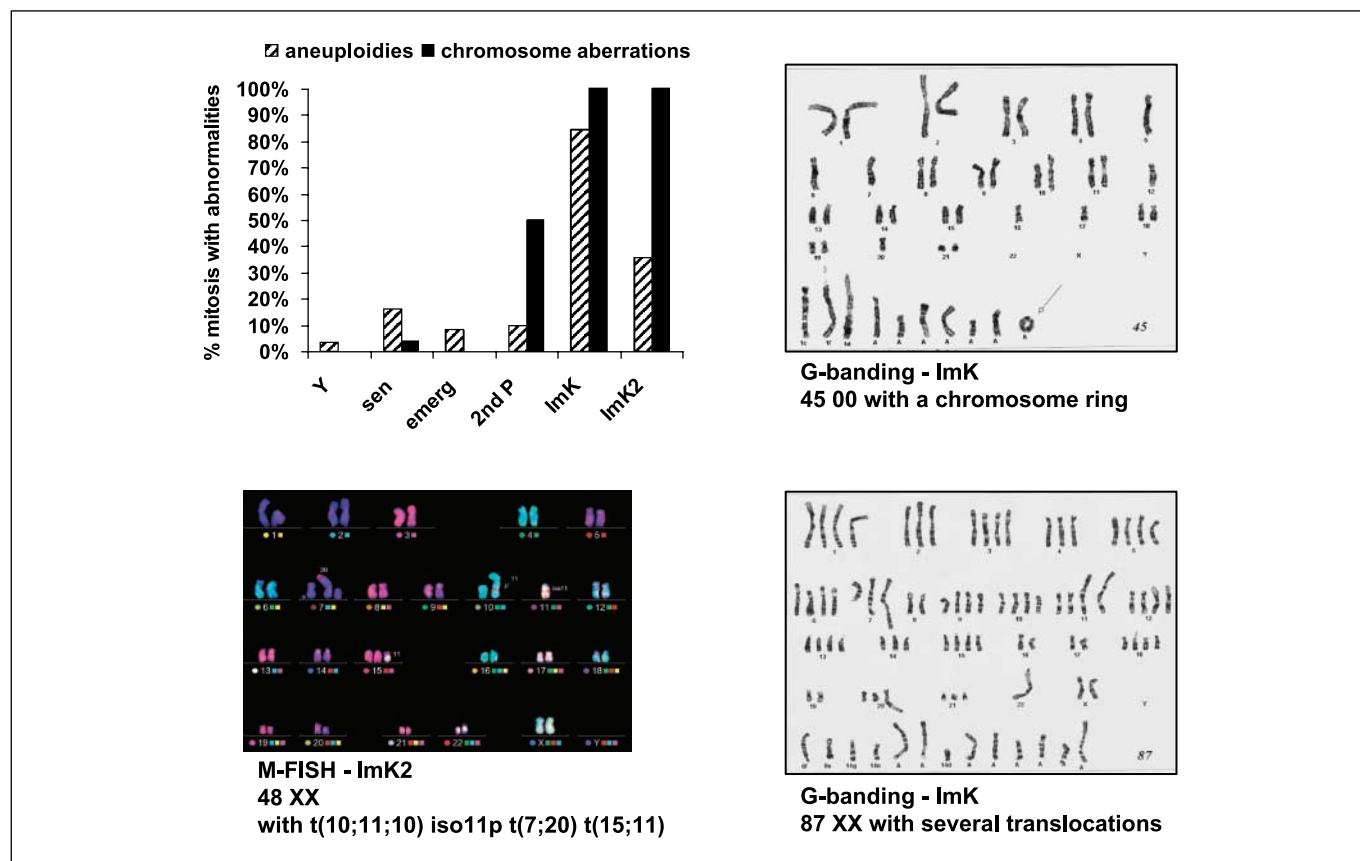


Figure 2. Karyotypic analysis of NHEKs at the different growth phases. Eleven to 28 metaphases from young (Y), senescent (sen), post-senescent-emerging (emerg), second plateau (2nd P), ImK, and ImK2 cells were analyzed by G-banding and/or multiplex fluorescence *in situ* hybridization (M-FISH). "Aneuploidies" regroups polyploidies and less severe chromosome gain or loss; "chromosome aberrations" regroups translocations, fusions, chromosome rings, and chromosome minutes. Photographs of representative ImK and ImK2 karyotypes.

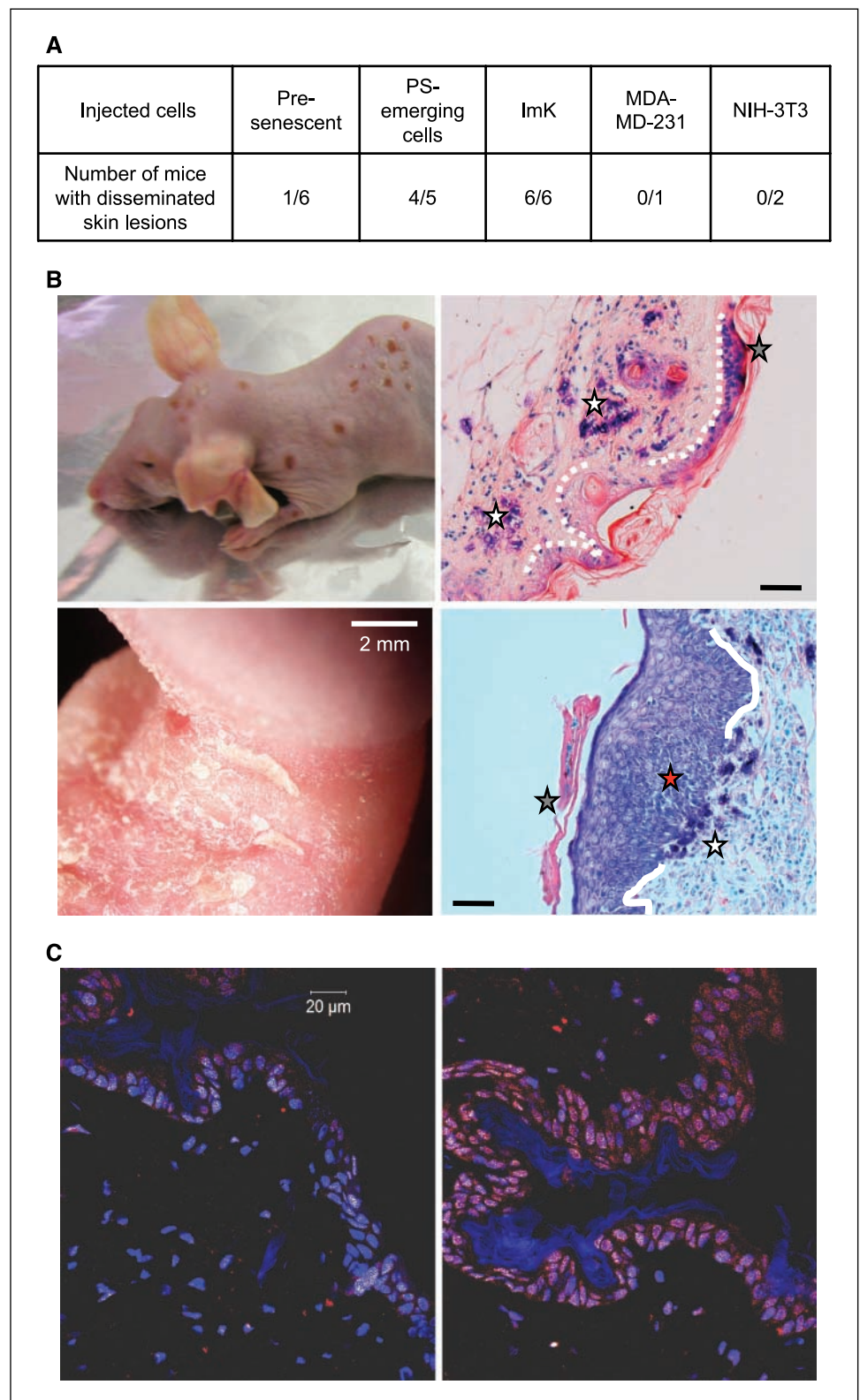


Figure 3. *In vivo* tumorigenicity of post-senescent-emerging cells and ImKs. Eight hundred thousand of either pre-senescent, post-senescent (PS)-emerging cells, ImKs, MDA-MB-231, or NIH-3T3 embedded in a plug of collagen/Matrigel were injected in the flank of 18-week-old female BALB/c nude mice. **A**, number of mice displaying disseminated skin lesions 19 wk onward after injection. **B**, macroscopic phenotypes of the disseminated skin lesions (*left column: top, entire mouse; bottom, detail of an ear*) and corresponding May-Grunwald-Giemsa staining of lesion sections (*right column*) of ImK-injected animals. Most lesions display epidermal hyperplasia (*red star*), actinic keratosis (*gray stars*), and moderate to very strong mastocytosis (*white stars*) at the level of basal lamina ruptures (*dotted white line*). **C**, confocal analysis of *in situ* hybridization for *Alu* sequences of two different lesions. The hybridization signal (*red spots* or *white spots* when merged with Hoechst staining) reveals the presence of human cells inside the mouse epidermis. When not specified, bar represents 50 μ m.

these experiments indicate that, although overall growth of the culture is arrested at the senescence plateau, senescent keratinocytes have still a division potential regarding their telomere length and are cell cycle arrested but not irreversibly and some senescent cells actually divide.

We next wondered by what cell division mechanism a senescent cell, very enlarged and littered with damaged components, gives rise to small cells with a clear cytoplasm. Multiple microscopic examinations suggested that senescent cells generate emerging cells by an unusual asymmetric mitosis mechanism, remaining

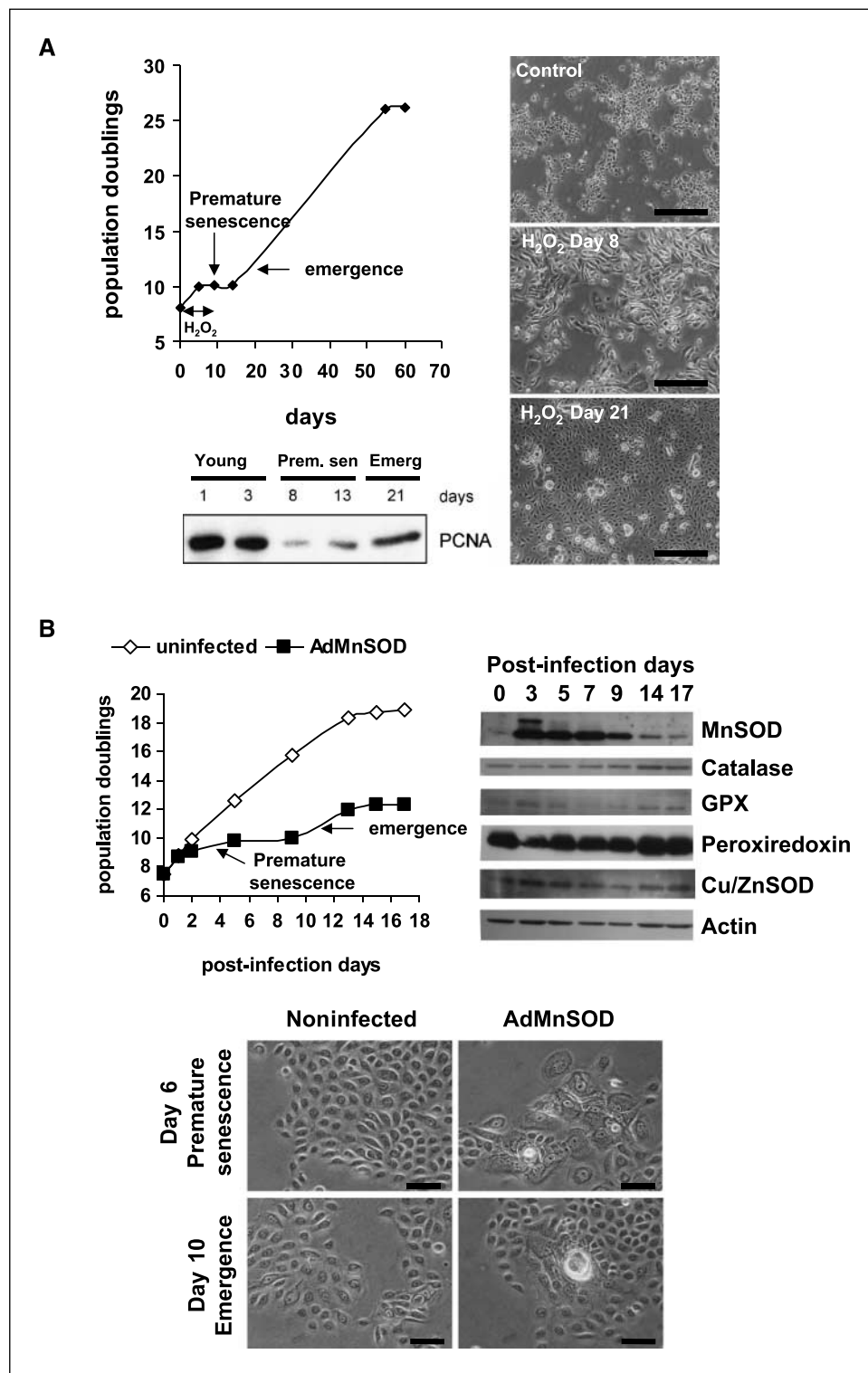


Figure 4. H₂O₂ is a motor of post-senescence emergence. **A**, young NHEKs were treated with 30 μ mol/L H₂O₂ during 2 h every 3 d until displaying the senescent phenotype (9-day total treatment). The treatment was then stopped and emergence was monitored. Growth curve, proliferating cell nuclear antigen (PCNA) expression, and representative images of cell morphologies. *Bar*, 400 μ m. **B**, young NHEKs were infected or not with AdMnSOD. AdMnSOD-infected cells underwent premature senescence (plateau from 3 to 10 d after infection) and emergence afterwards. As seen in the Western blot analysis, MnSOD was overexpressed on days 3 to 14 post-infection, the adenoviral genome remaining episomal. Expression of other antioxidant enzymes did not significantly change. Morphologies of infected and uninfected cells are shown on days 6 and 10 post-infection. *Bar*, 30 μ m.

budding yeast cells. First, on examining trypsin-dissociated fluorescence-activated cell sorted senescent cells by phase-contrast microscopy, we observed large cells with one, two, or three attached buds (Supplementary Fig. S7A). Second, in routine cultures on plastic, emerging cells were almost always observed gathered around a large senescent cell, to which some appeared still attached by a pedicle (Supplementary Fig. S7B). To formally

evidence this attachment, we analyzed by confocal microscopy the cytokeratin 14 network. Optical transverse sections revealed cytoskeleton continuity between the senescent cell and some surrounding emerging cells (Supplementary Fig. S7C). Finally, despite the challenge due to the low fraction of senescing cells actually producing emerging cells, we managed videomicroscopy and succeeded in capturing three sequences of images showing two

slightly different mechanisms of budding mitosis. In two cases, a large multinucleated senescent cell generated several small daughter cells by budding cytokinesis (data not shown). In the third case, we observed a typical senescent cell with already several nuclei, among which three underwent an additional full mitosis generating a daughter cell budding out of the senescent mother (Supplementary Fig. S8 and Supplementary Video).

NF- κ B > MnSOD > hydrogen peroxide pathway is causal in both senescence and emergence. We have shown previously that NHEK senescence arises in part through hydrogen peroxide (H_2O_2) accumulation. This accumulation was shown to result from an activation of NF- κ B and ensuing up-regulation of MnSOD, responsible for the dismutation of $O_2^{\cdot -}$ to H_2O_2 . Without any co-up-regulation of downstream H_2O_2 -degrading enzymes, this leads to H_2O_2 accumulation (23). H_2O_2 being mutagenic, we hypothesized here that it might also contribute to emergence.

To test the involvement of the NF- κ B > MnSOD > H_2O_2 pathway in emergence, we first treated young NHEKs with a concentration of H_2O_2 that we previously established as inducing premature senescence (23), monitored cells until they all displayed the senescent phenotype, and then stopped the treatment and waited for potential emergence. Emerging clones did appear ~ 10 days later at a frequency of $\sim 10^{-4}$ (Fig. 4A). We then examined whether MnSOD overexpression might have the same effect. Young NHEKs were infected with an adenoviral vector encoding MnSOD. A premature senescence phenotype arose after ~ 3 days followed 10 days later by emergence. We checked that both senescence and emergence occurred without any change in expression of several other antioxidant enzymes (Fig. 4B). We finally examined whether antioxidants or NF- κ B inhibitors could inhibit emergence. As antioxidant, we used catalase, which specifically degrades H_2O_2 and was already shown to delay keratinocyte senescence (23). We also used *N-tert-butyl-hydroxylamine*, a more general antioxidant shown to target mitochondria and to reduce nuclear DNA damage (24). To inhibit NF- κ B activity, we used sulfasalazine and gliotoxin, two weak inhibitors we have shown previously to delay senescence without inducing massive apoptosis (23). A few days after having treated pre-senescent cells with one of these drugs, emerging clones appeared in 85% of the control wells compared with 50% of those treated by catalase, 0% of the wells with (10 μ mol/L) *N-tert-butyl-hydroxylamine*-treated cells, and 0% of the wells containing NF- κ B inhibitors (Fig. 5).

Taken together, these results show that a H_2O_2 accumulation resulting from an activation of NF- κ B and an unbalanced antioxidant enzyme expression is sufficient to induce a post-senescence emergence similar to the spontaneous one.

To further test the hypothesis that H_2O_2 induces emergence through its mutagenicity, we searched for mutagenic oxidative DNA damage in senescent cells. We first investigated DNA single-strand breaks (SSB), which are known to be induced by H_2O_2 (25, 26), by comet assays that unable to distinguish SSB from double-strand breaks (DSB). The results show that SSB were predominant, increasing ~ 2 -fold with senescence and affecting $\sim 20\%$ of cells. Cells displaying DSB were rare, even at senescence. H_2O_2 did not induce any change in DSB but induced a dramatic increase in SSB (Fig. 6A). Accordingly, a catalase treatment decreased ~ 2 -fold the number of SSB per cell (Fig. 6B). H_2O_2 is also known to induce oxidation of bases in nuclear and mitochondrial DNA and in the nucleotide pool, the most common being 8-oxoG (27). By immunofluorescence, $\sim 20\%$ of senescent cells were found to display 8-oxoG, in their cytoplasm and nucleus,

compared with only 3% of young cells (Fig. 6C). When cells were treated with catalase, the percentage of affected senescent cells was reduced 4.25-fold (Fig. 6C). Conversely, treatment of young cells with H_2O_2 increased the percentage of 8-oxoG-positive cells ~ 5 -fold (Fig. 6C). Thus, at least two types of oxidative mutagenic damage, SSB and 8-oxoG, accumulate during senescence in correlation with the H_2O_2 level.

Discussion

Numerous studies have reported that senescence is an irreversible growth arrest associated with telomere shortening. This has led to viewing senescence as a tumor-suppressing phenomenon. However, most of these studies were done with human fibroblasts, which are not the most relevant cell model for studying the molecular links between tumorigenesis and ageing, because sarcomas are rare in humans and their incidence does not depend on age (National Cancer Institute statistics). Using normal human keratinocytes, we show that cells with moderate transformed and tumorigenic characteristics systematically and spontaneously emerge from senescence. We have observed a similar emergence with epithelial mammary cells, as already described by others (28–30), although these emerging cells never gave rise to a second emergence (data not shown). Therefore, we propose this *in vitro* post-senescence emergence as a model for studying the very first steps of carcinogenesis. However, it is not yet clear whether this model can be generalized to all carcinomas, because we never observed any emergence with prostatic epithelial cells under standard culture conditions (data not shown).

It is often assumed that bypassing senescence is an obligatory step for tumorigenesis. We show here that emerging cells have not bypassed senescence but are instead generated from fully senescent cells via an unusual budding-mitosis mechanism. Although largely ignored by the scientific community, this kind of cell division, specific to senescent or DNA-damaged cells, has

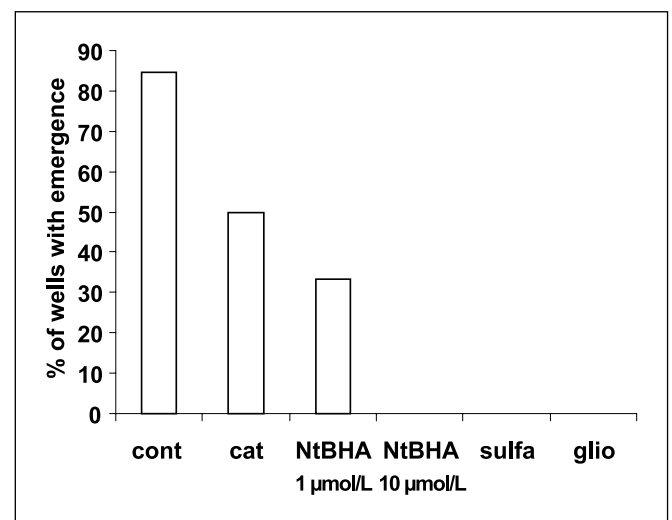


Figure 5. Antioxidants and NF- κ B inhibitors inhibit post-senescence emergence. Pre-senescent NHEKs were seeded into 24-well culture plates at the limit density for emergence (10,000 cells per well) and treated or not with catalase (*cat*) at 100 units/mL, *N-tert-butyl-hydroxylamine* (*NtBHA*) at 1 or 10 μ mol/L, sulfasalazine (*sulfa*) at 0.5 mmol/L, or gliotoxin (*glio*) at 0.05 μ mol/L. A few days later, the wells with emerging clones were counted and their percentages were calculated.

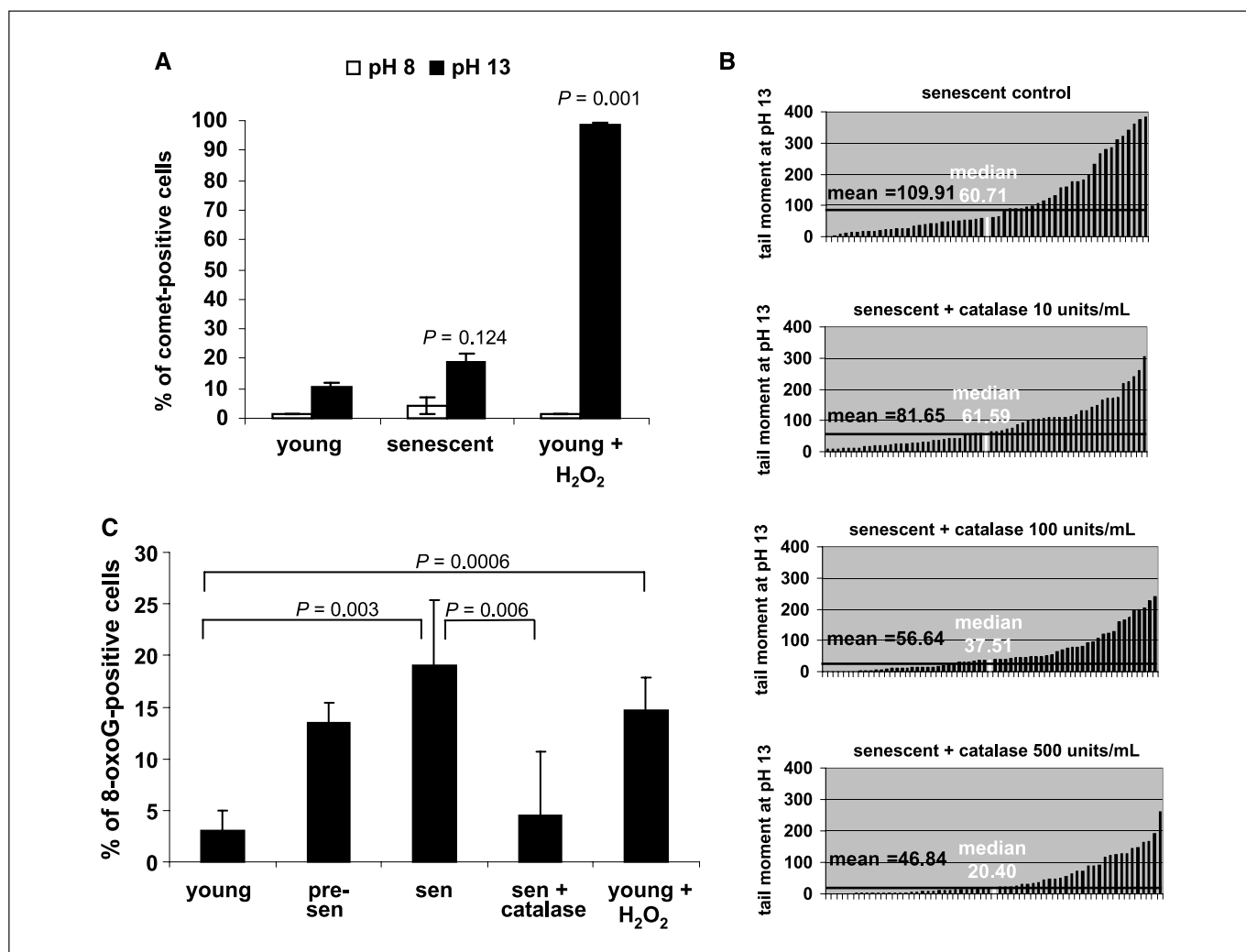


Figure 6. Post-senescence emergence is linked to the level of mutagenic oxidative DNA damage in senescent cells. *A*, comet assays on young and senescent NHEKs treated or not with 30 $\mu\text{mol/L}$ H₂O₂ done at pH 8 to detect DSB and at pH 13 to detect both DSB and SSB. Comet-positive cells were independently counted twice. Mean \pm SD percentages of comet-positive cells. *B*, comet assays on senescent NHEKs treated or not with 10, 100, or 500 units/mL catalase at pH 13. Tail moments (a value taking into account the percentage of DNA in the tail and the length of the tail) of 56 to 72 cells in each case are given (in arbitrary unit) along with mean and median values. *C*, NHEKs at different stages, treated or not with 30 $\mu\text{mol/L}$ H₂O₂ or 100 units/mL catalase, were subjected to immunofluorescence staining with an antibody against 8-oxo-guanine. 8-Oxo-guanine-positive cells were counted in five independent microscopic fields. Mean \pm SD. *P* values were calculated using *t* tests.

already been described by two groups (31, 32) and called “neosis.” We highlight here the importance of such a mechanism by showing that cells generated in this way are tumorigenic. We have ruled out the hypothesis that emerging cells come from an initial subpopulation of already transformed cells. On this last point, our results are in disagreement with those of Tlsty (33), suggesting, in the case of epithelial mammary cells, that emerging cells come from cells preexisting in explants and having a hypermethylated p16 promoter. We show here that the decrease in p16 expression in post-senescence-emerging keratinocytes is only transient, because p16 is again up-regulated at the second plateau. Only in ImKs does expression of p16 decrease to a very low level compatible with epigenetic extinction.

Through this study and a preceding one (23), we show that the NF- κ B > MnSOD > H₂O₂ oxidative stress pathway is causal in both senescence and emergence. This conclusion is based on the observations that NF- κ B inhibitors and antioxidants delay the occurrence of the senescence plateau and decrease the emergence

frequency, whereas, conversely, NF- κ B or MnSOD overexpression or H₂O₂ treatment induces premature senescence followed by emergence. We report the presence, in senescent cells, of at least two types of oxidative DNA damage, SSB and 8-oxoG, both potentially causing point mutations (27, 34, 35). That they play a causal role in emergence is supported by the consistent correlation between the percentage of cells affected by these damages and the emergence frequency: this percentage rises on H₂O treatment (which triggers senescence and emergence) and drops after senescence-delaying antioxidant treatments. Furthermore, the proportion of cells with SSB and 8-oxoG was always much higher than the proportion of emerging cells, making it statistically possible for these alterations to affect a favorable cocktail of oncogenes, tumor suppressor genes, and/or other crucial regulators of adhesion, migration, cell cycle, or cell death, as suggested by the transcriptomic changes observed in emerging cells. The stochastic nature of the events leading to emergence is supported by the fact that emergence is always multiclonal, each clone having

its specificities as regards morphology, doubling time, and life span (data not shown). In accordance with this mutagenic motor role of oxidative stress for emergence, it was shown that the spontaneous immortalization of mouse embryonic fibroblasts after senescence is accompanied by a 3-fold increase in point mutations resulting from oxidative stress (36). It was also reported that a mutation in codon 61 of the Ha-ras gene spontaneously occurs in mouse keratinocytes that, similarly to human keratinocytes, form emerging foci at the senescence plateau (37). We checked for such a mutation in four clones of post-senescent-emergent NHEKs but did not find it (data not shown). This suggests that emergence cannot rely on a unique mutation, even of a major oncogene, but probably necessitates multiple genomic alterations.

In conclusion, the results presented here suggest that the initiating events in tumorigenesis may result from the mutagenicity of the oxidative stress to which senescing cells are subject. Hence, senescence and its associated oxidative stress might be viewed as endogenous carcinogens, this providing a molecular explanation of the link between advanced age and increased cancer incidence. The presence of cells with senescence markers has been evidenced in some premalignant lesions of young people, such as congenital naevi and benign prostate hyperplasia (38, 39). Thus, oxidative stress, whether it results from normal aging, from a special local

hormonal environment as in the prostate, or from inherited oncogene activation as in congenital naevi, may generate (prematurely) senescent cells from which cancer-initiated cells have a high risk of emerging.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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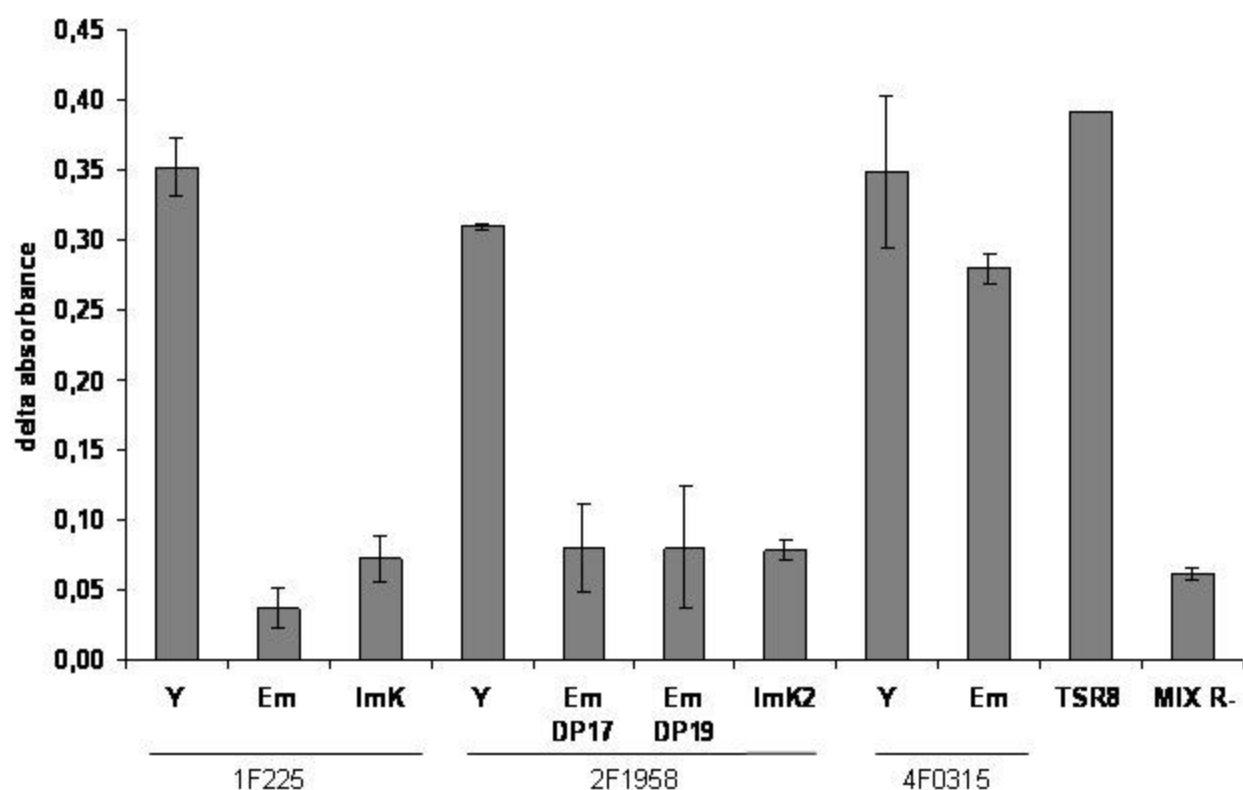
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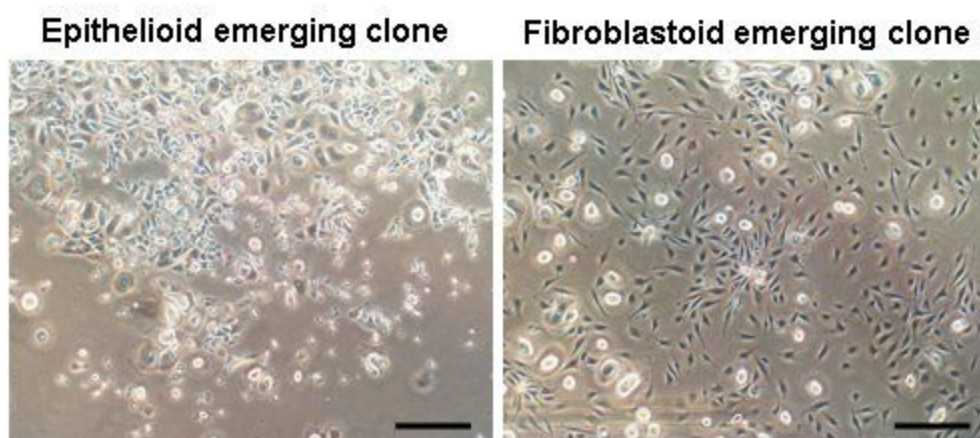
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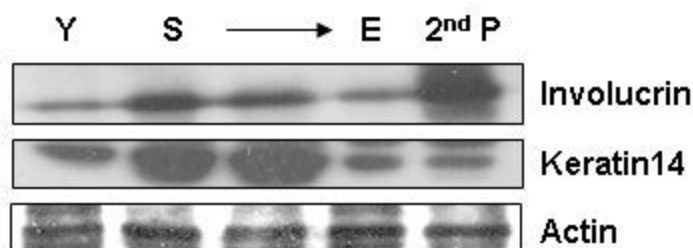
Supplementary Figure 1: Telomerase activity in NHEKs at the different growth stages

Young (Y), PS emergent (Em) and ImK NHEKs of three different donors (1F225, 2F1958, and 4F0315) were analyzed for telomerase activity by TRAP assay. Each sample was analyzed as a native extract and after heat inactivation, in duplicate. Results are given as the difference of the mean absorbance of the native extract minus that of the heat inactive extract +/- standard deviation. TSR8 is a positive internal control for PCR/ELISA. MIX R- is a negative control without cell extract.

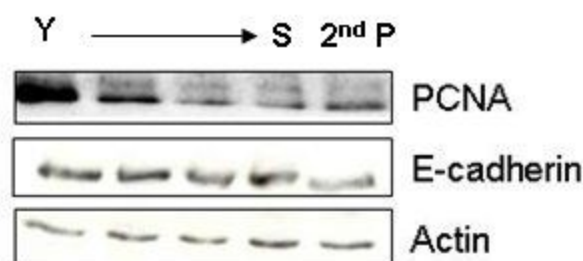
A.



B.

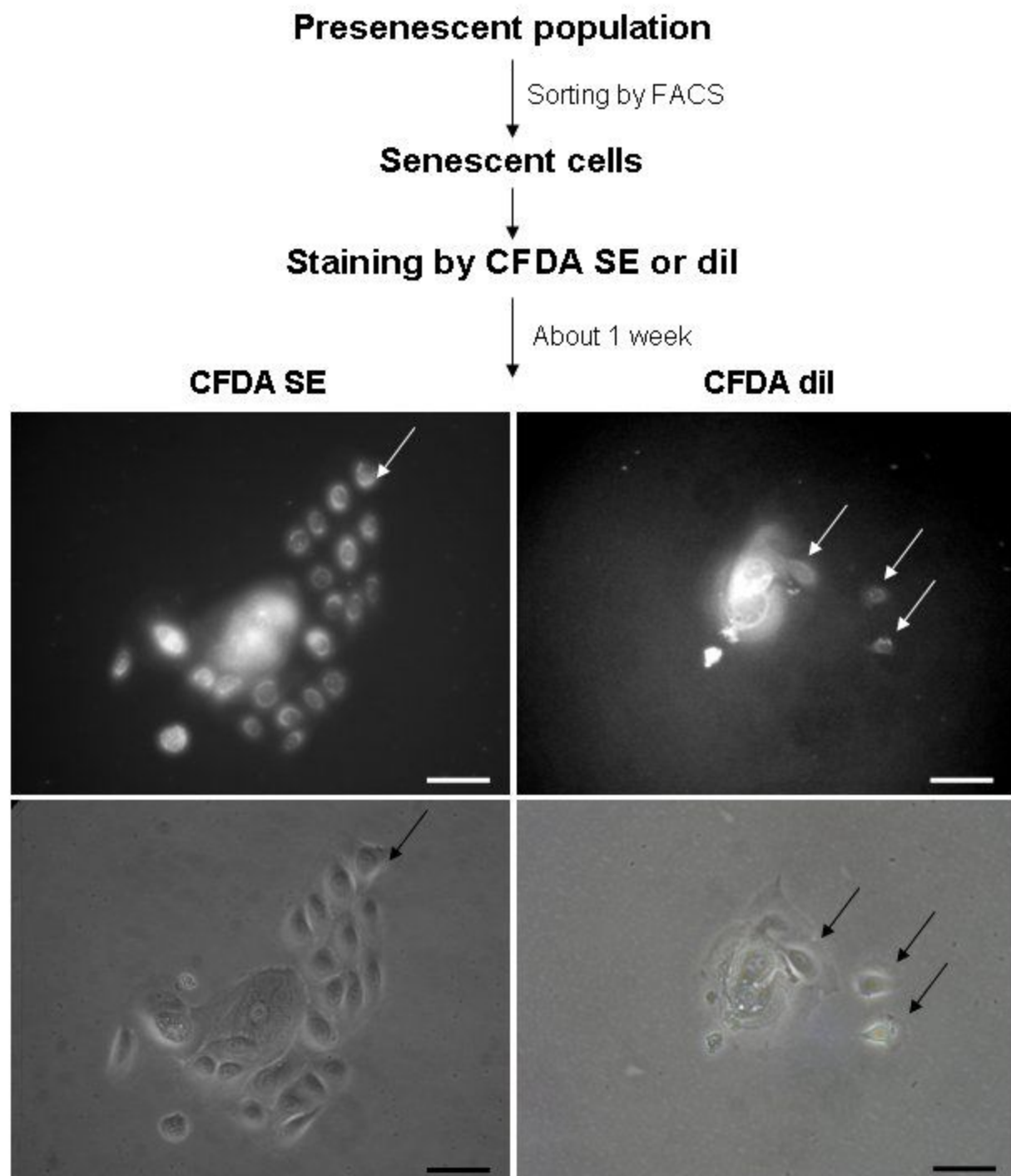


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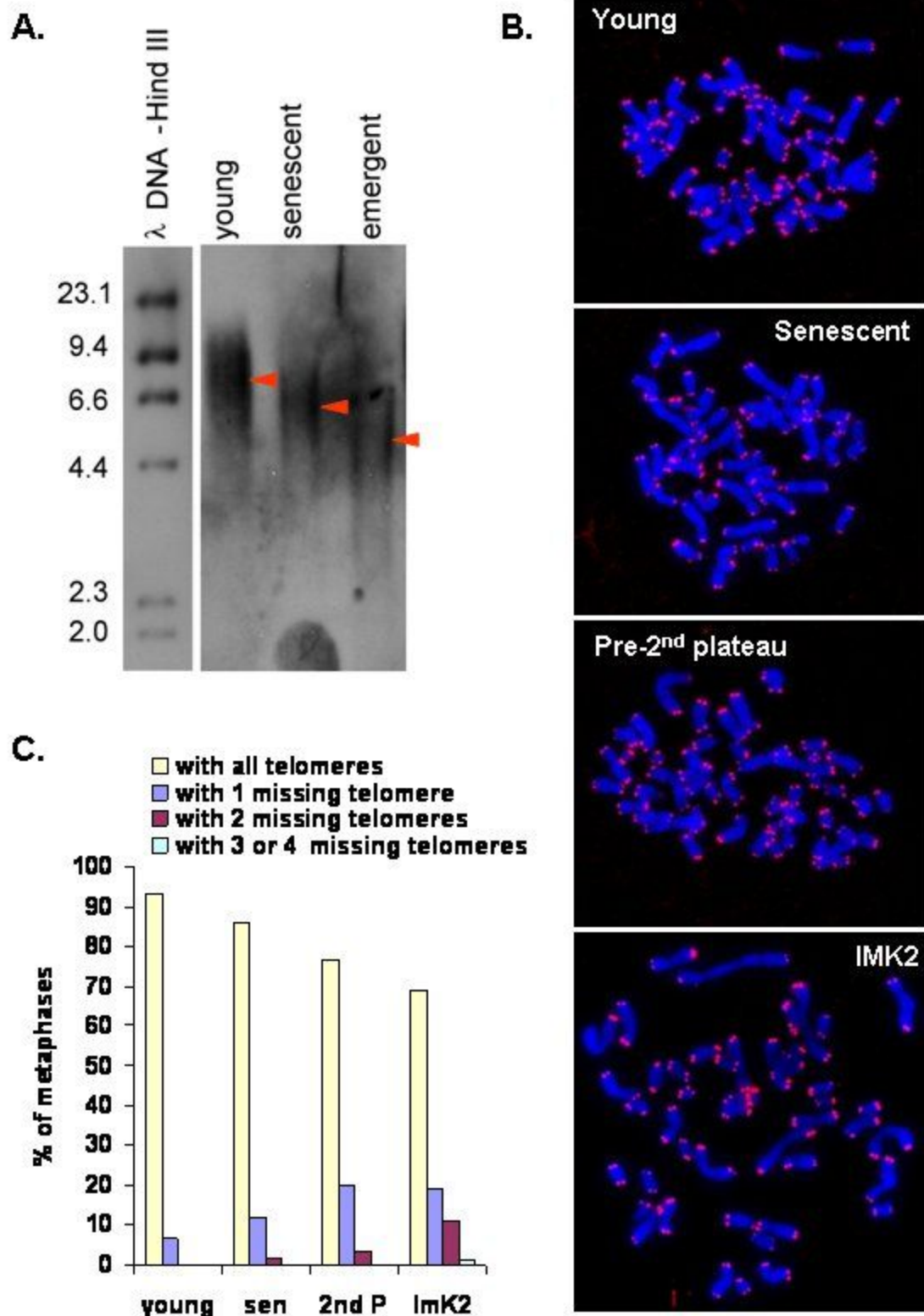
Supplementary Figure 2: Post-senescent emergent cells are partially transformed

(A) Observation by phase-contrast microscopy of 2 PS emerging clones representative of many routine observations done with different donors. The clone of the left panel is the most commonly observed; it has still an epithelioid morphology. That of the right panel is rarer; it has a fibroblastoid morphology. Bars represent 60 μ m. (B and C) Western blot analysis of some markers of keratinocyte differentiation, involucrin, cytokeratin14 and E-cadherin, in total cell extracts of young (Y), senescent (S), PS emergent (E) and 2nd plateau (2nd P) NHEKs.



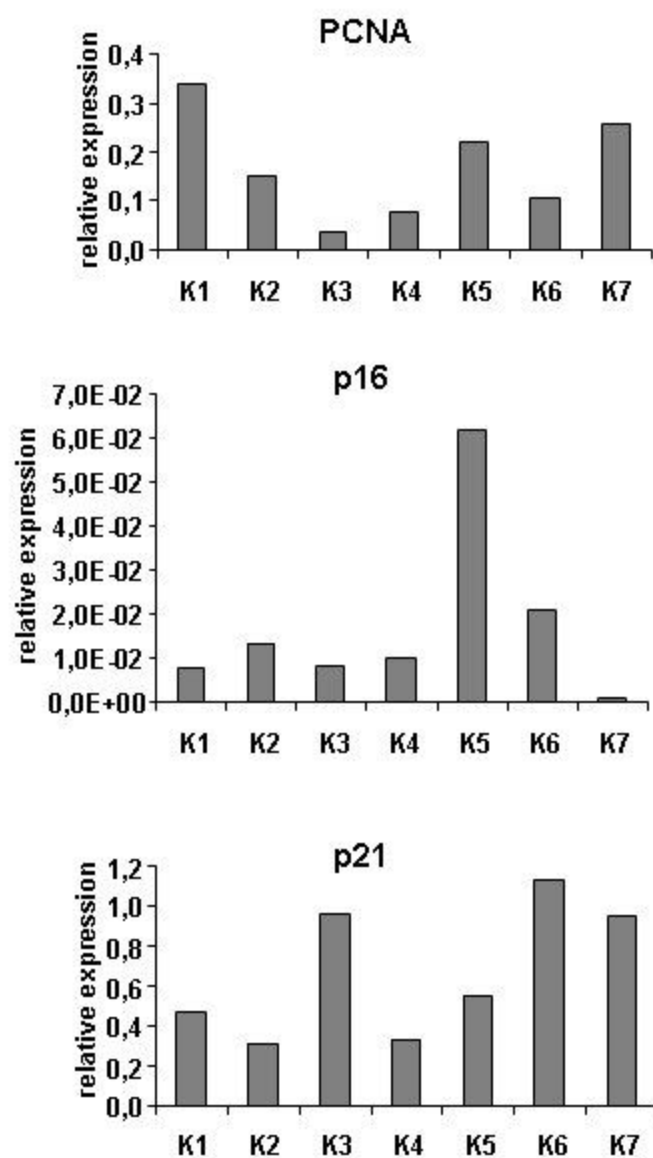
Supplementary Fig.3: Evidence of filiation between senescent and PS emergent cells

Senescent NHEKs were sorted by FACS as described in Experimental Procedures and placed again in culture at low density. After plating, the quality of the sort was checked by careful microscopic observation (not shown). The cells were then stained with the fluorogenic tracer CFDA SE or dil, washed, and monitored every day for emergence. Emergence occurred after about a week. Emerging clones were analysed under an epifluorescence microscope. One can see that emerging cells (arrows) are stained by the tracers like the parental senescent cells. Bars represent 30µm.



Supplementay Figure 4: NHEKs at the growth plateaus have telomeres only slightly shortened

(A) Young, senescent and PS emergent NHEKs were processed for telomere length analysis by southern-blot. The red arrowheads indicate the approximate median telomere length. (B) Young, senescent, 2nd plateau and IMK cells were processed for telomere analysis by teloFISH. Representative results are illustrated. Chromosomes without telomeres are very rare. (C) Thirty to 74 metaphases in each case were analysed for chromosomes with undetectable telomeres.

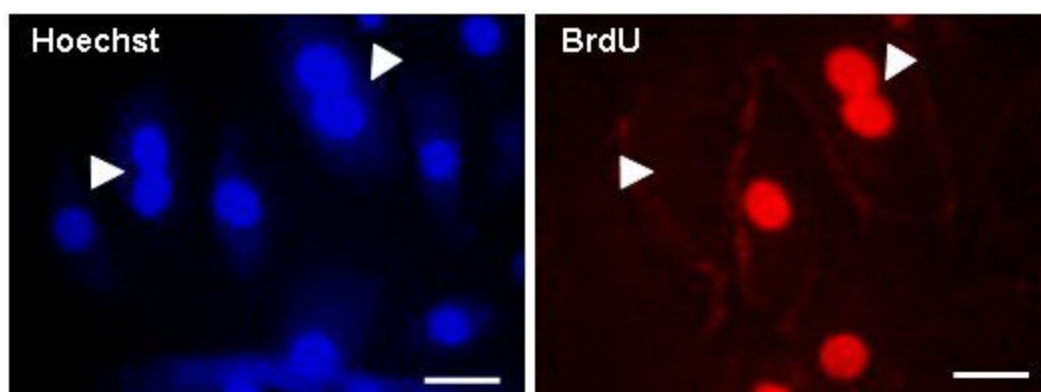


K1: young
 K2: presenescent
 K3: senescent
 K4: end of senescence and beginning of PS emergence
 K5: end of PS emergence and beginning of 2nd plateau
 K6: 2nd plateau
 K7: ImK

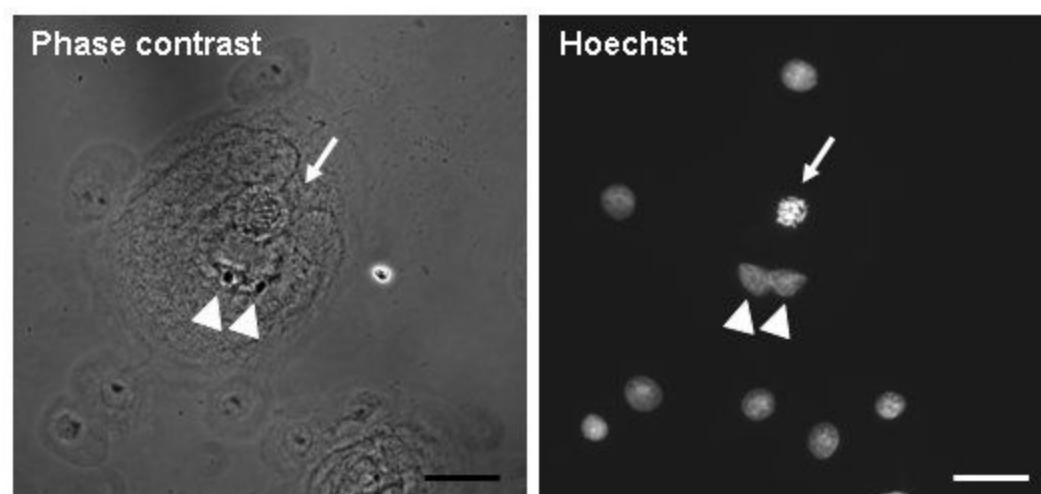
Supplementary Figure 5: Expression of p16 and p21 at the different growth phases

Total RNAs were extracted from NHEKs at different growth stages. RNAs for PCNA, p16 and p21 were quantified by Reverse Transcription and Quantitative-Polymerase Chain Reaction and normalized to the geometric average of 3 internal controls, ARNpol2, RPL13a and Actine b.

A. BrdU-incorporation assay



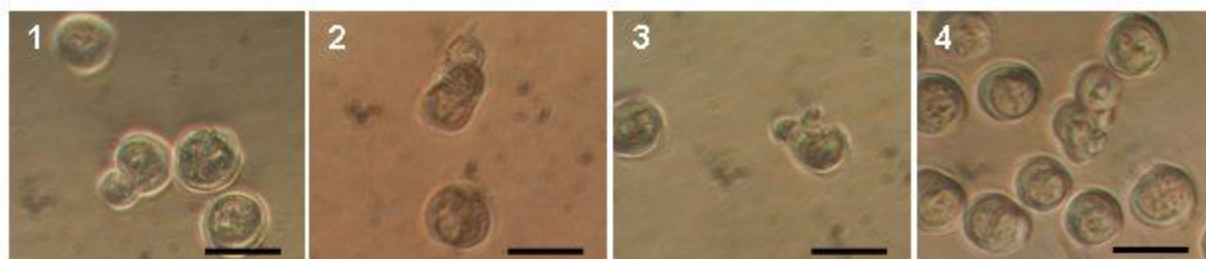
B. After colcemid



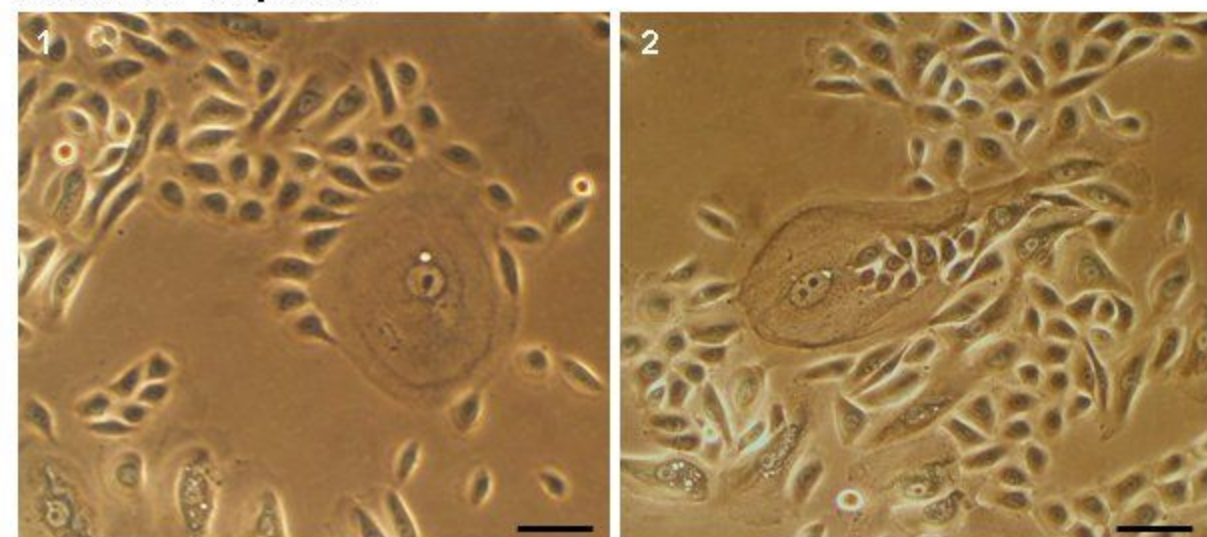
Supplementary Figure 6: Senescent keratinocytes are still able to divide

(A) Representative images of BrdU incorporation assays performed on a senescent population of NHEKs. Arrowheads indicate binucleated senescent cells. One is BrdU-negative; the other is BrdU-positive. Bars represent 6 μ m. (B) Images of a typical senescent cell treated by colcemid in order to block metaphase plates. This cell displays 3 nuclei, two interphasic (arrowheads), and one in metaphase (arrow). Bars represent 15 μ m.

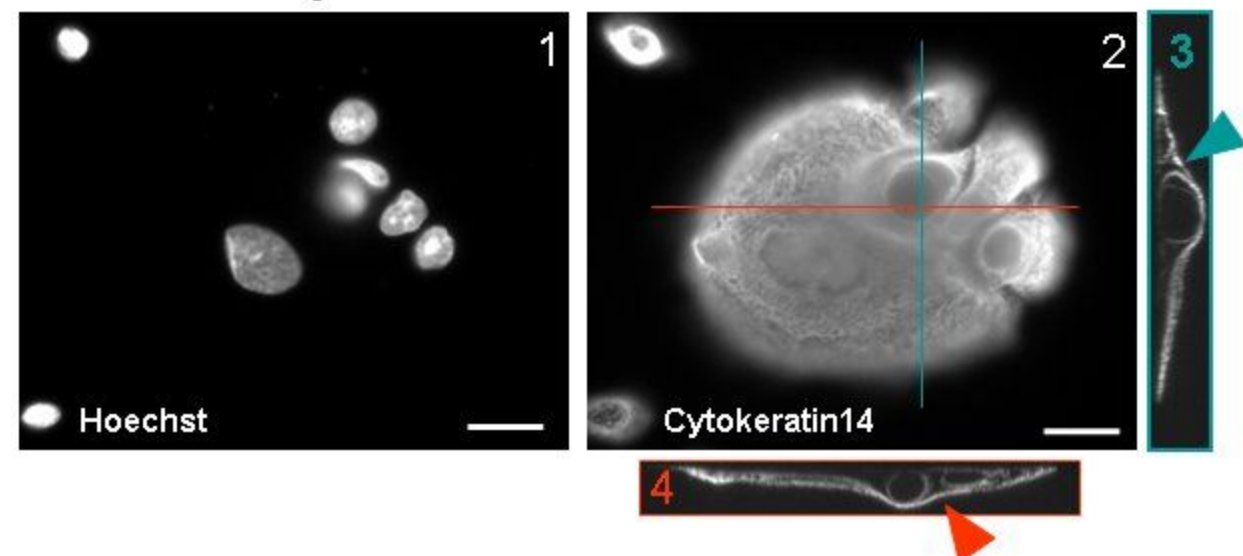
A. Trypsin-dissociated and FACS-sorted senescent cells



B. Culture on plastic

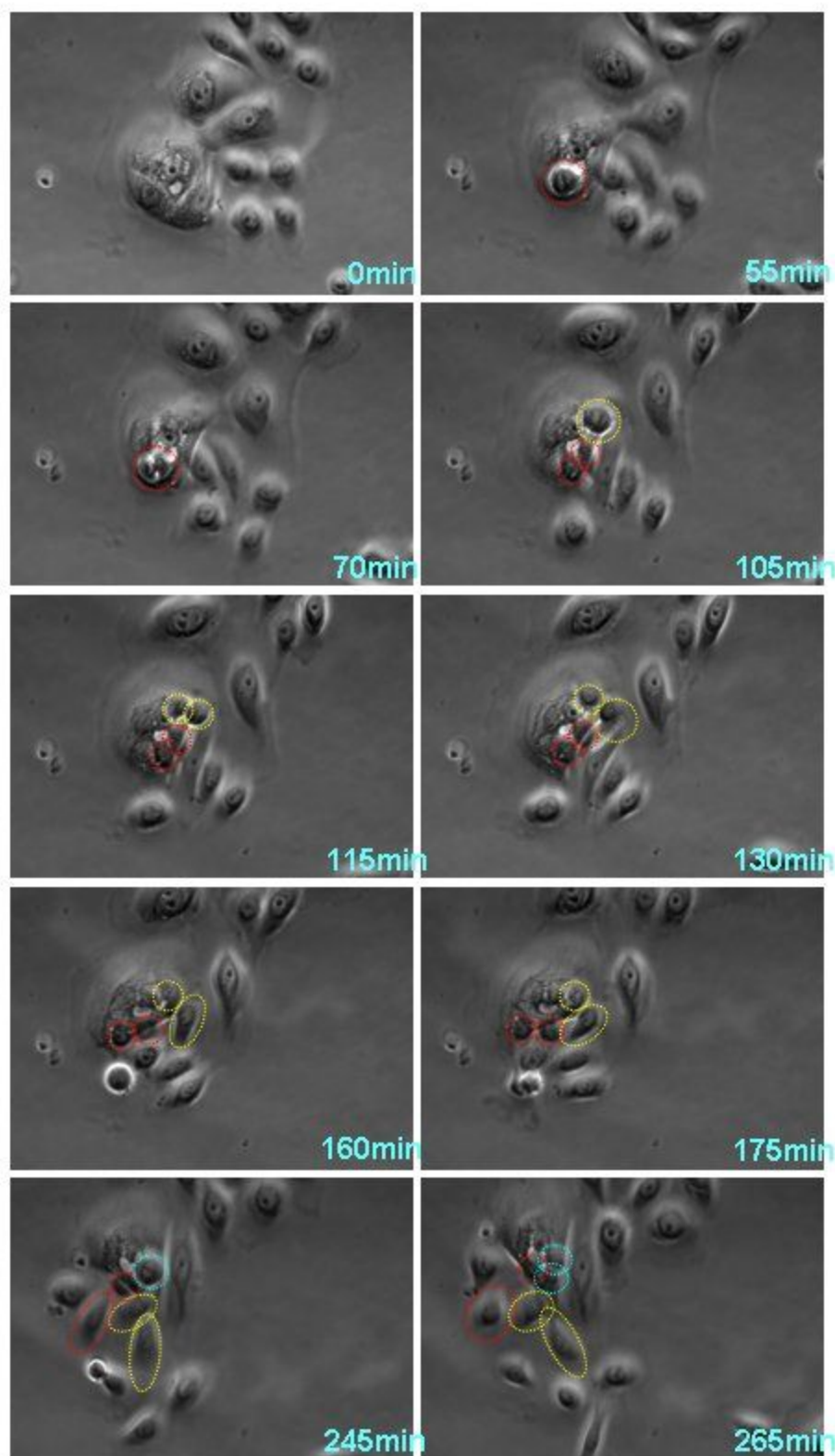


C. Confocal analysis



Supplementary Fig.7: Senescent cells produce emergent cells by budding

(A) Phase-contrast images of trypsin-dissociated, FACS-sorted senescent cells. In panel 1, one cell has an attached bud; in panels 2 and 3, one cell seems to have two attached buds; in panel 4, one cell seems to have at least 3 attached buds. Bars represent 10 μm . (B) Representative phase-contrast images of routine NHEK cultures on plastic dishes at the PS emergence stage. In panel 1, several emerging cells still seem attached by a pedicle to the senescent mother cell; in panel 2, the emerging cells seem to come from the inside of the senescent mother cell. Bars represent 30 μm . (C) NHEKs at the PS emergence stage were processed for immunofluorescence with an antibody against cytokeratin14, in order to stain the cytoskeletons of both senescent and emerging cells. Panels 1 and 2 are general views of the staining pattern observed with an epifluorescence microscope. Panels 3 and 4 are optical cross-sections viewed with a confocal microscope. In both panels; the continuity of the cytoskeleton between the large senescent cell with a large nucleus and two of the emerging cells is marked by an arrowhead. Bars represent 20 μm .



Supplementary Fig.8 : Time-lapse phase-contrast videomicroscopy of a senescent cell producing emergent cells by budding mitosis

NHEK cultures at the PS emerging stage were monitored by time-lapse phase-contrast videomicroscopy. Pictures were taken at 5-min intervals. The timing is indicated on each photograph. The large senescent cell with at least 3 visible nuclei and some vacuole-like structures undergoes three successive mitoses. The first (metaphase plate at 55 min and telophase at 70 min highlighted in red) produces one daughter cell that will individualize at 245 min and one daughter nucleus that will remain in the senescent mother cell. The second mitosis (metaphase plate highlighted in yellow) occurs at 105 min; it produces two daughter cells that will have individualized by 265 min. Note that at 130 min, a lamellipodia is observable in front of one daughter cell. The third mitosis (metaphase plate highlighted in blue) occurs at the end of the video. Note that throughout these divisions, the senescent mother cell remains spread on the dish and its central nucleus with a large nucleolus seems to remain quiescent.

Supplementary Methods

Measure of telomerase activity by Telomerase Repeat Amplification Protocol (TRAP)

Cells were collected and kept as frozen pellets. Pellets were suspended in ice-cold 1X CHAPS lysis buffer (10mM Tris-HCl, pH7.5, 1mM MgCl₂, 1mM EGTA, 0.1mM benzamidine, 5mM β-mercaptoethanol, 0.5% CHAPS, and 10% glycerol) containing RNase inhibitor at a final concentration of 150U/mL. After 30mn incubation on ice, the lysate was centrifuged at 14,000 g for 25mn at 4° C, and the supernatant was transferred into a fresh tube. Protein concentrations of the extract were measured by the Bradford method (Bio-Rad, Hercules, CA), and an aliquot of extract containing 1μg of protein was used for each TRAP assay. TRAP assays were performed using TRAPeze ELISA kit (S7750.Chemicon Int.,Temecula,CA), according to manufacturer's instructions with minor modifications. Twenty μL of reaction mixture containing 1μg of protein extract, 4μL of 5X TRAP reaction mix (Tris buffer, primers, biotinylated TS primer and RP primer, dNTPs and DNPdCTP, and oligomer mix for amplification of 36-bp internal control band), and 1 unit of amplitaqGOLD Taq DNA polymerase (Applied Biosystems Foster City, CA,), was incubated for 30mn at 30° C and subsequently subjected to tree-step PCR (after activation of the hot start Taq) at 94° C for 30 seconds, 56° C for 30 seconds and 72°C for 66 seconds for 40 cycles. Each assay design included (i) a test extract of 1μg of protein in duplicate, (ii) a heat-inactivated lysate at 85° C for 15mn before the assay, (iii) a telomerase-positive control cell extract MDA-MB 231 corresponding to a range of 125 to 31 cells, (iv) a primerdimer/PCR contamination control where 2μL of 1X CHAPS lysis buffer was substituted to the extract, and (v) the TSR8 PCR/ELISA-positive control supplied in the kit. Nonradioactive detection of the telomerase products was performed by ELISA protocol. TRAP products (fifteen percent) were immobilized onto streptavidin-coated microtiter plates and then detected by anti-DNP

antibody conjugate to horseradish peroxidase (HRP). The amount of TRAP products was determined by means of the HRP activity using 3,3',5,5'-tetramethylbenzidine substrate and subsequent color development. Absorbance was measured at 450nm and 690nm and given as $A_{450}-A_{690}$.

FACS sorting of senescent cells and staining for cell filiation tracing

Senescent cells were electrostatically sorted in air, out of a presenescent population and according to their forward and scatter factors, on a Coulter EPICS XL-MCL or a Coulter EPICS ALTRA. The 25% cells with the highest factors were collected in complete culture medium and again placed in culture. After plating, cells were incubated with 10 μ M Vybrant CFDA SE (Molecular Probes V-12883) for 15 min at 37°C or with Vybrant DiI cell-labelling solution (1/200 dilution, Molecular Probes) for 30 min at 37°C, washed, and monitored for emergence.

Transcriptomics analysis

cDNA preparation - Total RNAs were extracted using RNeasy mini-columns (QIAGEN). Double stranded cDNA was synthesised from total RNA using a SMART protocol (1). Two first strand reactions were set up starting with 500ng of total RNA. Four microlitres of each first strand reaction was used in the amplification step which was performed using 17 rounds of cycling. The ds cDNA was checked on a 1.2% agarose gel before the samples were purified using Qiagen Qiaquick clean up columns. The amount of cDNA amplified was then checked using the Nanodrop ND1000.

cDNA labelling and microarray hybridisation - Using the Bioprime labelling kits (Invitrogen), 10ul of purified cDNA was labelled, where 2 μ l of Cy5 dye (GE) was incorporated. After incubating for 3 hr at 37°C, the labels were purified using ProbeQuant

G50 micro columns (GE). Incorporation rates were determined using a Nanodrop ND1000 before specific labels were pooled and dried down to completion. The labels were re-suspended in 40µl of hybridisation buffer (40% deionised formamide; 5x Denhart's; 5x SSC; 1mM Na pyrophosphate; 50mM Tris pH 7.4; 0.1% SDS) and hybridised onto a RNG-MRC mouse set 25K microarray printed on GE Codelink slide (<http://www.mgu.har.mrc.ac.uk/facilities/microarray/rng.html>), overnight at 48°C in a water bath using the Corning hybridisation chambers. After hybridisation, the arrays were then washed initially in 2xSSC until the coverslip had come off, then 5 min with vigorous shaking in 0.1x SSC; 0.1% SDS and then finally in 0.1xSSC for 2 min with vigorous shaking. The arrays were then spun dry and scanned using a ProScanArray HT (Perkin Elmer, Beaconsfield, UK) at 7 different PMT gain settings from 40 to 70. The images were then processed using ImaGene 6.0.1 (Bio Discovery, El Segundo, CA, USA), where all 14 images were overlaid and gridded and the feature data extracted.

Analysis of microarray - The RNA data were processed using Mavi 2.6.0 (MWG Biotech AG, Ebersberg, Germany), which increases the dynamic range whilst avoiding saturation problems. The data was then loaded into R Project for Statistical Computing (<http://www.r-project.org>) for further analysis. A two dimensional loess normalisation, from the YASMA5 (Yet Another Statistical Microarray Analysis) library, was performed on each array to correct for any spatial variation within the slide. The LIMMA library (Linear Models for Microarray Analysis (2) from the BioConductor software project (<http://www.bioconductor.org>) was used to further normalise the data and to select differentially expressed genes. In brief, a linear model is fitted to the data for each gene to fully model the systematic part of the data and provide estimates for each coefficient (samples in this case). These coefficients can then be compared and differentially expressed genes selected using an empirical bayes moderated t-statistic. Differential genes were selected for the comparisons of interest based on their

moderated t-statistic (3) after using a false discovery rate control of 5 % (4). The fitted values for each sample were then converted back to red and green intensities and loaded into GeneSpring GX (Agilent technologies, Stockport, UK) to allow for easy comparison of lists of differential genes.

Telomere length determination

We used the TeloTAAAG telomere length assay kit (Roche), according to manufacturer's instructions. Briefly, genomic DNA was isolated using a standard SDS-proteinaseK protocol. Two μg of purified DNA was digested with a HinfI/RsaI enzyme mixture 2hrs at 37°C and separated by an overnight electrophoresis on a 0.8% agarose gel in TBE buffer at 5Volts/cm. The Southern capillary transfer was done using 20xSSC buffer on positively charged nylon membranes. The blot was hybridized with a digoxigenin-labeled probe specific for telomeric repeats and incubated with a digoxigenin-specific antibody coupled to alkaline phosphate. For TeloFISH, slides were prepared as for M-FISH. The hybridization mixture, containing 70% formamide, the nucleic acid probes labelled with Cy3 at 0.3 $\mu\text{g}/\mu\text{L}$ (Perceptive Biosystems, Ramsey, MN), 1% (W/V) blocking reagent (Boehringer-Mannheim, Gmbh) in 10mM Tris pH7.2, was laid down, a coverslip was added and DNA was denatured for 3min at 80°C. After 2hrs hybridization at RT, slides were washed with 70% formamide/10mM Tris pH 7.2 (2*15 min) and with 0.05M Tris 0.15M NaCl pH 7.5 containing 0.05% Tween-20 (3*5 min). Slides were then counterstained with 1 $\mu\text{g}/\text{mL}$ DAPI and mounted in antifade solution (VectaShield, Vector Laboratories Inc., Burlingame, CA).

Reverse Transcription and Quantitative-Polymerase Chain Reaction

Total RNAs were extracted using RNeasy mini-columns (QIAGEN). One μg of RNA was reverse-transcribed using random hexamers, Superscript III and dNTPs (Invitrogen) in a final

volume of 20 μ l according to the manufacturer's instructions. The quantitative PCRs were performed on an Opticon 2 Thermocycler (Bio-Rad) with the following primers : p21 (fd 5'-ATGAAATTCACCCCCTTTCC; rs 5'- CCCTAGGCTGTGCTCACTTC), p16 (fd 5'-TGCCTTTTCACTGTGTTGGA; rs 5'-GCCATTTGCTAGCAGTGTGA); PCNA (fd 5'-TCTCAGCCATATTGGAGATG ; rs 5'-CAGGTACCTCAGTGCAAAAG), ARNpol2 (fd 5'- GTGCGGCTGCTTCCATAA; rs 5'-GCACCACGTCCAATGACAT), RPL13a (fd 5'-AGCTCATGAGGCTACGGAAA; rs 5'- CTTGCTCCCAGCTTCCTATG), Actine β (fd 5'-TCCCTGGAGAAGAGCTACGA ; rs 5'- AGCACTGTGTTGGCGTACAG). PCRs were performed in duplicate on 10ng of cDNA using Invitrogen (for p16 and PCNA) and Epicentre reaction mixture with L buffer (for p21), with H buffer (for ARNpol2, RPL13a and Actine β) following the producer instructions. Cycle conditions were set as follows: 94°C for 15s and 60°C to 66°C (depending on the primers) for 1min, 40 cycles. Gene expression levels were normalized by geometric averaging of the 3 internal control genes, ARNpol2, RPL13a and Actine β , as recommended by Vandesompele et al.(5). Gene expression stability of ARNpol2, RPL13a and Actine β was validated with two softwares: geNorm(5) and NormFinder(6).

BrdU-incorporation assays

BrdU (Roche) was added to cell cultures at 10 μ M for 6hrs. Cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100, and incubated with 40U/ml DNase I (Promega) and 20U/ml Exonuclease III (Roche) for 30min at 37°C. BrdU was revealed with anti-BrdU mouse IgG (Dako) and Rhodamine Red-conjugated antimouse IgG (Jackson Immunoresearch laboratories).

Metaphase assays

Presenescent cells were plated in Lab-tek II Chamber slide (Nalge Nunc International) and let grown until senescence. Slides were incubated with Karyomax Colcemid (Invitrogen

Corporation) 1hr at 37°C, incubated in 60mM KCl solution at 37°C, fixed with 4% paraformaldehyde in PBS for 20min, and stained with 1µg/ml Hoechst 33258 for 3min.

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Genes involved in adhesion/migration up- or downregulated in emergent vs. young keratinocytes

t-test		Genbank	Name	Alternate name	Full name	Function
0.010164129	up	NM_020403	PCDH9		protocadherin 9	Localized to synaptic junctions
5.770558409	down	NM_018937	PCDHB3	PCDH-BETA3	protocadherin beta 3	Cell-cell adhesion
0.020029928	up	AK054903			moderately similar to mouse fat 1 cadherin	Protocadherin involved in polarization and directed migration
0.024951525	up	NM_018836	AJAP1	MOT8; SHREW1	adherens junction associated protein 1	Localizes to the baso-lateral membrane of polarized epithelial cells
5.728123444	down	NM_000299	PKP1		plakophilin 1 (ectodermal dysplasia/skin fragility syndrome).	Localizes to desmosomes. Links cadherin to intermediate filament. Also localizes to nuclei
7.667061664	down	NM_020435	GJC2	Cx47; GJA12; CX46.6	gap junction protein, gamma2	Component of gap junctions
5.670256384	down	NM_003829	MPDZ	MUPP1	multiple PDZ domain protein.	Associated with tight junctions
7.515596763	down	NM_002212	EIF6	EIF3A; p27BBP; ITGB4BP	translation initiation factor 6. Also integrin-beta4 subunit	Translation initiation factor. Also a component of hemidesmosomes
5.448743723	down	NM_002210	ITGAV	CD51; MSK8, VNRA	integrin, alpha V (vitronectin receptor)	Adhesion to extracellular matrix
6.265892046	down	NM_002414	CD99			Cell adhesion glycoprotein
5.90671464	down	NM_003247	THBS2	TSP2	thrombospondin 2	Cell-cell and cell-matrix interactions. Migration.
0.016774709	up	NM_005560	LAMA5		laminin alpha 5	Component of the basal membrane. Component of the anchoring complex connecting keratinocytes to the underlying dermis. Secreted by keratinocytes
6.54405465	down	NM_022763	FNDC3B	FAD104; PRO4979	fibronectin type III domain containing 3B; factor for adipocyte differentiation 104	
5.541533027	down	NM_005099	ADAMTS4	ADMP-1; ADAMTS-2; ADAMTS-4	ADAM metalloproteinase with thrombospondin type 1 motif, 4	Degradation of aggrecan and brevican, two components of the extracellular matrix
5.322172089	down	NM_015466	PTPN23	HDPTP; HD-PTP	protein tyrosine phosphatase, non-receptor type 23	Involved in trafficking of endosomes

Genes involved in cytoskeleton structure and dynamics up- or downregulated in emergent vs. young keratinocytes

t-test		Genbank	Name	Alternate name	Full name	Function
0.013092094	up	NM_001069	TUBB2A	TUBB; TUBB2	tubulin beta 2A	Component of microtubules. A nuclear form exists, that binds Notch
0.002168978	up	XM_067193			similar to microtubule-associated proteine 6	
0.00520902	up	NM_005909	MAP1B	MAP5; FUTSCH	microtubule-associated protein 1B	Microtubule assembly
0.013093579	up	NM_002475	MYL6B	MLC1SA	myosin, light chain 6B, alkali, smooth muscle and non-muscle	ATPase motor protein
8.832965442	down	NM_024430	PSTPIP2	MAYP	proline-serine-threonine phosphatase interacting protein 2	PCH family. Cytoskeletal-associated protein. Regulates F-actin bundling. Enhances filopodia and motility
7.381029035	down	NM_004411	DYNC111	DNC11, DNCIC1	dynein, cytoplasmic 1, intermediate chain 1	Binds to dynein

Genes involved in senescence/oxidative stress/DNA damage up- or downregulated in emergent vs. young keratinocytes

t-test		Genbank	Name	Alternate name	Full name	Function
0.006297251	up	NM_016258	YTHDF2	HGRG8, NY-REN-2	YTH domain family, member 2; high glucose regulated protein 8	

6.538781448	down	NM_006936	SUMO3		SMT3 suppressor of mif two 3 homolog 3	SUMO2/3 conjugate with high molecular proteins following stress.
0.020843235	up	NM_012331	MSRA		methionine sulfoxide reductase A	Reduction of methionine sulfoxide to methionine. Repair of oxidative damage to proteins
0.024998491	up	NM_175614	NDUFA11	B14.7	NADH dehydrogenase ubiquinone 1 alpha subcomplex 11	complex I of the respiratory chain
0.007309126	up	NM_003298	NR2C2	TR4; TAK1; TR2R1	nuclear receptor subfamily 2, group C, member 2	Orphan nuclear receptor. Stimulated by oxidative stress through FOXO3a. Apoptosis modulator via Bcl2 regulation
0.000522895	up	NM_002833	PTPN9	MEG2; PTPMEG2	protein tyrosine phosphatase, non-receptor type 9	Protein tyrosine phosphatase family (PTP). Role in secretory pathway. Modulator of insulin-dependent FOXO1 nuclear exclusion
0.008673079	up	NM_031296	RAB33B		RAB33B, member of RAS oncogen family	GTPase, retrograde traffic between Golgi and ER. Golgi resident. Interacts with Atg16L and modulates autophagosome formation
0.011705083	up	NM_014664	N4BP1		Nedd4 binding protein 1	Nedd4: E3 ubiquitine ligase. Also binds and inhibit Itch, a Nedd4 related E3. Nedd4 are involved in lysosomal and proteasomal degradation. Itch controls the stability of p63 and p73
8.169165982	down	NM_000122	ERCC3	XPB; BTF2; GTF2H, RAD25; TFIIH	excision-repair cross-complementing rodent repair deficiency, complementation group 3 (xeroderma pigmentosum group B complementing)	ATP-dependent helicase that functions in nucleotide excision repair. Also the 89 kda subunit of TFIIH

Genes involved in cell cycle progression or cell death up- or downregulated in emergent vs. young keratinocyte

t-test		Genbank	Name	Alternate name	Full name	Function
6.747502842	down	NM_014456	PDCD4		programmed cell death 4 -neoplastic transformation inhibitor)	Localized to the nucleus in proliferating cells. Role in apoptosis but not defined
0.003394879	up	NM_005134	PPP4R1	PP4R1; PP4(Rmeg)	protein phosphatase 4, regulatory subunit 1	Phosphatase whose substrates are: HDAC3, NFkB p65. Pro-apoptotic. DNA damage response. Centrosome maturation
0.009699459	up	NM_001353	AKR1C1	C9; DD1; DDH; H-37; MBAB; HAKCR; 2-ALPHA-HDS; 20-ALPHA HDS	aldo-keto reductase family 1, member C1; dihydrodiol dehydrogenase 1, 20-alpha (3-alpha)-hydroxysteroid dehydrogenase	Degrades progesterone
0.007374601	up	NM_001952	E2F6		E2F6	Transcription factor of the E2F family. Repressor? Anti-apoptotic via BRCA1 inhibition, counteraction on E2F1 activity
5.262452266	down	NM_152431	PIWIL4		piwi-like 4.	Belongs to argonaute family. Involved in chromatin remodelling by inducing histone methylation. Induces methylation at the p16 locus
0.018466307	up	NM_006341	MAD2L2	REV7; MAD2B	MAD2 mitotic arrest deficient-like 2	Component of the mitotic spindle assembly chekpoint that prevents anaphase
0.019704145	up	NM_001201	BMP3	BMP-3A	bone morphogenetic protein 3	Structurally divergent member of BMP family. Limits BMP and TGFbeta1 signalling
12.61036282	down	NM_014184	CNIH4	HSPC163	cornichon homolog 4	Transmembrane protein. Involved in TGFbeta/EGF secretion
0.020256547	up	BC027302	FLT4	PCL; VEGFR3	fms-related tyrosine kinase 4	VEGF C and D receptor. Involved in lymphangiogenesis and maintenance of the lymphatic endothelium

Genes involved in cancer up- or downregulated in emergent vs. young keratinocytes

t-test		Genbank	Name	Alternate name	Full name	Function
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0.002253894	up	NM_006279	ST3GAL3	ST3N; SIAT6; ST3GALIII	beta-galactoside alpha-2,3-sialyltransferase 3	Type II membrane protein that catalyzes the transfer of sialic acid to galactose-containing substrates. Localizes to Golgi.
0.008105121	up	NM_000782	CYP24A1	CP24; CYP24	cytochrome p450, family 24, subfamily A, polypeptide 1	Monoxygenase which catalyzes the degradation of 1,25-dihydroxyvitamin D3, the active form of vitamin D3. Localizes to mitochondria
5.311795768	down	S69025	HOX A1		homeobox A1	Transcription factor
5.385202954	down	NM_021220	OVOL2	movol2; movo2	ovo-like 2	Zinc-finger transcription factor. Role in epithelial and germ cell development by modulating the balance between proliferation and differentiation of progenitor cells
0.01994811	up	NM_003173	SUV39H1	MG44; KMT1A; SUV39H1	suppressor of variegation 3-9 homolog 1	Histone methyl transferase with a chromodomain and a SET domain. Moves to centromeres during mitosis. Required for pericentric heterochromatin organization, chromosome segregation, mitotic progression
0.019471283	up	NM_020892	DTX2	RNF58	deltex homolog 2	E3 ubiquitin ligase. Notch signalling
0.018608707	up	NM_002516	NOVA2	ANOVA; NOVA3	neuro-oncological ventral antigen 2	RNA-binding protein. Splicing factor. Expression restricted to astrocytes.
0.02136094	up	NM_013956	NRG1	GGF; HGL; HRG; NDF; ARIA; GGF2; HRG1; HRGA; SMDF	neuregulin1	ErbB ligand. Involved in growth and differentiation of several cell types, including epithelial cells
10.26533153	down	NM_005423	TFF2	SP; SML1	trefoil factor 2/spamolytic protein1	Stable secreted protein with a trefoil motif. May protect mucosa
9.639400736	down	NM_030774	OR51E2	PSGR; PSGR2; OR52A2; OR51E3P	olfactory receptor, family 51, subfamily E, member 2	prostate-specific G protein-coupled receptor

	Number of wells with one cell	Number of wells with emergence	% emergence
Experiment 1	42	31	73.8
Experiment 2	117	91	77

Table 1: Almost all young NHEKs have an emergence potential

Young NHEKs were diluted to one cell per well and cultured to senescence and emergence. The numbers of cultures performed and the percentages of cultures having developed emerging clones by the end of the experiment are given.

2. Impact du sécrétome des fibroblastes sénescents sur l'initiation cancéreuse des NHEK

Les travaux antérieurs réalisés par l'équipe ont pu démontrer que des cellules néoplasiques émergent à partir de l'état de sénescence des kératinocytes. Les fibroblastes de derme sont des partenaires privilégiés pour contrôler les activités biologiques des kératinocytes par l'intermédiaire des facteurs solubles qu'ils sécrètent. En culture la croissance des fibroblastes primaires de derme humains (NHDF) est radicalement différente des kératinocytes. En effet les NHDF effectuent 50 à 60 doublements de population (selon les donneurs) pendant une longue phase de croissance (4 mois au minimum) avant d'entrer au plateau de sénescence. Le blocage du cycle cellulaire, principalement causé par le raccourcissement télomérique détecté comme des cassures de l'ADN double brin, est irréversible. En effet, les NHDF sénescents peuvent être maintenus en culture pendant plus de deux ans sans qu'il y ait d'émergence de cellules transformées. Bien que bloqués dans leur prolifération, les NHDF sénescents demeurent néanmoins métaboliquement actifs et sécrètent des facteurs solubles pouvant contribuer à la progression tumorale. Dans d'autres modèles que la peau (prostate, sein...), il a été montré que des fibroblastes sénescents peuvent stimuler la croissance et l'invasion de cellules cancéreuses via un sécrétome inflammatoire.

Le risque de développer des carcinomes augmente fortement avec l'âge et il est probable que le micro-environnement vieillissant participe aux étapes les plus initiales de la transformation de cellules épithéliales. Pourtant très peu de données existent sur ces mécanismes faute de modèles cellulaires et moléculaires d'initiation cancéreuse. Nous avons émis l'hypothèse que les NHDF sénescents qui s'accumulent dans le derme pourraient contribuer à l'initiation tumorale des kératinocytes de peau.

Pour initier cette étude, nous avons remplacé 10% de milieu de culture des kératinocytes (KGM) par du milieu conditionné de fibroblastes jeunes (YF-CM) ou sénescents (SF-CM), ou par du milieu de culture des fibroblastes (FGM), comme contrôle (figure 20). Nous avons choisi de ne remplacer que 10% du KGM car les kératinocytes sont très sensibles aux fortes concentrations de calcium contenues dans le milieu de culture des fibroblastes (FGM). Nous avons débuté le conditionnement des NHEK dès le stade de croissance pré-sénescence, ce qui nous a permis de voir les effets du sécrétome de fibroblastes sénescents sur la survenue du plateau de sénescence puis sur l'émergence. Comme décrit dans l'*article n°1*, les kératinocytes cultivés dans leur milieu

classique (KGM) atteignent rapidement le plateau de sénescence puis après une semaine d'arrêt de croissance, de petites cellules émergentes post-sénescence (PSE) apparaissent à une faible fréquence et effectuent quelques doublements de population supplémentaires. Ces cellules atteignent alors un second plateau de sénescence, et meurent rapidement probablement par autophagie comme cela a été démontré pour les cellules au premier plateau de sénescence.

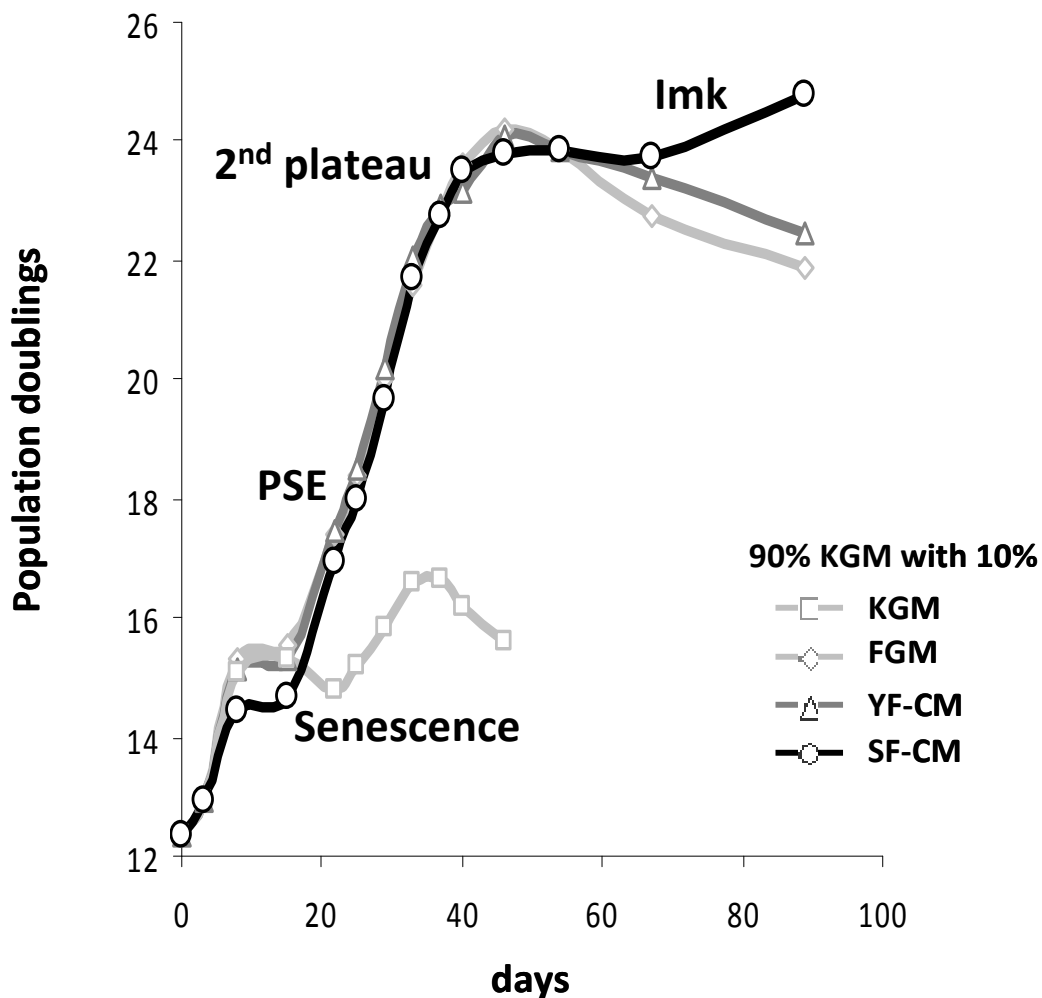


Figure 20 : Courbes de croissance des kératinocytes primaires de peau humains (NHEK) cultivés dans 90% de KGM (Keratinocytes Growth Medium) + soit 10% de KGM ; soit 10% de FGM (Fibroblasts Growth Medium) ; soit 10% de milieu conditionné de fibroblastes jeunes (YF-CM) ou sénescents (SF-CM).

Le remplacement de 10% de KGM par du FGM, du YF-CM ou du SF-CM provoque tout d'abord une sénescence prématurée. Cet effet, généralement plus marqué par le conditionnement du SF-CM, est induit par une augmentation des ROS dans les NHEK (comme nous avons pu l'analyser par FACS après avoir marqué les cellules au DCFA qui est fluorescent en présence de ROS). Puis, alors que les kératinocytes cultivés en KGM émergent uniquement sous forme de clones épithélioïdes, le conditionnement par le FGM, le YF-CM et le SF-CM peut permettre une émergence des kératinocytes sous forme de clones fibroblastoïdes. Comme cela est décrit dans l'**article n°2**, le SF-CM favorise particulièrement la fréquence d'émergence de clones fibroblastoïdes. Néanmoins après quelques passages en culture, les kératinocytes-PSE fibroblastoïdes sont sélectionnés pour former une population homogène. La prolifération de ces kératinocytes-PSE conditionnés par le FGM, le YF-CM et le SF-CM est fortement stimulée par rapport au contrôle KGM (figure 20).

En se basant sur les courbes de croissance des populations globales, des effets marqués du milieu conditionné de fibroblastes sénescents (SF-CM) interviennent sur les étapes de second plateau de sénescence et de deuxième émergence des kératinocytes (ImK). En effet le SF-CM **(i)** provoque une anticipation de la survenue du second plateau de sénescence des kératinocytes par rapport aux conditions FGM et YF-CM, **(ii)** favorise l'apparition d'une seconde émergence de cellules ImK, **(iii)** et stimule la prolifération de ces cellules Imk (figure 20). Les études réalisées dans l'article n°1 montrent que les cellules ImK présentent des caractères très transformées.

Bien que les courbes de croissance montrent peu de différences sur la prolifération des kératinocytes-PSE entre les différents conditionnements (FGM, YF-CM et SF-CM), une analyse phénotypique et moléculaire plus précise révèle que les fibroblastes sénescents renforcent la transformation des kératinocytes dès l'initiation de l'émergence post-sénescence.

Ces analyses font l'objet de l'**article n°2** actuellement en soumission dans Cancer Research : ***Senescent fibroblasts improve the early skin carcinogenesis via the paracrine MMP/PAR-1 axis***

Article n°2

*Senescent fibroblasts improve the early skin
carcinogenesis via the paracrine*

MMP/PAR-1 axis

Senescent fibroblasts improve early skin carcinogenesis

via the paracrine MMP-PAR-1 axis.

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Running title: MMP-PAR-1 axis in senescence-promoted early events of carcinogenesis.

Keywords: Senescence / Stroma / Epithelial-Mesenchymal Transition / MMP-PAR-1 / Cell migration

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ABSTRACT

The incidence of carcinoma greatly increases along ageing, but the cellular and molecular mechanisms underlying this correlation are only partly known. While senescent fibroblasts were shown to promote the malignant progression of already transformed cells, we investigated here the impact of senescent fibroblasts secretome on the very initial stages of transformation. We used an in vitro culture model of primary human epidermal keratinocytes displaying the emergence of cells with transformed and tumorigenic properties from the senescence plateau. We show in this model that the secretome of autologous senescent dermal fibroblasts exacerbated the epithelial-mesenchymal transition and migratory properties of these post-senescent neoplastic keratinocytes.

Using in vitro co-cultures of primary keratinocytes in presence of fibroblast-conditioned media, pharmacological inhibitors, siRNA transfections, blocking antibodies, immunocytofluorescence, in gel and in situ zymography as well as migration assays, we demonstrate that MMP-1 and MMP-2, two matrix metalloproteinases known to participate in late stages of cancer invasion and metastasis, are responsible for this increase in early carcinogenic characteristics of post-senescence emergent keratinocytes. In addition, we show that MMP act through the activation of the protease-activated thrombin receptor, PAR-1, whose expression is specifically increased in post-senescence emergent cells. The relevance of the involvement of the MMP-PAR-1 axis was demonstrated in biopsies from ageing skins. Our results suggest that the MMP-PAR-1 axis could participate in the initiation of non-melanoma skin cancers through the co-activation of both the dermal (stromal) and epidermal components of aged skin, as could also happen for other organs.

INTRODUCTION

Carcinomas are the most frequent human cancers. Their incidence is strongly correlated with advancing age amongst carcinomas (NCI and WHO data).

Changes in the ageing stroma are thought to contribute to increased carcinoma incidence among older adult age groups. Indeed, senescent fibroblasts are found in human tumour biopsies and share effects in stimulating growth and invasion of already transformed cells (1-5). For instance, senescent prostatic and lung fibroblasts were shown to overexpress several factors that could promote cancer (1) setting a complex (mainly inflammatory) senescence-associated secretory phenotype (SASP) (6, 7). However, whether an adult ageing stroma could contribute to the earliest stages of carcinogenesis, hence explaining why the incidence of carcinoma increases with age, was never investigated.

We investigated here the role of the ageing stroma in enhancing the first stages of carcinogenesis in the skin. Indeed, non-melanoma skin carcinomas are the commonest, and their incidence in developed countries is on the increase in association with rising life expectancy (8). They are the cause of considerable morbidity, notably squamous cell carcinoma (9). In addition, their study could highlight general features of initial mechanisms of carcinogenesis in association with ageing as they are associated to increased risk of developing other primary carcinoma (10).

Senescent cells appear in both the dermal and epidermal compartments along normal ageing skin (11, 12). Interestingly dermal and epidermal cells behave differently when in primary cultures. Indeed, we have precedently described (13) that as for other types of human fibroblasts, dermal fibroblasts enter a replicative senescence growth plateau (14) (15) after 50-60 population doublings, which appears irreversible. In contrast, we also showed that proliferating normal primary epidermal keratinocytes (NHEKs) accumulate oxydative stress and rapidly reach the senescence growth plateau. While incremental oxidative damage results in the death of most NHEKs by autophagy (16) in an *in vitro* primary culture, a fraction of the cell population (10^{-2} - 10^{-4}) emerges from this senescence plateau and grows as neoplastic cells. Post-senescence emerging keratinocytes (PSE-NHEK) display a modified transcriptome (17) indicating spontaneous cancer

initiation. Such post-senescence emergence was also shown from mammary epithelial cells (18). We showed that xenografts of in *nude* mice can form disseminated skin hyperplasias and carcinomas when animals had aged (17). Therefore this *in vitro* emergence of neoplastic cells from the senescence growth plateau of normal primary keratinocytes provides a suitable model to test the hypothesis according to which skin senescence generates a microenvironment promoting the inception of carcinogenesis from PSE keratinocytes. We unprecedentedly show that long-lasting culture of NHEKs in the presence of factors secreted by autologous NHDFs specifically enhances the epithelial-mesenchymal transition (EMT) of PSE-NHEKs. High levels of active matrix metalloproteinases (MMP-1; -2; -3 and -9) were specifically recorded in the secretome of senescent NHDFs. By pharmacological and genetic manipulations we demonstrate that MMP-1 and MMP-2 specifically promote the early migratory capacity of PSE-NHEKs, by activating the protease-activated receptor-1 (thrombin receptor, PAR-1), up to now known to be involved in more advanced stages of cancer aggressiveness. MMP activity and the MMP-PAR-1 axis could thus be crucial age-related mediators of early cooperative switches in the emergence and aggressiveness of carcinomas.

MATERIALS AND METHODS

Cell culture

Autologous (donor 2F1958) sets of normal primary human epithelial keratinocytes (NHEKs, CC-2501) and dermal fibroblasts (NHDFs, CC-2511) were from Clonetics. NHEKs were grown at 37°C in 5% CO₂ in KGM-2 BulletKit medium® (CC-3107 Lonza, herein called KGM) as already published (17). NHDFs were grown in FGM-2 BulletKit medium®, herein designated FGM (CC-3132, Clonetics) (19). Senescence was monitored as described (see supplementary methods) (14, 17)

Cultures in presence of conditioned medium

To investigate the impact of fibroblast secretions on NHEKs, these latter were grown in 90% KGM + 10% of either fresh FGM or conditioned medium (CM) prepared from 300,000 young or senescent fibroblasts in 6 ml FGM for 3 days (respectively denominated YF-CM and SF-CM). The supplemented KGM (FGM- or CM-supplemented KGM) thus contained 0.2% of serum, a level low enough to avoid keratinocyte differentiation. Media were renewed every 2 days.

Western blotting

Cells were lysed on ice in loading Laemli buffer and such total cellular extracts were boiled for 5 min. All extracts were from the same number of cells and loadings of relative protein contents were compared to GAPDH levels. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Hybond-C Extra, Amersham). Primary antibodies were: rabbit anti-human E-cadherin (Santa Cruz), mouse anti-human vimentin (Santa Cruz), mouse anti-human twist1 (Ab Cam), mouse anti-human PAR-1 (ATAP-2) (Santa Cruz), and mouse anti-rabbit GAPDH (Santa Cruz). Secondary antibodies were peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Peroxidase activity was revealed with the ECL (enhanced chemiluminescence) kit from Amersham Biosciences.

In-gel Zymography

MMP secretion and activation were assessed on the basis of gelatinolytic activity. SDS-PAGE of fibroblast-conditioned media was performed as described (20) under non-reducing conditions in 10% polyacrylamide gels containing 0.1% gelatin (or 0.25% casein for detection of MMP-3 activity). In such artificial conditions both active and latent proteinase forms are observable. White bands indicate digestion of the substrate.

In situ zymography

Paraffin-embedded sections of skin biopsies from young (ages 26-38 years) or old (60-89 years) human healthy donors were deparaffinized in toluene and rehydrated in graded alcohols. In situ zymography was adapted from (21). Details may be found in supplementary methods.

Immunofluorescence analyses

PSE-NHEKs were seeded on coverglasses and fixed with 2% paraformaldehyde in PBS. Antigen retrieval was performed using a microwave oven. After blocking (2% BSA in PBS), coverglasses were incubated with mouse anti-human PAR-1 primary antibody (ATAP-2) (Santa Cruz Biotechnology). Alternatively, buffer was used instead of primary antibody as control. Rhodamine Red-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories) was used as secondary antibody. Nuclei were stained with Hoechst 33258 (Sigma-Aldrich) at 1 µg/ml for 5 min, and coverglasses mounted in Glycergel® mounting medium (Dako). Image captures are detailed in supplementary methods.

Quantitative reverse-transcription and real-time PCR.

RNA was isolated from cultured cells with the Nucleospin® kit (Macherey-Nagel). The primers used are given in supplementary Table 1. The PCR protocol was that recommended for the Mx3005P Real-time PCR System® (Stratagene). Technical details are provided in supplementary methods.

MMP-1, MMP-2 and PAR-1 knockdown by RNA interference

Twenty nM of pools of 4 siRNAs were used for inhibition of MMP-1, MMP-2 in senescent NHDFs or of PAR-1 in PSE-NHEKs (on-target plus SMARTpool®, L-005951-00-0005, L-005959-00-0005 and L-005094-00-0005 respectively, Dharmacon). Specific and control transfections (siGENOME RISC-Free

Control siRNA, Dharmacon) were prepared as recommended by the suppliers and performed with Lipofectamine™ RNAiMAX transfection reagent (Invitrogen) in opti-DMEM (Gibco). After 6-h incubation at 37°C, the transfection medium was replaced with the culture media. When necessary, conditioned media or protein extracts were collected after 72 h and RNA isolated with the Nucleospin® kit (Macherey-Nagel).

Migration assays

PSE-NHEKs cultured with or without added CM were starved in KBM (i.e. basal medium without any supplement). When necessary, cells were pre-treated with PAR-1 blocking antibodies or alternatively transfected by control or PAR-1 specific siRNAs. Cells in KBM were then seeded onto Transwell® cell culture inserts with 8-µm pore size (Falcon). Various culture media of interest were placed in the lower chambers as chemo-attractants. After 30 hours of incubation at 37°C, migration was analysed on each filter using fluorescence imaging (details in supplementary methods).

Activation of PAR-1 on NHEKs

PSE-NHEKs were starved in KBM and subsequently re-stimulated for 5 to 60 minutes with various culture media or alternatively with activated recombinant MMP-1 or MMP-2 (50 nM) (see supplementary methods for activation protocol). Cells were then lysed for western blotting analysis. Alternatively, the activation protocol was used prior to immunofluorescence staining of PAR-1 within cells.

MMP activation

Recombinant pro-MMP-1 and pro-MMP-2 (R&D systems) were activated by incubation with 1 mM 4-aminophenylmercuric acetate (APMA, Sigma) in assay buffer (50 mM Tris pH 7.4, 10 mM CaCl₂, 150 mM NaCl) at 37°C for 2 hours. Diluted solutions of activated MMPs (1 ng/µl) were then stored at -80°C.

Immuno-histo-detection of PAR-1

Deparaffinized and rehydrated sections of skin biopsies from young (ages 26-38 years) or old (60-89 years) human healthy donors were treated as specified in supplementary methods. Briefly, slices were incubated with primary (Monoclonal mouse anti-Par-1, Santa Cruz Biotechnology, 1/100) and secondary (Biotinylated

rabbit anti-mouse, Dako, 1/100) antibodies in conditions promoting specific staining through inhibition of endogenous biotin and peroxydases. Control was isotype-specific primary antibody (Santa Cruz Biotechnology). Detection was amplified using streptavidin-peroxydase (Jackson ImmunoResearch).

Repetitions and statistical analyses

Precisions regarding reproducibility and repetitions of the different results are presented in supplementary methods. Statistical analyses were done using the Student-t test. (**: $p < 0.01$, *: $p < 0.05$).

RESULTS

Conditioned medium from senescent fibroblasts promotes PSE-keratinocyte early transformation and migration capacity in vitro.

To test if secreted factors from senescent skin fibroblasts might promote spontaneous transformation of autologous NHEKs, we replaced 10% of these latter usual medium (KGM) with either fresh fibroblast culture medium (FGM), young fibroblast-conditioned medium (YF-CM), or senescent fibroblast-conditioned medium (SF-CM). As previously described (17), a fraction of NHEKs emerges in control medium by a peculiar budding mitosis mechanism as small, transformed PSE-NHEK. Replacing 10% of the regular KGM culture medium by FGM, YF-CM or SF-CM slightly accelerates the occurrence of the senescence and strongly promoted PSE-NHEK growth (population doublings) (figure 1a and supplementary figure 1a). Addition of SF-CM specifically stimulated the total number of emerging PSE-NHEK clones within the cultures. Moreover, PSE-NHEK clones induced in presence of SF-CM were exclusively of fibroblastoid shape, on the contrary to other conditions that rather displayed also PSE-NHEK with epithelioid shapes (figure 1b). This shift of the morphology of PSE-NHEK towards a fibroblastoid morphology could suggest a reinforcement of the transformed state by an epithelial-to-mesenchymal transition mechanism (EMT). Western blotting analysis of the expression of EMT markers showed dramatically reduced E-cadherin and promoted vimentin expression levels in the populations of PSE-NHEKs cultivated with SF-CM (figure 1c) while such expressions were not affected in young keratinocytes (i.e. at the beginning of the co-culture) (supplementary figure 1b). Twist-1, a transcriptional factor associated to EMT and migration was also overexpressed (figure 1c). Acquisition of migratory ability being among the early manifestations of cancer initiation (22), we investigated whether a senescent microenvironment could render PSE-NHEKs capable of migration. In Transwell® assays, PSE-NHEKs cultivated in presence of SF-CM were particularly sensitized to migrate compared to cells arising in other culture conditions and SF-CM was the most efficient promoter of this migration (figure 1d). In comparison, young NHEK do not display any migratory ability regardless the original culture condition (supplementary figure 1c).

Thus, conditioning by senescent fibroblasts secretions increases the post-senescence emergence frequency and reinforces the neoplastic properties of the newly emerged cells particularly their epithelium-to-mesenchyme transition and migratory capacities.

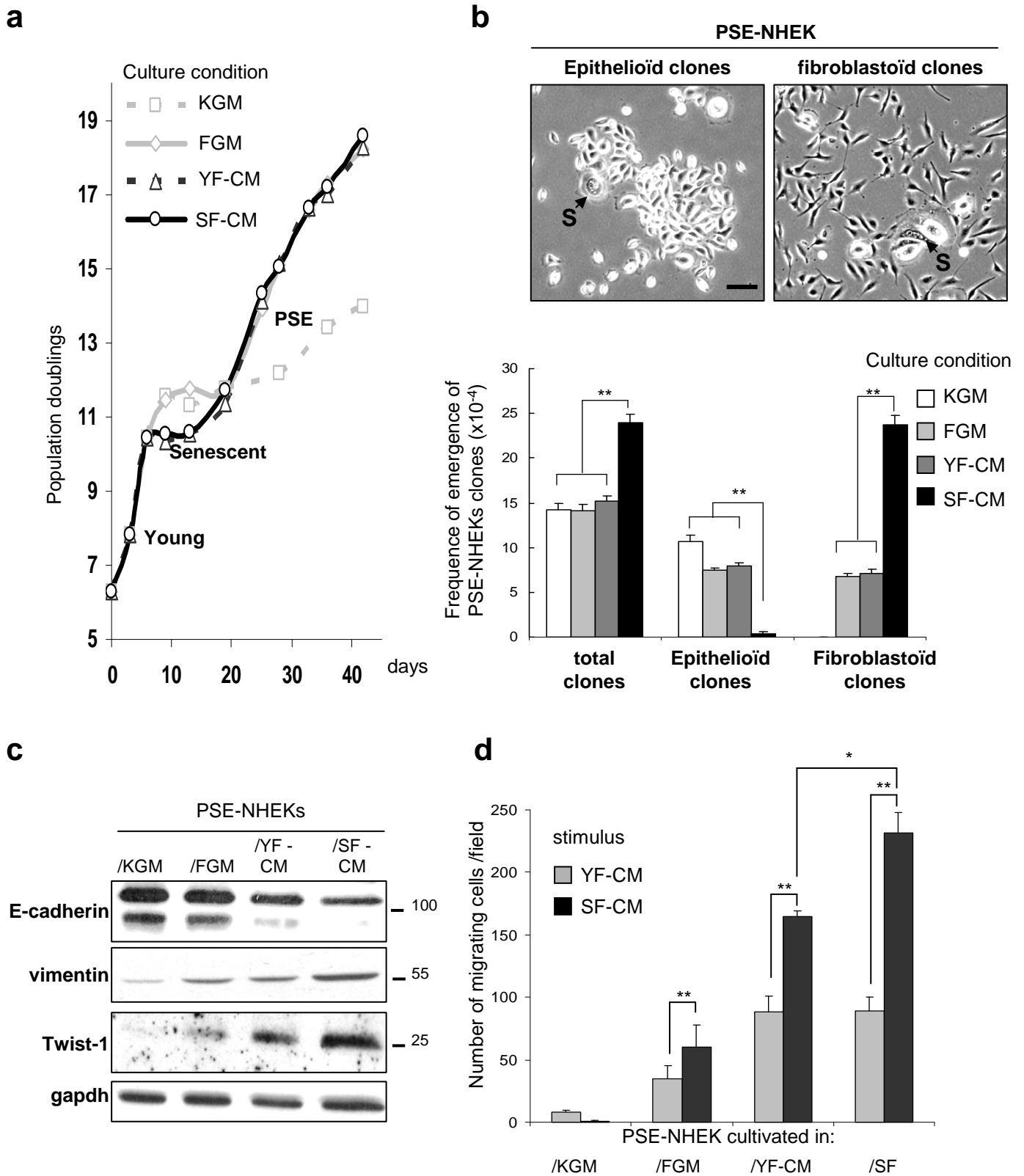


Figure 1

FIGURE LEGENDS

Figure 1: Influence of senescent fibroblast secretions on keratinocyte growth, transformation, and migration.

a. Growth curves (population doublings) of NHEKs cultured in control medium (KGM) or supplemented medium ((90% KGM + 10% fresh fibroblast growth medium (FGM), or young fibroblast-conditioned medium (YF-CM), or senescent fibroblast-conditioned medium (SF-CM)). The senescence growth plateau and post-senescence emergence (PSE) are indicated. Similar results were obtained with cells from other donors. **b.** Micrographs of cell cultures on day 20, depicting the epithelioid or fibroblastoid shape of PSE-NHEKs arising within the different culture media. Senescent cells are spotted by the letter S. Bar = 100 μ m. Graph displaying the respective number of total, epithelial or fibroblastoid PSE-NHEK clones counted by microscopic analysis in the different culture conditions. Each condition in triplicate. **c.** Western blotting experiment showing the expression of EMT markers (E-cadherin loss, and increase of vimentin and Twist-1 expressions) by PSE-NHEKs depending on their culture conditions. **d.** Migratory capacities of starved PSE-NHEKs from different culture conditions and stimulated to migrate by YF-CM- or SF-CM-supplemented KBM. Results (n=30) are means \pm SD.

MMPs secreted by senescent skin fibroblasts stimulate migration of PSE-NHEKs.

Searching for which component of the senescent fibroblast secretome was responsible for the effects described above, we focused our search on MMPs. Indeed, while they were already known as major actors of cancer progression and aggressiveness, they also had been described as major components of the secretome of senescent prostate fibroblasts (1). Also less known, MMPs were also involved in early stages of tumorigenesis (23). By RT-qPCR and western blotting of cell lysates, we recorded here critically enhanced metalloproteinase expression in senescent dermal fibroblasts (supplementary figure 2a and b). When conditioned media were analysed by in-gel zymography, remarkable increase in proteolytic activity in SF-CM was also recorded due to enhanced secretion and activation of MMP-1 and MMP-2. We also detected an increase in activation but not secretion of MMP-3 and MMP-9 in these same media (figure 2a). To assay the in vivo relevance of these results, we performed in situ fluorescent zymography. We observed an increase in gelatinolytic activity in skin samples from old versus young donors. Very interestingly, this activity was observed in the dermal compartment close to the basal lamina, i.e. the area the most proximal to epidermis (figure 2b).

In order to determine whether MMPs could participate in the stimulation of PSE-NHEK migration by senescent fibroblast-conditioned medium, MMP activities were reduced in fibroblast-conditioned media by pre-incubation with the broad-spectrum MMP inhibitor GM6001 and the media used as attractant in transwell® migration assays (figure 2c). Only the reduction of proteinolytic activities in SF-CM reduced the migratory response of PSE-NHEK/SF cells to SF-CM-supplemented KBM (Figure 2c). To confirm this result, and to determine which MMP could mediate the effect, we knocked-down MMP-1 and MMP-2 expressions by siRNAs in senescent fibroblasts. We verified that MMP expressions and proteinolytic activities were decreased in the conditioned media (supplementary figure 2d and e) that were harvested to challenge them in migration assays. Both activated recombinant MMP-1 and MMP-2 were able to confer a full migratory phenotype to PSE-NHEK/SF cells when used as sole stimulus (figure 2d). The strong stimulation of PSE-NHEK/SF migration by SF-CM medium was totally abolished when either MMP-1 or MMP-2 was absent (figure 2e).

We thus demonstrate that activated MMP-1 and MMP-2 produced by senescent fibroblasts are necessary and sufficient for the activation of migratory capacity of cancer-initiated PSE-NHEKs arisen in the presence of senescent fibroblast secretions.

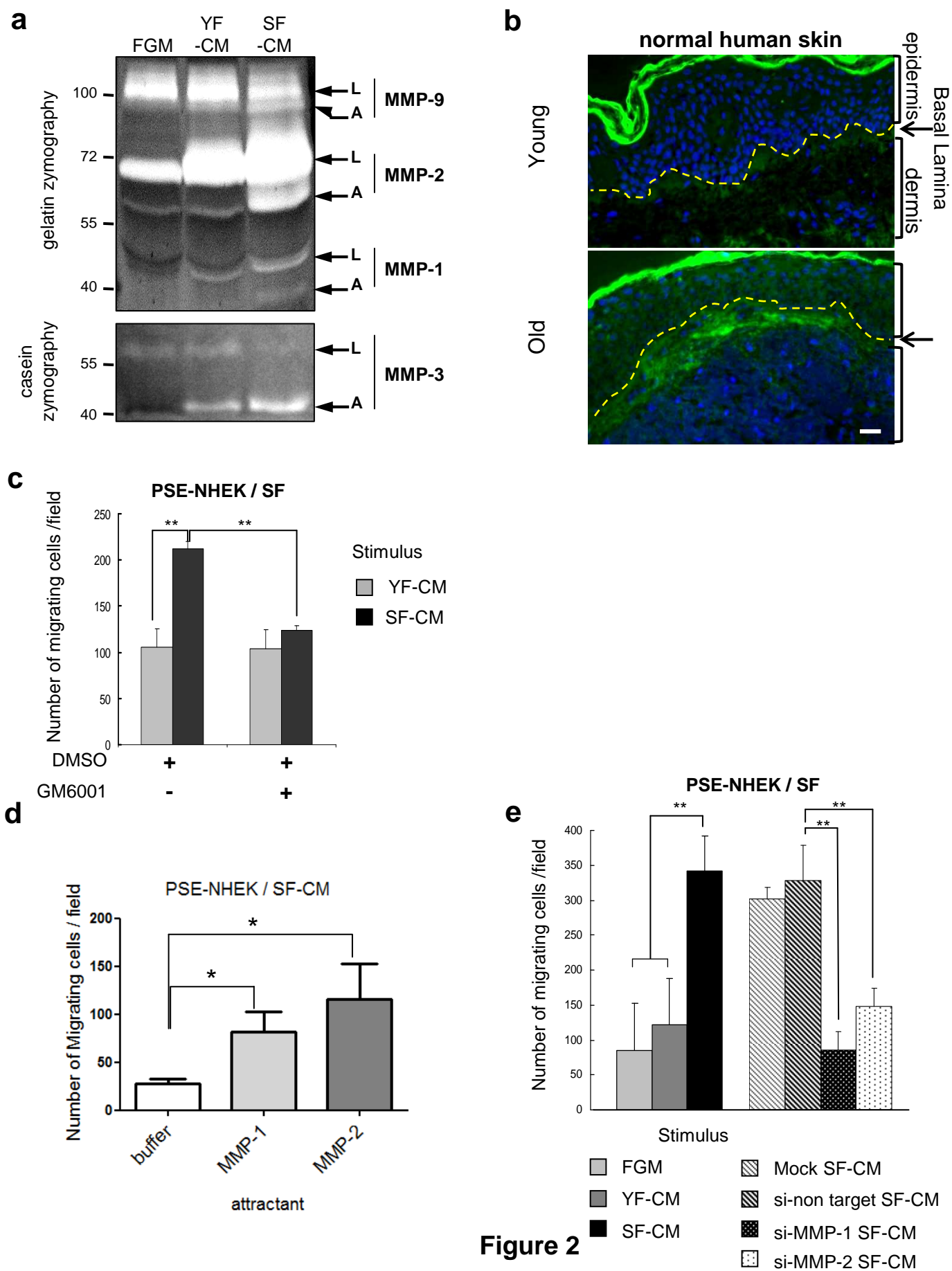


Figure 2

FIGURE LEGENDS

Figure 2: Manipulating MMP-1 and -2 synthesis and activity influences the migration of PSE keratinocytes exposed to senescent fibroblast secretions.

a. MMPs in young (YFCM) and senescent (SF-CM) fibroblast-conditioned media were analysed by gelatin (MMP-1, -2, -9) and casein zymography (MMP-3). Fresh FGM was used as a control. Secretion and activation of MMP-1 and MMP-2 are clearly enhanced in senescent fibroblast-CM. Latent forms of MMP-9 and MMP-3 present in the culture medium were activated by senescent fibroblasts. **b.** In situ zymography highlighting enhanced MMP digestion activity close to the basal lamina of epidermis (dotted line) in aged (65y.o) human healthy skin regarding young (34y.o.) samples. Similar results were obtained from 3 and 5 different young and healthy donors, respectively. Bar= 25 μ m. **c.** Transwell® assays testing the migration capacity of PSE-NHEKs cultured in SF-CM-supplemented KBM (PSE-NHEK/SF). Lower chambers were loaded with YF-CM- or SF-CM-supplemented medium pre-treated with GM6001 (12.5 μ M) or DMSO. Results (n=36) are means \pm SD (**: p<0.01). **d.** Effect of activated MMPs on the migratory capacity of PSE-NHEK cells arising from SF-CM-supplemented cultures. Cells were starved 8 hours in fresh KBM and assayed for migration during 30 hours using KBM supplemented with either control buffer or activated MMP-1 or MMP-2 as chemo-attractant. Results shown (n=20) are means \pm SD (*: p<0.05). **e.** siRNA-mediated depletion of MMP-1 or MMP-2 in SF-CM reduced the migratory response of PSE- NHEK/SF toward the different supplemented KGM culture media or control media (mock transfection, transfection with non-target-specific or target-specific siRNA). Results shown (n=36) are means \pm SD.

The PAR-1 thrombin receptor mediates MMP-1 and MMP-2 promotion of cancer-initiated PSE keratinocyte early migration.

We then undertook to investigate how MMP-1 and MMP-2 could mediate the promotion of PSE-NHEK migration. We focused on a possible MMPs fixation to and their activation of the thrombin receptor PAR-1. PAR-1 (Proteases Activated Receptor -1) is a G protein-coupled receptor expressed in several types of advanced cancers (breast, prostate, melanoma...), and correlated with the degree of invasiveness (24, 25). PAR-1 activation results from irreversible proteolytic cleavage of its amino-terminal exodomain exposing a tethered ligand (26). MMP-1 was shown to be able to activate PAR-1 (27-29). MMP-1 could be the mediator of PAR-1 activation for breast cancer cell migration and metastasis (27). We first examined whether PAR-1 was expressed in keratinocytes. RT-qPCR revealed a strong increase in PAR-1 transcripts in control PSE-NHEK (KGM) and was further enhanced in co-culture conditions (figure 3a). Western-blot analysis of PAR-1 (80kDa) confirmed the increase of PAR-1 expression in PSE-NHEK compared to the young-NHEK. However the levels of PAR-1 detection in PSE-NHEKs cultivated with SF-CM were repeatedly lower than when the cells were cultivated in YF-CM (figure 3b and supplementary figure 3a without starvation). This could be explained by the fact that PAR-1 receptors are activated by proteolytic cleavage and rapidly internalized and targeted to lysosomes for degradation(30). Hence, upon continued influence of senescent fibroblast-CM, the pool of PAR-1 receptors could undergo a continuous rate of degradation indicative of its continuous activation. To test this hypothesis, the different PSE-NHEK populations were starved in fresh KBM (i.e. basal keratinocyte culture medium without any supplement). This led to the recovery of PAR-1 expression (supplementary figure 3a). Cells were then submitted again to the influence of SF-CM; this led to the re-degradation of the receptor within only 15 minutes to one hour as depicted by the loss of its detection by western-blotting, confirming PAR-1 active activation/degradation when PSE-NHEKs were conditioned by SF-CM (figure 3c). By comparison, re-stimulation by SF-CM after starvation of PSE-NHEK cells conditioned by FGM, showed initiation of PAR-1 activation after one hour (supplementary figure 3b).

We then compared the impact of restimulating the starved cells by FGM, YF-CM or SF-CM for an hour (Figure 3d). While re-stimulation with FGM and YF-CM did not induce PAR-1 degradation in any PSE-NHEKs cells, re-stimulation by SF-CM specifically caused a rapid decrease of PAR-1 in PSE-NHEK/SF cells (figure 3d). To confirm the PAR-1 receptor activation, we monitored the subcellular location of PAR-1 in PSE-NHEKs before and after starvation, and after a very short re-stimulation with SF-CM (Figure 3e and supplementary figure 3c). Prior to starvation, PAR-1 staining was faint at the plasma membrane but clear in cytosolic vesicles in PSE-NHEK/SF cells (figure 3e left). Post-starvation, those cells displayed PAR-1 staining mostly restricted to the cell surface, indicating the reduced activation (and therefore less degradation to cytosolic vesicles) of this receptor (Figure 3e middle). A rapid redistribution of the PAR-1 staining to the intracellular vesicle compartment in response to a brief re-stimulation by SF-CM was observed in PSE-NHEK/SF cells (Figure 3e right). Although starvation also improved membranous staining of PAR-1 in PSE-NHEK/FGM cells, the brief re-stimulation by SF-CM did not induced PAR-1 re-location to cytosolic vesicles (supplementary figure 3c). Treatment with Bafilomycin (inhibitor of lysosomal activity) led the accumulation of PAR-1 in cytosolic vesicles in both cell types confirming that activated receptors are targeted to lysosome for degradation but also showed much stronger lysosomal staining of PAR-1 in PSE-NHEK/SF cells (Supplementary figure 4).

To evaluate the implication of PAR-1 receptor in the migratory capacity of PSE-NHEK/SF cells migration, we first blocked its activity by pre-treating the cells with specific receptor-blocking antibodies, either ATAP-2, or WEDE-15 or both. This dramatically reduced their migratory response to senescent fibroblast-CM (figure 4a). We also specifically abolished PAR-1 expression by siRNA tranfection in PSE-NHEK cells co-cultivated with FGM, YF-CM or SF-CM (supplementary figure 3b). This strategy reduced the moderate capacity of PSE-NHEK/FGM or PSE-NHEK/YF to migrate upon SF-CM stimulation, and strikingly abolished the strong migratory response of PSE-NHEK/SF cells highlighting the importance of PAR-1 expression and activity for the migratory capacity of cells conditioned in presence of senescent fibroblast secretions (figure 4b).

To confirm that MMPs accounted for SF-CM activation of PAR-1 providing the conditioned cells with migratory capacities, we re-stimulated starved PSE-NHEK/SF-CM cells with activated recombinant MMPs (Figure 5). Western blot analysis revealed a reduced level of PAR-1 in PSE-NHEK/SF cells after only 10 min of re-stimulation with recombinant MMP-1 or MMP-2 (Figure 5a). Immunofluorescence analysis also confirmed that MMP-1 and MMP-2 could trigger re-distribution of PAR-1 to intracellular vesicles similarly to Senescent Fibroblast-CM (Figure 5b).

Altogether, cancer-initiating PSE-NHEKs display a higher level of PAR-1 thrombin receptor expression and are subject to specific PAR-1 activation by MMPs secreted by senescent fibroblasts leading to the acquisition of a greater migratory capacity. PAR-1 detection by immuno-histochemistry in human young versus old healthy skin human samples showed PAR-1 expression in some foci of the epidermis of old samples. In these foci, cellular organization appeared disturbed with loosened intercellular cohesion of the epidermal cells that could initiate dissemination from the basal stratum of keratinocytes (supplementary figure 6). Interestingly those foci are close to the MMP-enriched dermis area shown in figure 2, supporting physio-pathological relevance of our results.

FIGURE LEGENDS

Figure 3: Culture in the presence of SF-CM particularly induces PAR-1 activation and routing to vesicles.

a. qRT-PCR analysis showing only minor variation of PAR-1 transcript levels in young keratinocytes (left) cultured under different conditions (KGM: control medium, or KGM supplemented with FGM, YF-CM, or SF-CM). PSE-NHEKs (right) displayed strongly elevated PAR-1 transcript levels in the control medium that were further increased in the supplemented media. Each condition run in triplicate. **b.** Western blot analysis comparing PAR-1 detection in cell lysates from young NHEKs (upper panel) and PSE-NHEKs (lower panel) from the different culture conditions and showing reduced PAR-1 protein detection in lysates of PSE-NHEKs cultured in SF-CM-supplemented KGM. **c.** Western blot analysis of PAR-1 in lysates of PSE-NHEKs cultured in SF-CM-supplemented KGM (PSE-NHEK/SF). Cells were starved in KBM to restore PAR-1 detection (second lane) and then re-stimulated for the indicated times with SF-CM-supplemented KBM. PAR-1 detection was reduced after 15 min and almost abrogated after 60 minutes re-stimulation in PSE-NHEKs arising in SF-CM-supplemented culture medium. Results were similar in two independent experiments. **d.** Western blotting of cell lysates from PSE-NHEKs arisen in different culture conditions. Cells were starved to stop PAR-1 activation and consequently protect it from lysosomal degradation (second lanes). Cells were eventually re-stimulated by a one-hour incubation in their corresponding original supplemented media. PSE-NHEKs conditioned by SF-CM (PSE-NHEK/SF) specifically showed reduction of PAR-1 expression, depicting high sensitivity to activating SF-CM. **e.** Immunofluorescence analysis of PAR-1 before and after starvation of PSE-NHEK/SF cells to reduce receptor activation and its degradation. Cells were alternatively re-stimulated for only 10 minutes by SF-CM-supplemented KBM. While starvation promoted membranous expression of PAR-1, re-stimulation by SF-CM specifically restored the vesicular localization of PAR-1 in the PSE-NHEK/SF population. Scale bar =10 μ m. Insets show representative sites magnified 2.5-fold.

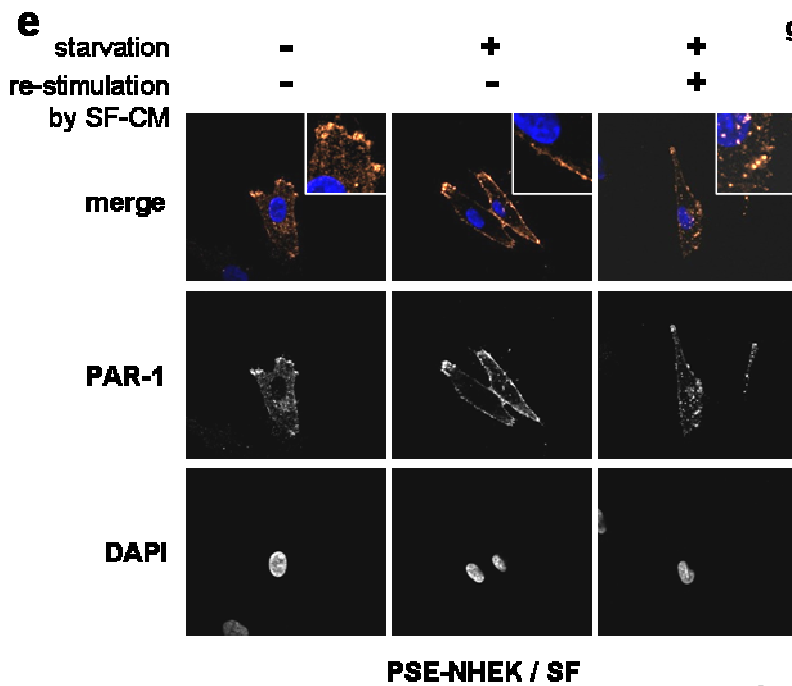
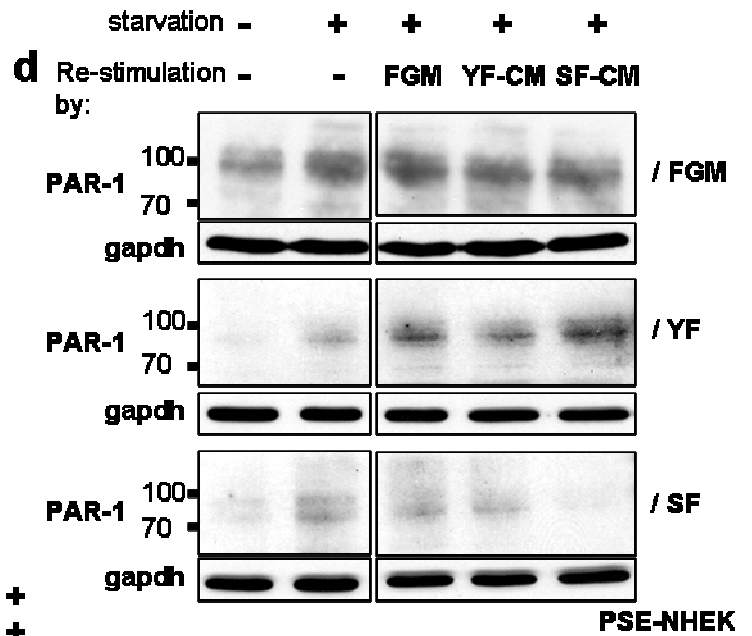
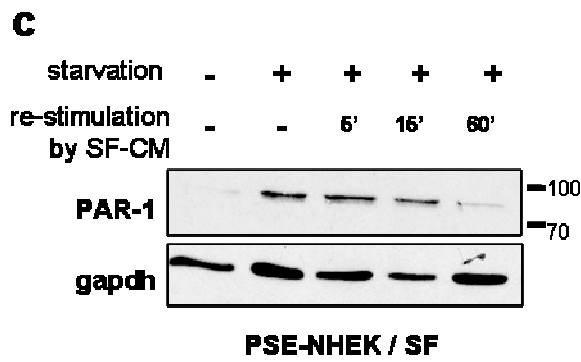
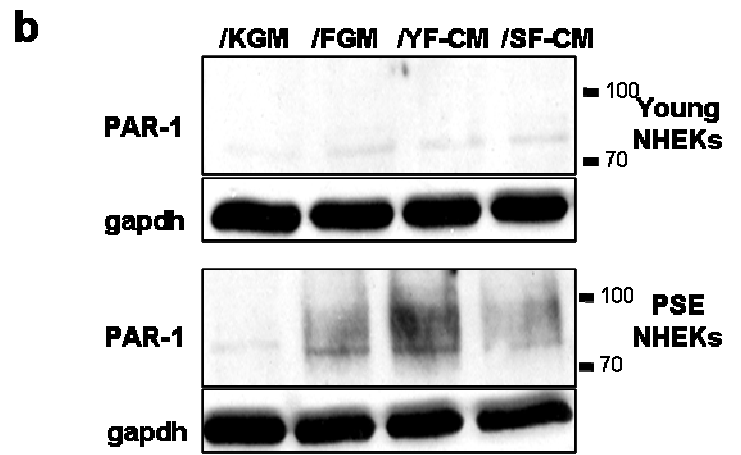
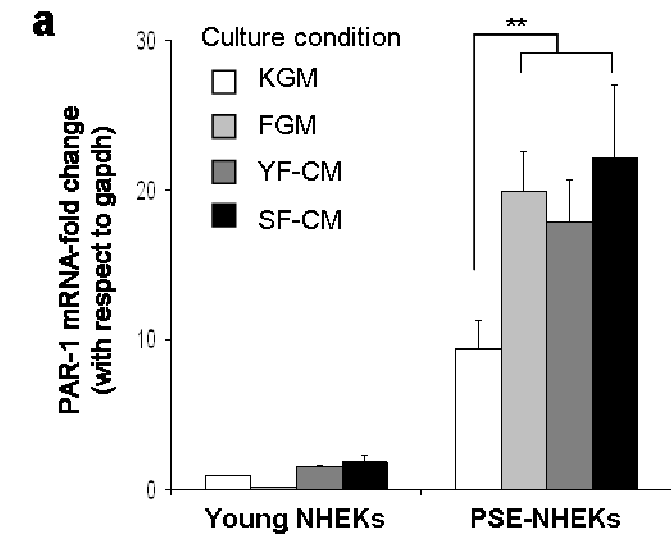


Figure 3

FIGURE LEGENDS

Figure 4: Interfering with PAR-1 expression or activation reduces the migratory response of PSE-NHEK/SF cells to senescent fibroblast-conditioned medium.

a. Treatment of PSE-NHEK/SF cells with PAR-1-blocking antibodies considerably reduced their migration toward MMP-rich SF-CM-supplemented KBM. Results shown (n=36) are means \pm SD. **b.** PSE-NHEK cells from cultures with SF-CM were transfected by pools of siRNAs targeting PAR-1, starved overnight and assayed for migration during 30 hours using either YF-CM- or SF-CM supplemented KBM stimulus. Results shown (n=36) are means \pm SD.

Figure 5: Recombinant MMP-1 and MMP-2 reproduce PAR-1 activation and relocation by MMP-rich senescent fibroblast-CM.

a. Western blot analysis of PAR-1 in lysates of PSE-NHEKs cultured in SF-CM-supplemented KGM (PSE-NHEK/SF). Cells were first starved in KBM to restore PAR-1 detection (first lane) and then re-stimulated for the indicated times with activated recombinant MMP-1 or MMP-2 (50 nM) or control buffer. **b.** Immunofluorescence analysis of PAR-1 expression in PSE-NHEK/SF starved in KBM and then re-stimulated for 10 min or 60 min with activated recombinant MMP-1 or MMP-2 (50 nM) or control buffer. Recombinant MMP-1 and MMP-2 specifically induced membrane-to-vesicle relocation of PAR-1. Scale bar =10 μ m. Insets show representative sites magnified 2.5-fold.

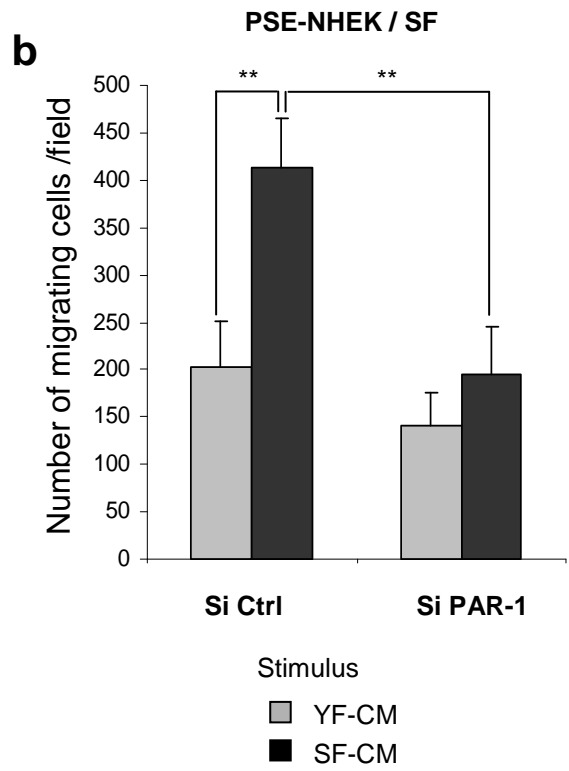
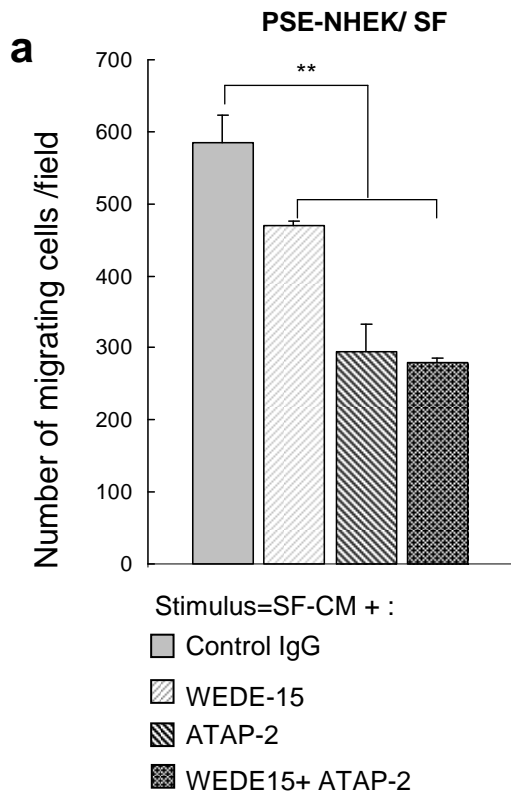
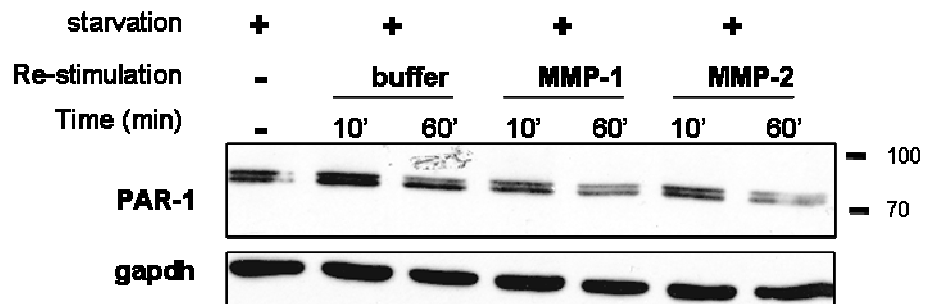
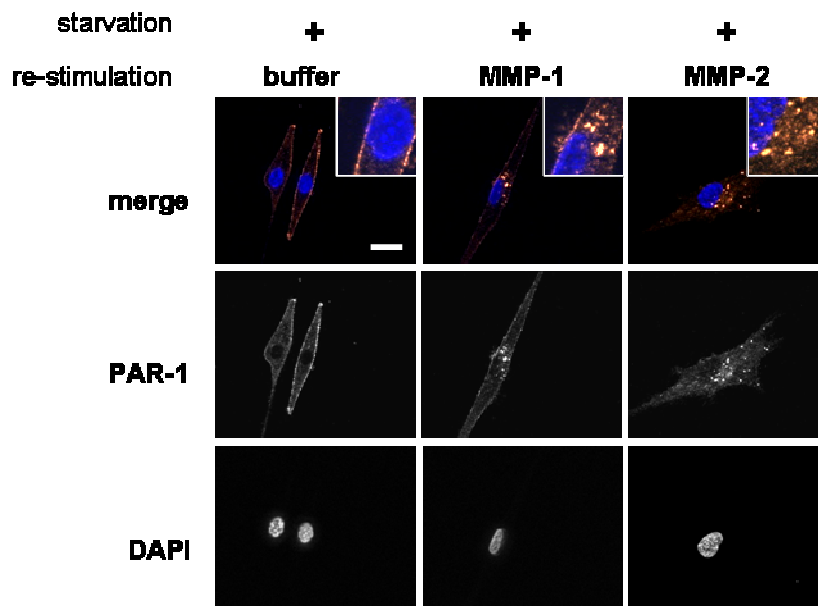


figure 4

a**b****Figure 5**

DISCUSSION

To test the hypothesis that conditioning by a senescent stroma could favor the promotion of early carcinogenesis, we used an *in vitro* model based on our earlier demonstration that senescent keratinocytes cultures can give rise to neoplastic cells displaying many alterations of cancer-related genes, reduced E-cadherin and involucrin expressions, and able to generate skin hyperplasias and small carcinomas *in vivo* (17).

We show that replacing 10% of their culture medium by conditioned medium from senescent fibroblasts favoured the carcinogenic transformation of post-senescence keratinocytes (PSE-NHEK). In addition to previous studies that showed that the SASP of senescent fibroblasts enhanced the development of cancers, we show that conditioned medium of human senescent dermal fibroblasts (SF-CM) promotes by several ways the very initial phases of carcinogenesis. On the first hand, conditioning with SF-CM increased the frequency of emergence of post-senescence neoplastic clones. We showed that the levels of reactive oxygen species (ROS) in keratinocytes controlled cell death and potential post-senescence emergence (31). An increase in ROS levels in cells cultivated with SF-CM could account for the increase in emergence of PSE-NHEK clones (data not shown). On the second hand, SF-CM promoted EMT of PSE-NHEK cells, with a strong decrease in cell-cell adhesion molecules expression while that of a marker of fibroblasts increased. The expression of Twist, a transcription factor associated to EMT was also enhanced. Cell shape changed to fibroblastoid and cells acquired migratory capacities.

Here we show that EMT could then be acquired very early in cancer initiation under the influence of senescing stroma. Carcinogenesis is a multistep process from hyperplasia to carcinoma diagnostic in aging populations. Our results are relevant to the observations that transformed cells undergoing EMT had already been shown to arise from hyperplasias and disseminate (22, 32, 33).

We demonstrate that cultured senescent dermal fibroblasts strongly synthesize MMP-1 and MMP-2 and that these MMPs are the molecules on which the migration-promoting properties of SF-CM rely. Increased expression of MMP-1 and MMP-2 from the peritumoural stroma are rather known to contribute to skin

carcinoma invasion and aggressiveness(34, 35). But MMPs were also demonstrated to be involved in early cancer initiation (36), to trigger EMT and induce genomic instability (37). MMP-1, -2, and -3 were also reported among the most increased factors in senescent breast and prostate fibroblast secretomes (1, 38). The activation of MMP-3 that we detected in addition to MMP-1 and -2, may also account for EMT initiation as described (39). The potential of the conditioned medium from senescent fibroblasts to increase EMT or cell migration is associated to long-lasting culture of PSE-NHEKs with SF-CM. PSE-NHEKs were then remodelled by SF-CM and prepared to respond to stimulatory messages. We therefore searched what could render PSE-NHEKs responsive to MMPs. We focused on membrane receptors activated by proteolytic activities and whose activation is known to be involved in cell migration. .

The G protein-coupled receptor PAR-1 responds to these criteria. It may trigger different biological pathways among which those governing cell shape and motility (26). Mammary cancer cell lines selected for strong EMT characters showed PAR-1 overexpression (40). MMP-1 activate PAR-1, in SV-40-transformed COS-7 fibroblast cells transiently transfected by PAR-1(27), and in ovarian cancer cell lines (28, 29). MMP-1 could be the mediator of PAR-1 activation for breast cancer cell migration and metastasis (27).

PAR-1 activation had been associated to EMT-related migration in primary cells (41, 42). We show that PAR-1 expression itself is endogenously strongly enhanced at the transcript level in cancer-initiating PSE-keratinocytes and further increased in the presence of media conditioned by senescent fibroblasts. Western blotting and immunostaining experiments highlight that activation of the PAR-1 receptor on the PSE-keratinocyte plasma membrane was specifically exacerbated by senescent stroma as depicted by rapid internalization and targeting of PAR-1 to lysosomes. In addition, we provide proof that activated recombinant MMP-1 but also MMP-2 could resume the effect of senescent fibroblast-conditioned medium. Our results demonstrating the involvement of the MMP-PAR-1 axis in the initiation of non-melanoma skin cancer find in vivo relevance at two levels. First, we show that MMP activities increase in the dermal compartment of skins of old donors compared to young ones, and particularly at the interface between dermal and epidermal compartments suggesting that MMPs could influence keratinocytes. Second, Par-1 was detected in keratinocytes within disorganized foci of the basal layer of aged skin samples confirming

increased MMP-1 and MMP-2 expressions in human dermal papillary fibroblasts in aged biopsy samples (43). We speculate that these keratinocytes could be further initiated to carcinogenesis and dissemination by MMPs of the surrounding senescent stroma. Hence, some cancer-initiated NHEKs could early disseminate in aging human skin microenvironment via the involvement of the MMP/PAR-1 axis as recently suggested for initiation of melanoma (33)

Thus initial switches along the process of carcinogenesis may occur through PAR-1 activation, during long-lasting exposure of post-senescent keratinocytes to MMP-1 and MMP-2 overexpressed in secretions of autologous senescent fibroblast. Persistence of high MMP levels in the senescent stroma could finally sustain tumour growth as shown in xenografted mammary tumours (23), probably through prolonged inflammation.

Non-melanoma skin cancers represent a fast growing, underestimated challenge for public health management (44, 45). The present results using normal primary skin cells could provide clues of common mechanisms for age-related carcinoma initiation as diagnosis of non-melanoma skin cancer is associated with an increased risk of developing other primary carcinomas (10).

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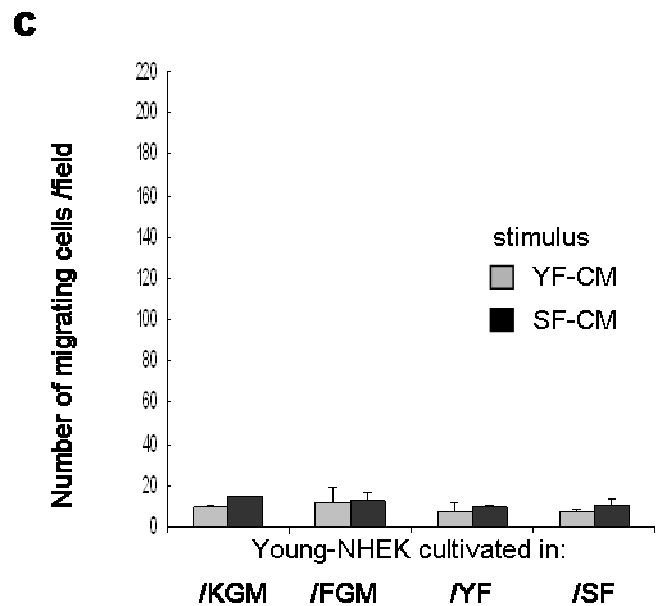
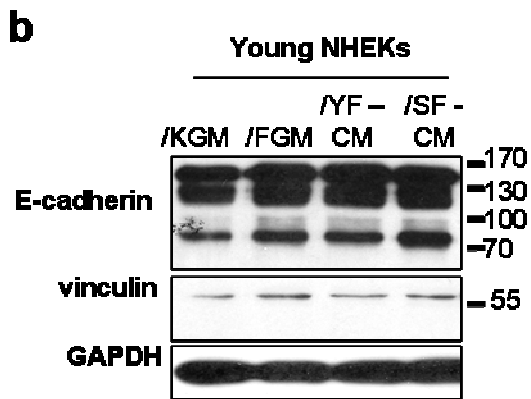
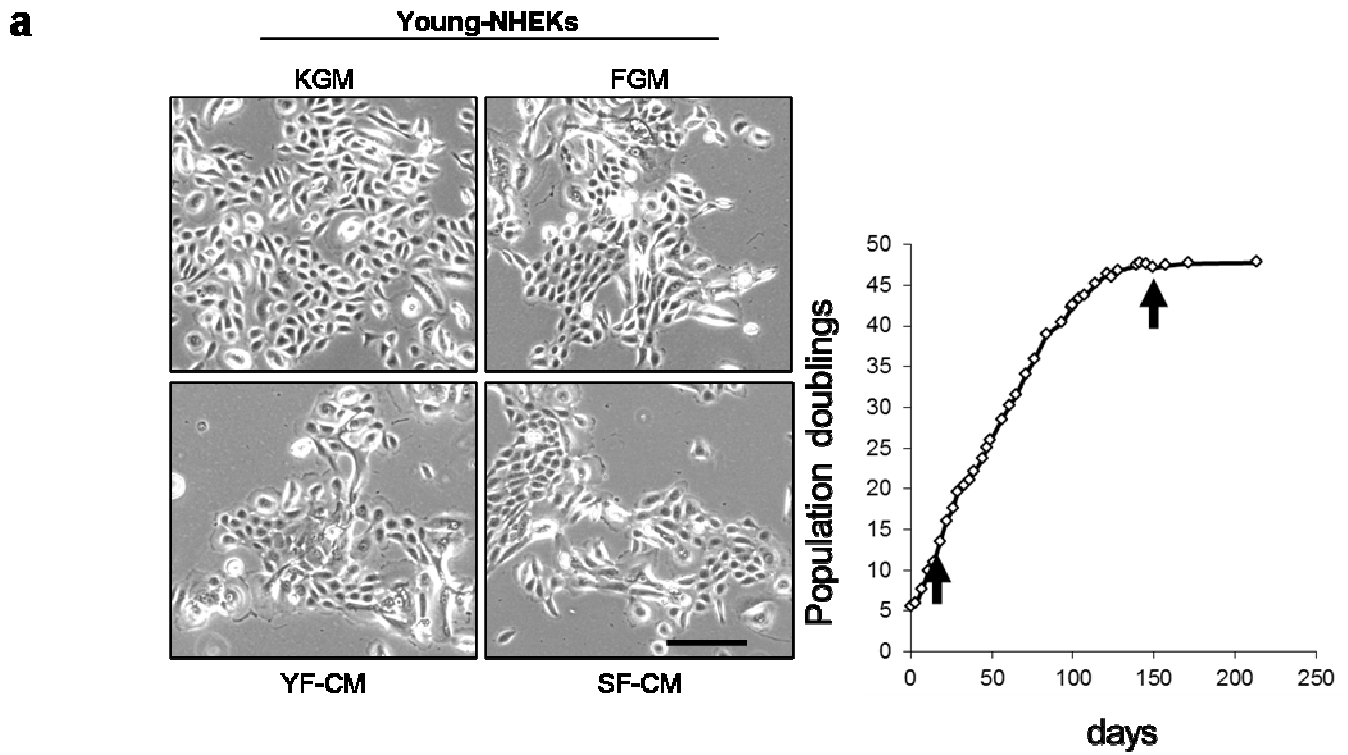
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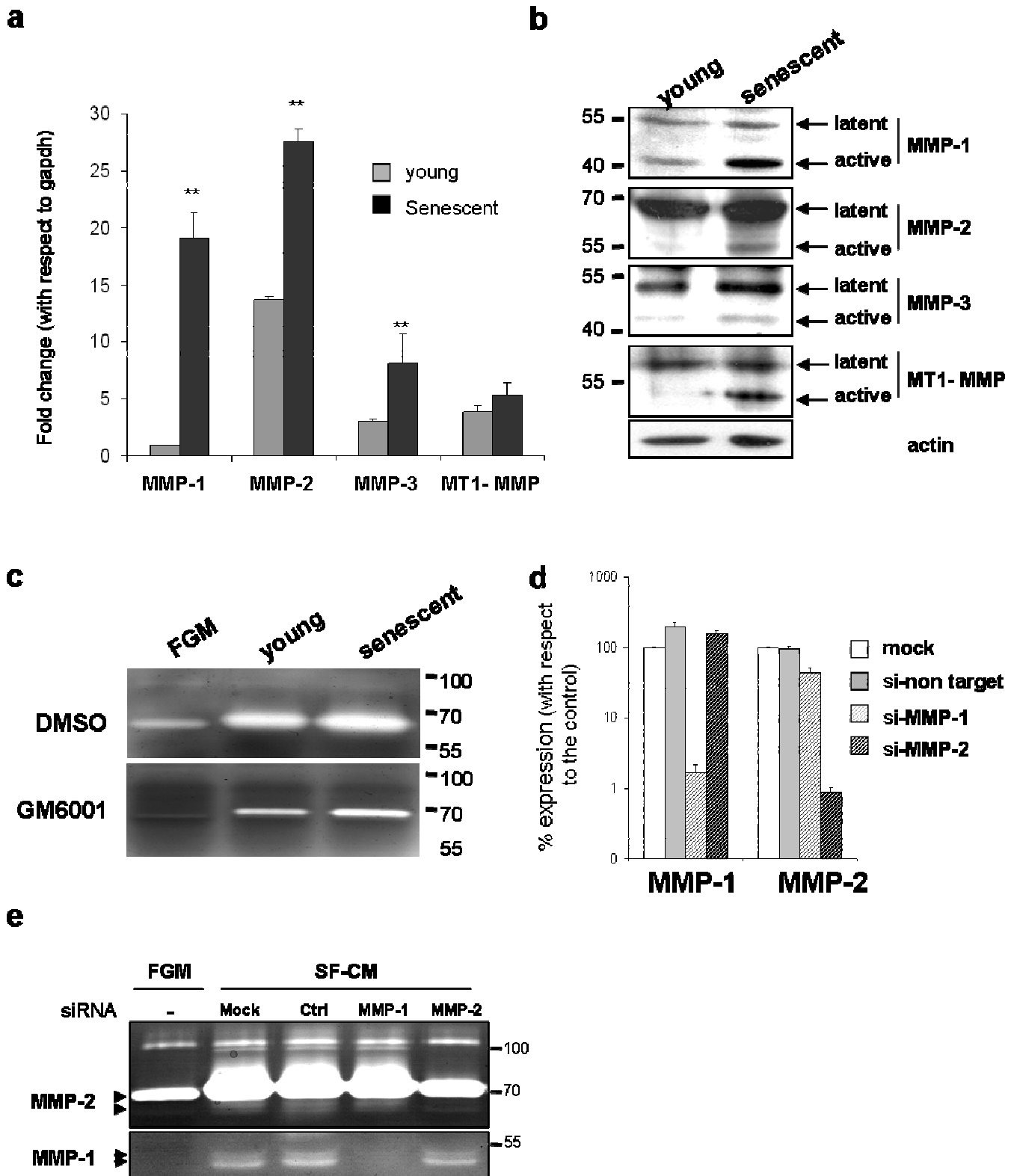
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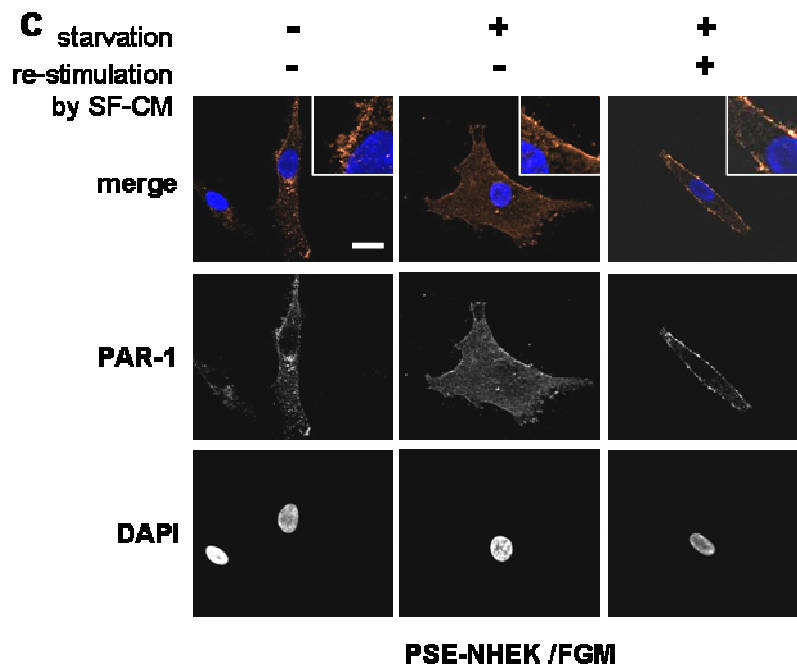
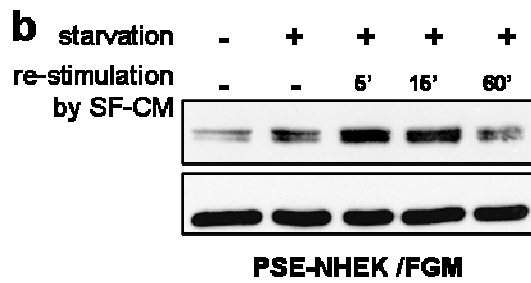
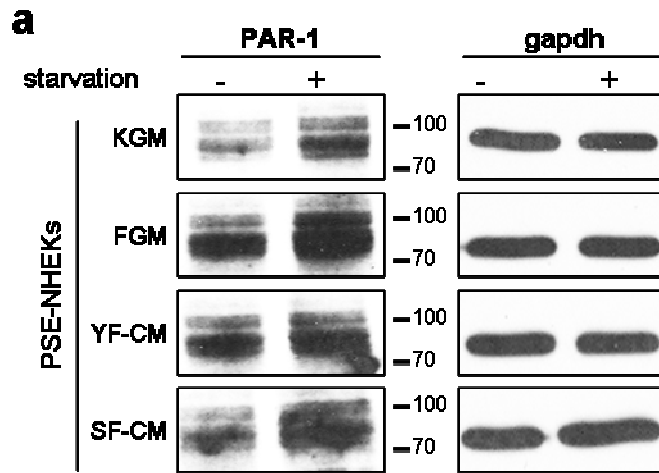
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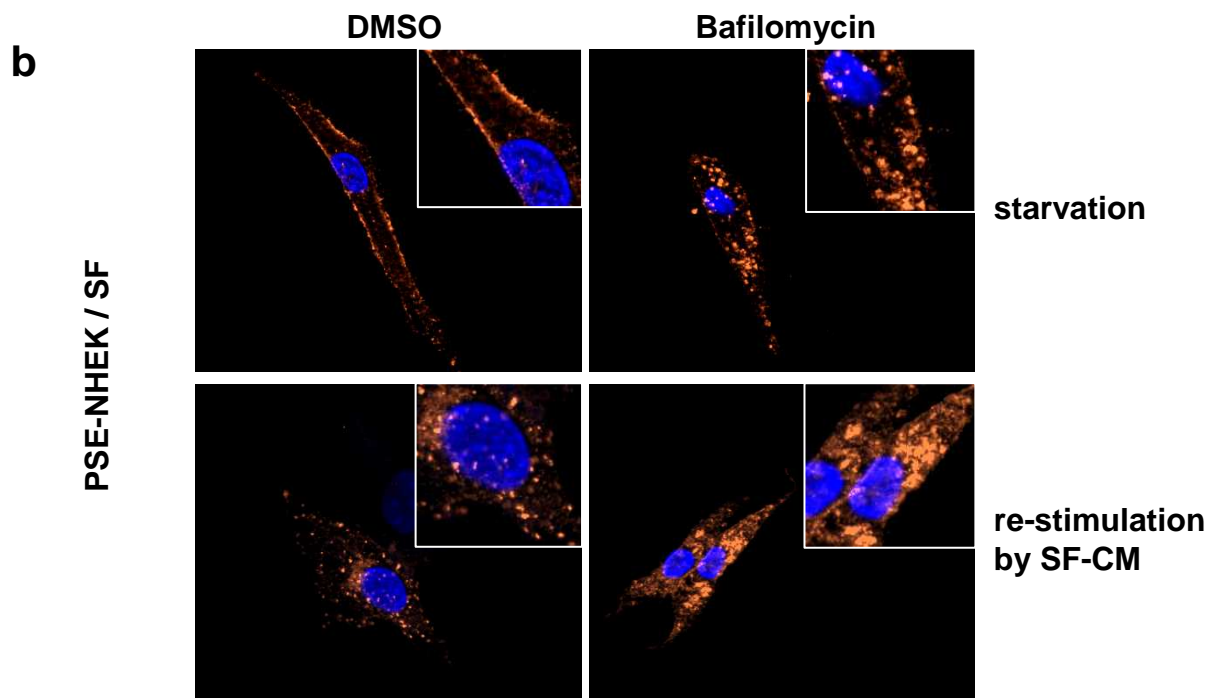
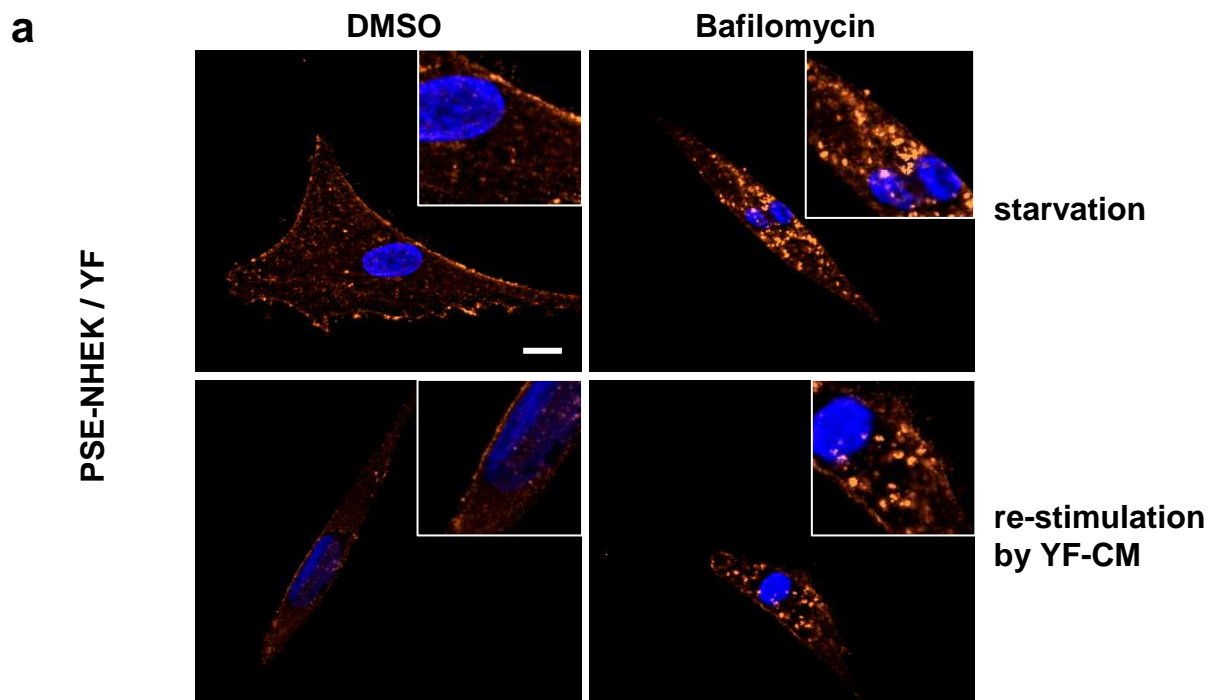
Supplementary figure 1



Supplementary figure 2

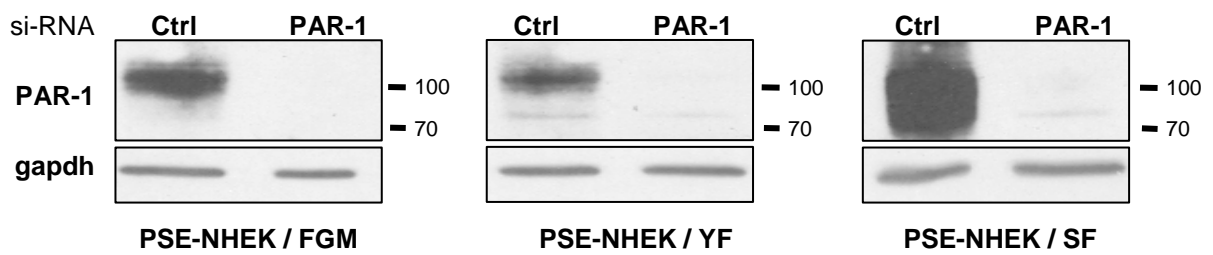


Supplementary figure 3

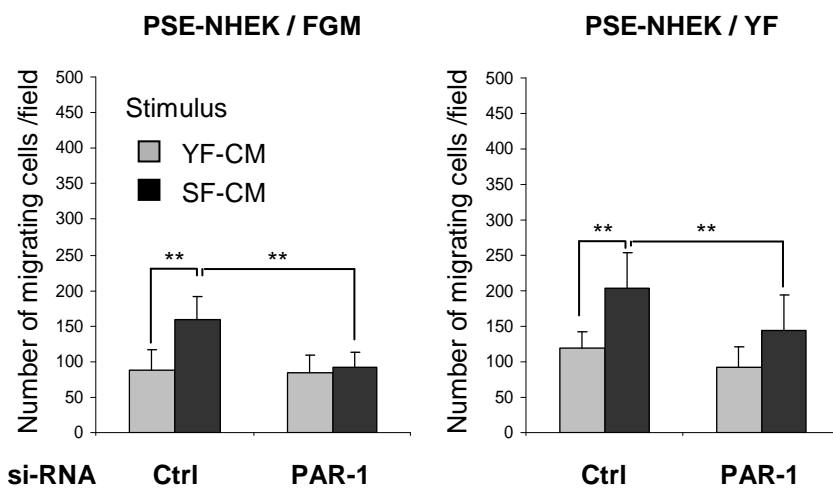


Supplementary figure 4

a



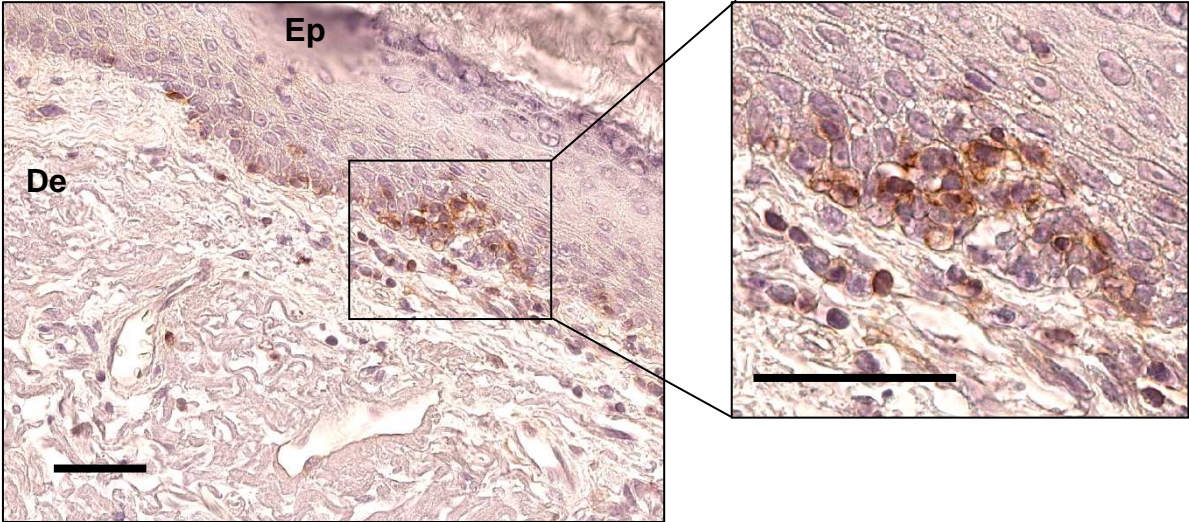
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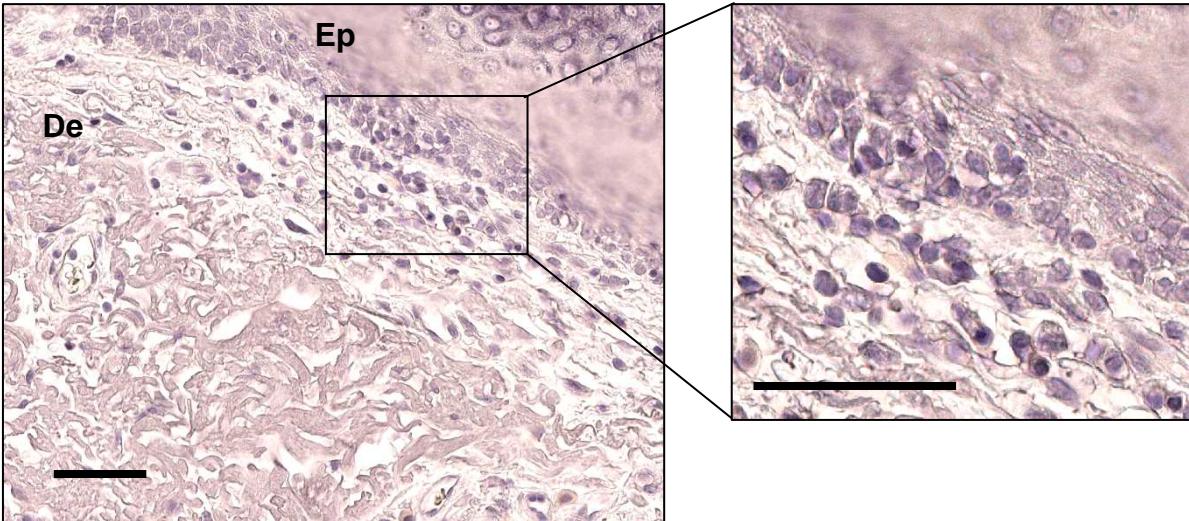
Supplementary figure 5

Old skin donor

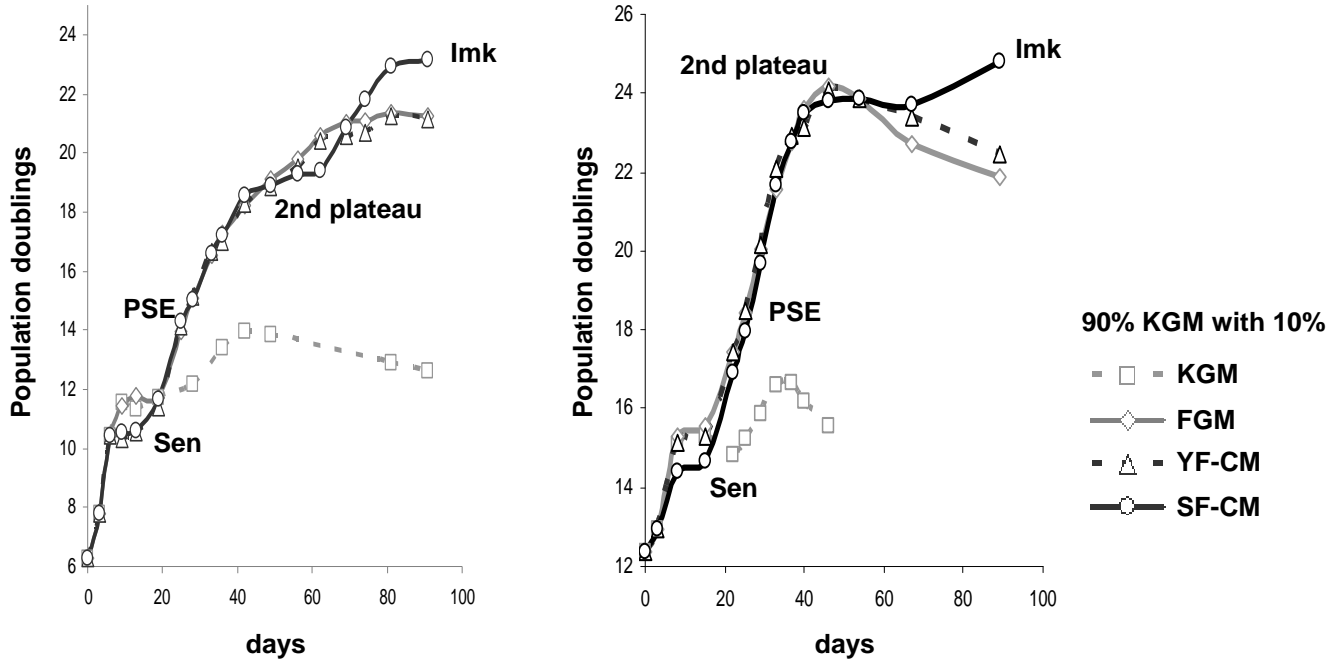
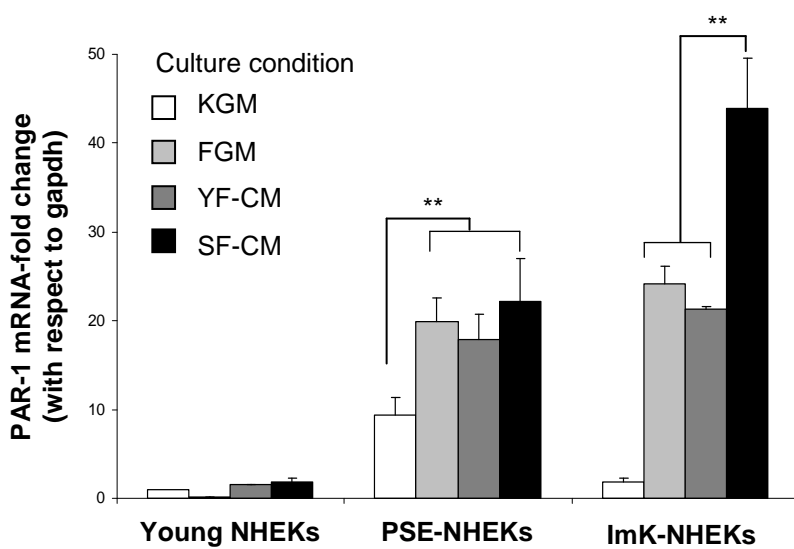
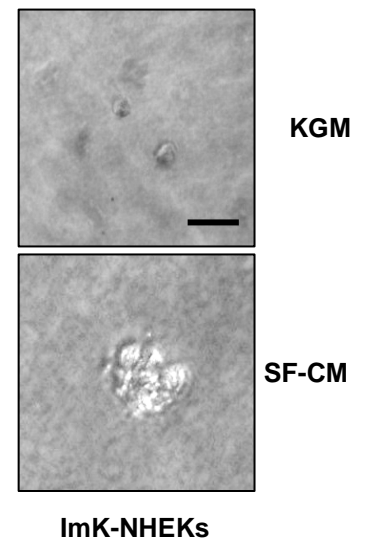
PAR-1



IgG



Supplementary figure 6

a**b****c****Supplementary figure 7**

Supplementary Figure Legend

Supplementary Figure 1: Interchanging a fraction of culture medium by different conditioned media had no influence on young proliferating keratinocytes.

a. Left: Micrographs at the beginning (day 3) of co-cultures of very early passages young NHEK cells from the same donor as in figure 1 panel (a) depicting the exclusively epithelioid shape of cells whichever the control KGM medium or supplementation of 90% of the latter by 10% of either fresh (FGM-), young or senescent fibroblast-conditioned medium (YF-CM and SF-CM respectively). Bar = 100 μ m. Right: Indication (arrows) of the typical times at which fibroblast-conditioned media were prepared **b.** Western blot analysis of E-cadherin and vinculin expressed by young NHEKs cultured in various conditions, 10% of the usual NHEK culture medium (KGM) being replaced either with fresh FGM or with conditioned medium (CM) from young- or senescent-fibroblast cultures (YF-CM, SF-CM). The 80-kDa and 120-kDa forms of E-cadherin are not affected. These results were corroborated using a second cell lysate preparation. **c.** The migratory capacity of young NHEKs cultured in panel control or FGM-, YF-CM-, or SF-CM-supplemented medium was analysed in Transwell® assays. Cells were starved for 8 hours in fresh KBM basal medium and assayed for migration during 30 hours using KBM supplemented with YF-CM or SF-CM as chemoattractant. Migration data are representative of two or more experiments. Results (n=30) are means \pm SD (**: p<0.01).

Supplementary Figure 2: Activated MMPs are critical components of the stimulation of PSE-NHEK migration by senescent fibroblast-CM.

a. qRT-PCR analysis of MMP transcripts in young and senescent fibroblasts. Levels of MMP-1, MMP-2, and MMP-3 transcripts were respectively 19-fold, 2-fold, and 2.7-fold higher in senescent than in young fibroblasts mRNA levels for the membrane protein MT1-MMP did not differ

significantly, and MMP-9 transcripts were below the detection threshold. Data are from a representative experiment of three carried out independently on cells from the same donor, each condition measured in triplicate (mean \pm SD). Analyses from three donors showed similar expression changes upon cell senescence (**: $p < 0.01$). **b.** Western blot analysis of cell lysates confirmed the absence of MMP-9 protein (not shown) but showed increased levels of the MMP-1(mouse anti-human MMP-1, Calbiochem), MMP-2(mouse anti-human MMP-2, Santa cruz), and MMP-3 (rabbit anti-human MMP-3, Santa Cruz) active forms in senescent fibroblasts. Although MT1-MMP (rabbit anti-human MT1-MMP, Chemicon International) was not regulated at transcript level, its active form, which is crucial for cross-activation of other MMPs, was only detected in lysates of senescent fibroblasts. Actin detection was performed as loading control (mouse anti-human actin, Sigma). Each panel is representative of 3 to 5 independent experiments. **c.** Compared to its solvent alone (DMSO), the broad-spectrum MMP inhibitor GM6001 (12.5 μ M) dramatically reduced the highest MMP activities observed by gelatin zymography in fibroblast media (results shown for MMP-2). This effect was corroborated in a replicate experiment. **d.** Specific inhibition of MMP-1 and MMP-2 expression by siRNA in senescent fibroblasts. Results shown are means \pm SD from a representative qRT-PCR experiment of two carried out independently, each condition being tested in triplicate. **e.** Gelatin zymography of conditioned media from senescent fibroblasts transfected with pools of siRNAs targeting MMP-1 or MMP-2. Results were similar in two independent experiments.

Supplementary figure 3. Controls of PAR-1 expression status and of the effects of starvation for the various PSE-NHEKs from the different culture conditions.

a. Western blot analysis showing that starvation in KBM reduced strong degradation of the activated PAR-1 receptor and restored its detection in PSE-NHEK/SF. **b.** Western blot analysis of PAR-1 in lysates of PSE-NHEKs cultured in fresh FGM-supplemented KGM (PSE-NHEK/FGM). Cells were

starved in KBM to restore PAR-1 detection (second lane) and then re-stimulated for the indicated time with SF-CM-supplemented KBM. **c.** Immunofluorescence analysis of PAR-1 before and after starvation (second column) of PSE-NHEK/FGM cells to reduce receptor activation and its degradation. Cells were alternatively re-stimulated for only 10 minutes by SF-CM-supplemented KBM (third column). Scale bar =10 μ m. Insets show representative sites magnified 2.5-fold.

Supplementary figure 4. PAR-1 degradation in PSE-NHEK/SF cells is associated to the lysosomal compartment.

Inhibition of lysosomal activity was used as a mean to evaluate the accumulation of PAR-1 in intracellular vesicles prior to their lysosomal degradation, as an indicator of the activation rate. PSE-NHEKs from cultures in KGM supplemented with young fibroblast-CM or senescent fibroblast-CM were starved, or not, for 12 hours in KBM in presence of Bafilomycin (5nM), or its solvent DMSO respectively. Control DMSO (-) Bafilomycin-treated cells were starved in KBM overnight and subsequently stimulated for 10 minutes with their respective initial KGM medium, i.e. supplemented either by young or senescent fibroblast-CM. Cells were processed for PAR-1 immuno-staining as described in the Materials and Methods section

a. While starvation of PSE-NHEKs cultured in young fibroblast-CM (YF-CM) improves the membrane staining of PAR-1 (upper left in a) as for cells cultured in presence of FGM (see figure 4), re-stimulation for 10 minutes with their initial culture medium (YF-CM) did not provide critical changes in PAR-1 staining (lower left in a). Bafilomycin treatment stopped lysosomal degradation of PAR-1 and enriched the cells in PAR-1-containing vesicles with persistent membrane staining.

b. Starvation of PSE-NHEKs cultured in senescent fibroblast-CM (SF-CM) induced the recovery of membrane localization of PAR-1 (upper left in b) while re-stimulation for 10 minutes by SF-CM induced the complete loss of membrane staining of the receptor and clear vesicular staining (lower left in b). Bafilomycin treatment during starvation led to very strong accumulation of exclusively

vesicular (endosomal) staining of PAR-1 in association with high degradation rate of the receptor under re-stimulation by medium containing senescent fibroblast-CM. Scale bar (10 μ m) applies to all the larger images in the montage. Insets show representative sites magnified 2.5-fold. (Image processing as in Materials and Methods).

Supplementary figure 5. Validation of PAR-1 silencing.

a. The efficiency of siRNA-mediated extinction of PAR-1 expression was verified by western blotting of cell lysates. RNA interference against PAR-1 transcripts fully abrogated the PAR-1 expression that is exacerbated in PSE-NHEK cells that arise in the various co-culture conditions, even in cells arising in SF-CM-supplemented culture medium, that display the highest PAR-1 expression level. Results were reproduced twice in independent experiments. **b.** PSE-NHEK cells from the different culture conditions were transfected by pools of siRNAs targeting PAR-1, starved overnight and assayed for migration during 30 hours using either YF-CM- or SF-CM supplemented KBM as chemo-attractant. Results shown (n=36) are means \pm SD.

Supplementary figure 6. PAR-1 expression is restricted to disorganized foci of epidermis in skin samples from aged donors.

Immunohistodetection of PAR-1 in healthy skin samples from aged people highlighted PAR-1 expression in basal and suprabasal areas of epidermis (ep) at disseminated sites where inter-cellular junctions were less obvious and displaying less compact organization.(de: dermis).

Deparaffinized and rehydrated sections of skin biopsies from young (ages 26-38 years) or old (60-89 years) human healthy donors were treated by 1%H₂O₂ in PBS to block endogenous peroxydases. Aspecific binding was prevented by incubation in PBS + 5%BSA and 10% rabbit serum.

Endogenous avidin and biotin were inhibited using the specific Avidin/Biotin Blockin Kit from Vector Labs. Primary (Monoclonal mouse anti-Par-1, Santa Cruz Biotechnology, 1/100) and secondary (Biotinylated rabbit anti-mouse, Dako, 1/100) antibodies were respectively diluted in PBS + 5%BSA+ 10% rabbit serum and PBS+2% BSA. Control was run in a serial section using isotype-specific primary antibody (Santa Cruz Biotechnology). Detection was amplified using streptavidin-peroxydase (Jackson Immunoresearch), and staining was performed using DAB detection Kit (Vector Labs). Slices were counterstained with Gill's hematoxylin in tap water and mounted in Dako Glycergel®. Control was run in a serial section using isotype-specific primary antibody Scale bar=50µm.

Supplementary Table I: List of primers used in qRT-PCR experiments.

	Forward primer (5'->3')	Reverse primer (5'->3')
MMP-1	GAGCAAACACATCTGAGGTACAGGA	TTGTCCCGATGATCTCCCCTGACA
MMP-2	AGATCTTCTTCTTCAAGGACCGGT	GGCTGGTCAGTGGCTTGGGGTA
MMP-3	GATCTCTTCATTTTGGCCATCTCTTC	CTCCAGTATTTGTCCTCTACAAAGAA
MT1-MMP	GGATACCCAATGCCCATTTGGCCA	CCATTGGGCATCCAGAAGAGAGC
PAR-1	GTGAAGCGGAGCAGCCCGA	GGCCGCACAGACTGAAGCA
GAPDH	ATCTCTGCCCCCTCTGCT	GCAGGAGGCATTGCTGAT

Monitoring of cell cultures and of senescence

Senescence was monitored on growth curves and validated at the morphological and biochemical level by SA-beta galactosidase staining (19, 22). Cultures were always split at 70% confluence and the number of population doublings (PD) was systematically determined as follows: $PD = \ln(\text{number of collected cells}/\text{number of plated cells})/\ln 2$.

For the determination of the proportion and morphology of PSE-NHEKs, senescent keratinocytes (10.000 per 10cm dish) were seeded in their respective culture condition (100% KGM, 90% KGM + 10% FGM, +10% YF-CM or +10% SF-CM). Medium was renewed every 2 days. After 1 week, the total number of post-senescence emergent (PSE) clones per dish was counted from pictures taken from each dish, discriminating epithelial from fibroblastoid clones.

Adapted protocol for in situ zymography

DQ™ Gelatin from pig skin (1mg/ml) (Molecular Probes) was diluted (1/50) in reaction buffer (50 mM Tris-HCl pH 7,6, 150 mM NaCl, 5 mM CaCl₂) and deposited on the top of dewaxed and rehydrated tissue sections, covered with a parafilm® and incubated 4 hours at 37°C in a dark humidity chamber. After removing the parafilm®, sections were rinsed with Milli-Q water and fixed in 4% PFA for 10 min. Nuclei were stained with Hoechst 33258 (Sigma-Aldrich) at 1 µg/ml for 5 min, and coverglasses mounted in Glycergel ® mounting medium (Dako). In presence of MMPs, digestion of the gelatin layer released fluorescence from the quenched substrate.

Immunofluorescence images capture settings.

Except when specified, images from fluorescence experiments were taken using an Axioplan2 (Zeiss, Germany) microscope equipped with an Apotome device and either Plan Neofluar (5x,

NA: 0.16), or Apochromat (20x, NA:0.8) or Plan Neofluar (40x, NA: 1.3) objectives. Images (12 bits, 1388/1040 pixels, no binning) were recorded using an AxioCam HRc camera (Zeiss) (3200°K) and using AxioVision® Software for microscopy image analysis (Zeiss). Images for figures with combined panels shared the same initial properties and gamma (i.e.:gamma:1) and were exported as 8 bits TIFF files. Common software brightness and contrast adjustments were finally applied under PowerPoint®.

Details for quantitative reverse-transcription and real-time PCR.

RNA was isolated from cultured cells with the Nucleospin® kit (Macherey-Nagel). For reverse transcription, 1µg polyA-RNA was used in a reaction with oligo dT primers and Superscript II reverse transcriptase (Invitrogen; 200 units) for 1 h at 55°C. The primers used (supplementary Table 1) were designed with the NCBI-Primer-BLAST software at <http://blast.ncbi.nlm.nih.gov/>. The PCR protocol was that recommended for the Mx3005P Real-time PCR System® (Stratagene). Accumulation of PCR products was measured by SYBR green® fluorescence (SYBR Green® master mix; Applied Biosystems). Raw data analysis was performed with the MxPro® software (Agilent). For each sample, Ct(gene) - Ct(GAPDH) was calculated, and this value was used to calculate the ratio of test gene mRNA to the GAPDH internal control mRNA.

Migration assay complements

PSE-NHEKs (day 20 of culture) cultured with or without added CM were starved in keratinocyte basal medium (KBM) for at least 8 h. When applicable, cells were pre-treated for 15 min at 37°C with PAR-1 blocking antibodies: ATAP-2 (10 µg/ml, Santa Cruz Biotechnology) or WEDE-15 (20 µg/ml, Beckman Coulter). Alternatively they could be transfected by control or Par-1 specific siRNAs. Cells in KBM were then seeded onto Transwell® cell culture inserts with 8-µm pore size (Falcon), at a density of 30,000 cells per

well (24-well format). Different chemo-attractants were placed in the lower chambers: 90%KBM + 10% FGM, YF-CM, or SF-CM or also variations of the latter medium harvested from senescent fibroblasts that had been transfected with MMP-1- or MMP-2-targeting or nontargeting siRNAs. In some cases, the CMs were pre-treated with an MMP inhibitor (12.5 μ M GM6001, Chemicon). After 30 hours of incubation at 37°C, non-migrating cells were removed from the top face of the inserts by scraping, while cells that had migrated to the lower face were fixed in methanol and stained with Hoechst 33258. Migration was analysed on each filter using fluorescence imaging (see above). Each condition was tested in triplicate. Ten to twelve images of randomly chosen optical fields were captured and migrating cells were counted with the Colony1.1® software.

Immuno-histo-detection of PAR-1

Deparaffinized and rehydrated sections of skin biopsies from young (ages 26-38 years) or old (60-89 years) human healthy donors were treated by 1% H₂O₂ in PBS to block endogenous peroxidases. Aspecific binding was prevented by incubation in PBS + 5% BSA and 10% rabbit serum. Endogenous avidin and biotin were inhibited using the specific Avidin/Biotin Blockin Kit from Vector Labs. Primary (Monoclonal mouse anti-Par-1, Santa Cruz Biotechnology, 1/100) and secondary (Biotinylated rabbit anti-mouse, Dako, 1/100) antibodies were respectively diluted in PBS + 5% BSA+ 10% rabbit serum and PBS+2% BSA. Control was run in a serial section using isotype-specific primary antibody (Santa Cruz Biotechnology). Detection was amplified using streptavidin-peroxydase (Jackson Immunoresearch), and staining was performed using DAB detection Kit (Vector Labs). Slices were counterstained with Gill's hematoxylin in tap water and mounted in Dako Glycergel®.

3. Résultats complémentaires sur la régulation des mécanismes de migration des NHEK-PSE conditionnés par le MC de fibroblastes sénescents

Au travers de l'étude réalisée dans l'*article n°2*, nous avons pu démontrer que le sécrétome des fibroblastes sénescents renforce la TEM et induit les capacités migratoires des kératinocytes néoplasiques PSE. Nous avons également démontré que le récepteur PAR-1 exprimé dans les kératinocytes-PSE est l'intermédiaire de la stimulation de la migration des cellules par les MMPs sécrétées par les fibroblastes sénescents. La surexpression de PAR-1 est associée à l'émergence néoplasique des kératinocytes PSE quelque soit leur conditionnement (KGM, FGM, YF-CM ou SF-CM). Pourtant l'activation de PAR-1 par les MMPs n'est possible que dans les kératinocytes PSE ayant été modifiés par le sécrétome des fibroblastes sénescents (NHEK-PSE /SF-CM). Les données bibliographiques suggèrent que seule la thrombine est capable de se fixer directement à PAR-1 pour l'activer (Vu *et al*, 1991b ; Liu *et al*, 1991). Pour que les autres protéases (ex : les MMPs) puissent activer PAR-1, elles doivent être recrutées par d'autres récepteurs membranaires. C'est pourquoi nous avons formulé l'hypothèse qu'un ou plusieurs partenaires membranaires de PAR-1 pourraient contrôler son activation en régulant le recrutement des MMPs à la membrane des kératinocytes PSE.

Le déroulement du processus migratoire des cellules nécessite l'intervention de différents récepteurs membranaires qui interagissent directement avec le micro-environnement. Ainsi le récepteur à l'acide hyaluronique CD44 et la famille des intégrines sont des acteurs clés de la migration non seulement pour leur capacité à ancrer les cellules aux différents supports matriciels mais aussi pour leur effet sur la régulation des activités métaboliques des cellules. CD44 et les intégrines sont capables (i) de recruter des MMPs, (ii) d'interagir avec d'autres récepteurs membranaires et de participer à leur activation et (iii) de participer au processus de transition épithélium-mésenchyme et migratoire des cellules. Nous avons envisagé que CD44 et/ou les intégrines puissent être des partenaires membranaires de PAR-1 participant à la régulation de son activation spécifique par les MMPs.

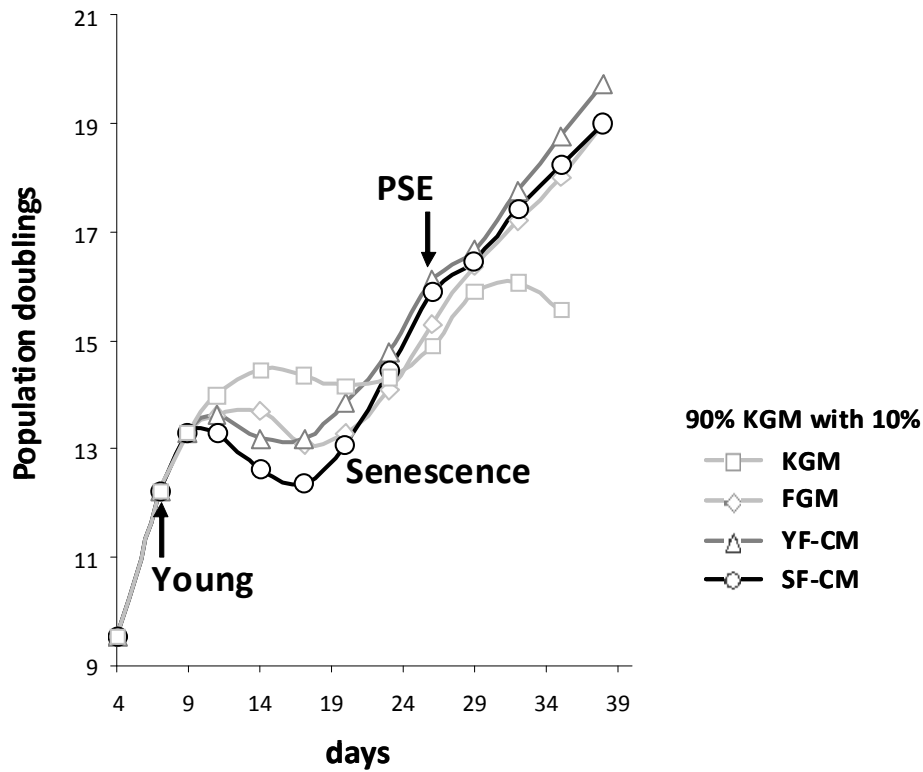
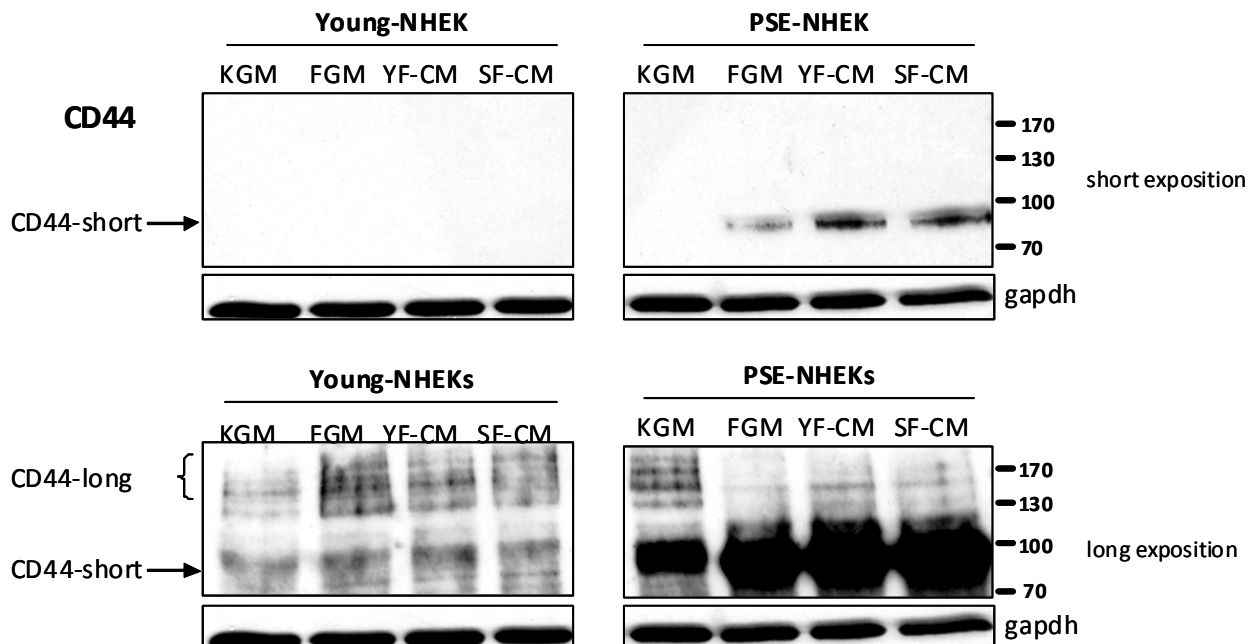
A**B**

Figure 21 : Impact des milieux conditionnés de fibroblastes sur l'expression de CD44 par les kératinocytes jeunes et post-sénescence émergents

(A) Le conditionnement des NHEK par 10% de FGM; de YF-CM et de SF-CM induit une émergence post-sénescence (PSE) exacerbée par rapport au contrôle KGM. Les extractions d'ARN et de lysats protéiques des kératinocytes jeunes et PSE ont été effectuées aux passages indiqués par les flèches dans les 4 conditions de culture. (B) Le western-blot de CD44 (souris anti-CD44 total, cell signalling) sur les lysats protéiques récupérés montre une perte d'expression des isoformes longues de CD44 (CD44-long) dans les NHEK-PSE conditionnés par le surnageant de fibroblastes au profit d'une isoforme courte (CD44-short).

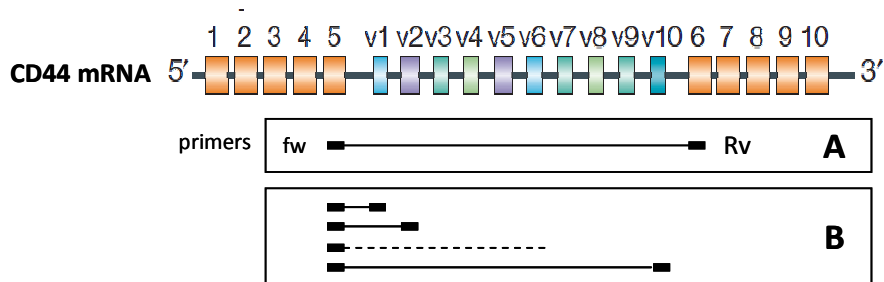
3.1. Rôle de CD44 dans la migration des NHEK-PSE

Nous avons donc commencé par étudier l'expression de CD44 dans les kératinocytes jeunes (Young-NHEK) et post-sénescence émergents (PSE-NHEK) cultivés avec les différents conditionnements décrits dans l'*article n°2* et dans la figure n°20 (figure 21 A).

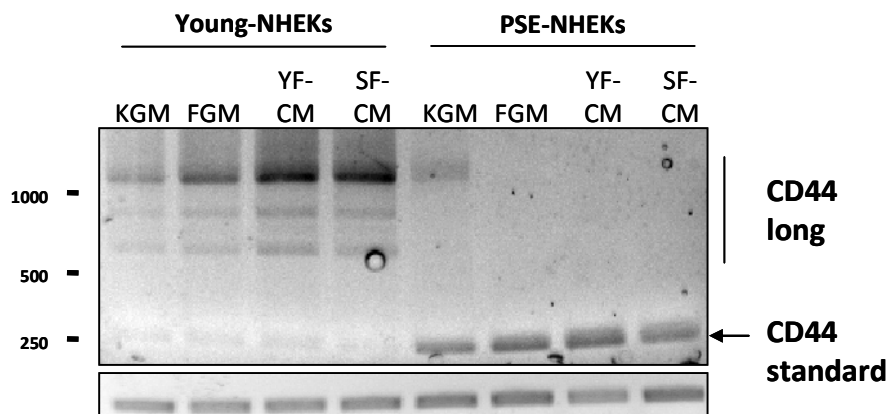
Après avoir extrait des lysats protéiques aux stades jeunes et PSE (au jour marqué par une flèche sur la courbe de croissance de la figure 21 A) au cours de la culture de kératinocytes nous avons réalisé des Western-blot de CD44. Ces analyses montrent des changements radicaux d'expression protéique des isoformes de CD44 provoqués par le conditionnement des NHEK-PSE. En effet les kératinocytes jeunes expriment des isoformes de CD44 de haut poids moléculaires d'environ 150 à 200kDa (CD44 long) (Figure 21 B). L'expression de ces isoformes longues est complètement perdue dans les NHEK-PSE conditionnés au profit d'une isoforme courte (environ 80kDa) (Figure 21B). Cette isoforme courte augmente principalement dans les kératinocytes-PSE conditionnés par les sécrétomes de fibroblastes jeunes et sénescents ce qui corrèle avec l'acquisition des capacités migratoires de ces cellules.

Les nombreuses isoformes de CD44 décrites dans la littérature sont obtenues par épissage alternatif de l'ARNm. La forme minimale (CD44-standard) est composée uniquement des 10 exons constants. 10 exons variants (v1 à v10) peuvent être sélectionnés en différentes combinaisons pour former de nombreuses isoformes appelées CD44-variant. Afin de mieux caractériser les isoformes exprimées dans les kératinocytes nous avons analysé la structure des ARNm de CD44 par RT-PCR.

Dans un premier temps nous avons réalisé des RT-PCR sur de ARNm des NHEK jeunes et NHEK-PSE issus des différents conditionnements en utilisant des amorces sens et anti-sens situées respectivement dans les exons constants n°5 et n°6 qui délimitent le domaine variant (figure 22 A). Trois amplicons de grandes tailles (CD44-long) sont amplifiés par la RT-PCR dans les NHEK jeunes, quel que soit leurs conditions de culture (KGM ; FGM ; YF-CM et SF-CM), tout comme dans les NHEK-PSE cultivés en KGM (figure 22A). La taille des amplicons suggère que les isoformes de CD44 exprimées dans ces cellules contiennent plusieurs exons variants. Les isoformes les plus longues de CD44 sont totalement perdues dans les NHEK-PSE cultivés en présence de FGM, de YF-CM et SF-CM alors qu'un amplicon de taille d'environ 250 pb est amplifié indiquant que le CD44 exprimé par ces cellules ne contient aucun exon variant (CD44-standard) (figure 22A).



A



B

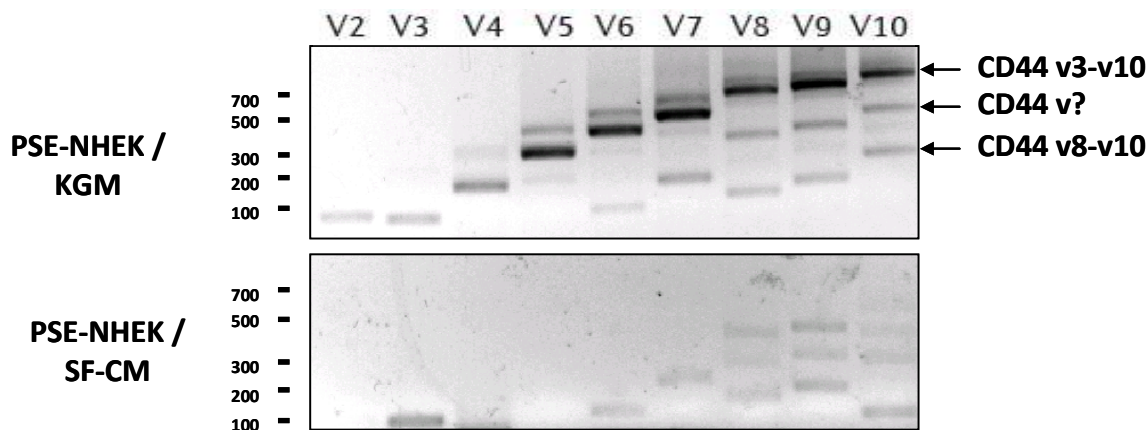


Figure 22 : Identification des isoformes de CD44 exprimées dans les kératinocytes.

(A) Analyse par RT-PCR des ARNm issus des kératinocytes jeunes et PSE co-cultivés (extractions des ARN marquées par des flèches sur la courbe figure 21 A) en utilisant des amorces qui encadrent le domaine variant de CD44 (amorces sens (Fw) situées dans l'exon 5 et anti-sens (Rv) dans l'exon 6). Les ARNm de CD44 des kératinocytes jeunes et NHEK-PSE/KGM contiennent des exons variants (CD44 long) alors que les kératinocytes PSE conditionnés (FGM, YF-CM et SF-CM) expriment un CD44 sans exon variant (CD44 standard). (B) Comparaison de la structure des domaines variants des ARNm de CD44 exprimés par les kératinocytes PSE cultivés en KGM et en présence de 10% de SF-CM. Chaque RT-PCR permet d'identifier la présence d'un exon variant en utilisant l'amorce sens située dans l'exon 5 avec une amorce anti-sens reconnaissant spécifiquement un exon variant (de v2 à v10). La taille des amplicons permet de définir la présence ou non des exons précédents. Les isoformes longues exprimées dans les kératinocytes PSE cultivés en KGM sont principalement CD44v3-v10, CD44v8-v10 et CD44 v ?. L'isoforme CD44v3-v10 est complètement perdue dans les kératinocytes PSE conditionnés par SF-CM.

Dans un deuxième type de RT-PCR nous avons utilisé l'amorce sens reconnaissant l'exon constant n°5 avec des amorces anti-sens reconnaissant chaque exon variant (v2, v3, etc.. jusqu'à v10) nous permettant ainsi de déterminer la présence ou non de chaque exon variant (figure 22B). La taille des amplicons obtenus augmente avec la présence des exons précédents nous permettant de définir la structure des ARNm de CD44. Dans les NHEK-PSE cultivés en KGM, l'isoforme principalement exprimée contient les exons variants v3 à v10 (CD44v3-v10). Deux autres isoformes sont exprimées dont le CD44v8-v10 et une autre dont on ne peut pas définir précisément toute la structure (CD44v ?). Nous confirmons également que ces isoformes sont complètement perdues dans les NHEK-PSE cultivés en présence de surnageant de fibroblastes sénescents (figure 22B).

L'ensemble des résultats obtenus par RT-PCR montre premièrement que les kératinocytes jeunes (quelque soit les conditions de culture) et les kératinocytes PSE cultivés en KGM expriment des isoformes longues de CD44 dont CD44v3-v10 et CD44v8-v10. Ces isoformes sont habituellement associées aux cellules épithéliales et kératinocytes normaux (Brown *et al*, 1991). Deuxièmement l'isoforme courte exprimée dans les kératinocytes PSE conditionnés par les sécrétomes de fibroblastes correspond au CD44 standard. De manière intéressante, il a été montré que la perte d'isoformes longues au profit du CD44-standard est essentielle lors de la transition épithélium-mésenchyme (TEM) de cellules mammaires participant à leur progression tumorale (Brown *et al*, 2011). Le changement d'expression d'isoformes est donc probablement associé au renforcement de la TEM des kératinocytes PSE par le sécrétome des fibroblastes sénescents et pourrait participer aux capacités migratoires de ces cellules.

Afin de démontrer l'implication des isoformes de CD44 dans la migration des kératinocytes PSE néoplasiques nous avons modulé leur expression (figure 24). Premièrement nous avons inhibé l'expression de l'ensemble des isoformes de CD44 par la transfection de si-RNA, dans les kératinocytes PSE cultivés en KGM (NHEK-PSE/KGM) et en présence de milieu conditionné de fibroblastes sénescents (NHEK-PSE/SF-CM) (figure 23A).

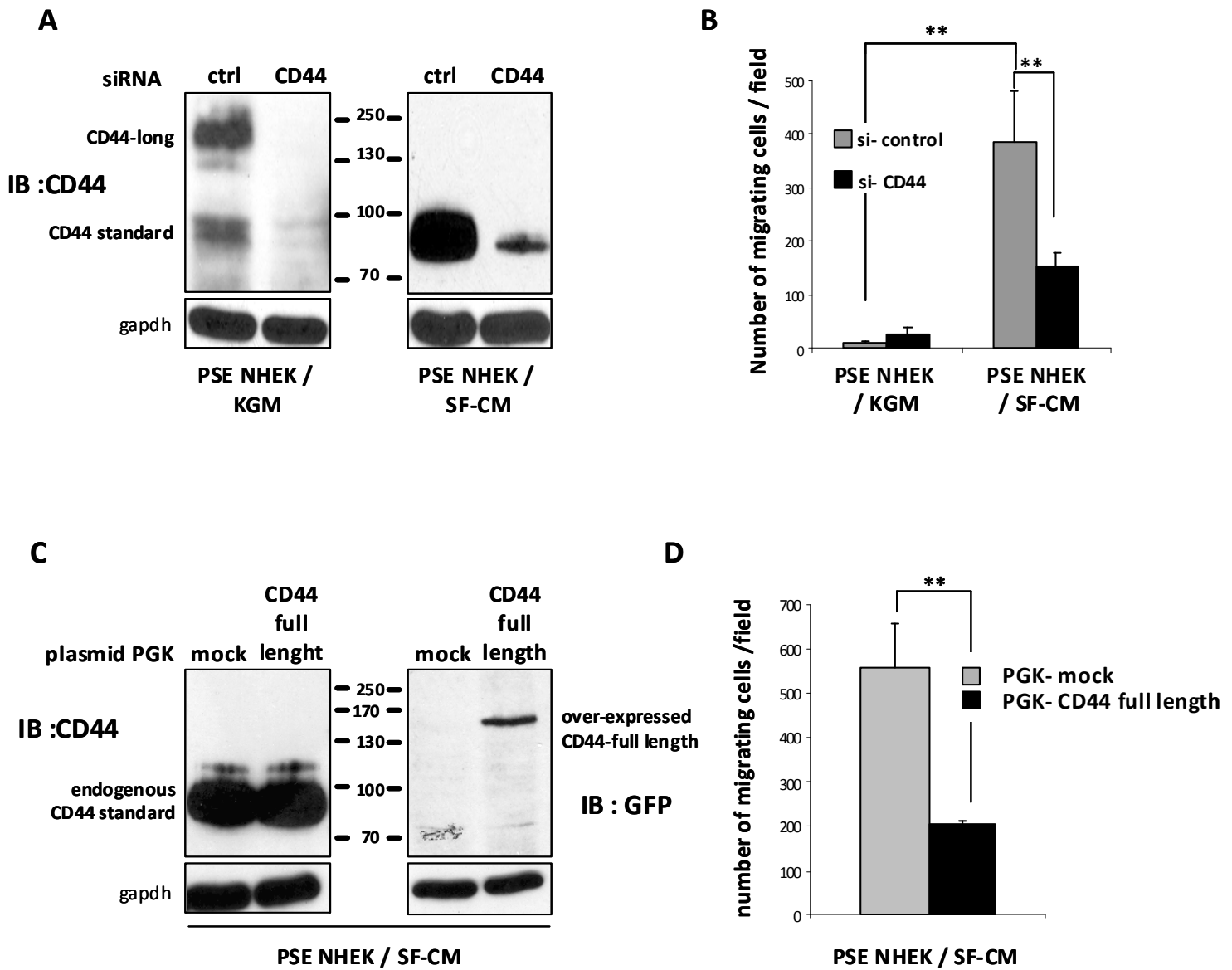


Figure 23: Impact de la modulation d'expression des isoformes de CD44 sur les capacités migratoires des NHEK-PSE

(A) L'inhibition de l'expression des isoformes de CD44 par la transfection de siRNA dirigé contre CD44 (ON-TARGET plus, smart-pool L-009999-00-0005, thermo scientific) (siRNA contrôle = siGenome RISC free, D-001220-01-20) dans les kératinocytes PSE cultivés en KGM (NHEK-PSE/KGM) et avec 10% de milieu conditionné de fibroblastes sénescents (NHEK-PSE/SF-CM) est validé par western-blot (anti-CD44 total, cell signalling). (B) Ces cellules sont ensemencées dans la chambre supérieure de la cupule de migration en transwell® et soumises à l'attraction du milieu conditionné de fibroblastes sénescents pendant 30H. Le comptage des cellules ayant migrées dans la partie inférieure du transwell montre que l'augmentation des capacités migratoires des NHEK-PSE/SF-CM par rapport aux NHEK-PSE/KGM observée dans les conditions siRNA-control est fortement atténuée par la transfection du siRNA dirigé contre CD44. (C) La sur-expression de l'isoforme pleine longueur CD44 (v2-v10) par la transfection (Lipofactamine LTX- Invitrogene) du plasmide PGK-T7-CD44 full-length couplé à la GFP (PGK-CD44 full length) (fourni par le Pr Günthert) dans les NHEK-PSE/SF-CM est validée par Western-blot (souris anti-CD44 total, cell signalling et souris anti-GFP, Santa Cruz). La transfection d'un plasmide PGK-T7-vide (PGK-mock) est utilisée comme témoin négatif. (D) Le test de migration par transwell® (même protocole qu'en B) montre que la sur-expression de CD44 full length réduit les capacités migratoires des NHEK-PSE/SF-CM soumis à l'effet attracteur du milieu conditionné de fibroblastes sénescents.

Nous avons utilisé ces cellules transfectées par les si-RNA dans des tests de migration par transwell® dans lesquels les cellules ont été soumises à l'effet attracteur du milieu conditionné de fibroblastes sénescents. Nous confirmons tout d'abord l'augmentation des capacités migratoires des NHEK-PSE/SF-CM par rapport aux NHEK-PSE/KGM transfectés avec des siRNA-control (non target) (figure 23 B- histogrammes gris). La réduction de l'expression du CD44-standard dans les NHEK-PSE/SF-CM réduit fortement leur capacité migratoire à répondre à un stimulus (figure 23 B- histogrammes noirs). Ce résultat suggère donc que l'expression de l'isoforme CD44-standard est nécessaire à la migration des NHEK-PSE/SF-CM en réponse à l'effet attracteur du sécrétome des fibroblastes sénescents.

Alors que CD44-standard participe au processus migratoire des NHEK-PSE néoplasiques conditionnés par le SF-CM, des données bibliographiques suggèrent des isoformes longues pourraient contrecarrer ces mécanismes (Harrison *et al*, 2006). C'est pourquoi nous avons cherché à ré-exprimer CD44 pleine longueur dans les NHEK-PSE/SF-CM (figure 23 C et D). Pour cela nous avons utilisé un plasmide (PGK-T7) exprimant l'isoforme « full-length » de CD44 (v2-v10) couplé à la GFP généreusement fourni par le Pr Günthert (Université de Basel, Suisse). La transfection du plasmide PGK-CD44 full length, permet d'obtenir des NHEK-PSE/SF-CM exprimant à la fois le CD44-standard endogène et le CD44 full-length exogène (figure 24 C). En test de migration par transwell®, l'expression de l'isoforme full-length suffit à réduire les capacités migratoires des NHEK-PSE/SF-CM en réponse au stimulus du milieu conditionné de fibroblastes sénescents (figure 24 D). Ces résultats suggèrent donc qu'en plus de l'expression du CD44-standard, la perte des isoformes longues de CD44 dans les NHEK-PSE/SF-CM est également nécessaire à l'acquisition de leur capacité migratoire. Nous avons pu montrer que CD44, et plus particulièrement ses changements d'isoformes, est un autre acteur important des processus migratoires précoces des kératinocytes PSE néoplasiques soumis au conditionnement du sécrétome des fibroblastes sénescents.

Néanmoins nous n'avons pas pu encore déterminer si CD44 intervient dans les mécanismes de régulation de l'activation de PAR-1 lors des étapes d'initiation cancéreuse des kératinocytes. Nous pensons que les isoformes longues de CD44 pourraient empêcher l'activation de PAR-1 et/ou que le CD44-standard pourrait favoriser cette activation. Pour vérifier ces hypothèses, nous étudierons l'activation de PAR-1 dans les NHEK-PSE suite à la modulation d'expression des isoformes de CD44 décrites précédemment (inhibition par siRNA et ré-expression de CD44-full length par infection) et la capacité des différents isoformes de CD44 à recruter les MMP-1 et -2 sécrétées par les fibroblastes sénescents.

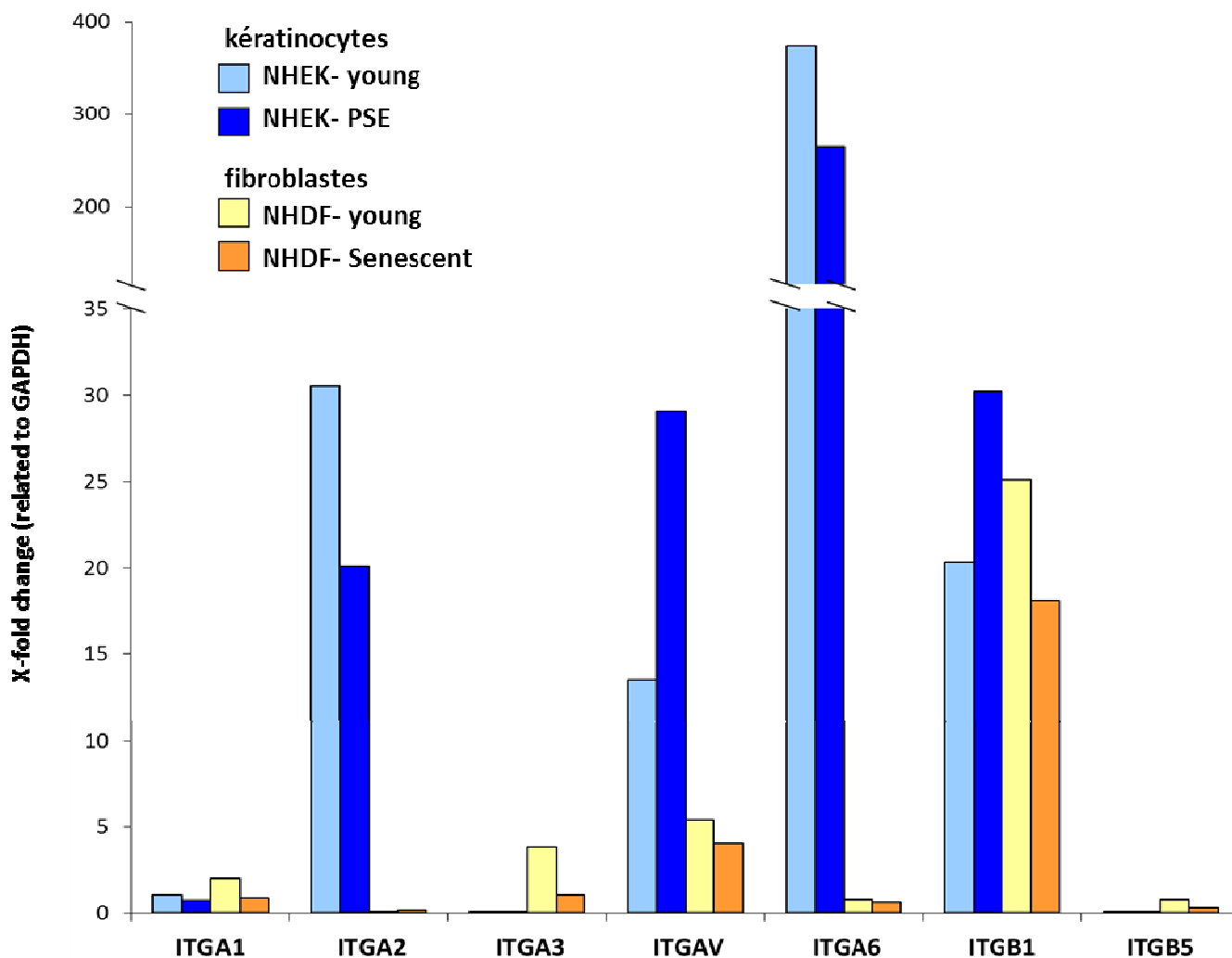


Figure 24 : Comparaison de l'expression des transcrits d'intégrines chez les kératinocytes et les fibroblastes de peau humains.

Analyse de l'expression des transcrits de sous-unités d'intégrines $\alpha 1$ (ITGA1), $\alpha 2$ (ITGA2), $\alpha 3$ (ITGA3), αV (ITGAV), $\alpha 6$ (ITGA6), $\beta 1$ (ITGB1) et $\beta 5$ (ITGB5) par RT- qPCR dans les kératinocytes jeunes et post-sénescence émergents (PSE) cultivés en KGM et des fibroblastes jeunes et sénescents cultivés en FGM. L'expression relative entre les différents gènes est rapportée à la GAPDH.

3.2. Impact du sécrétome des fibroblastes sénescents sur l'expression des intégrines dans les NHEK-PSE

Les intégrines représentent une grande famille de glycoprotéines membranaires (au moins 24 membres décrits chez l'homme) essentielles pour l'ancrage des cellules à la MEC et pour les processus migratoires. Les intégrines sont des hétérodimères composés d'une sous-unité α (18 sous-unités α différentes) et d'une sous-unité β (8). Parmi toutes ces sous-unités, nous avons sélectionné celles dont l'expression est relevante dans les cellules de la peau. Ainsi les intégrines $\alpha 6 \beta 4$ sont les principales responsables de l'ancrage des kératinocytes à la membrane basale (Nguyen *et al*, 2000) alors que les fibroblastes situés dans le derme adhèrent aux collagènes via $\alpha 1 \beta 1$ et $\alpha 2 \beta 1$ (Gailit *et al*, 1993 ; Lange *et al*, 1994).

Afin de mieux caractériser les intégrines exprimées dans les kératinocytes et les fibroblastes de derme nous avons étudié l'expression des transcrits des sous-unités $\alpha 1$, $\alpha 2$, $\alpha 3$, αV , $\alpha 6$, $\beta 1$ et $\beta 5$ par RT-qPCR. Nous avons ainsi comparé les ARNm issus de kératinocytes jeunes et PSE et de fibroblastes jeunes et sénescents cultivés dans leur milieu basal respectif (KGM et FGM) (figure 24). Les principales informations de ces analyses sont que les sous-unités $\alpha 2$ (ITGA2) et $\alpha 6$ (ITGA6), fortement exprimées dans les kératinocytes, sont absentes dans les fibroblastes et qu'inversement les sous-unités $\alpha 3$ (ITGA3) et $\beta 5$ (ITGA5) faiblement exprimées dans les fibroblastes sont absentes dans les kératinocytes cultivés en KGM. Le patron d'expression des transcrits des intégrines est spécifique du type cellulaire et nous pouvons donc utiliser ITGA2 et ITGA6 comme des marqueurs épithéliaux et ITGA3 et ITGA5 comme des marqueurs de cellules fibroblastiques.

Comme les intégrines sont des acteurs clés des processus migratoires normaux et tumoraux, nous avons supposé que certaines d'entre elles pourraient être impliquées dans la stimulation de la migration des kératinocytes néoplasiques conditionnés par surnageant de fibroblastes sénescents. C'est pourquoi nous avons comparé par RT-qPCR l'expression des sous-unités $\alpha 1$, $\alpha 2$, $\alpha 3$, αV , $\alpha 6$, $\beta 1$ et $\beta 5$ dans les NHEK-PSE issus des différentes conditions de culture décrites précédemment (KGM, FGM, YF-CM et SF-CM) (figure 25 A).

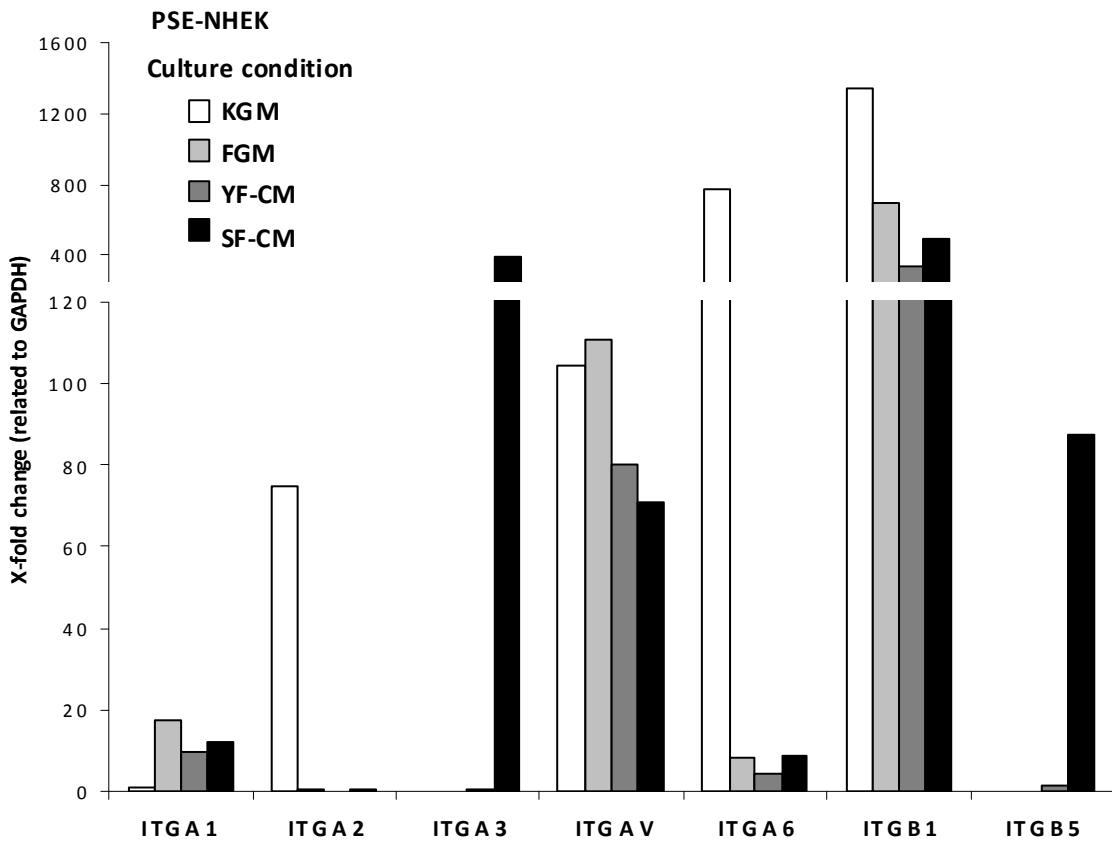
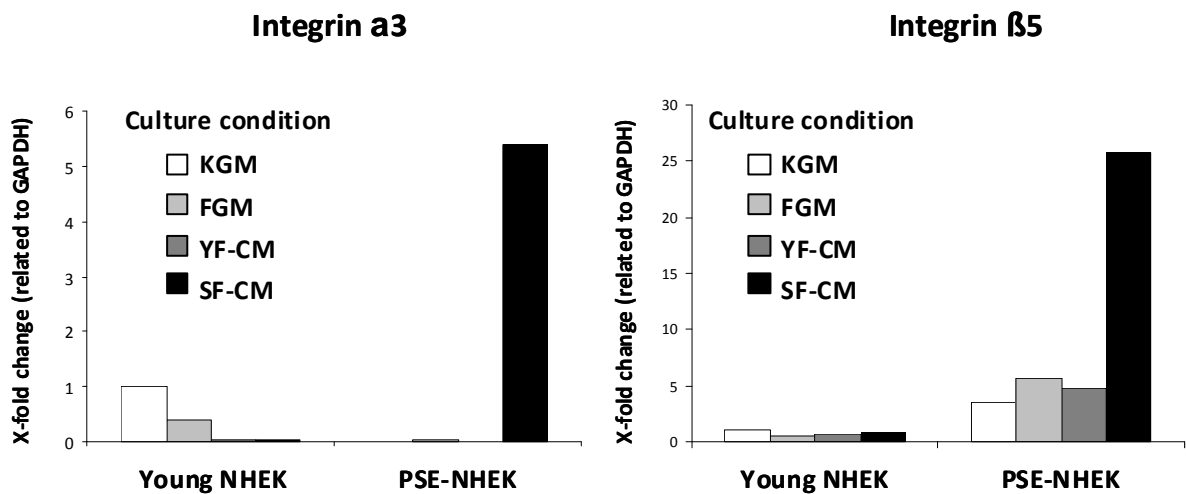
A**B**

Figure 25 : Effet de la co-culture sur l'expression des intégrines dans les kératinocytes PSE

Analyse de l'expression des sous-unités d'intégrines $\alpha 1$ (ITGA1), $\alpha 2$ (ITGA2), $\alpha 3$ (ITGA3), αV (ITGAV), $\alpha 6$ (ITGA6), $\beta 1$ (ITGB1) et $\beta 5$ (ITGB5) par RT- qPCR dans les kératinocytes jeunes et post-sénescence émergents (PSE) cultivés en KGM, avec 10% de FGM, 10% d de milieux conditionnés de fibroblastes jeunes (YF-CM) et sénescents (SF-CM). Les extractions d'ARN des kératinocytes jeunes et PSE ont été effectué au passage indiqué par les flèches de la figure ?. L'expression relative entre les différents gènes est rapportée à la GAPDH.

Premièrement l'expression des sous-unités $\alpha 2$ (ITGA2) et $\alpha 6$ (ITGA6) fortement exprimées dans les kératinocytes cultivés en KGM chute drastiquement avec le conditionnement par le FGM, YF-CM et SF-CM (figure 25A). La chute de l'expression de ITGA2 et ITGA6 semble donc être associée avec le mécanisme de transition épithélium-mésenchyme (TEM) de ces cellules néoplasiques. De plus, l'expression de l'intégrine $\alpha 2\beta 1$ peut inhiber les processus invasifs des cellules cancéreuses (Ramirez *et al*, 2011).

De manière plus intéressante encore, l'expression des sous-unités $\alpha 3$ (ITGA3) et $\beta 5$ (ITGB5) augmente spécifiquement dans les NHEK-PSE conditionnés par le sécrétome des fibroblastes sénescents (figure 25 A et B, histogrammes noirs). Nous confirmons que l'expression de ITGA3 et ITGB5 reste très faible dans les kératinocytes normaux (jeunes) quel que soit leur condition de culture ainsi que dans les kératinocytes PSE cultivés en KGM en FGM et en YF-CM (figure 25 B). ITGA3 et ITGB5 pourraient donc être des acteurs privilégiés associés aux étapes de transition épithélium-mésenchymateuse (TEM) et aux capacités migratoires des NHEK-PSE cultivés en présence de SF-CM.

L'ensemble de ces résultats indique que la co-culture a un impact important sur le patron d'expression des intégrines dans kératinocytes PSE néoplasiques avec notamment la perte d'expression d'intégrines « épithéliales » (ITGA2 et ITGA6) et la sur-expression d'intégrines « fibroblastiques » (ITGA3 et ITGB5). La sous-unité $\alpha 3$ est associée uniquement à $\beta 1$ pour former l'intégrine $\alpha 3\beta 1$ et la sous-unité $\beta 5$ et uniquement associée à αV pour former l'intégrine $\alpha V\beta 5$. Des données bibliographiques indiquent que les intégrines $\alpha 3\beta 1$ et $\alpha V\beta 5$ peuvent être impliquées dans les mécanismes de TEM et dans les processus de migration de cellules normales et tumorales (Kawahara *et al*, 1995; Natali *et al*, 1993 ; Borok, 2009; Kim *et al*, 2009).

Nous pensons que ces intégrines contribuent à la migration des kératinocytes PSE néoplasiques conditionnés par le sécrétome des fibroblastes sénescents et pourraient même intervenir dans les mécanismes de régulation de l'activation de PAR-1. Pour vérifier ces hypothèses, nous devons tout d'abord vérifier l'importance des intégrines $\alpha 3\beta 1$ et $\alpha V\beta 5$ dans la migration des NHEK-PSE par inhibition de ces intégrines (transfection de si-RNA) suivie de tests de migration par transwell. Puis nous étudierons l'impact de l'inhibition de ces intégrines sur l'activation de PAR-1 dans les NHEK-PSE) et sur la capacité à recruter les MMP-1 et -2 sécrétées par les fibroblastes sénescents.

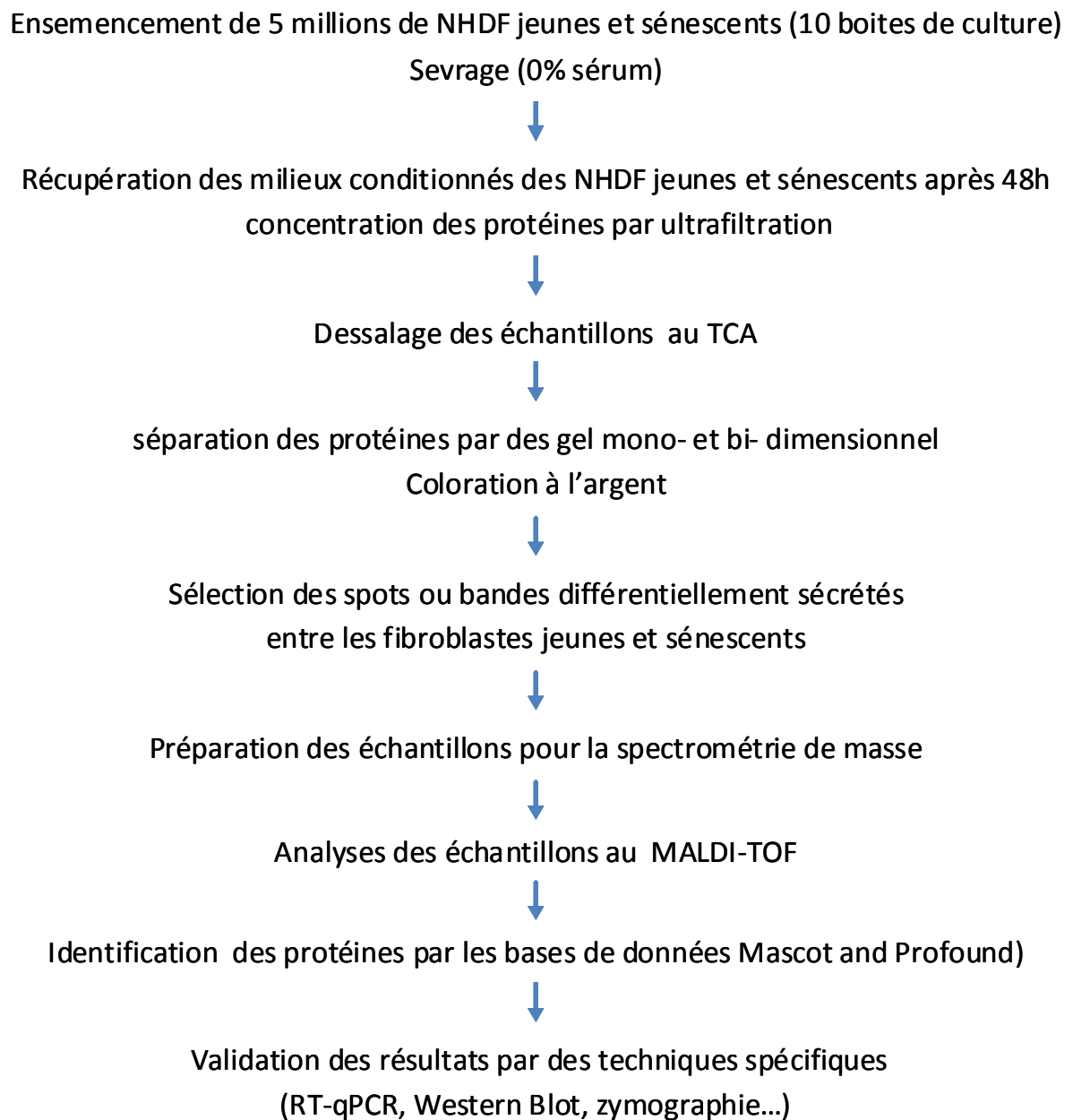


Figure 26 : Principales étapes du protocole expérimental pour la comparaison des sécrétomes de fibroblastes jeunes et sénescents de derme

B. Caractérisation du sécrétome des fibroblastes sénescents de derme

1. Etude comparative des milieux conditionnés de fibroblastes jeunes et sénescents par protéomique.

Afin de mieux comprendre les mécanismes associés aux contributions du sécrétome des fibroblastes sénescents de derme sur l'initiation cancéreuse des kératinocytes, il nous semblait important de bien caractériser les composantes moléculaires de ce secretome. Lorsque nous avons débuté ce projet, il existait peu de données bibliographiques sur les spécificités des sécrétomes de fibroblastes sénescents notamment de la peau. L'une des premières études globales sur les changements d'expression survenant lors de la sénescence a été réalisée en 2006 sur dans un modèle de fibroblastes prostatiques en utilisant des microarray de cDNA (Bavik *et al*, 2006). Elle a ainsi montré la sur-expression de facteurs de croissance (HGF/SF ; FGF-7...), de cytokines (IL-8, Gro-1...) et de protéases (MMP-2...).

Nous souhaitons décrire les changements survenant dans le sécrétome des fibroblastes de derme humains (NHDF) sénescents de manière globale. Nous avons choisi une approche expérimentale de protéomique pour comparer les milieux conditionnés de fibroblastes jeunes et sénescents puis analyser les différences par spectrométrie de masse. Les principales étapes de la démarche expérimentale sont résumées figure 26.

Par cette approche complexe nous espérons mettre en évidence dans les sécrétomes de NHDF sénescents des facteurs impliqués dans les interactions épithélium-stroma tels que des facteurs de croissance et des cytokines. Mais nous nous sommes heurtés à plusieurs difficultés :

- Obtenir 5 millions de NHDF sénescents
- Obtenir suffisamment de protéines sécrétées dans les milieux conditionnés
- Forte perte de quantité de protéines lors des étapes de préparation des échantillons pour la protéomique (précipitation au TCA)
- Quantité de protéines trop faible pour identifier des facteurs peu sécrétés
- Nombre de gels insuffisant pour réaliser des études statistiques.

Bien que toutes ces difficultés ne nous aient pas permis de mettre en évidence des facteurs de croissance ou des cytokines, nous avons quand même réussi à identifier des protéines parmi les plus sécrétées. En particulier nous avons pu montrer de manière originale et inédite un défaut de sécrétion de plusieurs glycoprotéines aux propriétés anti-tumorales dans les fibroblastes sénescents de derme.

Ces résultats font l'objet de l'*article n°3* actuellement en préparation : *ER-stress signalling pathway ATF-6 is associated to the pro-tumoral secretome of senescent dermal fibroblasts.*

Article n°3

*ER-stress signalling pathway ATF-6
is associated to the pro-tumoral secretome
of senescent dermal fibroblasts.*

**ER-STRESS SIGNALLING PATHWAY ATF-6
IS ASSOCIATED TO THE PRO-TUMORAL SECRETOME
OF SENESCENT DERMAL FIBROBLAST**

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ABSTRACT

Cellular senescence of fibroblasts is characterised by an irreversible growth arrest. However senescent fibroblasts could contribute to the tumoral progression of carcinoma by a specific secretome. In the skin model, we have previously demonstrated that senescent normal human dermal fibroblasts (NHDF) promote the early transformation of neoplastic keratinocytes. But the molecular characteristics of the senescent NHDF secretome are not well described. In this study we have investigated the pro-tumoral specificity of the senescent NHDF secretome.

We first showed that senescent NHDF secretome shares common pro-inflammatory features with those previously described for other tissues (breast, prostate...), in particular the secretion of pro-inflammatory cytokines like MCP-1 and IL-6. By a comparative proteomic analysis of young and senescent conditioned media, we showed that senescent fibroblasts present severe reduction of the secretion of several glycoproteins with anti-tumoral properties: Decorin, Secreted Protein Acidic and Rich in Cysteine (SPARC), and Pigment Epithelium-Derived Factor (PEDF). These alterations of glycoproteins secretion by senescent fibroblasts is correlated to the loss of SPARC and decorin expression in aged human skin samples.

Although the secretion of SPARC and decorin is altered in senescent fibroblasts conditioned media their expression within the cell lysates is not modified compared to the young fibroblasts. Senescent fibroblasts display activation of endoplasmic reticulum (ER) stress signaling and unfolded protein response (UPR) pathways. Those pathways were associated to the re-localisation of SPARC and DCN in lysosomes and the expression of pro-inflammatory cytokines (MCP-1; IL-6...). Inhibition of the ATF-6 pathways in senescent fibroblasts by siRNA transfection restores SPARC and Decorin secretion generating a young fibroblasts-like secretory phenotype. Thus we have highlighted ER-stress as contributing to the secretory phenotype of senescent dermal fibroblasts which could participate to this pro-tumoral potential

INTRODUCTION

Cellular microenvironment plays critical roles in epithelium homeostasis, wound healing and carcinoma initiation and progression (Maas-Szabowski et al., 1999; Matsumoto et al., 1990; Shirakata, 2010; Werner and Smola, 2001). While a normal and healthy microenvironment may prevent tumor progression of epithelial neoplastic cells and can revert tumor cells to a normal phenotype (Bissell and Hines, 2011; Bussard et al., 2010; Javaherian et al., 1998; Zhang et al., 2005), a peri-tumoral stroma could enhance the growth and invasion of cancerous cells (Fromigue et al., 2003). Carcinoma-associated fibroblasts (CAFs) produce a pro-inflammatory secretome with enhanced secretion of growth factors, cytokines and matrix proteinases (secretome named Senescence-Associated Secretory Phenotype - SASP). This highly inflammatory secretome could stimulate *in vitro* and *in vivo* growth and invasion of tumor-initiated epithelial cells (Ao et al., 2007; Olumi et al., 1999).

The risk to develop a carcinoma, the most frequent human cancers, is strongly correlated to advancing age (NCI and WHO data). Aging is characterized by alteration of tissue function, structural disorganization and by the increasing risk of developing age-associated diseases (Fenske and Lober, 1986; Kurban and Bhawan, 1990). Aging tissue alterations are caused by the accumulation of senescent cells (Dimri et al., 1995). Cellular senescence is essentially characterized by an irreversible growth arrest with activation of numerous tumour-suppressor genes (p53, p16, p21...) suggesting that mechanism as a potent barrier to tumorigenesis (Benvenuti et al., 2002; Campisi, 2001; Hayflick, 1965). Although blocked in the cell cycle, senescent cells remain metabolically active and produce a complex pro-inflammatory secretome composed of growth factors, cytokines and metalloproteinase

(Bavik et al., 2006; Benvenuti et al., 2002; Coppe et al., 2010; Coppe et al., 2008). Therefore senescent fibroblasts establish a chronic inflammatory microenvironment that could enhance cancer cells growth and invasion (Krtolica et al., 2001). However, the exact molecular pathways mediating the inflammatory process in the micro-environment remain unclear.

Emerging evidences from experimental systems and animal models highlighted endoplasmic reticulum (ER) signalling, an organelle responsible for protein folding, quality control and trafficking as influencing inflammatory pathways (Hotamisligil, 2010; McGuckin et al., 2010). Perturbations of the ER functions by micro-environmental stress result in an excess of misfolded proteins in the ER lumen. ER stress have been linked to several physiological and pathophysiological conditions including cancer, diabetes, infectious diseases (Yoshida, 2007). Alternatively, eukaryotic organisms can activate a pathway named the Unfolded Protein Response (UPR) to dampen stress conditions that compromise protein processing and folding in the ER and to restore correct protein expression (Schroder and Kaufman, 2005). The UPR response is induced, at least, by three ER sensors: PERK, ATF6, and the evolutionary conserved kinase/endoribonuclease IRE1 (Ron and Walter, 2007). IRE1 activation elicits its endogenous endoribonuclease activity to cleave the XBP1 mRNA by unconventional splicing. This reaction is required for translation of a transcriptionally active XBP1, activating a subset of ER chaperones and folding catalysts (Yoshida et al., 2001) distributed in three UPR branches. The UPR leads to the activation of JNK-AP1 and NF- κ B pathways through multiple and distinct mechanisms triggering expression of pro-inflammatory genes (Deng et al., 2004); (Hu et al., 2006). Although senescent fibroblasts are known to induce a chronic inflammatory environment during senescence (Coppe et al., 2010), the contribution of the UPR to the senescence and particularly to the inflammatory SASP phenotype onset has never been investigated yet.

In our laboratory, we demonstrated the impact of aging microenvironment to the early step of skin carcinoma development. Indeed senescent human dermal fibroblasts secretome promote the early transformation and migration of neoplastic keratinocytes (submitted). Here, we show that human dermal senescent fibroblasts (NHDF) secretome shares common features with those previously described for other tissues (Bavik et al., 2006; Coppe et al., 2008). Beside strong over-expression and activation of metalloproteinases that we already associated to the promotion of early carcinogenesis events, comparative proteomic analyses revealed an alteration of extra-cellular matrix (ECM) expression by senescent fibroblasts. Moreover, we have observed for the first time that senescent fibroblasts present severe reduction of the secretion of several glycoproteins with anti-tumoral properties: Decorin, Secreted Protein Acidic and Rich in Cystein (SPARC), and Pigment Epithelium-Derived Factor (PEDF). The analysis of the secretion defect highlighted alteration of the secretion rather than expression of SPARC, Decorin and PEDF in senescent fibroblasts. We demonstrated that senescent fibroblasts display activation of endoplasmic reticulum (ER) stress signaling and unfolded protein response (UPR) pathways. Those pathways were associated to the re-localisation of SPARC and DCN in lysosomes and the expression of pro-inflammatory cytokines (MCP-1; IL-6...). Inhibition of the ATF-6 pathway in senescent fibroblasts by siRNA transfection restores SPARC and Decorin secretion generating a young fibroblasts-like secretory phenotype. Thus, we have highlighted ER-stress as contributing to the secretory phenotype of senescent dermal fibroblasts which could participate to this pro-tumoral potential.

MATERIAL AND METHODS

Cell culture

Normal human dermal fibroblasts (NHDFs, CC-2511) were purchased from Clonetics. NHDFs were grown in FGM-2 BulletKit medium®, herein designated FGM (CC-3132, Clonetics). Senescence was monitored on growth curves and validated at the morphological and biochemical level (senescence-associated-β galactosidase assay) (Dimri et al., 1995). Cultures were always split at 70% confluence and the number of population doublings (PD) was systematically determined as follows: $PD = \ln(\text{number of collected cells}/\text{number of plated cells})/\ln 2$.

Preparation of fibroblast-conditioned media.

Young (~10 PDs) and senescent (~50 PDs) NHDF were seeded in ten 100 mm dishes ($5 \cdot 10^5$ cells per dish). After 12 hours, they were incubated in serum-free conditioned media (5 ml of FBM per dish) for 48 hours. The conditioned media was filtrated (0, 45 μm) and concentrated by an ultra-filtration system with a cut-off of 3kDa (Amicon®, Millipore). Protein concentration was measured with the Bio-Rad protein assay.

Protein separation and silver staining.

An equal quantity of proteins from young and senescent fibroblast-conditioned media (approximately 15 μg) was loaded and separated on SDS-PAGE gels with different percentages of acrylamide (7, 10 and 12%). The analytical gels were silver-stained according to the protocol previously described by *Shevchenko et al* (*Shevchenko et al., 1996*) with minor modifications. Briefly, gels were fixed overnight in 30% ethanol (v/v) and 5% acetic acid

(v/v) followed by 4 washes of 18 MΩ-H₂O. Gels were sensitized in 0.02% sodium thiosulfate (w/v), followed by two washes in 18 MΩ-H₂O, and then incubated in 0.2% silver nitrate (w/v) for 30 min. Proteins were then visualized using developing solution (0.028% formalin (v/v), 0.0125 sodium thiosulfate (w/v), 2.4% sodium carbonate (w/v) until an appropriate level of staining was achieved, after which reaction was stopped by adding 10% acetic acid (v/v).

Protein Identification by Mass Spectrometry.

Bands corresponding to differential secreted proteins from concentrated conditioned media of young or senescent fibroblasts were manually excised from a preparative silver-stained gel as previously described and washed with ultrapure water until totally destained. Bands were washed for 15 min with 100 μL of 50 mM ammonium bicarbonate solution and then twice for 15 min with 50 mM ammonium bicarbonate/50% ACN. They were then dried after adding 100 μL of ACN for 10 min. After the supernatant was discarded, tubes were left open for 10 min to complete solvent evaporation. Trypsin digestion was performed as previously described (Acosta-Martin et al., 2009). The spots were rehydrated with a solution containing Trypsin Enhancer (Promega) in 50 mM ammonium bicarbonate and 3 μL of 40 μg/mL Trypsin Gold (Promega) in 50 mM acetic acid. After overnight digestion at 37°C, peptide extraction was carried out in two steps according to the manufacturer's protocol. For acquisition of the mass spectra of the extracted and desalted peptides, we mixed 0.5 μL of the peptide solution with 0.5 μL of matrix solution (5 mg/mL of α-cyano-4-hydroxycinnamic acid dissolved in 0.1% TFA / 50% ACN) on the MALDI-TOF MS target. External calibration was performed with a peptide mixture resulting from the tryptic digest of BSA (0.5 μg/mL). MALDI-TOF MS was then performed with a Voyager DE STR mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with a 337.1 nm nitrogen laser and a delayed extraction facility (125 msec). All spectra were acquired in a positive ion reflector

mode under 20 kV voltage, 61% grid. Typically, 300 laser shots were recorded per sample. The mass spectra were then calibrated before protein identification by peptide mass fingerprinting, conducted by running the MASCOT web searcher (<http://www.matrixscience.com/>, Matrix Science, UK) against the NCBI nr 20100312 (10570301 sequences; 3602205473 residues) with the following parameters: Fixed modifications: Carbamidomethyl (C) and variable modifications: Oxidation (M); Peptide Mass Tolerance: ± 50 ppm; Peptide Charge State: 1+; Max Missed Cleavages: 1.

Western blotting

For preparation of cell lysates, an equal number of cells were seeded in each condition dish. After overnight, cells were directly lysed in loading denaturing Laemli buffer. An equal volume of this buffer was loaded in SDS-PAGE gel (Euromedex) (loading control was checked by GAPDH). To prepare conditioned media sample, an equal number of cells were seeded in dish and incubated in FGM during 48h. The conditioned media were filtrated (0, 45 μ m) and concentrated by an ultra-filtration system with a cut-off of 3kDa (Amicon®, Millipore). In parallel, the number of cells was evaluated (Thoma counting chamber). Volume of conditioned media corresponding to equivalent numbers of cells were loaded on the gel (loading control was checked by coomassie blue staining of a duplicate gel). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Hybond-C Extra, Amersham). Primary antibodies were: mouse anti-human SPARC (R&D systems); mouse anti-human Decorin (Santa cruz biotechnology), mouse anti-mouse GAPDH (Santa cruz biotechnology). Secondary antibodies were peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Peroxidase activity was revealed with the ECL (enhanced chemiluminescence) kit from Amersham Biosciences.

Lysotracker staining

Lysotracker (Molecular Probes) was added in cell culture medium (dilution 1:1000) at 37°C for 30 minutes as recommended by the supplier. Cells were rinsed in ice-cold PBS, fixed with 4% paraformaldehyde in PBS and used for immunofluorescence or directly observed on microscope.

Immunofluorescence

NHDF seeded on coverglasses were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton-X100. After 3 washes in PBS, non-specific sites were blocked with 2% of BSA in PBS during 1H. Slides were incubated with a primary antibody: mouse anti-human SPARC (R&D systems), mouse anti human Decorin (Santa Cruz biotechnology), rabbit anti human Giantin (Abcam), rabbit anti human Calnexin (Abcam). Cells were then washed 3 times with 0,05% Tween in PBS and incubated with the secondary antibody: Rhodamine Red-conjugated anti-Mouse IgG or FITC Green-conjugated anti-Rabbit IgG (Jackson ImmunoResearch Laboratories). Nuclei were stained by Hoechst 33258 at 1 µg/ml for 5 mn. Images from fluorescence experiments were taken using an Axioplan2 (Zeiss, Germany) microscope equipped with an Apotome device and either Plan Neofluar (5x, NA: 0.16).

IRE-1, PERK and ATF-6 knockdown by RNA interference

Senescents NHDF were seeded at 150,000 cells per well in six-well plates. Twenty nM of pools of 4 siRNAs were used for inhibition of IRE-1, PERK or ATF-6 (on-target plus SMARTpool®, respectively L-004951-02-0005; L-004883-00-0005; L-009917-00-0005, Dharmacon). Specific and control transfections (siGENOME RISC-Free Control siRNA,

Dharmacon) were prepared as advised by the suppliers and performed with Lipofectamine™ RNAiMAX transfection reagent (Life Technologies) in opti-DMEM (Gibco). After 6-h incubation at 37°C, the transfection medium was replaced with normal cell medium (FGM). Where applicable, supernatants or cell lysate proteins were collected and RNA was isolated from the cells with the Nucleospin® kit (Macherey-Nagel) after 72 h. The siRNA transfection efficiency was checked by qRT-PCR.

Quantitative reverse-transcription and real-time PCR.

RNA was isolated from cultured cells with the Nucleospin® kit (Macherey-Nagel). For reverse transcription, 1 µg of total mRNA was used in a reaction with the high capacity cDNA reverse transcription kit (Applied Biosystems). The primers used (see supplemental Materials, Table 1) were designed with the help of the NCBI-Primer-BLAST software at <http://blast.ncbi.nlm.nih.gov/>. The PCR protocol was that recommended for the Mx3005P Real-time PCR System® (Stratagene). Accumulation of PCR products was measured by SYBR green® fluorescence (SYBR Green® master mix; Applied Biosystems). Raw data analysis was performed with the MxPro® software (Agilent). The number of cycles required to reach the threshold level of PCR product (Ct) was determined. For each sample, Ct(gene) - Ct(h-gapdh) was calculated, and this value was used to calculate the ratio of test gene mRNA to the GAPDH internal control mRNA.

Immunohistochemistry

Deparaffinized and rehydrated sections of skin biopsies from young (ages 26-38 years) or old (60-89 years) human healthy donors were treated by 1% H₂O₂ in PBS to block endogenous peroxidases. Aspecific binding was prevented by incubation in PBS + 5% BSA and 10%

rabbit serum. Endogenous avidin and biotin were inhibited using the specific Avidin/Biotin Blockin Kit from Vector Labs. Primary (Monoclonal mouse anti-SPARC, R&D Systems, 1/100; Monoclonal mouse anti-Decorin, Santa Cruz Biotechnology, 1/100) and secondary (Biotinylated rabbit anti-mouse, Dako, 1/100) antibodies were respectively diluted in PBS + 5%BSA+ 10% rabbit serum and PBS+2% BSA. Control was run in a serial section using isotype-specific primary antibody (Santa Cruz Biotechnology). Detection was amplified using streptavidin-peroxydase (Jackson Immunoresearch), and staining was performed using DAB detection Kit (Vector Labs). Slices were counterstained with Gill's hematoxylin in tap water and mounted in Dako Glycergel®. Scale bar=50µm.

RESULTS

Characterization of human senescent dermal fibroblasts secretome.

Secretion of pro-inflammatory cytokines is among the hallmarks of senescence-associated secretory phenotype (SAPS) already described for prostatic and breast senescent fibroblasts (Bavik et al., 2006); (Coppe et al., 2008). In order to confirm that enhanced cytokines expression was conserved in the secretome of senescent normal human dermal fibroblasts (NHDF), we performed cytokines array analysis of young versus senescent fibroblasts conditioned media. As expected, several cytokines (MCP-1, IL-6, IL-8, MIF, Gro - 1...) are commonly strongly secreted by senescent fibroblasts, confirming the pro-inflammatory potential of their secretome (supplemental figure 1).

To complete the identification of factors potentially implicated in the contribution of aging micro-environment to the initiation and progression of skin carcinoma, we performed the proteomic analyses of the conditioned media of young (YF-CM) and senescent (SF-CM) human dermal fibroblasts (NHDF). Concentrated YF-CM and SF-CM have been separated in mono-dimensional poly-acrylamide gel electrophoresis at different concentrations of acrylamide (7%, 10% and 12%) (supplemental. figure 2). Generally, most expressions of secreted proteins were decreased in SF-CM compared to YF-CM, suggesting an important alteration of the dermis aging skin composition (supplemental. figure 2). Several bands of interest were selected and the corresponding peptides were analysed by mass spectrometry (MALDI-TOF).

Data bases (MASCOT and Profound) analyses of the peaks led to a list of candidate proteins with significant scores and therefore identified as differentially secreted between YF-CM and SF-CM (table 1). This list confirms the presence of extracellular matrix (ECM) components expressions alterations in SF-CM, particularly a decrease in collagen type I and VI and

laminin beta 2 (table 1). These alterations in the matrix components were concomitant with increased of proteinases activities. Indeed, the secretion of the major interstitial collagenase, MMP-1, was enhanced in SF-CM confirming our previous description of enhanced MMPs expression and activation in SF-CM (submitted). On the contrary, the protease inhibitors like tissue inhibitor of metalloproteinase-1 (TIMP-1) and plasminogen activator inhibitor-1 (PAI-1) were decreased (table 1). The decrease of PAI-1 and TIMP-1 secretion in SF-CM was confirmed by 2D-gels with significant scores (data not shown).

Altogether, these results showed that senescent fibroblasts display altered secretion of fibrillar components and an increase in remodelling potential of ECM. These results are in agreement with the strong alteration of extra-cellular matrix composition and destructuration observed in aging skin dermis (Mine et al., 2008).

Protein name		n° band	Protein database accession No.	Secretion by SF/YF	M (kDa) experimental (theoretical)	Pro-found scores (Z)	Mas-cot scores	% sequence coverage	No of matched peptide (total)
Secreted Protein, Acidic, Cysteine-Rich	SPARC	14	P09486	decrease	35 (40)	2	61	17	5 (28)
Pigment epithelium-derived Factor	PEDF	11, 12	P36955	decrease	44 (45)	2,37	152	23	11 (14)
Decorin	DCN	10	P07585	decrease	110 (40)	2,3	102	29	8 (21)
collagen, type VI, alpha 1	COL6A1	1, 9	P12109	decrease	108 (140)	2,37	147	13	12 (16)
collagen, type I, alpha 2	COL1A1	2, 5, 15	P02452	decrease	90 (94)	2,3	123	35	22 (65)
laminin beta 2 chain; S-laminin	LAMB2	9	P55268	decrease	204 (200)	2,26	77	7	12 (26)
matrix metalloproteinase 1 preproprotein	MMP-1	3	P03956	increase	55 (54)	2,36	233	38	18 (28)
Plasminogen Activator Inhibitor Type-1	PAI-1	4, 13	P05121	decrease	48 (43)	2,29	148	36	15 (59)
tissue inhibitor of metalloproteinase 1	TIMP-1	6, 16	P01033	decrease	27 (24)	1,77	41	20	5 (21)
Human Factor D, Complement Activating Enzyme	CFAD	8	P00746	decrease	24 (24)	1,78	70	25	7 (35)
triosephosphate	TPI1	17	P04789	decrease	27 (24)	1,22	74	21	7 (41)
manganese superoxide dismutase	MnSOD	18	P04179	increase	22 (21)	2,38	75	25	5 (11)
C-type lectin domain family 3, member B precursor - Tetranectin	CLEC3B - TETN	19	P05452	decrease	23 (20)	0,85	66	19	5 (23)
Ras-related protein Rab-1B	RAB1B	24	Q9H0U4	increase	18,5 (18)	0,9	66	34	6 (44)

Table 1

FIGURE LEGENDS

Table 1: Senescence-associated secretome of normal human dermal fibroblasts.

Table recapitulating the identification of proteins differentially secreted in young and senescent fibroblasts conditioned media after mass spectrometry (MALDI-TOF) analysis. Proteins contained in conditioned media were separated in mono-dimensional gels, silver stained and peptides excised from differentially expressed bands were prepared for mass spectrometry analysis. Proteins listed were identified with significant scores by Mascot and/or Profound databases (Mascot scores greater than 65 are significant ($p < 0.05$). Profound scores (Z-score) corresponding to the percentile of the search in the random match population (Z= 1.645- 95%; Z= 2.32 - 99.0%; Z= 3.0 - 99.9%)). Number of bands corresponds to the bands excised from silver-stained gels (supplemental figure 2). Two independent experiments were performed.

Senescent dermal fibroblasts show strong reduction of SPARC, decorin and PEDF glycoproteins secretion.

In addition to the altered expressions of ECM components, proteomic analysis highlighted the loss of three secreted glycoproteins in senescent fibroblasts conditioned media (SF-CM): Secreted Protein Acidic and Rich in Cystein (SPARC), Decorin and Pigment Epithelium-Derived Factor (PEDF) (table 1). In normal conditions, these secreted glycoproteins display important functions in the organization of the dermis, and present several anti-tumoral functions with direct (anti-proliferation, pro-differentiation, inhibitor of migration...) and indirect (anti-angiogenesis...) effects on cancerous cells. The loss of SPARC (figure 1A), Decorin (figure 1C) and PEDF (supplemental. figure 3A) secretions in senescent fibroblast conditioned media (SF-CM) was confirmed by western-blot.

Contrarily to PEDF, alteration of SPARC and decorin expression in aged human skin has never been documented (Francis et al., 2004). Investigating SPARC and decorin expressions in skin biopsies of young and aging healthy donors we detect a strong staining of SPARC in epidermis and around the cells of the dermis in young donor samples according to the importance of SPARC for cell-matrix interactions (figure 1B). In old skin tissue, however, SPARC expression is totally loss in epidermis and in most cells in dermis (figure 1B). Decorin was also strongly expressed in the whole extracellular matrix of the dermis in young skin while this expression was completely absent or very faintly detected around the basement membrane in old skin (figure 1D and data not shown). Although less present in SF-CM compared to YF-CM as depicted in western-blot analysis, we could not recorded differential expression of PEDF in young and aged samples (data not shown).

The loss of SPARC, Decorin and PEDF glycoproteins secretion by senescent fibroblasts could contribute to dermis alteration during ageing and to tumoral initiation and progression of skin carcinoma and melanoma.

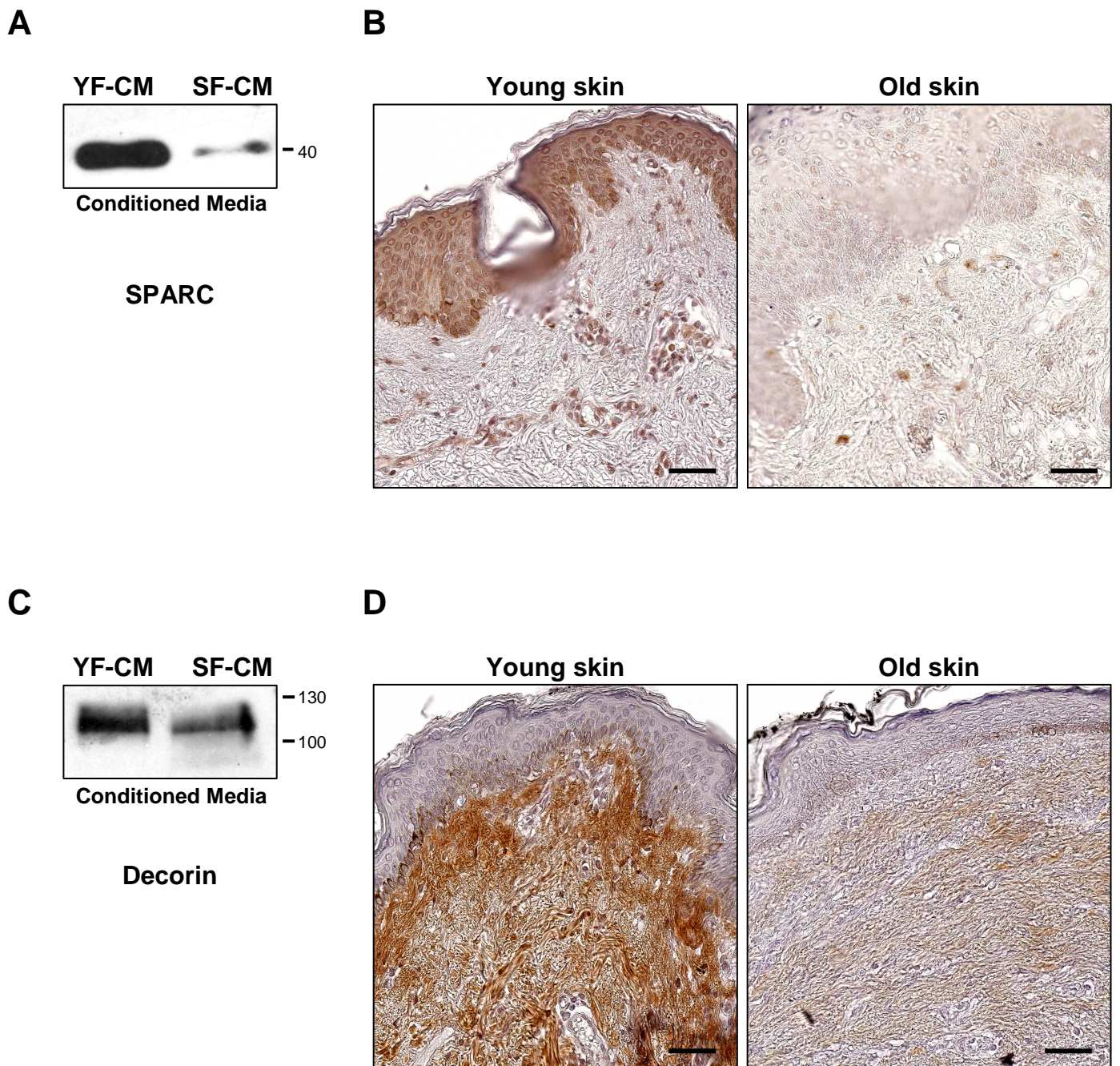


Figure 1

FIGURE LEGENDS

Figure 1: Alteration of SPARC and Decorin glycoproteins secretion in senescent dermal fibroblasts and aged human skin sample.

Western-blot analysis of SPARC (A) and decorin (C) proteins on young (YF-CM) or senescent (SF-CM) fibroblasts conditioned media showed a strong decreased secretion of these glycoproteins in SF-CM compared the YF-CM. Loss of SPARC (B) and decorin (D) expression in old human skin was demonstrated by immunohistochemistry analysis. Representative images of human skin sample obtained from young (n=4; aged 29 to 38 years) and old (n=8, aged 60 to 89 years) donors were captured with Axioplan2 microscope system (Zeiss) (axiovision software-mosaic program). Scale bar = 50 μ M

Even if expressed, non-secreted SPARC and Decorin are re-localized in lysosomal vesicles in senescent fibroblasts.

Western-blot analysis of SPARC, decorin and PEDF expression in cell lysates of young (YF-cells) and senescent (SF-cells) fibroblasts highlighted correct expression of the regular forms of SPARC (43kDa), decorin (150kDa) and PEDF (45kDa) in both cell types without difference (figure 2A and supplemental. figure 3B). However, shorter forms of SPARC (37 kDa) and decorin (45kDa) were promoted only in SF-cell (figure 2A).

To clarify the apparent contradiction between SPARC and decorin expressions in cell lysates and their strongly reduced secretions in senescent fibroblasts conditioned media, we investigated their subcellular localization by immunofluorescence comparatively in young and senescent fibroblasts. SPARC or decorin staining were specifically spread in vesicles in senescent fibroblasts (figure 2B and 2D). 50 to 60% of senescent fibroblasts displayed an accumulation of SPARC- or decorin-containing vesicles compared to less than 10% of young cells (figure 2C and 2E). Moreover, fibroblasts presenting SPARC- or decorin-vesicles accumulation presented 53BP1 foci in their nucleus, a marker of senescence (data not shown). We could not record PEDF apparent sequestration in vesicles in senescent fibroblasts, may be due to its very low expression in cell lysates (supplemental figure 3 B and C).

In order to clarify the mechanisms inducing the vesicular sequestration and the alteration of SPARC and decorin secretion in senescent fibroblasts we comparatively analyzed the structures of subcellular compartments implicated in the secretory process in young and senescent fibroblasts. Staining of calnexin (marker of endoplasmic reticulum (ER)) and giantin (Golgi apparatus) are compacted and correctly localized close to the nucleus in young

fibroblasts suggesting a functional state of these compartments (figure 3A, 3B and supplemental figure 4, green). Moreover, SPARC preferentially co-localized with calnexin (figure 3A) and decorin co-localized with giantin with decorin staining (figure 3C), suggesting a correct processing along the secretion of these glycoproteins in young fibroblasts. However senescent fibroblasts displayed a less dense and more scattered ER (figure 3A and supplemental figure 4A) while the Golgi apparatus was oversized and disorganized (figure 3B and supplemental figure 4B) without co-localization with SPARC. These observations suggest that the secretory machinery and SPARC processing are altered in senescent fibroblasts.

Our previous studies showed that senescent cells displayed an intense lysosomal and autophagic activity (Gosselin et al., 2009). We hypothesized that the deregulation of secretory machinery in senescent fibroblasts could account for the re-localization of SPARC and decorin glycoproteins toward the lysosomal degradation pathways. Using a lysosome specific fluorescent marker, lysotracker®, we recorded a strong lysosomal activity in senescent fibroblasts compared to the young (figure 4A, and supplemental figure 4C, red). A large fraction of the lysotracker positive vesicles were also co-localized with SPARC- or decorin-vesicles in senescent fibroblast (figure 4A, green; data not shown). The addition of lysosomal activity inhibitor Bafilomycin in the medium both abrogated the lysotracker staining and the co-localization with SPARC in senescent fibroblasts (figure 4B).

Altogether, our results show that the alteration of SPARC and decorin secretion by senescent fibroblasts is caused by a dysfunction during their secretory process and consequently their routing to lysosomal vesicles.

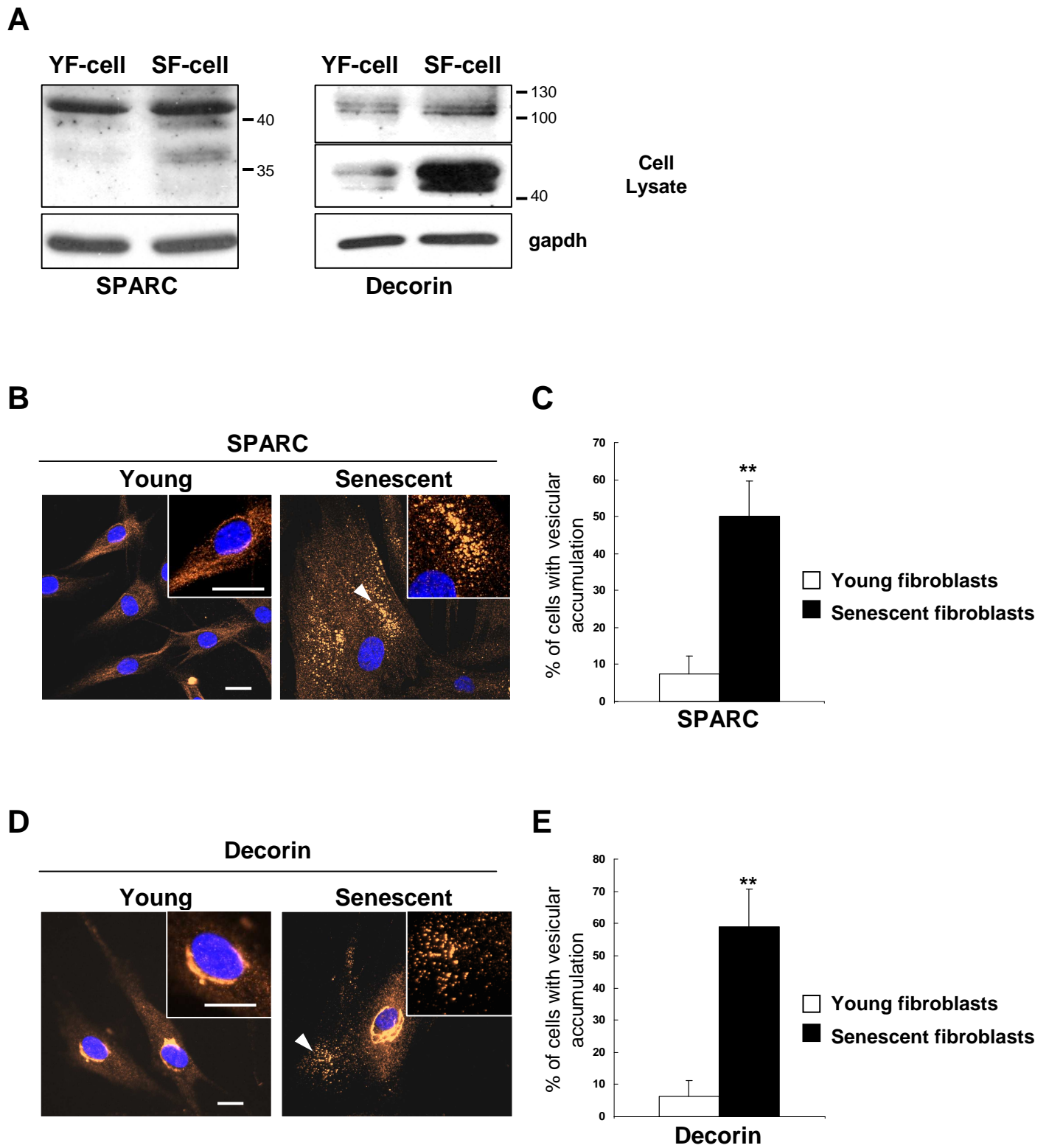


Figure 2

FIGURE LEGENDS

Figure 2: SPARC and Decorin glycoproteins are routed to peripheral vesicles in senescent fibroblasts.

(A) Western-blot analysis of SPARC and Decorin glycoproteins in young (YF-cell) and senescent (SF-cell) fibroblasts cell lysates showing an accumulation of short forms (35 kDa for SPARC and 40 kDa for decorin) in senescent fibroblasts (GAPDH was used as a loading control). Immunofluorescence analysis showed accumulation of (B) SPARC and (D) Decorin containing vesicles in peripheral of senescent fibroblasts. Nucli were stained with Hoechst (33258). Representative images were taken with the AxioImager Z1-Apotome system (Zeiss) (scale bar = 20 μ M). Percentage of cells with accumulation of vesicles containing (C) SPARC and (E) Decorin were set for young (white bars) and senescent (black bars) fibroblast populations. Each condition was in duplicate and at least 200 cells were counted per coverglass. Results are representative of 3 independent experiments Asterisks indicate statistically significant difference (**: $p < 0.01$).

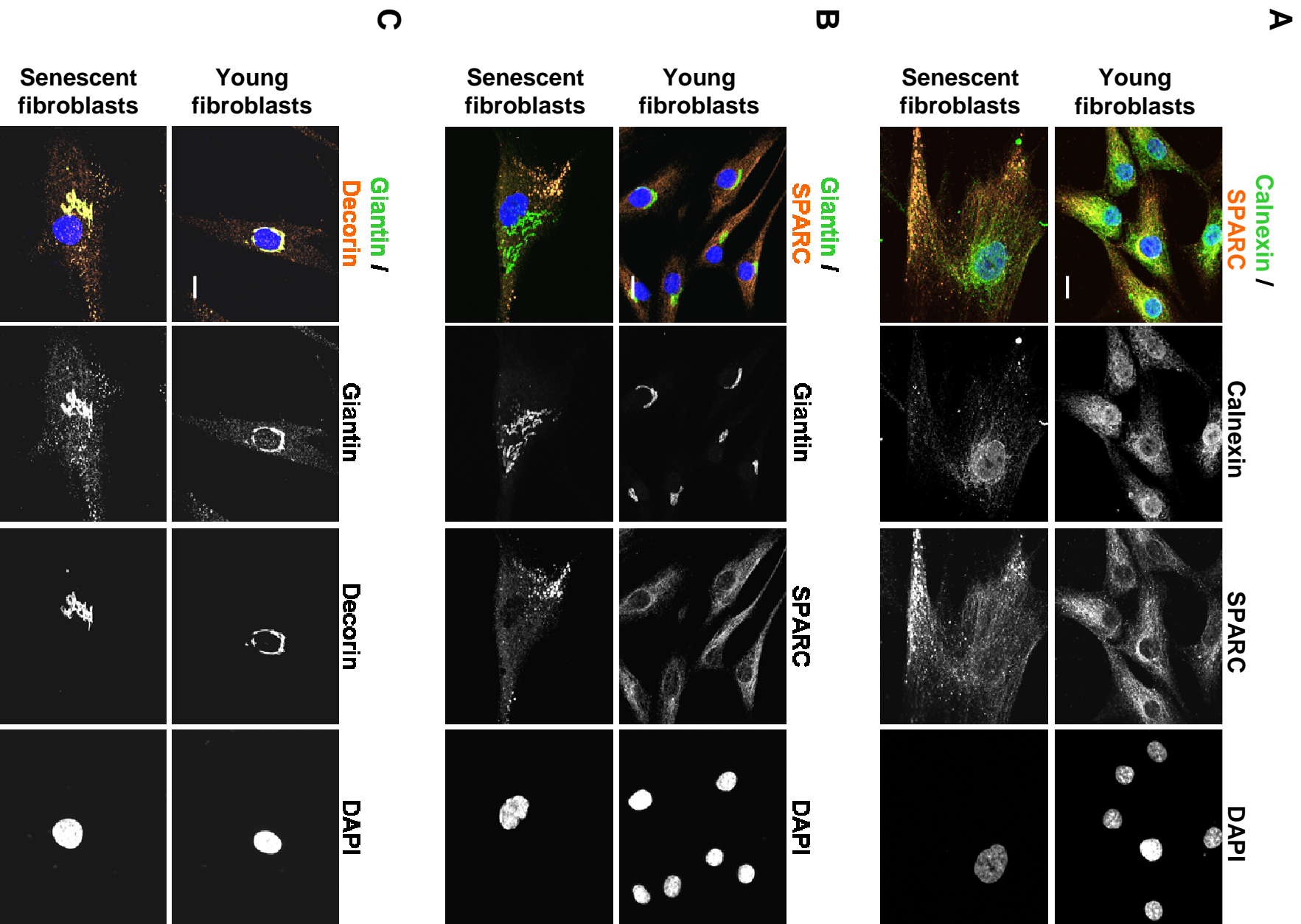


Figure 3

FIGURE LEGENDS

Figure 3: Disorganization of endoplasmic reticulum and golgi apparatus in senescent fibroblasts

(A) Immunofluorescence analysis showing co-localization of SPARC (red) and endoplasmic reticulum marker calnexin (green) staining in young fibroblasts. This co-localisation is lost in senescent fibroblasts where SPARC was routed to vesicles. Immunofluorescence analysis showing SPARC (red) (B) or decorin (C) were co-localized with Golgi apparatus marker giantin (green) only in young fibroblasts. In senescent fibroblasts SPARC and decorin were routed to vesicles. Nuclei were stained with Hoechst (33258). Representative images of the fluorescence staining were captured with the AxioImager Z1-Apotome system (Zeiss) (scale bar = 20 μ M). 3 independent experiments were performed.

Figure 4: SPARC is co-localized with lysosomal vesicles in senescent fibroblasts

(A) After pre-treatment with red LysoTracker®, immunofluorescence analysis showing a co-localization of SPARC (green) with vesicles positive for the lysosomal marker (red). (B) Bafilomycin (5nM) or its solvent DMSO were added overnight in culture media (FGM) of senescent fibroblasts. The LysoTracker staining (Red) is completely abolished in presence of bafilomycin and do not co-localized with SPARC positive vesicles (green). Nuclei were stained with Hoechst (33258). Representative images of the fluorescence staining were captured with the AxioImager Z1-Apotome system (Zeiss) (scale bar = 20 μ M). Two independent experiments were performed.

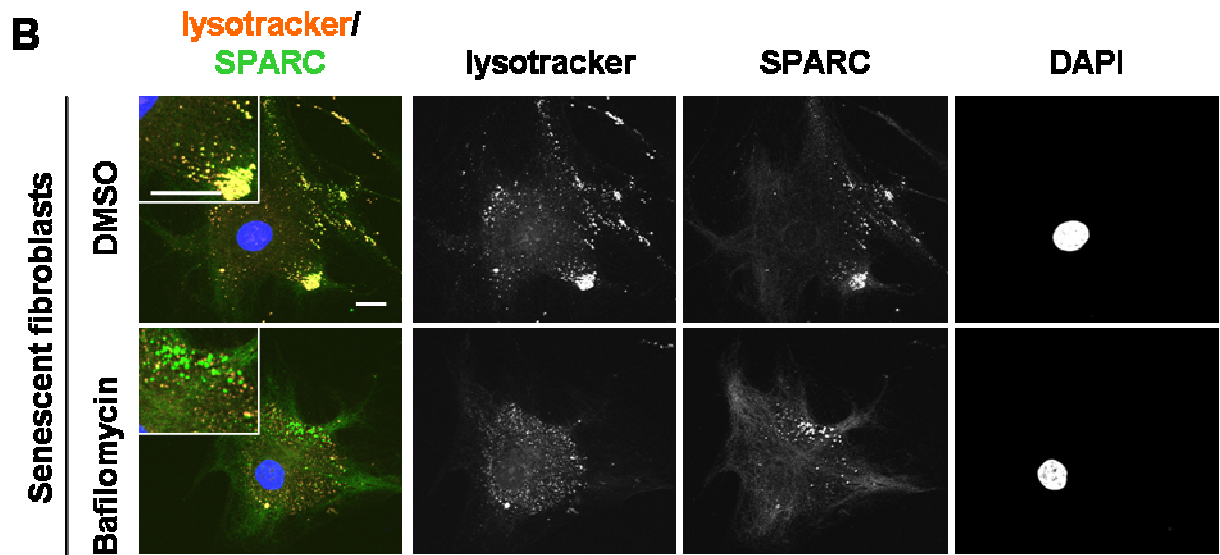
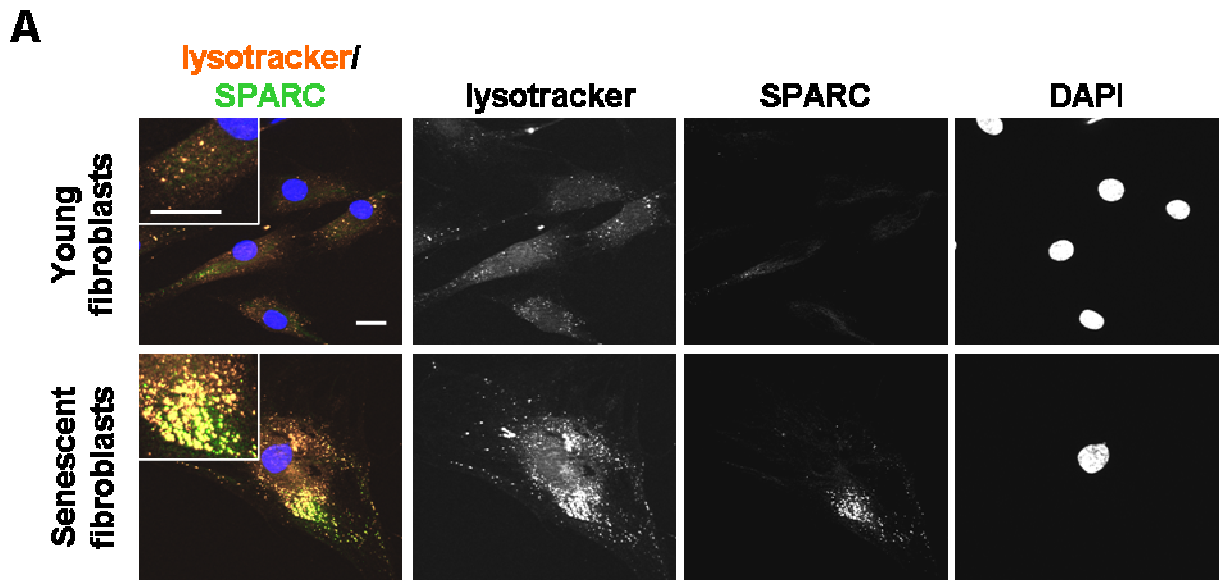


Figure 4

ER-stress induced UPR pathways are activated in senescent dermal fibroblasts.

Alteration of secretory processes could be associated to endoplasmic reticulum stress (ER-stress) inducing the degradation of misfolded proteins via proteasomal or lysosomal pathways. ER-stress signalling pathways initiated by the ER sensors IRE1, PERK and ATF6, lead to the activation of the Unfolded Protein Response (UPR). RT-qPCR experiments showed that IRE1, PERK and ATF6 mRNA were highly expressed in senescent fibroblasts compared to the young (figure 5A). Consequent activation of UPR pathways lead to the transcription of several target genes including BIP, HRD-1, CHOP, GRP94, HERP-UD1 and ERO-1L β . We have analyzed by RT-qPCR the respective expression of these genes in young and senescent fibroblasts. BIP, GRP94, CHOP, HERP, ERO-1L β and HRD-1 mRNA expression were all increased in senescent fibroblasts compared to the young (figure 5B). The activation of ER-stress sensor IRE-1 induces the splicing of XBP-1 (X-box-binding protein 1) mRNA. This splicing event produces a frame shift and results in the translation of the spliced form of XBP-1s (transcription factor that induces UPR effectors genes) (Back et al., 2005). Transcripts of unspliced XBP-1 (XBP-1u) were similarly express in both young and senescent fibroblasts while those of spliced XBP-1 (XBP-1s) were expressed in senescent fibroblasts (figure 5C). These results highlight that an endoplasmic reticulum stress leads to the activation of UPR pathways in senescent fibroblasts.

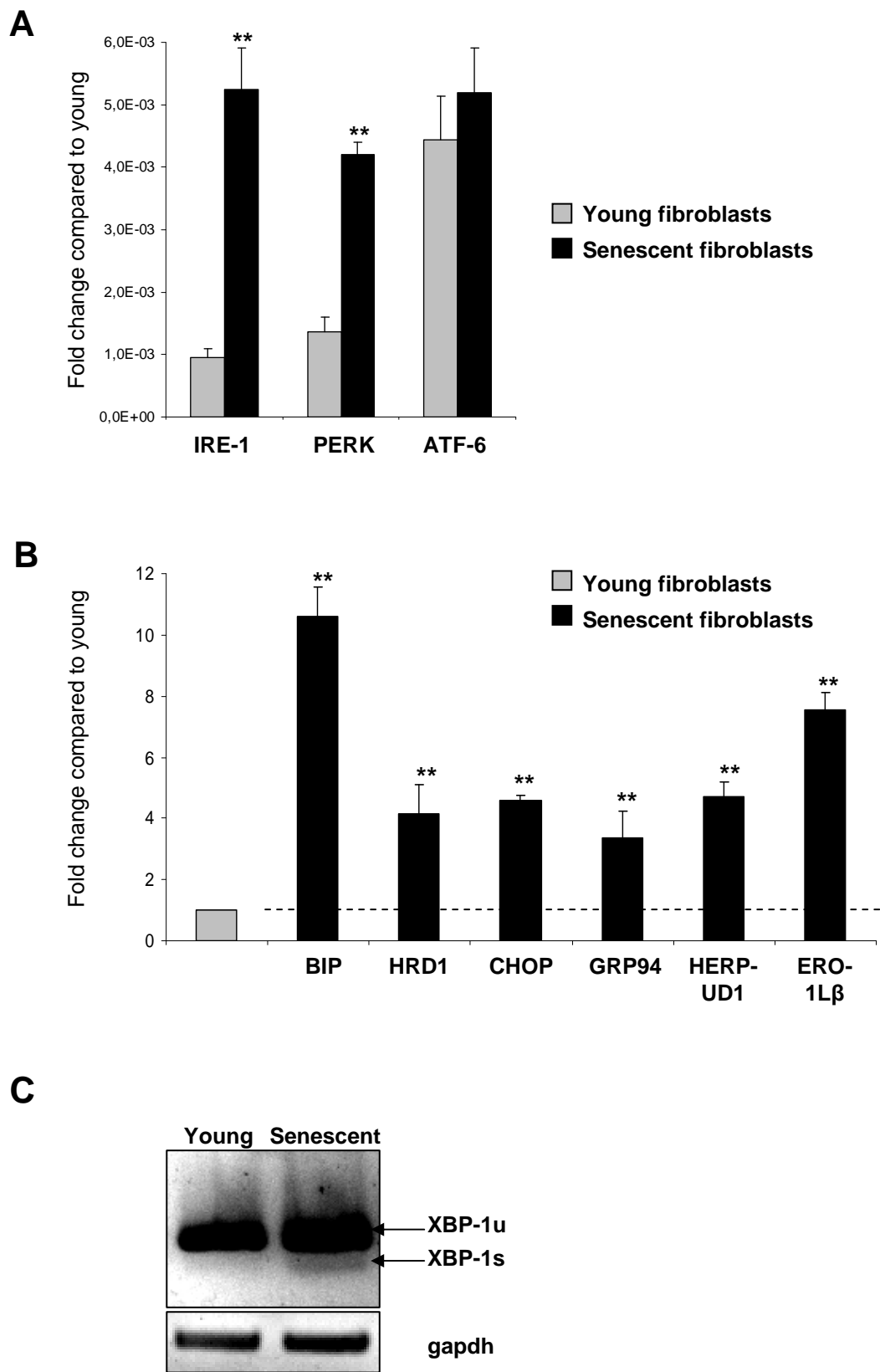


Figure 5

FIGURE LEGEND

Figure 5: Up-regulation of endoplasmic reticulum stress genes in senescent fibroblasts.

(A) Real Time RT-qPCR analysis of total RNA extracted from young and senescent fibroblasts population showing high expression of ER-stress sensors IRE1, PERK and ATF6 in senescent fibroblasts. (B) Real Time RT-qPCR analysis of total RNA extracted from young and senescent fibroblasts population showing increased of the expression of a set of genes activated during UPR responses (BIP, HRD-1, GRP94, CHOP, HERP and ERO-1L β) in senescent fibroblasts. Each bar represents the mean \pm S.D of 3 independent experiments (each in triplicate)(*: $p < 0.05$; **: $p < 0.01$). (C) RT-PCR analysis of XBP-1 and XBP-1 splicing in total mRNA extracted from young and senescent fibroblasts population. In senescent fibroblasts a specific splicing of XBP-1 (XBP-1s) appears indicating an activation of IRE1 pathways. GAPDH was used as an internal standard.

Activation of the UPR ATF-6 pathway in senescent dermal fibroblasts drives the shift from secretion to lysosomal degradation of SPARC and decorin.

To further investigate if the activation of UPR pathways in senescent dermal fibroblasts were associated to the disruption of glycoproteins secretion, we performed knockdowns of either IRE-1, PERK or ATF-6 in senescent fibroblasts by RNA interference and analyzed their consequence on sub-cellular localization of SPARC (figure 6). The percentage of senescent fibroblasts displaying SPARC accumulation in lysosomal vesicles was not modified by the inhibitions of PERK (si-PERK; 68%) or IRE-1 (si-IRE1; 45%) pathways compared to the control (si-Ctrl; 60%) (figure 6A and B). Inhibition of ATF-6 pathway, however, strongly reduced the percentage of senescent fibroblasts with accumulation of SPARC containing vesicles (20%) (figure 6 A and B). Moreover inhibition of the ATF-6 pathway (si-ATF6) in senescent fibroblasts restored the detection of SPARC within the endoplasmic reticulum as depicted for young fibroblasts transfected by control siRNA (figure 6A).

In the view of these results, we have focused our interest on the impact of ATF6 inhibition by siRNA (inhibition efficiency checked by RT-qPCR, figure 7A) on senescent fibroblasts phenotypes. Contrarily to PERK or IRE1 inhibition (data not shown) silencing of ATF6 in senescent fibroblasts re-established the transcription of UPR target genes BIP, HRD-1, CHOP, GRP94, HERP-UD1 and ERO-1L β at the same level of those observed in young fibroblasts (figure 7A).

Interfering with ATF-6 expression also restored low levels of lysosomal activity in senescent fibroblasts comparably to the young fibroblasts (figure 7B). The secretion of SPARC and decorin in conditioned media was clearly re-established in senescent fibroblasts transfected with ATF-6 siRNA (figure 7C) while their expression in cell lysate was decreased (figure 7D). Comparison with results in figure 2A highlighted that the expression of aberrant forms

of the glycoproteins are abolished in cell lysates probably depicting correct processing and good secretory activity as recorded in conditioned media (figure 7D). Beside the collapse of SPARC, decorin and PEDF secretion, we recorded over-expression of pro-inflammatory cytokines in senescent dermal fibroblasts secretome (supplemental figure 1). We then investigated a possible additional link between ER-stress and the pro-inflammatory components of the senescent fibroblasts secretome. Si-RNA mediated inhibition of the ATF-6 ER-stress signalling pathways decreased by 50 to 60% the transcription of some of the most over-expressed cytokines in senescent fibroblasts (i.e MCP-1, IL-6; IL-8 and MIF). As expected the transcription of PAI-1 (used as negative control) was unaffected (figure 7E).

Altogether our results showed that senescent dermal fibroblasts display acute ER-stress whose ATF-6 signalling pathway mediates aberrant routing of epithelial cancer inhibitory glycoproteins SPARC and decorin for lysosomal degradation and enhanced secretion of pro-inflammatory cytokines, hence being a crucial contribution to the dermal skin SASP.

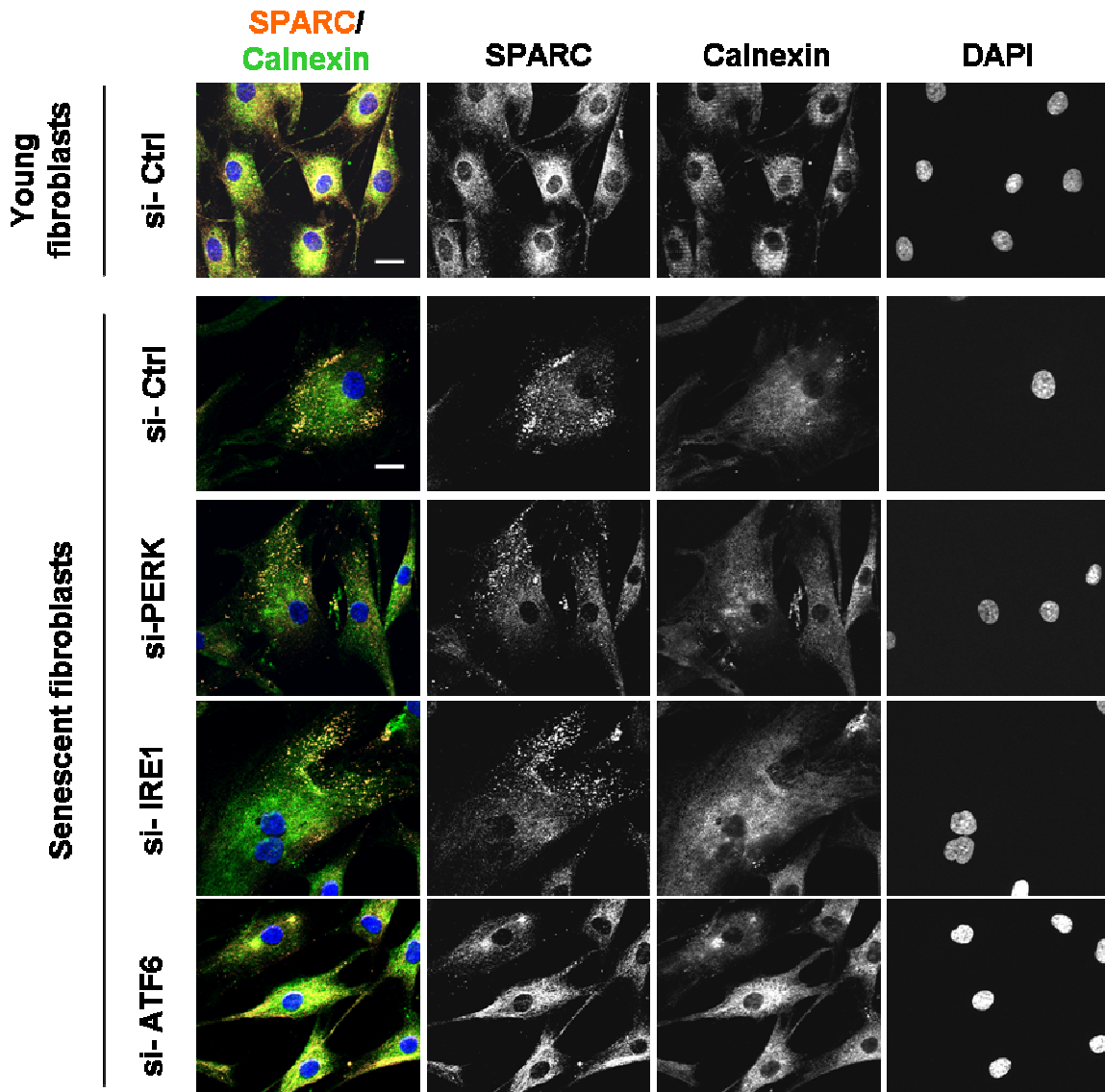
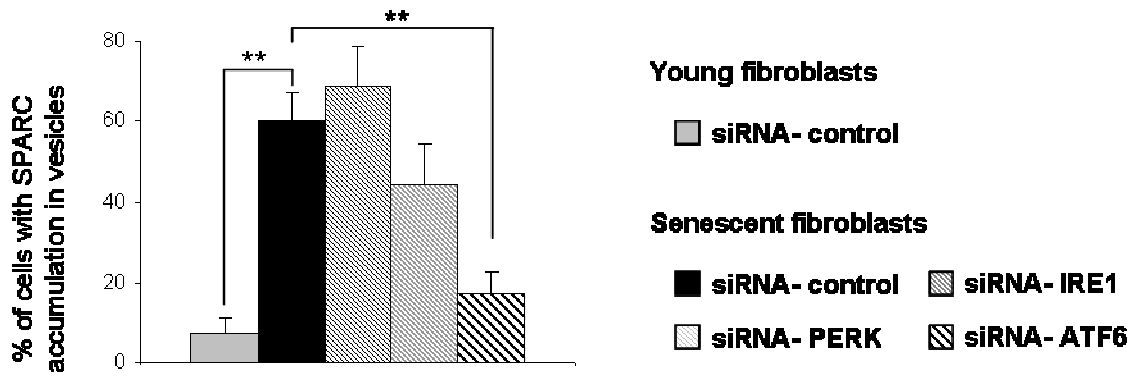
A**B****Figure 6**

FIGURE LEGEND

Figure 6: Inhibition of ER-stress signalling ATF-6 pathway restores appropriate SPARC sub-cellular localization and secretion in senescent fibroblasts.

ER-stress related UPR responses were selectively inhibited by RNA interference transfection targeting PERK (siRNA-PERK), IRE-1 (siRNA-IRE1), and ATF-6 (siRNA-ATF6) in senescent fibroblasts. Non target si-RNA were transfected in young and senescent fibroblasts as controls. **(A)** Co-Immunofluorescence analysis of SPARC (Red) and Calnexin (Green) showing that co-localization of SPARC and calnexin in young fibroblasts (si-control), is specifically restored in senescent fibroblasts after ATF-6 inhibition. Representative images of the fluorescence staining were captured with the AxioImager Z1-apotome system (Zeiss) (scale bar = 20 μ M). 2 independent experiments were performed. **(B)** Percentage of senescent cells accumulating SPARC vesicles were counted for each si-RNA transfection condition. For each condition, at least 200 cells were counted. Two independent experiments were performed. Each bar represents the mean \pm s.d of the total number of cell counted (**: $p < 0.01$).

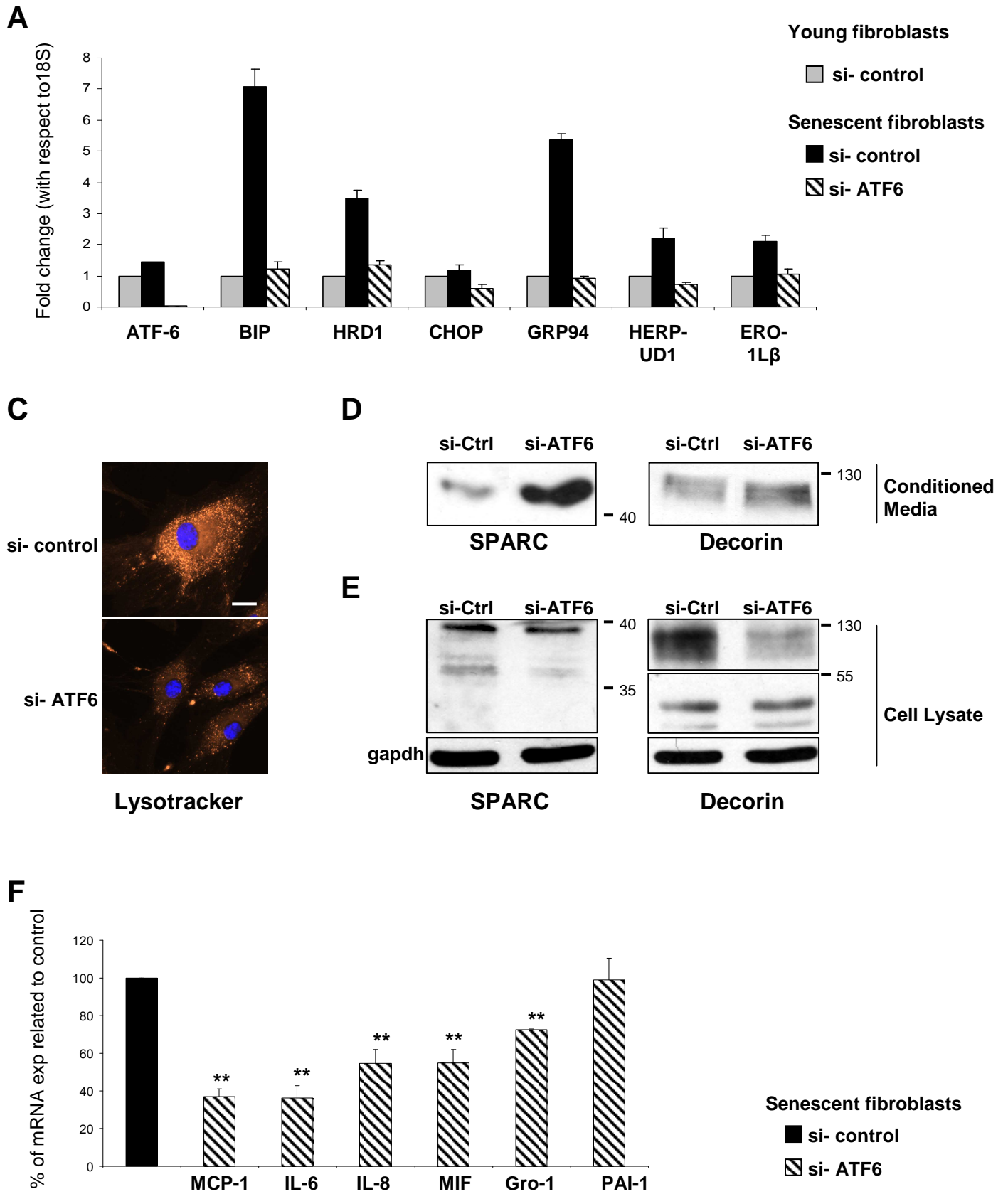


Figure 7

FIGURE LEGEND

Figure 7: ATF-6 drives the loss of SPARC and decorin secretion as well as the over-expression of pro-inflammatory cytokines by senescent fibroblasts.

(A) ATF-6 pathway was inhibited in senescent fibroblasts by RNA interference transfection targeting ATF-6 mRNA. Efficiency of ATF6 inhibition by siRNAs was checked by RT-qPCR analysis of total RNA on si-control (black lines) and si-ATF6 (hatched lines) condition. RT-qPCR analysis showed also a decrease of UPR target mRNA expression (BIP, HRD-1, GRP94, CHOP, HERP and ERO-1L β) in si-ATF6 (hatched lines) transfected senescent fibroblasts compared to si-control (black lines) condition. (B) Senescent fibroblasts were pre-treated with red LysoTracker® during 30 minutes and fixed with para-formaldehyde (4%). Fluorescence staining, captured with the AxioImager Z1-Apotome system (Zeiss), showed a reduction of lysosomal activity in senescent fibroblasts transfected with si-ATF6. (scale bar = 20 μ M). (C) Western-blot analysis showing that SPARC and decorin glycoproteins secretion is restored in conditioned media from senescent fibroblasts transfected by si-ATF6. (D) Western-blot analysis showing that expression of SPARC and decorin glycoproteins in cell lysate of senescent fibroblasts are decreased after ATF6 inhibition. GAPDH was used as a loading control. (E) RT-qPCR analysis showing a decrease of cytokines mRNA expression (MCP-1; IL-6; IL-8; MIF and GRO-1) in si-ATF6 transfected senescent fibroblasts (hatched lines) condition compared to si-control (black lines) condition. PAI-1 mRNA expression was checked as negative control. For RT-qPCR analysis: GAPDH was used as an internal standard. Each bar represents the mean \pm S.D of 3 independent experiments (each in triplicate) (*: p<0.05; **: p<0.01).

DISCUSSION

We investigated here the detailed composition of the secretome of senescent dermal fibroblasts that we already associated to the promotion of early carcinogenesis from autologous human keratinocytes in association with increased expression and/or activation of several metallo-proteinases among which MMP-1 and -2 (Malaquin et al. submitted).

We show here that comparatively to young ones, senescent NHDF over-expressed several cytokines among which MCP-1 (Monocyte chemo attractant protein 1) and IL-6 (interleukin-6) expressions were particularly enhanced. Interestingly, these two cytokines have strong potential to induce tumorigenesis and invasion of cancerous cells (Ohanna et al., 2011; Su et al., 2011). Regarding pro-inflammatory molecules, senescent dermal fibroblasts presented a secretory phenotype closely similar to the senescence associated secretory phenotype (SASP) described for breast and prostate senescent cells (Bavik et al., 2006); (Coppe et al., 2010; Coppe et al., 2008); (Kuilman and Peeper, 2009) and also shown to contribute to the growth and invasion of malignant epithelial cell in vitro and in vivo (Krtolica et al., 2001; Lawrenson et al., 2010; Liu and Hornsby, 2007).

Beside inflammation, aged skin is characterized by an alteration of epidermal and dermal structures and functions. These changes correlated with the increased number of benign and malignant epidermal neoplasms. In dermis, the number of fibroblasts decreases contributing to the loss of elasticity by reduction of major extracellular matrix components secretion (collagens, elastic fibres, proteoglycans) and their disorganization (Fenske and Lober, 1986; Kurban and Bhawan, 1990).

We highlighted, by comparative mass spectrometry analysis of young and senescent NHDF conditioned media, the loss of secretion of several fibrillar matrix proteins as collagen type I and type VI, laminin beta 2 confirming changes in insoluble factors constituting the extracellular matrix recorded in other cellular models (Bavik et al., 2006; Coppe et al., 2008;

Ohanna et al., 2011). For the first time however this approach pointed out the collapse of the expressions of decorin, SPARC (Secreted Protein, Acidic and Rich in cystine) and PEDF (Pigment Epithelium Derived Factor) glycoproteins. These glycoproteins contribute to the ECM organization and to the cells-matrix interactions but interestingly present powerful anti-tumoral activities.

SPARC is a extracellular matrix protein with diverse biologic functions. In normal “young” skin, SPARC is expressed both in the epidermal and in the dermal compartment (Hunzelmann et al., 1998). SPARC has a critical role of chaperone for the processing of procollagen I and collagen fibrillogenesis (Rentz et al., 2007). Its secretion defect could then contribute to the strong reduction of collagens secretion in the senescent fibroblast secretome. The role of SPARC in the skin carcinoma development remains unknown but skin wound healing was accelerated in SPARC null-mice (Bradshaw et al., 2002). While in some cancers SPARC could act as a tumor suppressor (Brekken et al., 2003), high levels of SPARC were associated to bad prognosis in several cancer types (melanoma, glioma, breast cancer...) (Chlenski and Cohn, 2009).

Decorin is a small leucine-rich proteoglycan which regulate the collagen matrix assembly. Decorin is the main proteoglycan in the skin and its alteration during the aging process may be involved in alteration of collagen matrix assembly (Nomura, 2006) (Carrino et al., 2011). Decorin, expression is altered in many types of cancer, and it is a potent inhibitor of tumor cell growth and migration by regulating many cell signalling pathways (Goldoni and Iozzo, 2008; Goldoni et al., 2008). For example, decorin can inhibits EGFR activity on the tumor cell surface by inducing its degradation through the lysosomal pathways (Zhu et al., 2005). In addition, decorin can induce apoptosis via activation of caspase-3 in human squamous carcinoma (A431) (Seidler et al., 2006). Finally, decorin can bind the transforming growth factor-beta 1 resulting in its inactivation (Hausser et al., 1994; Yamaguchi et al., 1990).

PEDF (Pigment epithelium-derived factor) is member of the serine protease inhibitor (SERPIN) superfamily, and displays broad anti-tumoral activities. PEDF is the most important endogenous inhibitor of angiogenesis activity (Dawson et al., 1999). PEDF could also induce tumor cells differentiation (Filleur et al., 2005) and could have direct tumor suppression effect by activating apoptosis pathways (Takenaka et al., 2005). Involvement of PEDF on skin carcinoma development remains unknown, but it has been recently shown that PEDF inhibits proliferation and migration of keratinocytes cells lines (HaCaT) (Li et al., 2011). PEDF (named also early population doubling level cDNA-1) expression is lost during skin aging in vivo, contributing to an excessive angiogenesis (Francis et al., 2004). Altogether the loss of SPARC, Decorin and PEDF secretions by senescent dermal fibroblasts could also significantly contribute to skin carcinoma initiation and development.

Our results pointed out specific re-localization of SPARC and decorin in lysosomal vesicles. This could account for the loss of secretion of these molecules via their strong lysosomal degradation after severe defects in secretion processing. Several studies highlighted the correlation between ER stress-signalling UPR activity and the impairment of protein processing and secretion. We show that transcription of some the ER-stress sensors but more clearly of most of their downstream effectors are overexpressed in senescent fibroblasts pointing a strong ER stress in those cells compared to young ones. PERK and IRE1 pathways are activated in senescent fibroblasts as their downstream target genes (BIP, HRD1, CHOP, GRP94...) are over-expressed and both PERK and IRE1 are transcriptionally enhanced. IRE1 was shown to mediate insulin mRNA cleavage and degradation in pancreatic beta cells via a regulated IRE1-dependent decay of mRNAs (Hollien and Weissman, 2006) in association with the reduction of insulin processing and secretion (Lee et al., 2011; Lipson et al., 2008). SPARC mRNA was shown to be similarly targeted by IRE1 in *D. Melanogaster*

(Hollien and Weissman, 2006; Lipson et al., 2008) although any impact on SPARC processing and secretion remains hypothetical up to now. Here we show that in senescent dermal fibroblasts, SPARC and decorin glycoproteins secretion defects are mainly consecutive to the ATF6 arm of the UPR activity. Strikingly we could revert the SPARC secretion defect of senescent dermal fibroblasts by reducing ATF-6 expression. The precise molecular and cellular mechanisms underlying how the UPR affected protein secretion in senescent cells remain to be clarified. Beside ATF-6 involvement, as the three ER sensors IRE1, PERK and ATF-6 present some functional interconnections (Vidal et al., 2012), one cannot exclude that the different ER stress-signalling pathways could be associated to additional specificities of the dermal senescent fibroblasts secretome. One possibility however is that some chaperones and enzymes, key components of the UPR, could be affected along ageing. Indeed, oxidative stress is a component of skin cells senescence, and reduced expressions and activities of UPR components were associated to progressive accumulation of oxidative stress resulting in a dysfunctional ER (Naidoo, 2009). Some ER enzymes important for correct protein conformation are also regulated by and sensitive to the oxydation-reduction status. For example, protein disulfide isomerase (PDI) is responsible for the formation of disulfides bonds in the ER. Oxydative stress could impact PDI activity and alter the secretion/folding of glycoproteins (Laurindo et al., 2012; Rutkevich et al., 2010), resulting in ER retention that in turn could sustain the UPR activation.

We also show for the first time that ATF-6 activity is associated to the control of the productions of IL-6, MCP1 and other inflammatory molecules by senescent fibroblasts. Interestingly, ER-stress sensors IRE-1 and PERK had recently been associated to atherosclerosis-induced ER stress in endothelial cells resulting in increased secretion of IL-6, MCP1, IL-8 and CXCL3, thus linking ER stress to inflammation induction (Gargalovic, 2009). In addition, ATF6 branch of the UPR was suggested to control inflammatory cytokines

secretion through the activation of the NF- κ B pathway (Nakajima et al., 2011; Yamazaki et al., 2009).

Our results show that ER stress modulates the panel of secreted proteins expressed by senescent fibroblasts compared to young ones. Indeed some are considerably reduced (SPARC, decorin) while other are exacerbated. Similarly ER stress was associated to decreased secretion of adiponectin and leptin, but increased secretion of IL-6 by adipocytes during obesity (Xu et al., 2010). Hence, the severity and duration of ER stress may shift the cell activities towards original secretory patterns, leading to the perturbation of tissue function. We have defined some characteristics of the senescent fibroblasts secretory phenotype that could contribute to the establishment of an environment permissive to the tumoral development and that ER stress-signalling ATF-6 pathway accounts for the onset of the senescent dermal fibroblast secretome.

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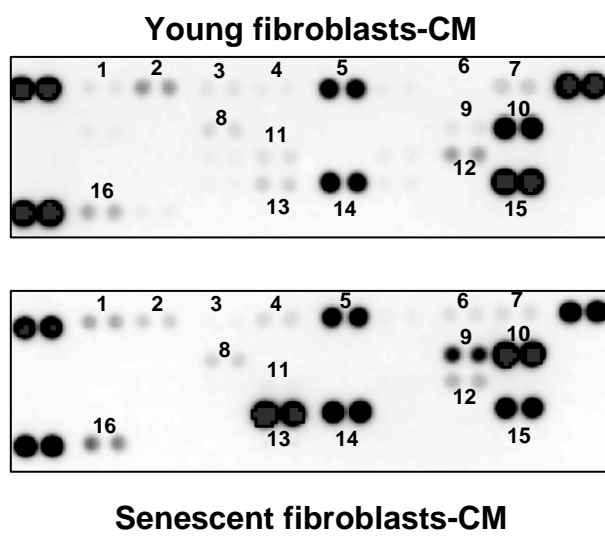
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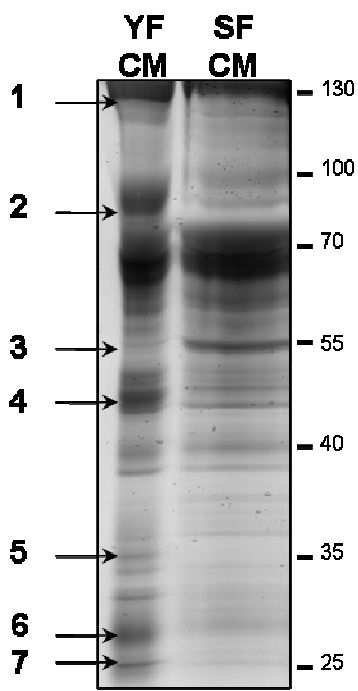


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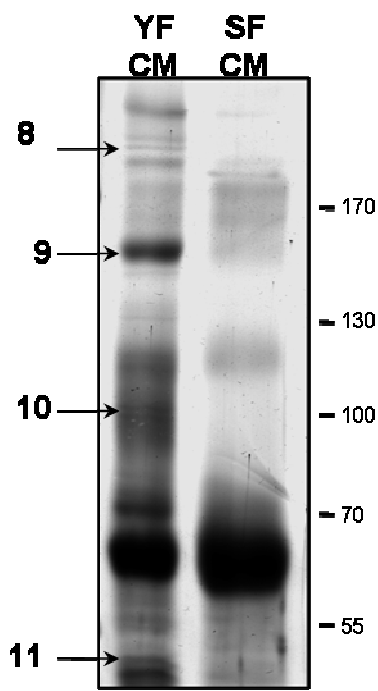
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IL-8 (CXCL8)	2,5	10
MIF	1,9	14
Serpin E1 (PAI1)	0,3	15
GRO- α (CXCL1)	1,2	5
IL-6	8,3	9
RANTES (CCL5)	1,7	16
IL-23	0,9	12
IL-16	0,8	11
C5/C5a	2,0	1
IFN- γ	0,8	7
sICAM-1	2,4	6
IL1-ra	1,2	8
GM-CSF	1,6	4
CD40 ligand	0,4	2
G-CSF	0,4	3

up-secreted in SF-CM
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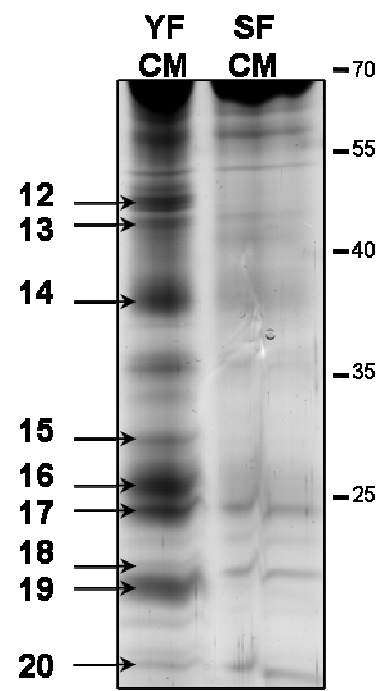
Supplemental figure 1



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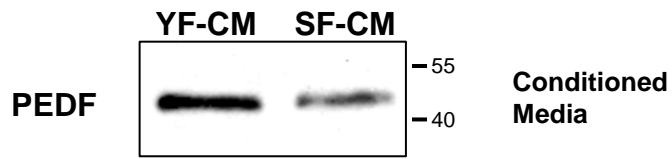
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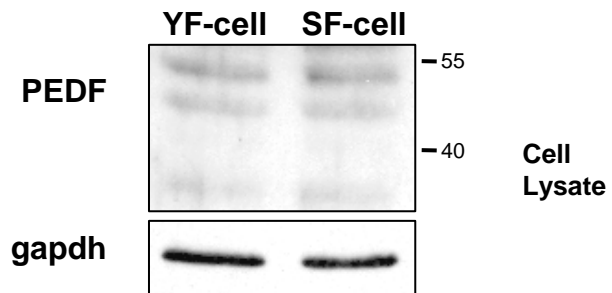
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Supplemental figure 2

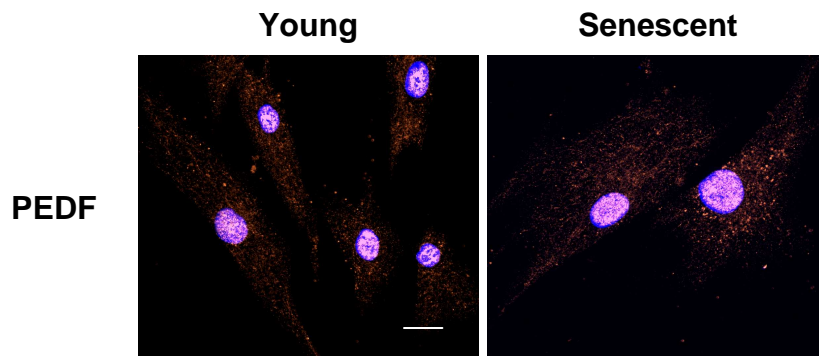
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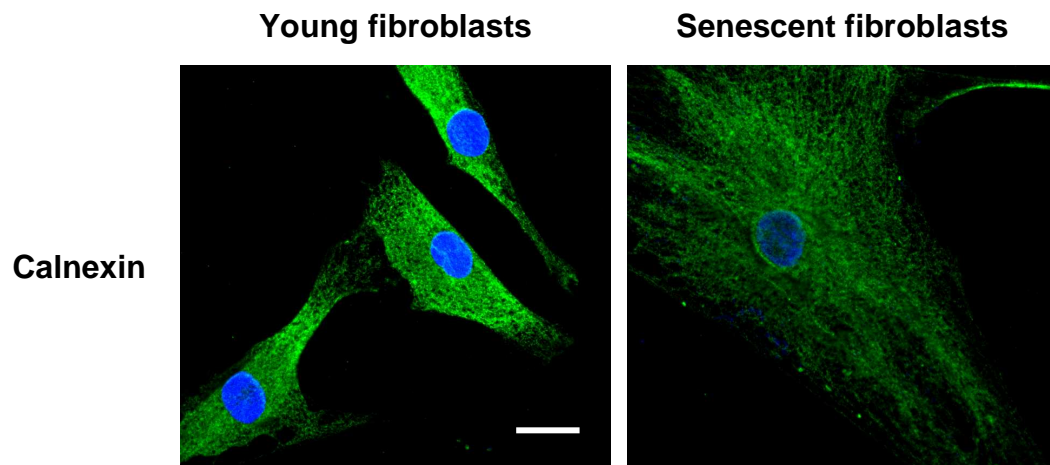


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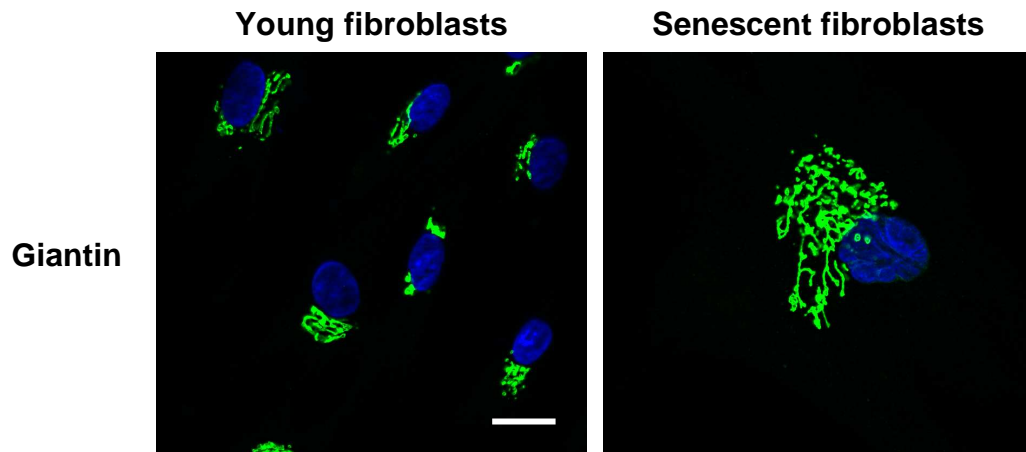


Supplemental figure 3

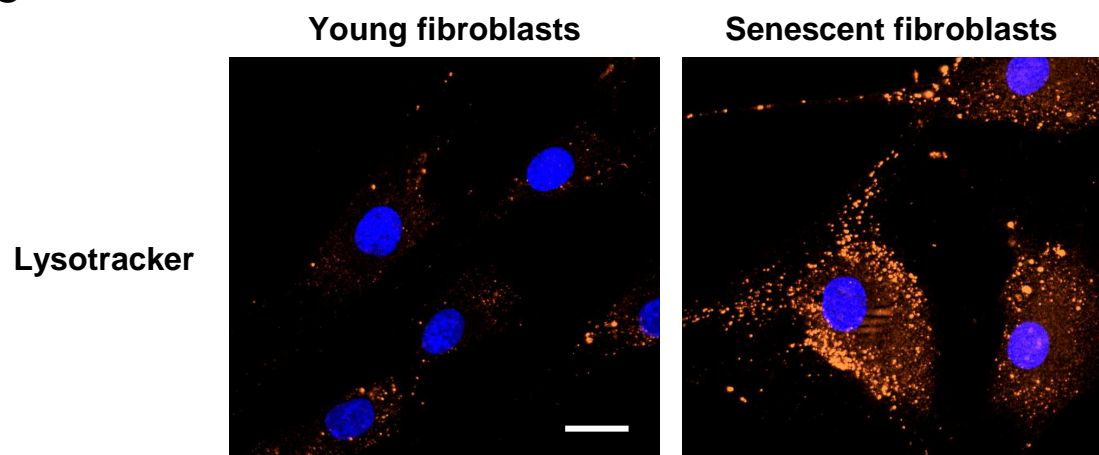
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C



Supplemental figure 4

SUPPLEMENTAL FIGURE LEGENDS

Supplemental figure 1: Over-expression of pro-inflammatory cytokines by senescent dermal fibroblasts

(A) Concentrated conditioned media corresponding to an equal cells number of young and senescent fibroblasts were used for cytokines array analysis (Human Cytokine Array Kit, Panel A, R & D systems) according to the manufacturer's protocol. Proteins were detected using enhanced chemoluminescence (ECL). Results were captured with a LAS3000 (Fujifilm) imager and quantification of spots were analysed with Multigauge software. (B) Cytokines are listed in order of their relative quantity in the senescent fibroblast conditioned medium.

Supplemental figure 2: Silver-staining of young and senescent dermal fibroblasts conditioned media

Serum-free conditioned media of an equal number of young and senescent fibroblasts were concentrated by ultra-filtration (Amicon, cut-off 3kDa). An equal quantity of proteins from young and senescent fibroblast-conditioned media (approximately 15 µg) was loaded and separated on SDS-PAGE gels with different percentages of acrylamide (7, 10 and 12%). The analytical gels were silver-stained according to the protocol previously described by (Shevchenko *et al.*, 1996). Bands corresponding to differential secreted proteins from concentrated conditioned media of young or senescent fibroblasts were manually excised from the silver-stained gel and proteins were prepared for mass spectrometry (MALDI-TOF) analysis. Number of bands corresponds to the proteins listed in table 1.

Supplemental figure 3: Reduced secretion of PEDF by senescent dermal fibroblasts

(A) Western-blot analysis of PEDF proteins on young (YF-CM) or senescent (SF-CM) fibroblasts conditioned media showing a decreased of their secretion in SF-CM compared the YF-CM. (B) Western-blot analysis of PEDF glycoproteins in young (YF-cell) and senescent (SF-cell) fibroblasts cell lysates showing no difference of their expression between these two conditions. (GAPDH was used as a loading control). (C) Immunofluorescence analysis of PEDF on young or senescent fibroblasts showing a nuclear localization of PEDF without difference between these two conditions. Fluorescence analyses were performed with Axioplan2 (Zeiss, Germany) microscope equipped with an Apotome device and either Plan Neofluar (5x, NA: 0.16).Nuclei were stained by Hoechst 33258 at 1 µg/ml for 5 mn. (scale bar = 20 µM)..

Supplemental figure 4: Abnormal structure of endoplasmic reticulum, Golgi apparatus and enhanced lysosomal activities in senescent dermal fibroblasts.

(A) Immunofluorescence analysis of the endoplasmic reticulum (ER) calnexin showing a less dense and more scattered ER in senescent fibroblasts compared to the young. (B) Immunofluorescence analysis of the Golgi apparatus marker giantin showing over-developed and disorganized golgi apparatus in senescent fibroblasts compared to the young. (C) Young or senescent fibroblasts were incubated 30 min with lysotracker® (Molecular Probes) (dilution 1:1000) at 37°C, and then cells were rinsed in ice-cold PBS and fixed with 4% paraformaldehyde. Fluorescence analyses were performed with Axioplan2 (Zeiss, Germany) microscope equipped with an Apotome device and either Plan Neofluar (5x, NA: 0.16).Nuclei

were stained by Hoechst 33258 at 1 $\mu\text{g/ml}$ for 5 mn. (scale bar = 20 μM). Strong lysosomal activities in senescent fibroblasts compared to the young were recorded.

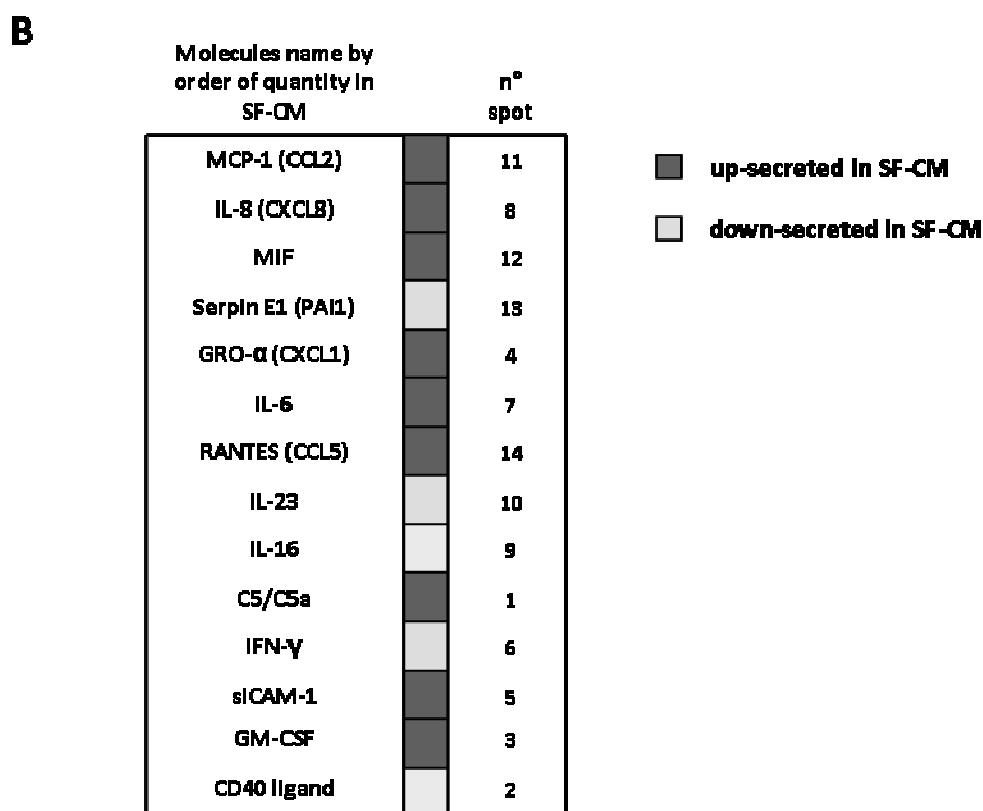
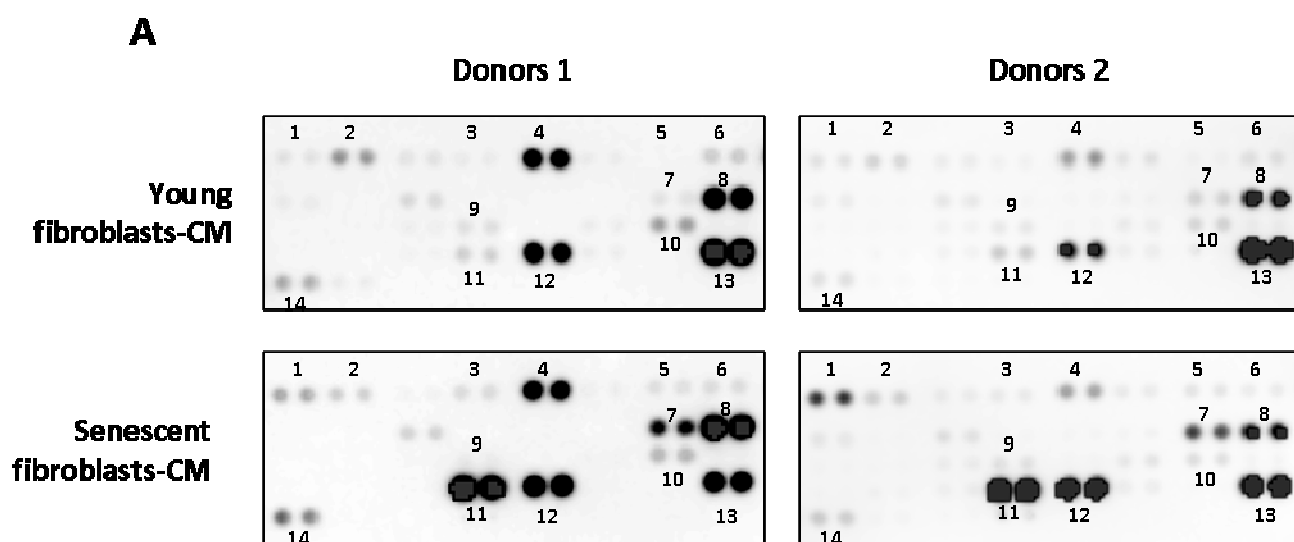


Figure 27 : Profil de sécrétion des cytokines pro-inflammatoires dans les fibroblastes jeunes et sénescents de derme

(A) Le milieu conditionné de 5.10^5 fibroblastes jeunes et sénescents est récupéré après 48H d'incubation à 37°C et concentré par ultrafiltration (Amicon, cut-off 3kDa). La sécrétion des cytokines est analysé par « array » (Human Cytokine Array Kit, Panel A ; R&D systems) selon le protocole du fabricant. Les fibroblastes sont issus de 2 donneurs différents (donneur 1 : 2F1966 ; donneur 2 : 1619). (B) L'analyse des intensités des spots de chaque cytokine par densitométrie (logiciel Multigaue) permet de classer les cytokines par ordre d'importance dans le milieu conditionné des fibroblastes sénescents (du donneurs n°1). Les augmentations de sécrétion de cytokines dans les MC de fibroblastes sénescents sont marquées en gris foncé et les diminutions en gris clair.

2. Résultats complémentaires sur la caractérisation du sécrétome des fibroblastes sénescents de derme

En marge de cette étude protéomique comparée des milieux conditionnés de fibroblastes jeunes et sénescents de derme, nous avons aussi voulu valider le caractère pro-inflammatoire du sécrétome des fibroblastes sénescents maintenant bien décrits dans la littérature pour d'autres modèles cellulaires (Coppé *et al*, 2010; Ohanna *et al*, 2011). Un sécrétome pro-inflammatoire est composé principalement de cytokines, de facteurs de croissance et des métalloprotéases (MMPs). Comme nous l'avons décrit dans *l'article n°2*, les NHDF sénescents sécrètent et/ou activent une grosse quantité de métalloprotéases dont les MMP-1 et -2.

2.1. Sécrétion de cytokines pro-inflammatoires par les fibroblastes sénescents

Pour étudier le caractère pro-inflammatoire des NHDF nous avons comparé le profil de sécrétion de cytokines aux stades jeune et sénescents par un kit de *cytokine array* (Human Cytokine Array Kit, Panel A ; R&D systems). Nous avons récupéré le milieu conditionné d'un même nombre de fibroblastes jeunes et sénescents (2 donneurs différents) que l'on a concentré par ultrafiltration (cut-off de 3kDa) et utilisé pour les *cytokines arrays* en suivant le protocole recommandé par le fabricant. De manière globale, la sécrétion de cytokines pro-inflammatoires est fortement stimulée dans les fibroblastes sénescents de derme (figure 27 A et B). En particulier les cytokines MCP-1 (Monocyte chemoattractant protein 1) et IL-6 (interleukines-6) apparaissent spécifiquement dans le milieu conditionné de fibroblastes sénescents. La sécrétion d'autres cytokines déjà présentes dans le sécrétome des NHDF jeunes, comme IL-8 (interleukines-8) MIF (Macrophage migration inhibitory factor) GRO-1 et CCL-5 (RANTES), est fortement stimulée dans les fibroblastes sénescents (figure 27). Il est aussi intéressant de noter que la diminution de PAI-1 dans le milieu conditionné de fibroblastes sénescents observée en spectrométrie de masse est confirmée par les *cytokines arrays*.

Ces données confirment donc que les fibroblastes sénescents de derme participent à la mise en place d'un micro-environnement pro-inflammatoire propice au développement tumoral des carcinomes.

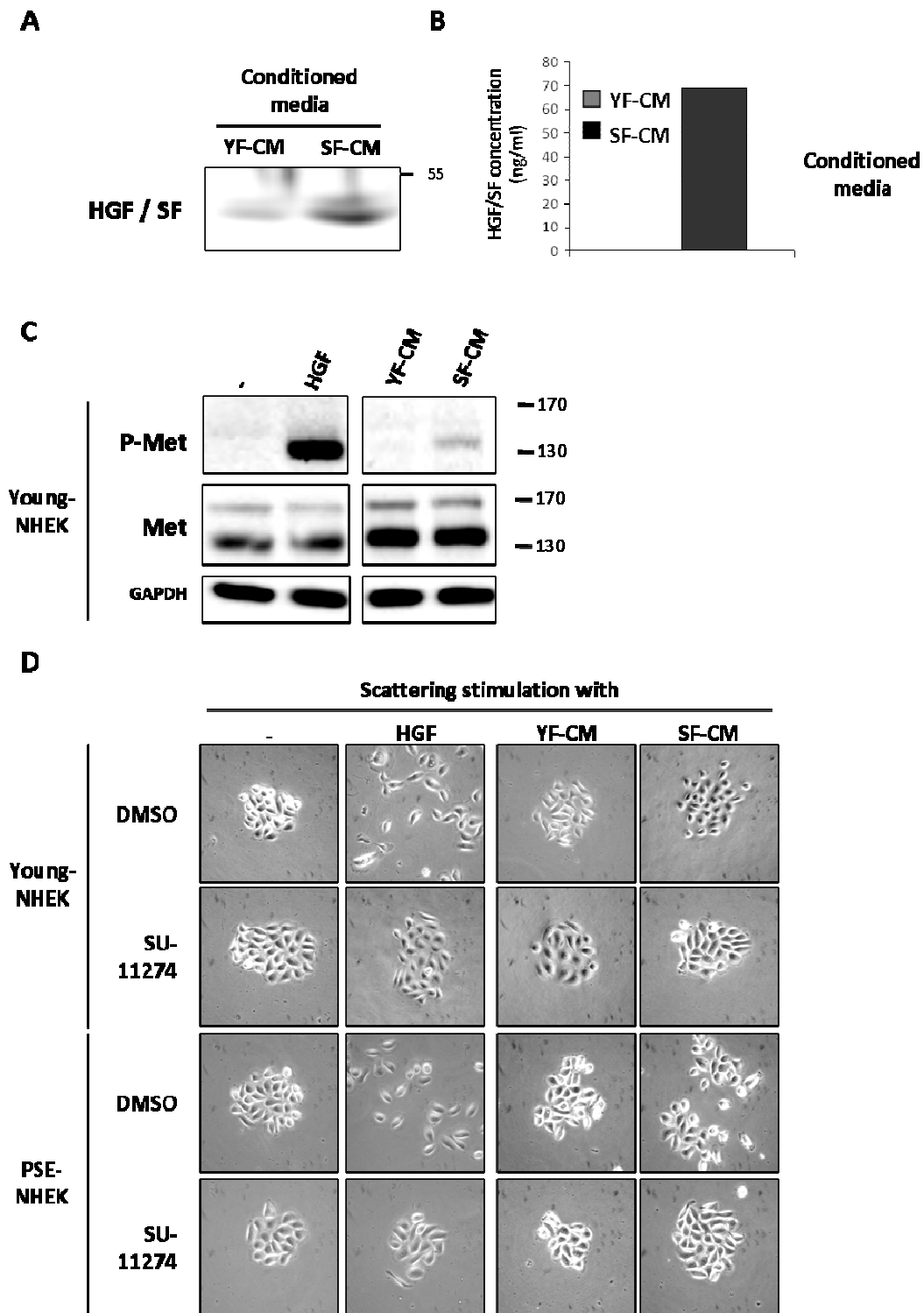


Figure 28 : L'HGF/SF sécrété par les fibroblastes sénescents de derme active le récepteur Met des kératinocytes cultivés en KGM pour induire leur dissémination

Les milieux conditionnés de $5 \cdot 10^5$ fibroblastes jeunes et sénescents sont récupérés après 48H d'incubation à 37°C et concentrés par ultrafiltration (Amicon, cut-off 3kDa). Ces milieux conditionnés concentrés sont utilisés dans les différentes expériences suivantes. Des analyses par (A) Western-blot et (B) dosage Elisa (Quantikine Human HGF Immunoassay kit, R&D system) montrent une augmentation de la sécrétion d'HGF/SF par les fibroblastes sénescents. (C) La stimulation des kératinocytes jeunes cultivés en KGM par l'HGF/SF sécrété par les fibroblastes sénescents et par de l'HGF/SF recombinant (30ng/ml) pendant 10 minutes activent le récepteur Met (l'activation est détectée par phosphorylation du récepteur (p-Met) en western-blot). (D) Les kératinocytes jeunes et PSE (cultivés en KGM) prolifèrent sous forme d'îlots. La stimulation de ces îlots (d'environ 10 cellules) par l'HGF recombinant (10ng/ml) et le milieu conditionné de fibroblastes sénescents induit la dispersion des cellules. L'inhibition de l'activité du récepteur Met par le SU-11274 annule cet effet de dispersion.

2.1. Sécrétion de facteurs de croissance par les fibroblastes sénescents : exemple de l'HGF/SF.

Les facteurs de croissance (FC) sont également fortement sécrétés dans un contexte pro-inflammatoire et participent activement aux interactions épithélium-stroma. Ainsi, les fibroblastes de derme peuvent sécréter de nombreux FCs lorsque les conditions d'homéostasie sont rompues (ex : cicatrisation). Par RT-qPCR nous avons pu montrer une augmentation d'expression de plusieurs FC dans les fibroblastes sénescents par rapport aux jeunes dont le KGF (Keratinocytes Growth Factor), le TGF- β 1 (Transforming Growth Factor), le VEGF (Vascular Endothelium Growth Factor) et l'HGF/SF (Hepatocellular Growth Factor / Scatter Factor) (données non présentées).

Nous nous sommes intéressés plus particulièrement à l'HGF/SF car il est souvent associé aux mécanismes de la TEM et de migration des cellules. Nous avons tout d'abord confirmé la forte augmentation de la sécrétion d'HGF/SF dans le milieu conditionné de fibroblastes sénescents (SF-CM) par rapport aux jeunes (YF-CM) par des analyses de western-blot (figure 28 A) et de dosage Elisa (figure 28 B). Le récepteur à haute affinité de l'HGF/SF, c-Met, est un récepteur de la famille tyrosine kinase qui est exprimé dans les kératinocytes jeunes (figure 28C) et PSE (données non présentées) cultivés dans leur milieu classique (KGM). L'HGF/SF sécrété par les fibroblastes sénescents est capable d'activer le récepteur Met exprimé sur les NHEK jeunes en induisant son auto-phosphorylation (p-Met) tout comme l'HGF/SF recombinant (figure 28 C). Enfin, nous avons pu montrer que l'HGF/SF recombinant et l'HGF/SF sécrété par les fibroblastes sénescents sont capables d'induire la dispersion des kératinocytes jeunes et PSE (cultivés en KGM) qui ont proliféré sous forme de petits îlots (figure 28 D). Cette dispersion est bien spécifique de l'activation du récepteur Met puis qu'elle est inhibée par l'ajout du SU-11274 (inhibiteur de l'activité kinase de Met) (figure 28D). Ces résultats montrent que l'HGF/SF participe à la migration de kératinocytes normaux (jeunes) et néoplasiques (PSE) mais seulement lorsqu'ils sont cultivés dans le KGM. En effet le conditionnement des kératinocytes-PSE (par le FGM, YF-CM et SF-CM) induit une perte de l'expression du récepteur Met et une perte de l'effet attracteur de l'HGF/SF (données non présentées).

Discussion

1. La sénescence des kératinocytes peut permettre l'initiation des carcinomes de la peau.

Les carcinomes non-mélanocytaires de la peau sont les cancers les plus courants dans la population vieillissante. Ils sont en augmentation constante représentant un véritable enjeu de santé publique et de coût pour la société d'autant plus que le risque de développer un autre type de carcinome est augmenté suite au diagnostic d'un carcinome non-mélanocytaire de la peau (Wheless *et al*, 2010). L'amélioration des diagnostics et des thérapies passe par une meilleure compréhension des mécanismes associés aux étapes initiales de la carcinogénèse. Les données épidémiologiques montrent clairement que les carcinomes représentent une pathologie liée à l'âge. Pourtant le vieillissement cellulaire, ou sénescence, est un mécanisme mis en place afin de limiter la transformation par un puissant blocage du cycle cellulaire (Hayflick, 1965 ; Collado *et al*, 2007). En effet, suite au raccourcissement télomérique (sénescence répllicative) ou à divers stress environnementaux (radiation, ROS, agents chimiques...) ou intrinsèques (activation d'oncogènes, sénescence prématurée), les voies de contrôle du cycle (p16^{INK4A}/Rb et p53/ p21^{CIP1/WAF1}) sont activées pour bloquer le cycle cellulaire.

Les cellules épithéliales de la peau ou kératinocytes, sont les cellules à l'origine des carcinomes de la peau et sont particulièrement exposées aux stress environnementaux mutagènes provoquant une accumulation de cellules en sénescence prématurée au niveau de l'épiderme (Dimri *et al*, 1995). Notre équipe a ainsi pu démontrer qu'en culture la sénescence des kératinocytes humains est induite, au moins partiellement, par une accumulation d'espèces réactives de l'oxygène (ROS) suite à la sur-expression de la Superoxyde Dismutase (MnSOD) elle-même induite par l'augmentation d'activité du facteur de transcription NF-κB (Bernard *et al*, 2001a, 2004). La survenue de la sénescence des kératinocytes est donc indépendante du raccourcissement télomérique. Ces évènements sont responsables de l'arrêt de prolifération de sénescence par l'intermédiaire de l'activation d'inhibiteurs du cycle cellulaire comme p21 (Sayama *et al*, 1999). Les ROS à l'origine de la sénescence des kératinocytes vont également activer les voies de la macro-autophagie qui vont induire la mort pour la grande majorité des kératinocytes sénescents (Gosselin *et al*, 2009a ; Deruy *et al*, 2010). Dans ce cas la sénescence joue bel et bien son rôle de suppresseur de tumeur.

Pourtant une sénescence prématurée induite par des stress peut bien être aussi à l'origine de l'initiation tumorale. En effet les agents inducteurs de sénescence peuvent être aussi responsables

d'instabilités génomiques à l'origine de la transformation cellulaire (Radisky *et al*, 2005). De plus dans cet état de sénescence, les cellules ont conservé des télomères suffisamment longs permettant de re-proliférer en cas d'échappement au blocage du cycle (Rheinwald *et al*, 2002; Jacobs & de Lange, 2004). Ainsi nous avons pu montrer qu'en culture, les kératinocytes primaires humains peuvent échapper spontanément de l'état de sénescence pour générer des clones de cellules post-sénescence émergentes (PSE) néoplasiques capables de proliférer à nouveau (**article n°1**- Gosselin *et al*, 2009b). Les cellules émergentes sont produites à partir des cellules sénescents polynucléées par un mécanisme original de mitose par bourgeonnement proche du mécanisme de division appelé *neosis* (Sundaram *et al*, 2004; Rajaraman *et al*, 2006). Nos études sur la caractérisation des cellules post-sénescence émergentes (PSE) démontrent leur caractère transformé : morphologie, nombreuses délétions aléatoires au niveau de génome (CGH-array), modifications du transcriptome, induction de lésions tumorales en souris NUDE, etc... Le stress oxydant lié à la sénescence des kératinocytes est donc à la fois suppresseur de tumeur (mort par autophagie) et promoteur de tumeur en provoquant une instabilité génomique par l'intermédiaire des cassures de l'ADN (Gosselin *et al*, 2009c). Le niveau de ROS et le niveau d'activité autophagique associé sont importants pour orienter l'issue des kératinocytes sénescents vers la mort ou vers l'émergence néoplasique (Deruy *et al*, article en préparation). Les kératinocytes néoplasiques PSE ne sont cependant pas immortalisés (car ils ne ré-expriment pas la télomérase) et atteignent un second plateau de sénescence en culture.

Selon les donneurs de cellules primaires, une seconde émergence de cellules appelée ImK (Immortal Keratinocytes) peut avoir lieu à partir des kératinocytes bloqués au second plateau de sénescence à une fréquence très faible. Les cellules ImK présentent des caractères de transformation plus marqués que les cellules PSE (translocations chromosomiques, morphologie fibroblastoïde plus marquée...). Jusqu'à présent les mécanismes associés au second plateau de sénescence et à la seconde émergence ont été moins décrits mais il est probable qu'ils fassent intervenir les mêmes acteurs que la première sénescence et émergence (ROS et autophagie...).

Les étapes successives de sénescence et d'émergence aggravent donc la transformation des kératinocytes néoplasiques ce qui constitue un modèle d'étude des mécanismes cellulaires et moléculaires très initiaux de la carcinogenèse de la peau lors du vieillissement. Ce mécanisme d'émergence post-sénescence intrinsèque a été observé également dans des cellules mammaires sénescents (HMEC) présumant que notre modèle d'étude des phases initiales de la transformation cellulaire peut être applicable dans d'autres types de carcinomes (Romanov *et al*, 2001 ; Tlsty *et al*, 2001). Ces émergences néoplasiques à partir de la sénescence pourraient permettre d'expliquer la présence de cellules sénescents au niveau de lésions tumorales

précoces (poumon, prostate, pancréas...) (Braig *et al*, 2005; Chen *et al*, 2005 ; Collado *et al*, 2005; Collado & Serrano, 2010).

Actuellement nous nous efforçons d'évaluer la présence de kératinocytes émergents post-sénescence (PSE) au sein de biopsies de peau de donneurs humains sains à différents âges et sur différents stades de la progression tumorale (dysplasies, BCC, SCC). Il est d'ores et déjà décrit que l'accumulation de kératinocytes sénescents participe à l'altération de l'architecture de l'épiderme au cours du vieillissement (Smith, 1989; Dimri *et al*, 1995). Nous avons observé la présence de nombreuses petites lésions microscopiques au sein des couches les plus basales de l'épiderme dans les tissus âgés. Nous pensons que les kératinocytes sénescents et PSE pourraient être à l'origine de certaines de ces altérations. Néanmoins notre difficulté est encore de pouvoir identifier ces cellules sénescents et PSE dans les tissus de peau humaine. Nous sommes actuellement à la recherche de marqueurs nous permettant d'identifier les cellules sénescents au sein d'un tissu. Nos pistes sont les marqueurs d'arrêt du cycle (p16, p53...), d'autophagie (LAMP-1...) et des voies de réponses aux dommages à l'ADN (DDR) (apparition de foyers γ H2AX, 53BP1, XRCC1...).

2. Les fibroblastes sénescents de derme établissent un environnement propice à l'initiation tumorale

Bien que l'initiation et le développement des carcinomes soient associés à des désordres moléculaires intrinsèques (mutations d'oncogènes et de gènes suppresseurs de tumeurs), il est maintenant bien établi que le micro-environnement cellulaire contribue activement à l'évolution tumorale (Liotta & Kohn, 2001). Ainsi les fibroblastes activés (CAF) présents dans le stroma péri-tumoral (Commandeur *et al*, 2011), tout comme les fibroblastes sénescents s'accumulent dans le micro-environnement vieillissant et présents à proximité des carcinomes (Dimri *et al*, 1995 ; Yang *et al*, 2006), peuvent stimuler la croissance et l'invasion des cellules déjà transformées par le biais notamment d'un sécrétome pro-inflammatoire (Olumi *et al*, 1999 ; Krtolica *et al*, 2001).

Un des objectifs de ma thèse était de mieux caractériser le sécrétome des fibroblastes normaux de derme humains (NHDF) sénescents pour lequel il existait peu de données. Tout d'abord j'ai pu montrer que ces NHDF sénescents produisent un sécrétome hautement pro-inflammatoire

composé de cytokines, de métalloprotéases et de facteurs de croissance (figure 29A) (HGF/SF ; TGF- β 1...) comme cela a pu être décrit pour d'autres modèles cellulaires (Coppé *et al*, 2008; Ohanna *et al*, 2011). Contrairement à la plupart des cytokines déjà présentes dans le milieu conditionné de NHDF jeunes, le MCP-1 (Monocyte chemoattractant protein 1) et l'IL-6 (interleukines-6) semblent être spécifiquement sécrétés à la sénescence. MCP-1 (appelé également CCL-2), est également le facteur majoritairement sécrété par les mélanocytes sénescents stimulant l'invasion et la tumorigenèse in vivo de cellules cancéreuses de mélanomes (Ohanna *et al*, 2011). Aussi, une forte production d'IL-6 est associée à un mauvais pronostic dans de nombreux cancers (prostate, poumon, ovaire...) (Alcover *et al*, 2010; Su *et al*, 2011; Lane *et al*, 2011). IL-6 est également ciblée dans de nouvelles approches thérapeutiques (Bayliss *et al*, 2011; Tawara *et al*, 2011). Ces cytokines fortement sécrétées par les fibroblastes sénescents de derme sont donc des facteurs critiques pour la mise en place d'un micro-environnement pro-tumoral. Le caractère pro-inflammatoire des NHDF sénescents est aussi renforcé par la sécrétion et l'activation de plusieurs métalloprotéases (MMPs) dont les MMP-1, -2, et -3 et l'activation de la MMP-9 déjà présente dans le milieu. L'activation de ces MMPs peut être issue de l'activité de la MT1- MMP à la surface membranaire des fibroblastes sénescents (Strongin *et al*, 1995; Cao *et al*, 1995).

Outre le caractère pro-inflammatoire, nous avons pu mettre en évidence des défauts de sécrétion de constituants de la matrice extra-cellulaire (collagène, laminine...) par les NHDF sénescents, contribuant ainsi à l'altération du derme vieillissant (figure 29A) (Lee *et al*, 2008; Kurban & Bhawan, 1990). Nos données issues de l'analyse comparée des milieux conditionnés de NHDF jeunes et sénescents par spectrométrie de masse montrent de façon inédite la perte de sécrétion de plusieurs glycoprotéines matricielles par les fibroblastes sénescents dont la décorine, SPARC (Secreted Protein, Acidic and Rich in cystein) et PEDF (Pigment Epithelium Derived Factor) (figure 29A). Ces glycoprotéines ont des rôles variés dans la production et l'organisation de la MEC ainsi que dans les interactions avec les cellules (Rentz *et al.*, 2007). De manière plus intéressante encore, ces glycoprotéines possèdent également de puissantes activités anti-tumorales. Ainsi le PEDF dont l'expression décroît avec l'âge au niveau de la peau est un puissant inhibiteur de l'angiogenèse, mais peut aussi induire la différenciation et l'apoptose des cellules épithéliales (Dawson *et al*, 1999; Francis *et al*, 2004; Li *et al*, 2011). SPARC dont l'expression a été démontrée dans les tissus de peau jeunes peut également inhiber le développement tumoral selon les modèles d'étude (Hunzelmann *et al*, 1998; Brekken & Sage, 2001; Said *et al*, 2007). Enfin la décorine inhibe la croissance et l'invasion de cellules

cancéreuses notamment en inhibant l'activité de récepteurs à tyrosine kinase (ex : EGFR, Met) (Seidler et al, 2006; Goldoni et al, 2008, 2009). L'altération au cours du vieillissement de la sécrétion de ce type de glycoprotéines matricielles aux propriétés anti-tumorales (Carrino *et al*, 2011, 2003) au niveau du derme papillaire (le plus superficiel) (Mine et al, 2008) peut donc participer non seulement à l'altération des tissus de derme mais aussi à la progression tumorale des carcinomes.

Les mécanismes associés à la production d'un sécrétome pro-inflammatoire par les cellules sénescents commencent seulement à être décrits. Les cassures à l'ADN et la persistance de l'activation des voies de réponse aux dommages à l'ADN (DDR) semblent être nécessaires tout comme l'activation du facteur de transcription NF- κ B (Rodier *et al*, 2009, 2011; Rodier & Campisi, 2011 ; Chien *et al*, 2011 ; Ohanna *et al*, 2011). Les réarrangements de la structure chromatinienne associés à la sénescence (SAHF) semblent être également impliqués dans la production d'un sécrétome pro-inflammatoire (Pazolli *et al*, 2012). Par contre il existe très peu de données sur les mécanismes associés à la sénescence contribuant aux défauts de sécrétion des glycoprotéines matricielles, comme SPARC et la décorine.

J'ai pu démontrer de manière inédite au cours de la fin de ma thèse que ces défauts de sécrétion peuvent être causés par un stress du réticulum endoplasmique (RE) induisant la re-localisation de SPARC et de la décorine vers les voies de dégradation associées aux lysosomes (dont les activités sont fortement augmentées en lien avec la sénescence). Lorsque les conditions micro-environnementales sont anormales (inflammation, hypoxie...), la conformation des glycoprotéines peut être incorrectement effectuée suite à un déséquilibre de l'homéostasie du RE (Hotamisligil, 2010). Cela active les 3 voies de l'UPR (Unfolded Protein Response), que sont ATF-6, PERK et IRE-1 qui vont induire un programme transcriptionnel afin de rétablir l'homéostasie du RE et d'éliminer les glycoprotéines mal conformées (par les voies du protéasome, ou de l'autophagie) (Schröder & Kaufman, 2005; Ron & Walter, 2007). Nous avons montré que les fibroblastes sénescents de derme présentent une forte expression des transcrits cibles de voies de l'UPR (ex : BIP, GRP94, HRD-1...) suggérant qu'ils sont en état de stress du RE. De plus, nos données indiquent que la voie ATF-6 de l'UPR a un rôle prédominant dans les défauts de sécrétion des glycoprotéines SPARC et décorine à la sénescence.

En général, les cellules ne réussissant pas à rétablir l'homéostasie du RE meurent par apoptose. Pourtant les fibroblastes sénescents de derme semblent rester de manière prolongée dans cet état contribuant à installer l'altération du stroma. Des données suggèrent qu'au contraire des voies

PERK et IRE-1, la voie ATF-6 est impliquée dans la survie cellulaire (Xu *et al*, 2005 Egawa *et al*, 2011). L'activation de la voie ATF-6 pourrait donc contribuer aux capacités étendues de survie des fibroblastes sénescents en culture et à la mise en place d'un micro-environnement pro-tumoral.

Une étroite relation entre l'inflammation et le stress du RE est décrite dans la littérature (Hasnain *et al*, 2012). Tout comme ce que nous montrons pour les fibroblastes sénescents de derme, il a été montré que les cellules dans un état de stress du RE vont produire une grande quantité de cytokines inflammatoires. Généralement l'activation du facteur de transcription NF-kappa B est associée à ce phénomène (Yamazaki *et al*, 2009). Mais une forte inflammation dans le micro-environnement peut également être à l'origine d'un stress du RE (Zhang & Kaufman, 2008). Donc le sécrétome pro-inflammatoire des fibroblastes sénescents de derme pourrait contribuer à l'entrée en stress du RE des cellules voisines et donc contribuer à épandre l'altération du tissu.

3. L'initiation tumorale des kératinocytes est promue par le micro-environnement généré par les fibroblastes sénescents

Alors que la contribution des CAFs (carcinoma associated fibroblasts) ou des fibroblastes sénescents sur la progression et l'invasion des cancers déjà établis est bien décrite, leur impact sur les étapes initiatrices du développement tumoral est encore très peu connu. Les CAFs étant activés par les cellules cancéreuses elles-mêmes, leur impact sur les étapes initiales du développement des carcinomes semble être peu probable (Fromigué *et al*, 2003 ; Mishra *et al*, 2008). Cependant contrairement aux CAFs, les fibroblastes sénescents s'accumulent dans le stroma vieillissant indépendamment (peut-être même antérieurement) de l'apparition des cellules cancéreuses et pourraient donc contribuer aux étapes initiatrices de la carcinogénèse.

Nous avons étudié l'impact du sécrétome des fibroblastes sénescents de derme sur l'initiation tumorale des kératinocytes en ne remplaçant que de 10% du milieu de culture des kératinocytes par du milieu frais de culture des fibroblastes (FGM), du milieu conditionné de fibroblastes jeunes (YF-CM) ou sénescents (SF-CM). Seuls 10% du milieu ont été remplacé car les kératinocytes sont très sensibles à l'effet différenciateur du calcium présent le milieu de culture des fibroblastes (FGM) (Tsao *et al*, 1982; Boyce & Ham, 1983).

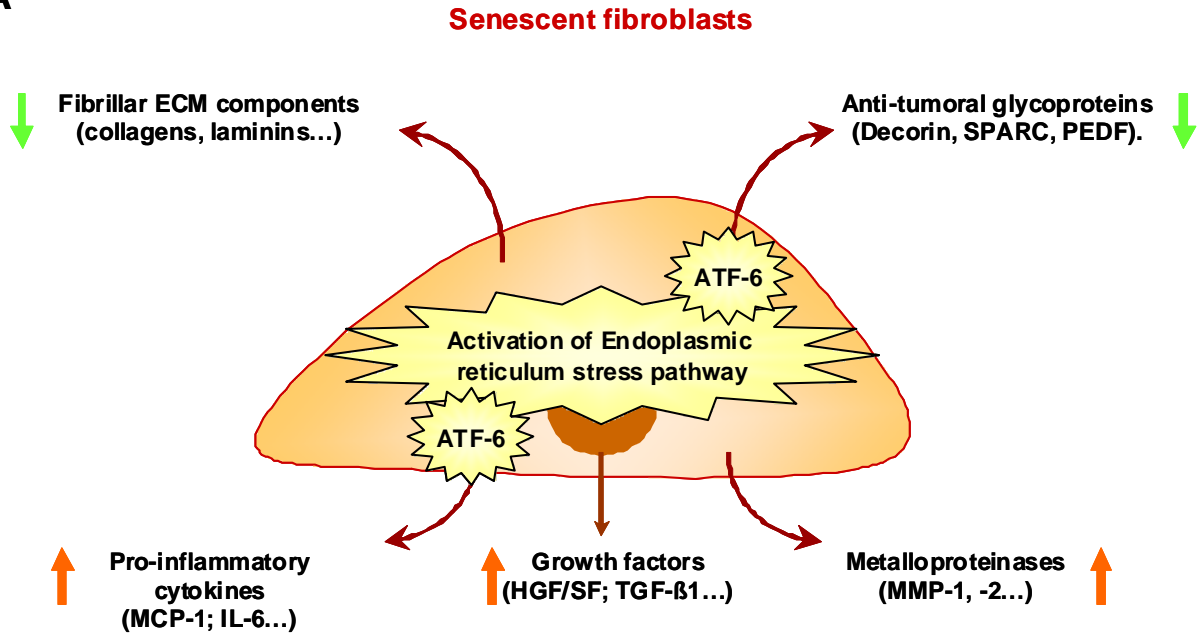
Nous avons mis en évidence que le SF-CM augmente d'une part la fréquence d'émergence post-sénescence des kératinocytes et d'autre part augmente la proportion de clones de kératinocytes PSE présentant une morphologie fibroblastoïde. Le FGM et le YF-CM permettent dans une moindre mesure, et un peu plus tard, l'apparition de clones de kératinocytes PSE fibroblastoïdes. Le TGF- β 1, puissant inducteur de la TEM (Zavadil & Böttinger, 2005) est plus fortement exprimé et activé par les fibroblastes sénescents de derme (données non montrées). Il est présent dans une moindre mesure dans le sérum du FGM et du YF-CM. Il est donc possible que l'apparition de clones de kératinocytes PSE fibroblastoïdes suite au conditionnement par le FGM et le YF-CM soit due à la fraction de sérum et de TGF- β 1 (Bertolero *et al*, 1986).

Des données bibliographiques montrent que la TEM peut permettre d'échapper au processus de sénescence prématurée (Ansieau *et al*, 2008a) et rend les cellules plus résistantes aux processus de mort (Robson *et al*, 2006). Si le conditionnement par le sécrétome des fibroblastes sénescents favorise d'abord l'émergence post-sénescence fibroblastoïde des kératinocytes, la succession des passages en culture pourrait finir par sélectionner artificiellement les kératinocytes PSE les plus résistants, en l'occurrence ceux qui ont subi une TEM. Cette sélection résulte en la production de populations homogènes de cellules fibroblastoïdes même dans les autres conditions de culture (FGM, YF-CM).

Le sécrétome des fibroblastes sénescents favorise ensuite la sélection et la prolifération des cellules en seconde émergence (ImK) toujours très fibroblastoïdes. Nos études précédentes indiquent que les ImK obtenus dans leur culture classique (KGM) présentent des caractères très transformés. Nous n'avons pas encore pu détailler les mécanismes par lesquels le sécrétome des fibroblastes sénescents renforce la transformation des kératinocytes PSE et ImK. Au vue des travaux réalisés par l'équipe nous supposons que le SF-CM pourrait agir sur le niveau de stress oxydant, les activités autophagiques et les dommages à l'ADN pour sélectionner les cellules les plus transformées.

Ces résultats d'impact du micro-environnement vieillissant sur l'initiation cancéreuse des kératinocytes obtenus *in vitro* peuvent être renforcés par nos expériences d'injection des kératinocytes PSE et ImK (cultivés en KGM) dans des souris immunodéficientes. En effet l'injection de ces cellules dans des souris jeunes induit des lésions tumorales (à distance du site d'injection) très tardivement (après 8 à 10 mois) lorsque les souris sont devenues vieilles. L'injection de ces mêmes cellules dans des souris âgées provoque des lésions tumorales beaucoup plus rapidement (3 à 4 mois). Ces résultats suggèrent donc que le micro-environnement vieillissant de la souris favoriserait le développement tumoral des kératinocytes.

A



B

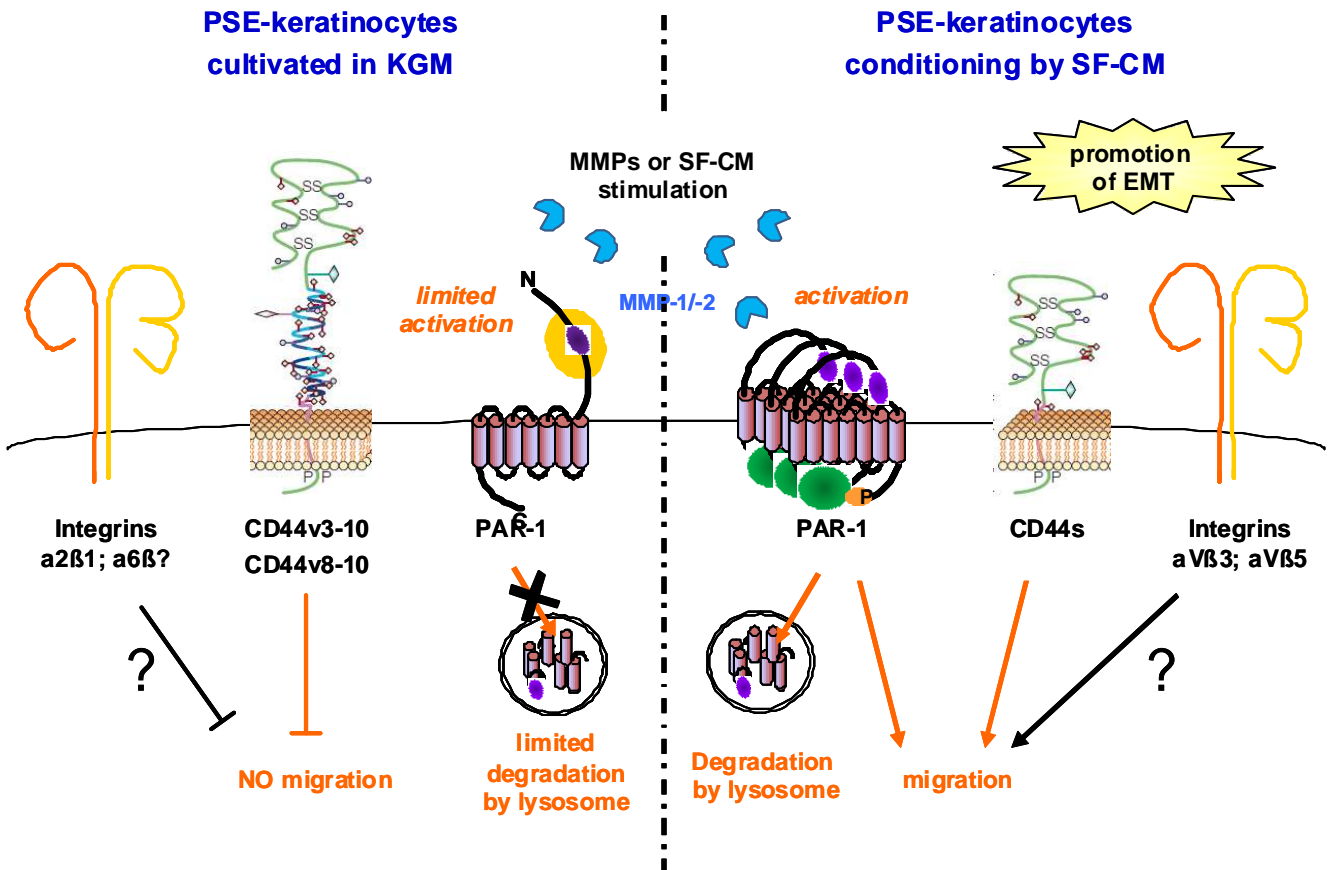


Figure 29 : Schéma récapitulatif des différents résultats obtenus (A) sur la caractérisation du sécrétome des fibroblastes sénescents de derme (↑ augmentations et ↓ diminutions de sécrétion à la sénescence); (B) sur l'impact du conditionnement par ce sécrétome envers les capacités migratoires des kératinocytes émergents post-sénescence (PSE).

4. L'activité de l'axe MMP/ PAR-1 est un nouveau marqueur de dissémination précoce.

Le renforcement de la TEM des kératinocytes PSE par le sécrétome des fibroblastes sénescents est accompagné d'une augmentation de leur capacité migratoire (en réponse au stimulus attractant de ce même sécrétome en tests de migration par transwell®). Ce résultat obtenu *in vitro* peut être mis en corrélation avec les données observées par l'injection de kératinocytes PSE dans le flanc des souris immunodéficientes. En effet, des lésions tumorales vont se former à partir de ces kératinocytes mais à distance du site d'injection suggérant que les cellules ont dans un premier temps disséminé.

Nous avons pu démontrer que les métalloprotéases (-1 et -2) sécrétées et activées par les fibroblastes sénescents sont des acteurs majeurs de l'activation des capacités migratoires des kératinocytes PSE soumis à l'attraction du milieu conditionné de fibroblastes sénescents (figure 29B). Outre le fait qu'elles peuvent induire une TEM, les MMPs sont souvent associées à la stimulation de la migration et de l'invasion des cellules (Egeblad & Werb, 2002). Nous avons montré que les MMPs stimulent la migration des kératinocytes PSE néoplasiques en activant le récepteur à la thrombine PAR-1 (protease activated receptor) (figure 29B). PAR-1, qui est surexprimé dans les kératinocytes PSE et plus encore dans les Imk en réponse au conditionnement des fibroblastes sénescents est généralement associé aux capacités invasives des cellules cancéreuses (Elste & Petersen, 2010). Plusieurs études montrent que l'axe MMP-1/PAR-1 peut être impliqué dans l'invasion des cellules cancéreuses (Boire *et al*, 2005 ; Fujimoto *et al*, 2008; Blackburn *et al*, 2009; Wang *et al*, 2011; Peng *et al*, 2012). Nos études suggèrent donc l'axe MMP/PAR-1, jusqu'ici décrit dans les processus métastatiques, pourrait être également associé à la contribution du microenvironnement aux phases les plus initiales de la transformation des cellules épithéliales lors du vieillissement.

Nous n'avons pas encore complètement décrit les mécanismes précis de la régulation de l'activation de PAR-1 par les MMPs. L'interaction des MMPs avec PAR-1 n'étant pas très spécifique, des partenaires membranaires pourraient intervenir pour favoriser l'activation de PAR-1. De nombreux récepteurs membranaires interviennent au cours des processus migratoires parmi lesquels CD44 et les intégrines sont capables à la fois de recruter des MMPs et d'interagir avec d'autres récepteurs membranaires (notamment des récepteurs à tyrosine kinase) pour les activer (Ivaska & Heino, 2000; Zöller, 2011). Ils sont donc des bons candidats pour participer à la régulation de l'activation de PAR-1 par les MMPs.

Nous avons montré que le sécrétome des fibroblastes sénescents modifie considérablement les isoformes de CD44 exprimées par les kératinocytes PSE (perte de CD44v3-v10 et v8-v10, isoformes spécialisées des kératinocytes normaux, au profit du CD44-standard) (figure 29B). Ce changement d'expression des isoformes de CD44 a été décrit pour être nécessaire à la TEM de cellules mammaires et à leur progression tumorale (Brown *et al*, 2011). De plus, les isoformes longues de CD44 comme CD44v3-v10 peuvent inhiber les processus invasifs des cellules (Harrison *et al*, 2006). Nous avons également montré que le patron d'expression des intégrines est modifié (perte des sous-unités $\alpha 2$ et $\alpha 6$; gain des sous-unités $\alpha 3$ et $\beta 5$) dans les kératinocytes PSE sous l'effet du conditionnement du sécrétome des fibroblastes sénescents (figure 29B). Des données bibliographiques indiquent que les intégrines $\alpha 2\beta 1$ peuvent inhiber les processus invasifs des cellules cancéreuses (Ramirez *et al*, 2011), au contraire des intégrines $\alpha 3\beta 1$ et $\alpha V\beta 5$ qui peuvent être impliquées dans les mécanismes de TEM et migratoires des cellules (Kawahara *et al*, 1995; Natali *et al*, 1993 ; Borok, 2009; Kim *et al*, 2009).

Tous ces changements d'expression de CD44 et d'intégrines corréleront donc avec la TEM que subissent les kératinocytes néoplasiques PSE sous l'effet du sécrétome des fibroblastes sénescents et sont favorables à la migration des cellules. Jusqu'à présent, aucune donnée bibliographique ne montre d'interaction directe entre CD44 et/ou les intégrines avec le récepteur PAR-1. Néanmoins nous pensons que CD44 et/ou les intégrines pourraient participer aux régulations de l'activation de PAR-1 par les MMPs matricielles.

L'axe MMP/PAR-1 n'est sans doute pas le seul impliqué dans les mécanismes de migration des kératinocytes PSE néoplasiques en réponse au sécrétome des fibroblastes sénescents. Ainsi les facteurs de croissance (HGF/SF ; TGF- $\beta 1$; KGF...) et les cytokines (MCP-1 ; IL-6...) sécrétés par les fibroblastes sénescents pourraient stimuler cette migration si leurs récepteurs spécifiques sont exprimés à la surface des kératinocytes.

La TEM et l'acquisition des capacités migratoires et invasives ont longtemps été associées uniquement aux stades tardifs du développement tumoral (métastases). Pourtant des données cliniques et expérimentales récentes suggèrent qu'une dissémination très précoce des cellules néoplasiques peut avoir lieu dès les premiers stades du développement tumoral (Ansieau *et al*, 2008b; Sabe, 2011; Weinberg, 2008). Une sur-expression du facteur de transcription Twist-1 peut être associée à cette dissémination précoce ainsi qu'un fort contexte inflammatoire (Hüsemann *et al*, 2008; Rhim *et al*, 2012).

Le traitement des carcinomes de la peau est de relativement bon pronostic car ils sont facilement détectables à l'examen externe et correctement réséqués par chirurgie, ce qui diminue le risque de développer des stades métastatiques. Pourtant nos données *in vitro* suggèrent que des kératinocytes néoplasiques PSE sur-exprimant Twist-1, pourraient disséminer dès les premiers stades de la carcinogénèse de la peau sous l'influence d'un micro-environnement vieillissant et pro-inflammatoire instauré par les fibroblastes sénescents.

La dissémination précoce des cellules néoplasiques peut permettre leur re-localisation dans d'autres organes dans lesquels elles peuvent persister en dormance (Páez *et al*, 2012; Eyles *et al*, 2010) augmentant le risque de développer des carcinomes par la suite. Ce concept permet d'expliquer l'apparition de métastases chez des patients qui n'ont jamais été diagnostiqués pour des tumeurs primaires (CUP : cancer of unknown primary) (Pavlidis & Fizazi, 2005). Aussi il pourrait expliquer pourquoi après avoir développé un carcinome de la peau, le risque de développer un autre type de carcinome augmente (Wheless *et al*, 2010).

Nous cherchons actuellement à vérifier si la dissémination précoce des kératinocytes en lien avec un micro-environnement vieillissant peut être décrite *in vivo*. Nous avons donc débuté l'étude de l'expression de PAR-1, de twist-1 et des MMPs dans des coupes de peau humaines issues de donneurs jeunes ou âgés. D'une part, nous avons observé une accumulation des activités métalloprotéasiques (par zymographie *in situ*) au niveau du derme réticulaire des peaux de donneurs âgés. D'autre part, sur ces mêmes coupes, nous avons également observé des marquages de PAR-1, par immunohistochimie, sous forme de petits foyers au niveau des couches basales de l'épiderme et au niveau de cellules qui ont pénétré dans le stroma. De même, un marquage de twist-1 peut être observé dans des noyaux de cellules situées à proximité des épithéliums de donneurs âgés. Il se peut donc que des kératinocytes néoplasiques puissent subir une TEM dans les épidermes de peaux de donneurs âgées, puis de disséminer dans leur micro-environnement du fait de sa richesse en MMPs.

Les études de la dissémination précoce des cellules néoplasiques restent difficile *in vitro* comme *in vivo*. La limite des études *in vitro* est de ne pas pouvoir facilement placer les kératinocytes et les fibroblastes dans leur contexte physiologique. En effet les interactions entre les cellules épithéliales et leur micro-environnement se font au sein d'un tissu complexe avec la présence d'une matrice extracellulaire et plusieurs types cellules autres que les fibroblastes (cellules endothéliales, cellules de l'inflammation). Les systèmes vasculaires et inflammatoires sont d'ailleurs fortement perturbés avec le vieillissement et participent activement à la progression

tumorale (Coussens *et al*, 1999; Alonso-Fernández & De la Fuente, 2011; Bennett *et al*, 2008; Chung & Eun, 2007). De plus les fonctions des molécules d'adhérence (ex : CD44, intégrines...) lors des processus migratoires peuvent dépendre du contexte micro-environnemental dans lequel se situent les cellules. Pour l'instant les études *in vivo* chez la souris restent donc des modèles plus relevant pour étudier la complexité du micro-environnement vieillissant sur l'initiation tumorale des kératinocytes. Mais la principale difficulté de ce modèle pour étudier une dissémination précoce de cellules isolées est de pouvoir suivre et identifier ces cellules au sein des souris entières.

C'est pourquoi nous cherchons à mettre au point des modèles expérimentaux *in vitro* plus relevant physiologiquement afin de mieux décrire les effets d'un micro-environnement vieillissant sur l'initiation tumorale des kératinocytes et sur leur dissémination précoce. Ainsi les modèles de peau reconstituée, dans lesquels les fibroblastes sontensemencés au sein d'un gel de collagène et où les kératinocytes sont déposés sur une fine couche de matrigel ou de collagène IV (lame basale), représentent une alternative intéressante pour nos études. Ces modèles expérimentaux nous permettrons d'une part de placer les kératinocytes et fibroblastes dans leur contexte matriciel respectif et d'autre part de pouvoir manipuler l'activité (inhibiteurs pharmacologiques, protéines recombinantes...) ou l'expression (inhibition ou sur-expression par des approches moléculaires) des acteurs moléculaires d'intérêt (ex : PAR-1, CD44, intégrines...) et d'en étudier les conséquences plus facilement que dans les études réalisées avec les souris.

Annexes

Article complémentaire n°1

*Levels of macroautophagy drive senescent keratinocytes
into cell death or neoplastic transformation*

Levels of macroautophagy drive senescent keratinocytes into cell death or neoplastic transformation

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Key words: autophagy, senescence, oxidative stress, cancer initiation

Abstract

Senescence is a non proliferative state reached by normal cells in response to various stresses including telomere shortening, oxidative stress or oncogene activation. In recent reports, we have highlighted that senescent human epidermal keratinocytes have two opposite outcomes: either they die by autophagic programmed cell death or they generate neoplastic cells called post-senescence (PS) emergent cells. Herein, we show that partially reducing macroautophagy in the all senescent population using 3-methyl adenine or anti-Atg5 siRNAs increases the emergence frequency, suggesting that senescent keratinocytes have to escape autophagic cell death to generate PS emergent cells. However, totally inhibiting macroautophagy drastically impairs emergence, indicating that senescent keratinocytes need to achieve basal macroautophagy for emergence to occur. In accordance to both results, we demonstrate that the progenitors of PS emergent cells display a level of macroautophagy slightly lower than that of the average senescent population. Macroautophagy thus plays antagonistic roles during senescence, inducing cell death or promoting neoplastic transformation, depending on its level of activation. We also show that this level of macroautophagic activation is directly correlated with the level of reactive oxygen species. Taken together, these data suggest that levels of oxidative damages and ensuing macroautophagic activity could be two main determinants of the very initial phases of neoplastic transformation occurring during aging.

Introduction

Initially described as the phase reached by human normal diploid fibroblasts after a limited number of serial passages in *in vitro* culture,¹ senescence is now recognized as a fundamental program activated whatever the cell type in response to various situations including telomere erosion or uncapping,^{2, 3} irreparable or excessive DNA damage,⁴ oxidative stress,⁵ or activation of oncogenes, such as Ras⁶ or NF-kappaB.⁷ Senescence can occur *in vitro* as *in vivo*, can be physiological or pathological, linked or not with chronological age, linked or not with the cell replicative past. The senescence program includes a cell-cycle arrest mainly mediated by the p53/p21 and p16/pRB pathways,⁸ changes in chromatin organization,⁹ major changes in transcriptome,¹⁰ proteome,¹¹ and secretome,^{12, 13} increase in cell volume and granularity,^{14, 15} and increase in macroautophagic activity.¹⁶⁻¹⁸

Oxidative stress seems to be one common denominator of several senescence inducers. It results from reactive oxygen species (ROS) accumulation either with chronological age,¹⁹ upon various stresses known to accelerate aging such as ionizing and UV radiations,⁵ or upon oncogenes such as Ras or NF-kappaB activation.^{7, 20} ROS damage organelles and macromolecules, including DNA, what results in a DNA Damage Response (DDR) which, by itself or in addition to the DDR activated by telomere uncapping, leads to the typical cell-cycle arrest encountered by senescent cells.^{21, 22} Moreover, the accumulation of ROS and oxidized cell components was shown to be one of the causes of the increase in macroautophagic activity.²³

Numerous reports claim that senescence corresponds to an irreversible growth arrest that cancer cells have to bypass to generate tumors. However, the cell-cycle arrest associated with senescence is not irreversible in all cell types, notably in epithelial cells that are at the origin

of the most frequent cancers in human. Indeed, we and others have shown that normal human epidermal keratinocytes²⁴ or mammary epithelial cells²⁵ having reached the senescence plateau, although displaying all the characteristics of senescent cells, can spontaneously reactivate a mitotic process to generate so-called post-senescence (PS) emergent cells which are transformed and able to form skin hyperplasia in nude mice. Several data from our group suggest that the oxidative DNA damages encountered by senescent cells could be the mutagenic motor of this neoplastic emergence.²⁴

Macroautophagy is a process that enables the isolation of cellular components inside a specific double-membrane vesicle, the autophagosome, and their degradation after the autophagosome has fused with a lysosome.²⁶⁻²⁸ The starting of the process is under the control of Atg6/Beclin-1 and a class III PI3 Kinase, hVps34.²⁹⁻³¹ The completion of the autophagosome formation and its fusion with lysosomes to form an autolysosome are driven by an Atg12-Atg5-Atg16 complex^{32, 33} and by the integration of Atg8/LC3 in the autophagosome membrane.³⁴ Macroautophagy has been until now primarily characterized as an in bulk degradative pathway induced by nutrient deprivation. In that situation, it is assumed to non-selectively degrade cytosolic components and organelles to produce new metabolites that will be used to synthesized indispensable components and generate energy, and hence help cell to survive. Although less well characterized, it is now clear that a basal constitutive macroautophagic activity also exists to contribute to selective disposal of misfolded aggregated proteins or altered organelles. This form of autophagy is often referred as housekeeping autophagy or quality control autophagy. This autophagic activity can be enhanced upon stresses such as oxidative stress to help cells to face damages and again survive. However, when it is overactivated, it could lead to an opposed outcome, i.e. cell death, through the excessive elimination of vital cell components such as mitochondria and

nucleus. This mechanism of cell death was shown to occur in various physiological and pathological situations, besides or instead of apoptosis. It is as such referred as type II programmed cell death (type I being apoptosis) or as autophagic programmed cell death.^{26,35}

We have recently shown that senescent epidermal keratinocytes experience an increase in macroautophagic activity whose excessive intensity leads to their death¹⁶. Therefore, two antagonistic outcomes are possible for senescent keratinocytes: autophagic programmed cell death for most cells, or mitotic activity recovery and PS emergence for less than 1% of cells. In the present report, we addressed the relationships between the macroautophagic activity and the PS emergence. We show that the senescent cells that are the progenitors of PS emergent cells display a moderate autophagic activity compared to the average senescent population and hence escape autophagic cell death. However, this moderate autophagic activity appears to be indispensable to senescent cells for their generation of neoplastic cells, probably because they need to assume quality control to resume mitosis and generate PS emergent cells. Therefore, these results indicate that the outcome of senescent keratinocytes is dependent, at least in part, on their level of macroautophagic activity. We also investigated the relationship between the oxidative stress encountered by senescent keratinocytes, their level of autophagic activity and their final outcome. Indeed, we have previously shown that the lethal autophagic activity of senescent cells is induced following oxidative damages to mitochondria and nucleus.²³ But, we also showed that oxidative stress is necessary and sufficient for PS emergence to occur, in correlation with the generation of mutagenic DNA damages including single-strand breaks and 8-oxo-guanines.²⁴ Here, we show that the macroautophagy level in senescent cells is directly correlated with their level of H₂O₂ and their probability of generating PS emergent clones, suggesting that the oxidative damages occurring during senescence and the way senescent cells face up to them using

macroautophagy are important parameters of the earliest steps of carcinogenesis occurring during aging.

Results

PS emergent cells have a lower macroautophagic activity than their senescent progenitors

In order to establish whether PS emergence involves an escape to autophagic programmed cell death, we first investigated the level of macroautophagy in PS emergent cells compared to their senescent progenitors. NHEKs were cultivated under standard conditions, and cells were taken during the initial exponential growth phase, or after the senescence plateau at the beginning of the growth of PS emergent cells (Fig. 1A). The composition of the different cell populations was checked by flow cytometry according to forward scatter factor which is indicative of cell size. The analysis revealed that the population of exponentially growing cells was highly homogenous in size, whereas that at the beginning of PS emergence was composed of two subpopulations with an about 10-fold difference in size (Fig. 1B). As already shown in previous studies,^{16, 23} the subpopulation of the largest cells corresponds to senescent ones, and consequently the subpopulation of cells with the smallest size to PS emergent cells. The two subpopulations were sorted according to their forward scatter factor value prior to protein extraction and western-blotting analyses. The growth stage of the sorted cells was checked by examining PCNA expression. PCNA down-regulation confirmed the senescent status whereas PCNA reexpression confirmed the emergent status of sorted cells (Fig. 1B). The expression of several macroautophagic markers was then examined. The expression of Atg6/Beclin-1 increased at senescence as already shown¹⁶ and decreased again in the population of emergent cells (Fig. 1B). Regarding Atg8/LC3, the only active cleaved form, LC3 II, was detected in NHEKs. The nature of the detected protein was validated using proteins extracted from Normal Human Dermal Fibroblasts (sup Fig. 1). The quantity of LC3 II in NHEKs increased at senescence and decreased again in the population of emergent cells

(Fig. 1B). Similarly, the expression of lysosomal associated membrane protein 1 (LAMP-1), a marker of lysosomes and autolysosomes, increased at senescence and decreased again at PS emergence (Fig. 1B). An immunofluorescence staining of LAMP-1 confirmed that PS emergent cells have a macroautophagic activity strongly reduced compared to their senescent progenitors (Fig. 1C). Taken together, these results suggest that PS emergent cells have lost the high and lethal macroautophagic activity of their senescent progenitors.

Lowering macroautophagy during senescence favors PS emergence

To further address the question of whether the generation of PS emergent cells involves an escape to autophagic cell death, we decreased macroautophagy in senescent cells and examined whether it affects the emergence frequency. NHEKs were induced in premature senescence by a sublethal H₂O₂ treatment (50 μ M) as previously described ⁷. After a 48 hrs treatment, once all cells had acquired the senescent phenotype, cells were transfected with siRNAs against Atg5 or control siRNAs at different concentrations. Twenty four hrs later, cells were seeded at low density. At 72 hrs post-transfection, the efficacy of siRNAs was checked by western blotting (Fig. 2A). In parallel, cells were monitored for PS emergence. PS emerging clones that appeared in the culture dishes were counted under microscopic observation after fixation and purple crystal coloration, and the emergence frequency was calculated by reporting the number of clones on the number of initially seeded senescent cells. The sole isolated clones containing a senescent cell amongst the emergent ones, as illustrated in Figure 2B, were taken into account, hence ensuring that these clones were *bona fide* PS emerging clones originating from a senescent cell and not from some putatively contaminating young cells. Cells transfected with control siRNAs underwent PS emergence about one week after having acquired the senescent phenotype, as already described, ²⁴

with an emergence frequency comprised between 2 to 4 10^{-5} (Fig. 2C) i.e. of the same range as that obtained with normal senescent cells ²⁴, despite the stress of transfection. Cells treated by siRNA against Atg5 underwent PS emergence at the same time with an about two fold higher frequency (Fig. 2C). We conclude from this experiment that senescent cells with a lowered macroautophagic activity, which consequently experience less cell death, ²³ become more prone to generate PS emergent cells.

Escaping autophagic cell death but maintaining housekeeping macroautophagy are both necessary for the generation of PS emergent cells

We then wanted to challenge the above results with normal senescent cells, and with another frequently used macroautophagic inhibitor, 3-methyladenine (3-MA), which blocks the activity of hVps34 involved in the initial autophagosome formation. ³⁶ NHEKs were taken at the senescent plateau, and analyzed by flow cytometry for forward scatter factor (Fig. 3A). According to our previous studies, the subpopulation of 15% cells with the highest forward scatter factor values were already autophagic dying cells (subpopulation D), whereas that of the 15% with just below values were fully senescent but still alive cells (subpopulation S). ¹⁶ Cells from the subpopulation S were sorted, seeded at low density, treated by 3-MA and monitored for PS emergence. Two concentrations of 3-MA were used in this assay, 5mM and 1mM. As shown in figure 3A, these two different concentrations gave opposite effects on the emergence frequency: at 1mM 3-MA, the emergence frequency was significantly increased, whereas it was significantly decreased at 5mM. We conclude from this experiment that partially reducing the macroautophagy in senescent cells increases their probability to generate PS emergent cells, whereas reducing it to a very low level impairs emergence. This suggests that to be able to generate emergent cells, senescent keratinocytes must display

a macroautophagic activity level not too high to avoid cell death, but enough high to ensure quality control activities and permit reentry in mitosis. To support this conclusion, we used Bafilomycine A1 which blocks the latest phases of macroautophagy both by inhibiting the fusion of autophagosomes with lysosomes and by inhibiting the activity of H⁺ pumps^{37, 38} and which was shown by us to lead to a huge accumulation of vesicles full of undigested material in senescent NHEKs.¹⁶ Here, Bafilomycine A1 was applied on senescent NHEKs sorted as above, and PS emergence was monitored. The results show that in this situation of macroautophagic flux blocage, PS emergence was almost completely abolished (Fig. 3A).

To confirm the dose effect of the inhibition of macroautophagy on PS emergence, we reiterated the experiments with 3-MA and Bafilomycine A1 on H₂O₂-induced senescence. The results were similar to that obtained with normal senescent cells (Fig. 3B), hence confirming that senescent cells have to escape autophagic cell death but maintain a housekeeping macroautophagic activity to generate emergent cells.

The progenitors of PS emergent cells display a moderate autophagic activity and a moderate oxidative stress

The above conclusion suggests that the progenitors of emergent cells could be found amongst the senescent cells with the lowest macroautophagic activity. To assay this assumption, we investigated the ability of senescent keratinocytes to generate PS emergent clones as a function of their level of macroautophagic activity. Since the macroautophagic activity of senescent cells is induced by their accumulation of oxidative damages,²³ we also measured in the same experiment the steady state level of ROS. For these goals, NHEKs at the senescent plateau were stained with LysoTracker, a cell-permeant probe that fluoresces in acidic

organelles, whose pattern of staining in senescent cells was shown by us to reflect autophagic vesicle accumulation,¹⁶ and with H2-DCFDA, a fluorescent H₂O₂ sensor. The cell population was analyzed according to forward scatter factor, and cells from the subpopulations S and D were delineated. The LysoTracker and H2-DCFDA staining intensity were also recorded. Each S and D subpopulation was divided in two new subpopulations regarding their LysoTracker staining, and named S1, S2, D1 and D2 (Fig. 4A). Cells from these four subpopulations were sorted, seeded at low density, and monitored for PS emergence as in the above experiments. As logically expected, the two subpopulations D1 and D2 of already dying senescent cells poorly generated emergent clones. The subpopulation S2 behaved similarly, and, interestingly, it was the subpopulation S1, i.e. that displaying the lowest autophagic activity, that generated the highest clone number (Fig. 4B). The analysis of H2-DCFDA staining intensity shows that the H₂O₂ steady state level in the four subpopulations was parallel to their macroautophagic activity level (Fig. 4C). Therefore, we can conclude that the progenitors of PS emerging cells are fully senescent cells, not too engaged in the death pathway, and displaying a moderate autophagic activity directly correlated to a moderate oxidative stress. Although moderate, these autophagic activity and H₂O₂ steady state level of PS emergent progenitors are much higher than that of cells at the beginning of the exponential growing phase, respectively 26 and 28 fold higher (Fig. S3). In comparison, differences between the S1 and S2 subpopulations are only respectively of 4 and 2 fold.

Discussion

Macroautophagy has two antagonistic roles, one helping cells to support various stresses (nutrient deprivation, lack of survival factors, oxidative stress, etc...) and survive, and the other inducing cell death. Recently, we and others have highlighted that macroautophagy plays also a role in senescence. This was shown for several normal cell types undergoing normal or stress-induced premature senescence, including normal senescent fibroblasts,³⁹ IMR90 fibroblasts overexpressing H-RasV12,¹⁷ long-term cultured and repeatedly stimulated T lymphocytes,⁴⁰ normal senescent epidermal keratinocytes,¹⁶ normal biliary epithelial cells,⁴¹ and also for cancerous cell lines reinduced in senescence upon various drug treatments.^{42, 43} One question opened by these data is what are the roles and consequences of the macroautophagic activity of senescent cells? Here we show that this senescence-associated macroautophagy determines the outcome of senescent cells, depending on its level of activation.

The outcome of senescent cells can differ from one cell type to another. In contrast to senescent fibroblasts that are irreversibly and permanently cell-cycle arrested and remain in this stage for several weeks and months,³ senescent NHEKs, as well as senescent normal human mammary epithelial cells (HMECs), either die or reenter mitosis to generate post-senescent (PS) emergent cells that display neoplastic properties.^{16, 24, 25} We demonstrate here that the NHEKs that are the most prone to generate PS emergent cells are those displaying a moderate autophagic activity compared to the average senescent population, what enables them to escape autophagic programmed cell death. However, this moderate level of macroautophagy is indispensable to generate PS neoplastic cells; it is probably required not only to ensure the elimination of the various accumulated altered components which could be deleterious for senescent cell survival, but also to provide basic metabolites and energy to

reenter mitosis and perform neosyntheses necessary to generate daughter cells, which will be themselves consequently properly equipped to survive and proliferate. Macroautophagy thus would play antagonistic roles in the determination of senescent keratinocytes outcome in relation with tumor initiation: when overactivated, macroautophagy would induce senescent-cell death, reinforcing the tumor-suppressive role of senescence already assigned to its cell-cycle arrest; when moderately activated, it would favor the new generation of neoplastic cells, hence contributing in contrast to a tumor-promoter role of senescence.

However, although escaping cell death and ensuring quality control are two necessary parameters of the mitosis reentry of senescent cells, they are not sufficient to explain the neoplastic phenotype of PS emergent cells. In this report, we show that the macroautophagic activity level of senescent cells is directly correlated to their ROS level. Therefore, the outcome of senescent keratinocytes could be primarily dictated by the level of oxidative damages they encompass, and, downstream, by the level of autophagic activity this induces. Previous results from our group have demonstrated that oxidative stress is not only responsible for keratinocyte senescence ⁷ and associated cell death, ²³ but also for the generation of PS emergent cells in correlation with the acquisition by senescent cells of oxidative DNA damages such as single strand breaks and 8-oxoguanines. ²⁴ Taken together, these results suggest that acquiring specific oxidative damages, such as damages affecting genome integrity, but keeping those affecting other molecules and organelles under control by autophagy could be the two key determinants of senescent cell outcome. The threshold of oxidative stress above which senescent cells shift from survival and emergence to death seems very subtle, since there is a difference of only a factor four between the S1 subpopulation which comprises the progenitors of emergent cells and the D2 subpopulation which contains dying cells, compared to a difference of more than 20 between young growing cells and the

average senescent population. Therefore, there would be a window just below their deleterious and lethal effects where ROS could act as a mutagenic factor enabling PS emergence. In addition, the fact that senescent cells escape cell death and hence acquire a life span longer than the average potentially gives them time to stochastically acquire the oxidative genetic alterations proper for transformation.

Are these results relevant to the very initial steps of carcinogenesis occurring *in vivo* during aging? In other terms, amongst the senescent cells that accumulate in epithelial tissues with age,⁴⁴⁻⁵⁰ could those with the lowest autophagic activity, the longest life span, and the presence of discreet oxidative DNA damages be the progenitors of the initial hyperplastic cells? The fact that senescent epithelial cells are present in benign tumors such as naevi,⁵¹ benign prostatic hyperplasia,⁵² papillary hyperplasia,⁵³ and metaplastic and dysplastic epithelium of the upper gastrointestinal tract⁵⁴ supports this possibility. It is to be noticed that senescent cells were demonstrated to be apoptosis-resistant,⁵⁵⁻⁵⁹ what could reinforce their persistence inside normal or hyperplastic tissues. Data regarding the role of macroautophagy in carcinogenesis are the subject of controversial interpretations. Several studies suggest that macroautophagy could be activated in cancer cells under nutrient deprivation and hypoxia resulting from limited angiogenesis.⁶⁰ In that sense, macroautophagy can be viewed as tumor promoter. In contrast, it was shown that several human cancers harbor inactivating mutations or deletions in several atg genes, including *atg6/beclin-1*,³⁰ its partner *UVRAG*,⁶¹ as well as *atg2B*, *atg5* and *atg9B*,⁶² what defines them as tumor suppressor genes. However, at least regarding *atg6/beclin-1* and *UVRAG*, the reported mutations were always monoallelic deletions. Moreover, homozygous knock-out *beclin1* mice were non viable, whereas heterozygous *beclin1*^{+/-} mutant mice were shown to spontaneously develop with age preneoplastic or malignant lesions,^{63, 64} suggesting that, in accordance with our present *in*

in vitro data, macroautophagy is tumor promoting only when occurring at a moderate level. Very interestingly, it was shown that *beclin1*^{+/-} mammary epithelial cells display more DNA damages than *beclin1*^{+/+} cells when subjected to metabolic stress,⁶⁵ suggesting that a moderate macroautophagic activity may promote tumorigenesis by keeping alive cells with genomic alterations. In conclusion, we propose that some of the senescent epithelial cells which accumulate in aged tissues could be a source for the generation of sporadic carcinomas, which incidence indeed dramatically increases with age.

Materials and methods

Cell culture

Normal human epidermal keratinocytes (NHEKs) were purchased from Clonetics (CC-2501) or Tebu-bio (102.05a). We used cells from 3 different female donors of different race and age (referred as 4F0315, 2F1958, and 13.20). Cells were obtained anonymously and informed consent of each skin donor was obtained by the supplier. Cells were grown at 37°C in an atmosphere of 5% CO₂ in the Epilife medium (MEPICF500 Invitrogen) with 0.15mM calcium supplemented with HKGS (S0015). Such a serum-free low-calcium medium was shown to minimize keratinocyte terminal differentiation.⁶⁶ Cells were routinely seeded at 3500 cells/cm² and always splitted at 70% confluence. The number of population doublings (PD) was calculated at each passage by means of the following equation: $PD = \log(\text{number of collected cells} / \text{number of plated cells}) / \log 2$.

Induction of premature senescence by H₂O₂

NHEKs at the exponential growth phase were treated by 50 μM H₂O₂ every 24 hrs. The senescent phenotype (growth arrest, cell enlargement and acquisition of the SA-β-Gal marker) was clearly established in all cells after 48 hrs, as already published.⁷

Inhibition of macroautophagy by siRNA or pharmacological inhibitors

SiRNAs were diluted in PrimeFect siRNA Transfection Reagent (Lonza) and incubated 15 min at room temperature before added to cells in fresh culture medium. Inhibition of Atg5

expression was performed using 1 to 50 nM of a pool of 4 siRNA (siGENOME SMARTpool, Dharmacon - GGAAUAUCCUGCAGAAGAA - CAUCUGAGCUACCCGGAUA - GACAAGAAGACAUUAGUGA - CAAUUGGUUUGCUAUUUGA). The control was a non targeting siRNA pool (siCONTROL Non Targeting siRNA Pool #2, Dharmacon). Transfections were stopped after 24 hrs by renewing the culture medium.

3-methyladenine (3MA) (Sigma-Aldrich M9281) and Bafilomycin A1 from Streptomyces griseus (B 1793), respectively diluted in water and DMSO, were directly added to the culture medium at 1 or 5mM for 3MA and 5nM for Bafilomycin and let for 48 hrs.

Western blotting

Cells were lysed in: Hepes 27.5mM pH 7.6, urea 1.1M, NaCl 0.33M, EGTA 0.5mM, EDTA 2mM, KCl 60mM, DTT 1mM and NP40 1.1%. The total protein concentration was measured with the Bio-Rad protein assay. Proteins were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore). Primary antibodies used were: GAPDH (Santa Cruz Biotechnology SC32233), PCNA (Dako cytometry M0879), LAMP-1 (Santa Cruz Biotechnology SC17768), LC3 B (Cell Signaling #2775), Atg5 (Cell Signaling #2630) and Beclin-1 (Cell Signaling #3738). Secondary antibodies used were peroxidase-conjugated anti mouse IgG (Jackson Immunoresearch Labs 115-035-146), or anti rabbit (Jackson Immunoresearch Labs 711-035-125)). Peroxidase activity was revealed using a ECL (enhanced chemiluminescence) or ECL advance kit. The luminescence was captured with a Fuji intelligent dark box camera. Quantifications were performed with the Multigauge V3.0 software.

Immunofluorescence

For LAMP-1 detection, cells were seeded onto microscopic coverslides. Twenty four hrs later, cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton-X100 and incubated with a primary antibody anti LAMP-1 (Transduction Labs L76620) for 1 hr at 37°C. Cells were then washed 3 times with PBS and incubated with the secondary antibody: Rhodamine RedX-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories). Nuclei were stained by Hoechst 33258 at 50ng/ml for 15mn. Slides were analyzed with a Zeiss Axioplan2 epifluorescence microscope.

Lysotracker and H2-DCFDA staining

Lysotracker and H2-DCFDA were purchased from Molecular Probes. Living cells were incubated with probes directly added to the culture medium at 37°C for 30 min as recommended by the supplier.

Flow cytometry analysis and fluorescence-activating cell sorting

Flow cytometry analysis were performed using a Beckman Coulter Epics XL-MCL. Collected data were exported to the WinMDI 2.9 software for detailed analysis. Sorting of NHEKs was performed on a BD FACS Aria or on a Coulter FACS Altra. Selected subpopulations with the ad hoc forward scatter factor and/or fluorescent staining intensity values were electrostatically sorted in air, collected in complete culture medium and put again in culture.

Measure of PS emergence frequency

Cells, either at the senescent plateau, sorted by FACS, or H₂O₂-treated were plated at 150 cells/cm². in order to be completely isolated. Culture dishes were scrutinized every day for the appearance of emerging clones. When having appeared at a sufficient number in control condition, dishes were fixed and stained with purple violet. Clones were manually counted under a binocular microscope. The sole clones that were taken into account were those well isolated and containing a senescent cell amongst emergent ones. The emergence frequency was then calculated by reporting the number of clones on the number of initially seeded senescent cells.

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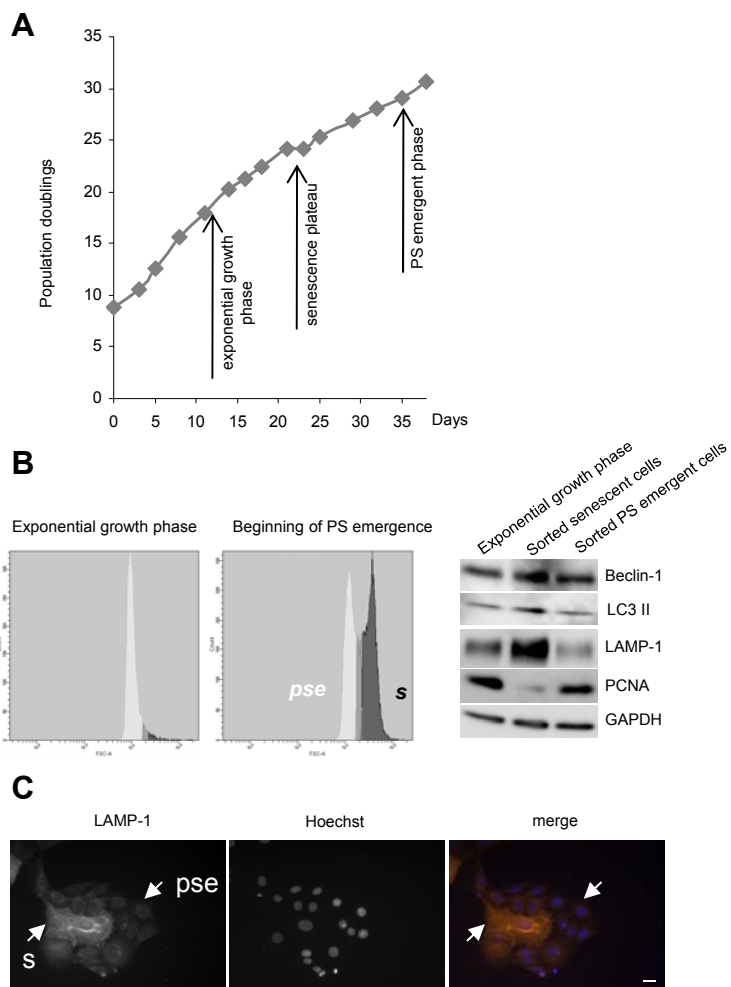
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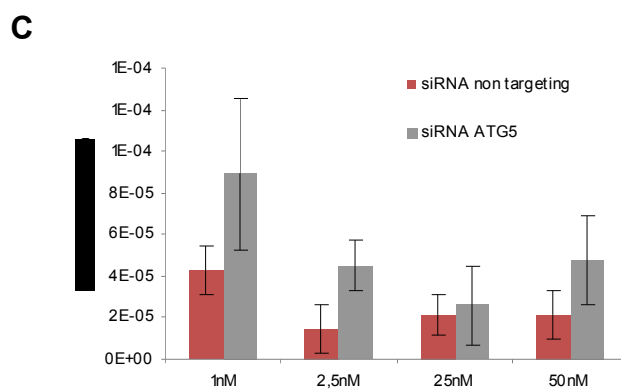
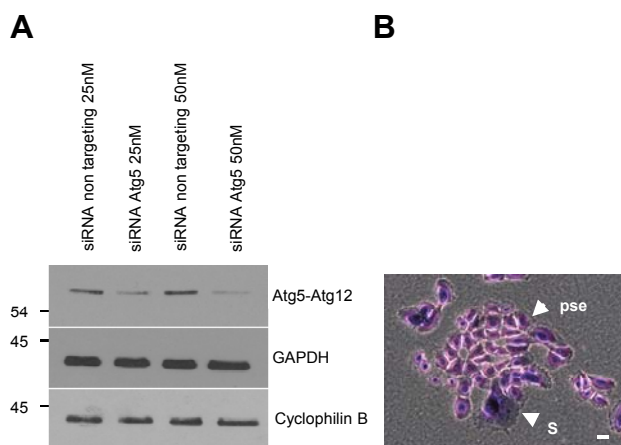
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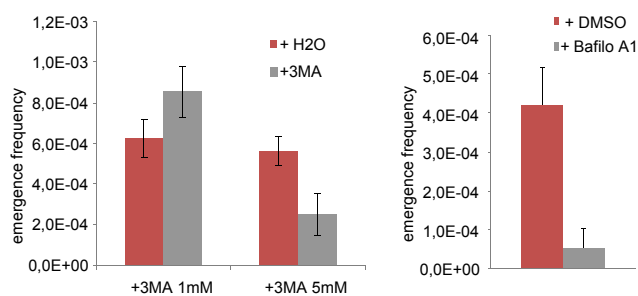
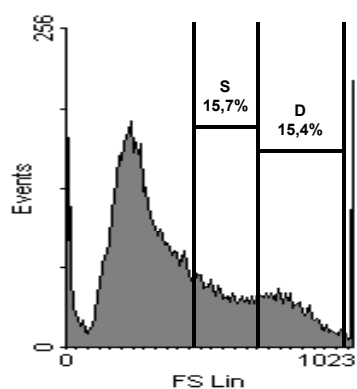
Fig. 1





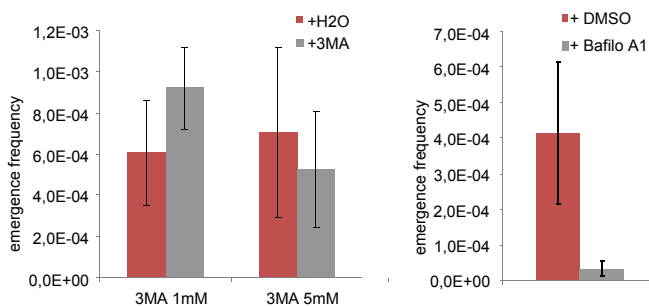
Fold increase		2,08	3,08	1,21	2,21
p	Student	0,01238	0,00029	0,58749	0,01765
	Wilcoxon	0,03021	0,00079	1,00000	0,01541

A



		3MA 1mM	3MA 5mM	Bafilo A1
Fold change		1.37	2.25	7.88
p value	Student	0.04522	0.00625	0.00057
	Wilcoxon	0.08143	0.02843	0.01141

B



		3MA 1mM	3MA 5mM	Bafilo A1
Fold change		1.52	1.34	12.40
p	Student	0.03210	0.37016	0.01820
	Wilcoxon	0.02747	0.68490	0.01066

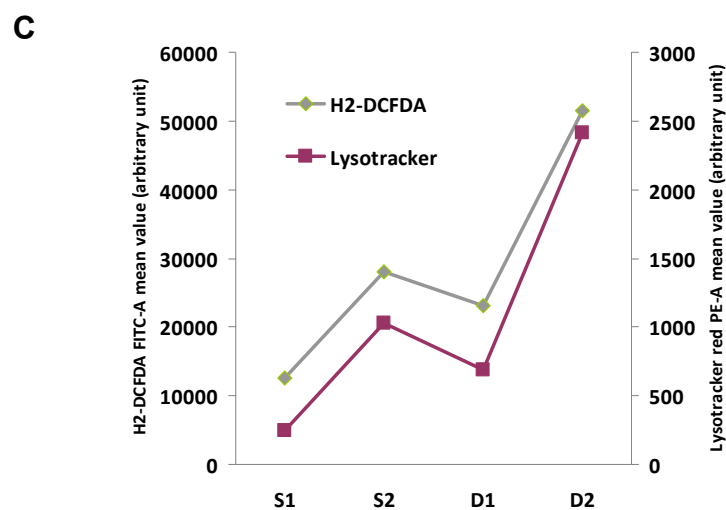
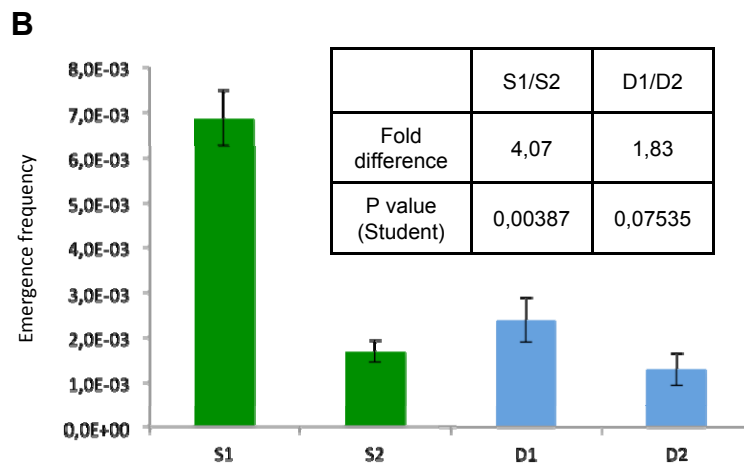
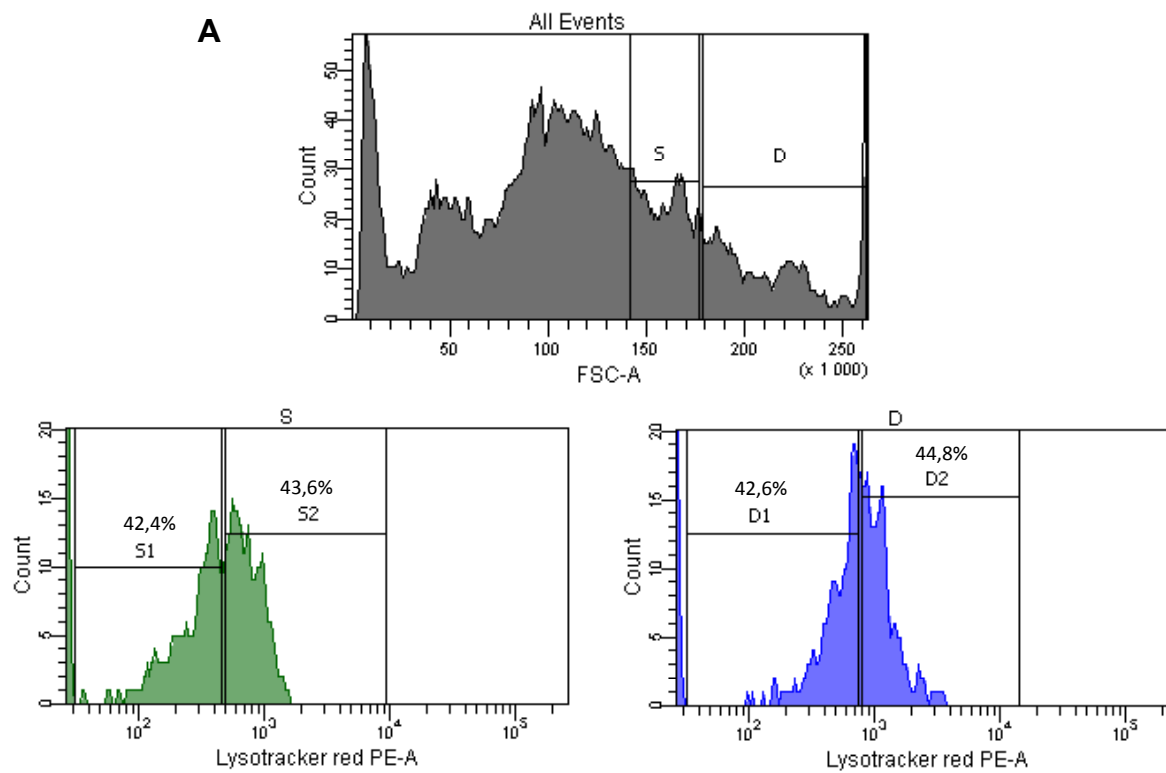


Figure legends

Figure 1: PS emergent cells display a lower macroautophagic activity than their senescent progenitors

(A) Growth curve of NHEKs showing the exponential growth phase, the senescence plateau, and the post-senescence (PS) emergent phase. (B) Flow cytometry analysis of cells taken at the exponential growth phase and at the beginning of PS emergence as a function of forward scatter value, indicative of cell size. The population of cells at the beginning of PS emergence was composed of both large cells, the senescent ones (s), and smaller cells, the PS emergent ones (pse). Senescent and PS emergent cells were sorted according to their forward scatter factor value. Proteins were extracted, separated in SDS-PAGE and expression of Beclin-1, LC3 II, LAMP-1 and PCNA was analyzed by western blot. Band intensity quantifications are given in Fig. S2. Results are representative of two independent sortings. (C) LAMP-1 immunofluorescence assays performed on cells at the beginning of the PS emergence phase. Bar represents 20 μ m.

Figure 2: Lowering macroautophagy in H₂O₂-induced premature senescent NHEKs favors PS emergence

H₂O₂-induced premature senescent NHEKs were transfected with siRNAs targeting Atg5 or control siRNAs at different concentrations. Twenty four hrs later, cells were seeded at low density and the PS emergence frequency was measured. (A) Verification of the efficiency of siRNAs by western blot performed 78 hrs post-transfection. The anti-Atg5 antibody reveals the covalent Atg5-Atg12 complex. (B) Representative image of a PS emerging clone after

fixation and coloration with purple crystal. S points a senescent cell and pse the clone of PS emergent cells. Bar represents 10 μ m. (C) Emergence frequency. The counts were performed in 8 independent culture dishes. The given results are the mean +/- standard deviation of all counts. The indicated fold increase corresponds to the ratio of the means at each concentration of siRNAs. P values were calculated using both student and Wilcoxon tests. This experiment is representative of 3 independent ones.

Figure 3: Lowering macroautophagy during normal and H₂O₂-induced senescence favors or limits PS emergence depending on the inhibitor concentration

NHEKs were taken at the beginning of the senescent plateau and analyzed by flow cytometry as a function of forward scatter factor value. Cells of the S subpopulation were sorted, seeded at low density, treated with pharmacological inhibitors of macroautophagy and monitored for their ability to generate PS emergent clones. (A, upper panel) Flow cytometry histogram. The X axis corresponds to forward scatter factor. The delineation of S and D subpopulations is indicated. (A, lower panels) PS emergence frequency obtained with the sorted S subpopulation treated with 3-methyladenine (3MA) or its diluent H₂O, or with Bafilomycin A1 or its diluent DMSO. (B) Emergence frequency of H₂O₂-induced premature senescence NHEKs treated as above by 3-MA or Bafilomycine A1. The counts of emerging clones in (A) and (B) were performed in 4 to 8 independent culture dishes. The given results are the mean +/- standard deviation of all counts. The indicated fold increase corresponds to the ratio of the means at each concentration of inhibitors. P values were calculated using both student and Wilcoxon tests. These experiments are representative of two independent ones for (A) and four independent ones for (B).

Figure 4: The probability of senescent cells to generate PS emergent clones is linked to their macroautophagic activity and oxidative stress levels

NHEKs at the senescence plateau were stained with LysoTracker and H2-DCFDA and analyzed by flow cytometry according to forward scatter factor, LysoTracker intensity and H2-DCFDA intensity. Four subpopulations, S1, S2, D1 and D2 were delineated according to their forward scatter factor and LysoTracker intensity values, sorted, seeded at low density and monitored for their ability to generate PS emergent clones. (A, upper panel) Flow cytometry histogram of NHEKs at the senescence plateau according to forward scatter factor and showing the S and D subpopulations. (A, lower panels) Flow cytometry histograms of the S (in green) and D (in blue) senescent subpopulations according to LysoTracker staining intensity and showing the S1, S2, D1 and D2 subpopulations. (B) PS emergence frequency obtained with sorted cells of the four subpopulations. The counts of clones were performed in 4 independent culture dishes. The given results are the mean +/- standard deviation of all counts. The indicated fold difference corresponds to the ratio of the means of S1 on S2 and D1 on D2. P values were calculated using student tests. (C) Mean values of the LysoTracker and H2-DCFDA staining intensities of the four subpopulations. These results from A to C are representative of 3 independent experiments.

Fig S1

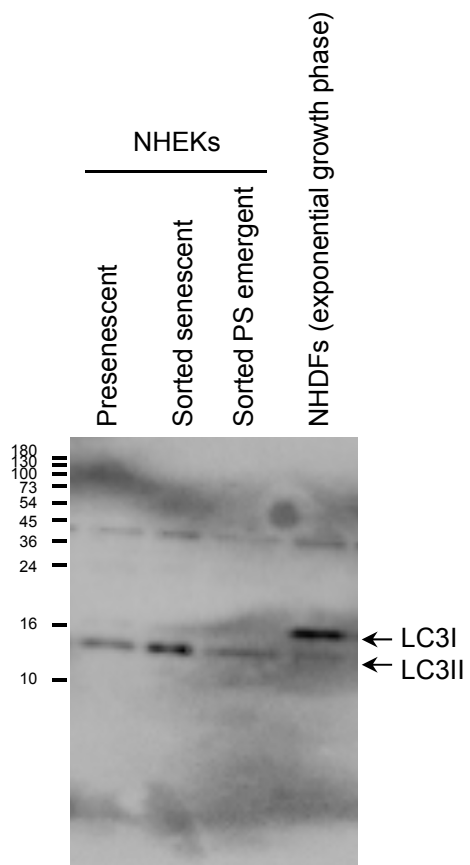
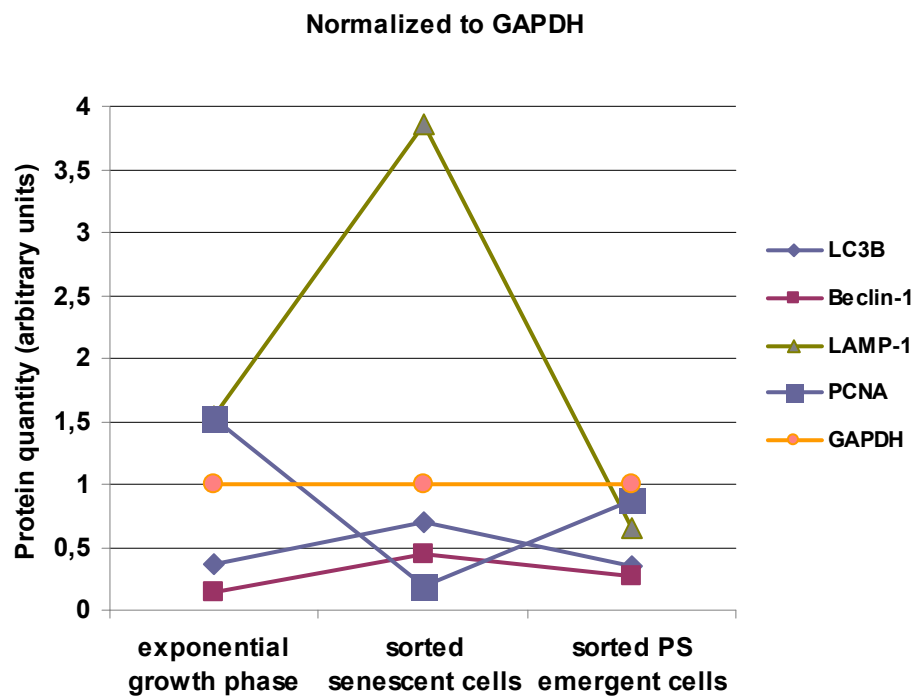
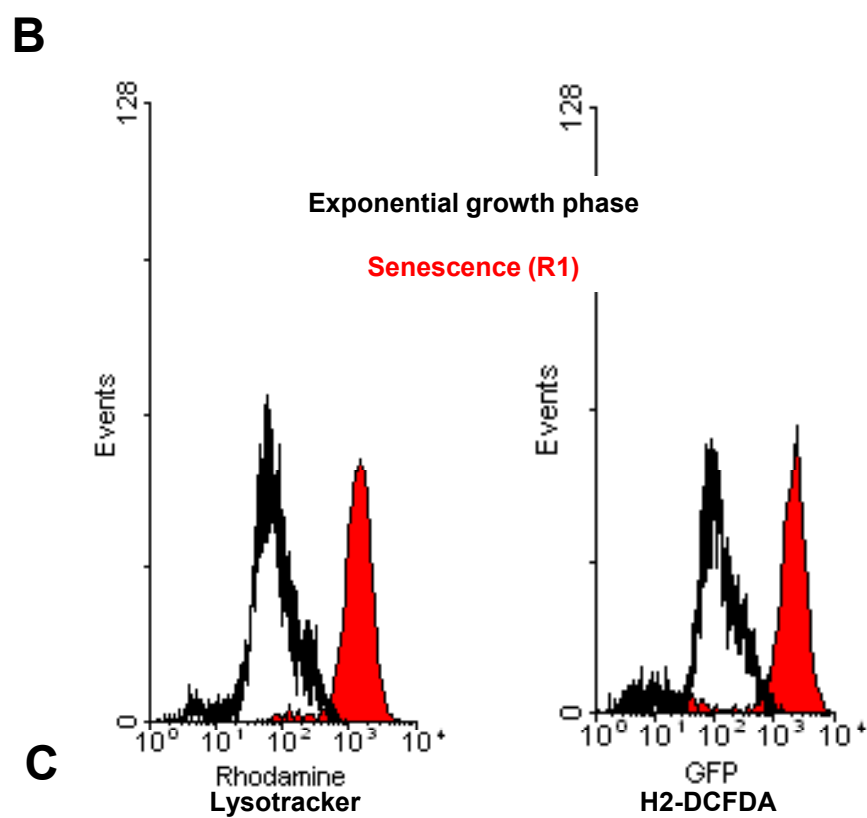
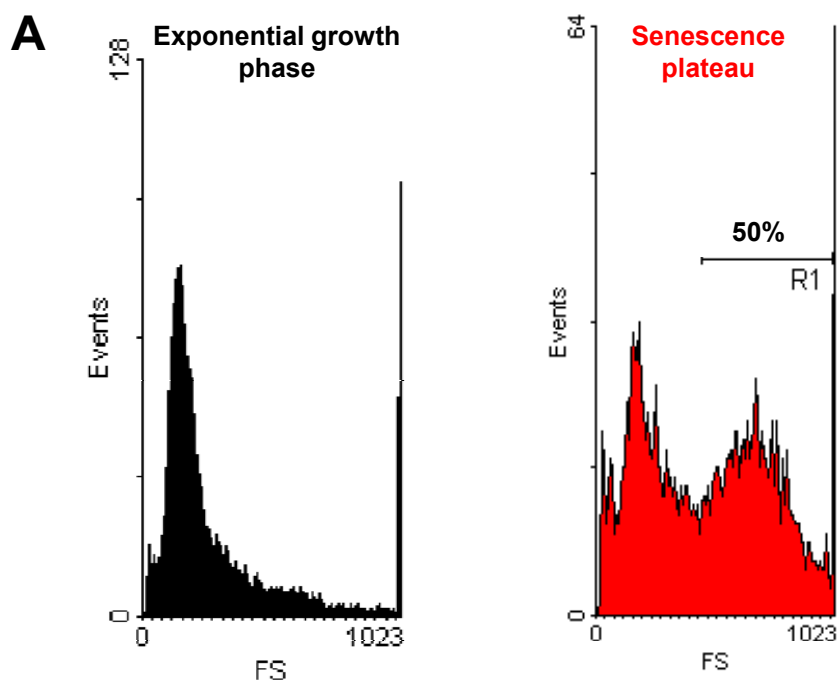


Fig S2





C

Peak values	Exponential growth phase	Senescence (R1)	Fold increase
Lysotracker	57.77	1553.84	26.89
H2-DCFDA	91.4	2571.32	28.13

Supplemental figure legends

Figure S1: Identification of the band corresponding to LC3II in western-blot

Senescent and PS emergent NHEKs were sorted by FACS as in Fig. 1. Proteins from these cells and from Normal Human Dermal Fibroblasts (NHDFs) at the exponential growth phase were extracted, separated in SDS-PAGE, and LC3 post-translational modifications were analyzed by western-blot. The non processed and processed forms of LC3, respectively LC3I and LC3II, were detected in NHDFs, whereas only LC3II was significantly detected in NHEKs, whatever the culture stage.

Figure S2: Quantification of the western-blot of Fig. 1

Figure S3: Autophagic activity and steady state levels of ROS in exponentially growing versus senescent NHEKs

NHEKs at the exponential growth phase (black) and senescence plateau (red) were stained with LysoTracker and H2-DCFDA and analyzed by flow cytometry. (A) Forward scatter factor analysis. The population at the senescence plateau shows two main peaks of size; the first one corresponds to residual small growing cells; the second one (R1) corresponds to senescent cells, including the S1, S2, D1 and D2 subpopulations of Fig. 4. (B) Histograms of LysoTracker (left panel) and H2-DCFDA (right panel) staining intensities of exponentially growing NHEKs (dark) and R1 senescent NHEKs (red). (C) Peaks values of the LysoTracker and H2-DCFDA stainings (values extracted using the WinMDI 2.9 software).

Article complémentaire n°2

Loss of Hypermethylated in Cancer 1 (HIC1) in Breast

Cancer Cells Contributes to Stress-induced Migration

and Invasion through β -2 Adrenergic Receptor

(ADRB2) Misregulation

Loss of Hypermethylated in Cancer 1 (*HIC1*) in Breast Cancer Cells Contributes to Stress-induced Migration and Invasion through β -2 Adrenergic Receptor (*ADRB2*) Misregulation^{*[5]}

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Background: The transcriptional repressor *HIC1* is a tumor suppressor gene epigenetically silenced in many human tumors.

Results: We identified β -2 adrenergic receptor (*ADRB2*) as a new direct target gene of *HIC1*.

Conclusion: Early inactivation of *HIC1* in breast carcinomas could predispose to stress-induced metastasis through up-regulation of the β -2 adrenergic receptor.

Significance: Characterization of *HIC1* target genes may explain how its inactivation contributes to tumorigenesis.

The transcriptional repressor *HIC1* (Hypermethylated in Cancer 1) is a tumor suppressor gene inactivated in many human cancers including breast carcinomas. In this study, we show that *HIC1* is a direct transcriptional repressor of β -2 adrenergic receptor (*ADRB2*). Through promoter luciferase activity, chromatin immunoprecipitation (ChIP) and sequential ChIP experiments, we demonstrate that *ADRB2* is a direct target gene of *HIC1*, endogenous in WI-38 cells and following *HIC1* re-expression in breast cancer cells. Agonist-mediated stimulation of *ADRB2* increases the migration and invasion of highly malignant MDA-MB-231 breast cancer cells but these effects are abolished following *HIC1* re-expression or specific down-regulation of *ADRB2* by siRNA treatment. Our results suggest that early inactivation of *HIC1* in breast carcinomas could predispose to stress-induced metastasis through up-regulation of the β -2 adrenergic receptor.

HIC1 (hypermethylated in cancer 1)⁶ is a tumor suppressor gene located at 17p13.3 on the short arm of human chromosome 17, in a region including the tumor suppressor gene p53 (17p13.1). This region is silenced in many human cancers by hypermethylation or deletions (1). *HIC1* is hemi-methylated in normal breast tissue and is found to be epigenetically or deletionally (loss of heterozygosity) inactivated in many cases of breast carcinomas (2, 3). Expression of *HIC1* is associated with an improved prognosis in human breast cancer (4).

HIC1 encodes a transcriptional repressor composed of two autonomous repression domains, an N-terminal BTB/POZ (Broad complex Tramtrack and Bric à brac/POXviruses and Zinc finger) domain and a central region, followed by five Krüppel like C2H2 zinc fingers able to bind a specific motif consisting of a 5'-(C/G)NG(C/G)GGGCA(C/A)CC-3' sequence centered on a GGCA motif named HiRE (*HIC1* Responsive Element) (5, 6).

HIC1 is able to recruit different co-repressor complexes to its target genes, using short motifs in its central region notably CtBP through a GLDLSKK motif (7), and MTA1, a component of the NuRD complex, through a SUMOylation-dependent Ψ KXEP motif (8). *HIC1* also recruits BRG1-ARID1A containing SWI/SNF complexes (9).

Although an increasing number have been described in the last several years, *bona fide* target genes of *HIC1* are still few. To date, ten target genes play supporting roles in developmental and cell cycle control: histone deacetylase *SIRT1* (10), the transcription factors *ATOX1* (11), *Sox9* (12), and Δ *Np73* (13), the G-protein coupled receptor *CXCR7* (14), *Cyclin D1* and *P57KIP2* (*CDKN1C*) (8) and *EFNA1*, a cell surface ligand for Eph tyrosine kinase receptors (15).

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[5] This article contains supplemental Figs. S1 and S2.

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⁶ The abbreviations used are: *HIC1*, hypermethylated in cancer 1; *ADRB2*, β -2 adrenergic receptor; GPCR, G-protein-coupled receptor; HiRE, *HIC1*-responsive elements.

ADRB2 Is a Direct Target Gene of HIC1

Depending on the cell type, re-expression of HIC1 leads to proliferation arrest, differentiation, and apoptosis (1, 14). Our recent results also demonstrate a role for HIC1 in the regulation of cell migration and invasion. These biological effects are partially mediated through transcriptional repression of the ligand/receptor couple *EFNA1* and *EphA2* in different cells (15, 34).

In this study, we demonstrate that *ADRB2* is a new direct target gene of HIC1. *ADRB2* encodes a G-protein-coupled receptor (GPCR) activated by adrenaline/noradrenaline, which are released *in vivo* under stress conditions (16). *Ex vivo*, *ADRB2* stimulation by agonists induces migration and invasion (17, 18, 19). *In vivo*, *ADRB2* activation promotes tumor growth and metastasis (16, 20, 21, 22 or 16, 20–22). A recent clinical study in breast cancer patients using β -blockers demonstrates a strong diminution of metastasis and heightened survival supporting the impact of stress in breast cancer progression (23).

Through molecular and biological approaches, we demonstrate that *ADRB2* is a new *bona fide* HIC1 target gene. Firstly, in WI-38 normal lung embryonic fibroblasts, endogenous HIC1 directly regulates *ADRB2* as demonstrated by chromatin immunoprecipitation (ChIP and sequential ChIP), siRNA targeting HIC1 and retroviral overexpression of HIC1. In MDA-MB-231, a metastatic breast cancer cell line expressing high levels of *ADRB2* and no HIC1, HIC1 re-expression strongly represses *ADRB2* expression and prevents its activation of migration and invasion. Furthermore, in these HIC1 re-expressing cells, concomitant expression of *ADRB2* partially rescues these phenotypes. Our results suggest that *HIC1* silencing, which occurs in the early stages of breast tumorigenesis, could also contribute to later stages of tumor progression such as metastasis.

EXPERIMENTAL PROCEDURES

Cell Culture—WI-38 cells (ATCC, passage 14) were grown in MEM (Invitrogen, Carlsbad, CA) supplemented with sodium pyruvate, NEAA, 10% fetal calf serum (FCS, Invitrogen) and gentamicin (Invitrogen). U2OS, the packaging cell line HEK293 GP and human mammary adenocarcinoma cells MDA-MB-231 were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% FCS and gentamicin. Cells were cultured at 37 °C in water-saturated 5% CO₂ atmosphere.

Western Blotting and Antibodies—Cells were washed twice with PBS and directly lysed in Laemmli buffer. Western blotting was performed as previously described (8). Results are representative of at least two experiments. Except for the anti-HIC1 325 polyclonal antibodies (7), commercial antibodies of the following specificities were used: *ADRB2* (sc-569), *EphA2* (sc-924), actin (sc-1616-R), *MTA1* (sc-17773X), and *CCND1* (sc-20044) were from Santa Cruz Biotechnology and CtBP2 from BD Biosciences (612044).

Vectors and Retroviral Infection—The pBABE-Puro-FLAG-HIC1 vector has been previously described (34). *ADRB2* coding sequence was cloned into the bicistronic pPRIG-GFP vector (24) in two steps. First, pcDNA3.1-*ADRB2* 3xHA-tagged (N terminus) (UMR cDNA Resource Center, Rolla, MO) was digested by XhoI and HindIII restriction enzymes and inserted in the pBluescript II KS (Stratagene). Then, a BamHI-XhoI

fragment containing the full-length *ADRB2* coding sequence 3xHA-tagged (N terminus) was cloned into the pPRIG-GFP vector. For the production of retroviruses, HEK293-GP cells were transfected with the pVSVG vector (expressing env) and with HIC1 and/or *ADRB2*-expressing retroviral vectors using the polyethyleneamine (Exgen 500) procedure (Euromedex). After 48 h, culture supernatants were collected, passed through 0.45 μ m filters and mixed with fresh medium (1/2) and polybrene at 8 μ g/ml to infect target cells. Then, infected cells were selected for 48 h by puromycin treatment at 0.5 μ g/ml for WI-38, 1 μ g/ml for U2OS, and 2 μ g/ml for MDA-MB-231.

***ADRB2* Promoter Cloning and Luciferase Assays**—The *ADRB2* promoter region was PCR-amplified from normal human genomic DNA (Clontech) using primers containing XhoI and HindIII restriction sites, respectively (forward primer: GGCTCGAGCTTTGTGCCGGATGGCTTCT; reverse primer GGAAGCTTCAGTCTGGCAGGTGAGCG). The PCR product was cloned in the PCR-TOPO-Blunt vector (Invitrogen) and verified by sequencing. After restriction digestion, the XhoI-HindIII fragment was cloned in the pGL3 basic reporter to generate the *ADRB2* promoter construct, pGL3 *ADRB2* -750/-6. Similarly, the pGL3 *ADRB2* -440/-6 promoter construct was obtained using the forward primer: GGCTCGAGGGGGCCAGCCAGGGTAGC.

U2OS cells were transfected in OptiMEM (Invitrogen) by the PEI (Euromedex) method in 12-well plates with 500 ng of DNA (14). Cells were transfected for 6 h and then were incubated in fresh complete medium. They were rinsed in cold phosphate buffer saline (PBS) 48 h after transfection and lysed with the Luc assay buffer. Luciferase and β -galactosidase activities were measured by using beetle luciferin (Promega) and the Galactolight kit (Tropix) respectively with a Berthold chemiluminometer. After normalization to β -galactosidase activity, the data were expressed as fold activation relative to the empty pGL3 basic control vector. The value obtained for each construct was divided by the repressive effect elicited by HIC1 on the empty pGL3 basic vector to obtain the final fold of activation. Results represented are the mean values and S.D. from a representative experiment performed in duplicate.

Quantitative RT-PCR—Total RNA was reverse transcribed using random primers and MultiScribe™ reverse transcriptase (Applied Biosystems). Real-time PCR analysis was performed with Power SYBR Green (Applied Biosystems) in a MX3005P fluorescence temperature cyler (Stratagene) according to the manufacturer's instructions. Results were normalized with respect to 18 S RNAs used as internal control. Primers were used at a concentration of 0.5 μ M. According to a melting point analysis, only one PCR product was amplified under these conditions. RNAs extracted from pBABE infected cells were used to generate a standard curve for each gene. Results were normalized with respect to the internal controls and are expressed relative to the levels found in pBABE-infected cells.

Relevant primers used are: HIC1 (for-CGACGACTACAAGAGCAGCAGC, rev-CAGGTTGTACCCGAAGCTCTC); *ADRB1* (for-TGCACAGCAGATAGAAAGACTTGT, rev-AGAGGCTCTGCGCTGGT); *ADRB2* (for-GCAAAGG-GACGAGGTGTGG, rev-AGACGCTCGAACTTGGCAAT);

ADRB3 (for-GGAGTGAGCTTGAGCCTTTGATA, rev-GCGTGGCTTTGCTACTCAATG); EphA2 (for-TGTGC-CAGGCAGGCTACG, rev-CTCCAAGCAGGGGCTCTCA); CyclinD1 (for-CATCTACACCGACAACCTCCATCC, rev-TG-TTCAATGAAATCGTGCGG); 18 S (for-GGCGCCCCCTC-GATGCTCTTAG, rev-GCTCGGGCCTGCTTTGAACAC-TCT).

Small Interfering RNA—WI-38 cells were reverse-transfected with RNAiMax according to manufacturer's instructions using 10 nM small interfering RNA targeting *HIC1* (HIC1 siGENOME Smart Pool M-006532-01, Dharmacon) or a scrambled sequence, as previously described (34). 72 h later, cells were lysed for RNA or protein extraction. MDA-MB-231 cells were forward-transfected with Lipofectamine 2000 according to manufacturer's instructions using 10 nM small interfering RNA targeting *ADRB2* (ADRB2 siGENOME Smart Pool M-005426-02, Dharmacon) or a scrambled sequence. 48 h later, cells were harvested for RNA/protein extraction or seeded for bioassays.

Chromatin Immunoprecipitation—ChIP was performed as previously described (8). Alternatively, we used the protocol previously described by Dahl and Collas (25). The purified DNAs were used for PCR analyses with Fast Start TaqDNA Polymerase (Roche) using the relevant primers for *ADRB2* (for-TCGGTATAAGTCTGAGCATGTCTG; rev-ACATTC-GGAAGGAAACGAGA), and *GAPDH* (for-TCCTCCTGTT-TCATCCAAGC; rev-TAGTAGCCGGGCCCTACTTT).

Type I Collagen and Fibronectin Coatings—Six-well plates were incubated with a solution of rat-tail Type I collagen at a concentration of 3.5 $\mu\text{g}/\text{ml}$ in PBS for 2 h at 37 °C or with human fibronectin (both from BD Biosciences, Bedford, MA) at 20 $\mu\text{g}/\text{ml}$ for 1 h at room temperature. Then, plates were washed twice with PBS(−/−) containing neither Ca^{2+} nor Mg^{2+} (Invitrogen) and stored at 4 °C in PBS(−/−) before use.

Adhesion Assay—WI-38 cells were serum-starved for 20 h, trypsinized, centrifuged, and resuspended in serum free medium containing PBS or 100 nM isoproterenol (Sigma-Aldrich). Then, 20,000 cells were seeded on 6-well plates pre-coated with fibronectin or type I collagen and incubated at 37 °C for 1 h 30 min on type I collagen or 2 h 30 min on fibronectin. Finally, cells were washed twice with PBS, fixed 10 min at −20 °C with ice-cold methanol, and conserved in PBS for later observation on a phase contrast microscope. Similarly, MDA-MB-231 cells were serum starved for 1 h and treated with 10 μM isoproterenol. Cells were then seeded in medium containing 0.5% FCS and incubated for 4 h on collagen or 5 h on fibronectin. Experiments were performed at least twice in triplicate.

Migration Assay (Wound Healing Assay)—Wound healing assays were performed using culture-insert μ -Dish (ibidi, Martinsried, Germany) composed of two chambers (growth area per well 0.22 cm^2) separated by a wall (width of 500 μm). Culture inserts were put in six-well plates precoated with fibronectin or type I collagen. MDA-MB-231 were starved in serum-free DMEM medium for 4 h, split and counted. When applicable, cells were pretreated with PBS or isoproterenol (10 μM) in 10% FCS-DMEM medium for 10 min, and then 30,000 cells were seeded into the chambers. After cell attachment overnight at 37 °C, culture-inserts were gently removed to form the cell-free gap. For each condition, pictures were taken at a precise local-

ization every 3 h to monitor the healing of the cell-free gap. To calculate the percentage of closure of the wound, 8-bit image analysis was performed with the Fiji software, an image-processing package based on ImageJ (NIH). First, cells were localized by edge detection. Then the mask of empty areas was created using background subtraction. Then, particle analysis enabled the detection and measurement of the uncolonized areas. Finally, the percentage of closure was obtained by subtracting the area at a given time by the original area at T0 in each condition.

Invasion Assay—MDA-MB-231 cells were starved in serum-free DMEM medium for 4 h, split and counted. When applicable, cells were pretreated with PBS or isoproterenol (10 μM) in serum-free DMEM medium for 10 min. Cells in serum-free DMEM medium were then seeded onto the BD BioCat™ growth factor reduced matrigel™ invasion chamber with 8- μm pore size (BD Biosciences), at a density of 50,000 cells per well (24-well format). For each treatment condition, cells were seeded in triplicate. Next, the cells were tested for their ability to invade the matrigel and migrate across the filters in response to chemoattraction of 10% FCS-DMEM medium placed in the lower chambers. After 24 h of incubation at 37 °C, non-migrating cells were scraped from the top face of the inserts, while cells that had migrated across the filter pores to the lower face were fixed in methanol and stained with Hoechst 33258. The number of cells that had migrated was analyzed on each filter using an Axioplan 2 (Zeiss, Germany) microscope. Ten images of randomly chosen optical fields were captured on each migration filter using AxioVision® Software for microscopy image analysis (Zeiss), and migrating cells were counted with the Colony1.1® software.

Statistics—Experiments were performed at least twice independently in duplicates or triplicates. Statistical analyses were performed by Student's *t* test. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, and NS indicates a non-significant variation.

RESULTS

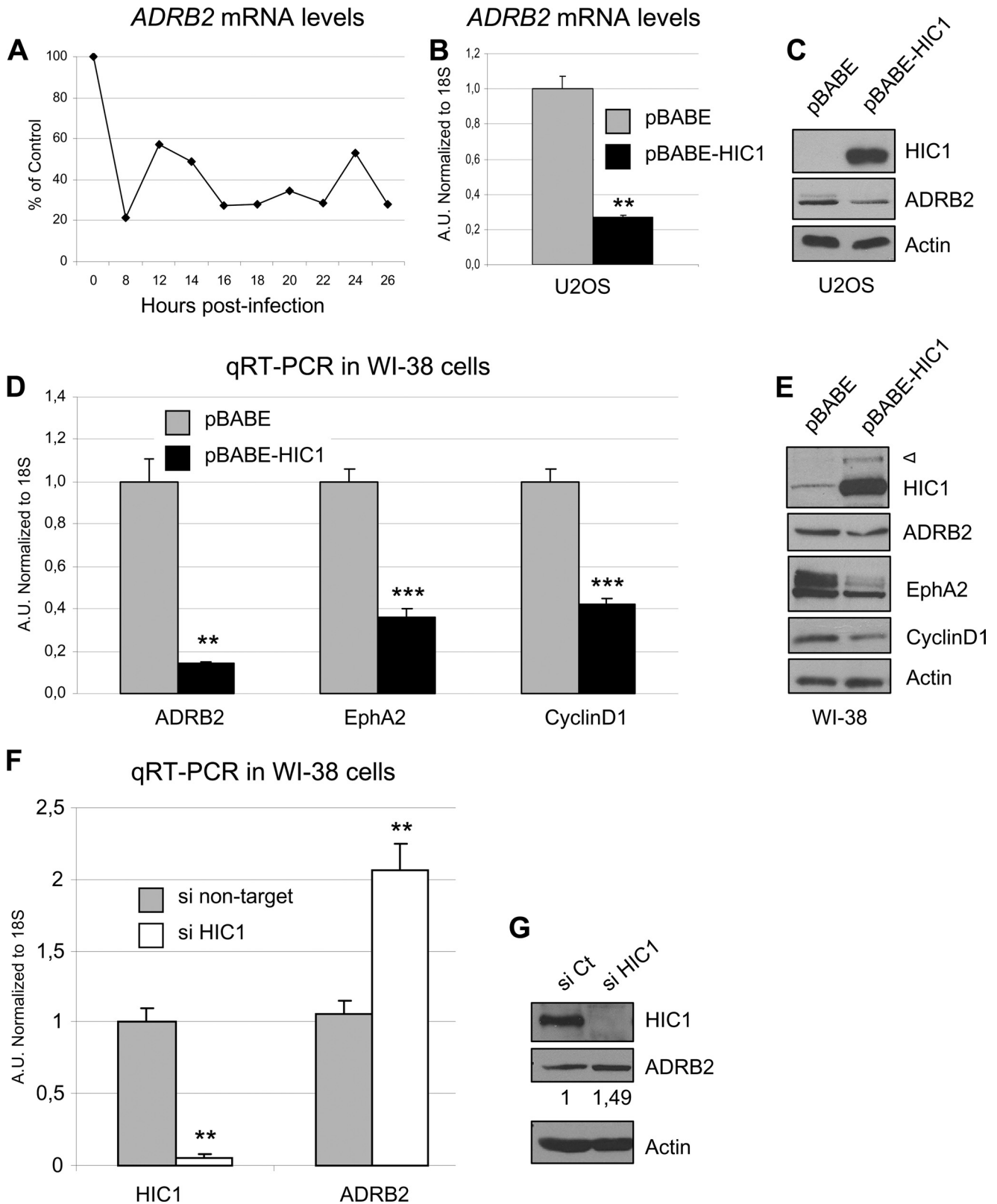
ADRB2 Is a Direct Target Gene of HIC1—We recently described the role of HIC1 in cell migration and invasion as partially relying on direct transcriptional repression of the tyrosine kinase receptor *EphA2* gene.⁷ Nevertheless, we hypothesized that other target genes could also be implicated in these important biological processes. In a previous report of gene expression profiling, we published a list of genes repressed in U2OS osteosarcoma cancer cells following adenoviral infection and reexpression of HIC1 (14). Among them, we already validated the receptors coding genes *CXCR7* and *EphA2* as new HIC1 target genes (14, 34). Another gene present in our list, *ADRB2*, coding for a G-protein-coupled receptor, was repressed 5-fold in Ad-HIC1-infected cells as compared with control infected cells as early than 8 h postinfection (Fig. 1A). In this current work, we first confirmed *ADRB2* down-regulation in U2OS cells infected with a retrovirus expressing FLAG-tagged HIC1. After puromycin selection, HIC1-expressing cells were harvested and then mRNAs and proteins were extracted. qRT-PCR and immunoblot with an antibody specific for ADRB2 confirmed the repression induced by HIC1 re-expres-

ADRB2 Is a Direct Target Gene of HIC1

sion (Fig. 1, B and C) although the effects are more significant at transcriptional levels.

Human lung embryonic fibroblasts (WI-38) are normal diploid cells expressing endogenous HIC1 (8, 10) and ADRB2 (Fig. 1E). Overexpression of HIC1 in these cells by retroviral infec-

tion induced a marked decrease of ADRB2 mRNA (Fig. 1D) and a slight decrease of protein levels (Fig. 1E). In these conditions, *Cyclin D1* and *EphA2* were also repressed (Fig. 1, D and E) confirming previous (8) and ongoing results (34). Conversely, inhibition of endogenous HIC1 expression in WI-38 cells by



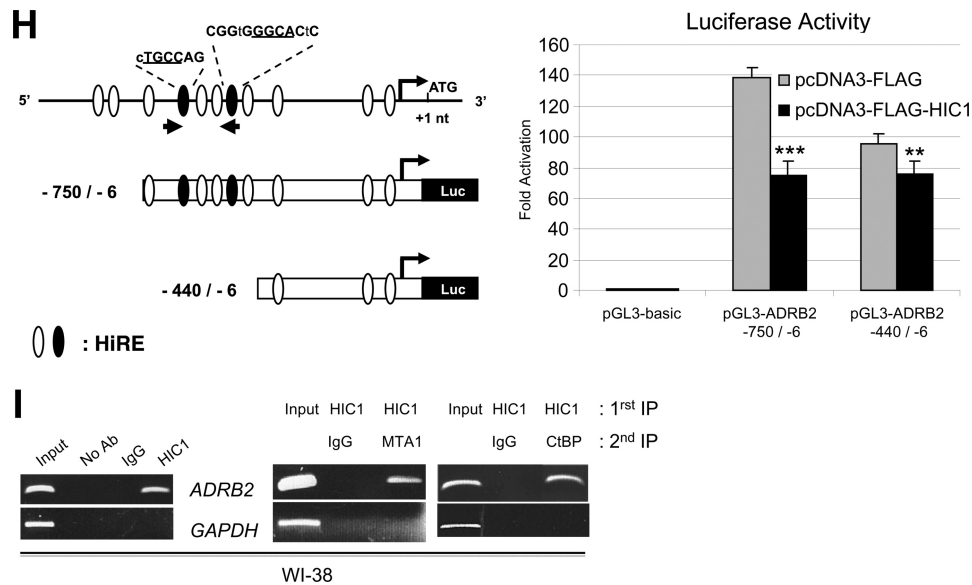


FIGURE 1—continued

siRNA resulted in a concomitant increase in *ADRB2* transcripts (Fig. 1*F*) and proteins (Fig. 1*G*).

To determine whether *ADRB2* is a direct target gene of HIC1, we first scanned its promoter for the presence of HIC1-responsive elements (HiRE) (5, 26). These analyses identified many putative HiRE, particularly 600 bp upstream of the translation start site (Fig. 1*H*, left panel). We cloned a length of genomic DNA corresponding to the region 750 bp upstream of the ATG codon into the pGL3 basic reporter vector and performed luciferase promoter-reporter assays in U2OS cells in the presence or absence of transiently transfected pcDNA3-FLAG-HIC1. Under former conditions, the promoter activity was repressed 2-fold (Fig. 1*H*, right panel) in accordance with our results showing similar repression of endogenous *ADRB2* following HIC1 re-expression in these cells (Fig. 1*A*). Furthermore, HIC1 was no longer able to repress a mutant with deletion of the region 600 bp upstream of the ATG and lacking a cluster of binding sites (Fig. 1*H*, pGL3-*ADRB2* -440/-6).

Finally, in WI-38 cells, chromatin immunoprecipitation (ChIP) experiments demonstrated the specific binding of HIC1 on the *ADRB2* promoter with primers flanking these HIC1

binding sites located 600 bp upstream of the translation start site (Fig. 1, *H* and *I*, left panels). Moreover, sequential ChIP showed concomitant fixation of HIC1 and at least two of its transcriptional co-repressors, MTA1, and CtBP on the *ADRB2* promoter (Fig. 1*I*, right panels) (7, 8). Taken together, these results demonstrate that *ADRB2* is a direct target gene of HIC1.

ADRB2-activated Primary Fibroblasts WI-38 Exhibit a Specific Adhesion Phenotype Suppressed by HIC1 Overexpression—We next attempted to decipher the functional link between HIC1 and this newly characterized target gene. Many studies performed on different tumor cell types have highlighted various important roles for epinephrine and norepinephrine, the natural ligands of adrenergic receptors, in biological processes (27). We focused on *ADRB2* function during cell adhesion on substrates like type I collagen and fibronectin as the natural microenvironment of normal fibroblasts. Indeed, a previous study showed that the activation of *ADRB2* with isoproterenol, a synthetic catecholamine targeting and activating β subtype adrenergic receptors, could accelerate the Ovar3 ovarian cancer cell's adhesion on fibronectin through a pathway involving integrins (28).

FIGURE 1. *ADRB2* is a direct target gene of HIC1. *A*, effects of HIC1 overexpression on expression of *ADRB2* in infected cells. Total RNAs from U2OS cells (HIC1 null) infected with Ad-FLAG-HIC1 and Ad-GFP were prepared at the indicated times (from 8 to 24 h) and Affymetrix HG U133A chips were used to measure the gene expression. Expression values were normalized to Ad-GFP-infected control cells at the same time points. % of control corresponds to the ratio between the expression levels of *ADRB2* measured in Ad-GFP and Ad-FLAG-HIC1-infected cells at each time point. *B*, quantitative Real Time PCR analyses of *ADRB2* and *C*) immunoblot assays of HIC1 and of *ADRB2* in U2OS cells infected with pBABE-FLAG-HIC1 (abbreviated as pBABE-HIC1 in all figures) or by pBABE-FLAG (pBABE) as control. Actin protein levels were used as a loading control. *D*, quantitative real time PCR analyses and *E*) immunoblot assays of HIC1 and of relevant target genes in WI-38 cells infected by pBABE-FLAG-HIC1 or by pBABE as control. ∇ , detection of SUMOylated forms of HIC1 (33). *F* and *G*, inactivation of endogenous HIC1 in WI-38 cells up-regulates *ADRB2*. *F*, qRT-PCR and *G*, Western blot analyses of HIC1 and of *ADRB2* in WI-38 cells following RNA interference of HIC1 or with non-targeting control siRNA. Quantification of *ADRB2* protein levels was realized using ImageJ by dividing the value obtained for *ADRB2* by the value of actin. ** indicates $p < 0.01$ and *** indicates $p < 0.001$. *H*, schematic drawing of the 5' promoter region and the unique coding exon of *ADRB2* are shown on the left. The transcription start site (bent arrow) as well as a portion of the coding exon are described in GenBank™ under accession number NG_016421; GI: 284520137. Numbering is relative to the *ADRB2* translational start site (ATG, nt +1) as in Ref. 26. The potential HiREs are shown as white ovals and as black ovals for those which are highly homologous to the consensus (5). The two small arrows indicate the position of the primers used to amplify the relevant region of *ADRB2* in the ChIP and sequential ChIP experiments. Below are shown schematic drawings of the two human *ADRB2* promoter constructs subcloned in the luciferase reporter plasmid pGL3 basic. Luciferase promoter reporter assay in U2OS cells (right). Results are expressed relative to a value of 1.0 for cells transfected with the pGL3 empty vector. Each condition was performed at least twice in duplicate and a representative experiment is shown. *I*, ChIP analyses of HIC1 on *ADRB2* promoter in WI-38 cells (left panels) and sequential ChIP experiments demonstrate that HIC1/MTA1 and HIC1/CtBP might form a stable complex on the *ADRB2* promoter (right panels). Normal WI-38 lung fibroblasts chromatin was immunoprecipitated with anti-HIC1 antibody (325) (7). The bound material was eluted, divided in two and subjected to a second round of immunoprecipitation with anti-MTA1 antibodies, anti-CtBP, or with normal rabbit IgG (8). PCR amplifications were performed with primers flanking HIC1 binding sites in *ADRB2*. *GAPDH* was used as an internal non-binding control.

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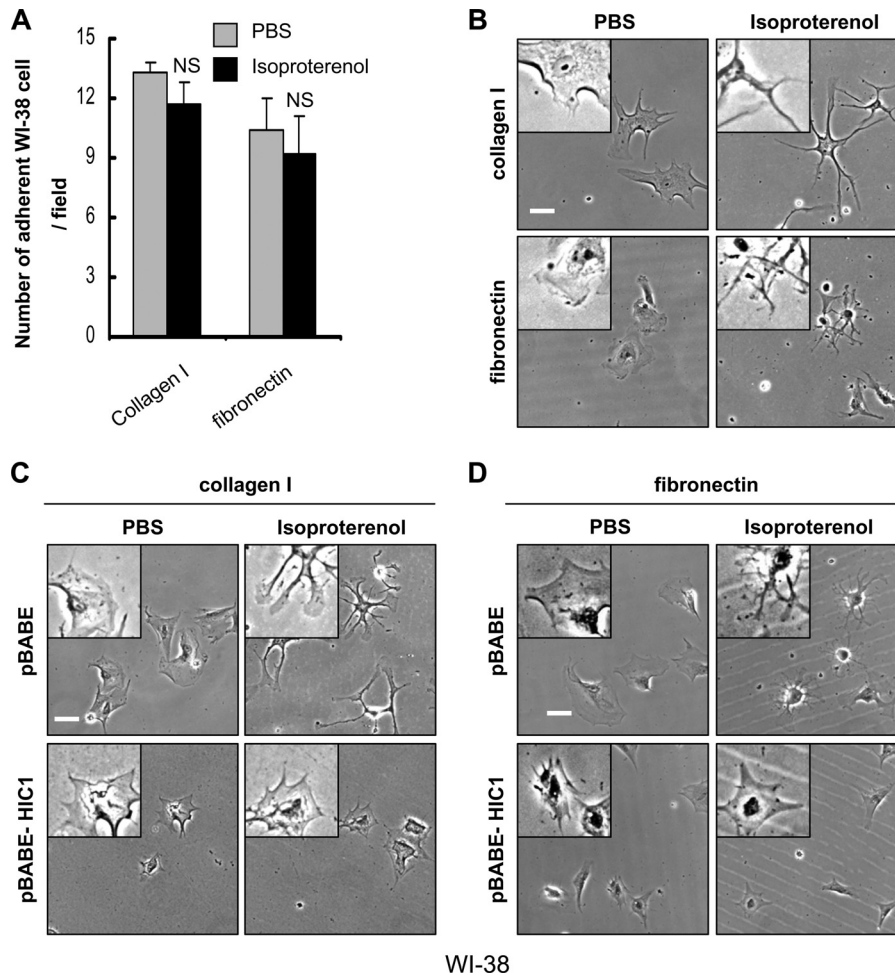


FIGURE 2. ADRB2-activated primary fibroblasts WI-38 exhibit a specific adhesion phenotype suppressed by HIC1 overexpression. *A*, ADRB2 activation does not affect cell number during adhesion. Ten fields per well were counted. Each condition was performed in triplicate. *NS* indicates non-significant. *B*, adhesion assay of WI-38 cells on Type I collagen and fibronectin-treated with PBS or isoproterenol (100 nM). In each condition, at *top left*, the magnification of a characteristic adherent cell. *C* and *D*, isoproterenol-induced phenotype is abolished by HIC1 overexpression on (*C*) type I collagen and (*D*) fibronectin. Scale white bar corresponds to 50 μm .

In normal WI-38 cells, isoproterenol did not affect adherent cell number on either type of coating (Fig. 2*A*), in contrast to previous results obtained in transformed cell lines (28). However, ADRB2 activation in WI-38 cells induced a particular adhesion phenotype illustrated by the establishment of focal adhesion sites usually associated with integrin recruitment (Fig. 2*B*, *right panels*). Following HIC1 retroviral over-expression in WI-38 cells, concomitant with a strong decrease of *ADRB2* levels (Fig. 1*F*), the isoproterenol-induced phenotype was completely abolished (Fig. 2, *C* and *D*, *bottom right panels*). Specific activation of ADRB2 could therefore induce localization of integrins on focal sites that are essential for cell migration. Based on these phenotypic results affecting adhesion, we hypothesized that other physiologic events could rely on ADRB2 activation and therefore could be regulated by transcriptional repression induced by HIC1.

Re-expression of HIC1 Decreases High ADRB2 Levels in MDA-MB-231 Breast Cancer Cells—Given our previous results and the link between norepinephrine and migration, we decided to focus on HIC1's impact on migration. To that end, we switched to a cellular model widely used to study migration properties, the metastatic breast cancer cell line MDA-MB-

231. As expected for MDA-MB-231 breast cancer cells, HIC1 was not detected by immunoblot (Fig. 3*A*) (2). Conversely, ADRB2, but not the related ADRB1 and ADRB3 receptors, is highly expressed in these cells (Fig. 3*B*).

We first established that ADRB2 activation had similar effects upon MDA-MB-231 cell adhesion and induced focal adhesion sites on type I collagen and fibronectin (supplemental Fig. S1*A*). In these cells, isoproterenol induced a slight increase in adherent cells (supplemental Fig. S1*B*). Again, re-expression of HIC1 induced a loss of the isoproterenol-induced phenotype (Fig. 3, *C* and *D*) correlated with a robust decrease of *ADRB2* transcripts (Fig. 3*E*) and protein (Fig. 3*F*) following HIC1 binding on the *ADRB2* promoter (Fig. 3*G*). In conclusion, *ADRB2* is the only member of the β -Adrenergic receptor family highly expressed in MDA-MB-231 breast cancer cells and re-expression of HIC1 in these cells is able to extinguish its expression.

HIC1 Blocks an ADRB2-mediated Boost of Migration and Invasion—We next verified that ADRB2 activation could stimulate migration of MDA-MB-231 as previously described (17). In a wound healing assay, pretreatment of cells with isoproterenol during adhesion could indeed accelerate migration on type I collagen and fibronectin (supplemental Fig. S1, *C* and *D* and

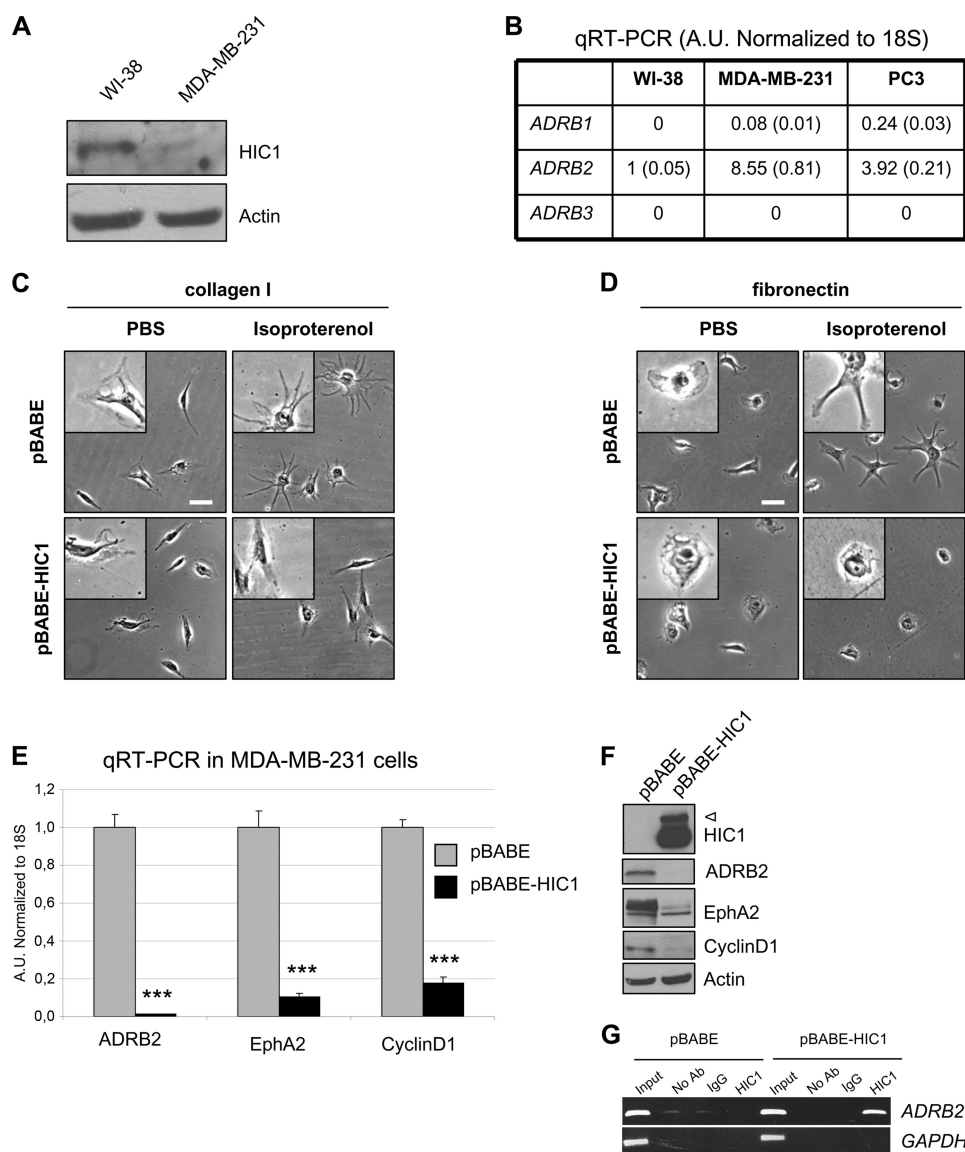


FIGURE 3. Re-expression of HIC1 decreases high ADRB2 levels in MDA-MB-231 breast cancer cells. *A*, immunoblot analyses of endogenous HIC1 in WI-38 and MDA-MB-231 cells. Actin was used as a loading control. *B*, quantitative real time PCR analyses of endogenous ADRB1, ADRB2, and ADRB3 mRNA levels in WI-38, MDA-MB-231, and PC3 cells. Results are compared with ADRB2 levels in WI-38 cells and standard deviations are in brackets. ADRB1 oligonucleotides efficiency was verified in PC3 cells (18). *C* and *D*, isoproterenol-induced phenotype during adhesion is abolished by HIC1 overexpression on (C) type I collagen and (D) fibronectin. In each condition, at top left, the magnification of a characteristic adherent cell is depicted. Scale bar corresponds to 50 μ m. *E*, quantitative real time PCR and *F*, immunoblot assays in MDA-MB-231 cells infected by pBABE-FLAG-HIC1. *** indicates $p < 0.001$. ∇ , detection of SUMOylated forms of HIC1. *G*, ChIP analyses of HIC1 on the ADRB2 promoter in MDA-MB-231 cells infected by pBABE-FLAG-HIC1. GAPDH was used as an internal non-binding control.

Fig. 4, *A* and *B*). Cells re-expressing HIC1 showed a diminution of migration compared with the empty vector-infected cells (Fig. 4, *A* and *B*, bottom right panels). The lack of isoproterenol effect in HIC1 re-expressing cells could therefore be caused by the loss of ADRB2 expression.

MDA-MB-231 cells are metastatic and highly invasive. In invasion assays, cells were seeded on the top of the invasion chamber (coated with a matrigel layer) and were stimulated with isoproterenol during the adhesion process. In agreement with the migration assays, we observed a significant increase of invasion (supplemental Fig. S1E). Again, HIC1 re-expression dramatically abolished these invasive properties upon isoproterenol stimulation and notably also in basal conditions (Fig. 4C). These results demonstrate that activation of β -subtype adrenoceptor ADRB2 enhances both the migration and inva-

sion of MDA-MB-231 breast cancer cells, and that both phenotypes are abolished by HIC1 re-expression.

Inhibition of ADRB2 in MDA-MB-231 Mimics the Effects of HIC1 Re-expression on Adhesion, Migration, and Invasion—To ensure that phenotypes observed following isoproterenol treatment and abolished in the presence of HIC1 are specific to ADRB2-mediated pathways, we inhibited ADRB2 by RNA interference in MDA-MB-231. Focal adhesion sites were severely impaired despite isoproterenol treatment but phenotypes were not fully abolished (Fig. 5A, bottom right panels). This could be explained by the presence of residual receptors on cells. Indeed, in our experimental conditions, ADRB2 levels were less decreased by siRNA ADRB2 treatment than following HIC1 re-expression (compare Fig. 3E and inset in Fig. 5C). Nevertheless, cellular migration was

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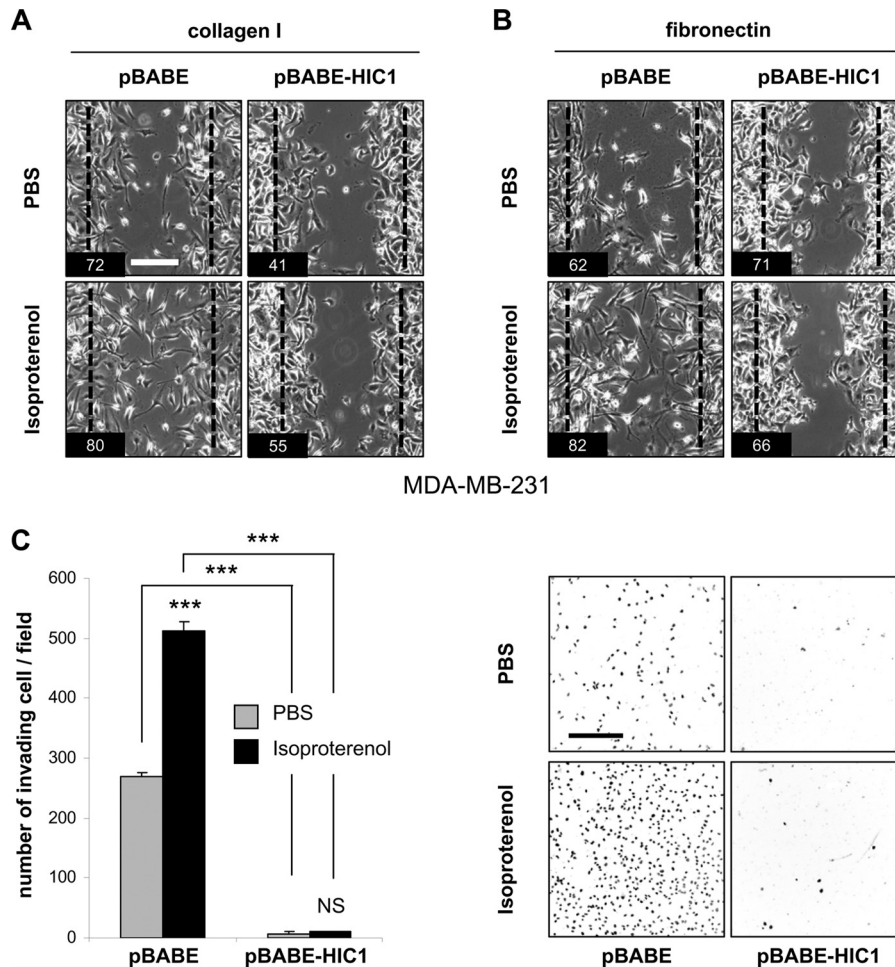


FIGURE 4. HIC1 blocks an ADRB2-mediated boost of migration and invasion. *A* and *B*, migration assays of MDA-MB-231 cells infected by pBABE-FLAG-HIC1 on (A) type I collagen for 7 h and (B) fibronectin for 25 h. Cells were pretreated with PBS or isoproterenol (10 μ M) during adhesion (overnight at 10% FCS). Migration assays were performed in 0.5% FCS. White bar, 200 μ m. Dotted lines correspond to the wound at $t = 0$. At the bottom left, the percentage of wound closure was established by image analysis as described under "Experimental Procedures." *C*, invasion assays in Matrigel of MDA-MB-231 cells infected by pBABE-FLAG-HIC1. Cells were seeded in absence of FCS with PBS or isoproterenol (10 μ M). After 24 h incubation at 37 $^{\circ}$ C, cells were fixed with cold methanol and Hoechst stained. Dark spots are nuclei of cells that had migrated to the other pole of the membrane. A representative field is shown for each condition. Ten fields per well were counted. Each condition was performed twice in duplicate. Black bar, 500 μ m. *** indicates $p < 0.001$, and NS indicates non-significant.

also severely impaired (Fig. 5*B*, compare bottom panels). Finally, invasion was also strongly inhibited in the presence and absence of isoproterenol, recapitulating the HIC1-induced phenotype (Fig. 5*C*).

ADRB2 Partially Rescues HIC1-induced Phenotypes—To more directly demonstrate that ADRB2 is a key target gene involved in phenotypes caused by HIC1 re-expression, we repeated invasion assays after reintroducing ADRB2 expression by retroviral infection. ADRB2 expression alone induced a 2-fold increase of invasion comparable to the results obtained with empty vector-infected cells treated with isoproterenol (Fig. 6, compare lanes 2 and 3). The lack of increased invasion in ADRB2 infected cells treated with isoproterenol (lane 4) could reflect saturation of the membrane due to the large number of cells. However, and in accordance with all of our previous results, co-expression of ADRB2 in HIC1-infected MDA-MB-231 cells partially rescued HIC1-induced abolition of invasion by a significant 3-fold increase in invading cells in the absence of isoproterenol (compare lanes 5 and 7). Furthermore, HIC1 and ADRB2 co-infected cells were statistically more invasive in the presence of isoproterenol (compare lanes 7 and 8). Alto-

gether these results demonstrate that phenotypes induced by re-expression of HIC1 in MDA-MB-231 breast cancer cells are in part due to transcriptional repression of one target gene, ADRB2, coding for a cell membrane receptor whose activation promotes migration and invasion.

DISCUSSION

In this study, we demonstrate that ADRB2 is a new direct target gene of HIC1, a tumor suppressor inactivated in many cancers, particularly in breast and prostate. In these tissues, HIC1 is already hemi-methylated in normal conditions and its extinction is correlated with the aggressiveness of tumors (2, 4). The identification of HIC1 target genes is thus a crucial step in understanding how its inactivation could contribute to tumorigenesis.

ADRB2 is a cell membrane GPCR, overexpressed in breast cancers whose activation by the stress-released hormones adrenaline/noradrenaline stimulates tumor growth, migration, and invasion. Recent studies and our results demonstrate that HIC1 re-expression strongly impairs these phenotypes in breast cancer cells (15, 34). ADRB2 repression consistently sup-

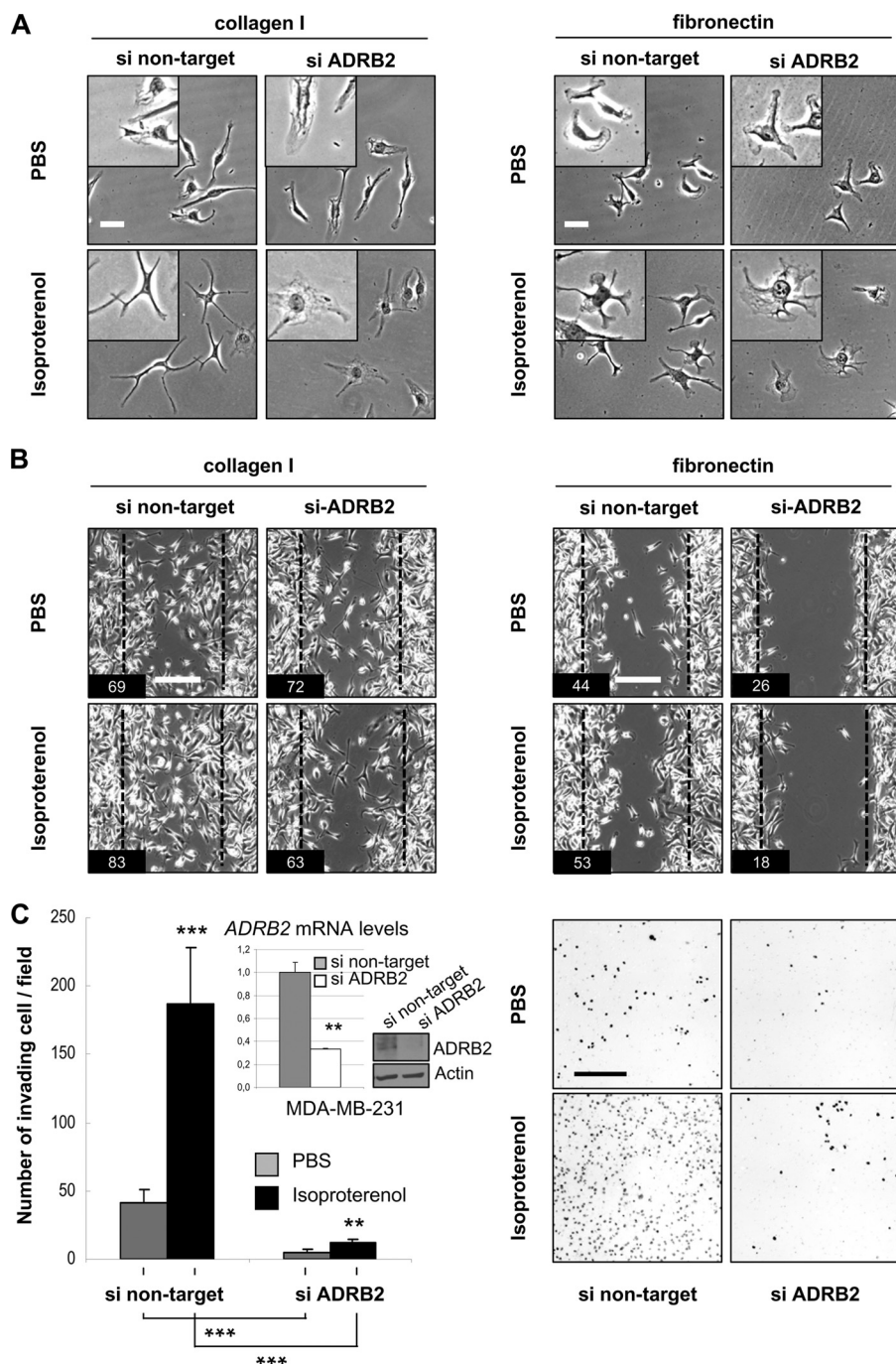


FIGURE 5. Inhibition of ADRB2 in MDA-MB-231 mimics the effects of HIC1 re-expression on adhesion, migration, and invasion. MDA-MB-231 cells were transfected with non-targeting control siRNA or with ADRB2 siRNA. After 48 h, cells were trypsinized, treated with PBS or isoproterenol (10 μ M) and seeded for (A) adhesion assay on type I collagen or fibronectin (Bar, 50 μ m), (B) wound test assay on type I collagen for 8 h and fibronectin for 11 h (Bar, 200 μ m) and (C) matrigel invasion assay as previously described in Figs. 3 and 4. Bar, 500 μ m. ADRB2 immunoblot and mRNA levels measured by qRT-PCR in cells treated by siADRB2 or control siRNA are shown as right inset. ** indicates $p < 0.01$, and *** indicates $p < 0.001$. In B, at the bottom left, the percentage of wound closure was established by image analysis as described under "Experimental Procedures."

ports these observations although re-expression of a transcription factor involves a network of multiple target genes, which in the case of HIC1, seem to be involved in cell cycle regulation (8, 15) and cell motility (14, 34).

We did not accumulate evidence for a role of ADRB2 in cell cycle progression but these effects seem to be highly cell type specific (29, 30). It is nevertheless conceivable that HIC1 could inhibit cell cycle progression by two means: directly by repress-

ing *Cyclin D1* (8) but also indirectly by decreasing ADRB2 levels. In our experiments with both WI-38 and MDA-MB-231 cells, expression of HIC1 induced a decrease of *Cyclin D1* transcripts and proteins levels in accordance with a retardation of proliferation (1, 14, 34).

In our experiments, we used isoproterenol, an adrenaline/noradrenaline mimetic, to activate ADRB2 as previously described (16, 20, 28, 29). Isoproterenol is also able to target

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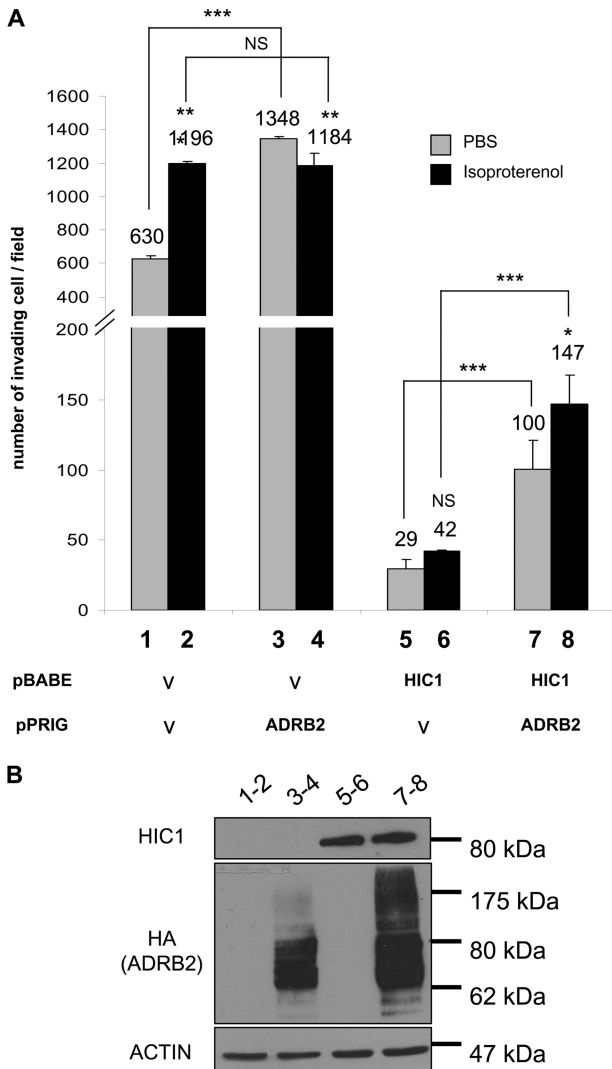


FIGURE 6. ADRB2 partially rescues HIC1-induced decrease of invasion. A, matrigel invasion assays of 50,000 MDA-MB-231 cells infected by a combinations of viruses coding for HIC1 and ADRB2 as indicated on the bottom panel and as previously described. v means “empty vector” used to clone coding sequences of HIC1 or ADRB2. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, and NS indicates non-significant. In the absence of bars, asterisks, or NS compare PBS and isoproterenol-treated conditions. B, immunoblot of MDA-MB-231-infected cells used for Matrigel invasion assays. Overexpression of ADRB2 proteins is detected as a ladder of multiple bands.

ADRB1 and ADRB3. Both were undetectable at the mRNA levels in WI-38 cells (Fig. 3B). In MDA-MB-231 breast cancer cells, ADRB3 was undetectable and ADRB1 was only slightly expressed and its transcription was not affected by re-expression of HIC1 (Fig. 3B and data not shown).

Surprisingly, ADRB2 stimulation during adhesion induced arborized shapes on matrix substrates, a phenotype abolished following HIC1 expression or specific inhibition of ADRB2. This phenotype has been previously described on fibronectin following dibutyryl cAMP treatment of transformed fibroblasts (BHK21 and NIH-3T3) (31) and is consistent with an increase in total cAMP in HEK-293 cells following ADRB2 activation (32).

To the best of our knowledge, our siRNA results are the first direct evidence that specific stimulation of ADRB2 is able to promote both migration and invasion of breast cancer cells. It is

noteworthy that in our experiments, preactivation of ADRB2 by isoproterenol occurred during cell seeding and adhesion and then had an impact on migration and invasion. These biological effects would thus be due to activation of pathways downstream of ADRB2. Previous studies have shown that stress results in higher levels of tissue catecholamine followed by increased levels of proangiogenic factors such as IL-8 (interleukine-8), VEGF (vascular endothelial growth factor), and MMP (matrix metalloproteinase) resulting in enhanced tumor vascularization (16, 21) and invasion (20).

In our experiments, the migration and invasion of MDA-MB-231 cells are affected after HIC1 re-expression in the presence but also in the absence of isoproterenol (Fig. 4C, right panels). Although other target genes must be involved in these phenotypes, it is essential to note that the same results are obtained after ADRB2 inhibition by siRNA even in the absence of isoproterenol (Fig. 5C), strongly suggesting that basal cell culture conditions are able to activate ADRB2 and that ADRB2 is a major player involved in migration and invasion of breast cancer cells. In agreement with this idea, ADRB2 overexpression increases invasion even in the absence of specific activation by isoproterenol treatment.

Our results are particularly significant in Matrigel invasion assays (Figs. 4 and 5). Wound-healing assays were more difficult to quantify because treatments effects are more visual and depend on the behavior of cells on the substrates. In particular, the HIC1-infected cells migrate differently on fibronectin (Fig. 4B). Nevertheless, on the whole, our results are consistent with the link established between HIC1 and ADRB2 on cell migration.

Strikingly, in immortalized normal mammary epithelial HMEC-hTERT cells, despite the presence of ADRB2, isoproterenol treatment did not induce any consistent phenotype during the adhesion process (data not shown). Furthermore, isoproterenol treatment delayed migration in wound healing assays. Thus, although siRNA mediated inhibition of HIC1 led to increased migration in wound healing assays as previously described in transwell migration assays (34), they were still delayed after isoproterenol treatment (supplemental Fig. S2). We speculate that some ADRB2 downstream effectors are absent in these normal cells, which in contrast with MDA-MB-231, have not undergone an epithelial-mesenchymal transition. Consistent with a multi step process of breast cancer progression, ADRB2 up-regulation could not favor cell motility in these normal cells. In conclusion, our data suggest that, in breast epithelial cells, loss of HIC1 in tumorigenesis could favor metastasis through up-regulation of β -2 adrenergic receptor.

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Supplementary Figure Legends

Supplementary Fig. 1. ADRB2 mediated boost of migration and invasion in MDA-MB-231 cells.

- (A) Cells were trypsinized, treated with PBS or Isoproterenol (10 μ M) and seeded for adhesion assay on type I collagen or fibronectin (Bar = 50 μ m). In each condition, at top left, is the magnification of a characteristic adherent cell.
- (B) ADRB2 activation slightly increases cell number during adhesion. Ten fields per well were counted. Each condition was performed in triplicate.
- (C) and (D) Isoproterenol induced boost of migration on (C) type I collagen and (D) fibronectin in wound healing assay (Bar = 200 μ m). At the bottom left, the percentage of wound closure was established by image analysis as described in the experimental procedures section.
- (E) Isoproterenol induced a boost of matrigel invasion. Cells were seeded in the absence of FCS with PBS or Isoproterenol (10 μ M). After 24 hours incubation at 37°C, cells were fixed with cold methanol and Hoechst stained. Left panels :Dark spots are nuclei of cells that had migrated to the other pole of the membrane. A representative field is shown for each condition. Right panels : Ten fields per well were counted. Each condition was performed twice in duplicate. * indicates $P < 0.05$, ** indicates $P < 0.01$ and *** indicates $P < 0.001$.

Supplementary Fig. 2. Inhibition of *HIC1* increases migration of HMEC-hTERT cells but is delayed by Isoproterenol treatment.

(A) and (B) Wound healing assays of HMEC-hTERT cells transfected with non-target siRNA control or with *HIC1* siRNA on (A) type I collagen for 26 hours and (B) fibronectin for 26 hours. Cells were pre-treated with PBS or Isoproterenol (10 μ M) during adhesion (overnight in complete medium). Migration assays were performed in medium not supplemented. Bar = 200 μ m. Dotted lines correspond to the wound at $t=0$. At the bottom left, the percentage of wound closure was established by image analysis as described in the experimental procedures section.

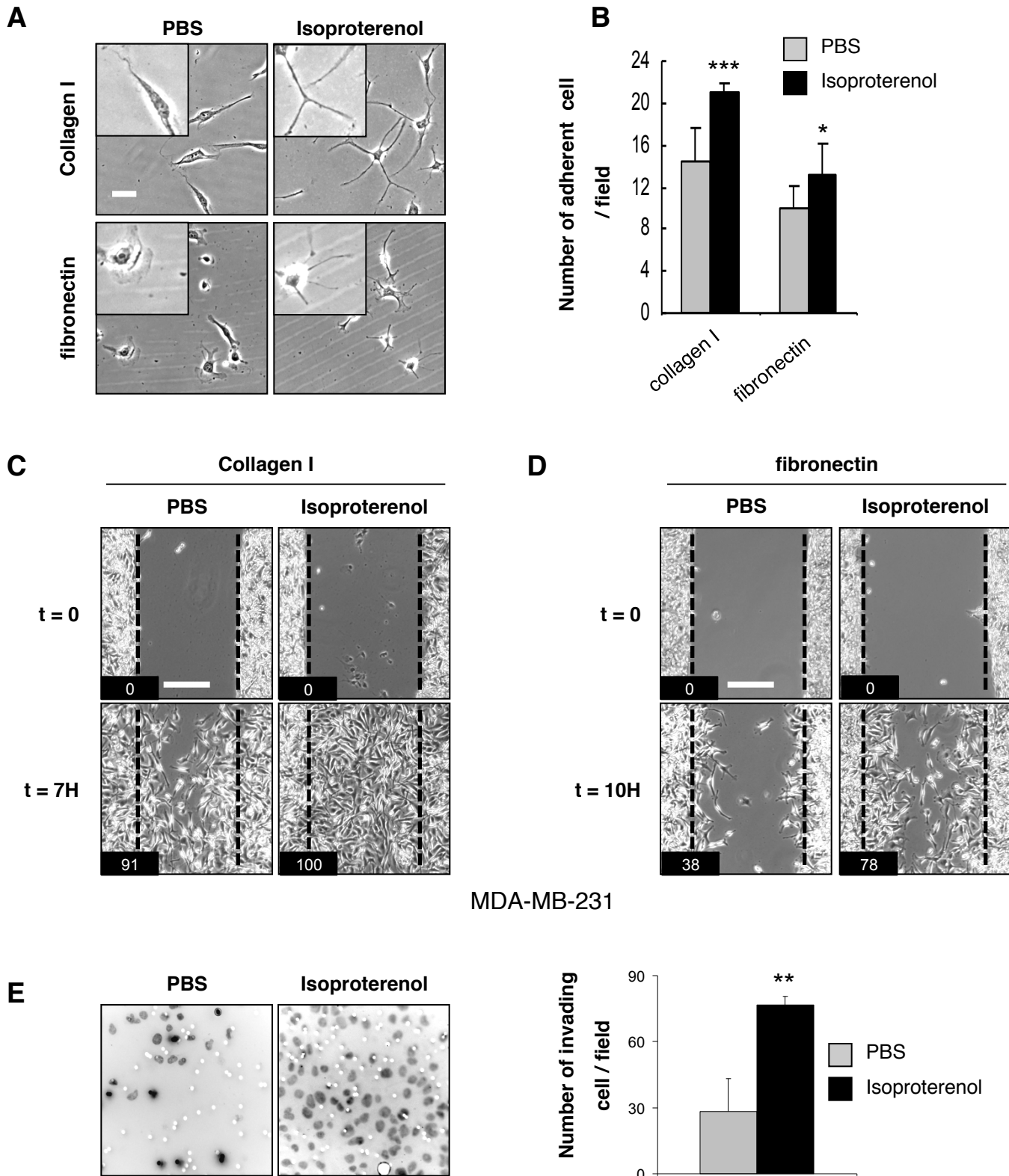
Supplementary Materials and methods

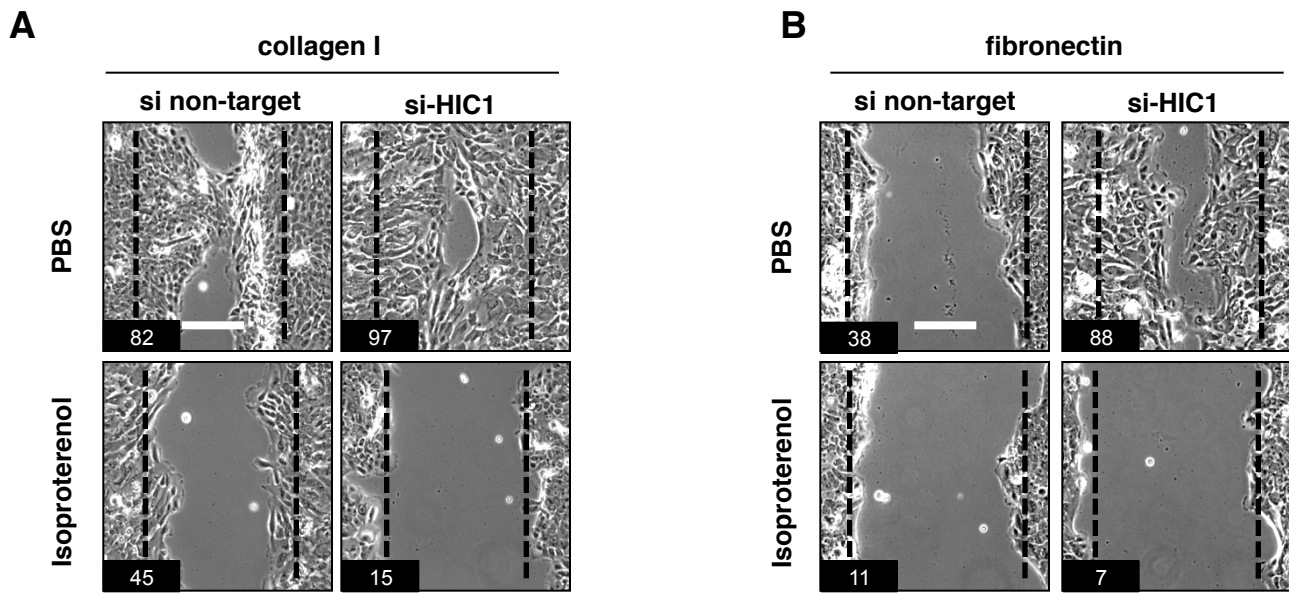
Cell culture

The normal mammary HMEC-hTERT cells were cultured in Mammary Epithelial Cell Growth Medium (C-21010, PromoCell, Heidelberg, Germany) supplemented with gentamicin and a mix (C-3911S) to obtain a final concentration of 0.004 ml/ml of Bovine pituitary extract, 10 ng/ml of epidermal Growth Factor (human recombinant), 5 μ g/ml of Insulin (human recombinant) and of 0.5 μ g/ml of Hydrocortisone.

Small Interfering RNA

HMEC-hTERT cells were reverse-transfected with RNAiMax according to manufacturer's instructions using 20 nM small interfering RNA targeting *HIC1* (*HIC1* siGENOME Smart Pool M-006532-01, Dharmacon) or a scrambled sequence, as previously described (Foveau *et al.*, in preparation). 72 hours later cells were lysed for RNA or protein extraction or seeded for bioassays.





HMEC-hTERT

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bibliographiques

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