Université de Lille 1 Sciences et Technologies

Ecole Doctorale Biologie-Santé de Lille Nord de France

Détermination des mécanismes d'échappement à la mort par autophagie lors des étapes très précoces de transformation de cellules sénescentes en cellules tumorales.

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Thèse de Doctorat

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Présentée et soutenue par

Emeric Deruy

A Lille, le 22 février 2010

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Devant un jury composé de

Pr Xuefen Le Bourhis	Président
Pr Olivier Coqueret	Rapporteur
Pr André-Patrick Arrigo	Rapporteur
Dr Patrice Codogno	Examinateur
Dr Pierre Golstein	Examinateur
Pr Corinne Abbadie	Directeur de thèse

A Emilie
A ma famille

Remerciements

Je tiens dans un premier temps à remercier les membres qui composent mon jury, éminences dans leurs domaines respectifs, qui ont bien voulu prendre sur leur temps pour évaluer mon travail de thèse.

Pr Xuefen Le Bourhis
Pr Olivier Coqueret
Pr André-Patrick Arrigo
Dr Patrice Codogno
Dr Pierre Golstein

Merci à **Yvan De Launoit**, directeur de l'UMR 8161 qui m'a permis d'intégrer l'équipe initialement IECE (équipe 5), puis ICE (équipe 6 ou 4 ou … à force, je ne sais même pas si quelqu'un en a la moindre idée) et qui a soutenu mes diverses demandes de formations.

J'aimerai remercier particulièrement **Corinne**, ma directrice de thèse. J'estime depuis le début de mon master que la relation entre un tuteur et son étudiant doit être une symbiose qui permet aux deux protagonistes de s'enrichir mutuellement. Vous m'avez appris énormément sur le métier de chercheur et j'espère moi aussi avoir joué le rôle que vous attendiez de moi en m'intégrant dans l'équipe il y a quatre ans. Merci donc, d'avoir pris ce temps pour moi, et notamment ces dernières semaines au cours desquelles, faute de financement pour une quatrième année, nous avons travaillé dur pour préparer la thèse dans les délais. J'ai encore beaucoup à apprendre, mais j'ai déjà énormément appris grâce à vous depuis mon Master recherche (notamment en français mais ça c'est une autre histoire).

Un grand merci à **Chantal**, la mémoire et l'expérience de notre équipe. Il faut avouer que tu m'as appris presque tout ce que je sais faire dans un laboratoire aujourd'hui (même jouer aux experts avec la carboglace…). Tu m'as toujours poussé à travailler plus, quitte parfois à te rajouter du travail quand j'étais pris ailleurs pour éviter que les projets ne s'arrêtent. Je vais regretter tes arrivées du matin les mains pleines de sucreries et autres gâteux hyper (pas-) diététiques (du tout) ainsi que toutes les discussions qu'on a pu avoir.

Merci à **Nico,** en Master et en thèse avec moi depuis le début, et très certainement l'un de mes meilleurs amis. On en aura passé des épreuves ensemble ! La thèse, c'est quand même bien plus facile quand on est deux et qu'on peut se soutenir autour d'une bonne bière dans les moments les plus durs. Ne t'inquiète pas, ils donnent aussi le diplôme aux gens qui travaillent sur les kératinocytes. Je ne peux décemment pas mentionner par écrit tout ce qu'on a fait au labo, alors merci pour tout. J'espère que tu auras l'occasion d'avoir le post doc que tu souhaites chez J. Campisi qui, je le précise, rivaliserait presque avec Clara Morgane tellement tu nous en as fait l'éloge. Occupes toi bien du p'tit Joe en mon absence mais je préférerai que tu attendes avant de lui parler de la sentence F.

Merci à tous les membres de l'équipe que j'ai pu côtoyer au cours de ma thèse. **Albin** tant pour votre sérieux et vos analyses toujours pertinentes des résultats pendant les réunions que pour votre bonne humeur et votre humour parfois (souvent) déplacé le reste du temps ; **Fatima**, ma voisine de paillasse avec qui j'ai pris grand plaisir à discuter depuis

son retour dans l'équipe, notamment sur des sujets comme la nourriture biologique ; **Joe**, le p'tit dernier, saches que dans ce métier, tout est une question de dosage… je te souhaite sincèrement de réussir ton Master pour que tu vives l'aventure que je termine aujourd'hui ; **Olivier** enfin, bienvenue dans l'équipe et surtout bon courage avec les kératinocytes (un type cellulaire qui révèle bien des surprises). Et les plus anciens ; **Sébastien** d'abord qui m'a appris beaucoup de choses, parfois scientifiques ;-), et qui mérite bien la sentence F ; **Alessandro**, grâce à toi Corinne garde une photo de moi du concours de Marshmallows, **Elisa**, probablement l'italienne la plus nordiste que je connaisse qui grâce à moi connaît entre autre la différence entre une profession et un hobby (pas la peine non plus d'entrer dans les détails sur ce point) ; et **Fazia**, dont j'ai toujours apprécié le sourire et la bonne humeur quotidienne qu'elle apportait.

Merci également à toutes les personnes appartenant à l'UMR8161 et aux autres équipes que j'ai connu et avec qui j'ai parfois eu la chance de collaborer ; **Fred et Béné**, bon courage dans *votre cabane au Canada* ; **Catherine**, désolé mais tu vas devoir défendre le Pas-De-Calais sans moi désormais ; **Jonathan**, le premier Master (thésard maintenant) que j'ai « martyrisé », bonne chance pour la suite ; **Julie Bertout** qui m'a initié à la cytométrie et au FACS ; **Didier Deslée** qui m'a appris à faire fonctionner et à comprendre tous les microscopes présents sur le campus ; etc. Une liste exhaustive entraînerait forcément des oublis, alors merci à tous.

Merci ensuite à tous mes amis avec qui j'ai passé du temps durant ces quatre années, notamment autour des pintes du vendredi soir ; **Gaylor(d)**, le p'tit gars d'Angers, troisième membre du trio de choc de la promotion master recherche 2006 que nous formons au R3D2, que je compte parmi mes meilleurs amis et avec qui j'ai passé toutes les étapes de la thèse. **Geo, Arnaud, et Rémy**, les autres potes de la promo et accessoirement les recordmen de l'arrivée en retard aux soirées tarot ; **aux « pasteuriens et pasteuriennes » Ludovic, Florent, Anaïs et Sophie** avec qui j'ai passé d'excellentes soirées même dans le cas des affrontements IBL/Pasteur des tournois de volley.

Merci à **tous les membres du bureau de l'association *BioAddoct*** (Association des docteurs et doctorants en Biologie santé du Nord de France) pour cette magnifique aventure dans laquelle nous nous sommes lancés tous ensemble et que j'ai eu grand plaisir à présider. Merci à **Jean-Jacques Hauser** pour son oreille attentive et son aide cruciale dans la création de l'association ainsi que dans toutes les démarches en rapport avec l'EDBSL.

Enfin, merci à ma famille. **Mes parents** d'abord, sans qui rien de cela n'aurait été possible. Les sacrifices qu'ils ont faits par le passé pour envoyer à la Fac le p'tit gosnaisien du fin fond du Pas-De-Calais que j'étais payent aujourd'hui. C'est donc un peu votre thèse que je soutiens aujourd'hui (par contre, je deviens chercheur, trouveur ça n'existe pas encore comme métier) ; Mes frères **Cédric et Maxime**, qui dès le début de ma thèse savaient que les cellules de la peau mouraient ! ; **Pépé et Mémé** qui ont réussi à comprendre ce que je faisais et qui, à leur manière, mon soutenu tout au long de mes études ; et enfin à **ma belle famille** qui m'ont apporté leur soutien sans vraiment savoir ce que je faisais, notamment dans les multiples weekends que j'ai passé au labo.

Enfin, et non des moindres, un grand merci à **Emilie**, ma p'tite femme qui m'a apporté tout l'amour et le soutien dont j'avais besoin alors même qu'elle préparait sa thèse en parallèle. D'autant que me supporter dans toutes les phases de la thèse n'a pas été chose aisée. Je ne pense pas qu'il existe un mot qui puisse définir ce que tu m'apportes tous les jours. C'est pourquoi tu as une page à part dans mes remerciements comme tu l'as dans mon cœur.

Abstract

Senescence is a non proliferative state that occurs in response to telomere shortening, oxidative stress or oncogenic activation. Whereas senescence is generally considered as an irreversible growth arrest, we recently reported, using Normal Human Epidermal Keratinocytes (NHEKs), that few senescent cells can spontaneously reactivate a mitotic process to generate so-called post-senescence emergent cells which are transformed and able to form skin hyperplasia in *nude* mice.

In the first part of this work, we have investigated the outcome of the majority of senescent cells that do not generate emergent cells. We highlighted that senescent cells massively die during the growth arrest. Interestingly, the death is not associated with apoptotic or necrotic features, but involves the elimination of numerous vital cell components by macroautophagy.

We next investigated the mechanism that activates the autophagic programmed cell death in senescent keratinocytes. We show that oxidative stress occuring during senescence causes numerous cellular damages, notably to nucleus and mitochondria, which activate the macroautophagic process to ultimately lead to the death.

In the last part of this work, we have investigated the relationship between oxidative stress and macroautophagy during the generation of post-senescence emergent cells. We show that progenitors of these neoplastic cells display less reactive oxygen species (ROS) production than other senescent keratinocytes, and hence escape autophagic cell death. However, in order to generate post-senescence emergent cells, they have to maintain a housekeeping autophagic activity.

Taken together, these results indicate that the outcome of a senescent cell is driven by its ROS level. A high ROS level induces a high and lethal activation of autophagy. At a lower ROS level, the cell activates a moderate autophagy that fails to induce death but favors the elimination of oxidized proteins and organelles. By this way, this cell becomes permissive to neoplastic evolution consecutively to the putative oxidative alteration of oncogenes, tumor suppressor genes or other crucial cell regulators.

Résumé

La senescence est un état d'arrêt prolifératif mis en place par les cellules en réponse à différents stress (raccourcissement des télomères, stress oxydant, ou activation d'oncogènes). Bien que la sénescence soit considérée comme irréversible, nous avons récemment montré, en utilisant des kératinocytes humains normaux d'épiderme, que certaines cellules sénescentes réactivent spontanément le processus mitotique pour générer des cellules proliférantes, baptisées émergentes post-sénescence, qui sont transformées et tumorigènes en souris *nude*.

Nous avons montré dans la première partie de ce travail que les cellules sénescentes qui ne génèrent pas de cellules émergentes meurent. La mort engagée à la sénescence n'est ni apoptotique ni nécrotique, mais implique l'élimination par macroautophagie de nombreux composés cellulaires vitaux.

Nous avons ensuite démontré que le stress oxydant, *via* les dommages qu'il crée, notamment aux niveaux nucléaire et mitochondrial, est responsable de l'activation de la mort cellulaire programmée par macroautophagie. Les cellules sénescentes progénitrices des cellules néoplasiques génèrent quant à elles moins d'espèces réactives de l'oxygène (ROS) que le reste des cellules sénescentes, ce qui leur permet d'échapper à la mort. Cependant, pour générer les cellules émergentes, elles doivent maintenir une activité macroautophagique de ménage.

L'ensemble de ces travaux démontre donc que le devenir des kératinocytes sénescents dépend de leur niveau de ROS. Un haut niveau de ROS induirait une activité macroautophagique élevée et létale, alors qu'un niveau plus bas induirait une activité trop faible pour induire la mort, mais suffisante pour éliminer les composés cellulaires oxydés. Dans cette situation, les cellules deviendraient permissives à l'évolution néoplasique si les dommages oxydants touchent l'ADN et affectent des oncogènes, des suppresseurs de tumeurs, ou d'autres régulateurs fondamentaux.

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Abréviations

8-OH-G	8-hydroxyguanine
ADN	Acide désoxyribonucléique
ALT	Alternative lengthening of telomeres
Ambra1	Autophagy/beclin1 regulator 1
AMP	Adénosine monophosphate
AMPK	AMP kinase
Atg	Autophagy related gene
ATP	Adénosine triphosphate
Bcl2/6/XL	B cell CLL/lymphoma 2/6/XL
BH3	Bcl2 homology domain
Bif-1	BMP-induced factor 1
CAF	Cancer associated fibroblast
CDK	Cyclin-dependent kinase
CGH	Comparative genomic hybridization
CKI	Cyclin-dependent kinase inhibitor
CMA	Chaperon mediated autophagy
CuZnSOD	Copper zinc superoxide dismutase
DAPK	Death-associated protein kinase 1
DDR	DNA damage response
E2F3	E2F transcription factor 3
eGFP	Enhanced green fluorescent protein
FACS	Fluorescence associated cell sorter
FADD	Fas-associated via death domain
FIP200	200kDa focal adhesion kinase family interacting protein
FoxO3	Forkhead box O3
GABARAP	Gamma aminobutiryc acid type A receptor associated protein
GATE16	Golgi-Associated ATPase Enhancer of 16kDa
H2-DCFDA	2,7-dichloro-dihydro-fluorescein diacetate
HDR	Homology directed repair
HMEC	Human mammary epithelial cell
HNE	4-hydroxynonenal
HSC70	Heat shock cognate 70
hTERT	Human telomerase reverse transcriptase
IMK	Immortal keratinocyte
Junk1	C-Jun NH2-terminal kinase 1
KLF4	Kruppel-like factor 4
LAMP1/2	Lysosomal-associated membrane protein 1/2
MAPK	Mitogen-activated protein kinase
MAP-LC3	Microtubule-associated protein 1 light chain 3
MDA	Malondialdéhyde
MDA-MB-231	Lignée cellulaire dérivée d'un adénocarcinome de glande mammaire

MEF	Mouse embryonic fibroblast
MnSOD	Manganese superoxide dismutase
mRFP	Monomeric red fluorescent protein
mTOR	Mammalian target of rapamycin
mTORC	Mammalian tor complex
NADPH	Nicotinamide adenine dinucleotide phosphate
NCI	National cancer institute
NFkappaB	Nuclear factor kappa B
NHEJ	Nonhomologous end joining
NHEK	Normal human epidermal keratinocyte
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 1/wild-type p53-activated fragment 1
p62/SQSTM1	Sequestosome 1
PAS	Preautophagosomal structure
PE	Phosphatidyléthanolamine
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol 3-kinase
PI3P	Phosphatidylinositol-3-Phosphate
PML	Promyelocytic leukemia
Pont AIP	Ponts 1-amino-3-iminopropène
Pot1	Protection of telomere 1
PTEN	Phosphatase and tensin homolog
RAP1	Repressor activator protein 1
RAPTOR	Target of rapamycin associated protein
Rb	Retinoblastoma
RICTOR	Rapamycin-insensitive companion of Tor
ROS	Reactive oxygen species
Rubicon	Run domain protein as Beclin-1 interacting and cystein-rich containing
RUNX2	Runt related transcription factor 2
SA.beta-gal	Senescence associated beta galactosidase
SIPS	Stress-induced premature senescence
SnoN/SKIL	Strawberry notch
STAT5A	Signal transducer and activator of transcription 5A
TBX2	T-box 2
Tin2	TRF1-interacting nuclear factor2
TNFα	Tumor necrosis factor alfa
TOR	Target of rapamycin
TRAIL	TNF-related apoptosis inducing ligand
TRF1/2	Telomere Repeat-binding Factor-1/2
ULK1/2	Unc51 like kinase 1/2
UVRAG	UV radiation resistance associated gene
VDAC	Voltage-dependent anion channel
Vps34	Vacuolar protein sorting factor 34

Introduction

1.1 La sénescence

Au début des années 1960, Léonard Hayflick décrit les propriétés prolifératives de fibroblastes extraits d'embryons humains. Il met alors en évidence qu'à la différence des lignées cellulaires qui sont immortalisées *in vitro*, les cellules normales ont un potentiel proliferatif limité à un nombre fini de divisions [1,2]. Il décrit que le comportement de cellules normales en culture se divise en trois phases : la phase I qui initie la culture et correspond à l'activation de la prolifération des cellules après leur décongélation ; la phase II durant laquelle les cellules croissent de manière exponentielle et nécessitent de nombreux passages ; et la phase III (ou phase terminale) qui se caractérise par un arrêt de la prolifération des cellules (Figure 1). Cette dernière phase, appelée sénescence, n'est pas spécifique aux fibroblastes et sera par la suite décrite pour de nombreux types cellulaires normaux issus de différentes espèces animales comme par exemple les kératinocytes [3], les cellules musculaires lisses [4], les cellules endothéliales [5], les lymphocytes [6], etc.

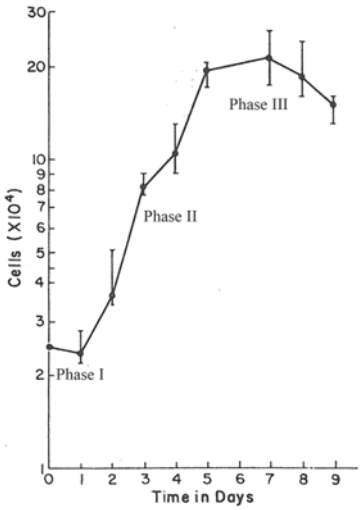

Figure 1 : Observation de la sénescence in vitro. *Courbe de croissance réalisée à partir d'une culture de WI-I (fibroblastes embryonnaires humains normaux de poumon) montrant les trois phases décrites par Hayflick et Moorhead. Adaptée de l'article original* [2].

Outre l'arrêt prolifératif, les cellules sénescentes présentent un certain nombre de modifications phénotypiques et moléculaires qui les distinguent des cellules en croissance exponentielle (Figure 2). Elles augmentent en taille, s'étalent sur leur support et diminuent leurs interactions cellule-cellule au profit d'une adhérence accrue à la matrice extracellulaire [7,8]. Leurs noyaux et nucléoles sont plus gros que ceux des cellules en phase exponentielle de croissance, la structure de leur chromatine change [9] et on met souvent en évidence une fraction non négligeable de cellules polynucléées dans la population sénescente [10,11]. C'est notamment le cas des cultures de kératinocytes au sein desquelles on dénombre environ 20% de cellules polynucléées durant l'arrêt de croissance (donnée du laboratoire). L'augmentation de la charge lysosomiale fait également partie des caractéristiques de la sénescence [12,13].

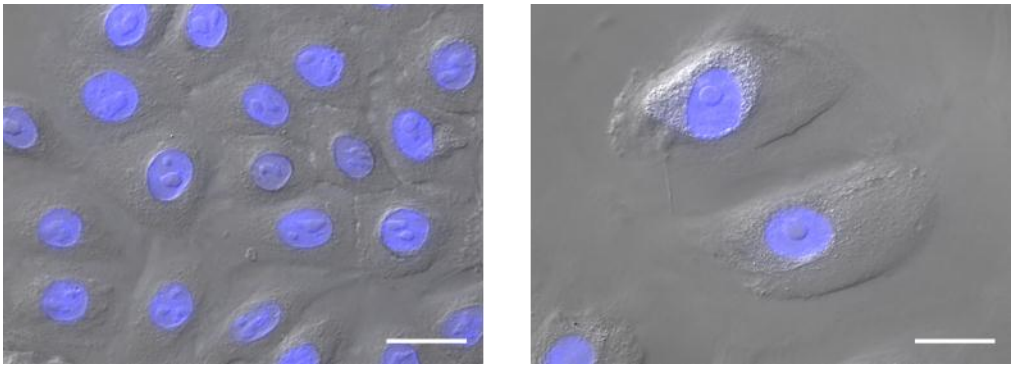

Figure 2 : Observation de la sénescence in vitro. *Image de gauche : kératinocytes humains normaux en phase II (croissance exponentielle) ; image de droite : kératinocytes en phase III (sénescence). Les noyaux ont été marqués au Hoechst 33342 (vital) et les cellules ont été observées en microscopie photonique. La barre représente 10µm.*

Au niveau moléculaire, les cellules sénescentes expriment un transcriptome [14] et un protéome [15] différents de ceux des cellules en croissance et ne répondent ni aux stimuli mitotiques, ni aux inducteurs d'apoptose [16-20]. De manière intéressante, elles accumulent un nombre important de dommages oxydants aux macromolécules qui conduiront notamment à la formation d'agrégats lipido-protéiques appelés lipofuscine qui s'accumulent dans les lysosomes (voir pour revue [21]).

En 1995, Dimri *et al* mettent au point une méthode de discrimination des cellules sénescentes basée sur l'augmentation de l'activité d'une enzyme lysosomiale (la beta-galactosidase) dont le pH optimal se situe à 4-4.5 et qui devient détectable à pH 6 lors de la sénescence [22]. Les travaux de Lee *et al* montreront par la suite que cette augmentation reflète l'augmentation de la charge lysosomiale associée à la sénescence [23]. Cette méthode, nommée test SA.beta-gal (pour *Senescence Associated beta-galactosidase*) est l'une des plus utilisées, car la plus universelle, pour détecter la sénescence dans les différents types cellulaires, *in vitro* comme *in vivo*.

Les voies moléculaires engagées dans l'induction de l'arrêt du cycle qui caractérise la sénescence mettent en jeu les protéines oncosuppressives p53 et Rb. Les expériences d'inactivation de l'un et/ou l'autre de ces gènes suppresseurs de tumeur conduisent à des conclusions complexes quant à leurs rôles respectifs durant la sénescence. Concernant p53, les données obtenues dans des fibroblastes embryonnaires murins (MEFs) montrent que son inactivation durant la phase de croissance exponentielle suffit à prévenir l'entrée en sénescence des cellules et à les immortaliser. L'inactivation de p53 durant la phase de sénescence promeut la réactivation de la prolifération et la sortie du plateau de sénescence, ce qui suggère que l'activité de p53 doit être maintenue dans les cellules sénescentes pour maintenir l'état non proliferatif [24]. La portée de ces conclusions est malgré tout à modérer puisque l'inactivation de p53 ne s'est révélée efficace pour repousser la survenue de la sénescence que dans les fibroblastes et non dans d'autres types cellulaires. Rb est quant à elle trouvée sous sa forme hypophosphorylée (active) durant la sénescence, ce qui lui permet d'interagir avec le facteur de transcription E2F pour en inhiber l'activité. Cela aboutit à la répression transcriptionnelle des gènes cibles de E2F, notamment ceux impliqués dans la progression du cycle [9]. La seule inhibition de Rb n'a cependant pas d'effet sur la survenue du plateau de sénescence. En effet, son expression peut être compensée par les autres membres de la famille des protéines à poche, p107 et p130 ; l'inhibition conjointe de ces protéines repousse la survenue de la sénescence [25,26]. De plus, comme pour p53, l'inhibition d'expression des protéines de la famille Rb durant la sénescence peut restaurer le phénotype proliferatif des cellules [27].

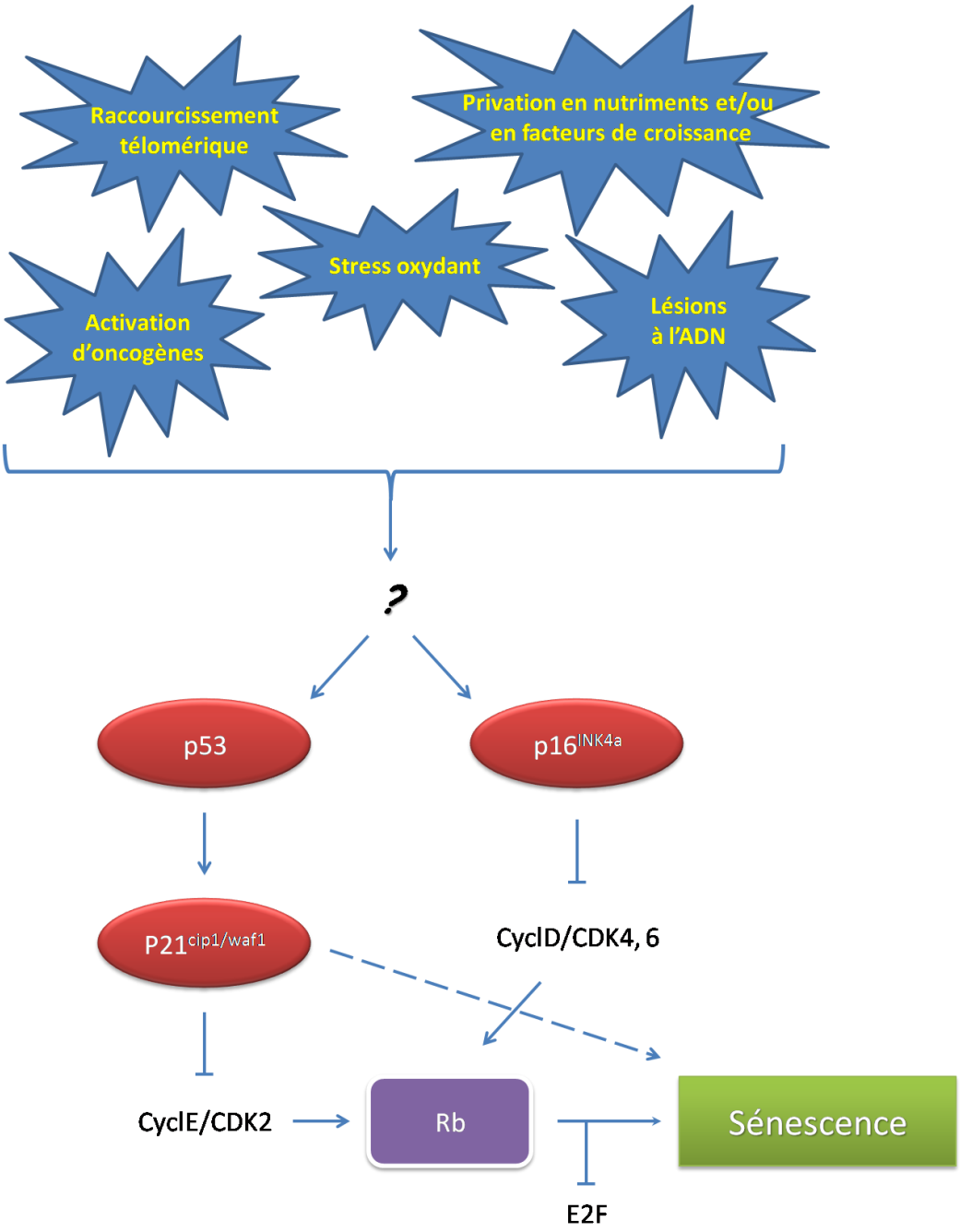

Figure 3 : Les voies d'activation de l'arrêt du cycle cellulaire associé la sénescence. *Adaptée de [28].*

L'état de phosphorylation de Rb pouvant être contrôlé par des complexes cycline/CDK (*Cyclin-Dependent Kinase*) comme le complexe cycline E/CDK2, la CKI $p21^{cip1/waf1}$ (*CDK Inhibitor*), gène cible de p53 [29], pourrait être un intermédiaire d'activation de la voie Rb par la voie p53. Et en effet, l'inhibition de $p21^{cip1/waf1}$ dans des fibroblastes humains s'est révélée efficace pour repousser la sénescence, démontrant qu'une activation de Rb par p53 *via* $p21^{cip1/waf1}$ est possible chez l'Homme [30,31]. Il s'avère néanmoins que l'inhibition de $p21^{cip1/waf1}$ n'a aucun impact sur l'entrée en sénescence de fibroblastes murins, suggérant donc que $p21^{cip1/waf1}$ n'est pas un intermédiaire crucial et qu'il existe des voies d'activation de Rb parallèles [32]. D'autres travaux effectués sur des fibroblastes humains montreront que l'inhibition indépendante de p53 ou Rb ne permet que de repousser la sénescence, alors que leur inactivation conjointe est nécessaire pour prévenir l'entrée en sénescence des cellules [33-35], suggérant ainsi la présence d'une voie parallèle d'activation de Rb indépendante de p53/$p21^{cip1/waf1}$ [36].

La protéine $p16^{INK4a}$ est également un inhibiteur majeur du cycle cellulaire qui agit sur la voie Rb *via* l'inhibition des complexes cycline D/CDK4, 6 [37]. Son expression est induite dans les cellules en réponse à divers stress et notamment lors de la sénescence[38-40]. L'inactivation de $p16^{INK4a}$ dans les cellules humaines prévient leur entrée en sénescence [41-44]. Cependant, l'inactivation de $p16^{INK4a}$ n'a pas d'impact sur la sénescence de cellules murines [45]. Il est également intéressant de constater qu'à la différence des fibroblastes, $p16^{INK4a}$ est particulièrement impliquée dans l'induction de la sénescence des cellules épithéliales humaines [42-44,46-50].

Deux voies moléculaires agissant en parallèle ou de facon interdépendante permettent donc aux cellules d'initier et de maintenir l'arrêt de prolifération caractéristique de la sénescence (Figure 3). Les divers travaux effectués à ce sujet montrent que l'engagement dans l'une ou l'autre des voies est déterminé par le contexte cellulaire et/ou le mécanisme inducteur de sénescence (voir pour revue [28,51]).

1.1.1 Les mécanismes inducteurs de sénescence

Depuis la description du processus de sénescence par Hayflick, de nombreux travaux ont tenté d'en déterminer le mécanisme inducteur. Il s'avère aujourd'hui que ce mécanisme n'est pas unique et qu'il faille considérer la sénescence comme un processus mis en place par les cellules en réponse à différents stress. Ces différents stress sont généralement regroupés en trois grandes catégories : ceux conduisant à un dysfonctionnement télomérique, le stress oxydant, et le stress consécutif à l'activation d'oncogènes.

1.1.1.1 Raccourcissement et dysfonctionnement télomérique

1.1.1.1.1 Structure des télomères

Les télomères (du grec *telos* « fin » et *meros* « partie ») représentent la partie terminale de l'extrémité des chromosomes. Ils sont composés de complexes nucléoprotéiques dont la principale fonction est de préserver les extrémités libres de l'ADN de l'action des exonucléases ainsi que des systèmes de signalisation et de réparation des cassures double-brin. Outre ces fonctions de protection de l'intégrité du génome, les télomères sont également impliqués dans la localisation des chromosomes dans le noyau, leur ségrégation durant l'anaphase et la réparation des cassures double-brin en permettant à la cellule de distinguer la fin d'une molécule d'ADN d'une cassure double-brin (voir pour revue [52] et [53]).

L'ADN télomérique est un ADN double-brin composé de séquences répétées riches en guanine. Chez l'Homme, il s'agit d'un hexanucléotide $(TTAGGG)_n$ répété sur une longueur allant de 5 à 15kb [54,55]. Causé par l'incapacité de l'ADN polymérase à répliquer le brin retardé dans sa totalité, la toute extrémité du télomère se termine par une portion simple-brin débordant en 3' [56]. Le bout débordant permet à l'ADN télomérique de se replier sur lui-même pour former une structure en boucle, appelée boucle T, stabilisée par appariement de séquence dans la boucle D et par association à des protéines télomériques. C'est cette structure qui masque les extrémités chromosomiques [57,58] (Figure 4 A, B).

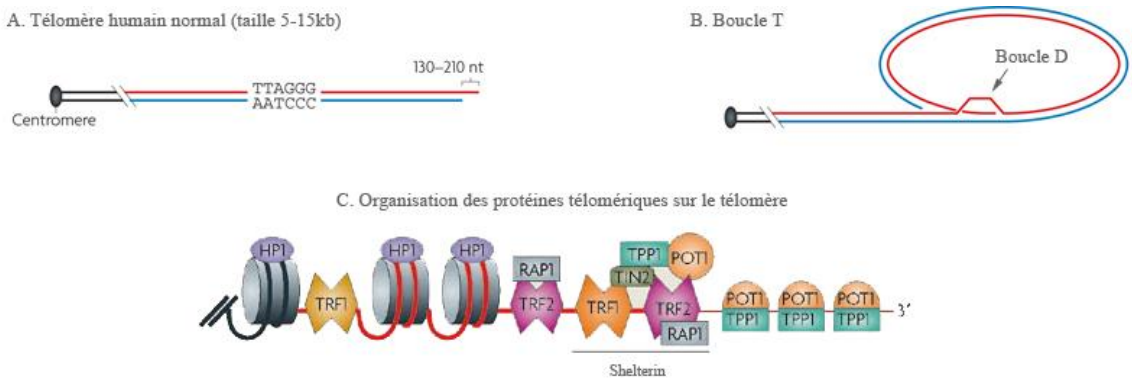

Figure 4 : Organisation des télomères humains. *Adaptée de [59,60]. A. Représentation linéaire d'un télomère mettant en évidence la séquence répétée (TTAGGG)n ainsi que l'extrémité débordante en 3'. B. Organisation du télomère en boucle T. C. Représentation du complexe protéique télomérique « shelterin » et organisation des protéines télomériques sur l'extrémité de l'ADN.*

Les télomères sont recouverts de complexes protéiques spécifiques. L'un de ces complexes, nommé « shelterin », se lie directement à l'ADN télomérique. Il se compose chez les mammifères de six protéines télomériques [61,62] : TRF1 (*Telomere Repeat-binding Factor-1*) [63,64], TRF2 [65,66], RAP1 (*Repressor/Activator protein 1*) [67], Tin2 (*TRF1-interacting nuclear factor2*) [68], TPP1 (aussi appelé TINT1/PTOP/PIP1) [69] et Pot1 (*Protection Of Telomere 1*) [70] qui stabilisent la boucle T et protègent les extrémités libres. Ces différentes protéines sont également importantes lors de la réplication et l'élongation des télomères (voir pour revue [71-73]). TRF1 et TRF2 forment des homodimères qui fixent l'ADN télomérique sur toute sa partie double-brin. RAP1 est associé spécifiquement à TRF2. TIN2 joue le rôle de pont protéique entre les homodimères de TRF1 et TRF2. Il est également associé à TPP1 ce qui permet de recruter Pot1 au complexe. Enfin, Pot1 est associé à TPP1 et fixe spécifiquement l'ADN télomérique simple-brin, notament au niveau de la boucle D [74] (Figure 4 C).

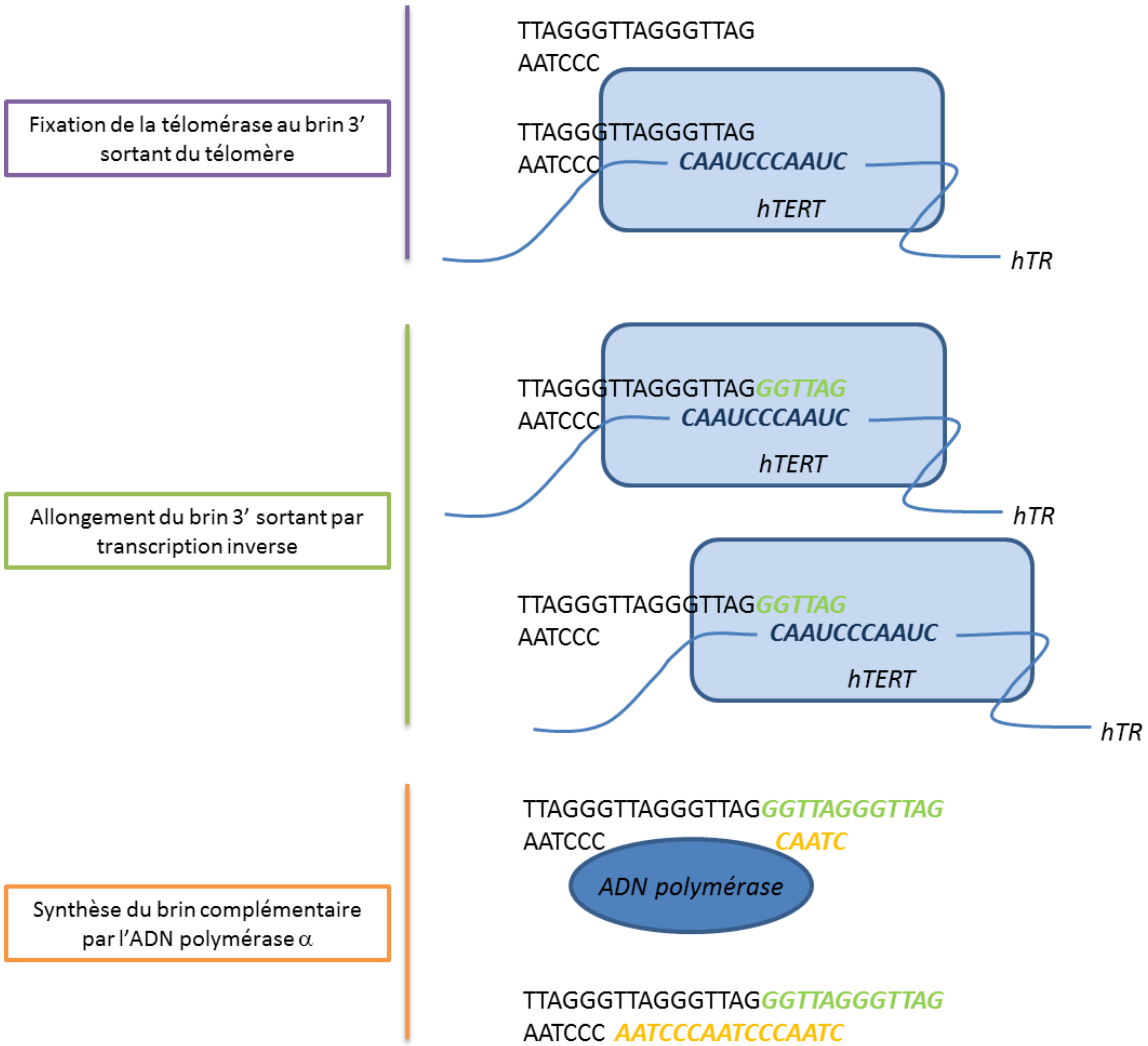

Figure 5 : Allongement des télomères par la télomérase. *Adaptée de* [75].

Mises à part celles-ci, d'autres protéines sont associées aux télomères mais ne font pas partie des shelterins, comme par exemple Rad50 ou PARP-2 [76]. Associées aux shelterins, elles ont des fonctions dans la maintenance et la stabilité des télomères mais ont également des fonctions indépendantes des télomères lors de la mitose, pour la détection du dommage à l'ADN et dans les mécanismes de réparation de l'ADN.

1.1.1.1.2 Maintenance de la longueur des télomères

Si le défaut de réplication par l'ADN polymérase alpha est inévitable, il peut néanmoins être compensé par l'activité transcriptase inverse d'une enzyme spécifique : la télomérase [77,78]. Cette enzyme est une ribonucléoprotéine composée d'une sous-unité protéique (appelée hTERT chez l'Homme) qui porte l'activité catalytique et d'une sous-unité ribonucléotidique longue de 451 nucléotides (hTR). Cette dernière sous-unité contient une séquence nucléotidique complémentaire à la séquence télomérique qui apporte la spécificité de l'enzyme pour les télomères. Une fois hybridée à l'extrèmité 3' sortante du télomère, cette séquence sert de matrice à la sous-unité catalytique pour rallonger le brin 3' [79,80]. L'ADN polymérase alpha génère ensuite le brin télomérique complémentaire (Figure 5).

Un mécanisme alternatif d'élongation des télomères appelé ALT (*Alternative Lengthening of Telomeres*) a plus récemment été décrit dans des cellules tumorales ou immortalisées en lignée qui n'expriment pas la télomérase. Basé sur la conservation de la séquence télomérique à l'extrémité de tous les chromosomes, ce mécanisme permet aux cellules d'entretenir la taille de leurs télomères par recombinaison homologue interchromosomique [59].

1.1.1.1.3 Rôle des télomères dans l'induction de la sénescence

Lors de la réplication, la synthèse du brin retardé est initiée par l'hybridation d'amorces ARN espacées de 200pb environ qui serviront à l'ADN polymérase pour générer le brin complémentaire bout par bout (également appelés fragments d'Okazaki) [81]. Par conséquent, si la distance entre le dernier fragment d'Okazaki généré et

l'extrémité de l'ADN est inférieure à 200pb, l'ADN polymérase sera, faute d'amorce, incapable de répliquer l'extrémité de l'ADN, entrainant un raccourcissement en 5' de 200pb ou moins à chaques cycle de réplication et générant un brin 3' sortant à l'extrèmité télomérique [82,83]. Arrivés à une taille critique, les télomères ne pourront plus former la boucle T. Les extrémités libres de l'ADN seront alors détectées comme des cassures double-brin qui initieront l'activation d'une voie de réponse au dommage à l'ADN (DDR - *DNA Damage Response*) et l'entrée des cellules en sénescence [84]. L'érosion des télomères à chaque cycle de division a donc conduit au développement du concept d'horloge mitotique qui détermine le nombre de divisions maximum qu'une cellule peut effectuer en l'absence d'une activation de la télomérase ou du système ALT [85,86]. De manière intéressante, la télomérase n'est exprimée chez l'Homme que dans les cellules germinales, les cellules embryonnaires, les cellules souches et les cellules cancéreuses et le système ALT n'a lui été décrit que dans les cellules cancéreuses et les cellules embryonnaires [87]. L'expression ectopique de la télomérase dans des cellules normales est suffisante pour prévenir de l'activation de la sénescence [88-90] et parfois pour les immortaliser *in vitro* [91-94] voire même pour acquérir un phénotype pré-malin [95]. Cependant, l'expression de hTERT ne suffit pas toujours à immortaliser les cellules, notamment dans le cas des cellules épithéliales, ce qui suggère que d'autres mécanismes inducteurs de sénescence sont mis en jeu dans ces cellules indépendamment de la taille des télomères [43,47,96].

Par ailleurs, les shelterins jouent également un rôle important dans le contrôle de l'entrée en sénescence des cellules. En effet, en l'absence des protéines télomériques, les extrémités de l'ADN seraient perçues par la cellule comme des cassures double-brin qui activeraient les voies de signalisation impliquant ATM et ATR et conduiraient à l'induction de la sénescence [97], ainsi qu'à l'activation des systèmes de réparation, soit par fusion non homologue des extrémités chromosomiques NHEJ (*Non Homologous End Joining*) ce qui causerait des fusions chromosomiques dites *end to end,* soit par des recombinaisons homologues HDR (*Homology Directed Repair*) (voir pour revue [74]). De plus, les protéines TRF2 et Pot1 sont également capables d'inhiber respectivement l'activité de ATM et ATR et d'en bloquer la signalisation [98].

1.1.1.2 Stress oxydant

Le stress oxydant est l'une des conséquences majeures de la vie aérobie des cellules et de l'utilisation du dioxygène (O_2) dans les diverses réactions d'oxydoréduction essentielles au fonctionnement cellulaire, notamment lors de la synthèse d'ATP par la chaîne respiratoire mitochondriale. En effet, la réduction du dioxygène en eau dans l'organite n'est pas parfaite et on estime à 4-5% la quantité d'O_2 qui sera partiellement réduite en espèces moléculaires dites réactives de l'oxygène (ou ROS - *Reactive Oxygen Species*). Si à faible concentration, les ROS sont nécessaires à la cellule et participent à certaines voies de signalisation, une trop forte accumulation constitue un stress constant dans la cellule, appelé stress oxydant, qui endommage les macromolécules et altère les fonctions cellulaires (voir pour revue [99-101]).

1.1.1.2.1 Les espèces réactives de l'oxygène

Il existe plusieurs de types de ROS dont l'anion superoxyde ($O_2°^-$), le radical hydroxyl (°OH), et le peroxyde d'hydrogène (H_2O_2). Ces espèces hautement réactives peuvent oxyder toutes les macromolécules (lipides, protéines et acides nucléiques) qui composent la cellule. La majorité de la production endogène de ROS est réalisée par la chaîne respiratoire mitochondriale. Cette chaîne est composée de quatre complexes multiprotéiques localisés au niveau de la membrane interne de la mitochondrie [102]. La fonction de cette chaîne est de transporter des électrons d'un complexe à l'autre tout en générant un gradient de protons entre l'espace inter-membranaire et la matrice qui sera utilisé par une ATP synthase pour générer de l'ATP (Figure 6). Les électrons transportés par les différents complexes protéiques sont utilisés pour réduire l'O_2 en H_2O au niveau du complexe IV (cytochrome C oxydase) selon la réaction suivante :

$$O_2 + 4H^+ + 4e^- \rightarrow 2\ H_2O \quad (1)$$

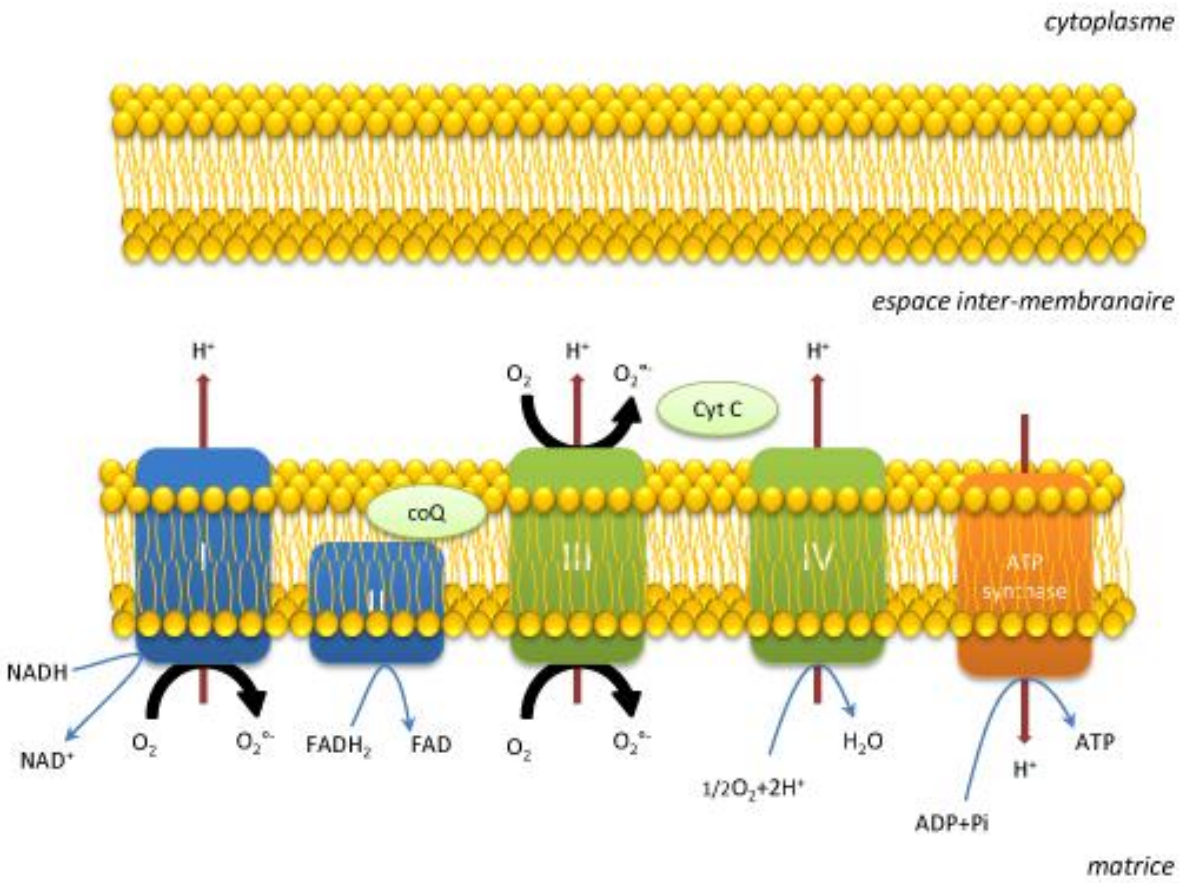

Figure 6 : Schéma simplifié de la chaîne respiratoire mitochondriale *mettant en évidence les quatre complexes protéiques composant la chaîne de transport des électrons et les zones de production d'anions superoxydes.*

En 2002 et 2003, les équipes de Andreyev et Lesnefsky mettent respectivement en évidence que la production d'anion superoxyde se fait au niveau des complexes I (NADH coenzyme Q reductase) et III (ubiquinol cytochrome C reductase) [103,104] (Figure 6). L'anion superoxyde étant chargé, il n'est pas capable de passer les membranes mitochondriales par diffusion simple mais peut toutefois emprunter les canaux VDACs (*Voltage Dependent Anion Channels)* de la membrane externe pour rejoindre le cytosol.

L'anion superoxyde, très réactif, peut être réduit en présence de H^+ pour produire du H_2O_2 (Figure 7 réactions 3 et 4). A la différence de l'anion superoxyde, le peroxyde d'hydrogène (H_2O_2) n'est pas chargé et a des propriétés physico-chimiques proches de celles de l'eau, ce qui le rend diffusible au travers des membranes cellulaires [105-107]. Le H_2O_2 peut ainsi réagir avec toutes les macromolécules partout dans la cellule. Le H_2O_2 peut de plus générer le radical hydroxyle (°OH) *via* la réaction de Fenton (Figure 7 réactions 7 et 8) qui nécessite des transferts d'électrons avec des métaux (Fe^{2+} et Cu^{2+}). Cette espèce oxydante est hautement réactive et d'autant plus dangereuse qu'il n'existe pas d'enzyme capable de la détoxifier [108].

Génération des espèces réactives de l'oxygène

$O_2 + e^- \rightarrow O_2^{\circ -}$ (anion superoxyde) (2)

$O_2^{\circ -} + H_2O \rightarrow HO_2^\circ$ (radical hydroperoxyl) (3)

$HO_2^\circ + e^- + H^+ \rightarrow H_2O_2$ (peroxyde d'hydrogène) (4)

$O_2^{\circ -} + e^- + 2H^+ \rightarrow H_2O_2$ (peroxyde d'hydrogène) (5)

$H_2O_2 + e^- \rightarrow OH^- + {}^\circ OH$ (radical hydroxyl) (6)

Réaction de Fenton

$H_2O_2 + Fe^{2+} \rightarrow OH^- + {}^\circ OH + Fe^{3+}$ (7)

$H_2O_2 + Cu^{2+} \rightarrow OH^- + {}^\circ OH + Cu^{3+}$ (8)

Figure 7 : Génération des espèces réactives de l'oxygène (adaptée de[109])

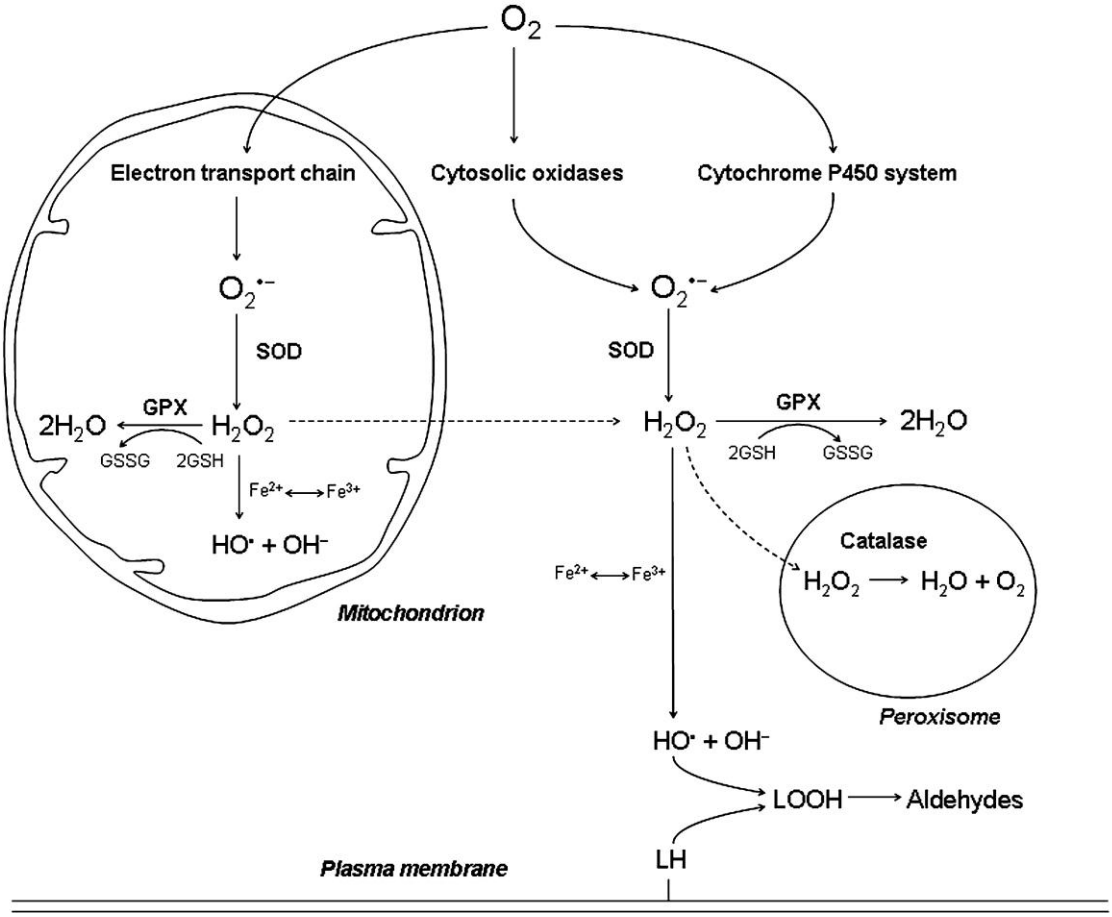

Figure 8 : Les différentes sources de ROS et systèmes antioxydants. *Adaptée de* [110].

Même si elles en sont la principale source, les mitochondries ne sont pas le seul site de production de ROS. En effet, toutes les oxydases et hydrolases cellulaires comme les acyl-CoA oxydase et xanthine oxydase des peroxysomes ou le cytochrome P450 du réticulum endoplasmique ainsi que de nombreuses réactions du métabolisme sont capables de produire du peroxyde d'hydrogène et de l'anion superoxyde (Figure 8)[110,111].

Les cellules inflammatoires (neutrophiles, éosinophiles et macrophages) peuvent également générer des ROS pour leurs propriétés cytotoxiques envers les bactéries et les parasites. Dans ce cas, c'est une NADPH oxydase qui, sous stimulation, réduit le dioxygène en $O_2^{\bullet -}$ qui sera à son tour pris en charge par une superoxyde dismutase (SOD) pour générer du H_2O_2 [112,113].

Les ROS sont également produits dans certaines voies de signalisation comme par exemple dans celle du TNFalfa où ils sont produits par une NADPH oxydase membranaire et agissent comme des messagers secondaires. Les voies moléculaires en aval de l'activation du TNFalfa sont complexes car peuvent promouvoir la mort comme la survie. La réponse cellulaire à l'activation du TNFalfa est en effet déterminée par un équilibre entre des signaux de survie (*via* l'activation de la signalisation NFkappaB) et des signaux apoptotiques. De manière intéressante, les ROS ont, dans cette signalisation, l'effet d'inhibiteurs des signaux prosurvie, favorisant l'induction de l'apoptose [114].

Outre les sources internes inhérentes au fonctionnement normal d'une cellule, les ROS peuvent être générées par des sources externes. C'est le cas des radiations ionisantes, des radiations UV, des agents xénotoxiques environnementaux ou de certains traitements thérapeutiques (notamment anticancéreux) qui augmentent la production de ROS directement ou indirectement en activant des sources endogènes [109].

1.1.1.2.2 Les défenses antioxydantes

Pour se prémunir des dommages oxydants faits par les ROS aux macromolécules, les cellules utilisent différents systèmes antioxydants. Certains sont non enzymatiques, comme par exemple l'acide ascorbique (Vitamine C), l'α-tocophérol (Vitamine E), le glutathion (GSH), les caroténoïdes, ou les flavonoïdes, qui agissent comme piègeurs de

ROS ou comme réductants d'autres espèces radicalaires [108]. Les cellules peuvent aussi utiliser des systèmes antioxydants enzymatiques (voir pour revue [101,115]).

La dismutation de l'anion superoxyde en peroxyde d'hydrogène est catalysée par une famille de métalloprotéines appelées superoxydes dimutases (SODs). Il existe quatre types de SODs (CuZnSOD, MnSOD, FeSOD et NiSOD) qui se différencient notamment par le métal co-facteur qu'elles utilisent dans leur site actif [116]. Seules deux SODs sont présentes chez l'Homme, la CuZnSOD (ou SOD1 - localisée dans le cytoplasme, le noyau et les peroxysomes) et la MnSOD (ou SOD2 - localisée dans la mitochondrie). Ces dismutases sont respectivement organisées en homodimère et homotétramère pour réduire l'anion superoxyde selon la réaction 9 [101] :

$$2O_2^{\circ-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (9)$$

Le peroxyde d'hydrogène formé peut ensuite être pris en charge par diverses enzymes antioxydantes qui le réduiront en eau. La catalase est une dismutase organisée en homotétramère d'une masse moléculaire d'environ 240kDa. Elle est principalement localisée dans les peroxysomes et les mitochondries où elle réduit le H_2O_2 (réaction 10).

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (10)$$

Les glutathion peroxydases (GPx) sont une famille de réductases qui utilisent des donneurs d'électrons pour réduire le H_2O_2 en $2H_2O$. Les GPx sont des homotétramères d'environ 80kDa qui contiennent 4 sélénocystéines. Elles utilisent deux molécules de glutathion (GSH) comme accepteur d'électron qu'elles oxydent en GSSG pour réduire le peroxyde d'hydrogène en eau. Cette réaction est couplée au cycle du glutathion dans lequel le GSSG est régénéré en GSH par une glutathion reductase qui utilise le NADPH comme réductant (réactions 11 à 13). De manière intéressante, les GPx peuvent également réduire des composés oxydés protéiques et lipidiques (ROOH et LOOH) en eau et en alcool.

$$2GSH + H2O2 \rightarrow GSSG + H2O \quad (11)$$

$$2GSH + ROOH \rightarrow GSSG + H_2O + ROH \quad (12)$$

$$GSSG + 2NADPH \rightarrow 2GSH + 2NADP^+ \quad (13)$$

De la même manière que les GPx, les peroxyredoxines forment une famille d'enzymes antioxydantes qui réduisent le H_2O_2 en H_2O en utilisant un cofacteur, la thioredoxine ($TrxS_2$) (réaction 14).

$$TrxS_2 + H_2O_2 \rightarrow Trx(SH)_2 + H_2O \quad (14)$$

1.1.1.2.3 Les dommages oxydants

Les espèces réactives de l'oxygène sont capables de réagir avec toutes les macromolécules (acides nucléiques, lipides, et protéines). D'une manière générale, l'oxydation de ces molécules altérera non seulement leur fonction dans la cellule mais pourra également en faire des intermédiaires réactifs qui amplifieront et propageront le stress oxydant. De plus, l'altération de ces composants primaires de l'architecture cellulaire endommagera le fonctionnement des cellules et de leurs organites, nécessitant la mise en place de systèmes de réparation spécifiques, voire l'activation de la mort cellulaire.

1.1.1.2.3.1 L'oxydation des protéines

D'une manière générale, tous les acides aminés peuvent être oxydés par les ROS. Cependant, la présence d'un atome de soufre dans les cystéines et méthionines en font les cibles les plus sensibles. En effet, l'oxydation des cystéines conduit à la formation de dérivés soufrés qui peuvent réagir entre eux et former des ponts disulfures intrachaînes, modifiant ainsi considérablement la conformation protéique, ou interchaînes, conduisant à la formation d'agrégats protéiques. L'oxydation des méthionines entraîne quant à elle des changements dans les propriétés physico-chimiques de la protéine, notamment en réduisant son hydrophobie et sa flexibilité [117]. Il n'existe que très peu de système de détoxification des protéines. Il s'agit des méthionine-sulfoxyde-réductases qui peuvent réduire les méthionines oxydées [118,119], ainsi que les thiorédoxines et glutarédoxines qui ciblent les ponts disulfures [120]. Néanmoins, plutôt que de réparer, la cellule éliminera généralement les protéines oxydées *via* le protéasome ou l'activation de l'autophagie (voir pour revue [119]) avant de les néo-synthétiser.

Initiation

LH + R° → RH + L°

Propagation

L° + O2 → LOO°

LOO° + LH → LOOH + L°

Figure 9 : Etapes d'initiation et de propagation de la peroxydation lipidique. Adaptée de [121]. LH, lipide ; L°, radical lipidique ; LOO°, radical peroxyl lipidique ; LOOH, hydroperoxyde lipidique ; R°, radical oxydant (par exemple °OH) ; RH, radical réduit.

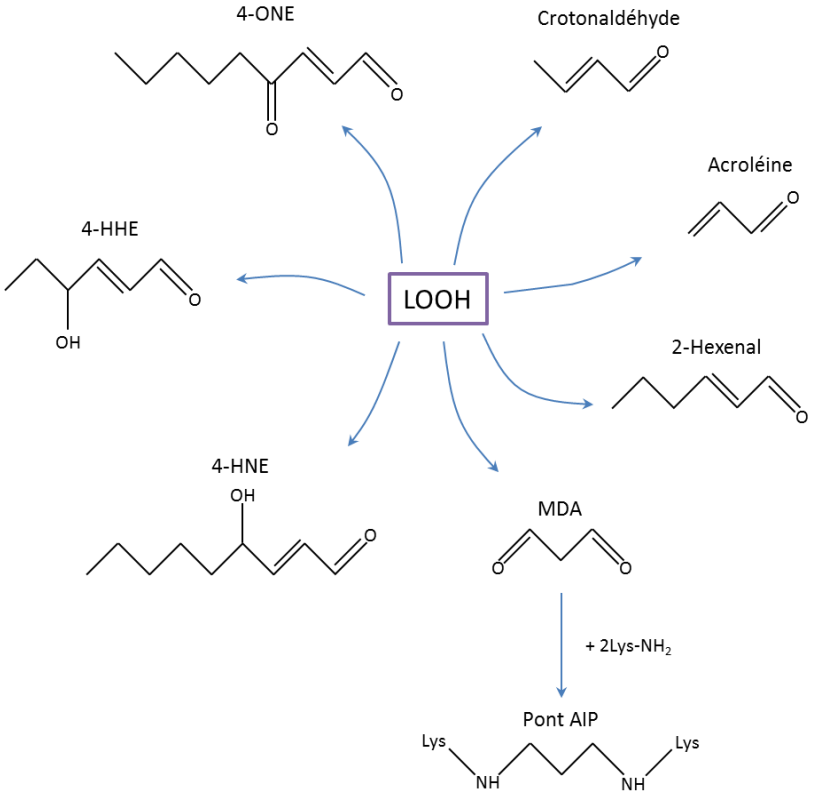

Figure 10 : Produits secondaires de la peroxydation lipidique issus de la réactivité de l'hydroperoxyde lipidique. *Adaptée de [122,123]. LOOH, hydroperoxyde lipidique ; MDA, malondialdéhyde ; HNE, 4-hydroxynonenal ; Lys-NH_2, lysine ; Pont AIP, pont 1-amino-3-iminopropène*

1.1.1.2.3.2 L'oxydation des lipides

La peroxydation lipidique est un processus complexe qui met en jeu les radicaux libres ($O_2°^-$ et °OH) et les acides gras polyinsaturés et qui se déroule en deux étapes. Une première étape (dite d'initiation) conduit à l'oxydation d'un lipide (LH) en radical lipidique (L°). Dans la seconde étape (dite de propagation), l'oxygène réagit avec L° pour former un radical peroxyl lipidique (LOO°). Ce dernier produit d'oxydation pourra réagir avec d'autres macromolécules et les oxyder. Il pourra notamment réagir avec un lipide voisin pour former un hydroperoxyde lipidique (LOOH) et un radical lipidique (L°). Commence alors une réaction en chaine qui ne nécessite plus d'apport en ROS et qui propage les dommages oxydants au sein des membranes [121] (Figure 9). La propagation de la peroxydation lipidique peut toutefois être enrayée par l'α-tocophérol (vitamine E) qui réduit les radicaux lipidiques et stoppe la réaction en chaîne [124,125].

L'hydroperoxyde lipidique formé au terme de ces réactions a des conséquences délétères pour la cellule. En effet, sa présence dans les bicouches lipidiques va perturber les propriètés de fluidité et de perméabilité des membranes. De plus, l'hydroperoxyde lipidique est une molécule instable qui conduit à la formation d'aldhéhydes, les principaux étant le malondialdéhyde (MDA) et le 4-hydroxynonenal (HNE) (Figure 10) [126]. Ces produits secondaires de la peroxydation lipidique sont également des éléments hautement réactifs envers les autres constituants cellulaires [122]. Le MDA est décrit pour former des adduits à l'ADN sur les bases adénines, guanines et cytosines pouvant être mutagènes [127]. Le MDA peut également réagir avec les acides aminés des protéines. Leur interaction au niveau des lysines et arginines entraîne la formation de ponts inter-protéines comme par exemple les ponts 1-amino-3-iminopropènes (AIP). En interagissant de la sorte avec les protéines, les produits de peroxydation lipidique peuvent entraîner la formation d'agrégats lipido-protéiques regroupés sous le nom de lipofuscine [128-130]. La lipofuscine est difficilement dégradable par les cellules qui l'accumulent dans leurs lysosomes ce qui en perturbe le fonctionnement [129].

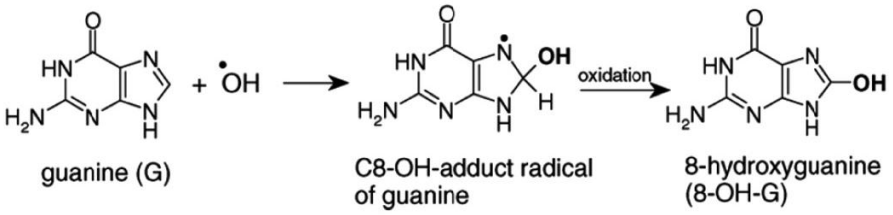

Figure 11 : Formation de la 8-hydroxyguanine. Adaptée de [109].

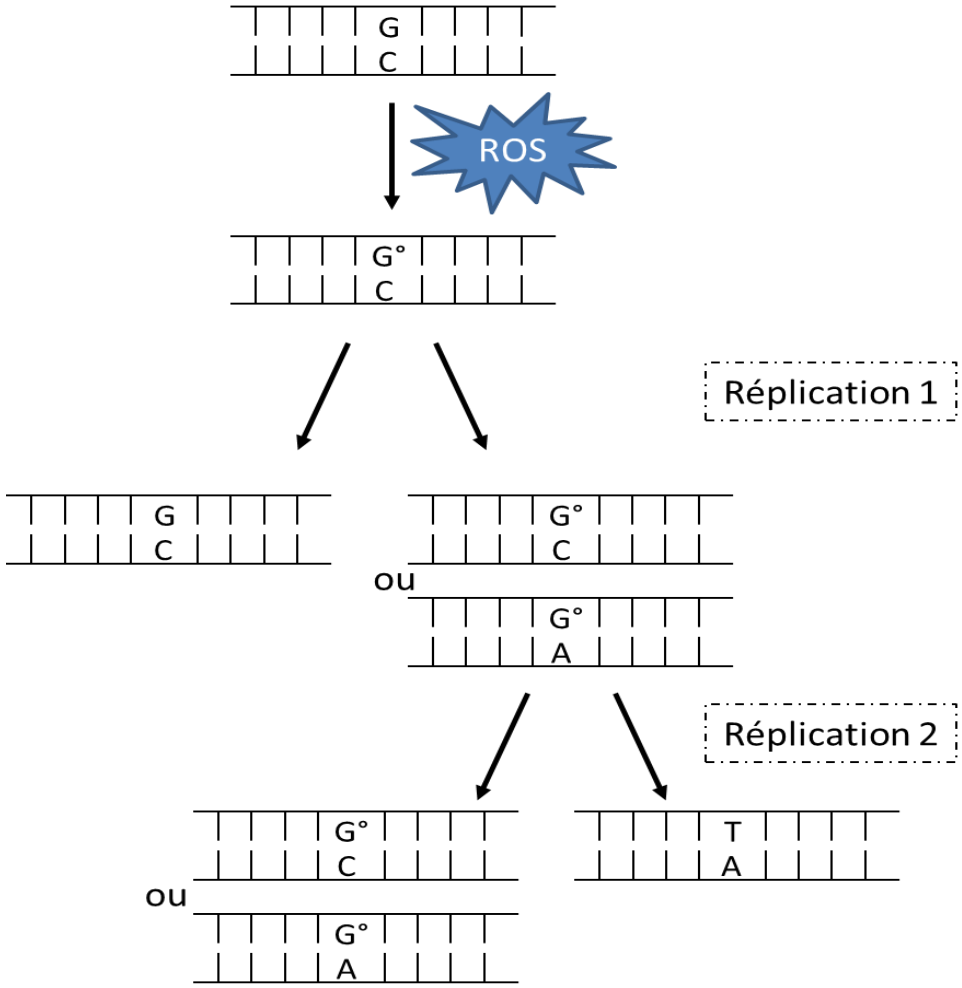

Figure 12 : Mécanisme de transversion mis en place après l'oxydation d'une guanine. *Adaptée de [131]. L'oxydation d'une guanine en 8-OH-G (G°) peut conduire lors de la réplication 1 à un mésappariement avec une adénine (A) qui sera responsable d'une transversion G : C vers T : A au cours de la réplication suivante.*

1.1.1.2.3.3 L'oxydation de l'ADN

Les dommages oxydants à l'ADN sont l'une des sources majeures de mutation chez les organismes vivants. On estime à environ 10 000 le nombre de dommages oxydants à l'ADN par cellule et par jour [132]. Plus d'une centaine de modifications oxydatives de l'ADN différentes ont été décrites touchant tant les bases que le squelette sucre-phosphate (voir pour revue [131]). Une fois encore, le radical hydroxyle est la cause principale de l'oxydation de l'ADN. Cependant, les propriétés de diffusion du H_2O_2 et sa capacité à générer du °OH *via* la réaction de Fenton en font un acteur majeur dans l'oxydation de l'ADN. En formant des adduits sur les bases, le MDA lui aussi est impliqué dans les lésions oxydatives de l'ADN [133]. Les effets de l'oxydation de l'ADN sont multiples : cassures simple-brin qui non réparées génèrent des cassures double-brin ou modifications de bases pouvant induire des erreurs de réplication (mutations ponctuelles), voire déstabiliser le chromosome altérant le fonctionnement cellulaire jusqu'à induire la mort ou la transformation des cellules [134,135].

Le dommage à l'ADN le plus abondant cible la base G et génère de la 8-hydroxyguanine (8-OH-G) (Figure 11). La propriété la plus délétère de cette base vient du fait qu'elle peut s'apparier avec les cytosines comme les adénines [136]. Par conséquent, l'appariement d'une 8-OH-G avec une adénine pourra causer une transversion G : C vers T : A au cycle de réplication suivant [137] (Figure 12). Les désoxyguanosines libres peuvent également être oxydées avant d'être incorporées et mésappariées dans l'ADN.

1.1.1.2.4 Rôle du stress oxydant dans l'induction de la sénescence

Le stress oxydant peut être considéré comme une cause de la sénescence indépendante et parallèle au raccourcissement des télomères. En effet, résultant de l'utilisation du O_2 dans leur fonctionnement normal, les ROS sont générées tout au long de la vie des cellules. Il en résulte alors un stress oxydant constant et chronique.

Le rôle des ROS dans l'induction de la sénescence a particulièrement bien été mis en évidence dans les fibroblastes embryonnaires murins (MEFs) qui présentent une

expression endogène de la télomérase [138]. Le raccourcissement des télomères n'a donc pas d'impact sur la survenue de la sénescence dans ces cellules qui ont un potentiel prolifératif illimité qui les immortalise *in vitro*. Les MEFs passent néanmoins par un court plateau de sénescence peu de temps après leur mise en culture avant de s'immortaliser [139]. Les travaux de Parrinello en 2003 ont mis en évidence que réduire le stress oxydant que subissent ces cellules en réduisant la concentration en O_2 de 20% à 3% inhibe la survenue de la sénescence [140]. De la même manière, de nombreuses études, dont celles de mon laboratoire d'accueil, ont mis en évidence que la culture des cellules en présence d'antioxydants repousse la survenue de la sénescence [141,142] et qu'à l'inverse, provoquer le stress oxydant en augmentant la pression en O_2 [143] ou par traitement au H_2O_2 [142,144] induit une sénescence prématurée phénotypiquement indifférenciable de la sénescence normale [145,146].

De façon étonante, il n'existe pourtant à ce jour que peu de données concernant le mécanisme moléculaire par lequel le stress oxydant induit la sénescence. On estime généralement de manière assez logique que les dommages cellulaires causés par les ROS sont les inducteurs de l'arrêt dans le cycle et de la sénescence ; les dommages à l'ADN entrainant l'activation des voies de DDR [84,97] et l'oxydation des lipides et des protéines entrainant la formation d'agrégats (lipofuscine) perturbant le fonctionnement cellulaire [128-130]. Les travaux de Frippiat *et al* en 2002 ont dégagé un rôle important de la protéine kinase P38MAPK [147]. Activée par le stress oxydant [148], P38MAPK active par phosphorylation le facteur de transcription ATF2 dont l'expression des gènes cibles (notamment du TGFβ1 et de son récepteur) est nécessaire à l'établissement de la sénescence induite par les ROS [147]. De manière intéressante, l'activation des voies de signalisation du TGFβ1 conduit en retour à l'activation de la P38MAPK ainsi qu'à la production de ROS par une NADH oxydase membranaire [149]. Cette boucle d'autoactivation contribue au maintien de l'état sénescent.

Les études portant sur les cellules en sénescence réplicative ont montré que le raccourcissement des télomères s'accompagne de la production de ROS et de dommages oxydants à l'ADN, aux lipides et aux protéines (8-OH-G, MDA, HNE, lipofuscine…) [150,151]. Ce stress oxydant a été décrit comme pouvant accélérer le

raccourcissement des télomères, suggérant que les deux causes puissent agir conjointement dans l'induction de la sénescence [143]. Comme décrit précédemment, les guanines, qui sont les bases les plus sensibles aux ROS, sont également majoritaires dans la séquence télomérique. De fait, les télomères peuvent donc être considérés comme une cible privilégiée des ROS. Il s'avère que les protéines télomériques TRF1 et TRF2 ont une faible tolérance pour les modifications de leur séquence consensus de fixation à l'ADN [152-154]. Par conséquent, les transversions G : C vers T : A causées par les ROS peuvent induire un défaut de fixation des protéines télomériques, entraîner la déstabilisation des télomères et accélérer la survenue de la sénescence [155]. De plus, des travaux réalisés sur les fibroblastes humains ont mis en évidence qu'un stress oxydant chronique entraîne une augmentation des cassures simple-brin au niveau des télomères entrainant leur raccourcissement précoce [156,157].

1.1.1.3 Sénescence induite par les oncogènes

Cause la plus récemment décrite, la sénescence peut également être induite en réponse à l'activation d'oncogènes. Cette sénescence est alors appelée OIS pour *oncogene-induced senescence*.

Les oncogènes appartiennent à une catégorie de gènes fréquemment mutés dans les cancers et dont l'effet sur la protéine produite apporte un gain de fonction (augmentation d'activité, surexpression…). *In vitro*, un gène est considéré comme un oncogène quand l'expression ectopique de sa forme modifiée accroit les propriètés de transformation et de tumorigénicité de cellules immortalisées et/ou prétransformées entretenues en lignée. Il est intéressant de noter que dans le cas des cellules normales, l'expression d'un oncogène seul ne suffit pas à les transformer. Plusieurs équipes ont en effet mis en évidence qu'une cellule normale doit subir plusieurs modifications génétiques affectant des oncogènes et gènes suppresseurs de tumeur, pour acquérir les propriètés d'une cellule pleinement cancéreuse et métastatique qui échappera notamment à l'induction de la mort cellulaire [158-160] et à la sénescence [161].

C'est initialement *via* la surexpression de l'oncogène *ras* [162] ou de l'un des effecteurs en aval de sa voie d'activation (RAF, MAP kinase, C/EBPbeta1 et PML [163-165]) que l'OIS a été découverte et est principalement étudiée. La mise en évidence d'un tel mécanisme inducteur de sénescence est d'autant plus intéressante qu'il semble avoir lieu *in vivo* dans certains contextes précancéreux comme c'est le cas des *nevi* (plus communément appelés grains de beauté). Ces tumeurs bénignes issues de mélanocytes cutanés ont la caractéristique de souvent présenter une mutation activatrice de l'oncogène BRAF (V600E). Malgré cela, ces tumeurs ne se développeront que très rarement et plusieurs années plus tard sous la forme de mélanomes extrêmement agressifs. Les travaux de Michaloglou ont notamment montré que durant la phase *nevi*, les cellules qui composent l'hyperplasie sont positives pour le marqueur SA.beta-gal [166]. Ces travaux, bien que discutables (car l'activité SA.beta-gal ne reflète que l'accroissement de la masse lysosomiale [167-169]), suggèrent que les mélanocytes normaux entrent en sénescence après l'activation de BRAF. Depuis ces travaux, quelques autres oncogènes ont été décrits activant l'OIS. C'est le cas de E2F3 [170], RUNX2 [171], SnoN [172], STAT5A [173] ou encore NFkappaB mis en évidence au laboratoire [142].

Plusieurs équipes ont cherché à comprendre comment une cellule normale peut détecter le dérèglement cellulaire causé par l'expression d'un oncogène. Il apparaît dans la plupart de ces études que les ROS sont une fois de plus au centre du processus. L'activation de Ras par exemple conduit à l'augmentation de la production de ROS par la cellule [174,175]. Les travaux précédant mon arrivée au laboratoire montrent également que la surexpression de l'oncogène *c-rel* (un facteur de transcription de la famille NFkappaB) induit une surexpression de la MnSOD. L'augmentation de l'expression de cette superoxyde dismutase spécifique des mitochondries conduit à l'augmentation de la production de H_2O_2 et à l'entrée en sénescence des cellules [142,176,177]. Un second mécanisme d'activation de l'OIS a été décrit pour *ras* suggérant que l'accroissement de la prolifération induite initialement par l'expression de cet oncogène s'accompagne d'une augmentation du nombre de fourches de réplication ce qui causerait un stress réplicatif suffisant pour activer la sénescence [178].

La notion d'OIS n'est cependant pas généralisable à tous les oncogènes. En effet, plusieurs études mettent en évidence que l'activation de certains oncogènes n'induit pas la sénescence mais pourrait au contraire en inhiber l'activation. C'est notamment ce qui a été décrit pour Jun [179], Myc [180], TBX2 [181], Bcl6 [182] et KLF4 [183] dont l'activation stimule la prolifération et repousse la sénescence alors que leur inhibition induit une sénescence prématurée. De manière intéressante, il apparaît que l'inactivation de Jun s'accompagne également d'une surproduction de ROS contrôlant l'entrée des cellules en sénescence [184].

La sénescence induite par l'activation d'oncogènes est donc un processus complexe qui dépend de la nature de l'oncogène et probablement en partie de sa susceptibilité à induire ou non la production de ROS.

1.1.2 Sénescence réplicative *versus* sénescence induite par des stress

Le raccourcissement des télomères à chaque cycle de réplication a conduit au développement du concept de sénescence réplicative. Dans ce contexte, en l'absence d'expression de la télomérase ou de l'activation du système ALT, les cellules atteignent la sénescence après un certain nombre de divisions (la limite de Hayflick) que l'on peut donc considérer comme représentant l'espérance de vie de la cellule. Dans le cas des sénescences induites par le stress oxydant ou par l'expression d'oncogènes, les données expérimentales suggèrent que la sénescence constituerait une réponse adaptative qui permet à une cellule de survivre plutôt que d'activer un processus de mort cellulaire programmée. Ainsi, malgré la similarité phénotypique, il est important de discriminer sénescence réplicative de sénescence induite par des stress (ou SIPS *Stress-Induced Premature Senescence* [185]). Les travaux de Dierick *et al* en 2002 ont d'ailleurs conforté cette idée en mettant en évidence des différences dans les profils protéomiques de fibroblastes en sénescence réplicative et en SIPS [186].

Les mécanismes moléculaires mis en jeu dans chacun des deux types de sénescence pourraient avoir des impacts très différents sur le devenir des cellules sénescentes, notamment dans certaines pathologies comme les cancers où les cellules

doivent surmonter des mécanismes qui limitent leur prolifération. Dans le cas de la sénescence réplicative, la taille critique des télomères conduit à l'activation des voies de réponse au dommage à l'ADN qui bloquent la progression des cellules dans le cycle cellulaire. Une cellule en sénescence réplicative doit donc acquérir un grand nombre de modifications lui permettant de stabiliser la longueur de ses télomères ainsi que d'outrepasser l'induction des voies DDR, afin de lui permettre de s'immortaliser et de se transformer suffisamment pour former une tumeur. Ces cellules doivent en effet activer un système d'élongation des télomères, en induisant par exemple la réexpression de la télomérase ou l'activation du système ALT, ainsi qu'inactiver les voies DDR, par inactivation des acteurs moléculaires engagés (comme par exemple BRCA1 [187,188]) ou mettre en place des systèmes de réparation inadéquats par fusion chromosomique *end to end*, générateurs d'instabilité génétique. Dans le cas de la sénescence induite par les stress, les télomères sont encore assez longs ce qui laisse théoriquement aux cellules sénescentes un certain potentiel prolifératif. Cumulé au fait que les stress inducteurs de sénescence sont de puissants agents carcinogènes (ROS et oncogènes), une cellule qui s'échapperait du plateau de sénescence pourrait donc devenir promotrice de tumeur.

1.1.3 Sénescence et vieillissement

Le vieillissement est un processus complexe généralement défini comme résultant de l'accumulation de changements délétères aux niveaux tissulaire et cellulaire, qui accroit la susceptibilité d'un organisme à développer des pathologies pouvant conduire à la mort [189]. A la suite de ses travaux sur le comportement prolifératif *in vitro* des fibroblastes embryonnaires humains, Hayflick écrit que le vieillissement pourrait être la conséquence de l'accumulation de cellules en sénescence dans les tissus [1].

Ce lien causal potentiel entre sénescence et vieillissement est notamment appuyé par la mise en évidence d'une augmentation du nombre de cellules présentant une activité SA.betagalactosidase dans des tissus provenant de patients âgés [22,190-195]. De plus, quelques équipes ont mis en évidence qu'une réduction sensible de la taille des télomères pouvait être corrélée au vieillissement d'un individu [196-200]. Cependant, il n'y a

pas de différences claires de capacités prolifératives *in vitro* entre les cellules issues de patients jeunes ou âgés [201,202]. Pourtant, l'inactivation de l'expression de la télomérase chez la souris entraîne un vieillissement prématuré des animaux ainsi que des pathologies associées. [203-206]. Par conséquent, même s'il n'en est pas la cause majeure, le raccourcissement des télomères peut induire le vieillissement d'un organisme, ce qui confirme que le vieillissement et la sénescence sont bien deux processus étroitement liés.

De nombreuses études ont également mis en évidence que le vieillissement des tissus s'accompagne de l'augmentation de la production de ROS par les cellules [207-209]. Source majeure de leur production, la mitochondrie en est également la première cible et l'accumulation de mutations dans l'ADN mitochondrial avec l'âge a aussi été rapportée. Ces altérations du génome mitochondrial endommagent alors le fonctionnement de l'organite et amplifient la production de ROS, initiant en conséquence un cercle vicieux qui accentue le stress oxydant avec le vieillissement [210-213]. De nombreuses expérimentations *in vivo* décrivent aussi que réduire le stress oxydant par l'expression d'antioxydants tels que les SODs et la catalase repousse le vieillissement des animaux. Inversement, l'induction d'un stress oxydant (par traitement au H_2O_2 ou par mutagenèses dirigées dans la chaîne respiratoire) avance la survenue du processus [214-217]. Il est également intéressant de noter que les animaux dits à durée de vie allongée, induite par une restriction calorique ou par des invalidations de gènes n'ayant aucun impact sur le stress oxydant (affectant par exemple les voies de l'IGF), se sont souvent révélés résistants au stress oxydant [218-222]. L'augmentation du stress oxydant avec l'âge s'accompagne de l'accumulation de dommages aux macromolécules cellulaires. L'une des manifestations bien connues de ces altérations cellulaires concerne l'apparition des « plaques de vieillesse », taches brunes tissulaires qui résultent de l'accumulation de lipofuscine dans les cellules [130]. Au niveau nucléaire, de nombreuses études ont mis en évidence que le vieillissement s'accompagne de l'accumulation de bases modifiées (adduits, oxydations) [223-225] probablement causée par l'augmentation de la production de ROS parallèle à une diminution d'efficacité des systèmes de réparation de l'ADN [226-231].

Il apparaît ainsi nettement que sénescence et vieillissement sont deux processus dont les causes sont communes (dysfonction télomérique, ROS, dommages à l'ADN). De manière intéressante, ces données expérimentales sont confirmées par l'étude des syndrômes progéroïdes. Ces pathologies, qui se manifestent par l'apparition d'un vieillissement prématuré des individus, résultent généralement de mutations monogéniques affectant un gène impliqué dans la réplication et/ou la réparation de l'ADN (voir pour revue [232,233]). Il a notamment été montré pour le syndrôme de Werner (résultant de la mutation du gène WRN codant une ADN hélicase impliquée dans la réparation et la réplication de l'ADN) que des fibroblastes de patients remis en culture entrent en sénescence prématurément [53]. Le syndrome de Down (ou trisomie 21) entraîne lui aussi un vieillissement prématuré. Il s'accompagne d'une augmentation d'expression de la SOD cytoplasmique (CuZnSOD) dont le gène est localisé sur le chromosome surnuméraire, ce qui abouti à la surproduction de ROS et notamment du H_2O_2 [234,235]. Néanmoins, toutes les études portant sur ce sujet ne permettent pour le moment que de corréler sénescence et vieillissement sans pouvoir établir formellement le lien causal de l'un des processus sur l'autre.

1.1.4 Sénescence et tumorigenèse

La grande majorité des travaux portant sur la sénescence la définit comme un processus suppresseur de tumeur qui bloque irrémédiablement la prolifération des cellules pour en prévenir la transformation maligne [236]. Différents types de données permettent cependant de partiellement remettre en cause le rôle strictement suppresseur de tumeur de la sénescence.

Premièrement, il est intéressant de constater que tous les inducteurs de sénescence décrits précédemment sont de potentiels stimulants oncogéniques susceptibles de favoriser l'instabilité génomique et de promouvoir le développement tumoral. Parmi ceux-ci, plusieurs études ont mis en évidence que les dommages oxydants à l'ADN, particulièrement dans les gènes suppresseurs de tumeur ou les oncogènes, sont clairement impliqués dans la tumorigenèse [237-239].

Deuxièmement, les données épidémiologiques du cancer montrent que l'incidence globale de survenue des cancers chez l'Homme s'accroit avec l'âge du patient (données issues du NCI - *Natinal Cancer Institute*). Il s'avère cependant que ces statistiques ne s'appliquent pas à tous les types de cancers et que ceux qui présentent l'incidence la plus liée au vieillissement sont les carcinomes (cancers épithéliaux). Or, la majorité des études visant à définir le rôle suppresseur de tumeur de la sénescence s'appuient sur des travaux effectués avec des fibroblastes, qui dans un contexte oncogénique, génèrent des sarcomes dont l'incidence est très faible et n'est pas influencée par le vieillissement (Figure 13).

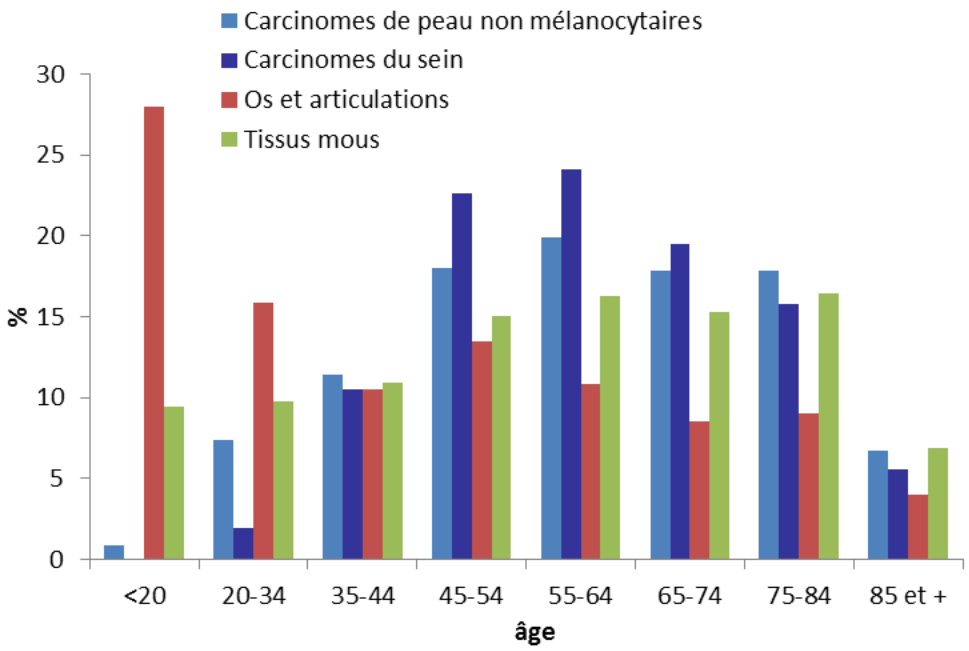

Figure 13 : Distribution des cancers par tranches d'âge *(d'après les données statistiques du NCI) montrant que les carcinomes (peau et sein) sont diagnostiqués préférentiellement entre 35 et 84 ans alors que les sarcomes (os, articulations et tissus mous) sont diagnostiqués de façon équivalente dans toutes les tranches d'âge, voire préférentiellement chez les individus de moins de 20 ans.*

Il existe donc un paradoxe entre les données épidémiologiques du cancer en lien avec le vieillissement et les études expérimentales définissant la sénescence comme un processus suppresseur de tumeur. En effet, si le nombre de cellules épithéliales sénescentes dans les tissus augmente avec le vieillissement [22,190-195] et si ces cellules sont

bien bloquées irréversiblement dans le cycle cellulaire, il est difficile de comprendre pourquoi l'incidence des carcinomes augmente avec l'âge. Des données récentes permettent toutefois d'avancer un mécanisme qui pourrait lever ce paradoxe. Il a en effet été montré que le profil de sécrétion des fibroblastes en sénescence réplicative ou induite par *ras* change au profit d'un sécrétome proche de celui de fibroblastes péritumoraux dits activés ou CAFs (*Cancer Associated Fibroblasts*) [240,241], conditionnés par les échanges tumeur-fibroblastes à sécréter des facteurs propices au développement du cancer [242]. On retrouve par exemple la sécrétion de facteurs inflammatoires comme certaines interleukines et chimiokines, de facteurs de croissance et angiogènes, de métalloprotéases (protéases impliquées dans la dégradation de la matrice extracellulaire), etc [243,244].

A côté de ce mécanisme paracrine lié au vieillissement du microenvironnement des tissus épithéliaux, des mécanismes intrinsèques et autonomes aux cellules épithéliales pourraient intervenir pour permettre aux cellules sénescentes de s'échapper de l'arrêt dans le cycle cellulaire. Les travaux de Romanov en 2001 ont les premiers mis en évidence l'existence d'une telle possibilité [245] en décrivant que des cellules épithéliales mammaires humaines (HMECs *Human Mammary Epithelial Cells*) sont capables de s'échapper du plateau de sénescence. Ces cellules présentent d'importantes altérations génétiques acquises spontannément et de façon autonome. Ces travaux suggèrent donc que non seulement la sénescence puisse ne pas être un processus suffisamment puissant pour prévenir les cellules épithéliales de la transformation cancéreuse, mais également qu'elle puisse en être responsable. Il est d'ailleurs intéressant de rappeler que les cellules sénescentes et les cellules cancéreuses ont des caractéristiques communes : stress oxydant élevé, altérations oncogéniques, télomères raccourcis, instabilité génétique, résistance à l'apoptose, ce qui appuie l'idée que la sénescence pourrait être, dans certains cas, un préalable à la transformation.

La relation entre sénescence et tumorigenèse est donc bien plus complexe qu'un « simple blocage » de la prolifération de cellules susceptibles d'évoluer vers une transformation cancéreuse. L'importance du rôle suppresseur de tumeur de la

sénescence est donc à modérer en fonction des contextes cellulaires et tissulaires au sein d'un organisme.

1.2 Autophagie et mort cellulaire programmée

Pour maintenir un fonctionnement optimal, les cellules doivent en permanence renouveler leurs constituants en les dégradant et en les néosynthétisant. Pour ce faire, elles utilisent des systèmes de dégradation adaptés au type, au degré d'altération et à la localisation du composé à éliminer. Le protéasome est le système de dégradation le plus connu. Ce système protéasique permet de dégrader des protéines mal conformées ou oxydées dans le cytoplasme et le noyau (voir pour revue [246-249]). Aux niveaux mitochondrial et peroxysomal, les protéines altérées sont dégradées par les Lon protéases associées en homo-oligomères de 4 à 8 composants (selon l'organisme) [250-252]. L'autophagie est un mécanisme de dégradation beaucoup plus large qui permet de dégrader macromolécules et organites cytoplasmiques en faisant intervenir la machinerie lysosomiale.

1.2.1 Macro-, micro- et autophagie médiée par les chaperonnes

Terme dérivé du grec qui signifie « se manger soi-même », l'autophagie a été initialement mise en évidence par Christian de Duve qui observe en 1966 que les organites peuvent être dégradés par les lysosomes [253]. Un regain d'intérêt pour ce processus a été suscité ces dernières années par l'identification chez la levure des molécules le régulant. On reconnaît aujourd'hui à l'autophagie une importance fondamentale dans la maintenance de l'homéostasie cellulaire et tissulaire normale. Son dérèglement est impliqué dans de multiples pathologies comme par exemple les cancers ou les maladies neurodégénératives [254].

Il existe trois types d'autophagie : la macroautophagie (généralement nommée simplement autophagie), la microautophagie, et l'autophagie médiée par des chaperonnes (ou CMA *Chaperon mediated autophagy*) (voir pour revue [255]). Ces types

d'autophagie se distinguent principalement par les cibles qu'elles visent et le moyen par lequel elles vont les diriger vers les lysosomes.

Des trois types d'autophagie, la CMA est probablement celle qui présente le plus de spécificité envers son substrat. La CMA ne dégrade en effet que les protéines prises en charge par HSC70, une chaperonne de la famille des *Heat Shock Proteins* (HSP), qui reconnaît un motif peptidique spécifique KFERQ [256,257]. Ce pentapeptide est présent sur au moins 30% des protéines cytoplasmiques [258] mais n'est accessible que lors de leur altération (oxydation, perte de conformation, dissociation d'un complexe protéique…) dans certaines conditions de stress [259,260]. Une fois reconnue, la protéine est dirigée vers un lysosome grâce à l'interaction spécifique de HSC70 avec LAMP2A (*Lysosomal Associated Membrane Protein 2A*) [261].

Comparées à la CMA, la micro- et la macroautophagie sont deux processus capables de dégrader les protéines comme les organites. Le mécanisme de reconnaissance des éléments à dégrader étant encore aujourd'hui peu compris, ils sont souvent considérés comme des processus de dégradation non spécifiques. Chez la levure, la dégradation d'un organite ou d'une protéine par microautophagie implique sa capture directement par la vacuole. Ce type d'autophagie n'a pas clairement été identifié chez l'Homme. La macroautophagie est un processus conservé jusque l'Homme ; la capture du composé cytoplasmique est réalisée par une vésicule spécifique qui fusionnera dans un second temps avec un lysosome afin d'en dégrader le contenu. N'ayant travaillé que sur la macroautophagie, je ne développerai ici que ce mécanisme.

1.2.2 Les mécanismes de la macroautophagie

La macroautophagie commence par la formation d'une double membrane appelée membrane d'isolation ou phagophore. Chez *Saccharomyces cerevisae*, le phagophore se forme à partir d'une structure cytoplasmique dite préautophagosomale qui n'existe pas chez l'Homme [262-264]. L'origine exacte de la membrane d'isolation chez les mammifères est aujourd'hui controversée, mais il semble qu'elle dérive du réticulum endoplasmique et/ou de l'appareil de Golgi [265,266]. Une fois générée, la membrane

d'isolation s'étend autour d'une portion cytoplasmique pouvant contenir organites et macromolécules et se referme pour former une vésicule à double membrane appelée autophagosome. La fusion de cette vésicule avec un endosome et/ou lysosome formera ensuite un autolysosome, forme mature de la vésicule autophagique dans laquelle le contenu séquestré sera dégradé par les enzymes lysosomiales [255,267]. Les produits de dégradation pourront être recyclés et réutilisés par la cellule comme matières premières pour des néosynthèses [268] (Figure 14).

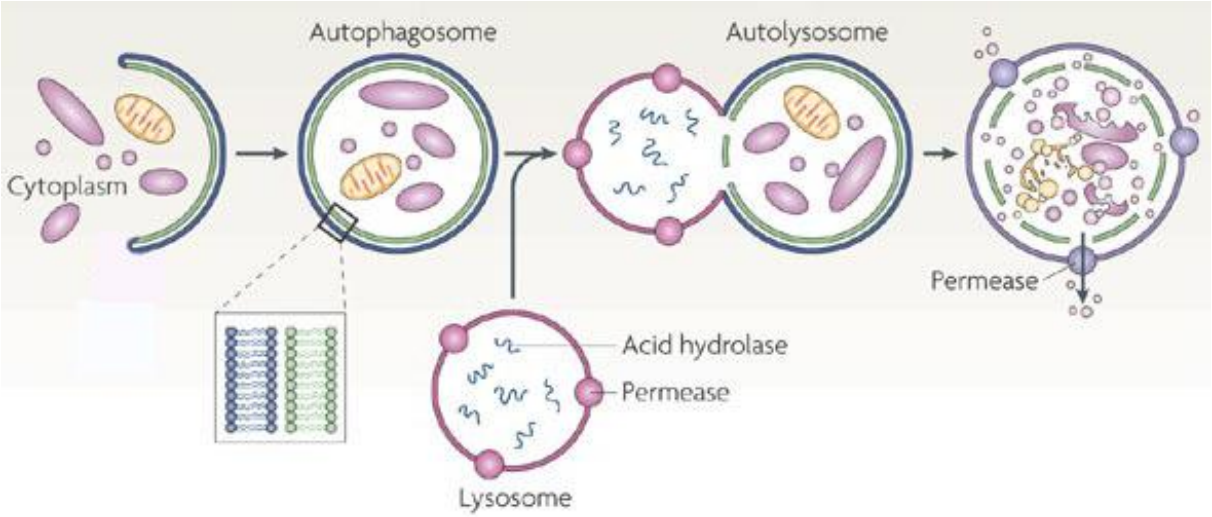

Figure 14 : Déroulement de la macroautophagie. *Adaptée de [262].*

La macroautophagie est un processus régulé par l'expression de gènes spécifiques, appelés Atg (*Autophagy related genes*), conservés pour la majorité de la levure à l'Homme. Ces gènes, au nombre d'une trentaine, régulent toutes les étapes du processus, de la nucléation et l'élongation de la membrane d'isolation à la clôture et la maturation de la vésicule autophagique (voir tableau 1 - voir pour revue [269]).

Etape du processus	*Noms*	*Rôle éventuel dans le cancer*	*Régulateurs positifs*	*Régulateurs négatifs*	*Réf*
Initiation	ULKs	ULK3 est impliquée dans l'OIS	PTEN AMPK	PI3K AKT mTOR	270-274
	mATG13	-			
	FIP200	-			
	Atg101	-			
Nucléation	Atg6 / Beclin1	Expression modulée dans les cancers du sein, de la prostate et des ovaires - Suppresseur de tumeur haploinsuffisant	Protéines pro-apoptotiques *BH3-only*	Protéines anti-apoptotiques de la famille Bcl2	275-285
	UVRAG	Expression modulée dans les tumeurs mammaires, colorectales et gastriques - Suppresseur de tumeur haploinsuffisant	-	-	
	Bif1	Expression diminuée dans les cancers gastriques et prostatiques - Souris Bif1$^{-/-}$ prédisposées au cancer	-	-	
	Ambra1	-	-	-	
	Atg14L	-	-	-	
	hVPS34	-	-	-	
Elongation	Atg12-Atg5	-	DAPK	-	286-294
	Atg16	Muté dans la maladie de Crohn	-	-	
	Atg8/LC3B	-	-	FLIP	
	Atg10	-	-	-	
	Atg7	-	-	-	
	Atg4	Souris Atg4C-/- développent des fibrosarcomes en réponse à des traitements carcinogènes	-	-	
	Atg3	-	-	-	
Maturation	Rab7	Expression aberrante de Rab7 dans les leucémies	UVRAG	RUBICON	279,295-298
	SNARE	-	-	-	
	LAMP1/2	-	-	-	

Tableau 1 : Protéines impliquées dans les différentes phases de la macroautophagie chez l'Homme. *Adapté de* [299]

L'initiation de la macroautophagie

Beaucoup des signaux inducteurs d'autophagie (restriction en acides aminés, en facteurs de croissance et en glucose, augmentation du ratio AMP/ATP et du stress oxydant) activent des voies de signalisation, notamment la voie PI3kinase/Akt, qui convergent au niveau de la protéine sérine-thréonine kinase TOR (*Target Of Rapamycin*) [300,301]. Chez l'Homme, la voie mTOR (*mammalian TOR*) agit sur l'activité des orthologues humains de Atg1 (Ulk1 et 2 - *Unc51 like kinase*), Atg13 et Atg17 (FIP200 - *200kDa focal adesion kinase familly interacting protein*) [302]. MTOR fonctionne au sein d'un complexe protéique appelé mTORC1 (*mTOR complex 1*) composé de RAPTOR, GβL/mLst8, PRAS40 et DEPTOR. En condition normale, ce complexe inactive la macroautophagie en inhibant l'activité des ULKs et mAtg13 par phosphorylation. Les voies de signalisation mises en jeu en réponse à un stimulus activateur d'autophagie conduiront à l'inhibition de l'activité de ce complexe. S'ensuivra alors l'autophosphorylation activatrice des ULKs qui phosphoryleront FIP200 et Atg13 pour induire la macroautophagie [273,303].

Un second complexe appelé mTORC2 (composé de RICTOR, GβL/mLst8, PRR5/PROTOR et DEPTOR) a également été décrit. MTORC2 a une action indirecte sur le processus autophagique en phosphorylant la sérine 473 de Akt [304,305]. Une fois activée, Akt phosphoryle le facteur de transcription FoxO3 pour en inhiber l'activité. Ce facteur de transcription pouvant induire l'expression d'un certain nombre d'Atg essentiels à la macroautophagie (Atg6/Beclin1, Atg8/LC3B, Atg4b, Atg12, hVps34, etc) [306], mTORC2 a donc, comme mTORC1, une action inhibitrice sur le processus.

L'étape de nucléation

En aval du premier complexe ULK-mAtg13-FIP200 entre en jeu l'activité de Vps34, une PI3kinase de classe III qui génère du PI3P (Phosphatidylinositol-3-phosphate) à partir de PI (Phosphatidylinositol), une production essentielle à la formation de la vésicule et au recrutement d'autres protéines Atg [307]. L'activité de Vps34 est régulée par la présence de Atg6/Beclin1 avec laquelle elle doit se complexer pour fonctionner [308].

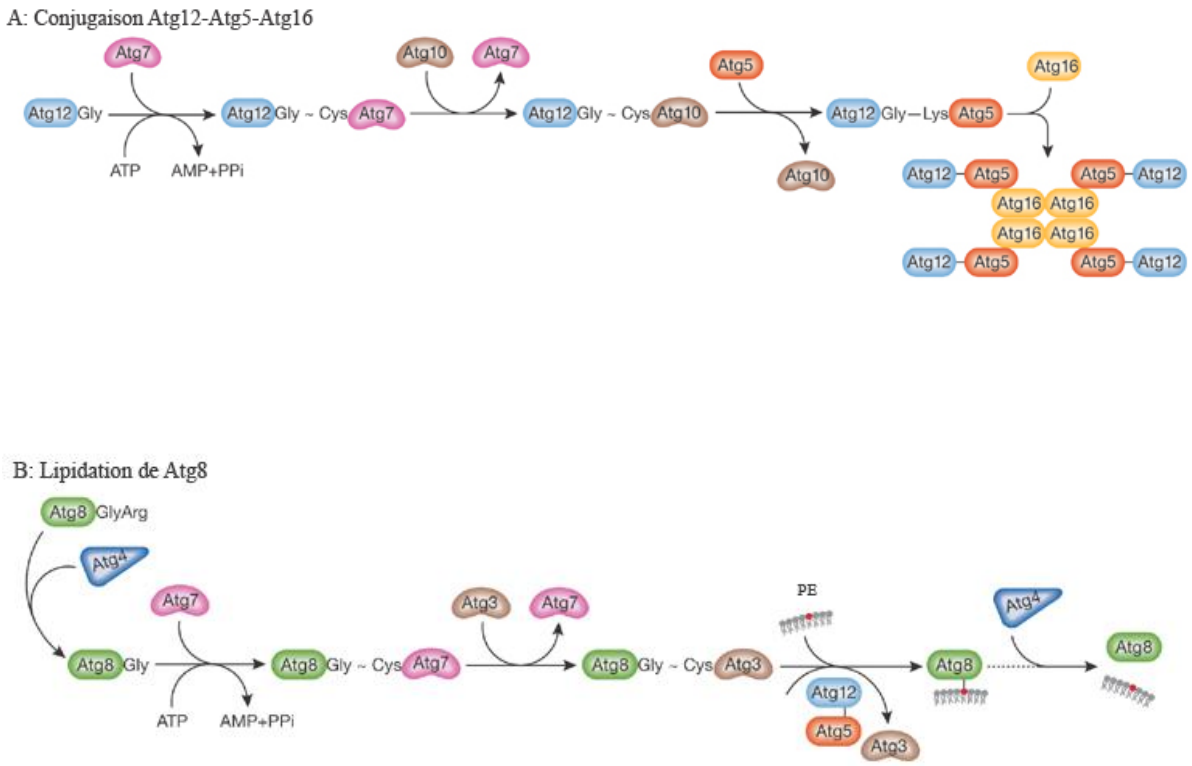

Figure 15 : Les systèmes de conjugaison ubiquitin-like mis en place lors de la macroautophagie. *Adaptée de [309]. PE = fragment membranaire contenant un Phosphatidyléthanolamine.*

De manière intéressante, la formation de ce complexe est régulée par Bcl2 et BclXL capables de se lier à Atg6/Beclin1 *via* son domaine BH3 pour la séquestrer dans le réticulum endoplasmique [282,310,311]. En cas de stress nutritif, les voies de signalisation activées conduisent à la phosphorylation de Bcl2 par Junk1, ce qui lève l'interaction entre Atg6/Beclin1 et Vps34 et initie la macroautophagie [312]. D'autres protéines ont été décrites pour se lier à et réguler le complexe PI3kinase. C'est notamment le cas de Bif-1, Atg14L et Ambra1 dont la présence stimule l'activité PI3kinase et favorise la formation initiale de la membrane d'isolation [277,297,298,313].

Elongation de la membrane d'isolation et formation de l'autophagosome

L'expansion de la membrane d'isolation autour de la portion cytoplasmique à dégrader est contrôlée par deux systèmes *ubiquitin-like*. Le premier conduit à la conjugaison de Atg5 sur Atg12. Pour cela, Atg7 (enzyme E1-*like*) active le résidu glycine de la partie carboxyterminale de Atg12 en utilisant de l'ATP. La protéine est ensuite prise en charge par Atg10 (enzyme E2-*like*) qui catalysera la création d'une liaison covalente entre Atg12 et Atg5 au niveau de sa lysine 130 [314,315]. La protéine de fusion ainsi générée recrute ensuite Atg16L pour former un complexe multimérique Atg12-Atg5-Atg16L qui intègre la membrane de la vésicule autophagique en formation [315,316] (Figure 15 A).

Parallèlement à la formation de ce premier complexe, la protéine Atg8/LC3B (ou MAP-LC3 pour *Microtubule Associated Light Chain 3*) est modifiée par un second système *ubiquitin-like* [317-319]. LC3B est exprimée dans la cellule sous forme d'une protéine inactive de pleine longueur de 18kDa. Sous induction de la macroautophagie, LC3B est clivée par la cystéine protéase Atg4, conduisant à la formation d'une protéine de 16kDa. Le clivage de LC3B met en exergue un résidu glycine en C-terminal qui sera activé par Atg7. LC3B est alors transférée à Atg3, une enzyme E2-*like* qui conjugue la protéine à un phosphatidyléthanolamine [320] (Figure 15 B). La protéine ainsi modifiée, appelée LC3B II, est ensuite prise en charge par les complexes Atg12-Atg5-Atg16L et intégrée à l'autophagosome en formation [321]. L'ensemble de ces partenaires moléculaires contribue à l'expansion et à la courbure de l'autophagosome en formation.

Deux autres protéines GABARAP (*gamma-aminobutyric acid type A receptor associated protein*) et GATE16 (*Golgi-Associated ATPase Enhancer of 16kDa*) sont prises en charge par la même voie que LC3B et sont aussi intégrées à l'autophagosome, mais le rôle qu'elles y jouent n'est pas clairement déterminé [321-323].

L'étape de maturation en autolysosome

La dernière étape de la macroautophagie qui consiste en la fusion de l'autophagosome avec un endosome et/ou lysosome n'est que peu étudiée. De manière inattendue, la bafilomycine A1, un inhibiteur des pompes H^+ lysosomiales, s'est révélée être un inhibiteur de la fusion autophagosome/lysosome, suggérant qu'un pH acide puisse être nécessaire à la fusion [324]. Il apparaît également que les protéines LC3B II présentes sur la membrane externe de l'autophagosome doivent en être extraites *via* l'activité protéasique de Atg4 avant la fusion avec le lysosome [317,325]. Des protéines endosomales et lysosomiales sont aussi nécessaires à la maturation de l'autophagosome. C'est notamment le cas de LAMP1, LAMP2 (*Lysosomial Associated Membrane Protein 1 et 2*) ainsi que de la GTPase Rab7 dont l'inhibition d'expression bloque la fusion autophagosome/lysosome [295,325-328]. Les microtubules jouent également un rôle essentiel dans la maturation des vésicules autophagiques [329-331]. De manière intéressante, le complexe Atg6/Beclin1-hVps34 régule également cette dernière étape : associé à UVRAG, il favorise la maturation de l'autophagosome, alors qu'associé à UVRAG et Rubicon il l'inhibe [297,298].

1.2.3 La macroautophagie, un processus de dégradation spécifique ?

Comme indiqué plus haut, la macroautophagie est un processus classiquement décrit comme étant non spécifique de son substrat, dégradant aléatoirement les constituants cytoplasmiques. Cette notion s'appuie principalement sur des observations en microscopie électronique où l'on voit notamment des autophagosomes contenant mitochondries, diverses petites vésicules, ribosomes, etc. Depuis quelques années, la

meilleure compréhension du processus aux niveaux mécanistique et moléculaire commence toutefois à remettre en cause l'aspécificité de la macroautophagie.

Jusque là, Atg8/LC3B était considérée comme une protéine structurant l'autophagosome et permettant sa maturation. Il s'est récemment révélé que Atg8/LC3B pouvait interagir avec la protéine p62/SQSTM1. L'intérêt de p62 est qu'elle peut fixer les protéines et agrégats ubiquitinylés. L'interaction p62/LC3B permet donc à la membrane de l'autophagosome en formation de cibler spécifiquement ce type de substrat [332,333].

La macroautophagie est également décrite comme étant spécifique de certains organites. Elle est alors déclinée en fonction de son substrat : la mitophagie (pour les mitochondries), la peroxyphagie (pour les peroxysomes), la réticulophagie (pour le réticulum endoplasmique), etc [334,335]. Concernant la mitophagie, des données récentes ont mis en évidence chez la levure un rôle important de la protéine Atg32. Cette protéine, localisée dans la membrane mitochondriale, est capable d'interagir avec Atg8/LC3B et Atg11. Cette interaction permet donc à l'autophagosome en formation de cibler spécifiquement les mitochondries [336-338]. Même s'il n'existe pas d'orthologue de Atg32 connu chez l'Homme, la perspective d'une autophagie ciblée des mitochondries chez les eucaryotes supérieurs est donc envisageable. C'est notamment ce qui ressort de travaux menés chez le rat et l'Homme qui semblent indiquer que le relargage de ROS associé à la perte de perméabilité mitochondriale puisse être un stimulus suffisant pour activer la dégradation spécifique des mitochondries altérées [339,340].

1.2.4 Macroautophagie et mort cellulaire programmée

La macroautophagie est largement décrite comme un processus favorisant la survie en permettant aux cellules de résister à différents stress. Elle leur permet notamment de résister à des privations nutritives ou en facteurs de croissance en dégradant des composés non essentiels ou altérés au profit de la production de métabolites essentiels [341]. L'activation de la macroautophagie permet également aux cellules de résister au stress oxydant en éliminant les organites altérés [342-344] et les

agrégats protéiques [345-347] qui pourraient affecter son fonctionnement. Elle défend aussi la cellule contre les infections bactériennes [348,349], parasitaires [350], voire virales [351].

Cependant, la macroautophagie est également décrite comme un processus de mort cellulaire programmée, dit de type II, en parallèle de l'apoptose dite de type I [352]. La caractérisation de la mort par autophagie remonte aux travaux de Schweichel et Merker en 1973 qui décrivent l'existence de plusieurs types de mort cellulaire lors du développement embryonnaire, se caractérisant notamment par la présence ou non de lysosomes [352-354]. Cette mort par macroautophagie joue un rôle crucial, notamment lors de la morphogenèse des membres des vertébrés [355] ou lors de la métamorphose de l'insecte [352,353,356]. La mort par macroautophagie peut également être induite en réponse à divers stimuli inducteurs de mort dans des contextes cellulaires variés (cellules cancéreuses, neurones, etc) [357-359].

Contrairement à l'apoptose dont le phénotype et la mécanistique sont très bien caractérisés, la mort par autophagie est moins bien décrite et nécessite encore beaucoup d'investigations. C'est pourquoi l'existence même d'un processus de mort cellulaire programmée non apoptotique impliquant la macroautophagie est assez controversée dans la littérature [360]. Le principal point en suspens concerne le mécanisme précis conduisant à la mort des cellules. C'est pourquoi, cette mort cellulaire programmée est souvent simplement décrite comme une mort indépendante de l'activation des caspases et s'accompagnant de l'accumulation de vésicules autophagiques. De plus, que le processus macroautophagique débouche sur la survie ou la mort cellulaire, les protéines impliquées dans la formation et la maturation des vésicules autophagiques sont les mêmes et aucun marqueur moléculaire permettant de différencier les deux voies n'est actuellement connu. Deux hypothèses non exclusives se dégagent toutefois de la littérature quant au mécanisme de mort par macroautophagie. D'un côté, un certain nombre de travaux ont mis en évidence que la mort des cellules peut résulter d'une dégradation massive du cytoplasme par macroautophagie [352,361]. C'est par exemple ce que décrivent les travaux de Lum *et al* dans lesquels ils mettent en évidence qu'une privation en facteur de croissance prolongée induit une accumulation massive de vésicules pouvant représenter un volume total supérieur ou égal au reste du

cytoplasme [361]. Il a également été montré que l'expression de la protéine Atg6/Beclin1 augmente lors de l'activation de la mort par macroautophagie induite par traitement à l'étoposide, alors qu'elle n'augmente pas lors d'une privation en nutriments visant à induire la macroautophagie de survie [362]. Il se pourrait donc que la mort puisse résulter d'une augmentation massive de l'activité autophagique directement contrôlée par le niveau d'expression de Atg6/Beclin1. D'un autre côté, la macroautophagie peut aussi dégrader plus spécifiquement des organites et/ou des protéines essentiels à la survie cellulaire. Les travaux de Yu *et al* montrent par exemple que la mort cellulaire par autophagie peut résulter de la dégradation spécifique de la catalase [363]. La dégradation sélective de cette enzyme antioxydante conduit alors à une accumulation de H_2O_2, potentiellement responsable de la mort des cellules. Une dégradation massive et spécifique des mitochondries pourrait également être un mécanisme conduisant à la mort des cellules [364].

Il est intéressant de constater qu'il existe un certain nombre d'interconnexions entre apoptose et autophagie. Il s'avère par exemple que beaucoup des stimuli inducteurs d'apoptose sont également inducteurs d'autophagie. C'est par exemple le cas des traitements à l'étoposide [362], interféron gamma [365], TRAIL [366], FADD [367], les céramides [368], etc. Il apparaît également que plusieurs acteurs moléculaires de l'apoptose sont impliqués dans la régulation de la macroautophagie. C'est le cas des protéines Bcl2 et BclXL qui sont capables d'interagir avec le domaine BH3 de Beclin1 pour prévenir de sa complexation avec Vps34 [283,310,311,369]. Bcl2 et BclXL inhibent donc l'activation de la macroautophagie comme celle de l'apoptose. D'autres membres *BH3-only* de la famille Bcl2 ont également été décrits pour influencer l'apoptose et la macroautophagie [370-372]. Ainsi, les mêmes acteurs conduisent au contrôle des deux types de mort, suggérant que les deux processus sont interconnectés et qu'ils puissent être induits ou réprimés de manière alternative ou simultanée, voire même qu'ils puissent influer l'un sur l'autre.

La simultanéité des deux processus de mort cellulaire programmée a par exemple été mise en évidence lors de la morphogenèse de l'intestin moyen et des glandes salivaires de *Drosophila melanogaster.* En effet, la mise en place de ces tissus

nécessite l'activation d'un processus de mort cellulaire présentant des critères morphologiques et moléculaires à la fois apoptotiques (fragmentation nucléaire) et autophagiques (présence de vésicules en microscopie électronique) [373-376]. D'autres études ont montré que les deux processus de mort peuvent être interdépendants et que leur activation, mutuelle et différée dans le temps, est nécessaire à l'induction de la mort. C'est ce qu'ont mis en évidence les travaux de Xue *et al* qui décrivent que l'induction de la mort par privation en NGF dans les neurones sympathiques active la macroautophagie en amont de l'apoptose. Dans ces travaux, les auteurs montrent également qu'inhiber l'autophagie repousse l'apoptose [359]. Inversement, les travaux de Martin et Baehrecke décrivent que l'activation des caspases est un préalable à l'induction de la mort macroautophagique induite par l'ecdysone durant la morphogenèse de la glande salivaire de *Drosophila melanogaster* [377]. Dans d'autres contextes, les deux processus peuvent compenser l'absence de l'autre. En effet, l'induction de la mort cellulaire dans des cellules incapables d'activer l'apoptose (déficientes pour la caspase 8) conduit à une mort cellulaire programmée dépendante de l'expression de Atg6/Beclin1 et Atg7 et qui s'accompagne de l'accumulation de vésicules autophagiques [378-380].

Concernant les voies de signalisation moléculaire engagées, il apparaît notamment que certaines protéines kinases impliquées dans l'induction de l'apoptose ont également un rôle majeur dans le contrôle de la macroautophagie. C'est par exemple le cas des protéines kinases JNK et DAPK qui, une fois activées, phosphorylent respectivement Bcl2 et Atg6/Beclin1 pour en favoriser la dissociation et activer l'autophagie [312,381,382]. Les travaux de Wei *et al* ont aussi mis en évidence que l'activation de la voie JNK en réponse à une privation en nutriments conduit dans un premier temps à la dissociation des complexes Bcl2/Beclin1 pour induire la macroautophagie avant l'activation de l'apoptose en second lieu (par dissociation des complexes Bcl2/Bax) [381].

Enfin, une relation antagoniste entre apoptose et macroautophagie a par exemple été décrite dans des études montrant que les protéines Atg4 et Atg6/Beclin1 sont des cibles des caspases, suggérant donc que ces protéines puissent être clivées durant l'apoptose pour inhiber la macroautophagie [383-385].

1.2.5 Macroautophagie et cancer

L'implication de la macroautophagie dans la survie comme dans la mort cellulaire lui confère un rôle complexe vis-à-vis de certaines pathologies. Dans le cas des cancers, on considère généralement que les cellules doivent résister à la mort et favoriser les signaux promoteurs de survie. Quelles sont les données concernant le rôle de la macroautophagie dans cette pathologie ?

Comme pour les cellules normales, la macroautophagie est activée dans les cellules cancéreuses en réponse aux privations en nutriments, facteurs de croissance, et oxygène généralement causées dans le contexte pathologique par le défaut d'approvisionnement sanguin au sein des tumeurs [386,387]. Activée dans ces contextes, la macroautophagie permet aux cellules de survivre en limitant le stress métabolique qu'elles subissent dans les zones hypoxiques tumorales [276]. Dans ce cas, la macroautophagie pourrait être considérée comme promoteur de tumeur [388].

Pourtant, certains gènes impliqués dans la macroautophagie sont fréquemment mutés dans les cancers, suggérant que le processus puisse être suppresseur de tumeur (Tableau 1). C'est notamment le cas de Atg6/Beclin1, essentiel à l'initiation de la macroautophagie [389], qui est souvent haplodélété dans les cancers humains du sein, de l'ovaire et de la prostate [310]. De manière intéressante, une délétion biallélique de *atg6/beclin1* n'a jamais été rapportée dans les cancers, suggérant que le gène puisse être un suppresseur de tumeur haploinsuffisant. La reconstruction d'une haplodéficience atg6/*beclin1*$^{+/-}$ dans un modèle murin conduit à un développement spontané de carcinomes pulmonaires et prostatiques, d'hyperplasies mammaires et de lymphomes chez les animaux [280,389]. Touchant le même stade du processus macroautophagique, il est intéressant de constater que l'expression de Bcl2, mise en place dans les cellules cancéreuses pour les protéger de l'apoptose, réprime également la macroautophagie [390]. De plus, la voie PI3kinase/AKT est généralement mise en jeu dans les cellules cancéreuses. En aval de cette voie, la macroautophagie est alors inactivée *via* l'activité de mTOR [391]. L'ensemble de ces études suggère qu'une inhibition partielle de la macroautophagie puisse être bénéfique à la progression tumorale, par

exemple en réduisant l'induction de la mort et en favorisant la survie de cellules présentant une instabilité génétique [387,392,393].

La relation entre autophagie et cancer apparaît donc extrêmement complexe. En réduisant les stress cellulaires (notamment oxydants) potentiellement oncogéniques, la macroautophagie promeut la survie des cellules cancéreuses mais bloque l'évolution tumorale [276]. La macroautophagie est donc un processus suppresseur de tumeur que les cellules cancéreuses doivent réduire pour favoriser le développement tumoral. Un des concepts qui ressort des différentes études pourrait également être que les cellules cancéreuses, qui doivent se prémunir des différents types de mort cellulaire, réduiraient leur activité autophagique, pâtissant ainsi d'une moins bonne élimination des composés altérés, mais évitant une trop forte activité autophagique susceptible d'être létale.

1.3 Objectifs de la thèse

La thématique générale de mon équipe d'accueil concerne l'étude des mécanismes précoces mis en jeu lors de la transformation de cellules normales en cellules cancéreuses, en lien avec le vieillissement. Dans ce cadre, nous utilisons un modèle *in vitro* de cellules épithéliales humaines, les kératinocytes de peau, qui nous permet d'étudier les mécanismes de sénescence et les voies impliquées dans leur échappement lors des phases très initiales de carcinogenèse. Il s'avère en effet que, après avoir atteint le plateau de sénescence, ces cellules normales génèrent spontanément des cellules néoplasiques, transformées et tumorigènes.

L'acquisition d'une résistance à la mort cellulaire étant généralement considérée comme une étape cruciale et précoce à la cancérisation, j'ai dans un premier temps cherché à comprendre comment les kératinocytes sénescents meurent afin de déterminer à quoi les cellules néoplasiques qui sont générées depuis la sénescence doivent échapper. Une partie de mes travaux montrera notamment que les cellules sénescentes ne meurent pas par apoptose mais que leur mort résulte d'une hyperactivation de la macroautophagie en réponse à l'accumulation de dommages oxydants.

Dans une seconde partie, j'ai axé mon travail sur la mise en évidence du mécanisme mis en place par les cellules sénescentes qui généreront des cellules néoplasiques pour échapper à la mort par autophagie. Il se dégagera de ces travaux la notion d'une relation complexe entre macroautophagie et sénescence, qui remet en cause les rôles suppresseurs de tumeur des deux processus.

Partie I :

Les dommages oxydants à l'ADN associés à la sénescence favorisent la génération de cellules néoplasiques

Pour étudier les mécanismes cellulaires et moléculaires très initiaux impliqués dans le développement cancéreux en lien avec le veillissement, nous utilisons un modèle de culture primaire de kératinocytes humains normaux d'épiderme (NHEKs *Normal Human Epidermal Keratinocytes*) issus de donneurs sains.

Les travaux antérieurs à mon arrivée au laboratoire ont mis en évidence que la sénescence *in vitro* des NHEKs est principalement causée par le stress oxydant. En effet, elle résulte d'une augmentation de l'activité des facteurs de transcription de la famille NFkappaB ainsi que de l'augmentation d'expression d'un de leurs gènes cibles, la MnSOD. Cette enzyme mitochondriale catalysant la dismutation de $O_2^{\bullet -}$ en H_2O_2, son augmentation d'expression en l'absence d'une corégulation des enzymes de dégradation du H_2O_2 comme la catalase ou les glutathion peroxydases, entraîne une accumulation de peroxyde d'hydrogène responsable de l'entrée des kératinocytes en sénescence [142,176,177].

La sénescence des NHEKs induite, au moins partiellement, par cette accumulation de H_2O_2, s'accompagne d'une augmentation de la taille des cellules, de leur étalement sur le support, de l'accumulation de vésicules cytoplasmiques, d'une augmentation de leur activité beta-galactosidase et également d'un arrêt du cycle cellulaire en lien avec une augmentation significative d'expression de $p21^{cip1/WAF1}$, et très faible de $p16^{INK4a}$. Cependant, dans le cas des kératinocytes, comme dans le cas des cellules épithéliales mammaires [245], cet arrêt dans le cycle cellulaire n'est pas irréversible. En effet, les kératinocytes sénescents réactivent spontanément le processus mitotique et génèrent une nouvelle population proliférante que nous avons baptisée émergente post-sénescence. Ce phénomène est multiclonal et a lieu à une fréquence de 10^{-2} à 10^{-4}, c'est-à-dire qu'environ 1 cellule sénescente sur 100 à 10 000 est capable de générer des cellules émergentes. Nous avons montré que les cellules émergentes ne sont pas issues de cellules déjà transformées qui auraient pu être présentes dans les explants d'origine, ou des cellules souches normales de l'épiderme, mais sont bien générées par des cellules pleinement sénescentes. De manière intéressante, les cellules émergentes sont produites par un mécanisme original de mitose par bourgeonnement, à partir de cellules sénescentes polynucléées. Les cellules émergentes post-sénescence

ne sont pas immortalisées et atteignent un second plateau de croissance, similaire mais pas strictement identique à la sénescence, après plusieurs cycles de division. Une autre population proliférante à durée de vie très allongée et morphologie plus transformée encore est susceptible de ré-émerger de ce second plateau. Ces cellules ont été baptisées IMKs, pour *IMmortal Keratinocytes*, mais contrairement à ce que nous pensions historiquement lors de leur découverte, les IMKs ne sont pas non plus immortalisées *in vitro* et atteignent un ultime plateau de croissance.

Les clones de cellules émergentes formés dans les deux stades d'arrêt de croissance sont phénotypiquement très hétérogènes : ils sont le plus souvent composés de cellules épithélioïdes qui restent localisées à proximité de la cellule sénescente qui les a généré et plus rarement de cellules fibroblastoïdes qui se dispersent dans les cultures. De plus, les cellules émergentes post-sénescence présentent un certain nombre de critères de transformation, avec par exemple la réduction d'expression de certains marqueurs de différenciation (involucrine et kératine14). Elles présentent également un profil transcriptomique différent de celui des kératinocytes en croissance exponentielle. Parmi les 100 gènes dont l'expression est la plus fortement modulée dans les cellules émergentes *versus* les cellules en croissance exponentielle, environ 50% codent des protéines impliquées dans l'adhérence et la migration, la structure et la dynamique du cytosquelette, la sénescence, le stress oxydant, le dommage à l'ADN, la progression dans le cycle et la mort cellulaire, et/ou la cancérogenèse. Les deux types de cellules émergentes possèdent encore des télomères longs (sans réexpression de la télomérase) et, de manière intéressante, seules les IMKs présentent des aberrations caryotypiques majeures, alors que les cellules émergentes post-sénescence ont un caryotype normal, identique à celui des cellules issues de la phase de croissance exponentielle. Injectées en souris *nude*, les cellules émergentes post-sénescence et les IMKs ne développent pas de tumeur au site d'injection, mais conduisent à la formation d'hyperplasies et de petits carcinomes cutanés après un long délai post-injection (supérieur à 19 semaines). Les cellules émergentes post-sénescence sont donc des cellules que l'on peut qualifier de néoplasiques.

Le stress oxydant étant le principal inducteur de la sénescence des kératinocytes, nous avons ensuite cherché à déterminer si, de par leurs propriétés mutagènes, les ROS pouvaient aussi être responsables de l'émergence post-sénescence. Nous avons montré que l'induction prématurée de la sénescence par traitement au peroxyde d'hydrogène ou après expression adénovirale de la MnSOD est également suivie d'une émergence, alors qu'à l'inverse, des traitements antioxydants repoussent la survenue du plateau de sénescence et diminuent la fréquence d'émergence. Enfin, nous avons également mis en évidence dans les cellules sénescentes des dommages oxydants à l'ADN comme des 8-OH-G et des cassures simple-brin, susceptibles, si elles affectent des oncogènes ou des gènes suppresseurs de tumeur, d'être responsables de l'émergence post-sénescence.

Le processus d'émergence était déjà bien caractérisé à mon arrivée au laboratoire et mon implication dans ce travail nous a notamment permis de montrer que les phénomènes et mécanismes mis en évidence n'étaient pas des particularités liés à un donneur de kératinocytes particulier, mais généralisables à tous les donneurs que j'ai manipulés. On peut cependant observer de petites différences entre les donneurs, notamment sur la durée du plateau de sénescence, le degré de transformation des cellules émergentes, la fréquence d'émergence, notamment de la seconde émergence, absente chez certains donneurs.

Tous ces travaux montrent donc qu'à la différence des études qui décrivent qu'une cellule doit échapper à la sénescence pour devenir tumorale, la sénescence peut aussi être, dans certains contextes cellulaires, une étape préalable et nécessaire à la génération de cellules néoplasiques.

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

Résultats complémentaires

Comme décrit dans l'article n°1, l'émergence du plateau de sénescence nécessite la production de ROS. Par conséquent, l'une des hypothèses les plus évidentes est que les ROS puissent favoriser l'émergence *via* leurs propriétés mutagènes. Nous avons donc recherché la présence de mutations dans les cellules émergentes post-sénescence. Comme nous avions déjà montré qu'elles ne présentent pas d'aberrations caryotypiques, nous avons recherché des altérations génomiques plus fines. Nous avons donc réalisé des analyses par array-CGH (*Comparative Genomic Hybridization*) qui permettent de détecter des amplifications et des délétions de petite taille. Ce travail a révélé que les cellules émergentes post-sénescence présentent un grand nombre de microdélétions (inférieures à 35kb) réparties aléatoirement sur le génome. En recoupant les données récoltées sur différents donneurs, nous avons mis en évidence que les gènes les plus fréquemment touchés par ces délétions sont impliqués dans le contrôle du cycle cellulaire, la transcription, le métabolisme et le cancer (oncogènes / suppresseurs de tumeurs) (Figure 16 - données non publiées du laboratoire).

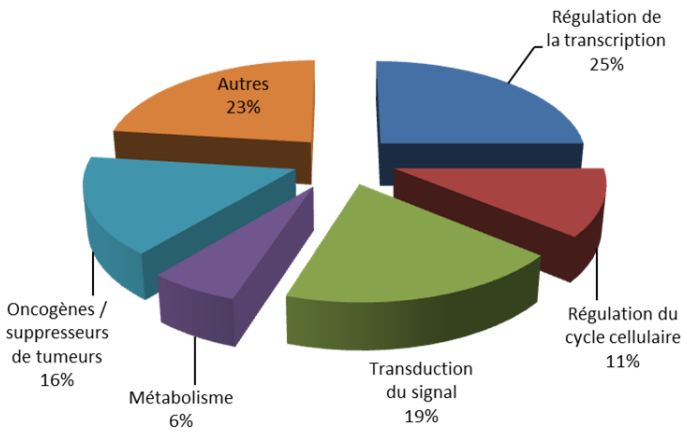

Figure 16 : Catégories de gènes affectés par des microdélétions dans les cellules émergentes post-sénescence *(données non publiées du laboratoire). Les données présentées ici correspondent aux gènes retrouvés délétés dans au moins trois clones d'émergence post-sénescence sur quatre issus de donneurs différents.*

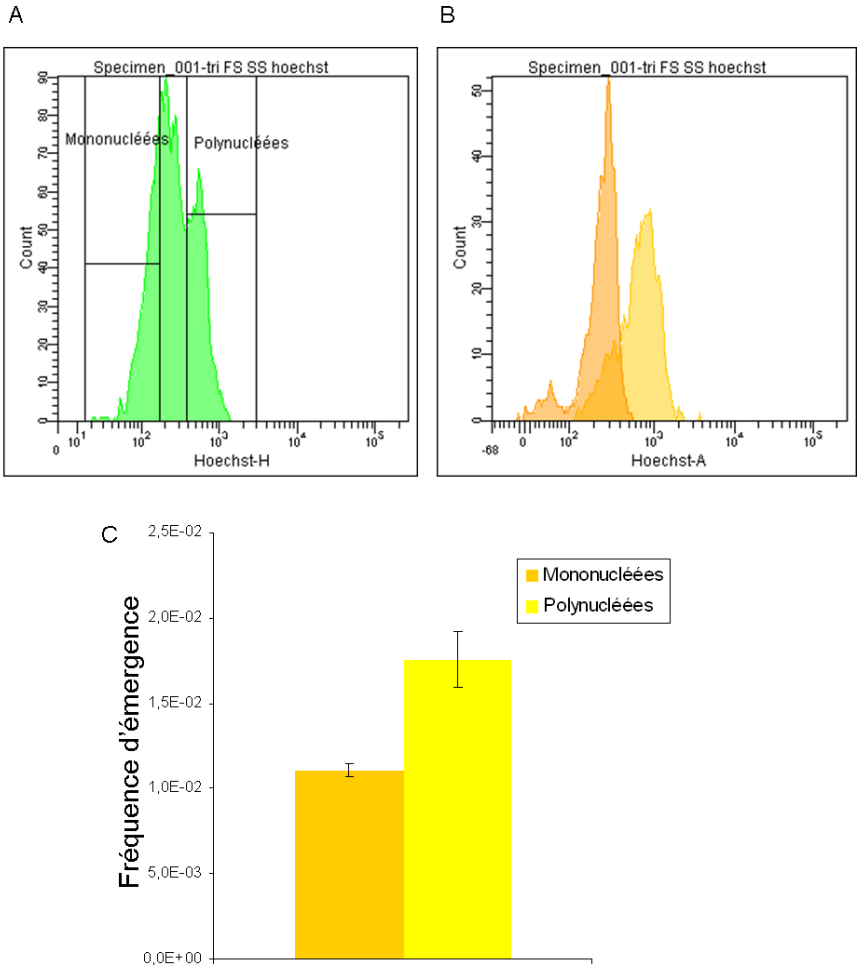

Figure 17 : Purification des cellules sénescentes progénitrices de cellules émergentes par FACS *(données non publiées du laboratoire) A. Analyse du marquage au Hoechst vital (33342) des kératinocytes sénescents. Les barres de sélection déterminent les cellules qui seront triées par FACS. B. Vérification de la pureté du tri des cellules mono- et poly-nucléées (respectivement orange et jaune). C. Estimation des fréquences d'émergence des deux populations.*

Nous avons par ailleurs initié des recherches sur le mécanisme de mitose mis en jeu dans la génération des cellules émergentes. En effet, ces cellules ne sont pas générées par une mitose classique des cellules sénescentes qui donnerait deux cellules filles identiques, mais par bourgeonnement de cellules sénescentes polynucléées. Ce que nous avons vu par vidéomicroscopie à contraste de phase en temps réel est que la génération des cellules émergentes à partir d'une cellule sénescente se fait en deux étapes : une étape de multiplication des noyaux dans le cytoplasme, suivie de l'étape de bourgeonnement. Pour vérifier que les kératinocytes sénescents progéniteurs de cellules émergentes sont bien polynucléés, nous avons tenté de trier spécifiquement les cellules sénescentes par FACS en fonction de leur niveau de polynucléation (déterminé par marquage au Hoechst vital 33342). Bien que l'histogramme montrant le marquage Hoechst de la population sénescente ne corresponde pas à un histogramme classique de cycle cellulaire, nous avons quand même trié les populations sénescentes dont les marquages au Hoechst étaient les plus extrêmes, pour enrichir les populations cellulaires récoltées en cellules mononucléées et polynucléées. Nous avons ensuite testé la capacité des deux sous-populations à émerger. Les résultats préliminaires obtenus semblent indiquer que les cellules polynucléées sont bien les cellules sénescentes les plus enclines à générer des clones de cellules émergentes (Figure 17). Il reste néanmoins à affiner les protocoles de marquage au Hoechst et de tri et à confirmer ces résultats avec plusieurs donneurs. A terme, la mise au point d'une telle méthode de purification des cellules sénescentes progénitrices de cellules émergentes nous permettra d'étudier spécifiquement les mécanismes d'émergence post-sénescence. Nous pourrons aussi typer ces progéniteurs, ce qui nous permettrait potentiellement de dégager des marqueurs précoces de la transformation cancéreuse en lien avec le vieillissement.

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

Karo Gosselin,[1,2,3,4,5] Sébastien Martien,[1,2,3,4,5] Albin Pourtier,[1,2,3,4,5] Chantal Vercamer,[1,2,3,4,5] Peter Ostoich,[7] Luc Morat,[7] Laure Sabatier,[7] Laurence Duprez,[8] Claire T'Kint de Roodenbeke,[9] Eric Gilson,[9] Nicolas Malaquin,[1,2,3,4,5] Nicolas Wernert,[10] Predrag Slijepcevic,[11] Marjan Ashtari,[11] Fazia Chelli,[1,2,3,4,5] Emeric Deruy,[1,2,3,4,5] Bernard Vandenbunder,[1,3,6] Yvan De Launoit,[1,2,3,4,5] and Corinne Abbadie[1,2,3,4,5]

[1]Université Lille Nord de France; [2]CNRS, UMR8161; [3]UDSL; [4]Institut Pasteur de Lille, Lille, France; [5]USTL; [6]CNRS, UMR3078, Villeneuve d'Ascq, France; [7]CEA Life Science Division, Fontenay-aux-Roses, France; [8]Laboratoire de Cytogénétique Erasme-ULB-CHU Brugmann, Brussels, Belgium; [9]CNRS, UMR5239, Faculté de Médecine Lyon Sud, Université Lyon 1, Pierre Bénite, France; [10]Institute of Pathology, University of Bonn, Bonn, Germany; [11]Brunel Institute of Cancer Genetics and Pharmacogenomics, Brunel University, Uxbridge, Middlesex, United Kingdom

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 MCBD153 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(number of collected cells / number of plated cells) / ln2. 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/).

K. Gosselin and S. Martien contributed equally to this work.

Requests for reprints: Corinne Abbadie, Institut de Biologie de Lille, 1 rue Calmette, BP 447, 59021 Lille Cedex, France. Phone: 33-3-20-87-11-02; Fax: 33-3-20-87-11-11; E-mail: corinne.abbadie@ibl.fr.

doi:10.1158/0008-5472.CAN-08-2510

(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 *Eco*RI and inserted into the pAdCMV2 vector at the *Xba*I 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 cometslide. 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 Tritek 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. 1*B*). 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. S2*A*). The expression of involucrin and keratin 14, two markers of keratinocyte differentiation, increased with senescence and decreased again with emergence (Supplementary Fig. S2*B*). That of E-cadherin, involved in cell-cell interaction, also slightly decreased in post-senescent–emerging cells (Supplementary Fig. S2*C*). 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).

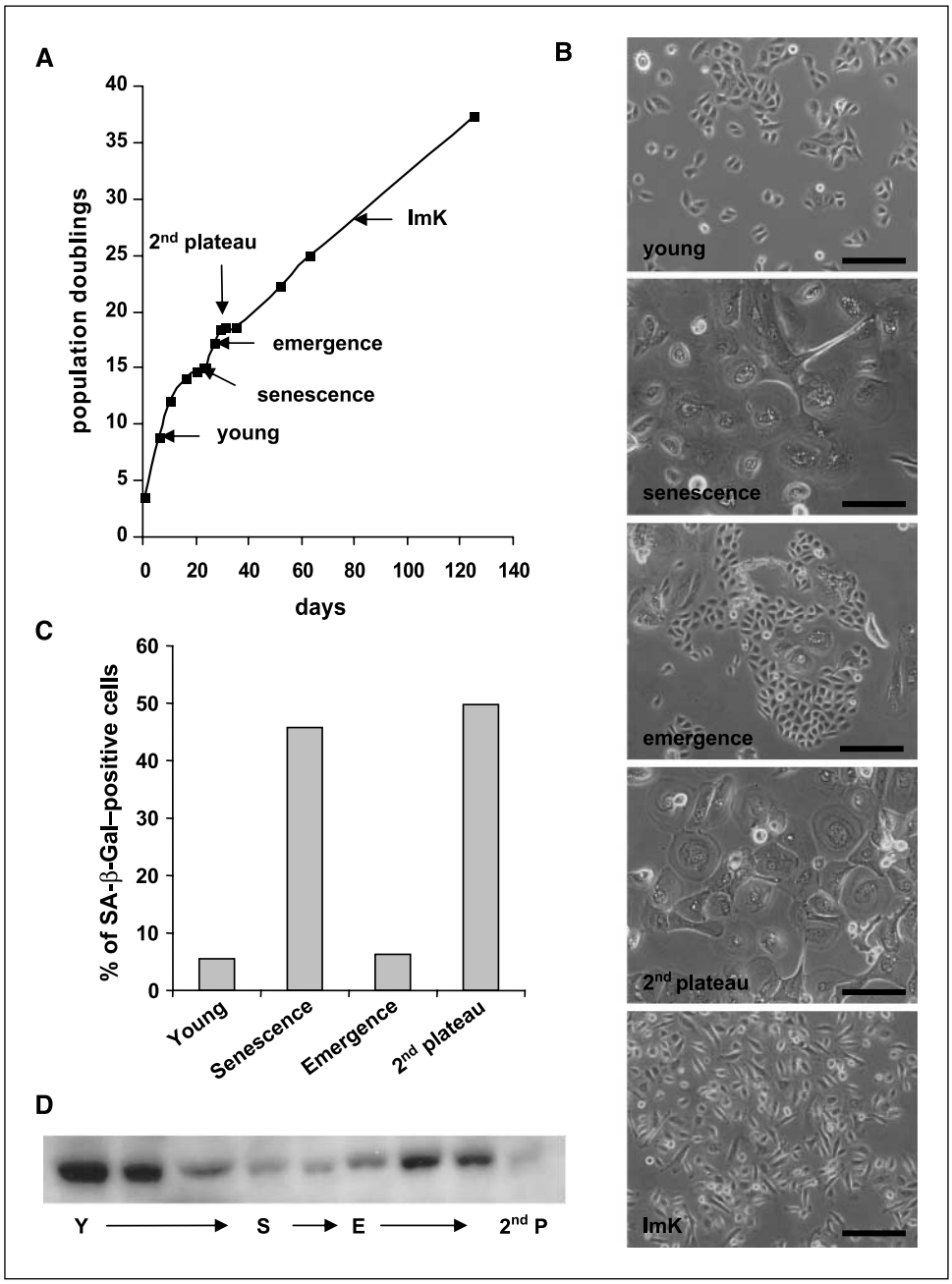

Figure 1. Senescence and emergence of NHEKs. *A,* growth curve. *B,* cell morphologies observed by phase-contrast microscopy. *Bar,* 80 μm. *C,* percentages of senescence-associated β-galactosidase–positive cells counted among 300 total cells. *D,* Western blot analysis of proliferating cell nuclear 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. 3*A*). Macroscopically, these lesions resembled early nonmelanoma skin carcinomas (Fig. 3*B*). 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. 3*B*). 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. 3*C*).

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 fluorigenic filiation tracers (Vybrant diI or Vybrant CFDA SE). After ~1 week, emerging cells arose around some senescent cells and were stained by the fluorigenic 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. S4*A*). 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. S4*B* 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. S6*A*). Moreover, we observed some typical large polynucleated senescent cells with one nucleus in metaphase after a colcemid treatment (Supplementary Fig. S6*B*). Taken together,

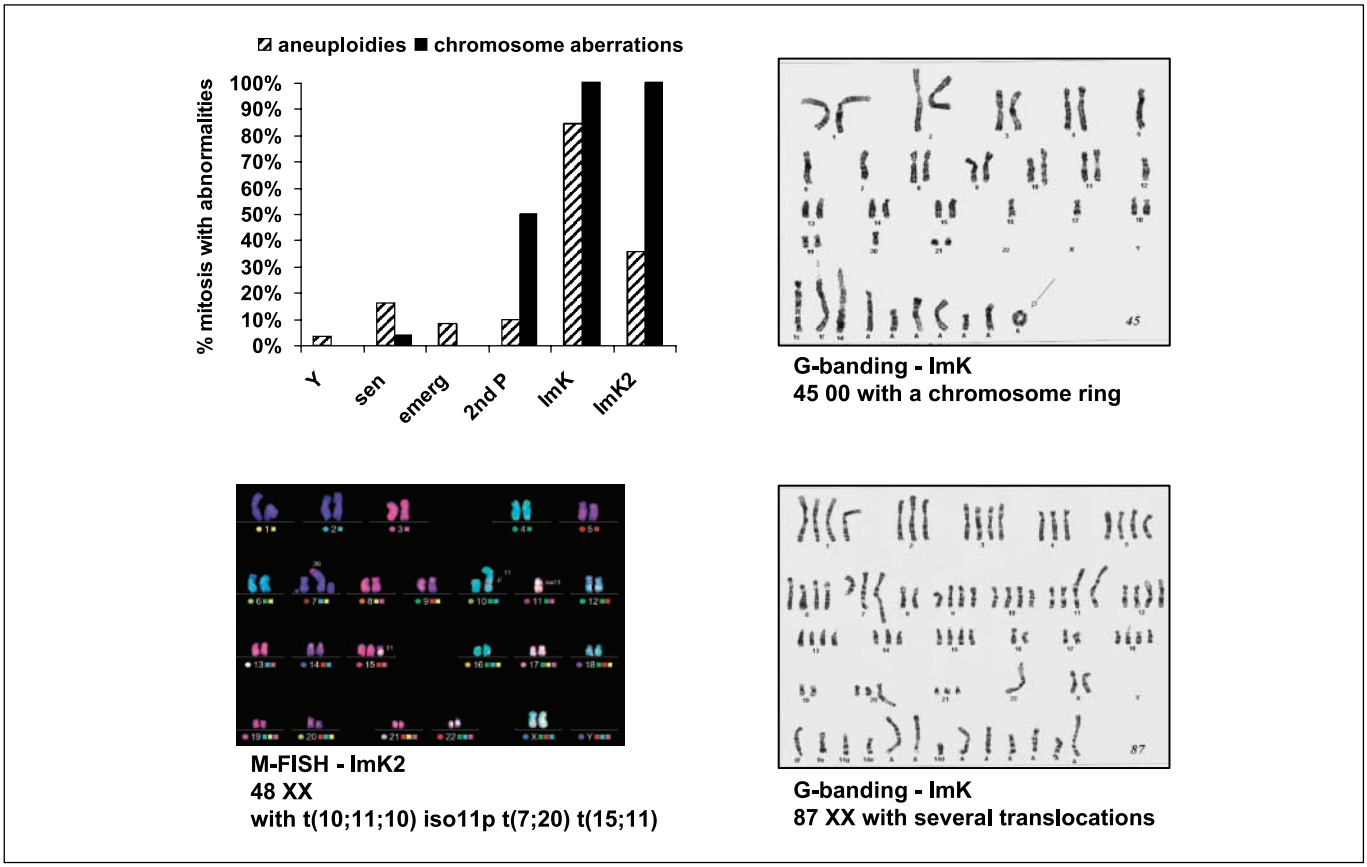

Figure 2. Karyotypic analysis of NHEKs at the different growth phases. Eleven to 28 metaphases from young (*Y*), senescent (*sen*), post-senescent–emerging (*emerg*), second plateau (*2nd P*), lmK, and lmK2 cells were analyzed by G-banding and/or multiplex fluorescence *in situ* hybridization (*M-FISH*). "Aneuploidies" regroups polyploidies and less severe chromosome gain or loss; "chromosome aberrations" regroups translocations, fusions, chromosome rings, and chromosome minutes. Photographs of representative lmK and lmK2 karyotypes.

A

Injected cells	Pre-senescent	PS-emerging cells	ImK	MDA-MD-231	NIH-3T3
Number of mice with disseminated skin lesions	1/6	4/5	6/6	0/1	0/2

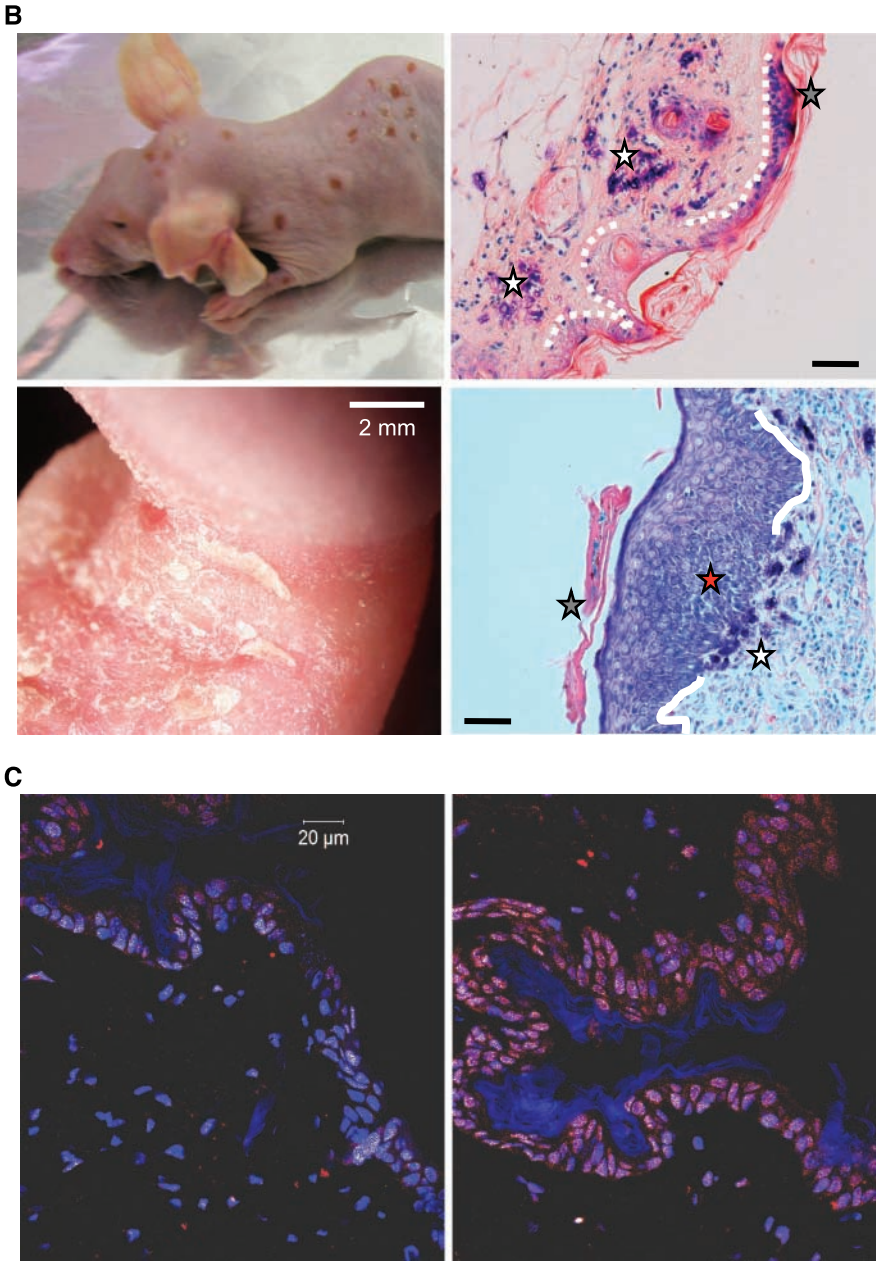

Figure 3. *In vivo* tumorigenicity of post-senescent–emerging cells and ImKs. Eight hundred thousand of either pre-senescent, post-senescent (*PS*)–emerging cells, ImKs, MDA-MB-231, or NIH-3T3 embedded in a plug of collagen/Matrigel were injected in the flank of 18-week-old female BALB/c nude mice. *A,* 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 μm.

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

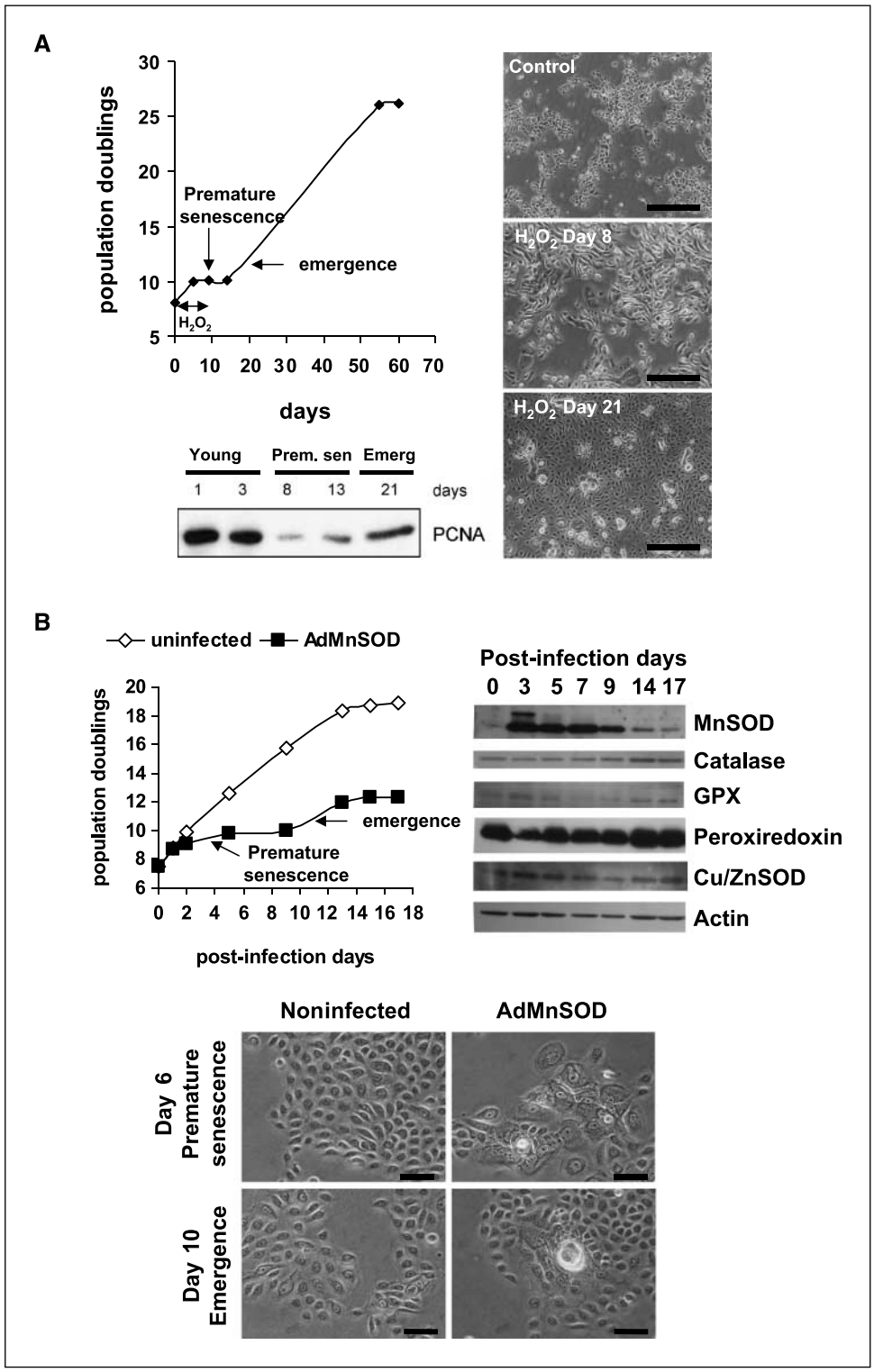

Figure 4. H_2O_2 is a motor of post-senescence emergence. *A,* young NHEKs were treated with 30 μmol/L H_2O_2 during 2 h every 3 d until displaying the senescent phenotype (9-day total treatment). The treatment was then stopped and emergence was monitored. Growth curve, proliferating cell nuclear antigen (*PCNA*) expression, and representative images of cell morphologies. *Bar,* 400 μm. *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 μm.

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. S7*A*). 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. S7*B*). 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. S7*C*). 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-κB > 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-κB 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-κB > 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 ~10^{-4} (Fig. 4*A*). 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. 4*B*). We finally examined whether antioxidants or NF-κB 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-κB 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 μmol/L) *N-tert*-butyl-hydroxylamine–treated cells, and 0% of the wells containing NF-κB inhibitors (Fig. 5).

Taken together, these results show that a H_2O_2 accumulation resulting from an activation of NF-κB 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 ~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. 6*A*). Accordingly, a catalase treatment decreased ~2-fold the number of SSB per cell (Fig. 6*B*). 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, ~20% of senescent cells were found to display 8-oxoG, in their cytoplasm and nucleus, compared with only 3% of young cells (Fig. 6*C*). When cells were treated with catalase, the percentage of affected senescent cells was reduced 4.25-fold (Fig. 6*C*). Conversely, treatment of young cells with H_2O_2 increased the percentage of 8-oxoG–positive cells ~5-fold (Fig. 6*C*). 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

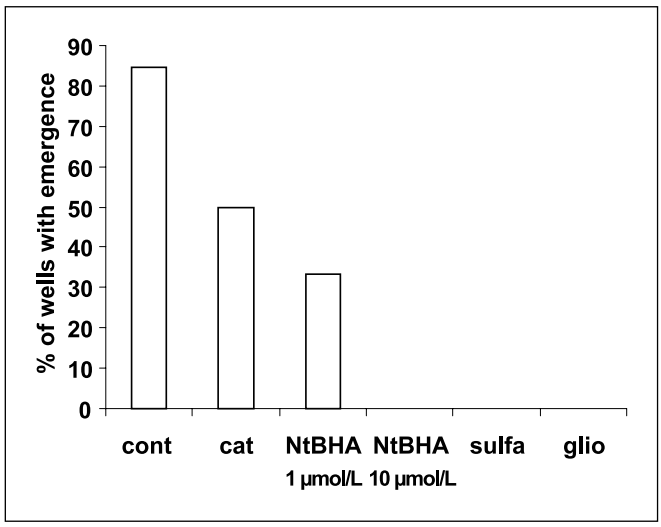

Figure 5. Antioxidants and NF-κB inhibitors inhibit post-senescence emergence. Pre-senescent NHEKs were seeded into 24-well culture plates at the limit density for emergence (10,000 cells per well) and treated or not with catalase (*cat*) at 100 units/mL, *N-tert*-butyl-hydroxylamine (*NtBHA*) at 1 or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

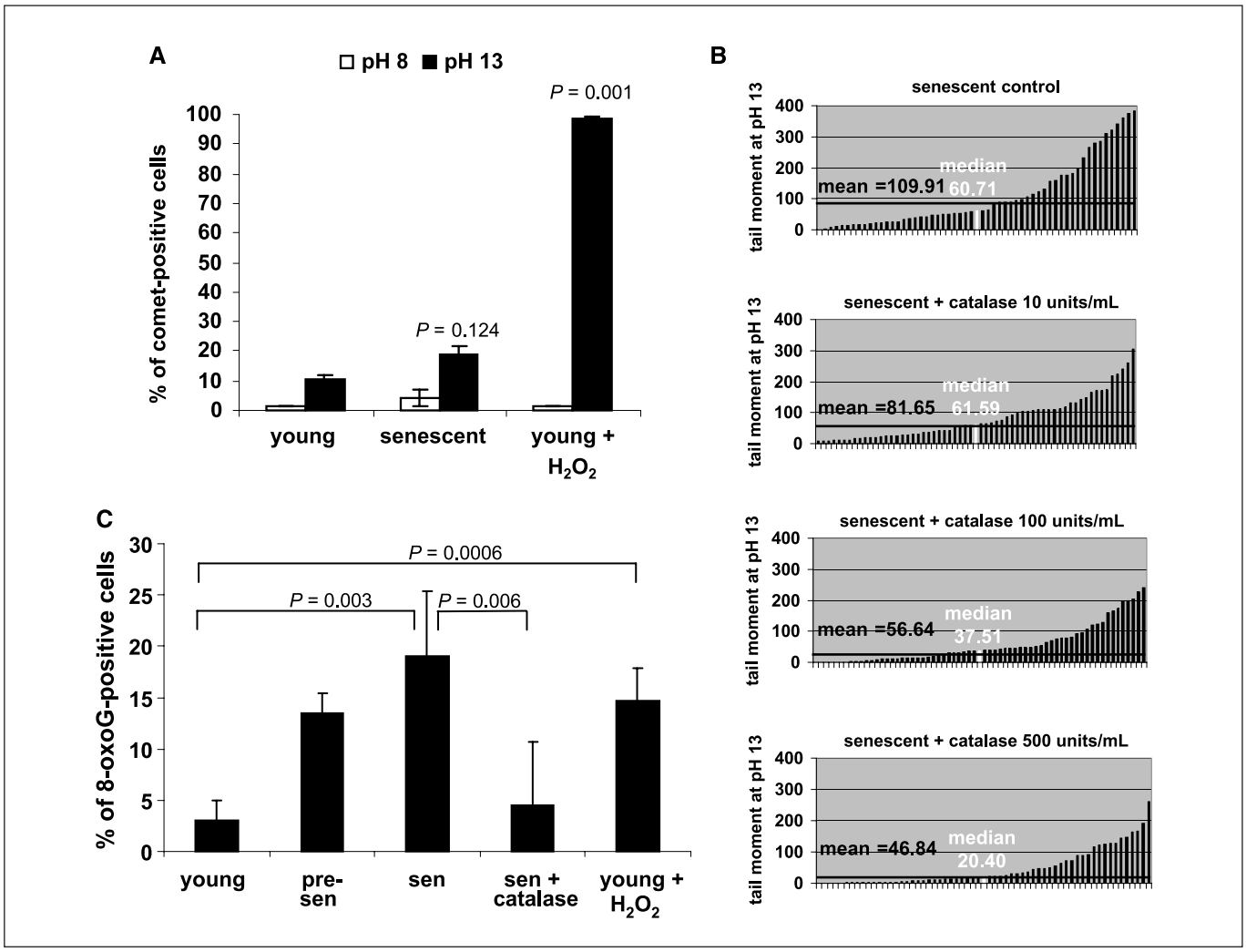

Figure 6. Post-senescence emergence is linked to the level of mutagenic oxidative DNA damage in senescent cells. *A,* comet assays on young and senescent NHEKs treated or not with 30 μmol/L H_2O_2 done at pH 8 to detect DSB and at pH 13 to detect both DSB and SSB. Comet-positive cells were independently counted twice. Mean ± 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 μmol/L H_2O_2 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 ± 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-senescent–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-κB > MnSOD > H_2O_2 oxidative stress pathway is causal in both senescence and emergence. This conclusion is based on the observations that NF-κB inhibitors and antioxidants delay the occurrence of the senescence plateau and decrease the emergence frequency, whereas, conversely, NF-κB or MnSOD overexpression or H_2O_2 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 H_2O 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.

Acknowledgments

Received 7/3/08; revised 6/30/09; accepted 8/12/09; published OnlineFirst 10/13/09.

Grant support: PPF Bioinformatique of Lille 1 University, Association pour la Recherche contre le Cancer, Ligue contre le Cancer, Conseil Régional NPdC, European Regional Development Fund, European Integrated Project RISC-RAD (FI6R-CT2003-508842), and Contract EDF V3-103. Institut Pasteur de Lille, Région NPdC, and Société Française du Cancer (K. Gosselin); Ministry of Research and FRM (S. Martien); Institut Pasteur de Lille (N. Malaquin); CEA (P. Ostoich); and Association pour la Recherche contre le Cancer (C. T'Kint de Roodenbeke).

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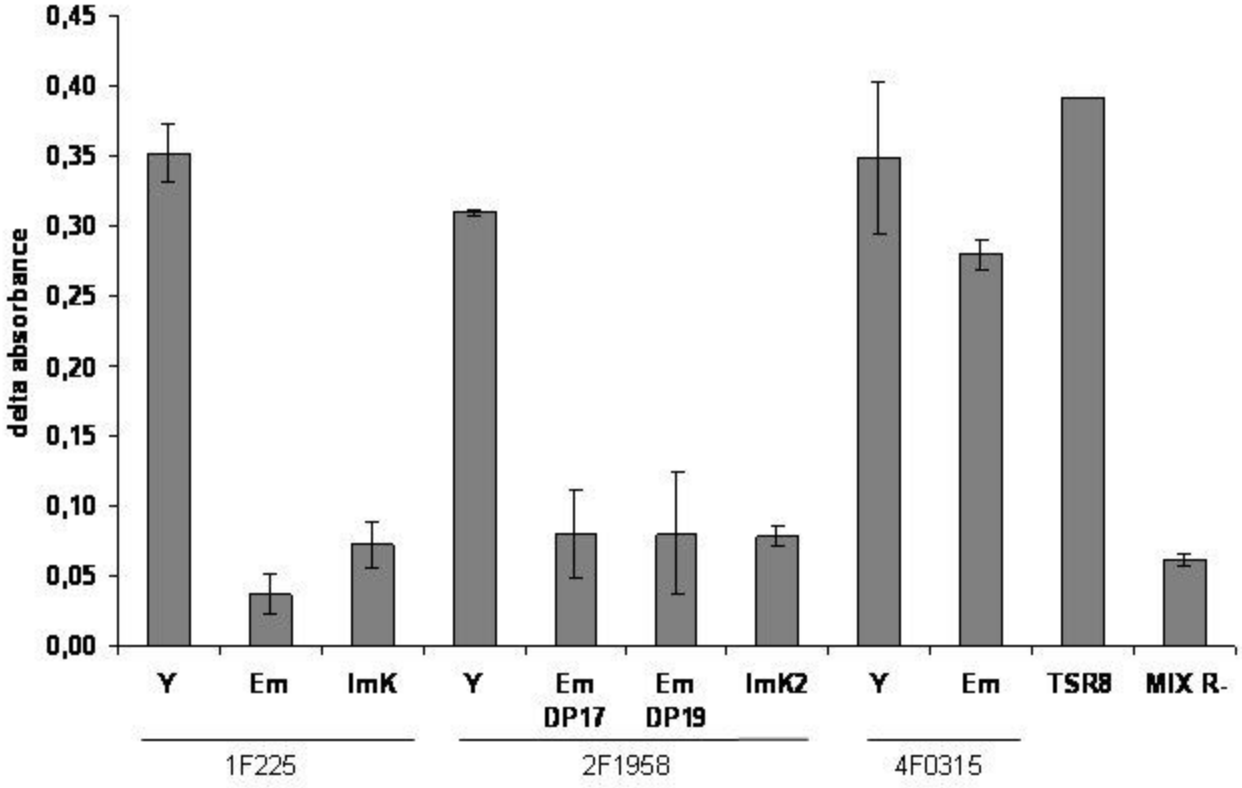
We thank Fabrice Nesslany for technical advice on comet assays, Nathalie Jouy and Brigitte Quatannens (FACS Facility of IMPRT-IFR114 and IBL, respectively), Didier Deslee, Elisabeth Werkmeister, and Antonino Bongiovanni (Microscopy Platform at the Institut Pasteur de Lille), and Géraldine Pottier and Debbie Williams for excellent technical help.

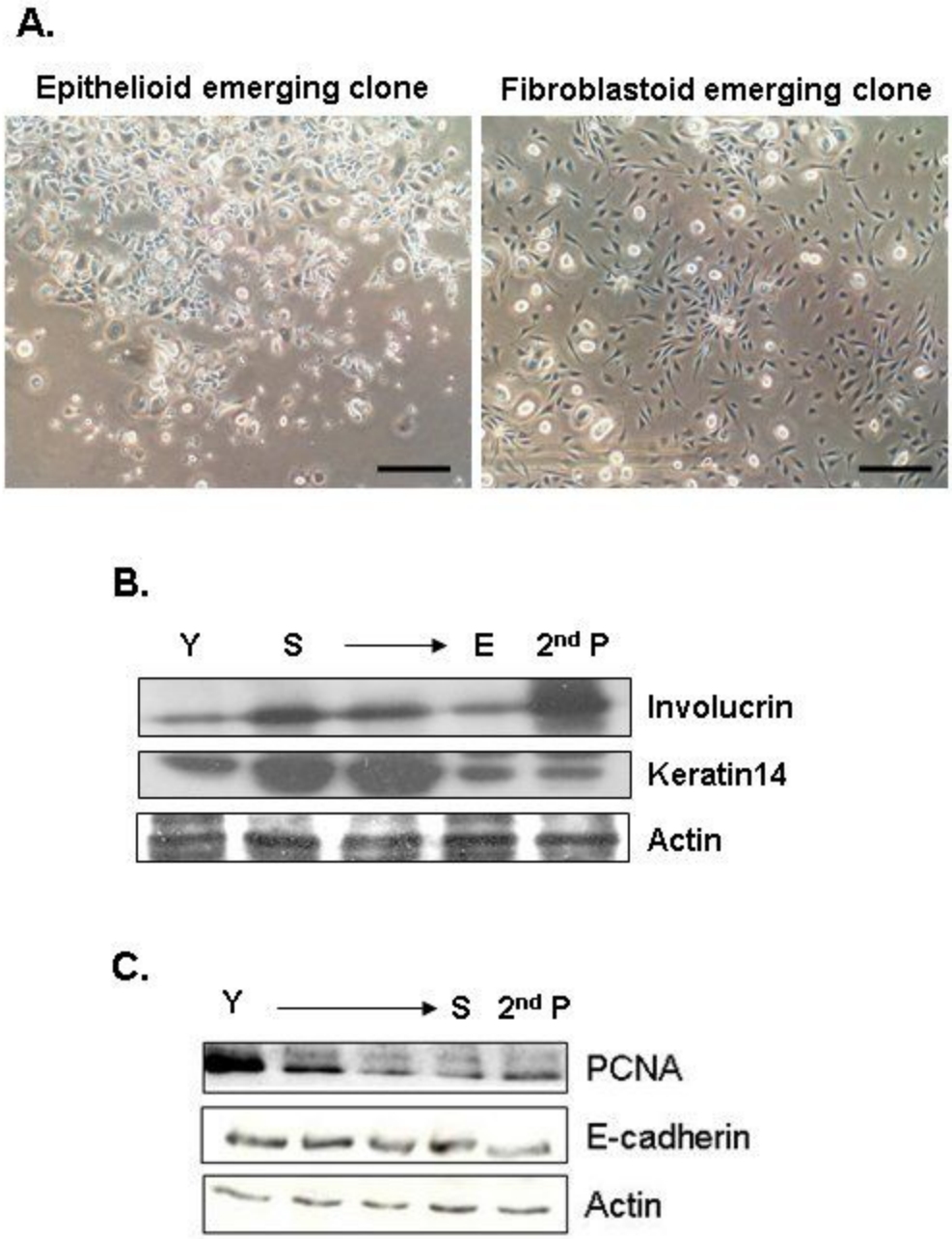
References

1. Hayflick L. The limited *in vitro* lifetime of human diploid cell strains. Exp Cell Res 1965;37:614–36.
2. Smith JR, Pereira-Smith OM. Replicative senescence: implications for *in vivo* aging and tumor suppression. Science 1996;273:63–7.
3. Cristofalo VJ, Lorenzini A, Allen RG, Torres C, Tresini M. Replicative senescence: a critical review. Mech Ageing Dev 2004;125:827–48.
4. Gosselin K, Deruy E, Martien S, et al. Senescent keratinocytes die by autophagic programmed cell death. Am J Pathol 2009;174:423–35.
5. Young AR, Narita M, Ferreira M, et al. Autophagy mediates the mitotic senescence transition. Genes Dev 2009;23:798–803.
6. Cuervo AM, Dice JF. When lysosomes get old. Exp Gerontol 2000;35:119–31.
7. Brunk UT, Jones CB, Sohal RS. A novel hypothesis of lipofuscinogenesis and cellular aging based on interactions between oxidative stress and autophagocytosis. Mutat Res 1992;275:395–403.
8. Chen Q, Fischer A, Reagan JD, Yan LJ, Ames BN. Oxidative DNA damage and senescence of human diploid fibroblast cells. Proc Natl Acad Sci U S A 1995; 92:4337–41.
9. Vaziri H, Benchimol S. From telomere loss to p53 induction and activation of a DNA-damage pathway at senescence: the telomere loss/DNA damage model of cell aging. Exp Gerontol 1996;31:295–301.
10. Lo AW, Sabatier L, Fouladi B, et al. DNA amplification by breakage/fusion/bridge cycles initiated by spontaneous telomere loss in a human cancer cell line. Neoplasia 2002;4:531–8.
11. Campisi J. Cellular senescence as a tumor-suppressor mechanism. Trends Cell Biol 2001;11:S27–31.
12. Puzianowska-Kuznicka M, Kuznicki J. Genetic alterations in accelerated ageing syndromes. Do they play a role in natural ageing? Int J Biochem Cell Biol 2005;37: 947–60.
13. Meydani M, Lipman RD, Han SN, et al. The effect of long-term dietary supplementation with antioxidants. Ann N Y Acad Sci 1998;854:352–60.
14. Boyce ST, Ham RG. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. J Invest Dermatol 1983;81:33–40s.
15. Dimri GP, Lee X, Basile G, et al. A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. Proc Natl Acad Sci USA 1995;92:9363–7.
16. Chartier C, Degryse E, Gantzer M, et al. Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. J Virol 1996;70:4805–10.
17. Schiedner G, Hertel S, Kochanek S. Efficient transformation of primary human amniocytes by E1 functions of Ad5: generation of new cell lines for adenoviral vector production. Hum Gene Ther 2000;11: 2105–16.
18. Shay JW, Wright WE. Quantitation of the frequency of immortalization of normal human diploid fibroblasts by SV40 large T-antigen. Exp Cell Res 1989;184:109–18.
19. Huschtscha LI, Holliday R. Limited and unlimited growth of SV40-transformed cells from human diploid MRC-5 fibroblasts. J Cell Sci 1983;63:77–99.
20. Dickson MA, Hahn WC, Ino Y, et al. Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. Mol Cell Biol 2000;20: 1436–47.
21. Bringold F, Serrano M. Tumor suppressors and oncogenes in cellular senescence. Exp Gerontol 2000; 35:317–29.
22. Darbro BW, Schneider GB, Klingelhutz AJ. Coregulation of p16INK4A and migratory genes in culture conditions that lead to premature senescence in human keratinocytes. J Invest Dermatol 2005;125:499–509.
23. Bernard D, Gosselin K, Monte D, et al. Involvement of Rel/NF-κB transcription factors in keratinocyte senescence. Cancer Res 2004;64:472–81.
24. Atamna H, Paler-Martinez A, Ames BN. N-t-butyl hydroxylamine, a hydrolysis product of α-phenyl-*N*-*t*-butyl nitrone, is more potent in delaying senescence in human lung fibroblasts. J Biol Chem 2000;275:6741–8.
25. Cunningham ML, Peak JG, Peak MJ. Single-strand DNA breaks in rodent and human cells produced by superoxide anion or its reduction products. Mutat Res 1987;184:217–22.
26. Baker MA, He SQ. Elaboration of cellular DNA breaks by hydroperoxides. Free Radic Biol Med 1991;11:563–72.
27. Sekiguchi M, Tsuzuki T. Oxidative nucleotide damage: consequences and prevention. Oncogene 2002;21: 8895–904.
28. Huschtscha LI, Noble JR, Neumann AA, et al. Loss of p16INK4 expression by methylation is associated with lifespan extension of human mammary epithelial cells. Cancer Res 1998;58:3508–12.
29. Romanov SR, Kozakiewicz BK, Holst CR, et al. Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes. Nature 2001;409:633–7.
30. Garbe JC, Holst CR, Bassett E, Tlsty T, Stampfer MR. Inactivation of p53 function in cultured human mammary epithelial cells turns the telomere-length dependent senescence barrier from agonescence into crisis. Cell Cycle 2007;6:1927–36.
31. Sundaram M, Guernsey DL, Rajaraman MM, Rajaraman R. Neosis: a novel type of cell division in cancer. Cancer Biol Ther 2004;3:207–18.
32. Walen KH. Spontaneous cell transformation: karyoplasts derived from multinucleated cells produce new cell growth in senescent human epithelial cell cultures. In Vitro Cell Dev Biol Anim 2004;40:150–8.
33. Holst CR, Nuovo GJ, Esteller M, et al. Methylation of p16(INK4a) promoters occurs *in vivo* in histologically normal human mammary epithelia. Cancer Res 2003;63: 1596–601.
34. Caldecott KW. Protein-protein interactions during mammalian DNA single-strand break repair. Biochem Soc Trans 2003;31:247–51.
35. Cheng KC, Cahill DS, Kasai H, Nishimura S, Loeb LA. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G-T and A-C substitutions. J Biol Chem 1992;267:166–72.
36. Busuttil RA, Rubio M, Dolle ME, Campisi J, Vijg J. Oxygen accelerates the accumulation of mutations during the senescence and immortalization of murine cells in culture. Aging Cell 2003;2:287–94.
37. Greenhalgh DA, Welty DJ, Strickland JE, Yuspa SH. Spontaneous Ha-ras gene activation in cultured primary murine keratinocytes: consequences of Ha-ras gene activation in malignant conversion and malignant progression. Mol Carcinog 1989;2:199–207.
38. Choi J, Shendrik I, Peacocke M, et al. Expression of senescence-associated β-galactosidase in enlarged prostates from men with benign prostatic hyperplasia. Urology 2000;56:160–6.
39. Michaloglou C, Vredeveld LC, Soengas MS, et al. BRAFE600-associated senescence-like cell cycle arrest of human naevi. Nature 2005;436:720–4.



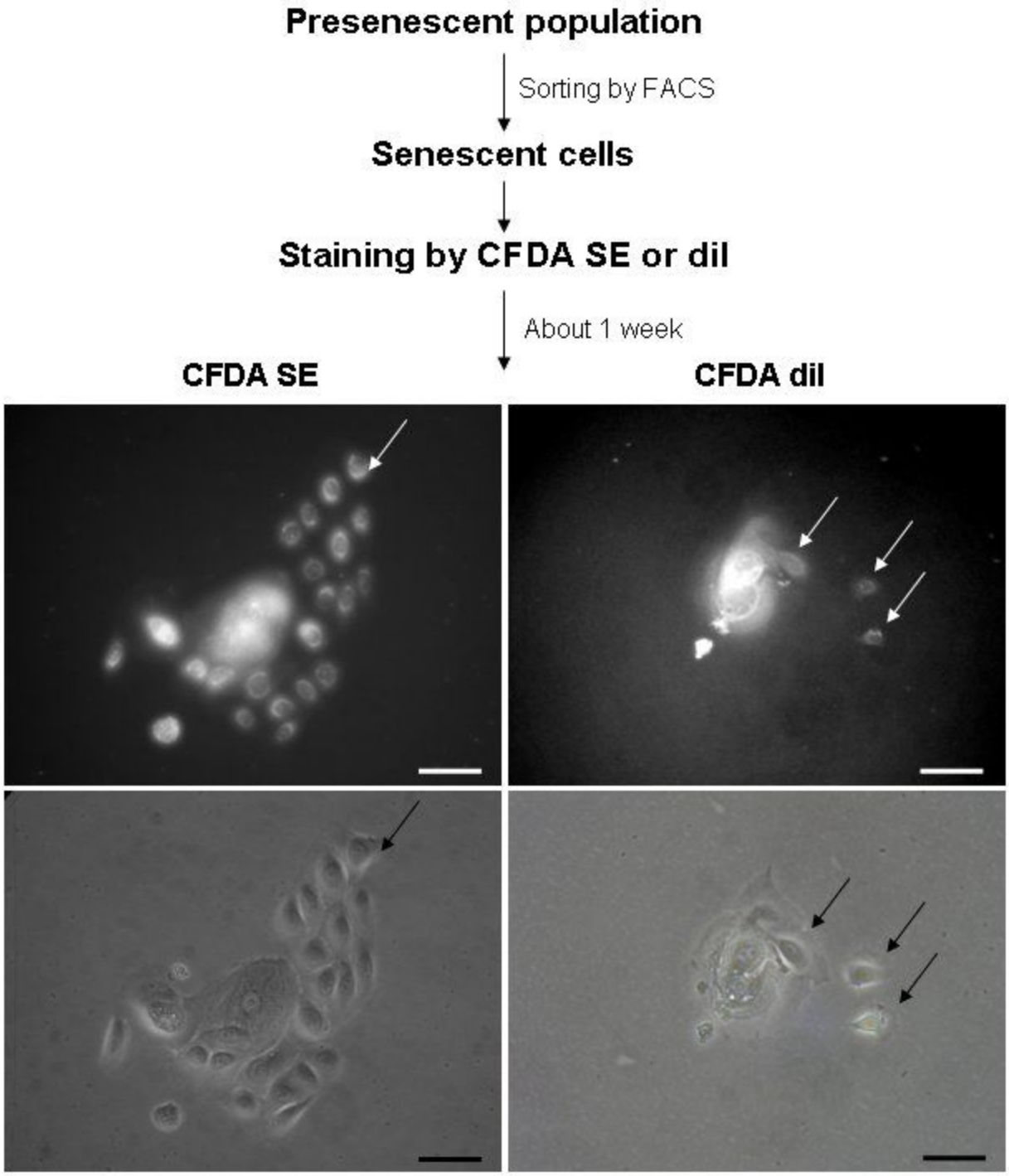
Supplementary Figure 1: Telomerase activity in NHEKs at the different growth stages

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. TRS8 is a positive internal control for PCR/ELISA. MIX R- is a negative control without cell extract.



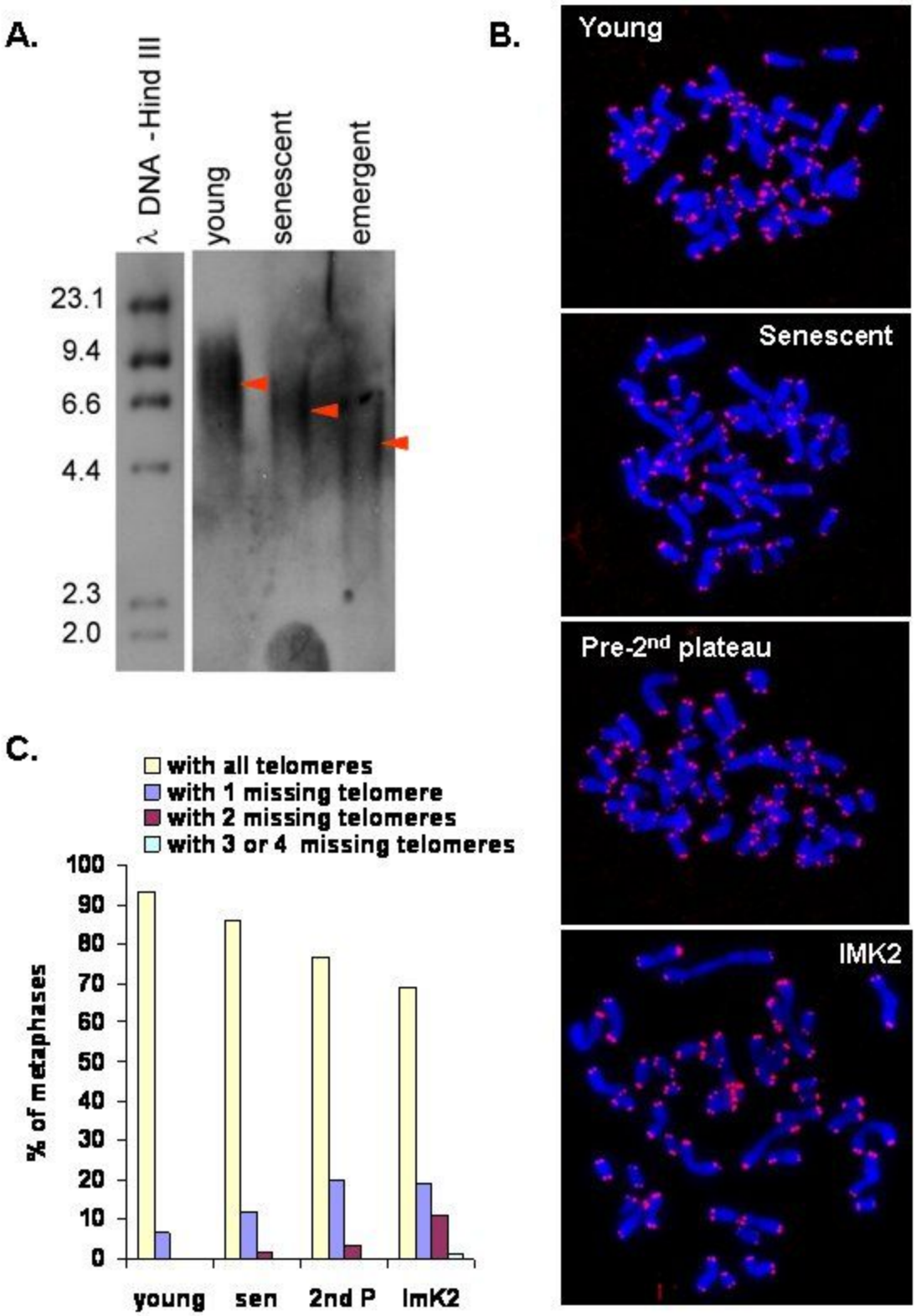
Supplementary Figure 2: Post-senescent emergent cells are partially transformed

(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 60µm. (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.



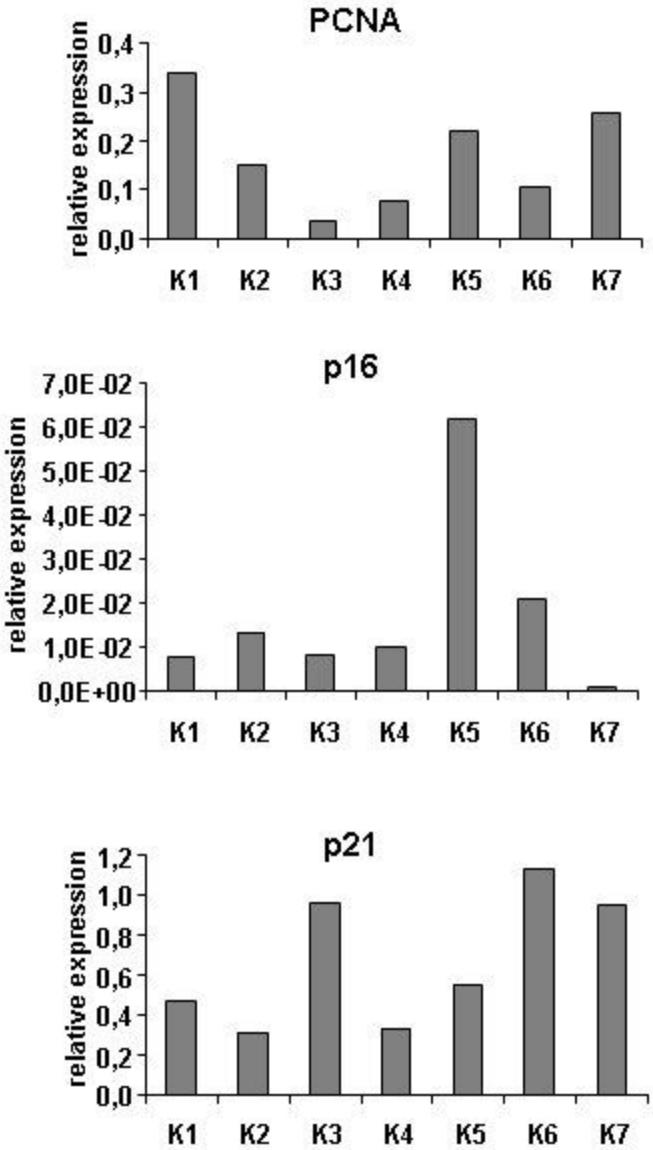
Supplementary Fig.3: Evidence of filiation between senescent and PS emergent cells

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.

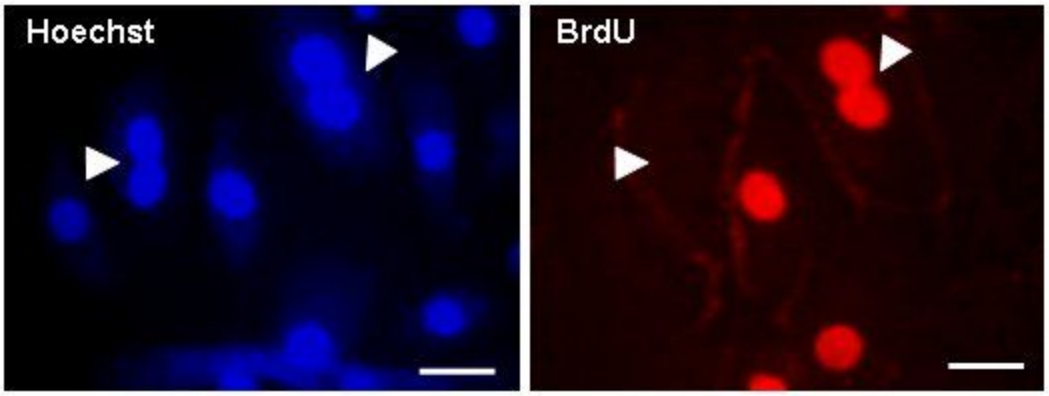


K1: young
K2: presenescent
K3: senescent
K4: end of senescence and beginning of PS emergence
K5: end of PS emergence and beginning of 2nd plateau
K6: 2nd plateau
K7: lmK

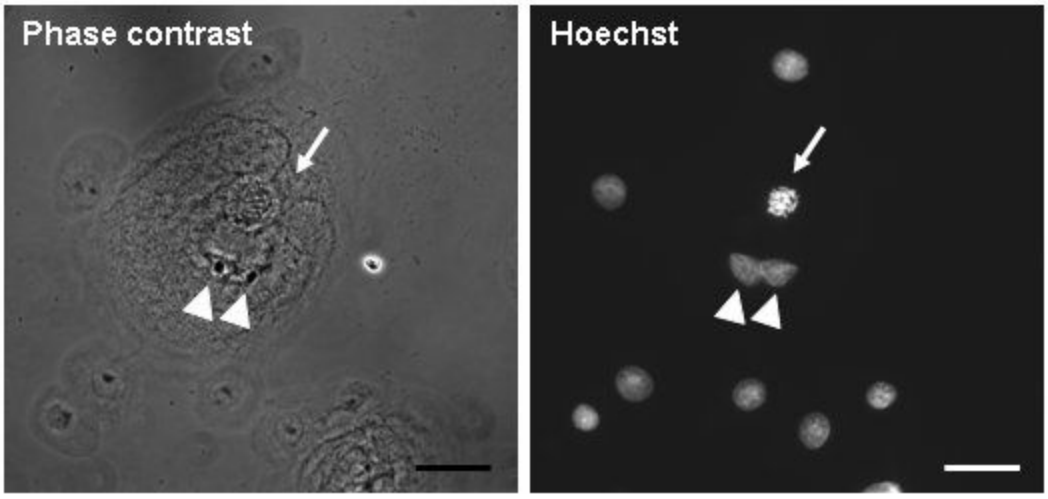
Supplementary Figure 5: Expression of p16 and p21 at the different growth phases

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

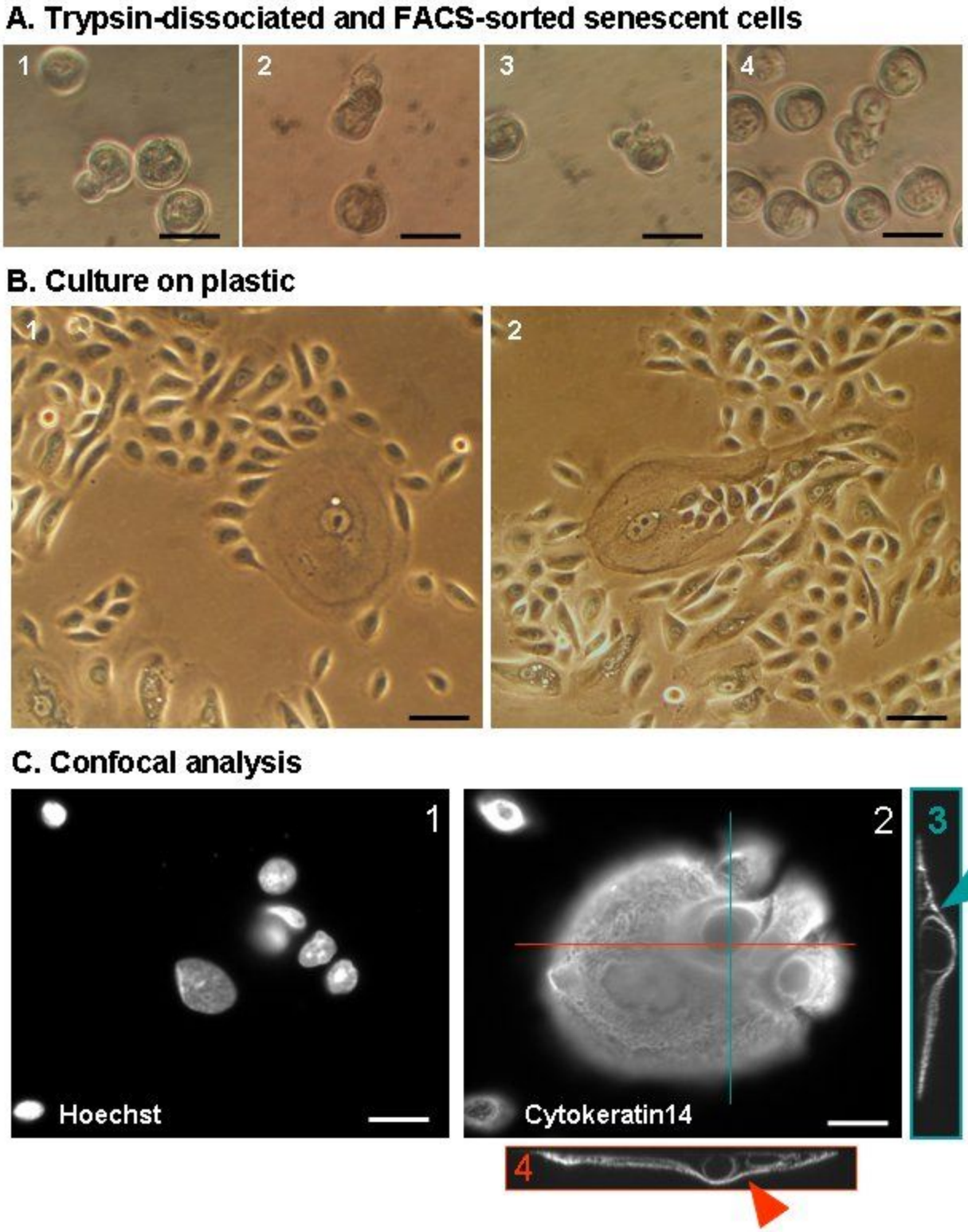


B. After colcemid



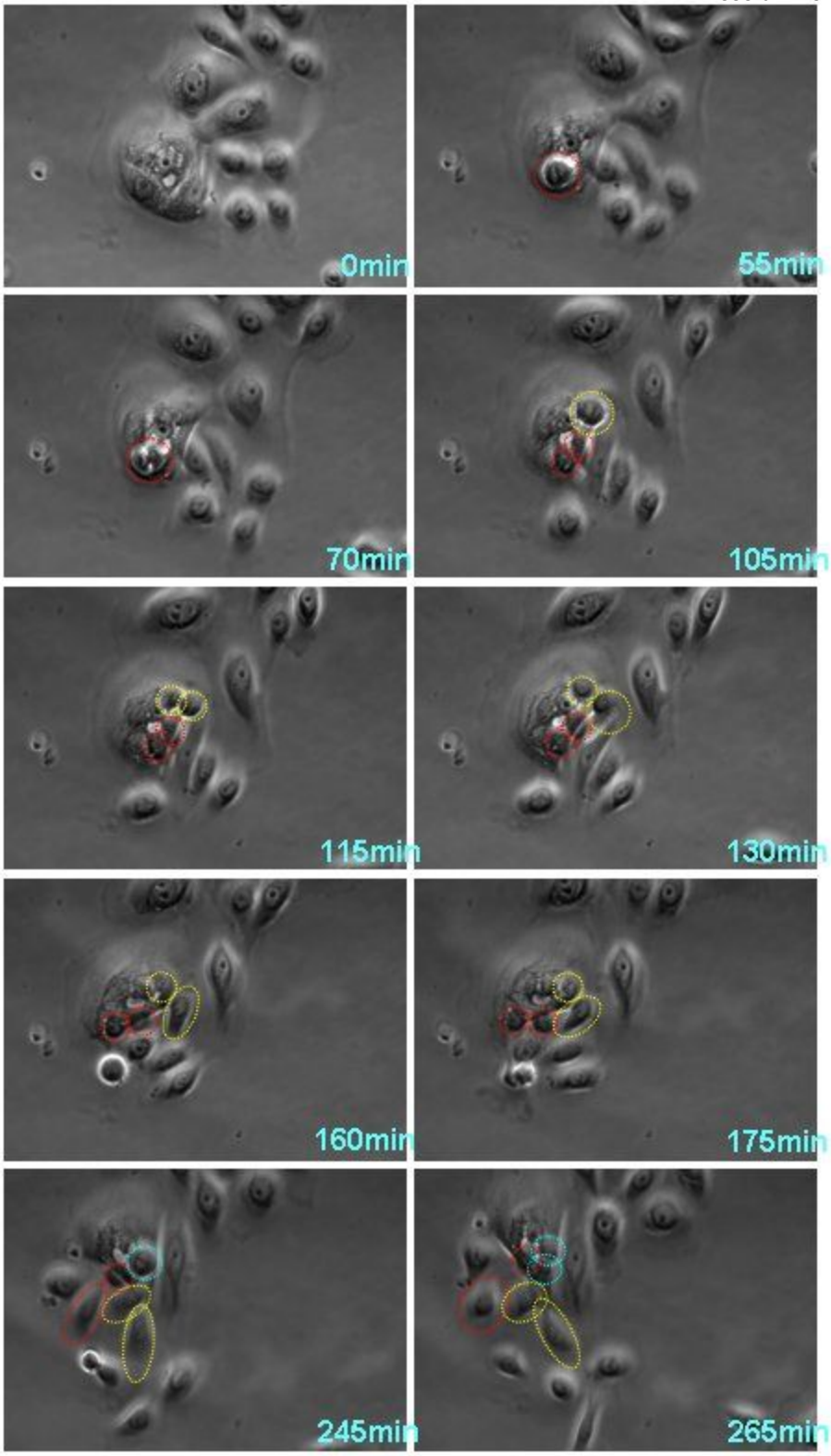
Supplementary Figure 6: Senescent keratinocytes are still able to divide

(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 6µm. (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 15µm.



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 20µm.



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.

Partie II :

Sénescence et mort cellulaire programmée de type II

Les kératinocytes sénescents meurent par autophagie

Il est communément admis que l'acquisition d'une résistance à la mort cellulaire programmée est une étape précoce et nécessaire à la transformation cancéreuse. Aucun type de mort cellulaire n'ayant été clairement décrit associé à la sénescence, nous avons cherché dans un premier temps à déterminer comment meurent les kératinocytes sénescents qui n'évoluent pas vers une émergence néoplasique, afin de comprendre à quoi les cellules émergentes post-sénescence doivent échapper.

Nous avons tout d'abord estimé la proportion de cellules mourantes au plateau de sénescence par une approche classique de marquage à l'annexine V et à l'iodure de propidium, respectivement spécifiques de l'externalisation des phosphatidylsérines et de la perméabilisation membranaire. Nous avons ainsi mis en évidence qu'environ 20% des kératinocytes au plateau de sénescence étaient positifs pour l'un et/ou l'autre des marqueurs, montrant ainsi qu'un mécanisme de mort apoptotique et/ou non apoptotique était induit lors du plateau de sénescence.

Phénotypiquement, ces cellules mourantes se distinguent du reste des cellules sénescentes : elles s'arrondissent et présentent une zone centrale réfringente en microscopie à contraste de phase, accumulent de nombreuses vésicules et sont souvent isolées des cellules sénescentes voisines. Pour évaluer la cinétique du processus de mort impliqué, nous avons suivi des cellules sénescentes par vidéomicroscopie à contraste de phase. L'analyse des résultats montre que les cellules réfringentes en contraste de phase s'isolent progressivement de leurs voisines et relarguent de nombreux composés denses, probablement par exocytose, avant de se détacher du support. Contrairement à l'apoptose qui tue les cellules en quelques heures, le processus que nous avons enregistré dure environ trois jours.

Nous avons ensuite examiné au microscope le marquage annexine V des cellules mourantes. L'analyse a révélé que la membrane plasmique n'était pas la seule cible du marquage comme c'est le cas lors de l'apoptose, mais qu'une structure intracellulaire périnucléaire, correspondant à la limite externe de la zone réfringente, était également

marquée. L'analyse du cytosquelette par immunofluorescence a révélé que cette zone centrale comprenant le noyau et probablement une partie des organites est délimitée par une cage de cytokératine. Aucun des marqueurs d'apoptose (activation des caspases, relargage du cytochrome C par les mitochondries, fragmentation internucléosomique de l'ADN…) ne s'est révélé mis en place durant la mort des NHEKs sénescents. Enfin, l'utilisation d'inhibiteur d'apoptose comme le zVAD (un inhibiteur des caspases) n'a pas eu d'effet sur l'incidence d'apparition de ces cellules mourantes. L'ensemble de ces résultats indiquent que les kératinocytes sénescents ne meurent pas par apoptose.

En revanche, l'utilisation d'un inhibiteur de la macroautophagie, la 3-méthyladénine, qui cible l'étape de nucléation de l'autophagosome en inhibant l'activité de Vps34, s'est révélée efficace pour repousser l'apparition des cellules mourantes dans les cultures, démontrant un rôle important de la macroautophagie dans la mort des NHEKs lors de la sénescence. Nous avons alors orienté nos recherches vers la mise en évidence d'un processus de mort cellulaire programmée impliquant la macroautophagie. Nous avons dans un premier temps montré que les cellules sénescentes accumulent une masse compacte de vésicules cytoplasmiques acides détectées par marquage au Lysotracker et au monodansylcadavérine [394]. L'analyse des cellules sénescentes par microscopie électronique à transmission révèlera ensuite que ces vésicules sont principalement des autophagosomes et autolysosomes. L'analyse par microscopie électronique nous a également permis de préciser l'anatomie très particulière des cellules mourantes : elles présentent une aire corticale dépourvue d'organites, séparée d'une aire centrale contenant noyau et organites par une cage de cytokératine. Nous avons ensuite recherché l'expression de marqueurs moléculaires de la macroautophagie lors de la mort des kératinocytes sénescents. Nous avons notamment mis en évidence que l'accumulation de vésicules autophagiques peut être détectée par immunofluorescence de Atg8/LC3. Nous avons aussi confirmé l'augmentation de l'activation de l'autophagie par western blot en mettant notamment en évidence une augmentation de l'expression des protéines Atg6/Beclin1 et LAMP1 parallèle à une diminution très forte d'expression de l'inhibiteur d'autophagie Bcl2.

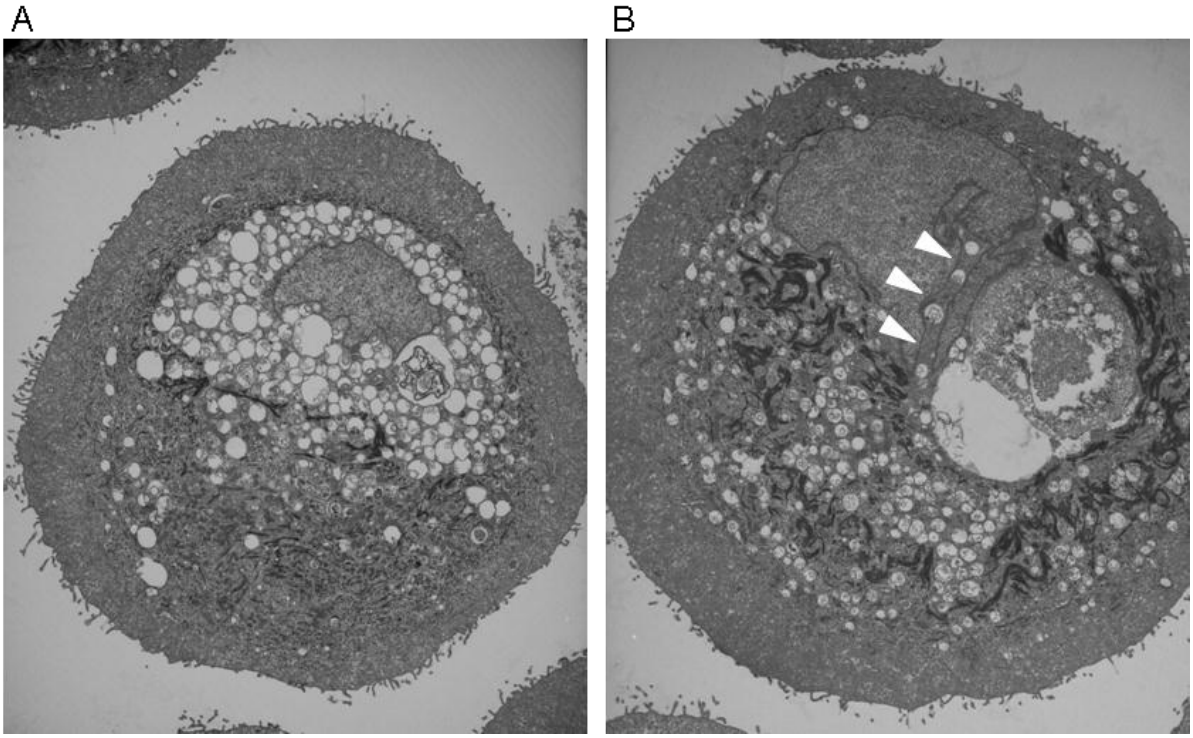


Figure 18 : Observation en microscopie électronique de l'impact de la macroautophagie sur le noyau lors de la sénescence *(données du laboratoire non publiées). L'accumulation de vésicules autophagiques déforme le noyau sénescent (A) allant jusqu'à créer des invaginations de son enveloppe (flèches blanches) (B).*

Nous avons donc conclu de cette étude que la sénescence s'accompagne d'une hyperactivation de la macroautophagie qui conduira à la mort des cellules. Ces travaux font l'objet de l'article n°2 : *Senescent keratinocytes die by autophagic programmed cell death.*

Travaux complémentaires concernant le mécanisme de mort par autophagie

Si nous avons pu décrire que les kératinocytes sénescents meurent par suractivation de la macroautophagie, aucune de ces expérimentations n'a cependant clairement mis en évidence le mécanisme précis par lequel ce processus tue les cellules.

Une hypothèse se dégage de l'analyse des informations structurales obtenues en microscopie électronique pour les cellules mourantes dans lesquelles tous les organites (notamment les vésicules autophagiques, les mitochondries altérées et le noyau) se retrouvent concentrés au centre de la cellule, retenus par une cage de cytokératine. Le noyau se retrouve alors en partie isolé du cytosol et comprimé par les vésicules autophagiques (Figure 18 A). Cet isolement, en limitant les communications nucléo-cytoplasmiques, pourrait suffire à entraîner la mort des cellules. Nous avons donc réalisé un certain nombre de vidéomicroscopies en fluorescence visant à suivre la répartition des vésicules autophagiques par marquage au Lysotracker durant la mort cellulaire associée à la sénescence. Nous avons alors pu mettre en évidence que le noyau va être comprimé par les vésicules autophagiques dans la cage de cytokératine en parallèle de l'acquisition du phénotype cadavérique (Figure 19). Ce phénotype semble ensuite relativement stable, du moins durant la durée d'observation.

Une autre hypothèse, non exclusive de la première, provient également de l'analyse attentive de l'ultrastructure des cellules sénescentes par microscopie électronique en transmission. On observe en effet que l'accumulation des vésicules autour du noyau le déforme énormément, créant parfois des invaginations de l'enveloppe nucléaire, invaginations dans lesquelles on retrouve des vésicules autophagiques (Figure 18 B). Ces altérations physiques du noyau sénescent pourraient aussi causer la mort des cellules.

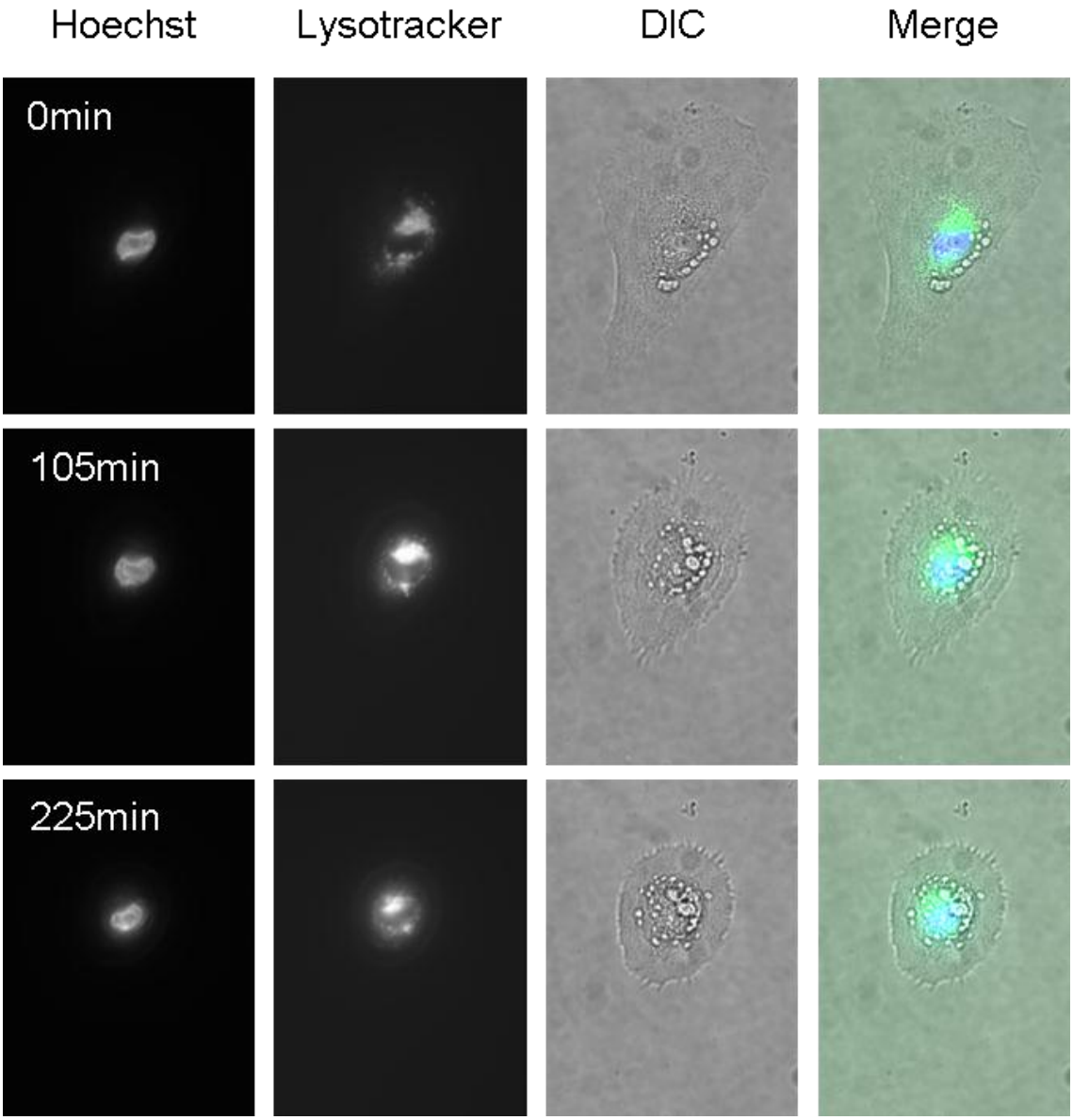


Figure 19 : Compression du noyau sénescent et des vésicules autophagiques lors de la mort par autophagie *(données non publiées). Observation en vidéomicroscopie en temps réel d'une cellule sénescente mononuclée évoluant vers la mort. La chromatine et les vésicules autophagiques ont respectivement été colorées au Hoechst vital 33342 (bleu) et au Lysotracker (vert). A T0, la cellule sénescente étalée sur le support présente un noyau altéré et un grand nombre de vésicules acides. Au cours du temps, les vésicules se concentrent autour du noyau en parallèle à l'acquisition du phénotype mourant (arrondissement). A 225min, on observe parfaitement la délimitation entre la zone corticale et la zone centrale contenant noyau et vésicules. Compressé par les vésicules au sein de cette zone centrale, le noyau est de plus en plus altéré au cours du temps. La cellule sénescente conservera ces caractéristiques phénotypiques de cellule mourante jusqu'à T480min (non montré).*

Nous avons également suivi l'activité autophagique des cellules sénescentes grâce à la transfection d'une protéine de fusion Atg8/LC3B couplée à une eGFP et une mRFP en N-Terminal [395]. Dans cette protéine de fusion, seule la partie mRFP, rouge, résiste au pH acide lysosomial. La protéine de fusion permet donc de suivre par imagerie cellulaire la maturation des vésicules autophagiques au cours du temps, qui se manifeste par une évolution d'un double marquage vert-rouge (pour les autophagosomes) vers un marquage rouge uniquement (pour les autolysosomes). Les cellules transfectées ont été analysées par microscopie confocale. Nous avons alors pu mettre en évidence une colocalisation des vésicules autophagiques matures (rouge) avec la chromatine (colorée au Hoechst) (Figure 20), ce qui suggère la présence de vésicules autophagiques dans le noyau. En plus de son isolement et de l'altération de sa structure, il pourrait donc y avoir une dégradation directe du noyau par macroautophagie.

Ces hypothèses sont uniquement basées sur des observations et nécessitent encore de nombreux travaux complémentaires pour être confirmées ou infirmées. Elles sont cependant appuyées par les travaux de deux équipes qui ont mis en évidence que le noyau pouvait, comme les autres organites, être la cible d'une dégradation par autophagie [396,397]. Dans ces études, Kovacs *et al* ont en effet décrit en 2000 qu'un large autophagosome peut être formé autour du noyau de cellules murines pour le dégrader, et Roberts *et al,* en 2003, montrent chez *Saccharomyces cerevisae* que le noyau peut être dégradé portion par portion directement par la vacuole, par microautophagie.

L'ensemble de ces travaux va à l'encontre des théories développées par l'équipe de Brunk selon lesquelles les capacités de dégradation des lysosomes décroient avec la sénescence et que c'est cette déficience qui entraîne la mort cellulaire [21]. Ici, avec l'accumulation de vésicules autophagiques rouges dans les cellules exprimant la protéine de fusion mRFPeGFPLC3 et avec les expériences d'inhibition à la 3-méthyladénine, nous avons mis en évidence que non seulement la macroautophagie est bien active dans les cellules sénescentes mais également qu'elle est responsable de la mort des NHEKs. Quelques mois après la publication de nos travaux, l'équipe de Narita décrivait que la sénescence de fibroblastes induite par l'expression de *ras* s'accompagne également d'une macroautophagie active, mise en évidence par l'augmentation de la

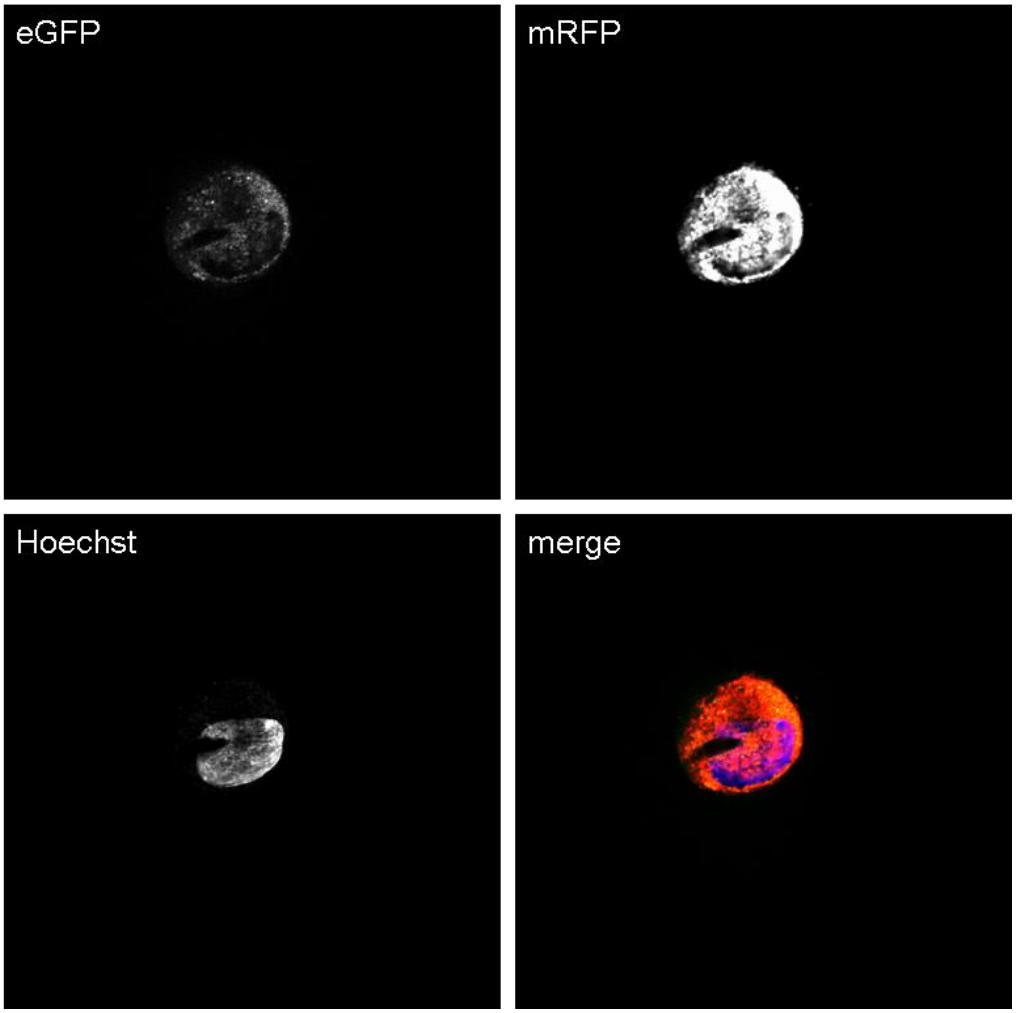


Figure 20 : Observation de l'activité autophagique dans une cellule mourante exprimant la protéine de fusion eGFPmRFPLC3 *en microscopie confocale mettant en évidence la présence d'autolysosomes (rouge) dans le noyau (bleu).*

dégradation des protéines à longue durée de vie [398,399].

Article n°2 : Senescent keratinocytes die by autophagic programmed cell death

The American Journal of Pathology, Vol. 174, No. 2, February 2009

DOI: 10.2353/ajpath.2009.080332

Cell Injury, Repair, Aging and Apoptosis

Senescent Keratinocytes Die by Autophagic Programmed Cell Death

Karo Gosselin,* Emeric Deruy,* Sébastien Martien,* Chantal Vercamer,* Fatima Bouali,* Thibault Dujardin,* Christian Slomianny,† Ludivine Houel-Renault,* Fazia Chelli,* Yvan De Launoit,* and Corinne Abbadie*

From the UMR8161 Institut de Biologie de Lille, CNRS/Universités Lille1 et Lille2/Institut Pasteur de Lille, Lille Cedex; and the INSERM U800 Laboratoire de Physiologie Cellulaire,† Université Lille 1, Villeneuve d'Ascq Cedex, France*

Normal cells reach senescence after a specific time and number of divisions, leading ultimately to cell death. Although escape from this fate may be a requisite step in neoplastic transformation, the mechanisms governing senescent cell death have not been well investigated. We show here, using normal human epidermal keratinocytes, that no apoptotic markers appear with senescence. In contrast, the expression of several proteins involved in the regulation of macroautophagy, notably Beclin-1 and Bcl-2, was found to change with senescence. The corpses occurring at the senescence growth plateau displayed a large central area delimited by the cytokeratin network that contained a huge quantity of autophagic vacuoles, the damaged nucleus, and most mitochondria. 3-methyladenine, an inhibitor of autophagosome formation, but not the caspase inhibitor zVAD, prevented senescent cell death. We conclude that senescent cells do not die by apoptosis, but as a result of high macroautophagic activity that targets the primary vital cell components. ***(Am J Pathol 2009, 174:423–435; DOI: 10.2353/ajpath.2009.080332)***

Senescence is described as a tumor suppressor mechanism that limits proliferation of altered cells.[1] It occurs *in vivo* with advancing age, as well as in culture because of both telomere erosion and increasing oxidative damage.[2] Despite these alterations, senescent cells are not dead cells: they maintain a metabolic activity, different from that of young cells.[3] However, they remain in this state only for a while, and finally die. Although escape from this fate could be a requisite step in neoplastic transformation, how cells die once they have become senescent has not been much investigated, and the results remain controversial.

Cell death can occur accidentally, in response to an insult, or as the result of a genetic program, activated for example during development or in response to a specific signal. Death following senescence fits with the definition of programmed cell death, because (i) it is time-scheduled and (ii) senescent cells express a genetic program different from that of young cells.[4–11] Two main types of programmed cell death have been described: apoptosis (type I), and autophagic programmed cell death (type II). Apoptosis involves characteristic morphological and biochemical changes, including cytoplasmic and nuclear condensation, blebbing, chromatin condensation, oligonucleosomal DNA degradation, and final cell fragmentation into apoptotic bodies. Apoptosis is attributed mainly to caspase activation.[12] Autophagic programmed cell death is accompanied by an increase in macroautophagic activity and is believed to occur through the ensuing degradation of many vital cell components.[13,14] The macroautophagic process starts with sequestration of a damaged cell component by a double membrane whose origin is controversial.[15–17] The autophagosome resulting from closure of this membrane then fuses with lysosomes to form an autophagolysosome, inside which the sequestered material is degraded by hydrolytic enzymes at acidic pH. The formation and migration of all these ves-

Supported by the Centre National de la Recherche Scientifique, the Université Lille 1, the Association pour la Recherche sur le Cancer, the Ligue contre le Cancer (Comités du Nord et de l'Aisne), the Institut Pasteur de Lille, the Conseil Régional Nord/Pas-de-Calais, and the European Regional Development Fund. KG had a fellowship from the Institut Pasteur de Lille, the Région Nord/Pas-de-Calais and from the Société Française du Cancer. SM has a fellowship from the French Research Ministry and the Fondation pour la Recherche Médicale. ED has a fellowship from the Institut Pasteur de Lille and the Région Nord/Pas-de-Calais.

Accepted for publication November 4, 2008.

Supplemental material for this article can be found on *http://ajp.amjpathol.org*

Address reprint requests to Abbadie Corinne UMR8161, Institut de Biologie de Lille, 1 rue du Pr. Calmette, BP 447, 59021 Lille Cedex, France. E-mail: corinne.abbadie@ibl.fr.

icles are orchestrated by about 30 Atg genes[17,18] and depend on the integrity of the cytoskeleton, which is in contrast degraded during apoptosis.[19] Caspase activation is not required[20] and may even inhibit the autophagic pathway.[21] Necrosis, although originally described as the process of accidental cell death, might be partly programmed. It is defined as a type of cell death lacking the characteristics of apoptosis and autophagy, but both its cellular manifestations and its molecular pathways are poorly described. It is generally recognized, however, that necrosis involves early plasma membrane lysis, organelle swelling and lysis, and some vacuolization.[22,23]

The purpose of this study was therefore to establish whether senescent cells die by apoptosis, autophagic programmed cell death, necrosis, or some other undescribed or mixed type of cell death. Throughout this text, we use the expression *senescent-cell death* to refer to the mechanism of death following senescence.

Materials and Methods

Cell Culture

Normal human epidermal keratinocytes (NHEKs), purchased from Clonetics (CC-2501), were collected from 6 different females of different races and ages. They were grown at 37°C in an atmosphere of 5% CO_2 in KGM-2 BulletKit medium consisting of modified MCBD 153 with 0.15 mmol/L calcium, supplemented with bovine pituitary extract, epidermal growth factor, insulin, hydrocortisone, transferrin, and epinephrin (CC-3107, Clonetics). Such a serum-free low-calcium medium has been shown to minimize keratinocyte terminal differentiation.[24] Cells were seeded as recommended by the supplier and split at 70% confluence. The number of population doublings (PD) was calculated at each passage by means of the following equation: PD = ln(number of collected cells/number of plated cells)/ln2. For positive controls of apoptosis, cells were treated for 6 to 18 hours with 20 ng/ml tumor necrosis factor alpha (TNFα) + 10 μg/ml cycloheximide (CHX), or 10 ng/ml tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) + 10 μg/ml CHX.

Flow Cytometry Analysis and Sorting, Annexin-V/Propidium Iodide Assays

NHEKs were analyzed on a Coulter EPICS XL-MCL based on their forward and side scatter factor values and, according to the experiment, subpopulations with different forward and/or side scatter factor values were electrostatically sorted in air. For Annexin-V/propidium iodide staining, cells were processed with an Annexin-V-Alexa 568 kit (Roche, Calbiochem) according to the manufacturer's recommendations. The results were analyzed with the WinMDI 2.9 software. Alternatively Annexin-V/propidium iodide assays were performed with the same kit on cells grown on glass slides.

Videomicroscopy

Time-lapse videomicroscopy was done with a Zeiss Axiovert 100M equipped with a warm stage. Cells were maintained under 5% CO_2 in closed flasks. Images were taken at 15-minute intervals for 24 to 48 hours.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton-X100. Slides were incubated with the primary antibody: anti-active caspase-3 (Cell Signaling Technology), anti-keratin 14 (Chemicon International), anti-cytochrome C (Pharmingen), anti-apoptosis inducing factor (AIF; Chemicon International), or anti-MAPLC3 (Santa-Cruz). They were then washed three times with PBS and incubated with the secondary antibody: Rhodamine red-conjugated anti-Mouse IgG or Rhodamine red-conjugated anti-Rabbit IgG (Jackson ImmunoResearch Laboratories). Nuclei were stained with Hoechst 33258 at 1 μg/ml for 3 minutes. Slides were analyzed with either a Zeiss AxioPlan2 epifluorescence microscope, or a Zeiss Axio Imager Z1-ApoTome epifluorescence microscope for optical sectioning.

Western Blotting

Cells were lysed in the following solution: 27.5 mmol/L Hepes pH 7.6, 1.1 M/L urea, 0.33 M/L NaCl, 0.1 M/L EGTA, 2 mmol/L EDTA, 60 mmol/L KCl, 1 mmol/L dithiothreitol, and 1.1% NP40. The total protein concentration was measured with the Bio-Rad protein assay. Proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Hybond-C extra, Amersham). Equal loading was checked after Ponceau red staining of the membranes. The primary antibodies used were: anti-active caspase-3 (Cell Signaling Technology), anti- poly(ADP-ribose) polymerase (PARP; Alexis Biochemical), anti Bcl-2 (Santa Cruz), anti-keratin-14 (Chemicon International), anti-Beclin-1 (Santa Cruz), anti-Bid (Pharmingen), anti-lysosomal-associated membrane protein 1 (Santa Cruz), and anti-actin (Santa-Cruz). The secondary antibody used was a peroxidase-conjugated rabbit anti-sheep IgG or a peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). Peroxidase activity was revealed with an enhanced chemiluminescence kit (Amersham). The films were quantified by scanning with a Syngene Chemi-Genius Bio Imaging System.

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling and Comet Assays

The terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed with the Apoptag Kit (Intergen) according to the manufacturer's recommendations. For comet assays, 10,000 cells were embedded in 80 μl of 0.5% low-melting-point agarose at 37°C and the suspension was immediately pipetted onto a TREVIGEN Inc. cometslide, which was then placed at

4°C in the dark for at least 30 minutes. The slides were immersed in prechilled Lysis Solution and left at 4°C for 60 minutes. They were then left in alkaline solution for 20 minutes at room temperature in the dark. The migration was performed at pH = 8 or pH >13 in Tris borate-EDTA buffer at 1 V/cm for 20 minutes. Quantitative analysis (%DNA in the comet tail) was performed with the Tritek Comet Score freeware.

Fluorescence Staining of Mitochondria, Lysosomes, and Autophagic Vacuoles

Monodansylcadaverine, Lysotracker green, and Mitotracker red were from Molecular Probes. Living cells were incubated at 37°C with monodansylcadaverine (MDC; 0.05 mmol/L), Lysotracker green (100 nmol/L), or Mitotracker red (25 nmol/L) added directly to the cell culture medium (respective incubation times: 10 minutes, 2 hours, and 30 minutes). Nuclei were stained with vital Hoechst 33342 at 1 μg/ml for 10 minutes at 37°C.

Transmission Electron Microscopy

Cell pellets were fixed with 2.5% glutaraldehyde in 0.1 M/L cacodylate buffer, pH 7.4 for at least 30 minutes at 4°C. After fixation, the specimens were thoroughly washed in 0.1 M/L cacodylate buffer and then postfixed with 1% osmium tetroxide in the same buffer for 1 hour at room temperature, stained *en bloc* with 2% uranyl acetate in distilled water for 15 minutes, dehydrated in graded acetonitrile, and embedded in Epon. Ultrathin sections (80 to 100 nm thick) mounted on 150-mesh grids were stained with 2% uranyl acetate solution and Reynolds lead citrate solution.[25] The electron micrographs were taken with a Hitachi H600 electron microscope at 75 kV.

Results

Kinetics of Senescence and Senescent-Cell Death

We first investigated the kinetics of senescence and senescent-cell death in two normal human cell types, dermal fibroblasts (NHDFs) and epidermal keratinocytes (NHEKs). Fibroblasts are the most commonly used cell type in senescence studies; keratinocytes are more relevant to tumorigenesis studies. We have used fibroblasts from three and keratinocytes from six different donors, all female, of different ages and races. Dermal fibroblasts grew for 50 to 60 PDs, according to the donor, and then reached a senescence growth plateau where they remained for several months, suggesting that the level and speed of cell death in this cell type were very low (data not shown). In contrast, keratinocytes reached a senescence growth plateau after only 15 to 25 PDs (according to the donor); this plateau lasted only a few days to 2 to 3 weeks (according to the donor), and then almost all cells apparently died massively and detached from the dish, while a few clones of partly transformed cells

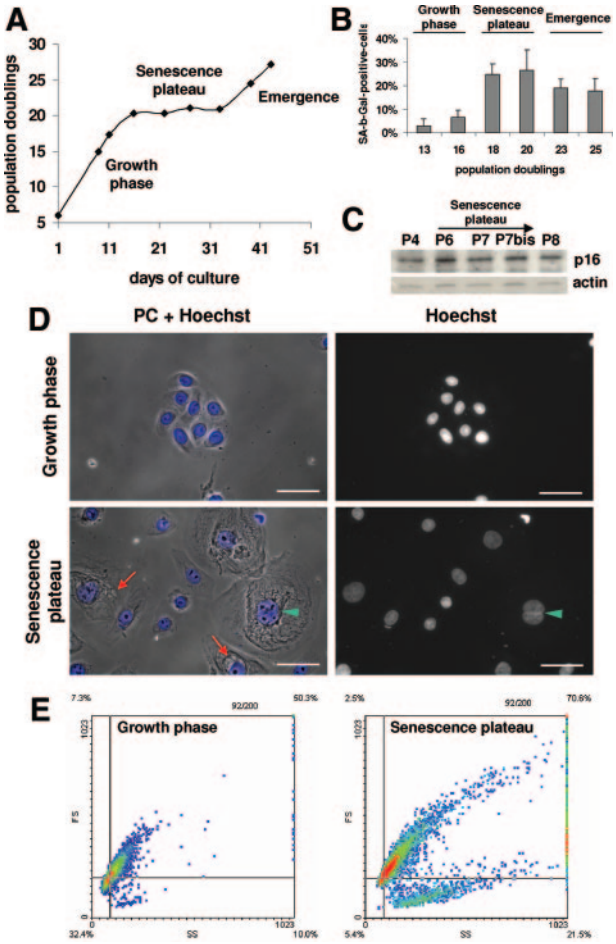


Figure 1. Growth curve and characteristics of young, senescent, and dying keratinocytes. **A:** NHEK growth curve under standard conditions. **B:** SA-β-Gal assays. SA-β-Gal-positive cells in each phase were counted among about a thousand cells in at least five microscopic fields. Results are given as means ±SD of all field counts. The percentages at 18 and 20 PD are statistically different from those at 13 PD ($P = 6 \times 10^{-7}$ and $P = 4 \times 10^{-10}$), and the percentages at 23 and 25 PD are statistically different from those at 20 PD ($P = 5 \times 10^{-05}$ and $P = 3 \times 10^{-05}$). **C:** Western-blot analysis of the CKI p16^{INK4} level in total cell extracts. Actin was used as a loading control. The quantification of the film is given in supplemental Figure S1 at *http://ajp.amjpathol.org*. **D:** Cells during the exponential growth phase and at the senescence plateau were stained with Hoechst and observed under phase contrast and by epifluorescence microscopy. Senescent keratinocytes display vacuole-like structures of different sizes generally close to the nucleus (red **arrows**); they are often binucleated (green **arrowhead**). Scale bars = 20 μm. **E:** Flow cytometry analysis of the side-scatter (X) and forward-scatter (Y) factors, ie, granularity and size respectively, of NHEKs during the exponential growth phase and at the senescence plateau. The colors represent the point densities. The two cursors help to see that at the senescent growth plateau the overall population shifts in size and granularity and becomes very heterogeneous. A subpopulation of cells of very low size but high granularity, probably corpses, appears at the senescence plateau. The results from (**A**) to (**E**) are representative of different experiments performed with cells from six different donors.

emerged (Figure 1A). Since the death of senescent keratinocytes was more massive and rapid than that of fibroblasts, and since emergence, possibly resulting from senescent-cell escape from death, was observed only with keratinocytes, we subsequently focused our studies on senescent-keratinocyte death.

Keratinocytes at the growth plateau displayed all of the characteristics of senescent cells: senescence-associated β-galactosidase activity at pH 6 (Figure 1B), up-

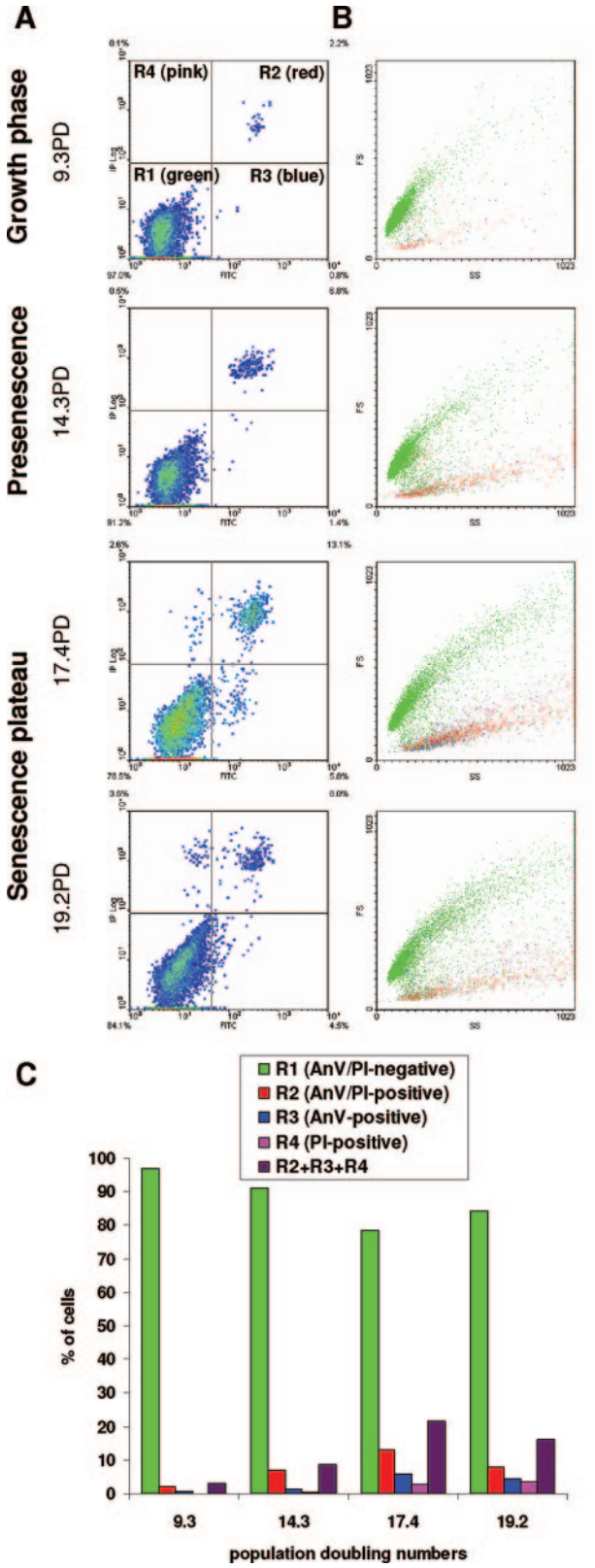


Figure 2. Flow cytometric analysis of cell death occurrence during NHEK culture. **A:** NHEKs grown under standard conditions were analyzed by flow cytometry after an increasing number of population doublings for Annexin-V (X) and PI (Y) staining. Colors represent point densities. Cursors were placed after considering autofluorescence (not shown). Different subpopulations are distinguishable: R1, AnV/PI-negative, ie, live cells, R2, AnV/PI-positive, ie, dead cells, R3, AnV-positive but PI-negative, ie, dying cells, and R4, AnV-negative PI-positive, also dead cells. **B:** Forward scatter (Y) and side scatter (X) factors of the four subpopulations R1, R2, R3 and R4. R1 (in green) is the main cell population, with a wide range of forward and side scatter factors values, ie, with a more or less developed senescent phenotype; they are alive. R2 (in red) is a subpopulation with a smaller size and a wide range of granularity; it appears with doubling and consists of corpses. R3 cells (in blue) are found in the whole population and display all sizes and granularities. They are senescent cells in the process of dying. R4 (in pink), like R2, consists of corpses. **C:** Proportions of cells in the different subpopulations.

regulation of the CKI p16^{INK4} (Figure 1C and supplemental Figure S1 at *http://ajp.amjpathol.org*), a 5- to 100-fold larger size than young cells, numerous dense particles (probably damaged components), and several vacuole-like structures of different sizes (Figure 1D). After Hoechst staining, their nuclei often appeared bigger than those of young cells, and about 20% of the cells were polynucleated (Figure 1D). Flow cytometry analysis of forward and side scatter factors, ie, size and granularity respectively, revealed dramatic changes in the cell population at the senescence plateau, with a global increase of these two parameters, a great variability within the population regarding these two parameters, and the appearance of a subpopulation of smaller cells with a high granularity, probably corpses (Figure 1E).

Morphological and Kinetic Features of Senescent-Keratinocyte Death

We then performed a more specific kinetic analysis of keratinocyte death by recording all types of cell death using Annexin-V/propidium iodide (AnV/PI) assays. Annexin-V is an appropriate marker of any type of cell death—apoptosis, autophagy, or necrosis—because cells undergoing either apoptosis[26] or autophagy[27] have been shown to externalize phosphatidylserines and because corpses generated by any mechanism ultimately lose their plasma membrane integrity. PI staining similarly reveals cells having lost their membrane integrity.

We first used flow cytometry to quantify AnV- and PI-positive cells in the whole population as the number of population doublings increased. The results revealed from the presenescent stage onward a general shift of the population toward an increase in AnV/PI staining, with the formation of three subpopulations (Figure 2, A and B). The first one is positive for both markers. It is composed of the smaller cells with high granularity, proving they are corpses. The second subpopulation is composed of AnV-negative/PI-positive cells. As the preceding ones, these cells are generally small and have a high granularity. They are therefore also corpses. The third subpopulation consisted of AnV-positive but PI-negative cells, and can therefore be classified as dying cells. They are found in all of the ranges of size and granularity, but mostly among the largest cells. At the senescence plateau, the proportion of dying and dead cells reached almost 20% of the total population (Figure 2C).

We then examined more precisely the morphology of the AnV-positive cells at the senescence plateau by a microscopic analysis. For comparison with the morphology of typical apoptotic cells, keratinocytes in the growth phase were treated with TNFα+CHX. These cells ap-

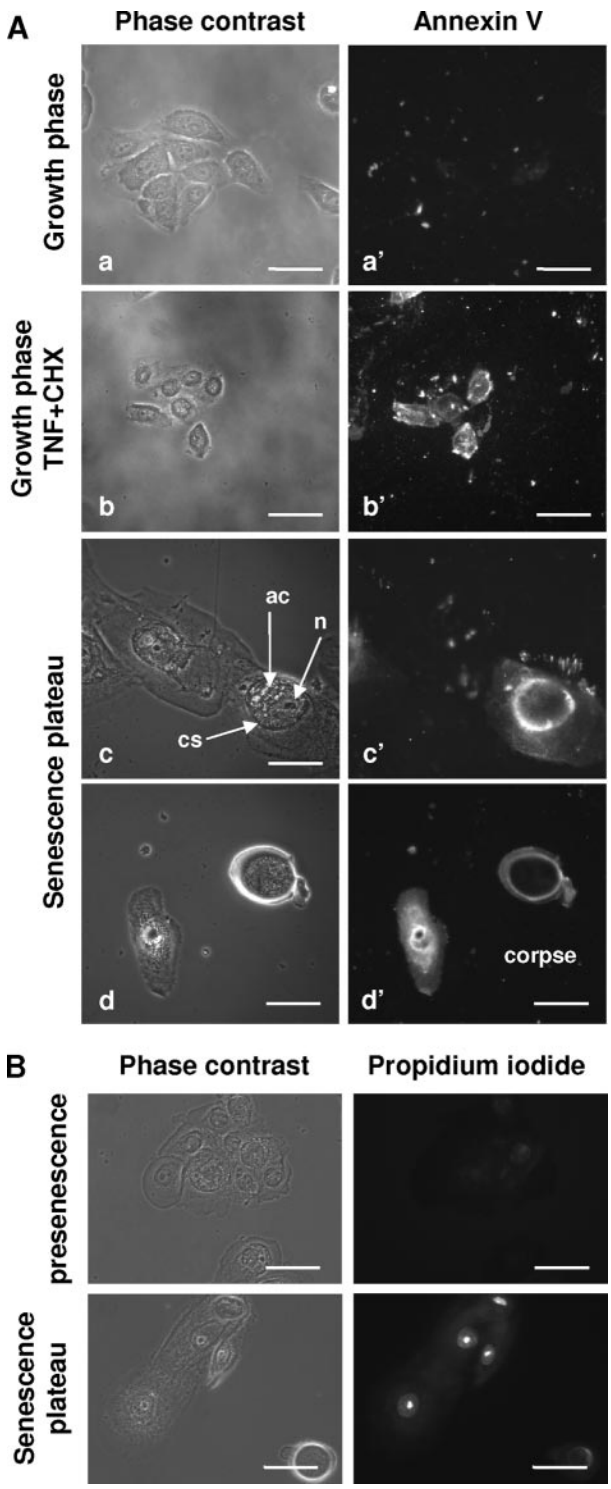


Figure 3. Morphological characteristics of dying and dead cells at the senescence plateau. **A:** NHEKs at different phases were processed for the Annexin-V assay. As a positive control for apoptosis, keratinocytes in the growth phase were treated with TNFα+CHX. Young keratinocytes (**a** and **a**′) are negative. Young apoptotic keratinocytes (**b** and **b**′) are stained at the level of the plasma membrane. The senescent keratinocyte (**c** and **c**′) on the right, but not the senescent keratinocyte on the left, displays some diffuse cytoplasmic staining and some staining inside a central structure (cs) containing a damaged nucleus (n) and various other altered components (ac). The corpse (**d** and **d**′) also displays some staining of its remaining cytoplasm and of its central structure. Scale bars = 20 μm. **B:** NHEKs at presenescence and senescence were processed for propidium iodide staining. Nuclear staining, indicative of nuclear membrane damage, was recorded only in cells at the senescence plateau. Scale bars = 30 μm.

peared stained at the level of the plasma membrane as expected, whereas untreated cells were negative (Figure 3A). At the senescence plateau, the AnV-positive cells typically display the morphology of senescent cells. Some diffuse Annexin-V staining was observable in their cytoplasm, but the highest staining localized to a central area that always contained a damaged nucleus and various other altered components (Figure 3A). The intracellular nature of this staining suggests that these senescent cells have their membrane permeabilized. PI staining confirmed the permeabilized state of the membrane in these large, flat senescent cells (Figure 3B), confirming they were dying, although they were still adherent. Among cells at the senescent plateau, corpses, identified by their small size and high density, also displayed some staining delineating a central area, with more diffuse staining of the remaining cytoplasm (Figure 3A).

To evaluate the kinetics of the death process at the single-cell level, we used time-lapse phase-contrast videomicroscopy. Pictures (Figure 4 and supplemental video, see *http://ajp.amjpathol.org*) clearly revealed typical senescent cells with a damaged nucleus evolving through progressive encircling of the nucleus by refringent components. Cells in this state progressively detached from the others and acquired a rounded shape, but remained attached to the dish and continued to move

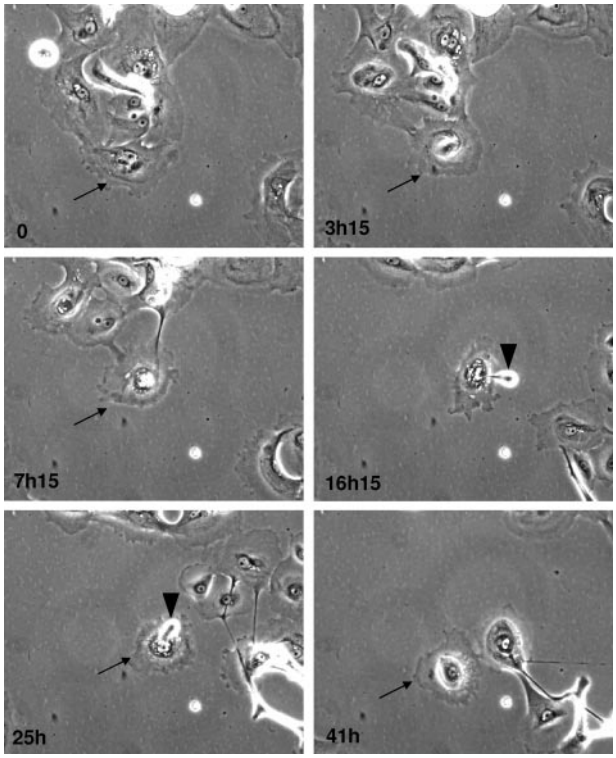


Figure 4. and supplemental video, see *http://ajp.amjpathol.org*. Time-lapse phase contrast videomicroscopy of senescent cells undergoing death NHEKs at the senescence plateau were followed by videomicroscopy. Pictures were taken at 15-minute intervals for 24 to 48 hours. Some images taken at the indicated times were extracted for the figure. The senescent cell that will evolve into a corpse is indicated by an **arrow**. Note that the nucleus, at first, seems surrounded by a big structure (conspicuous at 3 hours 15 minutes). Then the dying cell detaches from the other cells but remains attached to the support for several hours. The dying cell expulses some dense material (**arrowheads**) several times in the course of the process.

randomly for several tens of hours. Cells undergoing this process were found to secrete big, dense particles repeatedly, probably by exocytosis. On the basis of the videomicroscopy analysis, the duration of the death process was estimated at 24 hours to a few days.

In conclusion, dying cells and corpses at the senescence plateau appear morphologically different from apoptotic cells and apoptotic bodies, which are characterized by their condensed and fragmented nucleus and cytoplasm. In addition, the slowness of the senescent-cell death process does not fit with the duration of apoptosis, which is generally a few hours. PI-positivity might indicate that senescent cells die by necrosis; but no sign of cell swelling or lysis was observed. The presence of numerous vacuole-like structures in senescing cells is reminiscent, rather, of autophagic cell death.

Inhibiting Autophagic Cell Death, but Not Apoptosis, Decreases the Rate of Senescent-Cell Death

To test the hypothesis that the mechanism of senescent-keratinocyte death is autophagy rather than apoptosis, we examined whether inhibitors of apoptosis or autophagy would differentially affect the level or time course of the death process. zVAD-fmk, a pan-caspase inhibitor, was used to inhibit apoptosis, and 3-methyladenine (3-MA), an inhibitor of the class III phosphatidylinositol 3-kinase (class III PI3K) complex involved in initial autophagosome formation,[28] was used to inhibit autophagy. In a first experiment, zVAD or 3-MA was applied from the beginning and throughout the culture. 3-MA rapidly inhibited cell growth and induced a senescence-like phenotype (data not shown). This unanticipated effect of 3-MA, probably resulting from a lack of damaged component turnover, forced us to settle on a protocol in which the inhibitors tested were applied to pure populations of already senescent cells. For this purpose, NHEKs at the senescence plateau were analyzed by flow cytometry according to forward and side scatter factors values. The subpopulation with the highest forward and side scatter factors values and the subpopulation with values just below the highest were sorted and plated (Figure 5A). Microscopic observation of the subpopulation with the highest scatter factor values revealed that many of these cells were unable to adhere, suggesting that this subpopulation was engaged too far along the death pathway. The subpopulation with slightly lower scatter factor values was thus assumed to encompass pre-dying senescent cells suitable for this experiment. Cells of this subpopulation were therefore treated with z-VAD or 3-MA for 5 days, and corpses with the typical central area were counted daily. The percentage of corpses increased with time in control cells and, to the same extent, in z-VAD-treated cells. In 3-MA-treated cells it decreased (Figure 5B). These results indicate that senescent-cell death does not occur through a caspase-dependent mechanism but involves a class III PI3K, suggesting that senescent cells do not die by apoptosis but by autophagic cell death.

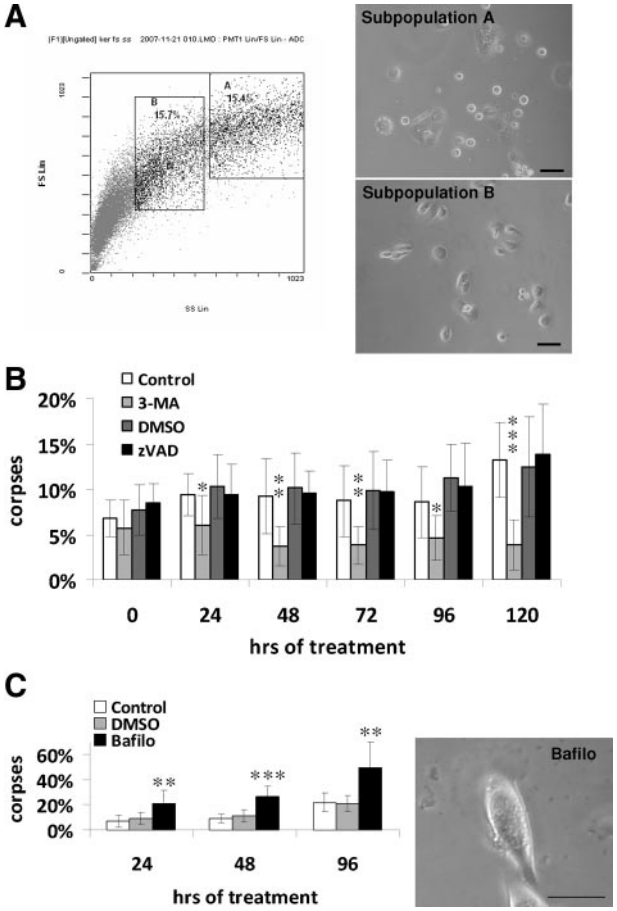


Figure 5. Inhibition of autophagy, but not of apoptosis, delays senescent-cell death. **A:** NHEKs at the senescence plateau were analyzed by flow cytometry according to their forward-scatter (Y) and side-scatter (X) factor values. Two subpopulations were sorted; subpopulation A comprised the 15% of the cells with the highest forward and side scatter factor values, and subpopulation B with the next-highest scatter-factor values. Sorted cells were plated in 12-well plates at 20,000 cells per well and observed 24 hours later under a phase contrast microscope. Many non-plated cells were observed in subpopulation A, indicating that it is enriched in dying cells. Therefore subpopulation B, less engaged in the death pathway, was chosen for the experiment. **B:** Cells of subpopulation B were continuously treated with 3-MA at 5 mmol/L or with its diluent H_2O as a control or with z-VAD at 20 μmol/L or its diluent DMSO. The number of typical corpses with a refringent central area was counted every day under the microscope. The results are means ± SD of counts in 5 random microscopic fields of each well, each condition being duplicated. **C:** Cells of subpopulation B were treated every 48 hours with 5 nmol/L bafilomycin A1 or its diluent DMSO. The number of corpses was counted as above. The photograph represents the morphology of bafilomycin-treated cells after 96 hours of treatment. Note the presence of a big central area full of very dense, granular material. *P* values were calculated with the *t*-test. $^*P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.0005$. Those indicated are between 3-MA and control and between bafilomycin and dimenthyl sulfoxide. The other differences are non-significant. Scale bars: 50 μm in (**A**); 40 μm in (**C**).

To confirm this conclusion, we examined whether bafilomycin A1, an inhibitor of the H^+ pump that decreases the efficacy of digestion inside autophagolysosomes, could freeze the death process in its last stage. Pre-dying senescent cells were sorted as above and treated with bafilomycin. Corpses with the typical central area rapidly accumulated to a high rate (40%) in bafilomycin-treated cultures (Figure 5C). To make sure this was not due to an inherent toxicity of bafilomycin, we applied bafilomycin under the same conditions to cells in the exponential growth phase and measured toxicity by

trypan blue exclusion: the percentage of trypan blue-positive cells reached only 23% (Supplemental Figure S2, see *http://ajp.amjpathol.org*). In addition to increasing the percentage of corpses, bafilomycin modified their appearance: the central area appeared to be full of very dense granular material (Figure 5C). Taken together, these experiments indicate that senescent-cell death involves class III PI3K-mediated autophagosome formation and cell component degradation in acidic compartments.

Senescent Keratinocytes Do Not Display the Hallmarks of Apoptosis, but Display Altered Mitochondria and Nuclei

To go beyond the above observations suggesting that senescent cells do not die by apoptosis, we checked for any activation of caspase-3, a major actor of apoptosis. Young cells treated with TNF-α or TRAIL plus CHX were used as a positive control. In immunofluorescence experiments with an antibody recognizing the cleaved active form of caspase-3, senescent cells were negative, in contrast to apoptotic cells (Figure 6A). In Western blots produced with extracts of cells harvested at different time points on the senescence plateau, the cleaved active form of caspase-3 was not detected, whereas it was detected in apoptotic control cells (Figure 6B). We also measured cleavage of PARP, a caspase target. Basal-level cleavage of PARP was observed, without any change during senescence (Figure 6B).

Another molecular hallmark of apoptosis is the DNA degradation to high molecular weight or oligonucleosome-length fragments elicited by caspase-dependent or independent pathways.[29] We searched for such DNA fragmentation by the TUNEL assay. Senescent cells with a damaged nucleus were TUNEL-positive, whereas cells with a nucleus still morphologically intact were negative (Figure 7A). Yet, as the TUNEL assay is not strictly specific to apoptosis since it detects both double-strand and single-strand breaks, we used comet assays to better characterize the type of DNA breaks encountered in senescent cells. In these assays, DNA fragments migrate out of a permeabilized nucleus in the form of a comet-like tail. At pH = 8, free DNA fragments result only from double-strand breaks, while under denaturing conditions (pH >13), they also result from single-strand breaks.[30] The analysis of cells at the growth phase and at the senescence plateau confirmed that the percentage of DNA breaks increases with senescence, and showed that breaks at senescence are mainly single-strand ones (Figure 7B). Thus, senescent cells do undergo DNA degradation, but not consequently to the activation of an apoptotic pathway.

We also investigated by immunofluorescence the potential release from mitochondria of cytochrome C and AIF, two markers of the intrinsic apoptotic pathway.[31] In cells at the growth phase, cytochrome C localized dis-

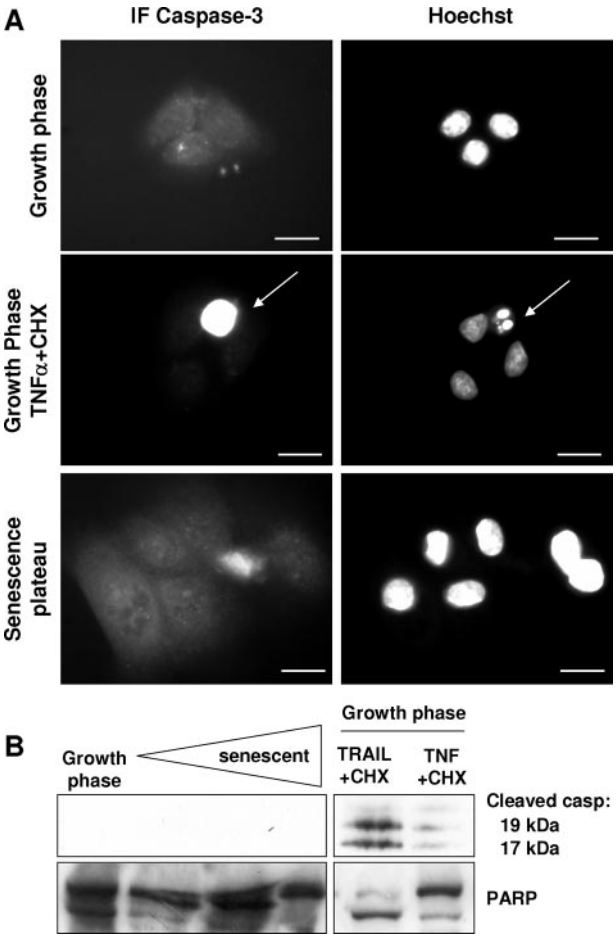


Figure 6. Caspase-3 is not activated in senescent keratinocytes. **A:** NHEKs in the growth phase or at the senescence plateau were processed for immunofluorescence with an antibody recognizing the activated form of caspase-3. As a positive control, NHEKs in the growth phase were treated with TNFα+CHX. Neither young nor senescent keratinocytes were positive. In contrast, typical apoptotic cells with condensed and fragmented nuclei were caspase-3 positive (**arrows**). Scale bars = 10 μm. **B:** Western blots. Cell extracts were produced with NHEKs in the growth phase and at different time points in the senescence plateau. Extracts of keratinocytes in the growth phase, induced to die by apoptosis with TRAIL+CHX or TNFα+CHX, were used as a positive control.

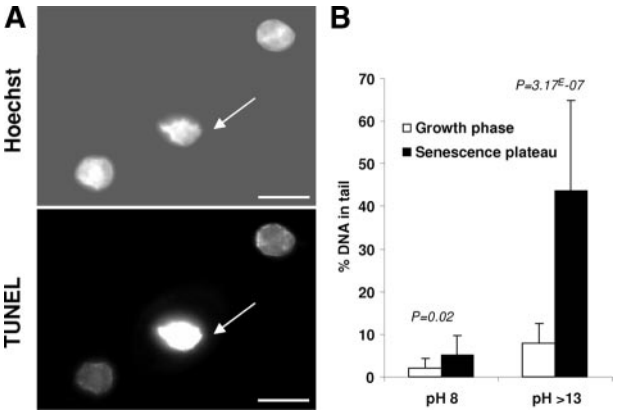


Figure 7. Senescent keratinocytes display DNA breaks. **A:** A TUNEL assay was performed on keratinocytes at the senescence plateau to detect DNA fragmentation. Positive signals were seen only on much-altered nuclei (**arrows**). Other large nuclei typical of still healthy senescent cells were negative. Scale bars = 5 μm. **B:** A comet assay was performed on NHEKs in the growth phase or at the senescence plateau in neutral (pH 8) versus alkaline conditions (pH >13) to detect only single- or single- plus double-strand breaks, respectively. The percentage (%) of DNA in the comet tail was assessed in each condition. Each bar represents the mean ±SD of 20 measures. *P* values were calculated with a *t*-test.

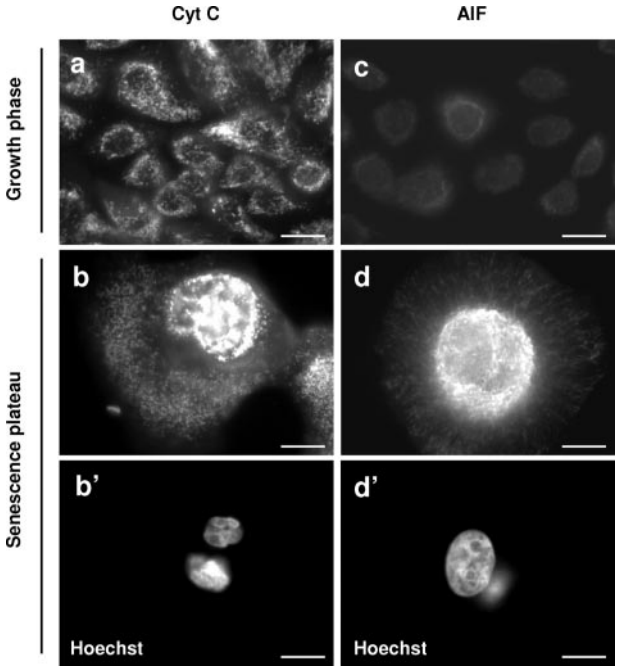


Figure 8. The mitochondria of senescent keratinocytes are altered but do not release cytochrome C or AIF. NHEKs in the growth phase or at the senescence plateau were processed for immunofluorescence with specific anti-cytochrome C and anti-AIF antibodies. Both signals increase with senescence. The cytochrome C-positive structures change from radiating stick-shaped structures in growth-phase cells (**a**) to vesicular ones agglutinated in the vicinity of the nucleus in senescent cells (**b** and **b′**, note that the shown senescent cell contains two pycnotic nuclei). The AIF staining is very faint in young cells (**c**). It increases greatly in senescent cells; AIF-positive structures are very numerous and agglutinated around the nucleus (**d** and **d′**, note that the nucleus of the shown senescent cell is very large). Senescent keratinocytes do not show clear translocation of AIF into the nucleus or cytochrome C release into the cytosol (b and d). Scale bars = 10 μm.

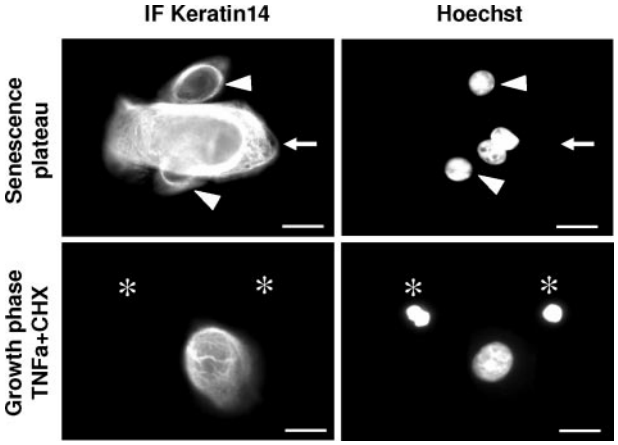


Figure 9. Fate of the cytoskeleton in senescent keratinocytes. The cytokeratin 14 network is preserved during senescent cell death. NHEKs at the senescence plateau were processed for immunofluorescence with a cytokeratin 14-specific antibody and compared with NHEKs in the growth phase undergoing apoptosis after TNFα+CHX treatment. The cytokeratin network appears totally preserved, even overdeveloped, in senescent cells (**arrows**) compared with the still-young cells visible in the microscopic field (**arrowsheads**), whereas apoptotic cells, identified by their condensed nuclei (**asterisks**), are completely cytokeratin14-negative. Scale bars = 10 μm.

creetly to small sticks (the typical appearance of mitochondria). AIF staining was very faint. In cells at the senescence plateau, the number of cytochrome C- and AIF-stained structures dramatically increased, these structures becoming vesicular and clustered in the vicinity of the nucleus. We never observed a clear translocation of cytochrome C or AIF into the cytoplasm or nucleus (Figure 8). These results indicate that senescent cells do display some mitochondrial alterations, but these alterations were unrelated to apoptosis.

Since one difference between apoptotic and autophagic programmed cell death is the fate of the cytoskeleton,[19] we performed cytokeratin 14 detections. In immunofluorescence experiments, the cytokeratin 14-network appeared completely preserved, even more developed, in senescent cells than in growing ones, whereas it was completely disintegrated in TNFα+CHX-induced apoptotic control cells (Figure 9).

Senescent Keratinocytes Display High Macroautophagic Activity

To more specifically document the likelihood that senescent cells die by autophagic cell death, we investigated different markers of macroautophagy by western blotting, flow cytometry, epifluorescence microscopy, and transmission electron microscopy (TEM). We first examined the expression of Atg6/Beclin-1, a subunit of the class III PI3K complex required for initial autophagosome formation.[17] Protein extracts were made from cells in the growing phase, cells in the presenescent stage, and from a fluorescence-activated cell sorting (FACS)-sorted subpopulation of cells at the senescence plateau with the highest forward and side scatter factor values (Figure 10A). We observed an increase in Beclin-1 expression as soon as presenescence (Figure 10B and supplemental Figure S3, see *http://ajp.amjpathol.org*). We also checked Bcl-2, the well-known anti-apoptotic protein, which is also an inhibitor of Beclin-1.[32] Bcl-2 expression dramatically decreased in sorted senescent cells (Figure 10B and supplemental Figure S3, see *http://ajp.amjpathol.org*). We also observed increased expression of lysosomal-associated membrane protein 1, as soon as the presenescent stage (Figure 10B and supplemental Figure S3, see *http://ajp.amjpathol.org*).

We further documented the increase in autophagic activity during senescence by using Lysotracker, a cell-permeant probe that fluoresces in acidic organelles, ie, lysosomes and autophagolysosomes (but also late endosomes). Cells were stained with Lysotracker and analyzed by flow cytometry. The subpopulation with the highest forward and side scatter factor values stained the brightest, while staining of the subpopulation ranked next on the scatter factor scale was less intensely stained (Figure 10C). Microscopic analysis confirmed the increase in Lysotracker staining with senescence and highlighted, as expected, a large quantity of small vacuoles (Figure 10D). Similar results were obtained with MDC (Figure 10D), also a marker of acidic compartments.[33,34]

We finally investigated the fine structure of senescent cells by TEM. NHEKs at the senescence plateau were sorted by FACS according to their forward scatter factor, and the subpopulation with the highest factor value was

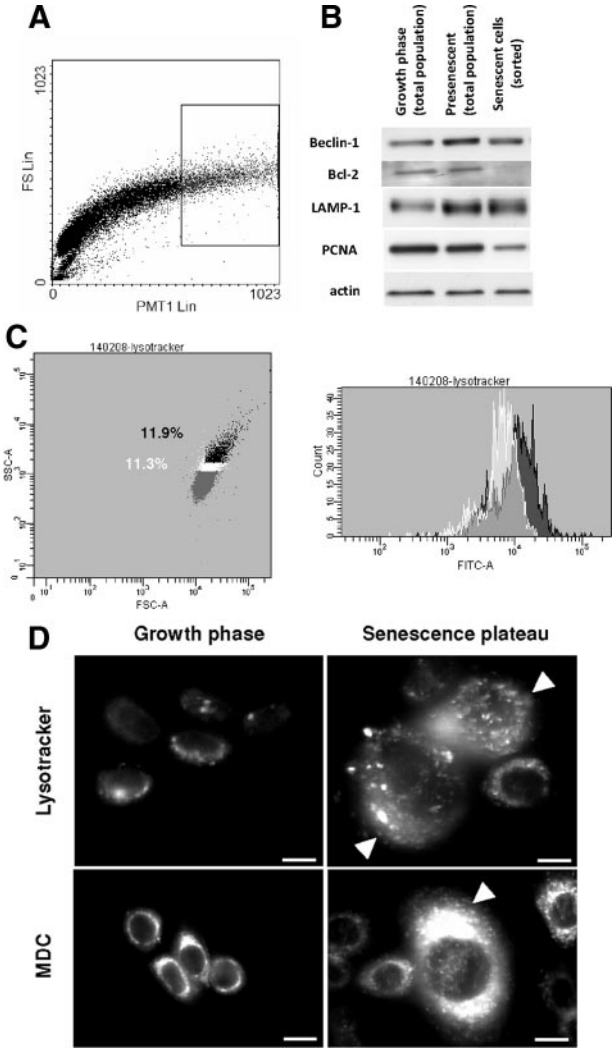


Figure 10. Expression of autophagic markers in senescent and dying keratinocytes. **A:** NHEKs at the senescence plateau were analyzed by flow cytometry according to their forward scatter (Y) and side scatter (X) factors. The subpopulation (10.9% in the frame) with the highest forward and side scatter factor values was sorted and used for protein extraction (**B**) Western-blot analysis of the expression of some markers of autophagosomes and lysosomes. Cell extracts were produced with keratinocytes in the growth phase, at the presenescent stage, and with senescent keratinocytes sorted by FACS in (**A**). PCNA was used as a sorting quality control and actin as a loading control. These results are representative of 3 independent experiments performed with cells from 2 different donors. **C:** NHEKs at the senescence plateau were stained with Lysotracker and analyzed by flow cytometry for their side scatter (X) and forward scatter (Y) factors. Then the staining intensity (FITC-A) in the subpopulation with the highest forward and side scatter factor (dark) was compared with that of the subpopulation ranking just below it in terms of scatter-factor values (white). The results show that the cells with the highest factors are the most Lysotracker-positive. **(D)** NHEKs in the exponential growth phase and at the senescence plateau were stained with either MDC or Lysotracker. The mass of both autophagic vacuoles and lysosomes increases in the large, flat senescent cells (**arrowheads**). Scale bars = 10 μm.

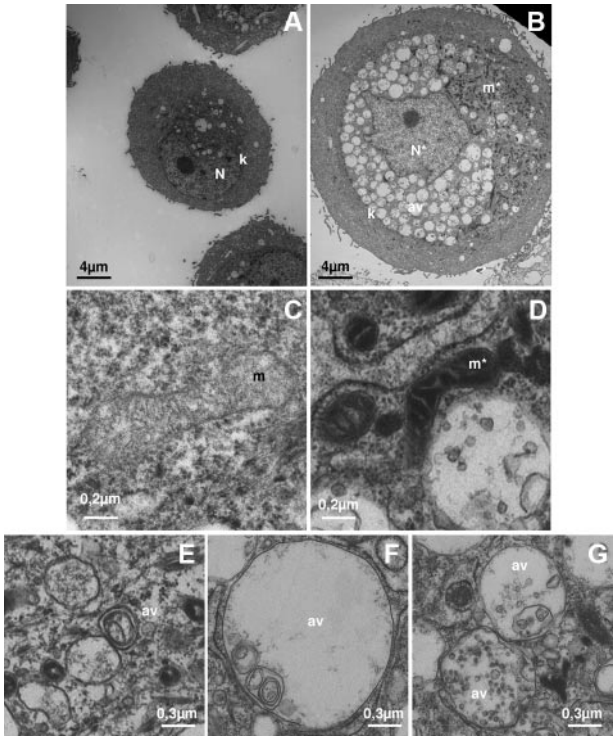


Figure 11. Ultrastructure of dying senescent cells. NHEKs at the senescence plateau (**B, D, E, F, G**) were analyzed by flow cytometry and sorted according to their forward side factor (not shown). They were then fixed for TEM analysis in parallel with cells in the exponential growth phase (**A** and **C**). **C** and **D** are details of mitochondria morphologies in NHEKs in growth phase (**C**) or at the senescence plateau (**D**), **E**, **F** and **G** are details of autophagic vacuole morphologies in NHEKS at the senescence plateau. N: nucleus, N*: deformed nucleus with less heterochromatin, k: cytokeratin network encircling the nucleus and the autophagic vacuoles, m: mitochondrion of a young cell, m*: mitochondria of senescent cells aggregated close to the nucleus and appearing very contracted, av: autophagic vacuoles with a simple (**F,G**) or a double membrane (**E**) full of membranous or non-membranous debris.

analyzed by TEM by comparison with cells in the exponential growth phase. Keratinocytes in the exponential growth phase displayed a normal organization[35] with a typical nucleus, several dispersed organelles, some rare vesicles, and a cytokeratin network (Figure 11A). In contrast, sorted keratinocytes from the senescence plateau displayed a highly partitioned substructure, with a cortical and a central area delimited by the cytokeratin network (Figure 11B). The cortical area appeared clear of any organelles, whereas the central area was full of autophagic vacuoles. The autophagic vacuoles generally contained debris, often membranous (Figure 11, C and D). They accumulated on bafilomycin treatment, and after this treatment they contained more debris than in the control situation (supplemental Figure S4, see *http://ajp.amjpathol.org*). The mitochondria were either dispersed in this central area or grouped together in an aggregate clustered with the nucleus (Figure 11B). Their morphology appeared altered compared with that of young cells (Figure 11, C and D). Some of these altered mitochondria were found inside autophagic vacuoles (supplemental Figure S4, see *http://ajp.amjpathol.org*). The nucleus was always found in the central area; it was often deformed and its chromatin often appeared clear, with levels of heterochromatin lower than in young cells (Figure 11B). These TEM observations are in total agreement with the observations made by epifluorescence microscopy with anti-cytokeratin 14 (Figure 9) and with Lysotracker and MDC (Figure 10). Note that the autophagic vacuoles concentrated in the central area of senescent cells might be responsible for the intracellular AnnexinV staining seen in Figure 3, a very recent study having shown that the cytosolic leaflets of endosomes and lysosomes are

rich in phosphatidylserine.[36] Taken together, these results indicate that senescence is accompanied by high autophagic activity.

Senescent Keratinocytes Die as a Result of High Autophagic Degradation of the Nucleus and Mitochondria

To determine whether this high autophagic activity in senescent keratinocytes might result in their death, we took a closer look at the corpses themselves. We performed triple staining experiments with lysosomal and autophagosomal markers (Lysotracker or antibodies against Atg8/LC3, a protein that associates with the membrane phagosome[37]), Hoechst to stain the nucleus, and Mitotracker to stain the mitochondria. The analysis was done by epifluorescence microscopy with a standard microscope or with a microscope equipped with the ApoTome system for optical sectioning. In corpses, the central area appeared Lysotracker-positive (Figure 12A) and full of numerous Atg8/LC3-positive punctate structures (Figure 12B). It always contained a nucleus, sometimes two or three, that could be pycnotic (Fig; 12A). It also contained almost all of the Mitotracker staining, which no longer delineated individual structures identifiable as mitochondria (Figure 12A). These Hoechst and Mitotracker staining patterns suggest that the mitochondria and nuclei were undergoing degradation.

To further investigate this potential nuclear and mitochondrial degradation, we performed a Western blot analysis of several mitochondrial and nuclear proteins at different stages of the senescent growth plateau and, at the latest stage, in dead floating cells. The results show that the nuclear protein PARP was degraded in cells having reached the senescence plateau, its complete degradation being reached in floating cells. The mitochondrial proteins Bcl-2 and Bid were also completely undetectable in dead floating cells, the disappearance of Bcl-2 beginning earlier than that of Bid. These nuclear and mitochondrial protein degradations were specific, since the quantity of cytokeratin 14 increased continuously throughout the senescence plateau until the latest stage (Figure 12C). Thus, senescent keratinocytes seem to die through massive and specific autophagic degradation of their vital components, notably their nuclei and mitochondria.

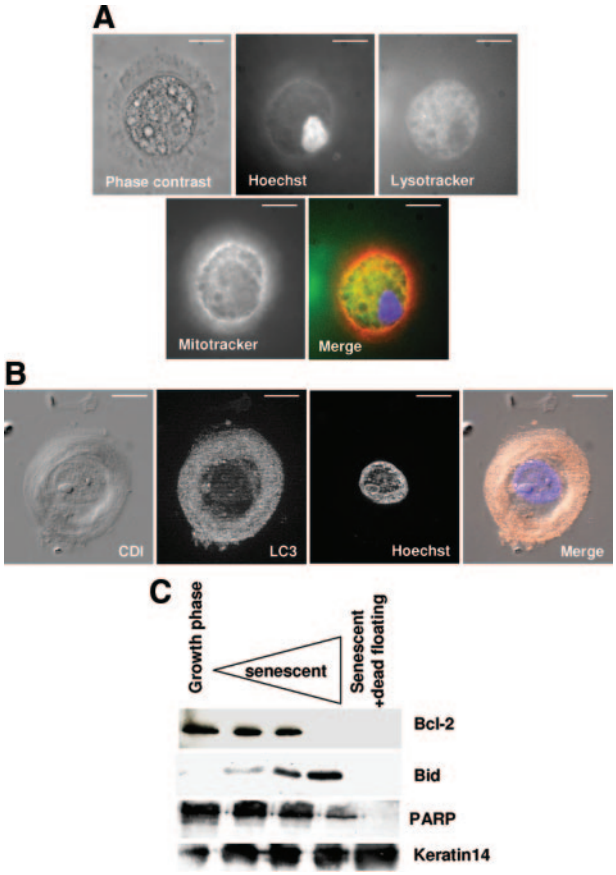


Figure 12. Corpses degrade their mitochondria and nuclei in a central area full of autophagic vacuoles. **A:** Triple staining of a corpse with Lysotracker (green), Hoechst (blue) and Mitotracker (red). The three stains colocalize within a central area that occupies most of the corpse volume. The nucleus appears pycnotic. Scale bars = 10 μm. **B:** Corpse immunostained with an antibody against LC3 (pink), co-stained with Hoechst (blue), and analyzed by circular dichroism and with the ApoTome system. The four images represent one optical section. The central area is full of LC3-positive punctate structures and contains a damaged nucleus. Scale bars = 10 μm. **C:** Western blots. Cell extracts were performed with NHEKs in the growth phase and at different time points in the senescence plateau. Last lane: extracts from still-adherent cells plus dead floating cells.

Discussion

Although senescence is a cell state now extensively studied because of its implication in aging and associated pathologies such as cancer, the final fate of senescent cells, ie, how they die, has not been clearly established. Here, on the basis of an investigation of several molecular and morphological markers of apoptosis and autophagy, we propose that the main cell death mechanism occurring in senescent keratinocytes is not apoptosis but autophagic cell death. This conclusion would appear to apply to other cell types as well, since we observed increasing vacuole formation in several other senescent epithelial cell types (mammary and prostatic epithelial cells, data not shown) and in dermal fibroblasts (and prostatic fibroblasts, data not shown). Although in our hands the process of senescent fibroblast death seems very slow, a study by another group has confirmed that an increase in subcellular modifications corresponding to autophagy occurs during MRC5 human fibroblast senescence.[38]

Autophagy as a Cell Death Mechanism in Senescent Keratinocytes?

Autophagy plays a number of different, apparently contradictory, roles. It is the normal mechanism for the turnover of long-lived proteins and organelles. It is also a survival process induced to enable cells to resist nutrient deprivation, and it can evolve toward cell death when the cytosol and organelles are excessively degraded.[39–41]

The increased autophagic activity we have evidenced in senescent cells might thus play a role other than in cell death. What are the arguments in favor of a role of autophagy in the death of senescent keratinocytes?

First of all, we show that when the initial phases of autophagy are blocked with 3-methyladenine, the death of senescent cells is delayed. Moreover, if the acidification required for the final degradation of cell components is blocked, autophagic vacuoles full of debris accumulate inside corpses.

Secondly, we show by fluorescence and electron microscopy that dying senescent cells acquire a particular intracellular organization. The cytokeratin network develops and partitions the cell into two main areas, a cortical one devoid of organelles and a central one in which are concentrated a huge quantity of autophagic vacuoles, most of the mitochondria, and the nucleus. We assume that nuclei and mitochondria could be degraded therein. This is supported by the altered morphology of the nuclei and mitochondria in this area, by the level and type of DNA degradation, and by our Western blot analysis showing that numerous mitochondrial and nuclear proteins are lacking in the subpopulation comprising adherent senescent cells and floating dead cells. Autophagic elimination of nuclei has been poorly studied, as regards both its mechanism and its inducers, except in yeast where nuclei appear to be degraded by so-called piecemeal microautophagy.[42] The nuclei of senescent cells could be targeted for autophagy because their DNA is broken, since inhibition of DNA-PK, a nuclear kinase involved in DNA-break signaling, sensitizes to autophagy,[43] suggesting that persistence of damaged DNA can activate the autophagic process. Similarly, both an increase in Beclin-1 expression and an increase in autophagic programmed cell death occur following treatment with the DNA-damaging agent etoposide.[44] Furthermore, DNA damage has been shown to accumulate in cancer cells deficient in autophagy.[45]

The elimination of a large quantity of mitochondria may also be crucial to rendering autophagy lethal. The mitochondria of senescent cells are damaged. This may activate their massive autophagic elimination, as shown in the case of nerve growth factor-deprived neurons.[46] Surprising is the increased number of mitochondria during senescence despite the damage these organelles undergo. Such an increase has already been documented in MRC5 fibroblasts following H_2O_2-induced premature senescence.[47] Also surprising is the lack of cytochrome C and AIF release from the damaged mitochondria. Actually, cytochrome C and AIF are probably released but, as suggested by Lemasters et al,[48] sequestration of the mitochondria in autophagic vacuoles would prevent them from diffusing toward the cytosol and nucleus, and hence from exerting their activity as apoptosis-inducing factors.

The last argument concerns Beclin-1 expression. This might be a key determinant of the switch from autophagy as a vital process of cell component turnover to autophagy as a lethal process. Unlike autophagy induced by serum or amino acid deprivation that involves beclin-1 but without any increase in expression, autophagic programmed cell death induced by etoposide appears to be associated with an increase in Beclin-1 expression.[44] We show here that Beclin-1 expression is induced at the presenescence stage, supporting the notion that autophagic cell death could follow.

A contrario, it has been suggested that senescent cells die because of a decreased ability to digest and evacuate the content of autophagic vacuoles.[49] Our videomicroscopic recordings and flow cytometric analyses show that senescent cells retract during their death process. If they died because of an inability to evacuate damaged components, they should instead continue to increase in size. Our videomicroscopic recordings also show that dying senescent cells do evacuate some dense material, probably corresponding to the non-degradable content of their autophagolysosomes. This suggests that the autophagic process is functional in senescent cells until late. Moreover, our electron micrographs show that the autophagic vacuoles accumulating in senescent cells are functional, since most of them contain only small pieces of degraded material. In contrast, when the degradation process is blocked with bafilomycin, these vesicles retain more material.

Senescent Cells Do Not Die by Apoptosis

We additionally show here that senescent cells do not die by apoptosis. This is supported by the morphology of the corpses appearing during senescence, which differ markedly from apoptotic bodies. This is also supported by the lack of several apoptotic markers in senescent cells or corpses observable at the senescence plateau. Furthermore, caspase inhibitors have no effect on the kinetics of senescent-cell death. Our conclusion is entirely consistent with the apoptosis-resistance of senescent cells, an unpopular fact that is nevertheless clearly established for several cell types and several apoptosis inducers.[50–53] Other groups have examined whether senescent cells die by apoptosis, and conflicting results have been published. In one study, senescent fibroblasts appeared caspase-3 positive, with only 2% of them showing other typical apoptotic changes.[54] In another study comparing human umbilical vein endothelial cells (HUVECs) with fibroblasts, senescent HUVECs displayed many signs of apoptosis but senescent fibroblasts did not.[55] The authors associated the death of HUVECs with the generation of oxidative stress during senescence[56] and proposed that fibroblasts do not die by apoptosis because they are more resistant to oxidative stress than HUVECs.[57] A study focusing on keratinocytes concluded, in accordance with our results, that apoptotic cells are a minority subpopulation and that their number does not change with passaging.[58]

Implications for Aging and Tumorigenesis

An interesting question is whether autophagy is involved in the death of senescent cells *in vivo* during aging, as it is in culture. The sole established universal marker of senescence, SA-β-Gal activity,[59] increases in the course of normal human and mouse aging.[59–62] Since a lysoso-

mal hydrolase has been shown to exert this activity,[63] this indicates that the autophagic activity of the cells increases during aging. Similarly, lipofuscin, the well-known marker of aged skin, has been shown to accumulate with advancing age inside autophagic vacuoles, as an aggregate of proteins having reacted with lipid peroxidation end-products.[64] This also supports the notion that autophagic activity increases with age. Therefore, one might speculate that during aging, most altered cells might die by autophagy.

Understanding the death pathway of senescent cells may be a key to understanding the relationship between aging and cancer. It is widely recognized that the appearance of apoptosis resistance is an important event in neoplastic transformation. The same might apply to resistance to autophagic cell death. There is evidence already that autophagy is down-regulated in cancer cells. Beclin-1 is often mono-allelically deleted in various carcinomas[38] and its heterozygous disruption in mice causes spontaneous tumors.[65,66] The tumor suppressor phosphatase and tensin homolog, which rivals p53 as the most frequently mutated gene in human cancer,[67] promotes autophagy in HT-29 colon cancer cells by blocking the Akt survival pathway, and mutations in phosphatase and tensin homolog result in inactivation of autophagy and tumor formation.[68] One might thus speculate that during aging, some cells escape autophagic cell death to evolve into long-lived transformed cells. Once formed, however, cancer cells might use autophagy in the opposite way, for example to survive under nutrient deprivation resulting from limited angiogenesis.[69]

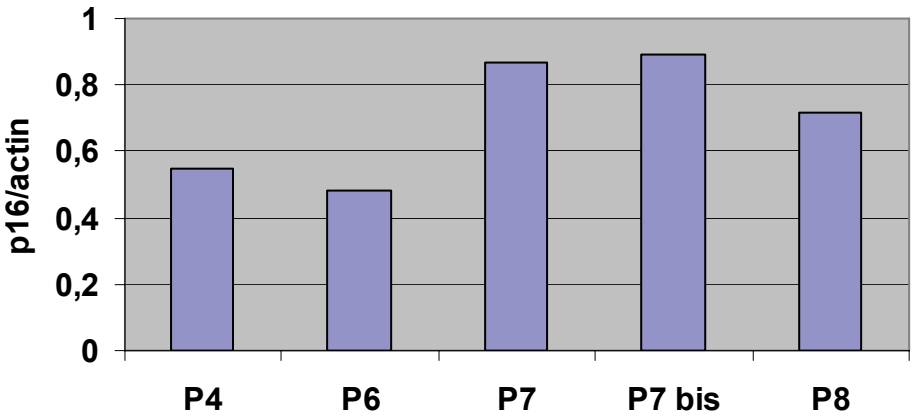
Acknowledgments

We thank Fabrice Nesslany for technical advice on comet assays, Nathalie Jouy at the Service commun de Cytometrie et de Tri cellulaire (IMPRT-IFR114), and Julie Bertout at IBL for FACS facility, Didier Deslee for Videomicroscopy Facility at the Institut Pasteur de Lille Campus, and Albin Pourtier for critical discussions and reading of the manuscript.

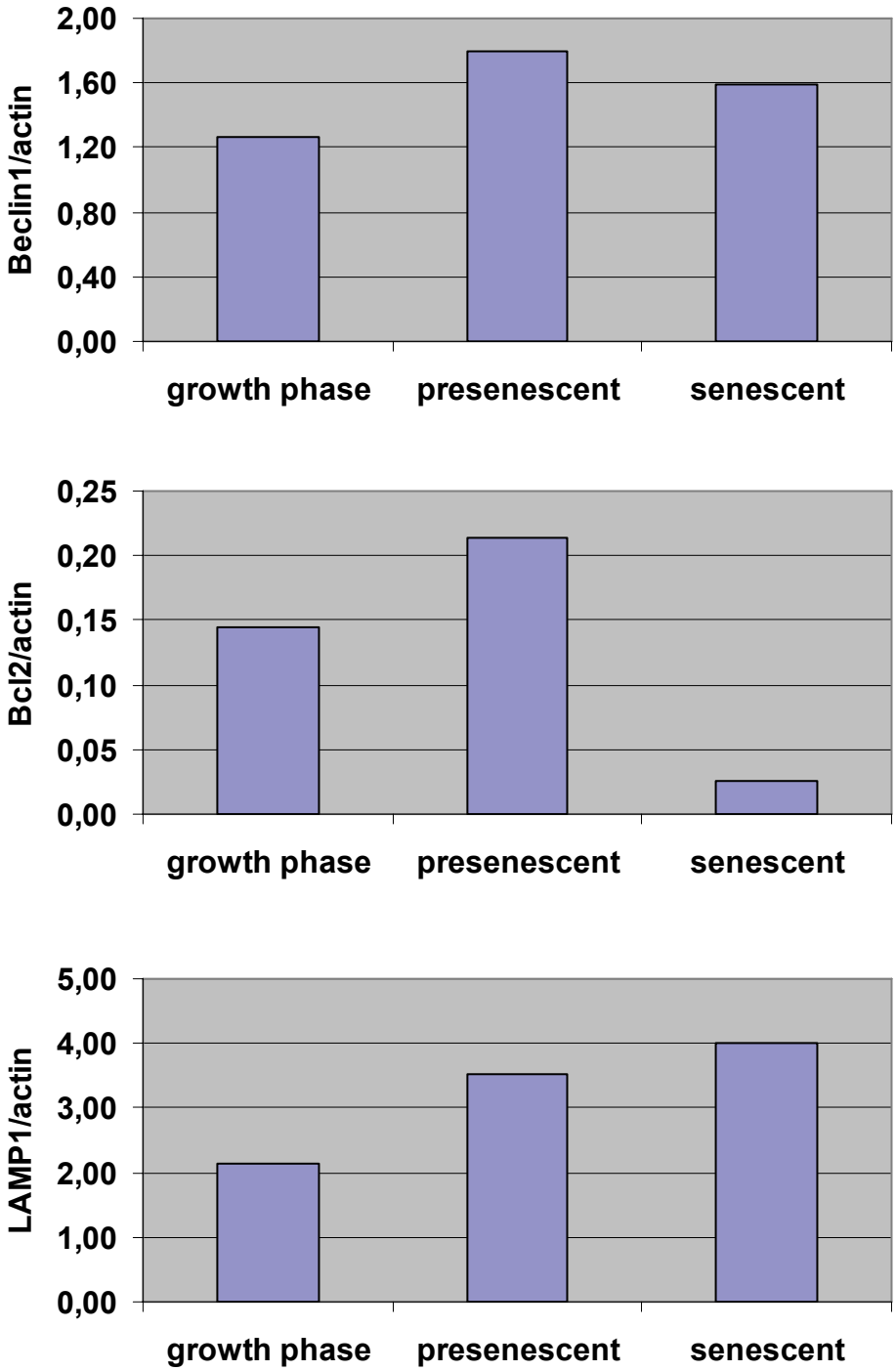
References

1. Campisi J: Cellular senescence as a tumor-suppressor mechanism. Trends Cell Biol 2001, 11:S27–S31
2. Cristofalo VJ, Lorenzini A, Allen RG, Torres C, Tresini M: Replicative senescence: a critical review. Mech Ageing Dev 2004, 125:827–848
3. Zwerschke W, Mazurek S, Stockl P, Hutter E, Eigenbrodt E, Jansen-Durr P: Metabolic analysis of senescent human fibroblasts reveals a role for AMP in cellular senescence. Biochem J 2003, 376:403–411
4. Yoon IK, Kim HK, Kim YK, Song IH, Kim W, Kim S, Baek SH, Kim JH, Kim JR: Exploration of replicative senescence-associated genes in human dermal fibroblasts by cDNA microarray technology. Exp Gerontol 2004, 39:1369–1378
5. Kang MK, Kameta A, Shin KH, Baluda MA, Kim HR, Park NH: Senescence-associated genes in normal human oral keratinocytes. Exp Cell Res 2003, 287:272–281
6. Linskens MH, Feng J, Andrews WH, Enlow BE, Saati SM, Tonkin LA, Funk WD, Villeponteau B: Cataloging altered gene expression in young and senescent cells using enhanced differential display. Nucleic Acids Res 1995, 23:3244–3251
7. Schwarze SR, DePrimo SE, Grabert LM, Fu VX, Brooks JD, Jarrard DF: Novel pathways associated with bypassing cellular senescence in human prostate epithelial cells. J Biol Chem 2002, 277: 14877–14883
8. Shelton DN, Chang E, Whittier PS, Choi D, Funk WD: Microarray analysis of replicative senescence. Current Biology 1999, 9:939–945
9. Untergasser G, Koch HB, Menssen A, Hermeking H: Characterization of epithelial senescence by serial analysis of gene expression: identification of genes potentially involved in prostate cancer. Cancer Res 2002, 62:6255–6262
10. Benvenuti S, Cramer R, Quinn CC, Bruce J, Zvelebil M, Corless S, Bond J, Yang A, Hockfield S, Burlingame AL, Waterfield MD, Jat PS: Differential proteome analysis of replicative senescence in rat embryo fibroblasts. Mol Cell Proteomics 2002, 1:280–292
11. Dierick JF, Kalume DE, Wenders F, Salmon M, Dieu M, Raes M, Roepstorff P, Toussaint O: Identification of 30 protein species involved in replicative senescence and stress-induced premature senescence. FEBS Lett 2002, 531:499–504
12. Degterev A, Boyce M, Yuan J: A decade of caspases. Oncogene 2003, 22:8543–8567
13. Gozuacik D, Kimchi A: Autophagy and cell death. Curr Top Dev Biol 2007, 78:217–245
14. Lockshin RA, Zakeri Z: Apoptosis, autophagy, and more. Int J Biochem Cell Biol 2004, 36:2405–2419
15. Dunn WA, Jr.: Studies on the mechanisms of autophagy: formation of the autophagic vacuole. J Cell Biol 1990, 110:1923–1933
16. Mizushima N, Yamamoto A, Hatano M, Kobayashi Y, Kabeya Y, Suzuki K, Tokuhisa T, Ohsumi Y, Yoshimori T: Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. J Cell Biol 2001, 152:657–668
17. Mizushima N: Autophagy: process and function. Genes Dev 2007, 21:2861–2873
18. Xie Z, Klionsky DJ: Autophagosome formation: core machinery and adaptations. Nat Cell Biol 2007, 9:1102–1109
19. Bursch W, Hochegger K, Torok L, Marian B, Ellinger A, Schulte-Hermann R: Autophagic and apoptotic types of programmed cell death exhibit different fates of cytoskeletal filaments. J Cell Sci 2000, 113: 1189–1198
20. Broker LE, Kruyt FA, Giaccone G: Cell death independent of caspases: a review. Clin Cancer Res 2005, 11:3155–3162
21. Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, Baehrecke EH, Lenardo MJ: Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. Science 2004, 304:1500–1502
22. Edinger AL, Thompson CB: Death by design: apoptosis, necrosis, and autophagy. Curr Opin Cell Biol 2004, 16:663–669
23. Golstein P, Kroemer G: Cell death by necrosis: towards a molecular definition. Trends Biochem Sci 2007, 32:37–43
24. Boyce ST, Ham RG: Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. J Invest Dermatol 1983, 81:33s–40s
25. Reynolds ES: The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 1963, 17:208–212
26. Kagan VE, Fabisiak JP, Shvedova AA, Tyurina YY, Tyurin VA, Schor NF, Kawai K: Oxidative signaling pathway for externalization of plasma membrane phosphatidylserine during apoptosis. FEBS Lett 2000, 477:1–7
27. Petrovski G, Zahuczky G, Katona K, Vereb G, Martinet W, Nemes Z, Bursch W, Fesus L: Clearance of dying autophagic cells of different origin by professional and non-professional phagocytes. Cell Death Differ 2007, 14:1117–1128
28. Seglen PO, Gordon PB: 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. Proc Natl Acad Sci USA 1982, 79:1889–1892
29. Robertson JD, Orrenius S, Zhivotovsky B: Review: nuclear events in apoptosis. J Struct Biol 2000, 129:346–358
30. Rojas E, Lopez MC, Valverde M: Single cell gel electrophoresis assay: methodology and applications. J Chromatogr B Biomed Sci Appl 1999, 722:225–254
31. Fadeel B, Orrenius S: Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. J Intern Med 2005, 258:479–517
32. Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, Packer M, Schneider MD, Levine B: Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 2005, 122:927–939

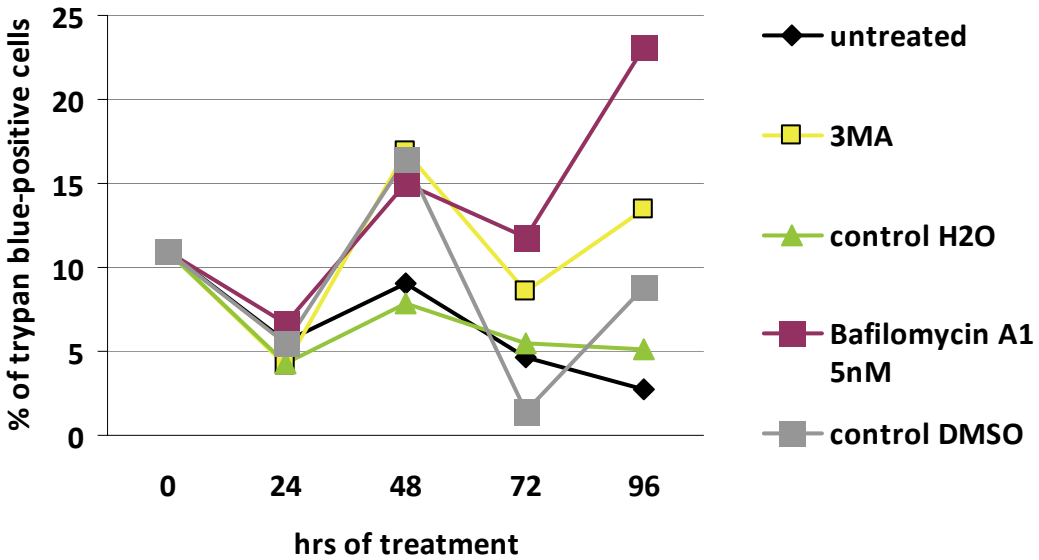
33. Biederbick A, Kern HF, Elsasser HP: Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles. Eur J Cell Biol 1995, 66:3–14
34. Mizushima N: Methods for monitoring autophagy. Int J Biochem Cell Biol 2004, 36:2491–2502
35. Allen DG, Riviere JE, Monteiro-Riviere NA: Analysis of interleukin-8 release from normal human epidermal keratinocytes exposed to aliphatic hydrocarbons: delivery of hydrocarbons to cell cultures via complexation with alpha-cyclodextrin. Toxicol In Vitro 2001, 15: 663–669
36. Yeung T, Gilbert GE, Shi J, Silvius J, Kapus A, Grinstein S: Membrane phosphatidylserine regulates surface charge and protein localization. Science 2008, 319:210–213
37. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T: LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 2000, 19:5720–5728
38. Gerland LM, Peyrol S, Lallemand C, Branche R, Magaud JP, French M: Association of increased autophagic inclusions labeled for beta-galactosidase with fibroblastic aging. Exp Gerontol 2003, 38: 887–895
39. Clarke PGH: Developmental cell death: morphological diversity and multiple mechanisms. Anat Embryol 1990, 181:195–213
40. Gozuacik D, Kimchi A: Autophagy as a cell death and tumor suppressor mechanism. Oncogene 2004, 23:2891–2906
41. Mizushima N, Levine B, Cuervo AM, Klionsky DJ: Autophagy fights disease through cellular self-digestion. Nature 2008, 451:1069–1075
42. Roberts P, Moshitch-Moshkovitz S, Kvam E, O'Toole E, Winey M, Goldfarb DS: Piecemeal microautophagy of nucleus in Saccharomyces cerevisiae. Mol Biol Cell 2003, 14:129–141
43. Daido S, Yamamoto A, Fujiwara K, Sawaya R, Kondo S, Kondo Y: Inhibition of the DNA-dependent protein kinase catalytic subunit radiosensitizes malignant glioma cells by inducing autophagy. Cancer Res 2005, 65:4368–4375
44. Shimizu S, Kanaseki T, Mizushima N, Mizuta T, Arakawa-Kobayashi S, Thompson CB, Tsujimoto Y: Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. Nat Cell Biol 2004, 6:1221–1228
45. Karantza-Wadsworth V, Patel S, Kravchuk O, Chen G, Mathew R, Jin S, White E: Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. Genes Dev 2007, 21:1621–1635
46. Kirkland RA, Adibhatla RM, Hatcher JF, Franklin JL: Loss of cardiolipin and mitochondria during programmed neuronal death: evidence of a role for lipid peroxidation and autophagy. Neuroscience 2002, 115:587–602
47. Lee HC, Yin PH, Lu CY, Chi CW, Wei YH: Increase of mitochondria and mitochondrial DNA in response to oxidative stress in human cells. Biochem J 2000, 348 Pt 2:425–432
48. Lemasters JJ, Nieminen AL, Qian T, Trost LC, Elmore SP, Nishimura Y, Crowe RA, Cascio WE, Bradham CA, Brenner DA, Herman B: The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. Biochim Biophys Acta 1998, 1366:177–196
49. Brunk UT, Terman A: The mitochondrial-lysosomal axis theory of aging: accumulation of damaged mitochondria as a result of imperfect autophagocytosis. Eur J Biochem 2002, 269:1996–2002
50. Wang E: Senescent human fibroblasts resist programmed cell death, and failure to suppress bcl2 is involved. Cancer Res 1995, 55:2284–2292
51. Seluanov A, Gorbunova V, Falcovitz A, Sigal A, Milyavsky M, Zurer I, Shohat G, Goldfinger N, Rotter V: Change of the death pathway in senescent human fibroblasts in response to DNA damage is caused by an inability to stabilize p53. Mol Cell Biol 2001, 21:1552–1564
52. Spaulding C, Guo W, Effros RB: Resistance to apoptosis in human CD8+ T cells that reach replicative senescence after multiple rounds of antigen-specific proliferation. Exp Gerontol 1999, 34:633–644
53. Chaturvedi V, Qin J-Z, Denning MF, Choubey D, Diaz MO, Nickoloff BJ: Apoptosis in proliferating, senescent, and immortalized keratinocytes. J Biol Chem 1999, 274:23358–23367
54. Ohshima S: Apoptosis in stress-induced and spontaneously senescent human fibroblasts. Biochem Biophys Res Commun 2004, 324:241–246
55. Wagner M, Hampel B, Bernhard D, Hala M, Zwerschke W, Jansen-Durr P: Replicative senescence of human endothelial cells in vitro involves G1 arrest, polyploidization and senescence-associated apoptosis. Exp Gerontol 2001, 36:1327–1347
56. Unterluggauer H, Hampel B, Zwerschke W, Jansen-Durr P: Senescence-associated cell death of human endothelial cells: the role of oxidative stress. Exp Gerontol 2003, 38:1149–1160
57. Hampel B, Malisan F, Niederegger H, Testi R, Jansen-Durr P: Differential regulation of apoptotic cell death in senescent human cells. Exp Gerontol 2004, 39:1713–1721
58. Norsgaard H, Clark BF, Rattan SI: Distinction between differentiation and senescence and the absence of increased apoptosis in human keratinocytes undergoing cellular aging in vitro. Exp Gerontol 1996, 31:563–570
59. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, Peacocke M, Campisi J: A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci USA 1995, 92:9363–9367
60. Ding G, Franki N, Kapasi AA, Reddy K, Gibbons N, Singhal PC: Tubular cell senescence and expression of TGF-beta1 and p21(WAF1/CIP1) in tubulointerstitial fibrosis of aging rats. Exp Mol Pathol 2001, 70:43–53
61. Martin JA, Buckwalter JA: The role of chondrocyte senescence in the pathogenesis of osteoarthritis and in limiting cartilage repair. J Bone Joint Surg Am 2003, 85-A Suppl 2:106–110
62. Keyes WM, Wu Y, Vogel H, Guo X, Lowe SW, Mills AA: p63 deficiency activates a program of cellular senescence and leads to accelerated aging. Genes Dev 2005, 19:1986–1999
63. Lee BY, Han JA, Im JS, Morrone A, Johung K, Goodwin EC, Kleijer WJ, DiMaio D, Hwang ES: Senescence-associated beta-galactosidase is lysosomal beta-galactosidase. Aging Cell 2006, 5:187–195
64. Yin D: Biochemical basis of lipofuscin, ceroid, and age pigment-like fluorophores. Free Radic Biol Med 1996, 21:871–888
65. Yue Z, Jin S, Yang C, Levine AJ, Heintz N: Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc Natl Acad Sci USA 2003, 100: 15077–15082
66. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y, Cattoretti G, Levine B: Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J Clin Invest 2003, 112:1809–1820
67. Sansal I, Sellers WR: The biology and clinical relevance of the PTEN tumor suppressor pathway. J Clin Oncol 2004, 22:2954–2963
68. Arico S, Petiot A, Bauvy C, Dubbelhuis PF, Meijer AJ, Codogno P, Ogier-Denis E: The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. J Biol Chem 2001, 276:35243–35246
69. Hippert MM, O'Toole PS, Thorburn A: Autophagy in cancer: good, bad, or both?. Cancer Res 2006, 66:9349–9351



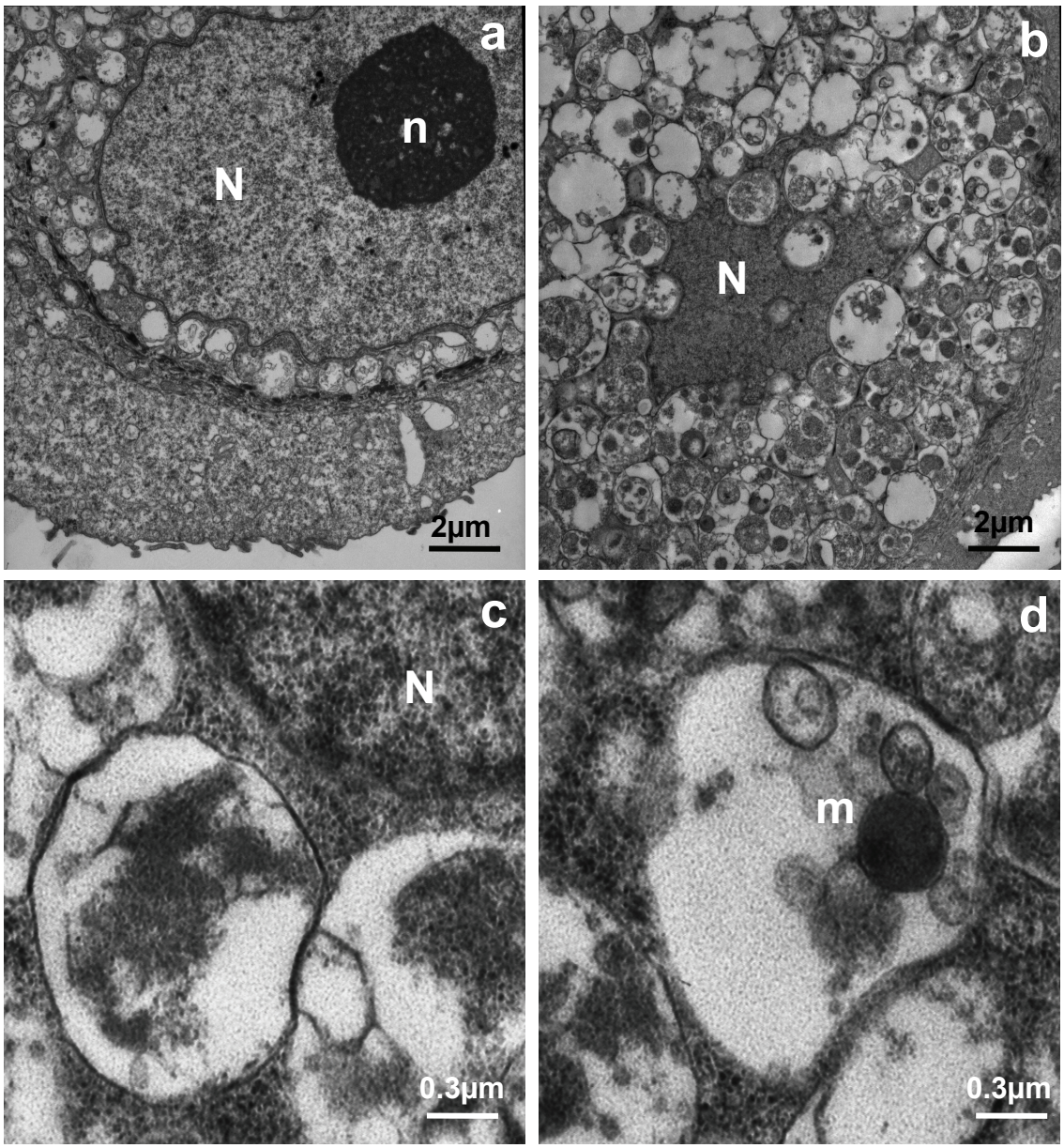
Supplemental Figure S1: Quantification of the western-blot of Fig1C



Supplemental Figure S2: Quantification of the western-blots of Fig10B



Supplemental Figure S3: Toxicity of 3-MA and Bafilomycine
Cells at the exponential growth phase were treated by 3-MA 5mM or its diluent (H2O) or Bafilomycine A1 5nM or its diluent (DMSO). Toxicity was evaluated by counting every day trypan blue-positive cells



Supplemental Figure S4: Bafilomycine induces an accumulation of autophagic vacuoles

Cells at the end of the growth phase were treated (b, c and d) or not (a) by Bafilomycin A1 5nM and analyzed by TEM. Bafilomycin-treated cells are full of autophagic vacuoles (b) which contain numerous various debris (b, c and). Some of these autophagic vacuoles contain elements resembling a mitochondrion with a double membrane (d). N=nucleus, n=nucleolus, m=michondrion.

L'augmentation d'expression de la manganèse superoxyde dismutase est responsable de la mort des kératinocytes sénescents par autophagie

L'équipe a montré en 2004 que la sénescence des kératinocytes est causée par le stress oxydant, principalement par le peroxyde d'hydrogène résultant de l'augmentation d'expression de la MnSOD [142]. Nous avons donc cherché à déterminer le rôle potentiel de cette voie dans l'activation de la mort par macroautophagie associée à la sénescence.

Sachant désormais distinguer les cellules mourantes du reste de la population sénescente, nous avons dans un premier temps montré qu'elles présentent de nombreux dommages oxydants (8-OH-G, ponts AIP) dans leurs noyaux et mitochondries, notamment lorsqu'ils sont présents dans la zone centrale, c'est à dire à proximité des vésicules autophagiques.

Pour confirmer l'impact du stress oxydant sur l'induction de la mort par autophagie, des kératinocytes en croissance exponentielle ont été induits en sénescence prématurée par surexpression adénovirale de la MnSOD ou traitement au peroxyde d'hydrogène. Comme pour la sénescence normale, l'induction prématurée de la sénescence s'accompagne de l'accumulation de cellules mourantes dans les cultures. Ces cadavres ont la morphologie typique de ceux précédemment décrits lors de la sénescence normale, à savoir réfringents en microscopie à contraste de phase, s'arrondissant et généralement isolés des cellules voisines. Nous avons ensuite recherché la présence de lysosomes dans les cadavres par marquage au Lysotracker dans le but de déterminer si cette mort s'accompagne également d'une augmentation de l'activité macroautophagique. L'analyse de ces marquages par microscopie et cytométrie en flux confirmera alors que cette mort associée à la sénescence induite par le H_2O_2 s'accompagne de l'accumulation de vésicules acides autour du noyau. Ces résultats ont pu être confirmés par l'expression de la protéine de fusion Atg8/LC3B couplée à une eGFP et une mRFP en N-Terminal [395]. Nous avons alors pu conclure que, comme pour la sénescence spontanée, la sénescence induite par traitement au H_2O_2

s'accompagne de l'accumulation d'autolysosomes (rouge), que l'on retrouve concentrés autour du noyau altéré dans les cadavres autophagiques.

Pour valider définitivement le rôle central des ROS dans l'activation de la mort par macroautophagie, des NHEKs induits en sénescence par traitement au H_2O_2 ont été traités avec la 3-méthyladénine ou transfectés par des siRNA ciblant spécifiquement l'expression de Atg5 (essentiel aux étapes précoces de la macroautophagie). Dans ces deux conditions, l'inhibition de la macroautophagie repousse l'apparition des cadavres dans les cultures. De plus, un traitement des cellules sénescentes normales (triées par FACS) avec la catalase (qui réduit le H_2O_2 en H_2O) est efficace pour diminuer l'apparition de cadavres.

Ces travaux concernant le rôle du stress oxydant lors de l'induction de la mort des NHEKs sénescents par macroautophagie font l'objet de l'article n°3 : *MnSOD upregulation induces autophagic programmed cell death in senescent keratinocytes.*

Article n°3 : MnSOD upregulation induces autophagic programmed cell death in senescent keratinocytes

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MnSOD Upregulation Induces Autophagic Programmed Cell Death in Senescent Keratinocytes

Emeric Deruy[1,2,3,4,5], Karo Gosselin[1,2,3,4,5], Chantal Vercamer[1,2,3,4,5], Sébastien Martien[1,2,3,4,5], Fatima Bouali[1,2,3,4,5], Christian Slomianny[1,3,6], Julie Bertout[1,2,3,4,5], David Bernard[7], Albin Pourtier[1,2,3,4,5], Corinne Abbadie[1,2,3,4,5]*

1 Université Lille Nord de France, Lille, France, **2** CNRS, UMR8161, Institut de Biologie de Lille, Lille, France, **3** USTL, Villeneuve d'Ascq, France, **4** UDSL, Lille, France, **5** Institut Pasteur de Lille, Lille, France, **6** INSERM, U600, Laboratoire de Physiologie Cellulaire, Villeneuve d'Ascq, France, **7** CNRS, UMR5238, Centre Léon-Bérard, Lyon, France

Abstract

Senescence is a state of growth arrest resulting mainly from telomere attrition and oxidative stress. It ultimately leads to cell death. We have previously shown that, in keratinocytes, senescence is induced by NF-kappaB activation, MnSOD upregulation and H_2O_2 overproduction. We have also shown that senescent keratinocytes do not die by apoptosis but as a result of high macroautophagic activity that targets the primary vital cell components. Here, we investigated the mechanisms that activate this autophagic cell death program. We show that corpses occurring at the senescence plateau display oxidatively-damaged mitochondria and nucleus that colocalize with autophagic vacuoles. The occurrence of such corpses was decreased by specifically reducing the H_2O_2 level with catalase, and, conversely, reproduced by overexpressing MnSOD or applying subtoxic doses of H_2O_2. This H_2O_2-induced cell death did occur through autophagy since it was accompanied by an accumulation of autophagic vesicles as evidenced by Lysotracker staining, LC3 vesiculation and transmission electron microscopy. Most importantly, it was partly abolished by 3-methyladenine, the specific inhibitor of autophagosome formation, and by anti-Atg5 siRNAs. Taken together these results suggest that autophagic cell death is activated in senescent keratinocytes because of the upregulation of MnSOD and the resulting accumulation of oxidative damages to nucleus and mitochondria.

Citation: Deruy E, Gosselin K, Vercamer C, Martien S, Bouali F, et al. (2010) MnSOD Upregulation Induces Autophagic Programmed Cell Death in Senescent Keratinocytes. PLoS ONE 5(9): e12712. doi:10.1371/journal.pone.0012712

Editor: Mikhail V. Blagosklonny, Roswell Park Cancer Institute, United States of America

Received October 30, 2009; **Accepted** July 21, 2010; **Published** September 14, 2010

Funding: This work was supported by the Centre National de la Recherche Scientifique, the Universite Lille 1, the Ligue contre le Cancer (Comites du Nord et de l Aisne), the Institut Pasteur de Lille. E.D. has a fellowship from the Institut Pasteur de Lille and the Region Nord/Pas-de-Calais. K.G. had a fellowship from the Institut Pasteur de Lille and the Region Nord/Pas-de-Calais and from the Societe Francaise du Cancer. S.M. had a fellowship from the French Research Ministry and the Fondation pour la Recherche Medicale. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: corinne.abbadie@ibl.fr

Introduction

In vivo as *in vitro*, normal human cells have a limited lifespan. After having performed a certain number of divisions, they enter a special state termed senescence [1] which is the consequence of both the telomere erosion occurring at each replication cycle and of oxidative damages which increase with time. Senescent cells are cell-cycle arrested and display numerous morphological, metabolic and genetic changes [2,3]. Recently, it was shown by us and others that senescence is associated with an increase in macroautophagic activity [4–6].

The macroautophagic process, here referred as autophagy, starts by encircling a damaged cell component inside a double membrane. The autophagosome resulting from the closure of this membrane then fuses with endosomes and lysosomes to form an autophagolysosome, inside which the sequestered material is submitted to an acidic pH and to the activity of various hydrolytic enzymes. The recognition of the altered material, and the formation, migration and fusion of the autophagic vacuoles at different stages involve about thirty Atg genes [7–9].

We have shown that the autophagic activity associated with senescence of normal human epidermal senescent keratinocytes (NHEKs) occurs at a so high level that it targets the main vital cells components and ultimately leads to cell death [4]. The dying senescent keratinocytes are characterized by an accumulation of a huge quantity of autophagic vacuoles and a particular intracellular organisation. Their cytokeratin network develops to form a cage that partitions the intracellular space in two areas: a cortical one completely devoid of organelles and a central one in which are concentrated mainly all the organelles including the nucleus and all the autophagic vacuoles. The nuclei and mitochondria localized in this central area display various degrees of morphological damages, suggesting they are degraded therein [4].

Since escaping senescent-cell death could be a requisite step in neoplastic transformation, it is important to establish which the inducers of the autophagic programmed cell death encountered by senescent cells are. The purpose of this study was hence to analyse the type of damages undergone by senescent cells and to investigate whether these damages are responsible for the activation of the autophagic process at a lethal level. We had established in a previous work that keratinocyte senescence partly results from an accumulation of hydrogen peroxide (H_2O_2) due to the up-regulation of MnSOD by NF-kappaB transcription factors. MnSOD is a

mitochondrial redox enzyme that dismutates O_2^- in H_2O_2; hence, since H_2O_2-degrading enzymes such as catalase or glutathione peroxidase are not upregulated during keratinocyte senescence in coordination with MnSOD, the increased MnSOD expression leads to H_2O_2 accumulation [10]. Here, we hypothesized and demonstrate that, in addition to be involved in the establishment of the senescent phenotype itself, this H_2O_2 accumulation sufficiently damages nuclei and mitochondria to target them for massive autophagic elimination, hence leading to the death of senescent cells.

Methods

Cell culture

Normal human epidermal keratinocytes (NHEK) were purchased from Clonetics (CC-2501). We used cells from 6 different female donors of different race and age. 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% CO_2 in the KGM-2 BulletKit medium consisting of modified MCBD 153, with 0.15 mM calcium, supplemented with bovine pituitary extract, EGF, insulin, hydrocortisone, transferrin and epinephrin (CC-3107, Clonetics). Such a serum-free low-calcium medium was shown to minimize keratinocyte terminal differentiation [11]. In all experiments, cells were seeded as recommended by the supplier at 3500 cells/cm^2. When necessary, they were split at 70% confluence. The number of population doublings (PD) was calculated at each passage by means of the following equation: PD = ln(number of collected cells/number of plated cells)/ln2.

Western-blotting

Cells were lysed in the following solution: Hepes 27.5 mM pH 7.6, urea 1.1 M, NaCl 0.33 M, EGTA 0.1 M, EDTA 2 mM, KCl 60 mM, DTT 1 mM 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 nitrocellulose membranes (Hybond-C extra, Amersham). Equal loading was verified after a Ponceau Red coloration of the membranes. Primary antibody used was an anti human MnSOD sheep IgG (Calbiochem), anti human Atg5 rabbit antibody (Cell Signalling) or anti human GAPDH mouse monoclonal antibody (Chemicon International). Secondary antibodies used were peroxidase-conjugated (Jackson ImmunoResearch Laboratories). Peroxidase activity was revealed using a ECL (enhanced chemiluminescence) or ECL advance kit (Amersham Biosciences).

Immunofluorescence

For detection of MnSOD, AIP bridges and LAMP1, cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton-X100. For 8oxoG immunodetection, cells were fixed in 4% paraformaldehyde for 15 mn at 4°C, dehydrated in 70% and 95% methanol for 3 mn followed by 99% methanol for 30 mn at −20°C. Finally cells were rehydrated by 3 mn incubation at −20°C in 95% and 70% methanol, and washed 3 times in PBS. Slides were incubated with a primary antibody: anti-MnSOD (Chemicon), anti-8oxoG (Trevigen), anti-LAMP1 (Santa Cruz) and anti-AIP that are mouse monoclonal antibodies produced by [12]. Cells were then washed 3 times with PBS and incubated with the secondary antibody: Rhodamine Red-conjugated anti-Mouse IgG or Rhodamine Red-conjugated anti-Rabbit IgG (Jackson ImmunoResearch Laboratories). Nuclei were stained by Hoechst 33258 at 1 µg/ml for 3 mn.

Fluorescent co-staining of mitochondria and lysosomes

Lysotracker green and Mitotracker red were from Molecular Probes. Living cells were incubated with Lysotracker green directly added in the cell culture medium at 37°C at 100 nM for 2 hrs, or with Mitotracker red at 25 nM for 30 mn. Nuclei were stained with the vital Hoechst 33342 at 1 µg/ml for 10 mn at 37°C.

Transmission electron microscopy

Cell pellets were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for at least 30 min at 4°C. After fixation, the specimens were thoroughly washed in 0.1 M cacodylate buffer and then postfixed with 1% osmium tetroxide in the same buffer for 1 h at room temperature, stained *en bloc* with 2% uranyl acetate in distilled water for 15 min, dehydrated in graded acetonitrile, and embedded in Epon. Ultrathin sections (80–100 nm thick) mounted on 150-mesh grids were stained with 2% uranyl acetate solution and Reynolds lead citrate solution [13]. The electron micrographs were taken with a Hitachi H600 electron microscope at 75 kV.

SA-beta-Galactosidase assays

SA-beta-Gal assays were performed as described by Dimri [14].

Annexin-V assays

Cells were processed with an Annexin-V-Alexa 568 kit (Roche, Calbiochem) according to manufacturer's recommendations.

Flow cytometry measurement of ROS levels

ROS levels were measured using non-fluorescent H_2-DCFDA (2′,7′-dichlorofluorescein diacetate) (D399, Molecular Probes) which diffuses across membranes and is oxidized to fluorescent DCF. Cells were rinsed in PBS, incubated with H_2-DCFDA diluted in medium at 5 µM for 30 min at 37°C. After that, cells were washed, trypsinized, and re-suspended in pre-warmed PBS at 37°C. They were analyzed for forward and side scatter factor values and fluorescence intensity using a flow cytometer (Coulter EPICS XL-MCL) with FITC filters. The results were analyzed with the WinMDI 2.9 software.

Flow cytometry measurement of acidic vesicles

Acidic vesicles levels were measured using Lysotracker green (Molecular Probes). Cells were incubated with Lysotracker directly added in the cell culture medium at 37°C at 200 nM for 15 min. After that, cells were washed, trypsinized, and re-suspended in pre-warmed PBS at 37°C. They were analyzed for forward and side scatter factor values and fluorescence intensity using a flow cytometer (Coulter EPICS XL-MCL) with FITC filters. The results were analyzed with the WinMDI 2.9 software.

Flow cytometry sorting of senescent cells. Antioxidant treatment

NHEKs were analyzed on a BD FACS Aria and the subpopulation with the ad hoc forward and side scatter factor values was electrostatically sorted in air, collected in complete culture medium and put again in culture. After plating, cells were treated either by Catalase (Sigma, C1345) or PEG-catalase (Sigma, C4963) diluted in PBS and directly added in the culture medium at different final concentrations.

Adenoviral vector encoding MnSOD

The human MnSOD cDNA was obtained after retrotranscription, amplified by PCR and inserted into the pcDNA3.1 as previously described [15]. The MnSOD cDNA was then digested by EcoRI and inserted into the pAdCMV2 vector between the XbaI sites after filling with Klenow polymerase. Recombinant adenovirus vectors (AdMnSOD) were obtained by homologous

recombination in *E. coli* BJ5183 as described in [16] (details are available on request). Viral stocks were amplified after infection of N52.E6 cells [17]. Recombinant adenoviruses were purified using ViraBind Adenovirus purification kit (Cell Biolabs Inc., San Diego, CA) and titrated using Adeno-X rapid titer kit (BD Biosciences Clontech, Palo Alto, CA, USA). Cells were infected by adding virus stocks directly to the culture medium at an input multiplicity of 200 viral particles/cell.

Inhibition of autophagy by RNA interference

NHEK at exponential growth phase were plated at 70,000 cells per well in six-well plates. The day of transfection, culture media were renewed and siRNA mixtures prepared using PrimeFect siRNA Transfection Reagent diluted 1/100 in PrimeFect diluent (purchased from Lonza) and incubated 15 minutes at room temperature before adding to cells. Inhibition of Atg5 expression was performed using 25 or 50 nM of a pool of 4 siRNA (siGENOME SMARTpool, Dharmacon - GGAAUAUCCUG-CAGAAGAA - CAUCUGAGCUACCCGGAUA - GACAAGA-AGACAUUAGUGA - CAAUUGGUUUGCUAUUUGA). Control transfection was performed using a non targeting siRNA pool (siCONTROL Non Targeting siRNA Pool # 2, Dharmacon). Transfections were stopped after 24 hrs by adding fresh culture medium.

Cell transfection with the mRFP-GFP-LC3 vector

NHEK at exponential growth phase were treated twice by 50 μM H_2O_2 at 48 hrs interval and then electroporated with the mRFP-GFP-LC3 [18] or the mRFP-GFP control vector using the Neon transfection system (Invitrogen) according to supplier recommendations. Briefly, 80,000 cells were suspended in 10 μL of R buffer containing 1 μg of plasmid and the electroporation was performed by 2 pulses at 1400 V for 20 ms. After electroporation, cells were plated in complete culture medium on microscopic slides. Forty eight hrs later, they were stained by Hoechst 33342 at 1 μg/ml for 10 mn at 37°C, mounted in PBS without any fixation, and analyzed under a confocal microscope (LSM710, Zeiss).

Ethics statement

Human cells used in this study provide from people whose informed consent was obtained by the cell supplier (Clonetics) Cells were obtained anonymously. No ethics approval was necessary for the experiments performed therein.

Results

Senescent keratinocytes and corpses at the senescent plateau display altered mitochondria and nuclei that colocalize with autophagic vacuoles

To study the inducers of senescent-cell death by macroautophagy, we used normal human epidermal keratinocytes (NHEKs). As we have shown in previous studies, NHEKs cultured in vitro reach a senescence growth plateau after 15–25 population doublings (PDs). At this plateau most of the cells display all the characteristics of senescence: senescence-associated beta-galactosidase activity at pH 6, upregulation of the CKI $p16^{INK4}$, a 5- to 100-fold larger size than young cells, numerous dense particles (probably protein aggregates), several vacuole-like structures of different sizes, and about 20% of polynucleation [4,10]. A flow cytometry analysis of AnnexinV- and Propidium Iodide-staining revealed that about 20% of cells at the senescence plateau are dying cells or corpses [4]. Microscopically, they are characterized by their round shape and the presence of a central area that was demonstrated by transmission electron microscopy to be delineated by a keratin cage and to be full of autophagic vacuoles [4].

In order to make a first investigation of the nuclear damages that could contribute to the induction of autophagic programmed cell death in senescent NHEKs, we performed a Hoechst staining at the senescence plateau. Nuclei of senescent cells are often larger than that of young cells and their chromatin organisation can appear slightly altered (Figure 1). In corpses, the nuclei found in the central area always display a damaged chromatin and are often deformed (Figure 1).

We also investigated damages to mitochondria in senescent cells and corpses by performing an immunofluorescence against MnSOD, a mitochondrial matrix enzyme. In cells at the exponential growth phase, MnSOD antibodies stained discreet small sticks or vesicles, the typical appearance of mitochondria. With increasing population doublings, the stained structures tend to increase in number, in size, to vesiculate and to delocalize toward the nucleus. In corpses, almost all the stained structures were concentrated inside the central area (Figure 2A). A transmission electron microscopy analysis confirmed the alteration of mitochondria in senescent cells which displayed very dense and thickened cristae (Figure 2B).

To confirm the final colocalisation of damaged nuclei and mitochondria with autophagic vacuoles inside the central area of corpses, we performed a triple staining with Hoechst, Mitotracker (a permeant probe that fluoresces in mitochondria upon oxidation) and Lysotracker (a permeant probe that fluoresces in the acidic organelles). The analysis revealed that the three stainings indeed colocalize inside a central area (Figure 3), suggesting that the damaged nucleus and mitochondria are addressed to the central area and are degraded therein by macroautophagy.

Mitochondria and nuclei of corpses display oxidative damages

Since oxidative stress is recognized as a main cause of senescence, we postulated that it could lead to the mitochondrial and nuclear damages we evidenced and, hence, to the induction of their degradation by macroautophagy. We therefore searched for the presence of oxidative damages in mitochondria and nuclei of corpses at the senescence plateau. We focussed first on 8-oxoguanines (8oxoG), the main form of oxidized base that can be detected in nuclear or mitochondrial DNA, as well as in the free nucleotides pool [19]. The quantity of 8oxoG-immunopositive cells dramatically increased from about 3% in cultures at the exponential growth phase to about 20% at the senescence plateau (Figure 4). In senescent cells, the fluorescence was mainly localized on cytoplasmic punctuated structures clustered around the nucleus, probably the damaged mitochondria (Figure 4B and C). In corpses, some staining was observed in the central area both on the nucleus when it appeared damaged, as well as on smaller components, probably damaged mitochondria (Figure 4D).

We also examined 1-amino-3-iminopropene (AIP) bridges, an oxidative damage resulting from the reaction of primary amino groups of proteins with malondialdehyde, an end product of lipid peroxidation [12]. Almost all cells in senescent cultures were AIP-positive (Figure 5). The staining was mainly localized in nuclei and also on punctuated cytoplasmic structures, probably mitochondria (Figure 5B and C). In corpses, the nuclei kept outside the central area were AIP-positive, whereas those inside the central area were negative (Figure 5C and D), suggesting that AIP bridges are enzymatically degraded in the central area.

Taken together, these results indicate that mitochondria and nuclei of senescent cells are indeed affected by oxidative damages. They are found in this state inside the central area of corpses in

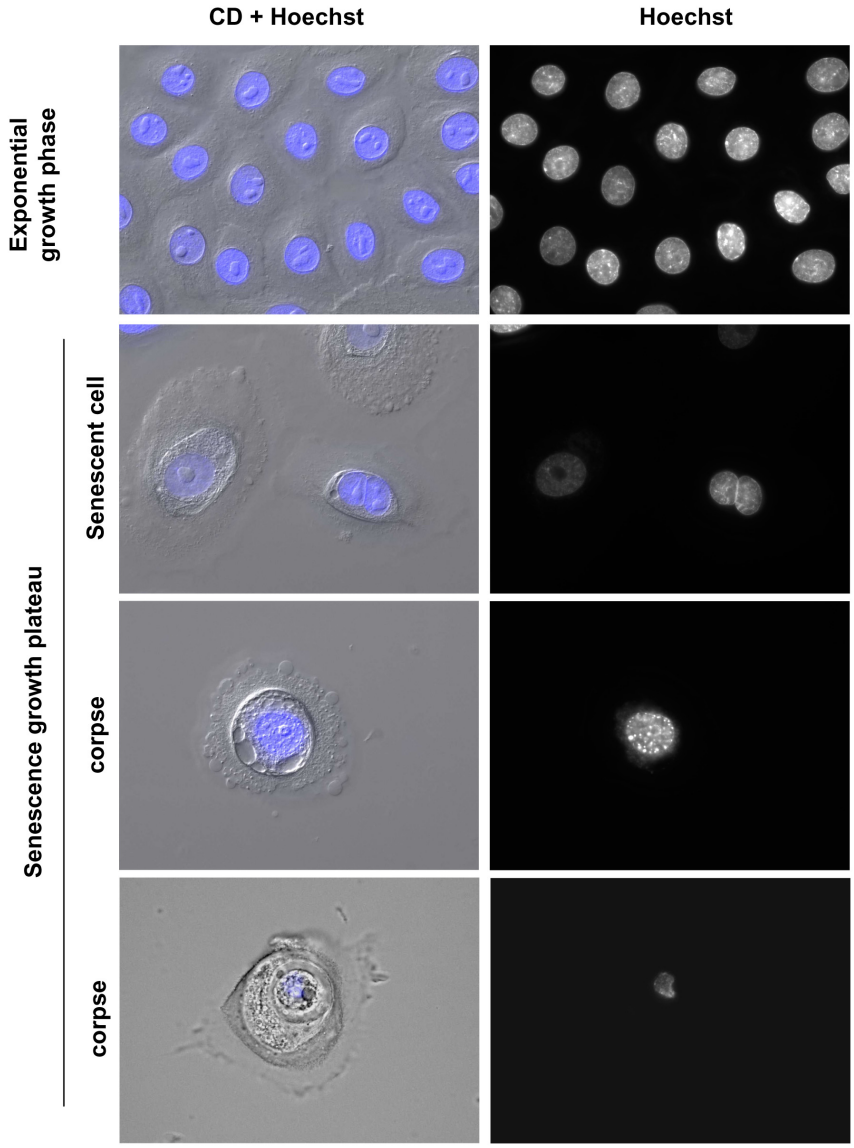


Figure 1. Nuclear damages in senescent keratinocytes and corpses. NHEKs at the exponential growth phase or at the senescence growth plateau were fixed, stained with Hoechst and observed under circular dichroism plus epifluorescent microscopy. Senescent keratinocytes display altered chromatin and are often polynucleated. Corpses are characterized by the presence of a central area always containing a much damaged nucleus. Images are representative of all the senescent cells and corpses visible at the senescence plateau of different cell donors. Scale bar = 20 μM.
doi:10.1371/journal.pone.0012712.g001

close contact with autophagic vacuoles, suggesting that autophagic programmed cell death of senescent cells could be initiated as a result of their oxidative damage.

Autophagic programmed cell death is initiated in senescent keratinocytes by MnSOD overexpression and H_2O_2 accumulation

To further demonstrate that autophagic cell death is induced in senescent cells following their oxidative damage, we provoked an oxidative stress in young cells that mimics that occurring during senescence and examined whether it induces autophagic cell death. We had previously established that the oxidative stress associated with NHEK senescence results from an activation of NF-kappaB transcription factors, that upregulate the expression of the manganese superoxide dismutase (MnSOD), a mitochondrial enzyme of the redox control, whose increased activity leads to increased hydrogen peroxide (H_2O_2) production [10]. Figure 2A confirms the accumulation of MnSOD in senescent cells versus young ones, and indicates in addition that MnSOD is also highly expressed in corpses. The accumulation of ROS in senescent cells was confirmed by a flow cytometry analysis using H_2-DCFDA, a cell permeant fluorigenic probe that fluoresces upon oxidation (Figure S1).

To assay the importance of this pathway in the induction of autophagic senescence-cell death, we overexpressed MnSOD in NHEKs at the exponential growth phase using an adenoviral vector (AdMnSOD), and we monitored the occurrence of senescence and autophagic cell death. Three days after infection, AdMnSOD-infected cells undergone a premature senescence growth plateau (Figure 6A), and from 4 days onwards, we observed cells with signs of autophagy, and corpses similar to those observed during normal senescence (Figure 6B). An immunofluorescence against MnSOD revealed that premature senescent cells

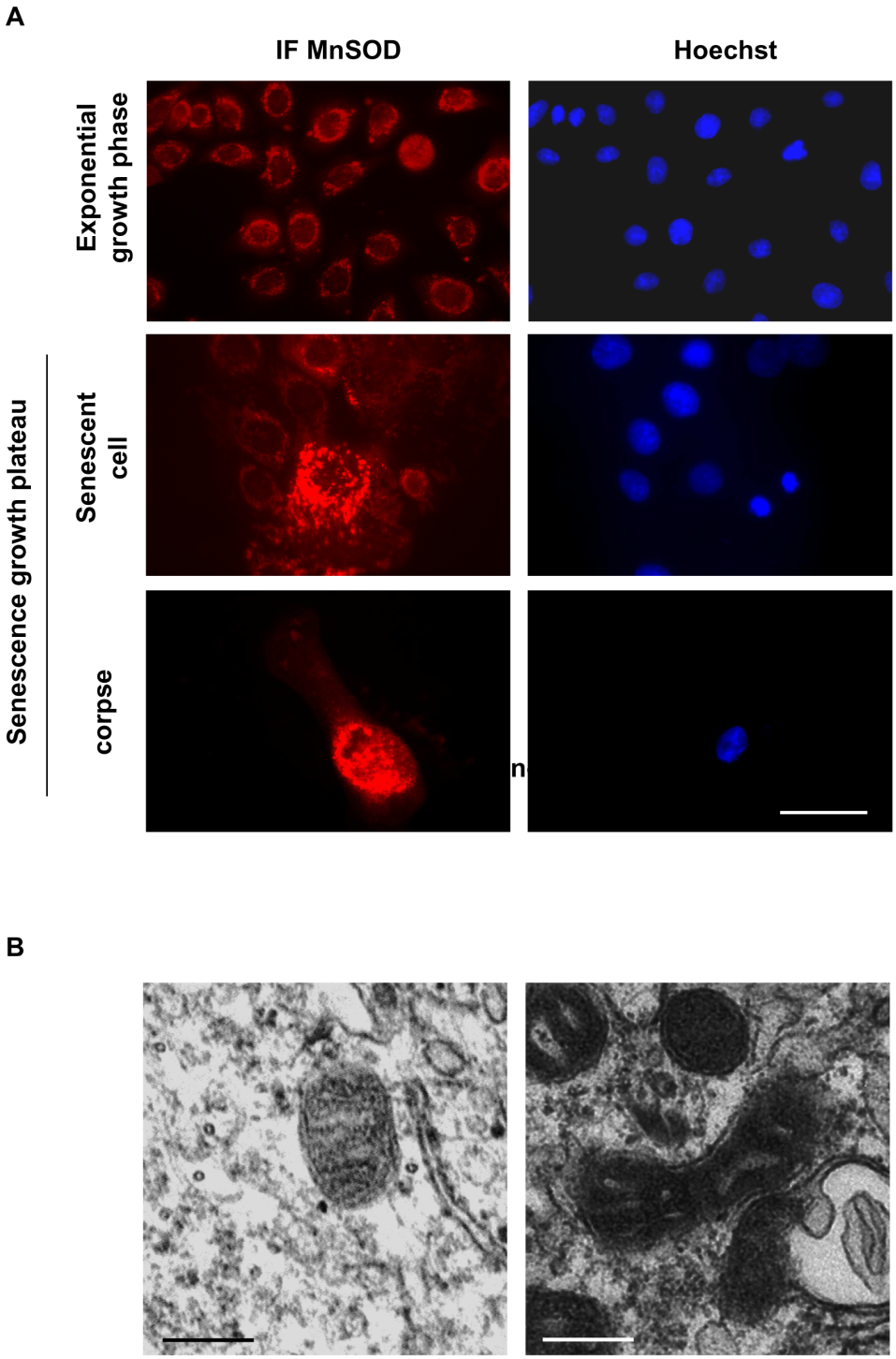


Figure 2. Mitochondrial damages in senescent keratinocytes and corpses. (A) NHEKs at the exponential growth phase or at the senescence plateau were immunostained with anti MnSOD antibodies. In almost all senescent cells, the stained structures (the mitochondria) are vesiculated and agglutinated in the vicinity of the nucleus. In corpses, the stained structures are concentrated in the central area which also contains the altered nucleus. Scale bars = 40 μM. (B) NHEKs at the exponential growth phase or at the senescence plateau were trypsinized and prepared for transmission electron microscopy. Details of mitochondrial morphology are shown. In young cells at the exponential growth phase, mitochondria have a normal morphology, whereas those of senescent cells display very dark and thickened cristae. Scale bars = 0.25 μM.
doi:10.1371/journal.pone.0012712.g002

and corpses express MnSOD at a very high level (Figure 6D). In corpses, most of the MnSOD staining was included into the central area (Figure 6D). These MnSOD-induced senescent cells and corpses contained a huge quantity of acidic vacuoles as indicated by a Lysotracker staining (Figure S2), intracellular Annexin-V staining (Figure S3A), 8-oxoG (Figure S3B) and AIP bridges (Figure S3C) similar to those of normal senescent NHEKs and corpses.

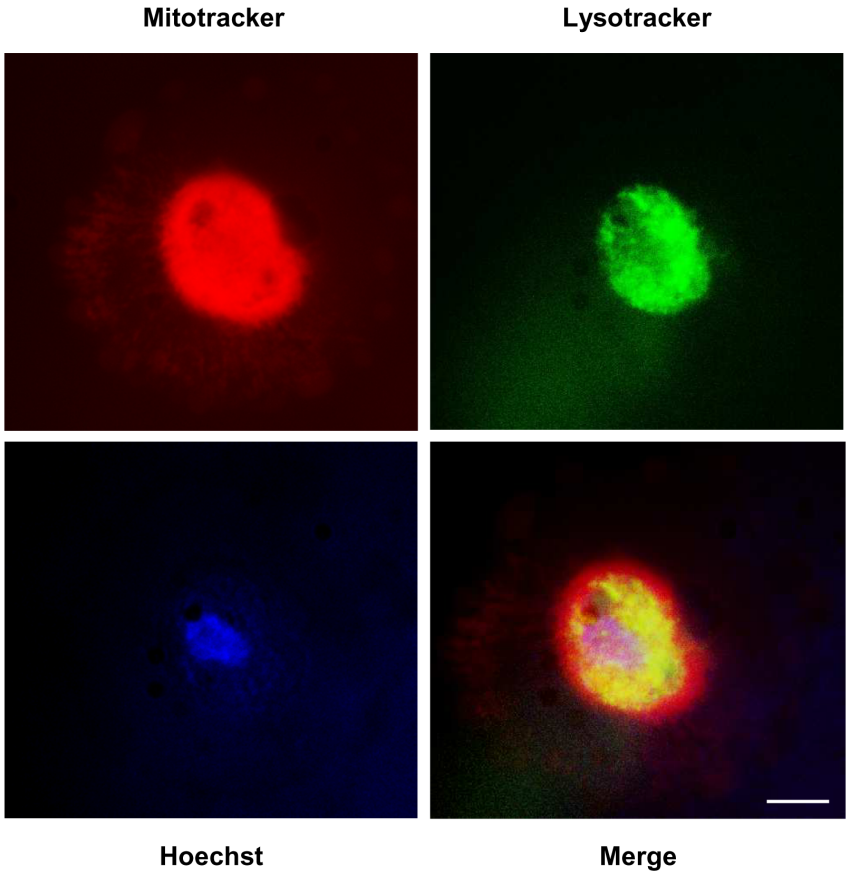
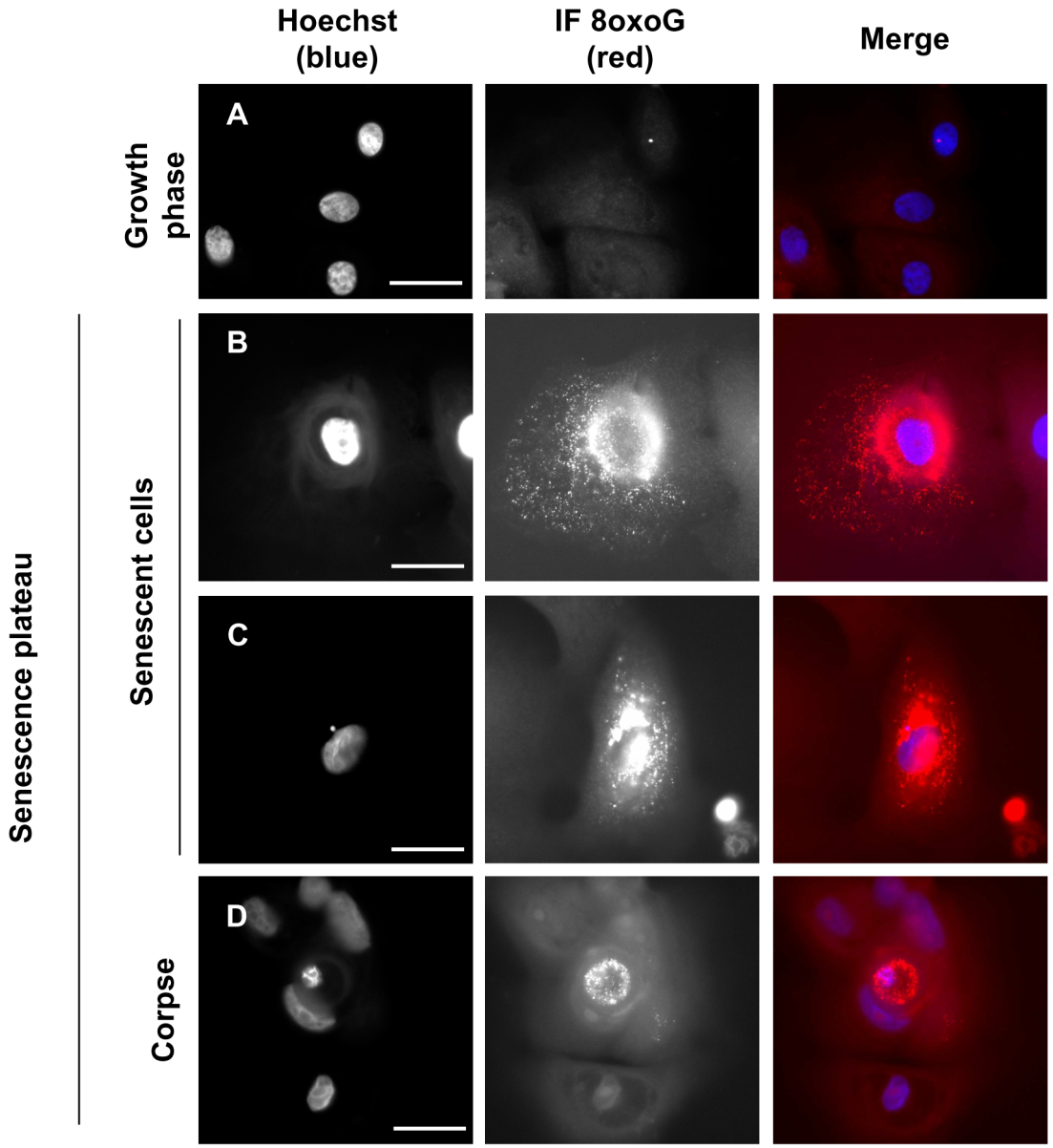


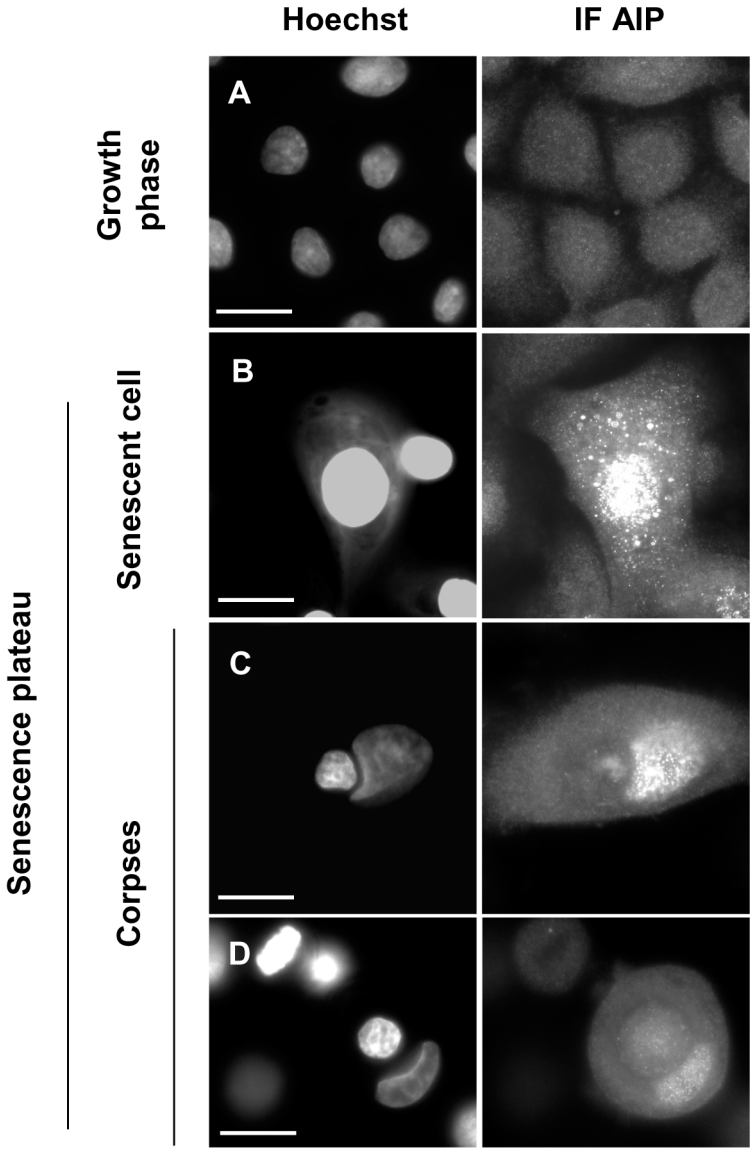
Figure 3. Corpses contain a central area in which mitochondria, nucleus and acidic vacuoles are colocalized. NHEKs at the senescence plateau were triply stained with Mitotracker (red), to stain mitochondria, Lysotracker (green), to stain the acidic organelles (including autophagic vacuoles), and Hoechst (blue) to stain nuclei. A representative image of the staining of corpses is given at high magnification (scale bar = 20 μm). The three stainings co-localize within a central area. The rest of the cell, visible as background in the Mitotracker image, is devoided of any mitochondria or acidic organelles. Note that the Lysotracker staining is vesiculated, as expected, and that the nucleus of the corpse is much damaged.
doi:10.1371/journal.pone.0012712.g003

To confirm these results and to support a direct role of H_2O_2 in the induction of autophagic cell death of senescent cells, we directly treated NHEKs at the exponential growth phase with 30 to 60 μM H_2O_2. This treatment induced from 3 days onward a growth arrest (Figure 7A), the appearance of the SA-beta-Gal marker (Figure 7B), and phenotypes morphologically resembling senescence, with large and spread cells, signs of autophagy with numerous vacuoles and corpses with a refringent central area (Figure 7C). We compared the morphology of these H_2O_2-induced corpses with that of those normally occurring at the senescence plateau by performing an analysis by circular dichroïsm microscopy and Lysotracker staining. Corpses occuring in both conditions appeared completely similar, with the same load in and distribution of acidic vacuoles (Figure 7D). The quantification of the effects of the H_2O_2 treatment was made by flow cytometry. Cells were treated by 50 μM H_2O_2 and analyzed 3 days later according to their forward and side scatter values and their Lysotracker staining. We had previously shown that the subpopulation R1 with the smallest forward scatter and side scatter values corresponds to normal young living cells, the subpopulation R2, the largest and most granular, comprises cells with senescent features, and the subpopulation R3 with a small size but a high granularity corresponds to corpses with altered membranes [4]. The analysis shows that the H_2O_2 treatment increases the subpopulation of cells with senescent features about 5 fold, and the subpopulation of corpses about 3 fold (Figure 8). The analysis of the Lysotracker fluorescence intensity indicates an increase of about 7 fold of the mass of acidic vacuoles per cell (Figure 8). To make sure that the acidic vacuoles induced by the H_2O_2-treatment were autophagic vacuoles, we performed an analysis by transmission electron microscopy. This analysis confirmed the presence of numerous vacuoles in H_2O_2-treated cells, that appeared autophagic in nature because full of various membranous and non-membranous debris (Figure 9). We also used a mRFP-GFP tandem fluorescent-tagged Atg8/LC3 that enables to see the formation of autophagosomes by the vesiculation of the green and red fluorescent staining, and to see the ensuing maturation in autophagolysosomes by the keeping of the sole red fluorescent in some vesicles, the GFP being sensitive and the mRFP resistant to the acidic pH of autophagolysosomes [18]. Cells were treated twice with H_2O_2 at 48 hrs interval, transfected by the vector, and their fluorescence was analysed 48 hrs later. The results indicate that non treated cells display both green and red vesicles, and the H_2O_2-treated ones that have become senescent or dying display vesicles more numerous, aggregated in the vicinity of the nucleus, and numerous ones redder than green (Figure 10). Taken together, these results prove that a H_2O_2 treatment induces an intense and fully active autophagic



	% 8oxoG-positive cells
Growth phase	**2.98** +/-1.95
Senescence plateau	**19.09** +/-6.33 *T-test P=0.003* *Wilcoxon P=0.036*

Figure 4. Mitochondria and nuclei of senescent keratinocytes and corpses contain oxidized guanines. NHEKs at the exponential growth phase or at the senescence plateau were processed for immunodetection of 8-oxo-guanines (8oxoG). Different representative images are given. Young cells at the growth phase are negative for 8oxoG (A). Cells at the senescence plateau display different staining patterns, according to their degree of damaging. In senescent cells not much damaged, 8oxoG are mainly localized on punctuated cytoplasmic structures, probably mitochondria (B). When the nucleus is damaged (as indicated by the presence of a micronucleus), 8oxoG are localized inside the nucleus and on punctuated cytoplasmic structures aggregated in the vicinity of the nucleus (C). When the cell is obviously dead and display a very much damaged nucleus inside the central area and another one pushed away by it, 8oxoG are localized inside the central area, inside the nucleus itself as well as inside other punctuated structures (D). Scale bars = 30 μm. The number of 8oxoG-positive cells (including corpses) was manually counted in 5 random microscopic fields amongst a total of 617 young or 123 senescent cells. Values are the mean percentages of 8-oxoG-positive cells +/− SD. Since the data are not strictly normally distributed, P values were calculated using both Student and Wilcoxon tests.
doi:10.1371/journal.pone.0012712.g004



	% AIP-positive nuclei
Growth phase	**7.19** +/-1.44
Senescence plateau	**87.72** +/-3.79 *T-test P=7.6^{E}-08* *Wilcoxon P=0.0079*

Figure 5. Mitochondria and nuclei of senescent keratinocytes and corpses contain oxidized lipids and proteins. NHEKs at the exponential growth phase or at the senescence plateau were processed for immunodetection of amino-imino-propene bridges (AIP). Young cells at the growth phase are negative for AIP (A). In senescent cells and corpses, AIP are found mainly in the nucleus and in some punctuated cytoplasmic structures (B and C). When the nucleus is localized in the autophagic central area, the staining disappears (C and D). Scale bars = 30 μm. The number of cells (including corpses) with AIP-positive nuclei was manually counted in 5 random microscopic fields amongst a total of 2068 young and 1040 senescent cells. Values are the mean percentages of cells with AIP-positive nuclei +/− SD. Since the data are not strictly normally distributed, P values were calculated using both Student and Wilcoxon tests. doi:10.1371/journal.pone.0012712.g005

flux, associated with senescence and cell death. We finally checked that H_2O_2-induced senescent cells and corpses do displayed oxidative damages similar to those observed in normal senescent cells and corpses, i.e. a membrane permeabilisation revealed by an intracellular Annexin-V staining (Figure S4), 8-oxoG (Figure S5A) and AIP bridges (Figure S5B).

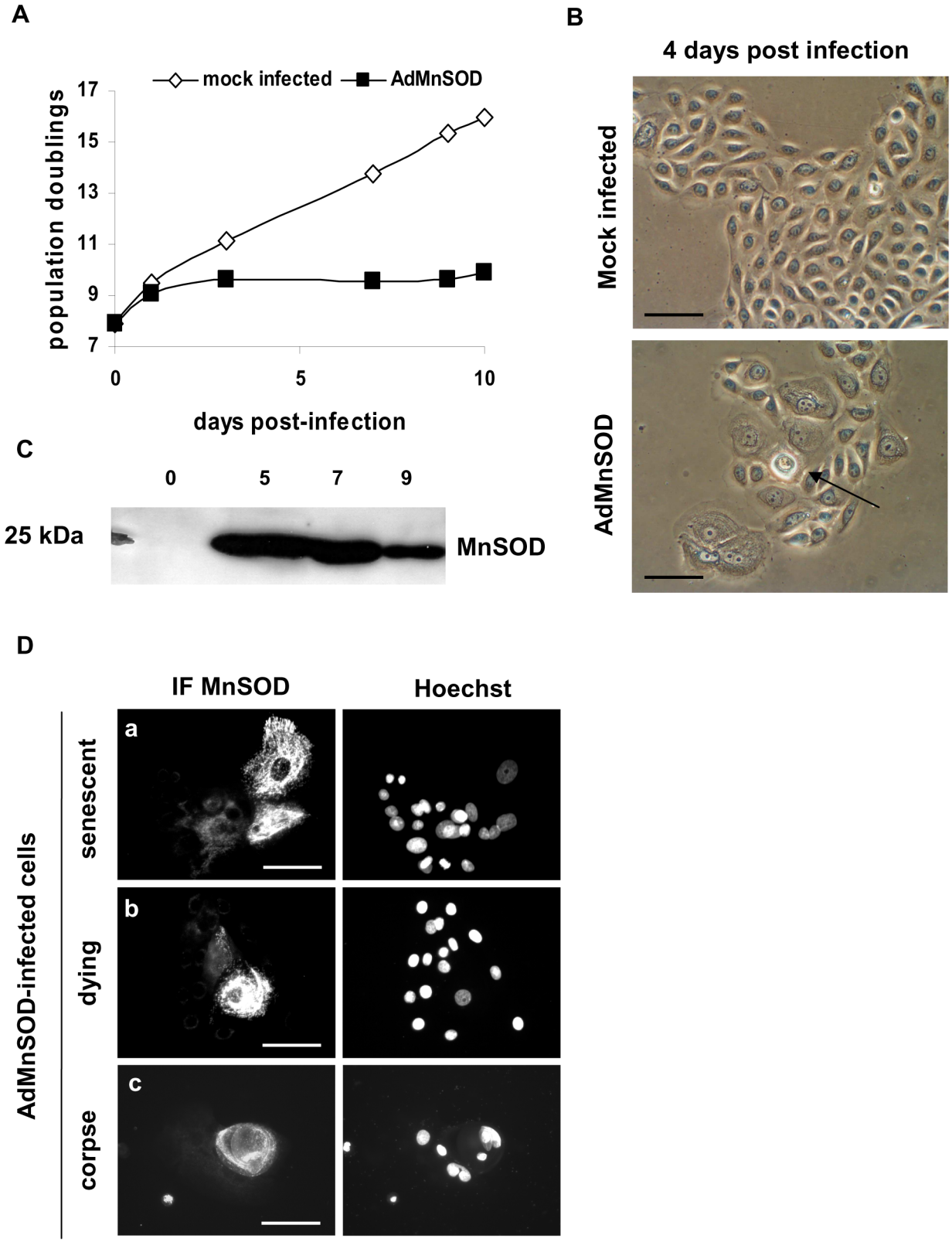


Figure 6. Overexpression of MnSOD induces premature senescence and autophagic cell death. NHEKs at the exponential growth phase were infected or not with an adenovirus encoding MnSOD (AdMnSOD) by directly adding virus particles in the culture medium at an input multiplicity of 200 viral particles/cell. (A) Growth curve of control and infected cultures. (B) Observation by phase contrast microscopy of the morphology of control and infected cells. Note that almost 100% of infected cells display a senescent-like morphology with spreading and polynucleation. One cell in the centre of the field resembles an autophagic corpse with a refringent central area (arrow). (C) Control of MnSOD expression in infected cells by western-blotting. (D) Immunofluorescence against MnSOD on AdMnSOD-infected cells. Amongst cells of an islet, cells expressing MnSOD at the highest level have either a marked senescent phenotype (a), a dying phenotype (b) or are already a corpse (c). Scale bars = 40 μM.
doi:10.1371/journal.pone.0012712.g006

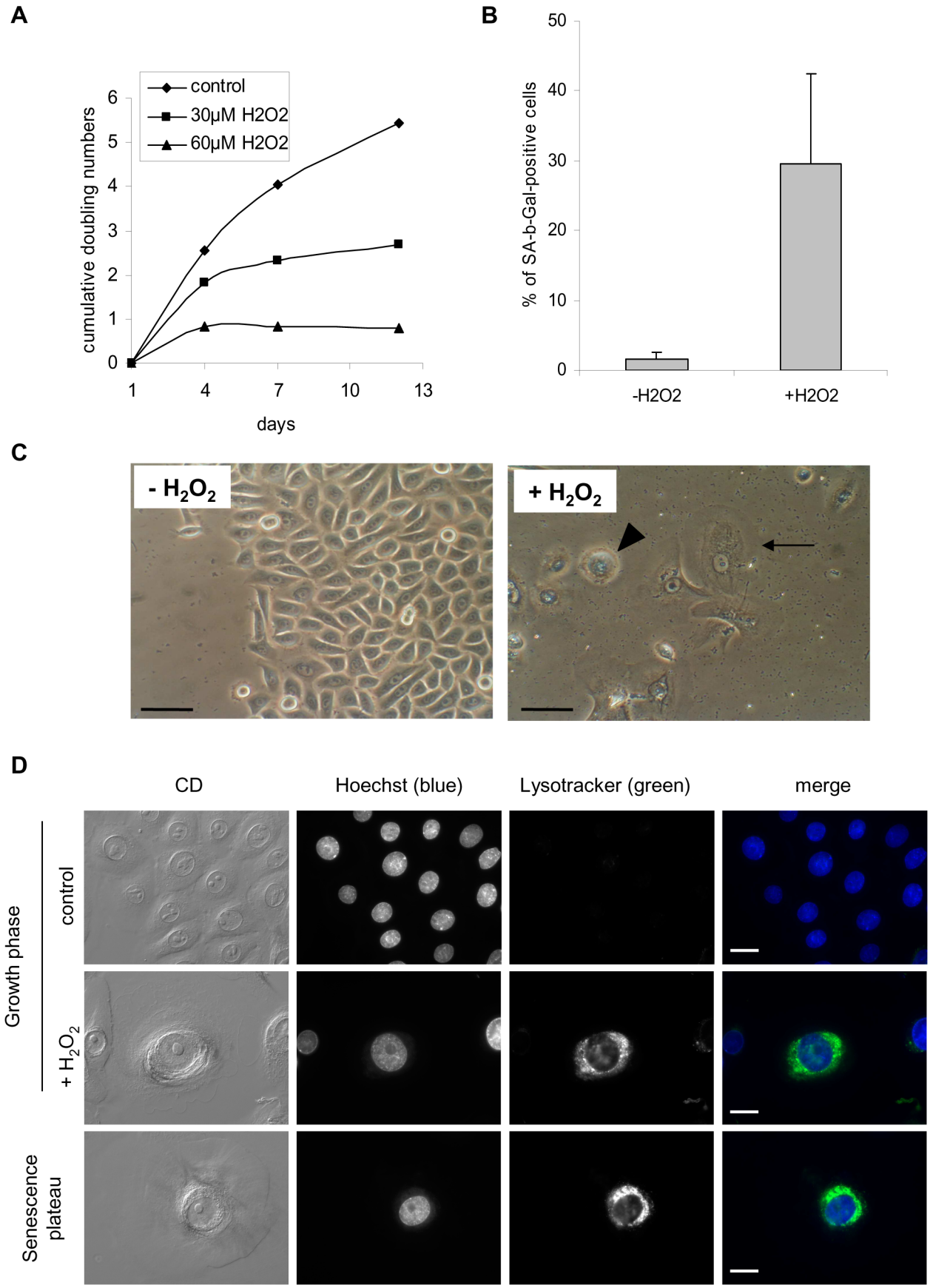


Figure 7. H_2O_2 induces premature senescence and signs of autophagic cell death. (A) NHEKs at the exponential growth phase were treated or not with 30 or 60 µM H_2O_2 for 2 hrs every 72 hrs. Cells were counted at different time points during the treatment in four independent wells, and the cumulative numbers of doublings were calculated using the mean of cell counts. (B) SA-beta-Gal assays were performed on control and 30 µM H_2O_2-treated cells at day 8.

SA-beta-Gal-positive cells were counted in 4 microscopic fields. Results are given as means +/− SD of all field counts. Since the data are not strictly normally distributed, P values were calculated using both Student and Wilcoxon tests. They respectively equal 0.03 and 0.028. (C) Cells were observed under phase contrast microscopy. The images illustrate control and 30 μM H_2O_2-treated cell morphologies at day 8. Note the presence in H_2O_2-treated cultures of large senescent cells (arrow) and corpses (arrowhead). Scale bars = 20 μM. (D) NHEKs at the exponential growth phase were treated with 50 μM H_2O_2 and analyzed three days later by Lysotracker staining and microscopic analysis in comparison with cells at a normal senescence plateau. The corpses induced by the H_2O_2 treatment were similar to the normal ones occurring at the senescence plateau regarding their morphology under circular dichroism (CD) and their Lysotracker staining. The Lysotracker staining concentrates in the central area of corpses. Scale bars = 30 μM.
doi:10.1371/journal.pone.0012712.g007

Taken together, these results indicate that mimicking the MnSOD>H_2O_2 pathway that contributes to senescence in keratinocytes resumes senescence-associated oxidative damages and autophagic programmed cell death.

To definitely prove that the cell death following senescence induced by oxidative stress is driven by autophagy, we checked whether cell death level or time course could be affected by autophagy inhibitors. We used 3-methyladenine (3-MA), an

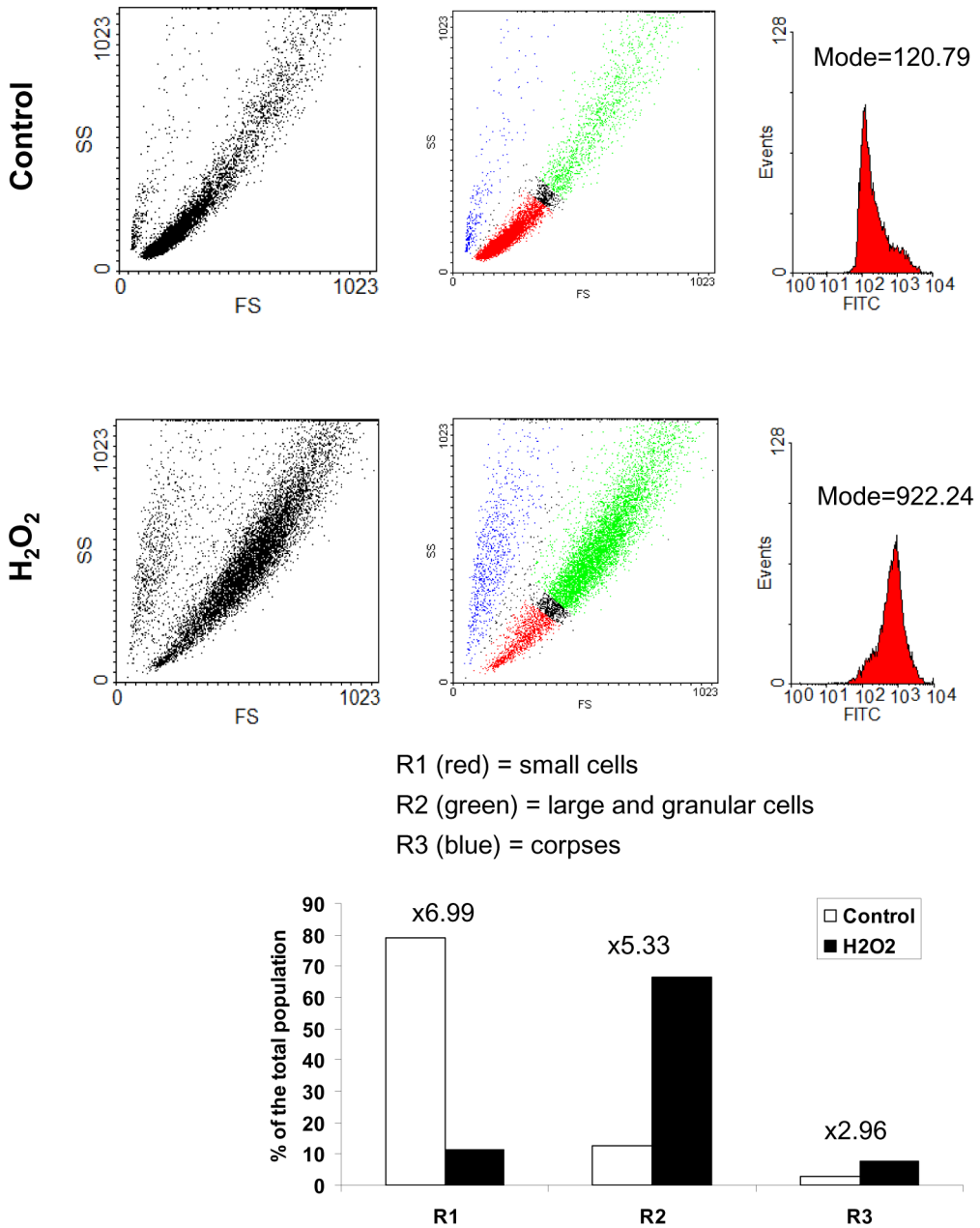


Figure 8. Quantification of the effect of the H_2O_2-treatement by flow cytometry and Lysotracker staining. NHEKs at the exponential growth phase were treated with 50 μM H_2O_2 and analyzed three days later by Lysotracker staining. (Left panel) Flow cytometry analysis of the cell population plot for forward factor (FS, indicative of size, in X) and side scatter factor (SS, indicative of granularity, in Y). (Middle panel) Quantitative analysis of the evolution of the subpopulations upon the H_2O_2 treatment using the WinMDI software. (Right panel) Measure of the intensity of the Lysotracker staining (FITC, in X) of the all population. The mode values of the fluorescence intensity are given.
doi:10.1371/journal.pone.0012712.g008

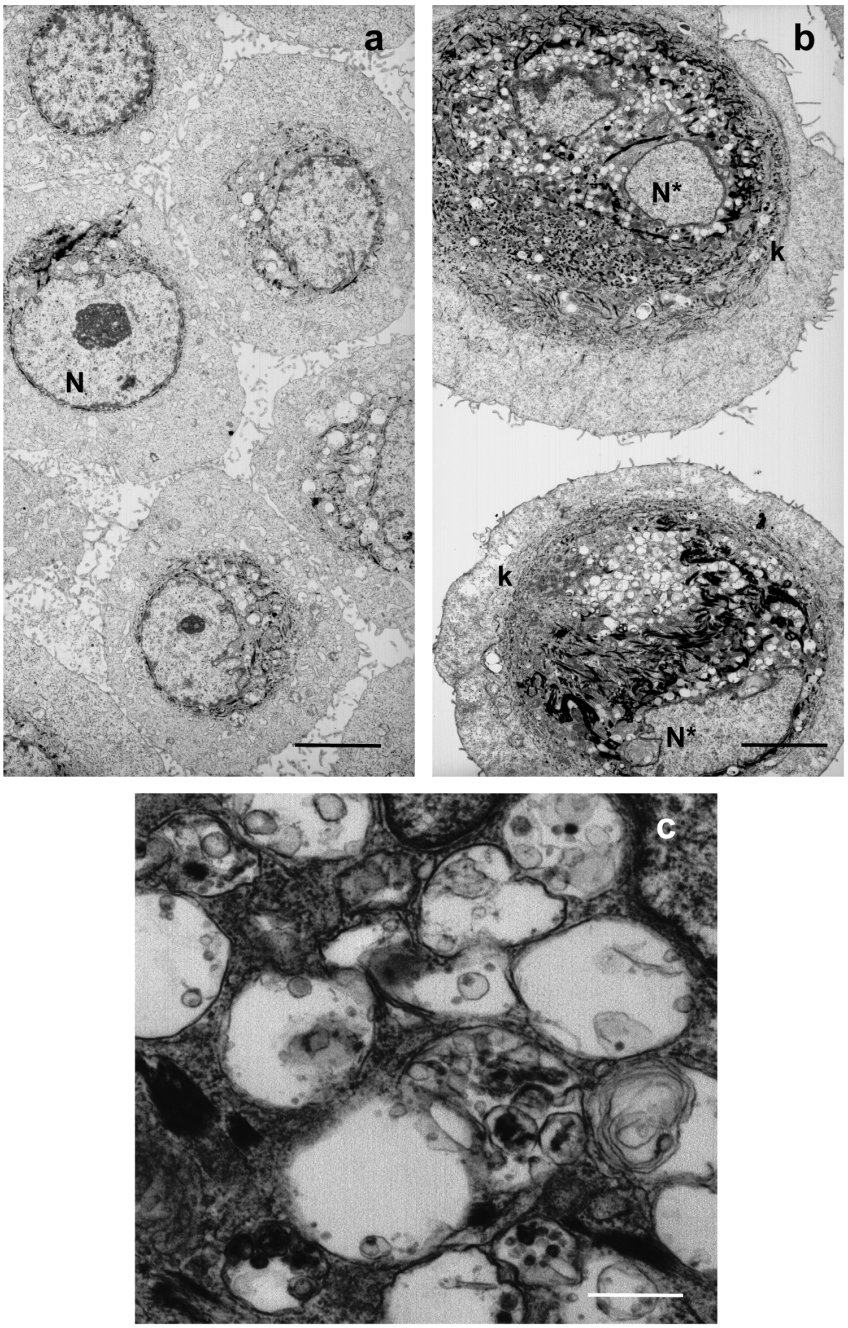


Figure 9. Ultrastructure of H_2O_2-induced senescent cells. NHEKs at the growth phase were treated with 50 μM H_2O_2 and processed 72 hrs later for transmission electron microscopy. (a) Control non treated cells. (b) H_2O_2-treated cells. N: nucleus, N*: deformed nucleus with less heterochromatin, k: cytokeratin network encircling the nucleus and the autophagic vacuoles. Scale bars = 5 μM. (c) Detail of autophagic vacuoles found in H_2O_2-treated cells. Scale bars = 0.4 μM.
doi:10.1371/journal.pone.0012712.g009

inhibitor of the class III phosphatidylinositol 3-kinase (class III PI3K) complex involved in initial autophagosome formation [20], and Bafilomycin A1 an inhibitor of the H^+ pump [21] that decreases the fusion of autophagosomes with lysosomes and the efficacy of digestion inside autophagolysosomes [22]. Because oxidative stress is also able to induce apoptosis, we also included in this assay zVAD, the specific caspase inhibitor. NHEKs at the exponential growth phase were treated by 50 μM H_2O_2 until almost 100% cells were senescent (after 48 hrs). Then, cells were either kept untreated or treated by 3-MA, Bafilomycin A1, z-VAD, or DMSO (the diluent of z-VAD and Bafilomycin A1). The treatments were repeated twice at 48 hrs intervals in order to enrich in corpses. Typical corpses with a refringent central area were counted under microscopic observation at the different time points. Their number increased with time in control cultures as well as in cultures treated with z-VAD. The number of corpses was significantly lower in cultures treated with 3-MA at 5 and 8 days of treatment (Figure 11A). These results suggest that NHEKs treated by 50 μM H_2O_2 die by autophagic programmed cell death, not by apoptosis. In cultures treated with Bafilomycin A1, we observed an accumulation of corpses with a very

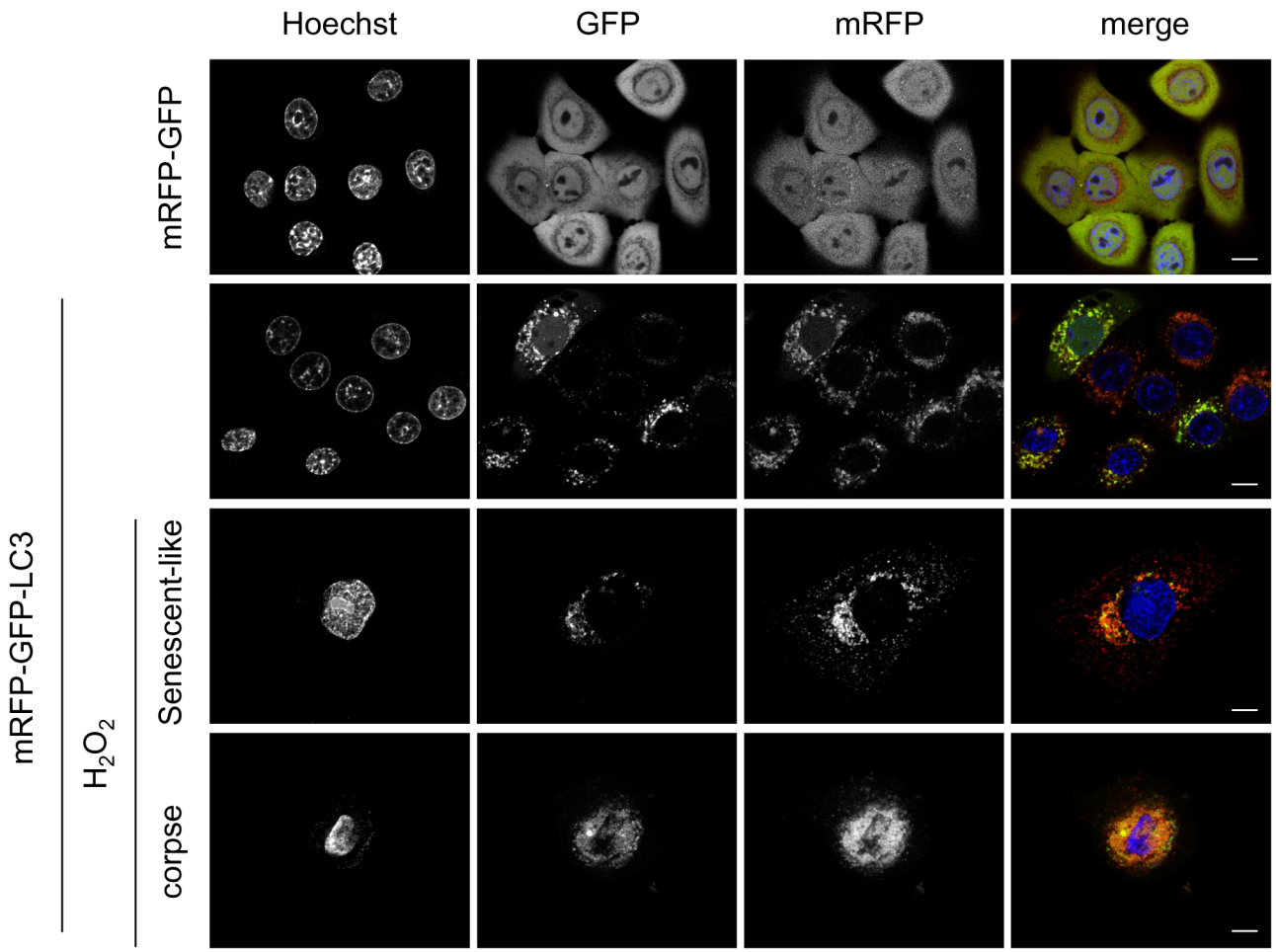


Figure 10. H_2O_2-induced senescent cells and corpses display a high and fully active autophagic flux. NHEKs at the exponential growth phase were treated or not twice with 50 μM H_2O_2 at 48 hrs interval and transfected with either the mRFP-GFP-LC3 or the control vector mRFP-GFP devoided of LC3. Forty eight hrs later, cells were analyzed under a confocal microscope. Non treated cells transfected with the control vector display a homogenous green (GFP) and red (mRFP) fluorescence. Non treated cells transfected by the mRFP-GFP-LC3 vector display some red and green vesicles. H_2O_2-treated cells transfected by the mRFP-GFP-LC3 vector display senescent or corpse features, with damaged nuclei and numerous vesicles whose majority are redder than green. In corpses, the vesicles are concentrated in the central area. Scale bars = 10 μM.
doi:10.1371/journal.pone.0012712.g010

refringent central area (Figure 11A). We have previously demonstrated by transmission electron microscopy that normal senescent cultures treated by Bafilomycin A1 evolve in corpses completely congested by numerous autophagic vacuoles, themselves full of various components incompletely degraded [4]. This accumulation of non degraded material inside autophagic vacuoles in corpses upon Bafilomycin A1 treatment confirms that the autophagic flux is active during NHEK autophagic death. Since pharmacological inhibitors are not completely specific, we performed a supplementary experiment using siRNA directed against Atg5, a protein involved in the lipidation of Atg8/LC3 [23] and hence in autophagosome formation. Cells were treated twice with 50 μM H_2O_2 at 48 hrs interval to induce premature senescence, and 24 hrs later transfected with anti-Atg5 siRNAs or non target siRNAs. The efficacy of siRNA was checked by western-blotting at day 4 after transfection, and the accumulation of corpses was quantified by counting under microscopic observation. Four days after transfection, corpses were about two fold less numerous in cells transfected by anti-Atg5 siRNAs than in cells transfected by non target siRNAs (Figure 11B), hence confirming that cell death following H_2O_2-induced premature senescence occurs through autophagy.

Lowering oxidative stress decreases the autophagic activity and the death of senescent keratinocytes

Finally, to definitely prove that autophagic cell death is activated in senescent cells in response to oxidative damage, we examined whether antioxidants could decrease senescent-cell death rate or kinetics. As antioxidant, we used catalase which specifically degrades H_2O_2 into H_2O. We used either native catalase since we previously showed that it is able to delay the NHEK senescence plateau [10] or PEG-catalase, a monomethoxy-polyethylene glycol conjugated catalase which is more efficient because more resistant to protease attacks [24] and able to bind plasma membranes [25]. In a first series of experiments, native catalase was applied to a subpopulation of still alive senescent cells sorted by FACS [4] (Figure 12A) every 24 hrs, during 4 days, and corpses were counted under a phase contrast microscope. The percentage of corpses in the non treated cell population increased about 3.33 fold in 4 days, whereas it increased only about 1.9 fold in the catalase-treated cell population, hence representing a 42.9% inhibition of senescent-cell death (Figure 12A). In a second series of experiments, PEG-catalase was applied to a total population of NHEKs at the

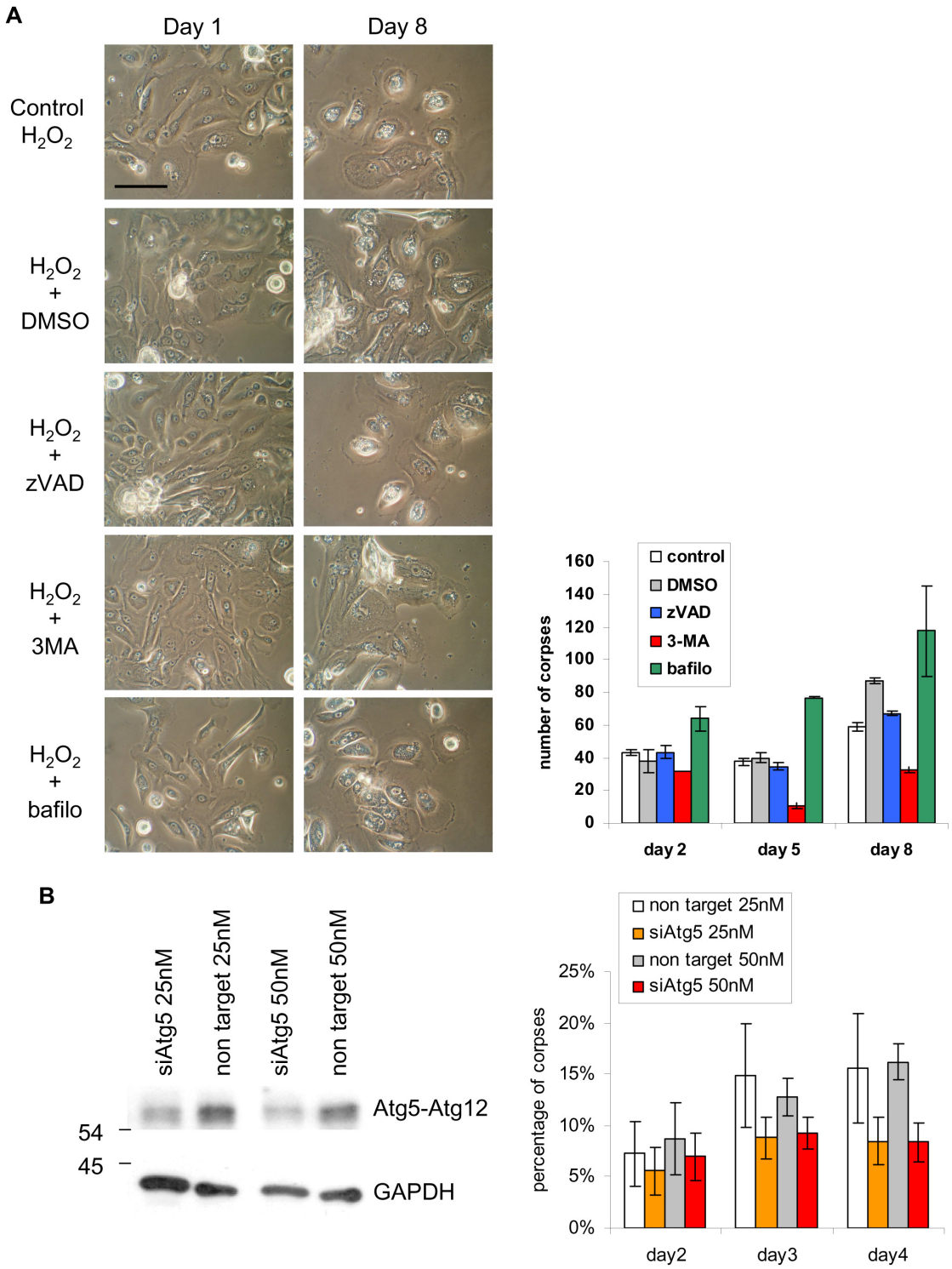


Figure 11. Inhibiting autophagy, but not apoptosis, delays the death of H_2O_2-induced senescent cells. (A) NHEKs at the exponential growth phase were treated by 50 μM H_2O_2 until almost 100% cells were senescent (after 48 hrs). Then, cells were either kept untreated for control, or treated by 3-MA at 5 mM, z-VAD at 20 μM, Bafilomycine A1 at 5 nM or DMSO, the diluent of z-VAD and Bafilomycine A1. Forty eight hours later, the H_2O_2-treatment followed by the inhibitor treatment was repeated a second time. (Upper panel) Cells morphologies observed under a phase-contrast microscope at different time points after the beginning of the inhibitor treatment. Scale bar = 50 μm. (Lower panel) The number of typical corpses with a refringent central area was counted under microscopic observation at the indicated time points after the beginning of the inhibitor treatment. The counts were performed in 20 microscopic fields of two independent culture dishes, each field comprising about 300 cells. The given results are the mean +/− standard deviation of all counts. Since the data are not strictly normally distributed, P values were calculated using both Student and Wilcoxon tests. The results are given Figure S6. This experiment is representative of two independent ones. (B) NHEKs at the exponential growth phase were treated twice by 50 μM H_2O_2 at 24 hrs interval until almost 100% cells were senescent (after 48 hrs). Then, cells were either transfected by anti-Atg5 siRNAs at 25 or 50 nM or by non target siRNAs at 25 or 50 nM. (Left panel) Western-blot analysis of Atg5 expression at day 4 after

transfection. The antiAtg5 antibody recognizes the Atg5-Atg12 conjugate. (Right panel) The number of typical corpses with a refringent central area was counted under microscopic observation at the indicated time points after transfection. The counts were performed in 10 microscopic fields, each field comprising about 100 cells. The given results are the mean +/− standard deviation of all counts. Since the data are not strictly normally distributed, P values were calculated using both Student and Wilcoxon tests. The results are given Figure S6. This experiment is representative of three independent ones.
doi:10.1371/journal.pone.0012712.g011

senescent plateau, and corpses were counted after 24 and 48 hrs. With that protocol, the inhibition of senescent-cell death was more efficient (58.7% inhibition) and more rapid (Figure 12B). Both these experiments demonstrate that senescent-cell death is induced consequently to H_2O_2 accumulation.

Discussion

Autophagy as a cell death mechanism in senescent keratinocytes

Macroautophagy is a process involved in proteins and organelles normal turnover; it is referred as such as constitutive autophagy. It is also a survival process induced to resist to nutrient deprivation, referred as starvation-induced autophagy [9]. However, macroautophagy is also a cell death process, referred as type II programmed cell death [26]. This type of cell death was described in several model organisms and seems to involve the same set of Atg proteins as those involved in constitutive and starvation-induced autophagy [27,28]. Therefore, how autophagy can shift from a survival process to a lethal one is not entirely clear. Several parameters seem important: the level of the autophagic activity that have to be high enough to destroy major portions of cytosol and organelles [29], the levels of expression of Atg5 and Atg6/Beclin-1 that do not change during starvation-induced autophagy but increase during autophagic cell death [30], and the possible selective degradation of some vital components or proteins, such as catalase, a major antioxidant enzyme [31]. We and others have demonstrated in recent works that autophagy is activated in normal or RAS-induced senescent cells, and that the autophagic flux is fully functional and contribute to the establishment of the senescent phenotype [4,5]. In addition, we have shown in keratinocytes that this autophagic activity is high enough to end up in the death of senescent cells. Notably, we have evidenced in normal senescent keratinocytes an increase in Atg6/Beclin-1, and an accumulation of a huge quantity of autophagic vacuoles [4]. We have not observed any increase in catalase degradation, either in the total population at the senescent plateau [10], or in senescent cells and corpses sorted by FACS (data not shown).

Oxidative stress as an inducer of autophagic cell death in senescent keratinocytes

Several data have suggested that the damaged cell components that are targeted for autophagy are those that are oxidized [32]. Moreover, diverse inducers of autophagic programmed cell death were shown to act through the generation of reactive oxygen species (ROS) [33], more specifically H_2O_2 which was shown to act as a signalling molecule able to activate the initial autophagosome formation, via the oxidation of a specific cystein of Atg4 [34]. We have demonstrated in a previous study that H_2O_2 is accumulated in senescent keratinocytes following the activation of NF-kappaB and the upregulation of MnSOD, and contributes to the occurrence of the senescent phenotype [10]. We complete here these data by showing that this oxidative stress pathway is also at the origin of a high autophagic activity that becomes fatal to senescent keratinocytes. Indeed, we show that mimicking this pathway by overexpressing MnSOD or directly adding H_2O_2 to cultures of young keratinocytes induced a premature senescence plateau followed by autophagic cell death; the level of this ROS-induced autophagic cell death is decreased in the presence of 3-MA or anti-Atg5 siRNAs. Conversely, treating senescent cells with the H_2O_2-degrading enzyme catalase reduced their level of autophagic cell death.

MnSOD being a mitochondrial enzyme, the first targets of the H_2O_2 it produces should be mitochondria, potentially explaining why they are swelled and aggregated in advanced senescent cells. A surprising point is the increase in mitochondria number during senescence despite their damaging. Such an increase was already documented in MRC5 fibroblasts following H_2O_2-induced premature senescence, suggesting that mitochondrial biogenesis is unaffected by oxidative damage [35]. Besides mitochondria, H_2O_2 can also affect nuclei. Indeed, being diffusible across membranes [36], H_2O_2 could easily reach the nucleus, all the more because damaged mitochondria are agglutinated in its vicinity. The presence of 8oxoG and AIP bridges on mitochondria and nuclei inside the central area of corpses where autophagic vesicles accumulate argues that mitochondria and nuclei of senescent cells are targeted to autophagic elimination because of their oxidative damages. Such an elimination of oxidized mitochondria was already documented in several models [37–39]. The possible elimination of nuclei by autophagy because of their oxidative damage is suggested by several studies: it was shown that H_2O_2-induced DNA breaks can induce autophagy through the activation of PARP-1 [40]; The inhibition of DNA-PK, a nuclear kinase involved in DNA break signalisation, was shown to sensitize to autophagy [41]; Treatments with an other DNA damaging agent, the etoposide, were shown to induce autophagy and senescence [5] and to result in autophagic programmed cell death through the increase in Beclin-1 [30]. Moreover, DNA damage has been shown to accumulate in cancer cells deficient in autophagy [42]. Despite this evidence of an autophagy-inducing activity of DNA damages, the direct autophagic elimination of nuclei by autophagy is up to now poorly documented. In yeasts, nuclei could be degraded by a process morphologically resembling microautophagy [43] but involving the core macroautophagy genes [44]. In mammalian cells, one study has illustrated by electron microscopy the possible nucleus encircling by a phagocytic double membrane [45]. Recently, another study gave evidence of nuclear components degradation by perinuclear giant autophagic vacuoles [46].

Therefore, we propose the following scenario: with time and doublings, senescent keratinocytes become cell-cycle arrested and accumulate ROS, especially H_2O_2, in part through the activation of NF-kappaB and the upregulation of MnSOD [10]. Oxidatively-damaged mitochondria loss their attachment to microtubules, hence aggregate in the vicinity of the nucleus, which favours its oxidative attack. In consequence to these oxidative damages, and potentially through the oxidation of Atg4 by H_2O_2, autophagy is highly activated and progressively eliminates all the oxidized vital cell components, leading to cell death.

Apoptosis versus autophagic cell death in senescent cells

In a previous work, we had demonstrated that normal senescent keratinocytes do not die by apoptosis [4]. Here, we add that

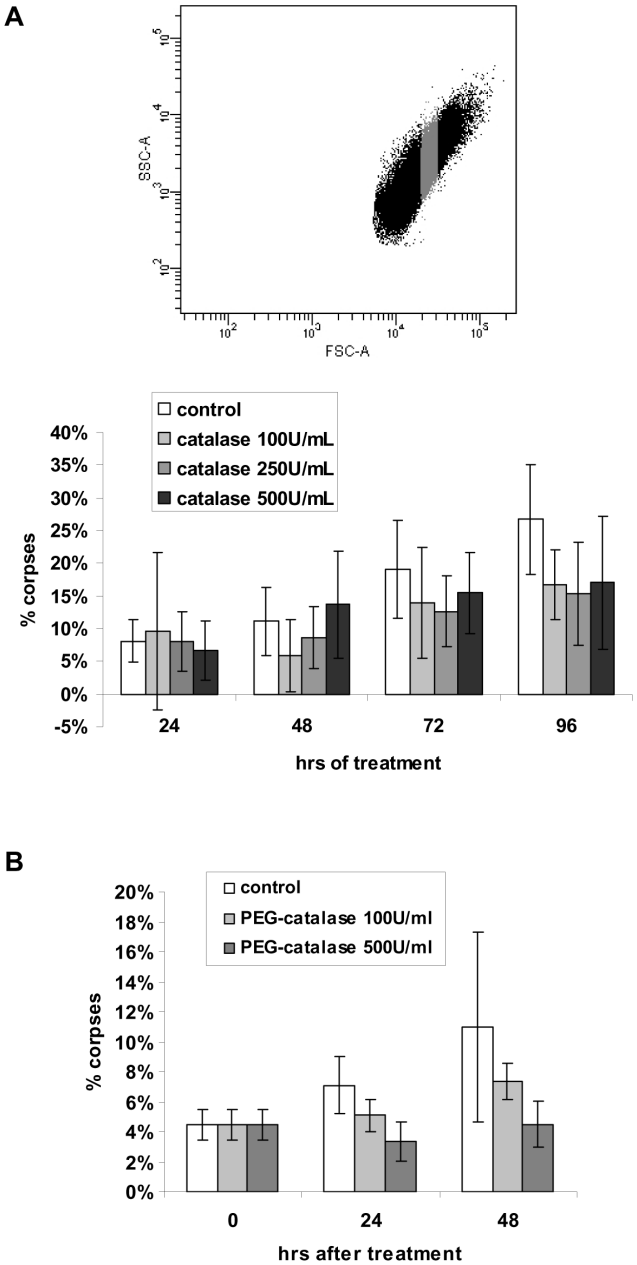


Figure 12. Enhancing H_2O_2 degradation delays senescent-cell death. (A) NHEKs at the senescence growth plateau were analyzed by flow cytometry according to size (FSC in X) and granularity (SSC in Y) and the subpopulation of still viable senescent cells (in grey) was sorted. Sorted cells were plated in 6-wells plates at 30.000 cells per well and treated or not by catalase at different concentrations. The medium +/− catalase was renewed every 24 hrs. The number of typical corpses with a refringent central area was counted under microscopic observation every day. The counts were performed in 12 microscopic fields in three independent culture wells, each field comprising about 10 cells. The given results are the mean +/− standard deviation of all counts. Since the data are not strictly normally distributed, P values were calculated using both Student and Wilcoxon tests. The results are given Figure S6. This experiment is representative of two independent ones. (B) NHEKs at the senescence growth plateau were plated in 6-wells plates at 500.000 cells per well and treated or not by PEG-catalase at different concentrations. The number of typical corpses with a refringent central area was counted under microscopic observation every day. The counts were performed in 6 microscopic fields comprising about 100 cells. The given results are the mean +/− standard deviation of all counts. Since the data are not strictly normally distributed, P values were calculated using both Student and Wilcoxon tests. The results are given Figure S6. This experiment is representative of two independent ones.
doi:10.1371/journal.pone.0012712.g012

H_2O_2-induced senescent-like keratinocytes do not die by apoptosis as well. However, oxidative stress is a well known apoptosis inducer. Actually, we observed an increase in apoptotic cells from 1–2% in the population of exponentially growing keratinocytes to 5–8% at the senescence plateau (data not shown). In parallel, we observed that H_2O_2 treatments induce different outcomes for keratinocytes according to the concentration used: as shown in this paper, low concentrations of H_2O_2 induced in a few days a senescent-like phenotype followed by autophagic programmed cell death, but higher concentrations induced in a few hours an apoptotic cell death characterized by typical membranous Annexin-V staining and cytoplasm/nuclear condensation (data not shown). A study comparing senescent endothelial HUVECs with senescent fibroblasts showed that senescent HUVECs display many signs of apoptosis, whereas senescent fibroblasts do not [47]. The authors associated the death of senescent HUVECs by apoptosis with the generation of oxidative stress during senescence [48] and proposed that senescent fibroblasts do not die by apoptosis because they are more resistant to oxidative stress than HUVECs [49]. Therefore, apoptosis is likely to co-exist with autophagic programmed cell death during senescence, but in low proportions, and only in the case of high level of oxidative stress or especially sensitive cell-types.

Implications in aging

Do old cells die through autophagic programmed cell death *in vivo* during aging as in culture because of oxidative damages? Probably yes. Indeed, the universal marker of senescence, the SA-beta-Gal activity [14], is actually an indirect marker of autophagy since it reflects the activity of a lysosomal enzyme, and hence the mass of lysosomes [50]. The number of cells positive for this marker was shown to increase during normal human and mouse aging [14,51–53], suggesting that the autophagic activity increases in cells during aging. Lipofuscin, the well-known marker of aged skin and other organs, is an aggregate of proteins having reacted with lipid peroxidation end-products that accumulates with advancing age inside autophagic vacuoles [54]. Hence, it is a marker of autophagy resulting from oxidative damage. Consequently, its accumulation in cells during aging not only confirms that the autophagic activity increases with age, but in addition that it does in response to oxidative damage.

Implications for tumorigenesis

An important consequence of our understanding of the role of oxidative stress in senescent-cell death concerns the relationship between aging and cancer. Indeed, there are numerous evidences that autophagy is down-regulated in cancer cells. Beclin-1 is often mono-allelically deleted in various carcinomas [29] and its heterozygous disruption in mice caused spontaneous tumours [55,56]. The tumour suppressor PTEN, that rivals p53 in being the most frequently mutated gene in human cancer [57], promotes autophagy in HT-29 colon cancer cells by blocking the Akt survival pathway. Mutations in PTEN result in inactivation of autophagy and tumour formation [58]. Therefore, one can speculate that during senescence, most oxidatively-altered cells would die by autophagy, but some cells would beneficiate from the mutagenicity of ROS to escape autophagic cell death and evolve in transformed long-lived cells. Hence, acquiring autophagic cell death resistance would be an event as important as becoming

apoptosis resistant for neoplastic transformation. However, this autophagic resistance should be only partial so that, once formed, cancer cells can normally use constitutive autophagy for turnover ensuring, or have recourse to starvation-induced autophagy in case of limited angiogenesis.

Supporting Information

Figure S1 Senescent keratinocytes accumulate reactive oxygen species NHEKs at the exponential growth phase or at the senescence plateau were suspended and stained with H2-DCFDA. They were then analyzed by flow cytometry for forward (FS, indicative of size, in Y in the dot plot) and side scatter (SS, indicative of granularity, in X in the dot plot) factors and H2-DCFDA fluorescence intensity (GFP on the histograms). The senescent population increases in size and granularity; its H2-DCFDA increases about ten fold.
Found at: doi:10.1371/journal.pone.0012712.s001 (0.31 MB TIF)

Figure S2 Senescent cells and corpses induced by MnSOD overexpression have an increased number of acidic organelles NHEKs at the exponential growth phase were infected with AdMnSOD as in Fig. 6 and 4 days later they were stained with Lysotracker as in Fig. 3. Note that in AdMnSOD-infected cultures, cells with a marked senescent phenotype (green arrows) and corpses (red arrows) display a high Lysotracker staining. Scale bars = 40 μM.
Found at: doi:10.1371/journal.pone.0012712.s002 (1.84 MB TIF)

Figure S3 Corpses induced by MnSOD overexpression have their membranes altered and display oxidative damages NHEKs at the exponential growth phase were infected with AdMnSOD as in Fig. 6. (A) Annexin-V assays performed 10 days post-infection. Both typical large senescent cells and corpses that appeared in the AdMnSOD-infected cultures display some staining of their endomembranes. Scale bars = 30 μM. (B) Immunodetection of 8-oxo-guanines (8oxoG) 5 days post-infection. The image shows an example of the high staining of a corpse and of a senescent cell (C) Immunodetection of amino-imino-propene (AIP) bridges 10 days post-infection. The image shows an example of the high staining of a corpse. Scale bars = 40 μM.
Found at: doi:10.1371/journal.pone.0012712.s003 (2.63 MB TIF)

Figure S4 H2O2-induced senescent cells and corpses have permeabilized membranes NHEKs at the exponential growth phase were treated with H2O2 as in Fig. 7 and processed for Annexin-V assay. H2O2-treated cells with senescent morphology and corpses display intracellular staining, revealing that their membranes are permeabilized. Scale bars = 40 μM.
Found at: doi:10.1371/journal.pone.0012712.s004 (2.02 MB TIF)

Figure S5 H2O2-induced senescent cells and corpses display oxidative damages NHEKs at the exponential growth phase were treated with 50 μM H2O2 and processed 48 hrs later for immunofluorescence against 8-oxo-guanines (8oxoG) and amino-imino-propene (AIP) bridges. Nuclei were counterstained with Hoechst. Cells were observed under epifluorescence microscopy and circular dichroism (CD). (A) 8oxoG staining. Both cells with a senescent-like morphology and corpses display some staining of cytoplasmic structures and some staining inside the nucleus. In corpses, the cytoplasmic staining is concentrated in the central area. (B) AIP bridges staining. Cells with a senescent-like morphology display a nuclear staining and some diffuse cytoplasmic staining. In corpses, the nuclear staining is very intense, and the cytoplasmic staining is concentrated in the central area. Scale bars = 20 μM.
Found at: doi:10.1371/journal.pone.0012712.s005 (2.93 MB TIF)

Figure S6 Statistical analysis of the results of Figures 11 and 12.
Found at: doi:10.1371/journal.pone.0012712.s006 (0.61 MB TIF)

Acknowledgments

We thank T. Yoshimori, Osaka University, Japan, for the mRFP-GFP-LC3 plasmid.

Author Contributions

Conceived and designed the experiments: ED KG SM CA. Performed the experiments: ED KG CV SM FB CS JB DB. Analyzed the data: ED KG CV SM CS DB CA. Contributed reagents/materials/analysis tools: CS. Wrote the paper: AP CA.

References

1. Hayflick L (1965) The limited *in vitro* lifetime of human diploid cell strains. Exp Cell Res 37: 614–636.
2. Cristofalo VJ, Lorenzini A, Allen RG, Torres C, Tresini M (2004) Replicative senescence: a critical review. Mech Ageing Dev 125: 827–48.
3. Zwerschke W, Mazurek S, Stockl P, Hutter E, Eigenbrodt E, et al. (2003) Metabolic analysis of senescent human fibroblasts reveals a role for AMP in cellular senescence. Biochem J 376: 403–11.
4. Gosselin K, Deruy E, Martien S, Vercamer C, Bouali F, et al. (2009) Senescent keratinocytes die by autophagic programmed cell death. Am J Pathol 174: 423–35.
5. Young AR, Narita M, Ferreira M, Kirschner K, Sadaie M, et al. (2009) Autophagy mediates the mitotic senescence transition. Genes Dev 23: 798–803.
6. Sasaki M, Miyakoshi M, Sato Y, Nakanuma Y (2010) Autophagy mediates the process of cellular senescence characterizing bile duct damages in primary biliary cirrhosis. Lab Invest.
7. Gozuacik D, Kimchi A (2007) Autophagy and cell death. Curr Top Dev Biol 78: 217–45.
8. Xie Z, Klionsky DJ (2007) Autophagosome formation: core machinery and adaptations. Nat Cell Biol 9: 1102–9.
9. Mizushima N (2007) Autophagy: process and function. Genes Dev 21: 2861–73.
10. Bernard D, Gosselin K, Monte D, Vercamer C, Bouali F, et al. (2004) Involvement of Rel/NF-kappaB transcription factors in keratinocyte senescence. Cancer Res 64: 472–81.
11. Boyce ST, Ham RG (1983) Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. J Invest Dermatol 81: 33s–40s.
12. Martinon O, Poulet JP, Chancerelle Y, Viret-Soropogui R, Mathieu J, et al. (1994) Immunization of mice with proteins reacted with malonic dialdehyde (MDA): comparison between autologous and heterologous modified proteins. Biochem Mol Biol Int 34: 135–45.
13. Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17: 208–12.
14. Dimri GP, Lee X, Basile G, Acosta M, Scott G, et al. (1995) A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. Proc Natl Acad Sci USA 92: 9363–9367.
15. Bernard D, Quatannens B, Begue A, Vandenbunder B, Abbadie A (2001) Anti-proliferative and anti-apoptotic effects of cRel may occur within the same cells via the up-regulation of MnSOD. Cancer Research 61: 2656–2664.
16. Chartier C, Degryse E, Gantzer M, Dieterle A, Pavirani A, et al. (1996) Efficient generation of recombinant adenovirus vectors by homologous recombination in Escherichia coli. J Virol 70: 4805–10.
17. Schiedner G, Hertel S, Kochanek S (2000) Efficient transformation of primary human amniocytes by E1 functions of Ad5: generation of new cell lines for adenoviral vector production. Hum Gene Ther 11: 2105–16.
18. Kimura S, Noda T, Yoshimori T (2007) Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. Autophagy 3: 452–60.
19. Sekiguchi M, Tsuzuki T (2002) Oxidative nucleotide damage: consequences and prevention. Oncogene 21: 8895–904.
20. Seglen PO, Gordon PB (1982) 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. Proc Natl Acad Sci U S A 79: 1889–92.
21. Bowman EJ, Siebers A, Altendorf K (1988) Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. Proc Natl Acad Sci U S A 85: 7972–6.
22. Yamamoto A, Tagawa Y, Yoshimori T, Moriyama Y, Masaki R, et al. (1998) Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion

between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. Cell Struct Funct 23: 33–42.
23. Hanada T, Noda NN, Satomi Y, Ichimura Y, Fujioka Y, et al. (2007) The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy. J Biol Chem 282: 37298–302.
24. Abuchowski A, McCoy JR, Palczuk NC, van Es T, Davis FF (1977) Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. J Biol Chem 252: 3582–6.
25. Beckman JS, Minor RL, Jr., White CW, Repine JE, Rosen GM, et al. (1988) Superoxide dismutase and catalase conjugated to polyethylene glycol increases endothelial enzyme activity and oxidant resistance. J Biol Chem 263: 6884–92.
26. Clarke PGH (1990) Developmental cell death: morphological diversity and multiple mechanisms. Anat Embryol 181: 195–213.
27. Tsujimoto Y, Shimizu S (2005) Another way to die: autophagic programmed cell death. Cell Death Differ 12 Suppl 2: 1528–34.
28. Kourtis N, Tavernarakis N (2009) Autophagy and cell death in model organisms. Cell Death Differ 16: 21–30.
29. Gozuacik D, Kimchi A (2004) Autophagy as a cell death and tumor suppressor mechanism. Oncogene 23: 2891–906.
30. Shimizu S, Kanaseki T, Mizushima N, Mizuta T, Arakawa-Kobayashi S, et al. (2004) Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. Nat Cell Biol 6: 1221–8.
31. Yu L, Wan F, Dutta S, Welsh S, Liu Z, et al. (2006) Autophagic programmed cell death by selective catalase degradation. Proc Natl Acad Sci U S A 103: 4952–7.
32. Kiffin R, Bandyopadhyay U, Cuervo AM (2006) Oxidative stress and autophagy. Antioxid Redox Signal 8: 152–62.
33. Scherz-Shouval R, Elazar Z (2007) ROS, mitochondria and the regulation of autophagy. Trends Cell Biol 17: 422–7.
34. Scherz-Shouval R, Shvets E, Fass E, Shorer H, Gil L, et al. (2007) Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. Embo J 26: 1749–60.
35. Lee HC, Yin PH, Lu CY, Chi CW, Wei YH (2000) Increase of mitochondria and mitochondrial DNA in response to oxidative stress in human cells. Biochem J 348 Pt 2: 425–32.
36. Bienert GP, Moller AL, Kristiansen KA, Schulz A, Moller IM, et al. (2007) Specific Aquaporins Facilitate the Diffusion of Hydrogen Peroxide across Membranes. J Biol Chem 282: 1183–92.
37. Kirkland RA, Adibhatla RM, Hatcher JF, Franklin JL (2002) Loss of cardiolipin and mitochondria during programmed neuronal death: evidence of a role for lipid peroxidation and autophagy. Neuroscience 115: 587–602.
38. Kim EH, Choi KS (2008) A critical role of superoxide anion in selenite-induced mitophagic cell death. Autophagy 4: 76–8.
39. Dagda RK, Cherra SJ, 3rd, Kulich SM, Tandon A, Park D, et al. (2009) Loss of PINK1 function promotes mitophagy through effects on oxidative stress and mitochondrial fission. J Biol Chem 284: 13843–55.
40. Huang Q, Shen HM (2009) To die or to live: the dual role of poly(ADP-ribose) polymerase-1 in autophagy and necrosis under oxidative stress and DNA damage. Autophagy 5: 273–6.
41. Daido S, Yamamoto A, Fujiwara K, Sawaya R, Kondo S, et al. (2005) Inhibition of the DNA-dependent protein kinase catalytic subunit radiosensitizes malignant glioma cells by inducing autophagy. Cancer Res 65: 4368–75.
42. Karantza-Wadsworth V, Patel S, Kravchuk O, Chen G, Mathew R, et al. (2007) Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. Genes Dev 21: 1621–35.
43. Roberts P, Moshitch-Moshkovitz S, Kvam E, O'Toole E, Winey M, et al. (2003) Piecemeal microautophagy of nucleus in Saccharomyces cerevisiae. Mol Biol Cell 14: 129–41.
44. Krick R, Muehe Y, Prick T, Bremer S, Schlotterhose P, et al. (2008) Piecemeal microautophagy of the nucleus requires the core macroautophagy genes. Mol Biol Cell 19: 4492–505.
45. Kovacs AL, Rez G, Palfia Z, Kovacs J (2000) Autophagy in the epithelial cells of murine seminal vesicle in vitro. Formation of large sheets of nascent isolation membranes, sequestration of the nucleus and inhibition by wortmannin and 3-ethyladenine. Cell Tissue Res 302: 253–61.
46. Park YE, Hayashi YK, Bonne G, Arimura T, Noguchi S, et al. (2009) Autophagic degradation of nuclear components in mammalian cells. Autophagy 5: 795–804.
47. Wagner M, Hampel B, Bernhard D, Hala M, Zwerschke W, et al. (2001) Replicative senescence of human endothelial cells in vitro involves G1 arrest, polyploidization and senescence-associated apoptosis. Exp Gerontol 36: 1327–47.
48. Unterluggauer H, Hampel B, Zwerschke W, Jansen-Durr P (2003) Senescence-associated cell death of human endothelial cells: the role of oxidative stress. Exp Gerontol 38: 1149–60.
49. Hampel B, Malisan F, Niederegger H, Testi R, Jansen-Durr P (2004) Differential regulation of apoptotic cell death in senescent human cells. Exp Gerontol 39: 1713–21.
50. Lee BY, Han JA, Im JS, Morrone A, Johung K, et al. (2006) Senescence-associated beta-galactosidase is lysosomal beta-galactosidase. Aging Cell 5: 187–95.
51. Ding G, Franki N, Kapasi AA, Reddy K, Gibbons N, et al. (2001) Tubular cell senescence and expression of TGF-beta1 and p21(WAF1/CIP1) in tubulointerstitial fibrosis of aging rats. Exp Mol Pathol 70: 43–53.
52. Martin JA, Buckwalter JA (2003) The role of chondrocyte senescence in the pathogenesis of osteoarthritis and in limiting cartilage repair. J Bone Joint Surg Am 85-A Suppl 2: 106–10.
53. Keyes WM, Wu Y, Vogel H, Guo X, Lowe SW, et al. (2005) p63 deficiency activates a program of cellular senescence and leads to accelerated aging. Genes Dev 19: 1986–99.
54. Yin D (1996) Biochemical basis of lipofuscin, ceroid, and age pigment-like fluorophores. Free Radic Biol Med 21: 871–88.
55. Yue Z, Jin S, Yang C, Levine AJ, Heintz N (2003) Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc Natl Acad Sci U S A 100: 15077–82.
56. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, et al. (2003) Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J Clin Invest 112: 1809–20.
57. Sansal I, Sellers WR (2004) The biology and clinical relevance of the PTEN tumor suppressor pathway. J Clin Oncol 22: 2954–63.
58. Arico S, Petiot A, Bauvy C, Dubbelhuis PF, Meijer AJ, et al. (2001) The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. J Biol Chem 276: 35243–6.

Figure S1

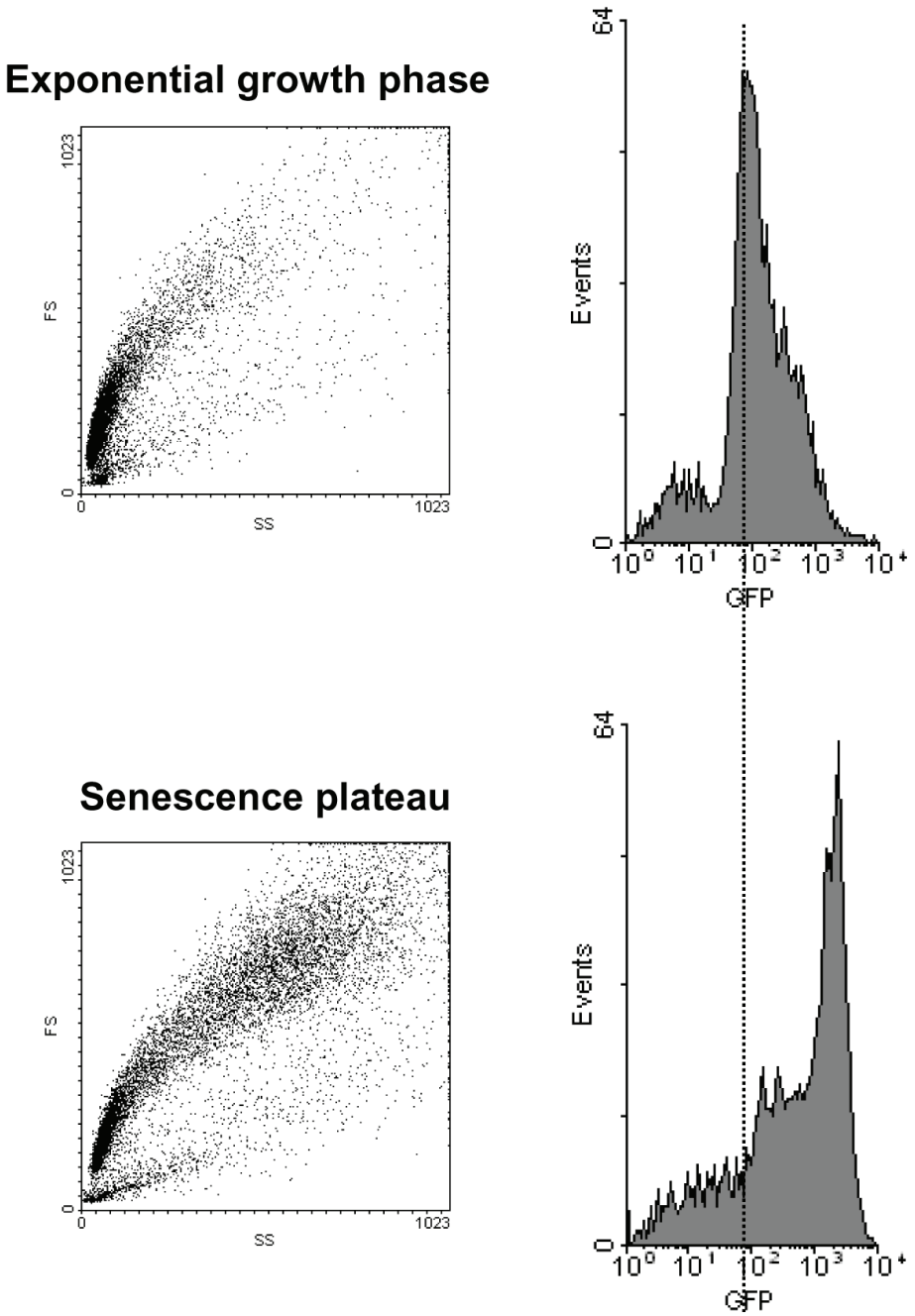


Figure S2

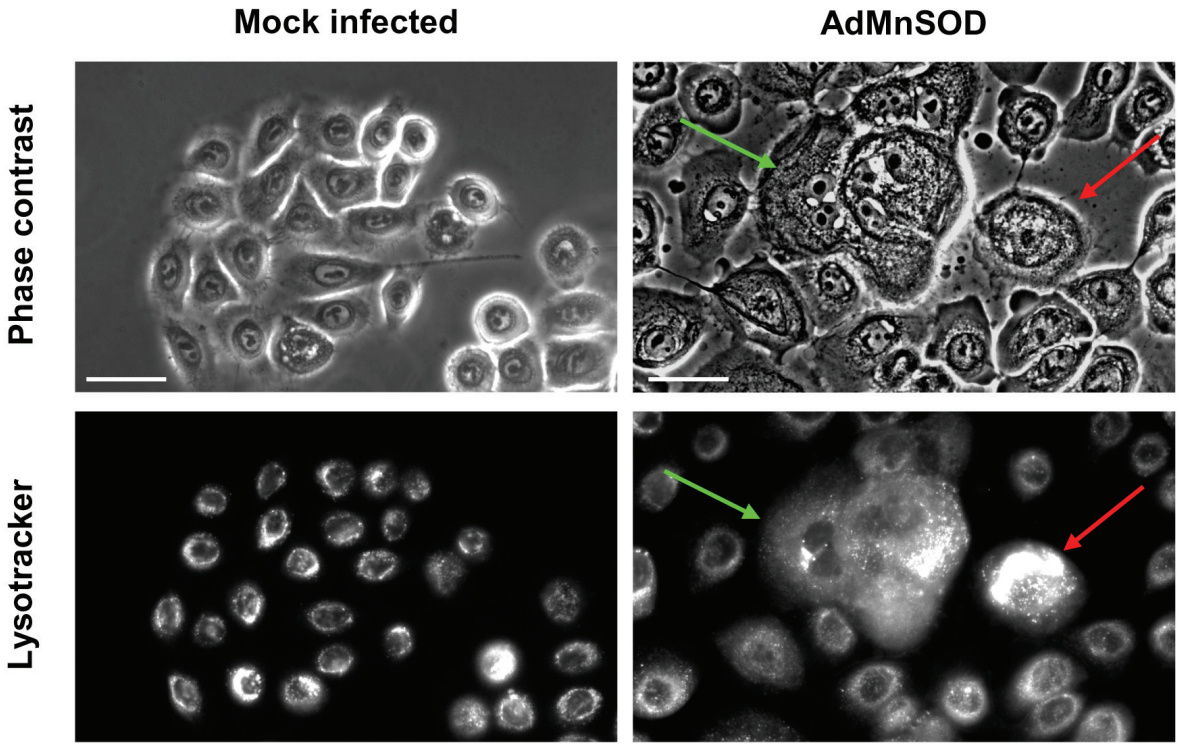


Figure S3

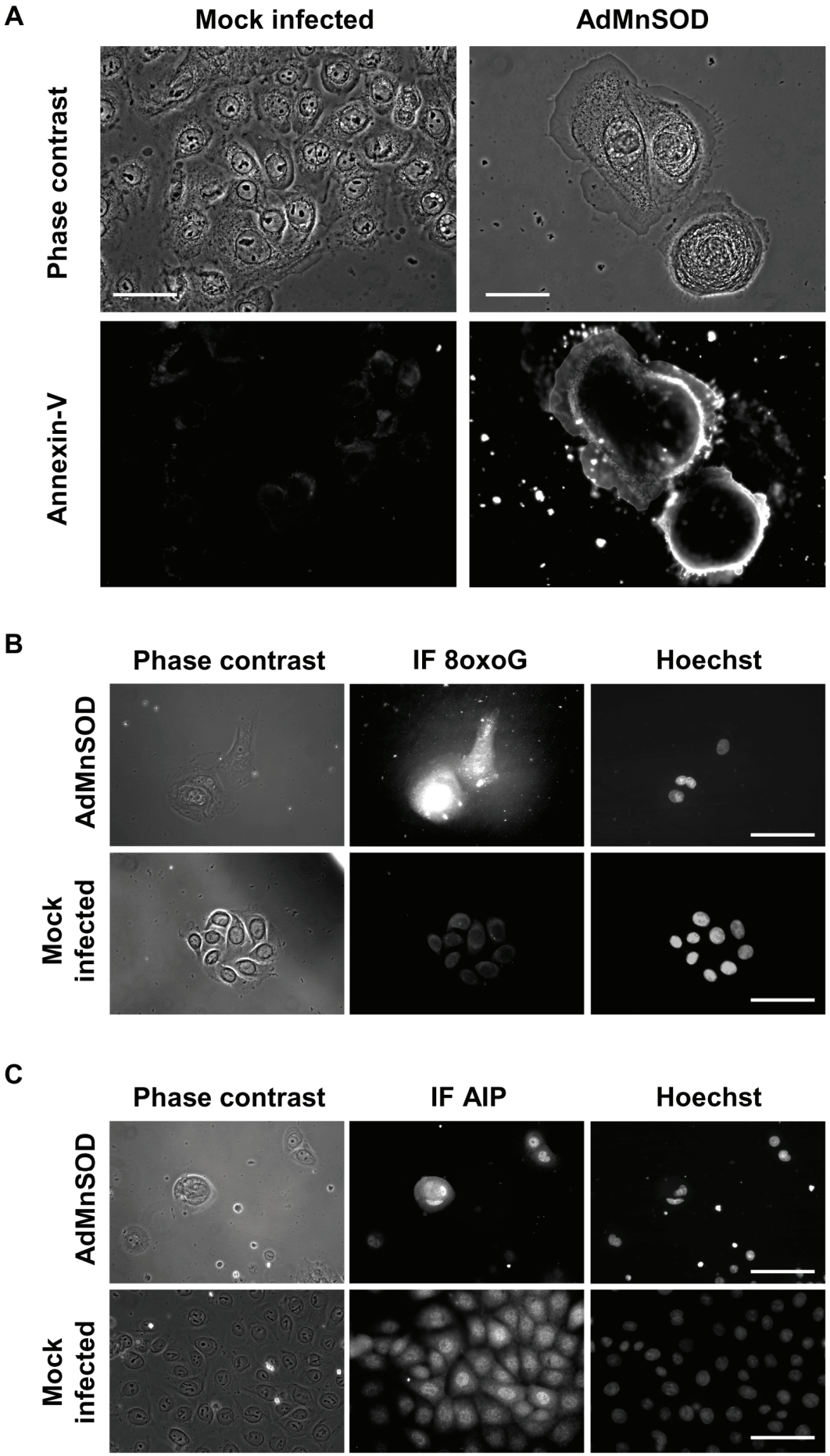


Figure S4

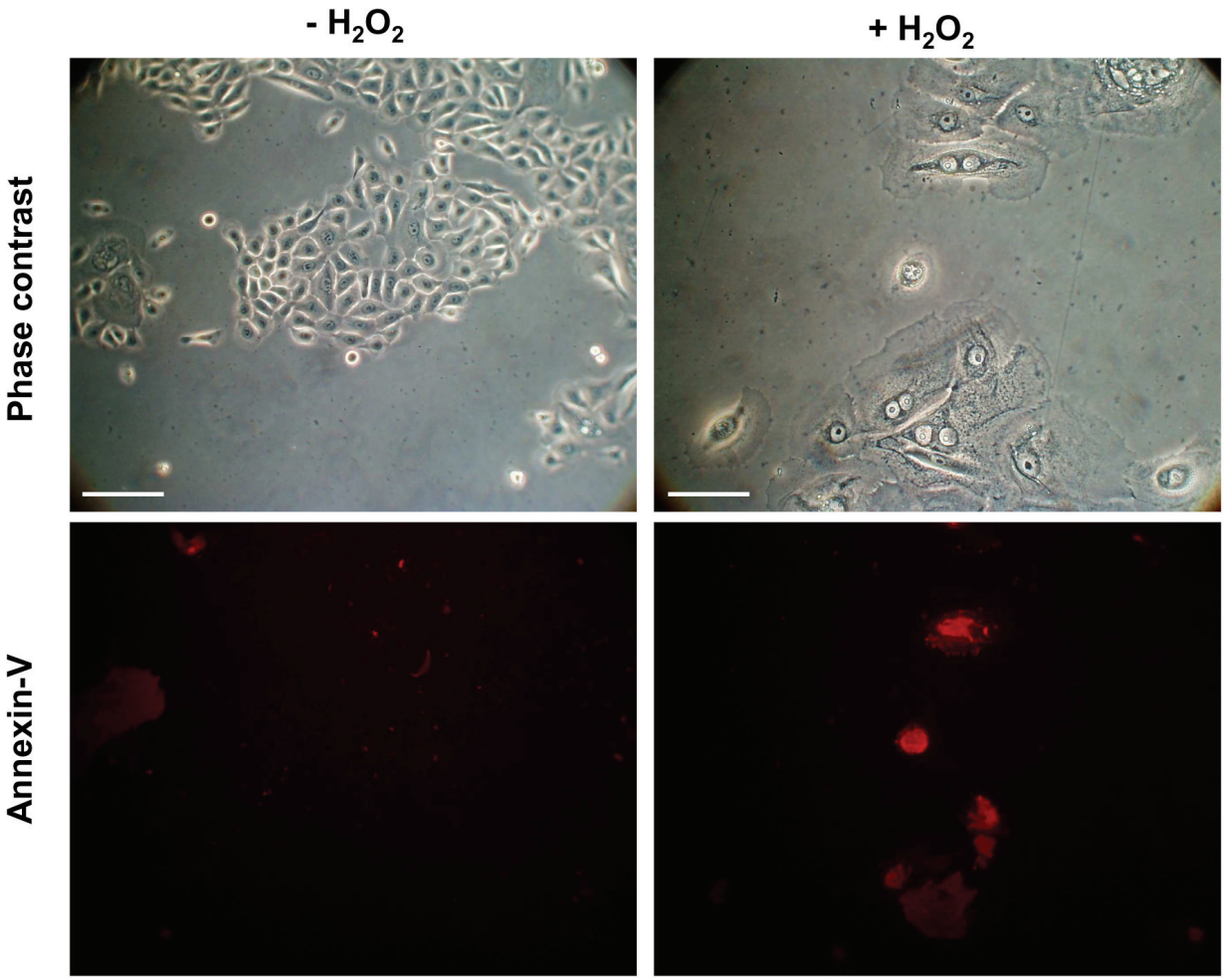


Figure S5

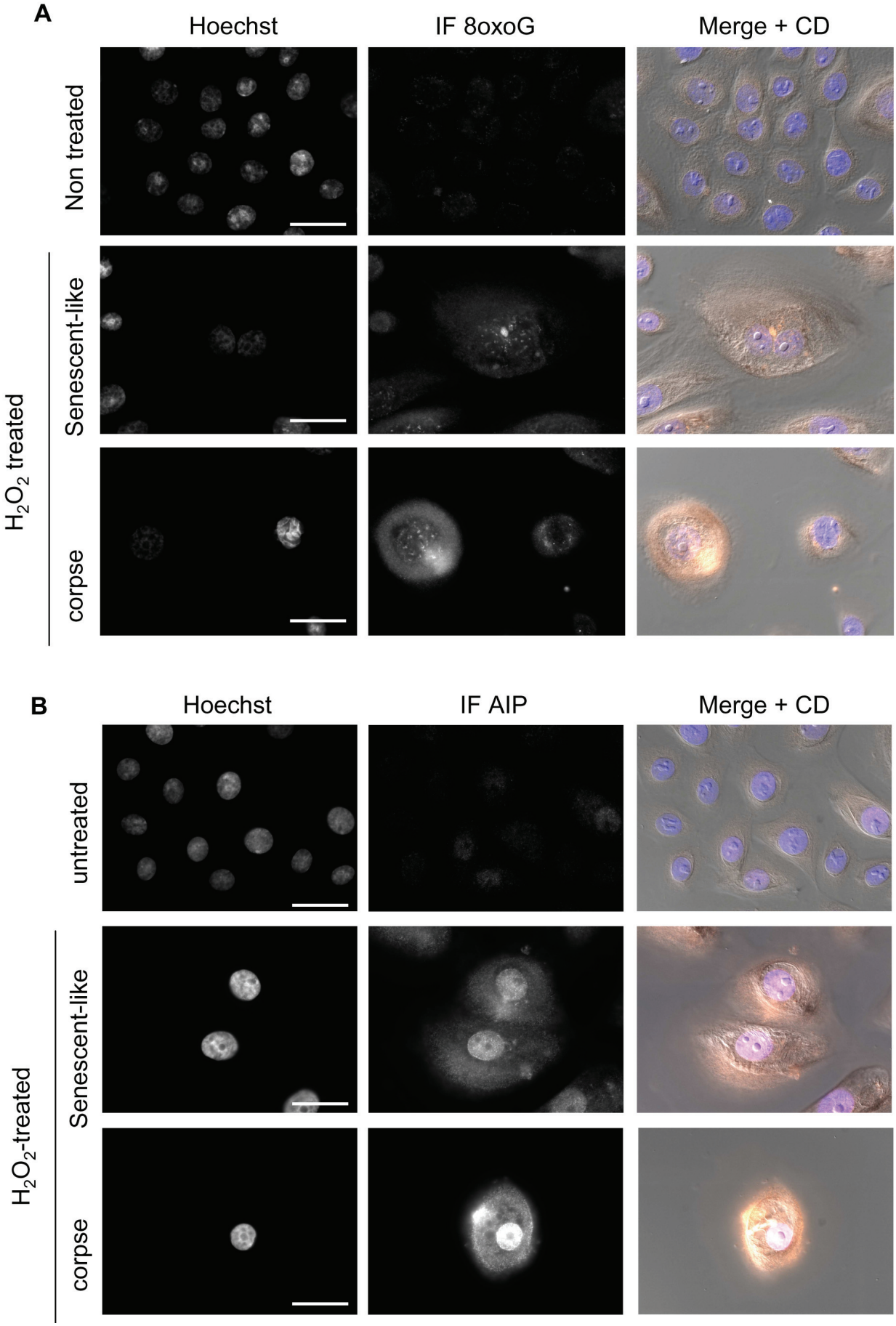


Figure S6

P values of Figure 11 panel A

		day 2	day 5	day 8
zVAD vs DMSO	t-test	0,20756	0,20457	0,08214
	Wilcoxon	0,2228	0,1207	0,02292
3 MA vs control	t-test	0,05259	2,7049E-08	0,00033
	Wilcoxon	0,08408	4,95E-07	0,00094
bafilo vs DMSO	t-test	0,01328	0,00040	0,01866
	Wilcoxon	0,01514	0,00029	0,03168

P values of Figure 11 panel B

		day 2	day 3	day 4
siAtg5 25 nM vs non target	t-test	0,20889	0,00612	0,00304
	Wilcoxon	0,3073	0,0115	0,00105
si Atg5 50 nM vs non	t-test	0,21773	0,00034	3,8839E-08
	Wilcoxon	0,1903	0,00049	0,00024

P values of Figure 12 panel A

		24 hrs	48 hrs	72 hrs	96 hrs
catalase 100U/mL vs relevant control	t-test	0,699	0,033	0,140	0,003
	Wilcoxon	0,644	0,028	0,094	0,006
catalase 250U/mL vs relevant control	t-test	0,974	0,243	0,031	0,003
	Wilcoxon	0,751	0,248	0,049	0,007
catalase 500U/mL vs relevant control	t-test	0,383	0,398	0,226	0,024
	Wilcoxon	0,105	0,686	0,174	0,018

P values of Figure 12 panel B

		24 hrs	48 hrs
PEG-catalase 100U/ml vs relevant control	t-test	0,077	0,264
	Wilcoxon	0,078	0,485
PEG-catalase 500U/ml vs relevant control	t-test	0,006	0,073
	Wilcoxon	0,009	0,009

Partie III :

Implication de la macroautophagie et du stress oxydant dans la génération de cellules néoplasiques lors de la sénescence

Les travaux précédents montrent que les kératinocytes sénescents accumulent de nombreux dommages oxydants et organites altérés qui conduiront à l'activation de la mort cellulaire par autophagie. Une des étapes prérequises à l'émergence post-sénescence serait donc que les kératinocytes sénescents progéniteurs de cellules émergentes échappent à la mort par macroautophagie. La dernière étape de mon travail a donc consisté à déterminer si tel était bien le cas. Il est important de rappeler que le stress oxydant, précédemment démontré comme étant l'inducteur majeur de la sénescence et de la mort cellulaire, est aussi essentiel à l'émergence. Les travaux présentés dans cette dernière partie traitent donc des rôles respectifs du stress oxydant et de l'autophagie lors de l'induction de la mort ou de son échappement lors de l'émergence.

Nous avons dans un premier temps pu mettre en évidence que les cellules émergentes post-sénescence (triées par FACS) ont une activité autophagique plus faible que les cellules sénescentes (réduction de l'expression des protéines Atg et en conséquence des vésicules autophagiques), mais toutefois pas totalement absente. Ces résultats suggèrent donc que l'émergence passerait par une diminution de l'activité macroautophagique permettant aux cellules progénitrices d'échapper à la mort.

Pour éprouver cette hypothèse, nous avons ensuite évalué l'impact de l'inhibition de l'autophagie sur la probabilité que des kératinocytes induits en sénescence prématurée par traitement au H_2O_2 génèrent des cellules émergentes. De manière intéressante, l'inhibition de l'autophagie par transfection d'un siRNA ciblant l'expression de Atg5 augmente la probabilité qu'une cellule sénescente génère des cellules émergentes, suggérant que les cellules sénescentes doivent en effet échapper à la mort par autophagie pour générer des cellules émergentes. Nous avons ensuite cherché à valider ce résultat sur des kératinocytes en sénescence spontanée. Pour éliminer tout biais causé par l'absence de synchronisation des NHEKs à entrer en sénescence, les kératinocytes sénescents ont d'abord été triés par FACS en fonction de leur taille avant d'être traités par la 3-méthyladénine. Dans cette expérience, nous avons utilisé deux concentrations d'inhibiteurs, 5mM (concentration précédemment utilisée pour inhiber la mort des NHEKs) et 1mM. L'utilisation de 1mM d'inhibiteur favorise la

génération de clones d'émergence à partir des cellules sénescentes, ce qui confirme donc la conclusion précédente. Il faudra néanmoins vérifier que l'utilisation de 1mM de 3-méthyladénine et de siRNA anti-Atg5 diminue bien la mort des NHEKs sénescents. De façon inattendue, l'utilisation de la concentration la plus forte de 3-méthyladénine (5mM) réduit la probabilité que les cellules sénescentes génèrent des clones d'émergence. Il semble donc que pour générer des cellules émergentes, les NHEKs sénescents doivent réduire leur niveau d'autophagie afin d'échapper à la mort tout en conservant un niveau d'activité minimal, probablement nécessaire à l'élimination de leurs composés altérés. Cette dernière conclusion est notamment soutenue par l'utilisation de la bafilomycine A1, un inhibiteur de la maturation des autophagosomes en autolysosomes, qui réduit très fortement la génération de cellules émergente post-sénescence. Afin de confirmer ces résultats avec des outils moléculaires plus spécifiques, je mets actuellement au point un système utilisant des shRNAs ciblant l'expression de Atg5. Le shRNA est intégré dans un plasmide contenant une cassette de résistance à la généticine (G418) afin de sélectionner les cellules transfectées. De plus, l'expression du shRNA est sous contrôle d'un promoteur tet-on qui nous permettra de contrôler de manière dose-dépendante l'inhibition de la macroautophagie lors de la sénescence avec des gammes de doxycycline.

Les résultats ci-dessus suggèrent donc que les cellules sénescentes progénitrices des cellules émergentes seraient préférentiellement celles présentant une activité autophagique modérée, pas trop intense pour échapper à la mort, mais suffisante pour maintenir l'homéostasie cellulaire. Afin de valider cette hypothèse, les kératinocytes sénescents ont été triés par FACS en fonction de leur niveau d'activité autophagique (estimé par marquage au Lysotracker), puis leur fréquence d'émergence a été évaluée. Comme attendu, les NHEKs sénescents à forte activité autophagique émergent peu, alors que ceux présentant une activité autophagique modérée développent des clones de cellules émergentes post-sénescence à une fréquence trois fois plus élevée. L'ensemble de ces résultats montre donc que la macroautophagie joue un rôle dual lors de la sénescence des kératinocytes, induisant la mort des cellules sénescentes ou favorisant la génération de cellules néoplasiques, ceci de façon dépendante de son niveau d'activation.

Le stress oxydant étant le principal inducteur de la mort par autophagie associée à la sénescence des kératinocytes, nous avons ensuite cherché à déterminer le rôle potentiel du H_2O_2 dans les différents niveaux d'activation de la macroautophagie. Pour cela, nous avons évalué la quantité de peroxyde d'hydrogène présente dans les cellules sénescentes prédisposées ou non à émerger (discriminées grâce au Lysotracker) par marquage au H2-DCFDA, une sonde fluorescente qui présente une affinité préférentielle pour le peroxyde d'hydrogène. De manière intéressante, les cellules sénescentes prédisposées à générer des cellules émergentes produisent moins de H_2O_2 que le reste des NHEKs sénescents. Le niveau d'activation de la macroautophagie lors de la sénescence semble donc étroitement corrélé au niveau de stress oxydant que les cellules subissent.

Le stress oxydant et l'activité macroautophagique qu'il induit jouent donc un rôle dual dans la sénescence et l'émergence. Durant la sénescence, les cellules accumulent de nombreux dommages oxydants qui conduisent à l'activation d'une macroautophagie importante responsable de la mort des NHEKs. La faible fraction de cellules sénescentes qui présentent un niveau de stress oxydant plus faible que la moyenne, échappent à la mort par autophagie et génèrent des cellules émergentes. Elles doivent néanmoins pour cela maintenir une activité macroautophagique de ménage pour favoriser l'élimination des composés cellulaires oxydés et la reprise de la prolifération.

C'est travaux font l'objet de l'article n°4 (en préparation) : *Levels of macroautophagy drive senescent keratinocytes into cell death or neoplastic transformation.*

Ce travail soulève un certain nombre de questions :

1- Concernant les cellules sénescentes progénitrices de cellules émergentes.

Ces cellules présentent un niveau de stress oxydant plus faible que le reste des cellules sénescentes mais supérieur à celui des cellules en croissance exponentielle. Une

de nos hypothèses est que les cellules progénitrices de cellules émergentes pourraient présenter plus de dommages mutagènes et moins de dommages délétères que les cellules sénescentes qui meurent. Pour tester cette hypothèse, il faudrait analyser qualitativement les dommages oxydants subis par les cellules issues des populations sénescentes (que l'on sait désormais enrichir en cellules prédisposées ou non à générer des cellules émergentes) notamment les 8-OH-G et les ponts AIP ainsi que les cassures à l'ADN. Si nous observions de telles différences qualitatives, il resterait à expliquer pourquoi. Une possibilité pourrait être que ces différents types de dommages résultent d'une production de ROS différente dans les sous-populations de cellules sénescentes prédisposées ou non à émerger. Il s'agira alors d'en analyser précisément la nature en utilisant des sondes à affinité variable pour les différentes espèces réactives de l'oxygène.

Nous avons également vu précédemment que les cellules émergentes sont générées par des kératinocytes sénescents polynucléés. Il serait donc intéressant d'évaluer le niveau d'activité autophagique et de stress oxydant que présentent les cellules polynucléées ; et inversement, d'évaluer la proportion de cellules polynucléées présentes dans la sous-population de cellules sénescentes présentant une activité macroautophagique modérée propice à l'émergence. A terme, l'utilisation de ces trois critères et des trois marqueurs correspondants (stress oxydant/H2-DCFDA, polynucléation/Hoechst vital et activité macroautophagique/Lysotracker) devrait nous permettre de trier spécifiquement les cellules progénitrices de cellules néoplasiques afin d'étudier la mécanistique cellulaire et moléculaire mise en place lors de l'émergence post-sénescence.

2- Concernant la résistance des cellules émergentes post-sénescence à la mort.

On considère généralement que lors de la transformation cancéreuse, les cellules doivent acquérir une certaine résistance à la mort cellulaire. Dans ce travail, nous avons établi que les cellules émergentes post-sénescence régulent à la baisse la macroautophagie. Il serait donc intéressant de déterminer si elles sont devenues

résistantes à l'induction de la macroautophagie par la rapamycine (inhibiteur de mTOR) ou traitement au H_2O_2. De plus, comme les kératinocytes sénescents sont résistants à l'apoptose induite par TRAIL + cycloheximide (donnée du laboratoire), il serait intéressant de déterminer si les cellules émergentes ont conservé cette faculté.

Article n°4 : Levels of macroautophagy drive senescent keratinocytes into cell death or neoplastic transformation

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

Emeric DERUY [1,2,3,4,5], Chantal VERCAMER [1,2,3,4,5], Nicolas MALAQUIN [1,2,3,4,5], Julie BERTOUT [1,2,3,4,5], Albin POURTIER [1,2,3,4,5] and Corinne ABBADIE [1,2,3,4,5*]

[1] Université de Lille Nord de France, F-59000 Lille, France.

[2] CNRS, UMR8161, Institut de Biologie de Lille, 1 rue Calmette, F-59021 Lille, France.

[3] USTL, F-59650 Villeneuve d'Ascq, France

[4] UDSL, F-59000 Lille, France

[5] Institut Pasteur de Lille, F-59000 France

*Corresponding author. Mailing address: UMR8161, Institut de Biologie de Lille, 1 rue du Pr. Calmette, BP 447, 59021 Lille Cedex, France. Phone: 33-3-20-87-11-02. Fax: 33-3-20-87-11-11.

e-mail: corinne.abbadie@ibl.fr

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: they die by autophagic programmed cell death or they generate neoplastic cells called post-senescence (PS) emergent cells. Herein, we demonstrate that the progenitors of PS emergent cells display a moderate level of macroautophagy compared to the average of the senescent population. 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. Macroautophagy thus plays antagonistic roles during senescence, inducing cell death or promoting neoplastic transformation, depending on its level of activation. We show in addition that the macroautophagic activity of senescent cells is directly correlated with their ROS level. Taken together, these data suggest that levels of oxidative damages and ensuing macroautophagic activity are the two main determinants of senescent keratinocytes outcome.

Introduction

Initially described by Hayflick in 1965 (Hayflick, 1965) as the phase reached by *in vitro* cultured human normal diploid fibroblasts after a limited number of serial passages, cellular senescence is now recognized as a fundamental program that can be activated whatever the cell type in response to various situations including telomere erosion or uncapping (Allsopp et al., 1992; d'Adda di Fagagna et al., 2003; Kruk et al., 1995), irreparable or excessive DNA damage (Chen et al., 2007), oxidative stress (Toussaint et al., 2000), or activation of oncogenes, such as Ras (Serrano et al., 1997) or NF-kappaB (Bernard et al., 2004; Bernard et al., 2001a). These situations of senescence induction 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 (Ben-Porath and Weinberg, 2005), changes in chromatin organization (Narita et al., 2003), major changes in transcriptome (Shelton et al., 1999), proteome (Benvenuti et al., 2002) and secretome (Coppe et al., 2010; Kuilman and Peeper, 2009), an increase in cell volume and granularity (Cristofalo and Kritchevsky, 1969; Greenberg et al., 1977), and an increase in the macroautophagic activity (Gosselin et al., 2009a; Young et al., 2009).

Oxidative stress seems to be one common denominator of senescence induction. It results from reactive oxygen species (ROS) accumulation with chronological age (Sohal and Dubey, 1994), or from ROS overproduction upon various stresses known to accelerate aging such as ionizing and UV radiations (Toussaint et al., 2000). Ras and its downstream effectors as well as NF-kappaB transcription factors lead to senescence induction also in part through the production of ROS (Atwood and Sealy, 2010; Bernard et al., 2004; Bernard et al., 2001a; Bernard et al., 2001b; Lee et al., 1999; Pearson et al., 2000; Zhu et al., 1998). Oxidative stress

induces senescence by damaging organelles and macromolecules, including DNA (Cadet et al., 2010; Djordjevic, 2004; Lu et al., 2001). The oxidative DNA damages result in the activation of a DNA Damage Response (DDR) which, by itself or in addition to the DDR activated by telomere erosion and uncapping, leads to the typical cell-cycle arrest encountered by senescent cells (Sedelnikova et al., 2004; Zhan et al., 2010). The accumulation of other oxidized cell components could explain the increase in cell size and granularity and was shown to be one of the causes of the increase in macroautophagic activity (Deruy et al., 2010).

Numerous reports claim that senescence corresponds to an irreversible growth arrest mechanism that cancer cells have to bypass to generate tumours. 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 keratinocytes (Gosselin et al., 2009b) or mammary epithelial cells (Romanov et al., 2001) 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) emerging cells which are transformed and able to form skin hyperplasia in nude mice (Gosselin et al., 2009b). Several data from our group suggest that the oxidative DNA damages encountered by senescent cells could be the mutagenic motor of this neoplastic emergence (Gosselin et al., 2009b).

Macroautophagy is a degradation system of proteins and organelles that involves the lysosomial machinery (Glick et al., 2010; Mizushima et al., 2008). It begins by the formation of an isolation membrane originating from the Golgi or the endoplasmic reticulum (Hayashi-Nishino et al., ; Hayashi-Nishino et al., 2009) that targets a cytoplasmic cargo, for example misfolded aggregated proteins or altered organelles (Ding and Yin, 2008). The resulting double-membrane vesicle, the autophagosome, next fuses with a lysosome to form an

autolysosome in which the sequestrated cargo will be degraded through the acidic pH and the lysosomial hydrolases activity. More than thirty genes named Atg (for autophagy related gene) are involved in all steps of vesicles formation and maturation (Eskelinen, 2008; Klionsky et al., 2003). Atg6/Beclin-1 is essential for the isolation membrane nucleation and curvature, hence for the starting of the process (Aita et al., 1999; Liang et al., 1999). It acts in a multi-protein complex with hVps34 (a class III PI3 Kinase), P150 (Vps15) and other adaptators (Fimia et al., 2007; Furuya et al., 2005; Itakura et al., 2008; Liang et al., 2006; Takahashi et al., 2007). The autophagosome elongation, closure and its fusion with lysosomes are driven by an Atg12-Atg5-Atg16 complex (Eskelinen, 2008; Mizushima et al., 2003; Mizushima et al., 1998) and by the integration of Atg8/LC3-II (processed form of Atg8/LC3) in the autophagosome membrane (Eskelinen, 2008; Kabeya et al., 2000; Kabeya et al., 2004; Sou et al., 2006).

A basal constitutive macroautophagic activity is assumed to contribute to cell homeostasis and is often referred as housekeeping autophagy. This autophagic activity can be enhanced to help cells to face nutrient deprivation, virus infection, or oxidative damages, thus favoring cell survival. However, when macroautophagy 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 (Mizushima et al., 2008; White and DiPaola, 2009).

We have recently shown that senescent epidermal keratinocytes encounter an increase in macroautophagic activity whose excessive intensity leads to their death (Deruy et al., 2010; Gosselin et al., 2009a). 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 address the question of whether these two cell destinies are independent or alternative, in other terms whether the PS emergence necessitates escaping autophagic cell death. We show that the progenitors of PS emergent cells display a moderate autophagic activity compared to other senescent cells and indeed escape autophagic cell death, but have to maintain a minimal level of macroautophagic activity in order to be able to resume mitosis and generate PS emergent cells. Therefore, these results indicate that the outcome of senescent keratinocytes is dependent, at least in part, of the level of their macroautophagic activity. In this report, we also investigated the role of oxidative stress in the senescent keratinocyte outcomes. Indeed, we have previously shown that the lethal autophagic activity of senescent cells is induced following oxidative damages to mitochondria and nucleus (Deruy et al., 2010). 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 (Gosselin et al., 2009b). Here we show that levels of macroautophagy in senescent cells death are directly correlated with their H_2O_2 steady-state levels. Taken together, these results suggest 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.

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 (Boyce and Ham, 1983). In all experiments, cells were seeded at 3500 cells/cm2. When necessary, they were split at 70% confluence. The number of population doublings (PD) was calculated at each passage by means of the following equation: PD=log(number of collected cells/number of plated cells)/log2.

Inhibition of macroautophagy by siRNA or pharmacological inhibitors

H2O2-induced senescent NHEKs were plated at 70,000 cells per well in six-well plates. SiRNAs were diluted in PrimeFect siRNA Transfection Reagent (Lonza) and incubated 15 minutes at room temperature before adding 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). Control transfection was performed using a non targeting

siRNA pool (siCONTROL Non Targeting siRNA Pool #2, Dharmacon). Transfections were stopped after 24 hours 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 for stock dilutions, were directly added to cells diluted in fresh culture medium at 1 or 5mM for 3MA and 5nM for Bafilomycin and let for 48 hours.

SA beta-galactosidase staining

SA-beta-galactosidase assays were performed as described by Dimri (Dimri et al., 1995).

Western blotting

Cells were lysed in the following solution: 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 cytomation M0879), LAMP-1 (Santa Cruz Biotechnology SC17768), Cyclophilin B (Abcam ab16045), 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 and

quantifications were performed with the associated Multigauge V3.0 software.

Immunofluorescence

For LAMP-1 immunofluorescent 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. Slides were incubated with a primary antibody anti LAMP-1 (Transduction Labs L76620) for 1 hour 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.

Lysotracker and H2-DCFDA staining

Lysotracker and H2-DCFDA were purchased from Molecular Probes. Living cells were incubated with probes directly added to the cell culture medium at 37°C for 30 minutes as recommended by the supplier. Nuclei were stained with the vital Hoechst 33342 at 100ng/ml for 10mn at 37°C.

Flow cytometry analysis and fluorescence-activating cell sorting

Flow cytometry quantifications of fluorescent probes staining were performed using a Beckman Coulter Epics XL-MCL. Collected data were exported to the WinMDI 2.9 software

for detailed analysis.

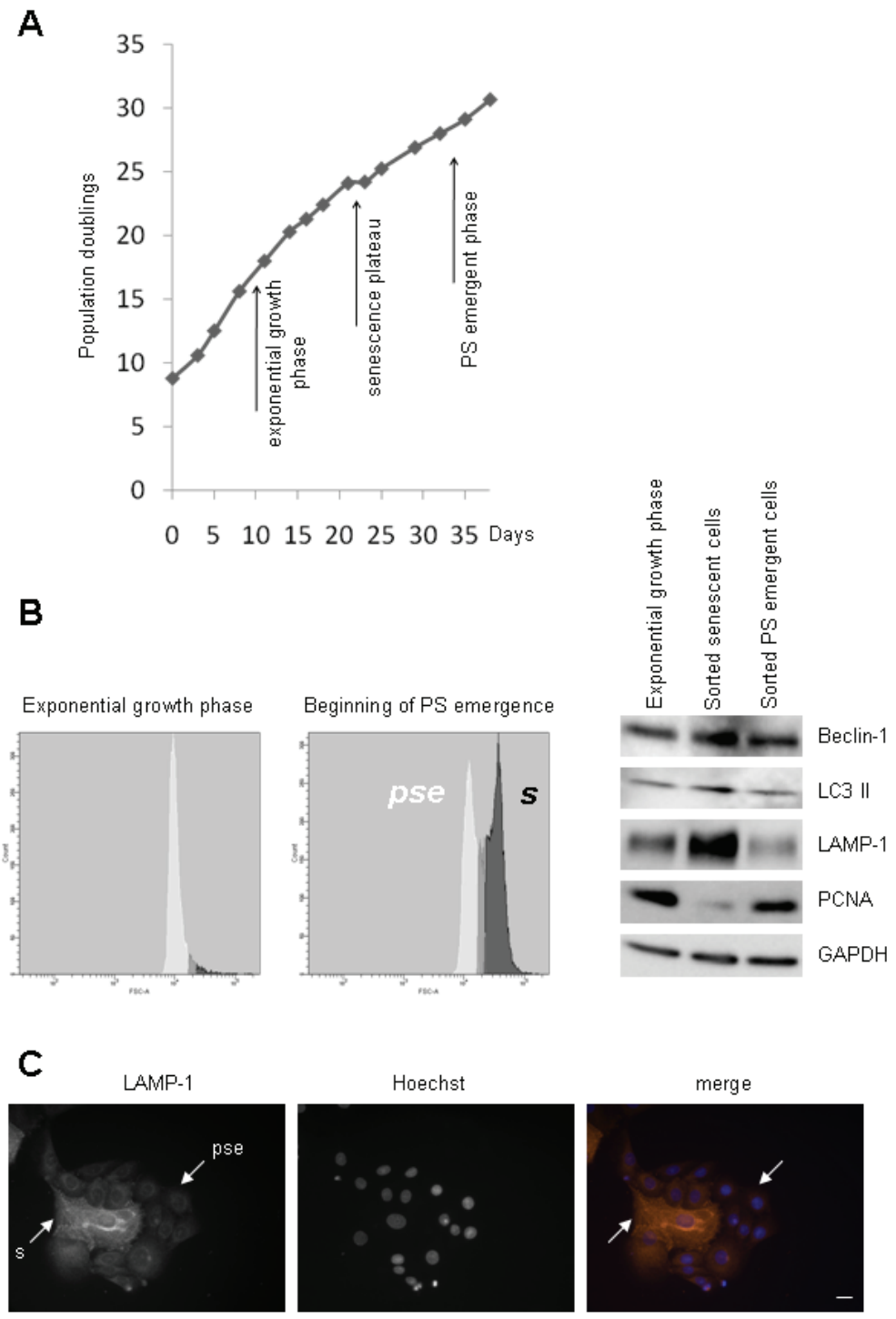
Sorting of NHEKs was performed on a BD FACS Aria or on a Coulter FACS Altra. Selected subpopulations with the ad hoc forward scatter factor and/or fluorescent probes staining intensity values were electrostatically sorted in air, collected in complete culture medium and put again in culture.

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 macroautophagic activity 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 and B). The composition of the different cell populations was checked by flow cytometry according to the forward scatter factor value 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 the PS emergence phase was composed of two subpopulations differing by an about 10-fold difference in size (Fig. 1B). As already shown in previous studies (Deruy et al., 2010; Gosselin et al., 2009a), the subpopulation of the largest cells corresponds to senescent cells, and consequently the subpopulation of cells with the smallest size was assumed to correspond 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 nature of the sorted cells was checked by examining PCNA expression. As expected, PCNA was down-regulated in sorted senescent cells compared to exponentially growing cells and re-expressed in sorted PS emergent cells (Fig. 1B). The expression of several macroautophagic markers was then examined. The expression of Atg6/Beclin-1 increased at senescence as already shown (Gosselin et al., 2009a) 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.

Fig 1

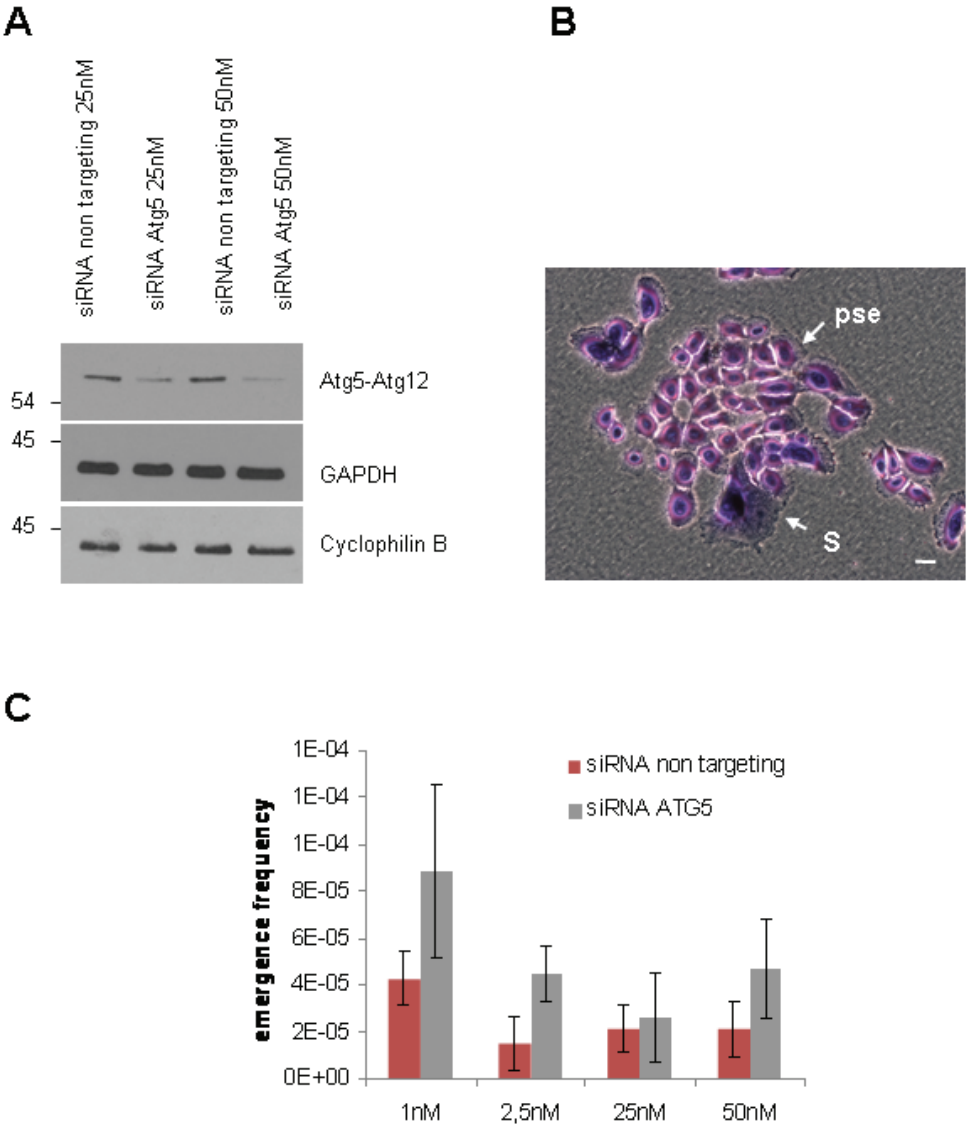


The nature of the detected protein was validated using proteins extracted from Normal Human Dermal Fibroblasts (sup Fig. 1). The quantity of LC3 II in NHEKs increased at senescence and decreased again in the population of emergent cells (Fig.1B). Similarly, the expression of lysosomal associated membrane protein 1 (LAMP-1), a marker of lysosomes and autolysosomes, increased at senescence and decreased again at PS emergence (Fig.1B). An immunofluorescence assay targeting LAMP-1 confirmed that PS emergent cells have a macroautophagic activity strongly reduced compared to their senescent progenitors (Fig.1C). Therefore, 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 the macroautophagic activity in the senescent population and examined whether that alters the frequency of PS emergence. NHEKs were induced in premature senescence by a sublethal H_2O_2 treatment (50 µM) as previously described (Bernard et al., 2004). 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.

Fig 2



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

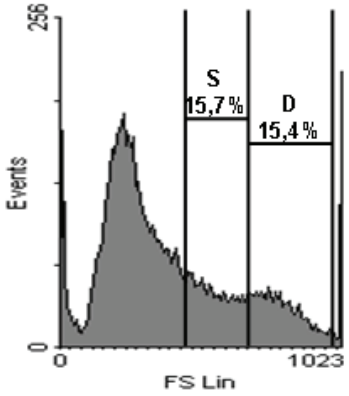
The sole isolated clones containing a senescent cell amongst the emergent ones as illustrated in Figure 2B were taken into account, hence ensuring that these clones were *bona fide* PS emerging clones originating from a senescent cell and not a clone formed from some putatively contaminating young cells. Cells transfected with control siRNAs underwent PS emergence about one week after having acquired the senescent phenotype, as already described (Gosselin et al., 2009b), with an emergence frequency comprised between 2 to 4 10^{-5} (Fig. 2C) i.e. of the same range of that obtained with normal senescent cells (Gosselin et al., 2009b), 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 a senescent population with a lowered macroautophagic activity, which consequently suffers from a lowered cell death level (Deruy et al., 2010), becomes more prone to generate PS emergent cells.

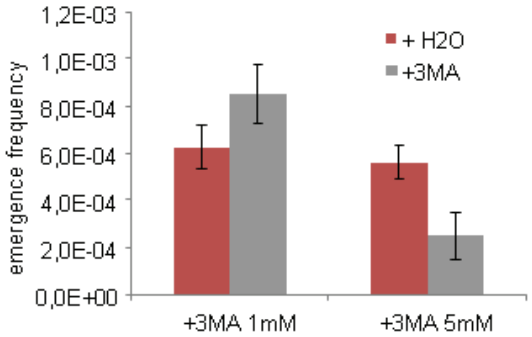
Escaping autophagic cell death but maintaining a housekeeping macroautophagic activity are both necessary for the generation of PS emergent cells

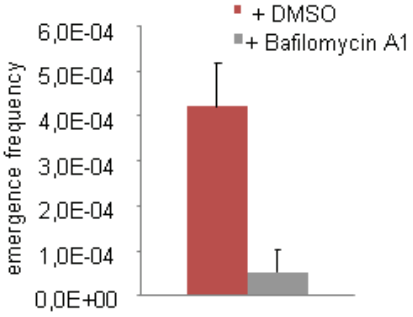
To strengthen the above conclusion, we reiterated a similar experiment, but with normal senescent cells, and with a macroautophagic inhibitor acting upstream Atg5, the 3-methyladenine (3-MA) which blocks the activity of hVps34 involved in the initial autophagosome formation (Blommaart et al., 1997). NHEKs were taken at the senescent plateau, and analyzed by flow cytometry for forward factor (Fig.3A). According to our previous studies, the subpopulation of 15% cells with the highest forward 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) (Gosselin et al., 2009a).

Fig 3

A



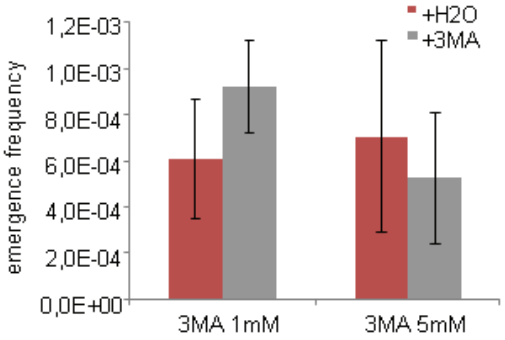


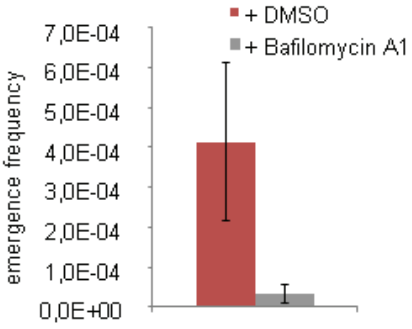


Fold increase		1.37	2.25
p value	Student	0.04522	0.00625
	Wilcoxon	0.08143	0.02843

Fold increase		7.88
p value	Student	0.00057
	Wilcoxon	0.01141

B





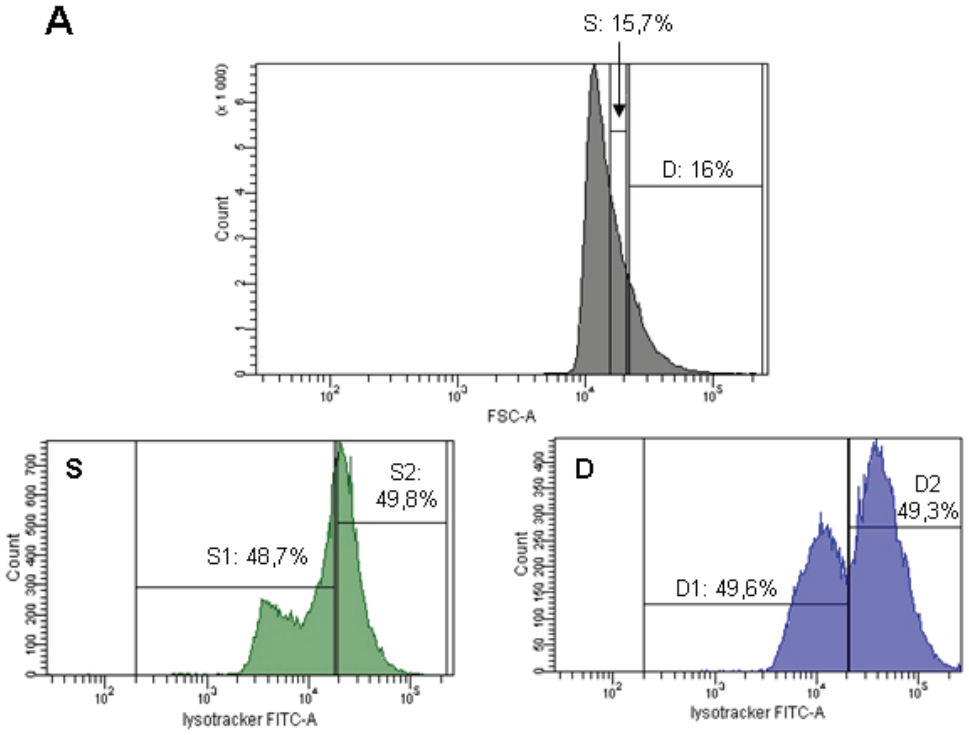
Fold increase		1.52	1.34
p value	Student	0.03210	0.37016
	Wilcoxon	0.02747	0.68490

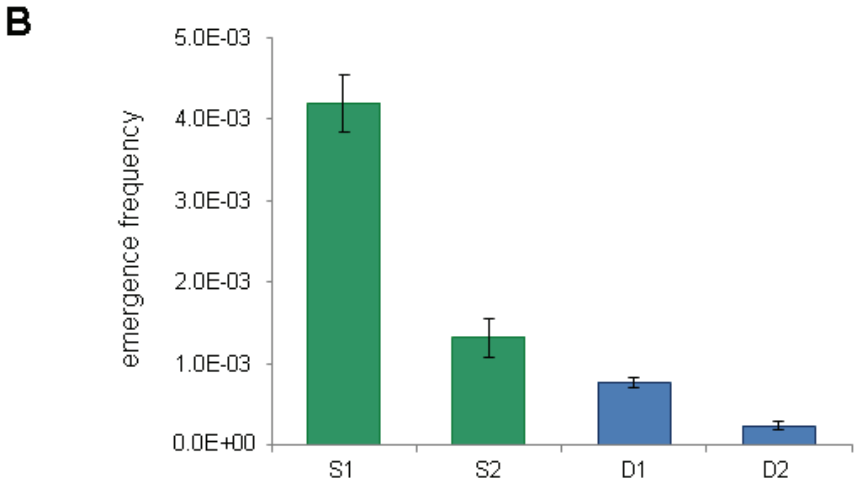
Fold increase		12.40
p value	Student	0.01820
	Wilcoxon	0.01066

Cells from the subpopulation S were sorted, plated at low density, treated by 3-MA and monitored for PS emergence. Two concentrations of 3-MA were used in this assay, 5 mM and 1mM. As shown in figure 3A, these two different concentrations had 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 macroautophagic activity in the senescent population increases its 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 not too high to avoid cell death but enough high to ensure housekeeping activities and be able to resume 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 (Bowman et al., 1988; Yamamoto et al., 1998). We have previously shown that applying Bafilomycine A1 to senescent NHEKs leads to huge accumulation of phagosomes and autolysosomes full of undigested material and blocks cells at the late stage of autophagic cell death. Here, Bafilomycine A1 was applied on sorted senescent NHEKs as above, and PS emergence was monitored. The results show that in this situation of complete blockage, the PS emergence was almost completely abolished (Fig.3A).

To further assay the dose effect of the inhibition of the macroautophagic activity on the generation of PS emergent cells, we reiterated the experiments with 3-MA and Bafilomycine A1 on H_2O_2-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.

Fig 4



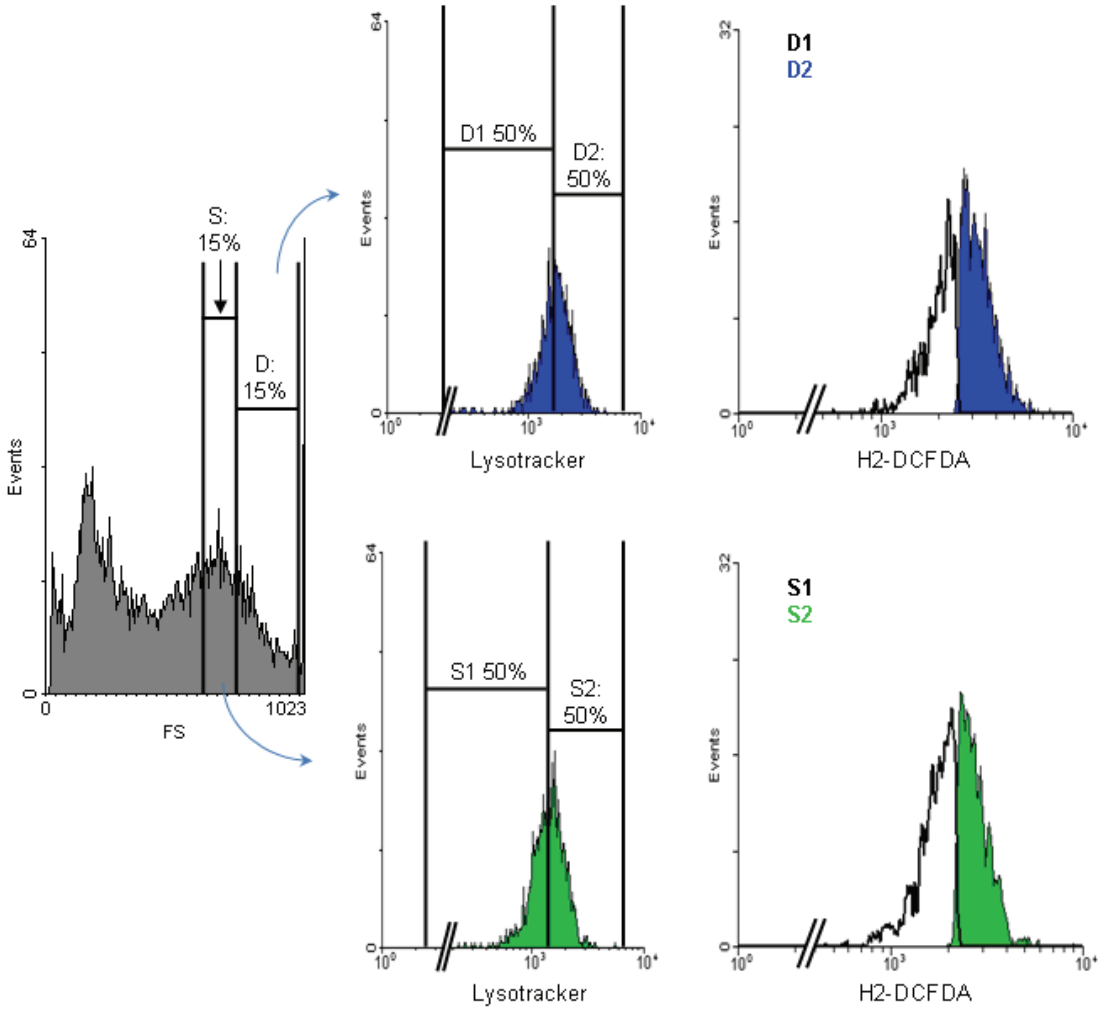


		S1/S2	D1/D2
Fold increase		3.20	3.23
p value	Student	0.00206	0.00142
	Wilcoxon	0.02857	0.04975

The progenitors of PS emergent cells display a moderate autophagic activity

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 hypothesis, we investigated the ability of senescent keratinocytes to generate PS emergent clones according to the level of their macroautophagic activity. For that purpose, 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 in a previous study to reflect autophagic vesicle accumulation (Gosselin et al., 2009a). The cell population was analyzed according to forward scatter factor value, and cells from the subpopulations S and D were sorted. During this sorting, the Lysotracker staining intensity was also analyzed, and each subpopulation was secondly separated in two equal subpopulations, the 50% of cells with the lowest Lysotracker staining and the 50% of cells with the highest Lysotracker staining respectively referenced as S1, S2, D1 and D2 (Fig 4A). Cells from the four resulting subpopulations were plated 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 generated lesser clones than the two subpopulations S1 and S2 of still alive senescent cells (Fig. 4B). But inside both categories, the cells with the lowest Lysotracker staining intensity showed to be those generating the highest clone number (Fig 4B). Therefore, the progenitors of PS emerging cells are fully senescent cells, not too engaged in the death pathway, and display a moderate autophagic activity (population S1). This moderate autophagic activity is, even though, about 27 fold higher than that of exponentially growing cells as shown in sup Fig 2B.

Fig 5

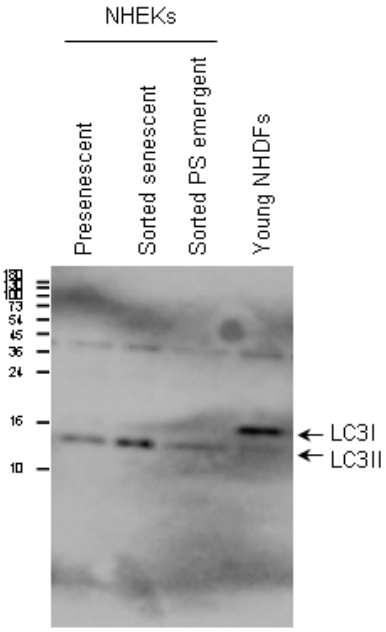


	S1	S2	D1	D2
H2-DCFDA peak values	2016,91	2371,37	2617,99	2788,13

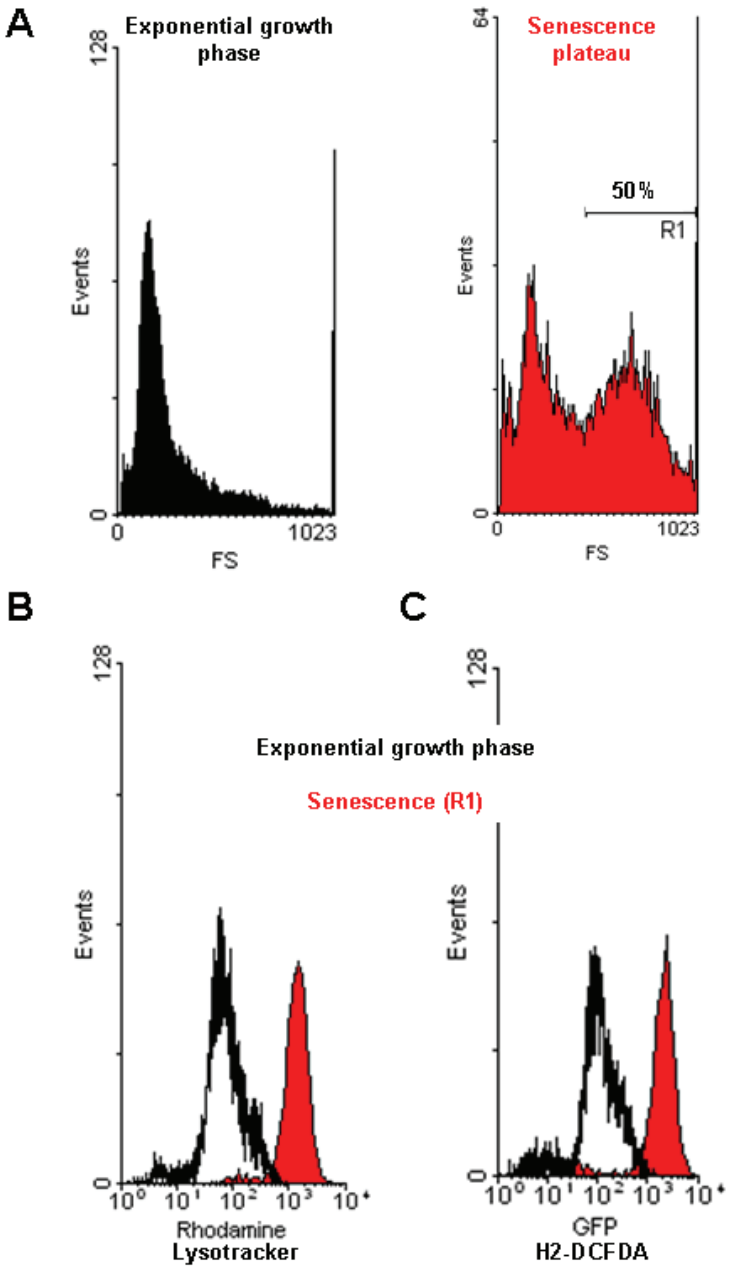
The levels of macroautophagy are directly correlated to the levels of oxidative stress associated to senescence

We have recently demonstrated that the oxidative stress associated to senescence is the inducer of the high macroautophagic activity that leads to senescent cell death (Deruy et al., 2010). Here, we wanted to determine whether or not the optimal levels of macroautophagy enabling PS emergent cells generation are directly correlated with the level of oxidative stress. To address this question, we analyzed the steady state level of reactive oxygen species in the four senescent cells subpopulations characterized above for their macroautophagic activity and their ability to generate PS emergent clones. For that purpose, NHEKs at the senescence plateau were doubly stained with Lysotracker and H2-DCFDA, a fluorescent H_2O_2 sensor, and analyzed by flow cytometry. The analysis shows that the H_2O_2 steady state level in the four subpopulations is inversely correlated with their macroautophagic activity and their ability to generate emergent clones (Fig. 5). Therefore, the moderate level of autophagic activity which is optimal for generating PS emergent cells would be the direct consequence of a moderate oxidative stress. Even so, this moderate H_2O_2 steady state level of PS emergent progenitors is 28 fold higher than the level measured in exponentially growing cells (Sup Fig 2C).

Sup Fig 1



Sup Fig 2



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

Discussion

Macroautophagy has two antagonistic roles, one helping cells to support various stresses (nutrient deprivation, lack of survival factors, etc...) and survive, and the other inducing cell death. We and others have shown that senescence is accompanied by a macroautophagic activity. This was shown for several normal cell types undergoing normal or stress-induced premature senescence, including normal senescent fibroblasts (Gerland et al., 2003), IMR90 fibroblasts induced in premature senescence upon H-RasV12 overexpression (Young et al., 2009), long-term cultured and repeatedly stimulated T lymphocytes (Gerland et al., 2004), normal senescent epidermal keratinocytes (Deruy et al., 2010; Gosselin et al., 2009a), normal biliary epithelial cells (Sasaki et al., 2010), and also for cell lines reinduced in senescence upon various drug treatments, such as mammary epithelial MCF-7 or MDA-MB231 lines treated by a microtubule polymerization inhibitor or doxorubicin (Arthur et al., 2007; Di et al.). One question opened by these data is what are the roles and consequences of the macroautophagic activity of senescent cells? Here, using normal human epidermal keratinocytes (NHEKs) undergoing spontaneous or premature H_2O_2-induced senescence, we show that macroautophagy determines the outcome of senescent cells, depending on its level of activation.

In contrast to senescent fibroblasts that are irreversibly and permanently cell-cycle arrested (d'Adda di Fagagna et al., 2003), senescent NHEKs, as well as senescent normal human mammary epithelial cells (HMECs), are able to reenter mitosis to generate post-senescent (PS) emergent cells that display neoplastic properties (Gosselin et al., 2009b; Romanov et al., 2001). We show 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, and that this moderate autophagic activity enables them to escape autophagic

programmed cell death. However, even if senescent cells must have a not too high level of macroautophagy to escape cell death, they have to maintain a minimal level of activity to be able to generate PS neoplastic cells. This minimal autophagic activity is, even though, more than 20 fold higher than that of young cells. It is probably required not only to ensure the elimination of the various accumulated altered components which could be deleterious for the senescent cell survival, but also to provide basic metabolites and energy to reenter mitosis and perform neosyntheses necessary to generate daughter cells, themselves properly equipped to survive and proliferate. Macroautophagy thus could play antagonistic roles in the determination of the senescent keratinocytes outcome in relation with tumor initiation. When overactivated, macroautophagy would induce 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.

Escaping autophagic cell death and eliminating altered cell components could be sufficient for senescent cells to reenter mitosis and generate daughter cells, but can not explain why these daughter cells have acquired transformed and tumorigenic characteristics. In this report, we show that the macroautophagic activity levels leading to either cell death or neoplastic cells generation are directly correlated to the ROS levels of senescent cells. Therefore, the outcome of senescent keratinocytes could be dictated by the level of oxidative damages they encompass and the downstream autophagic activity this induces. Interestingly, previous results from our group have demonstrated that oxidative stress is not only responsible for keratinocyte senescence (Bernard et al., 2004) and associated cell death (Deruy et al., 2010), 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

(Gosselin et al., 2009b), suggesting that despite their lethal effects, ROS could act as the mutagenic motor necessary for PS emergence, probably by affecting oncogenes and/or tumor suppressor genes. 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 fact that these cells escape cell death and hence acquire a life span longer than the average potentially gives them time to stochastically acquire the proper genome alterations favorable to transformation.

Are these results relevant to the very initial steps of carcinogenesis occurring *in vivo*? In other terms, amongst the senescent cells that accumulate in epithelial tissues with age (Dimri et al., 1995; Herbig et al., 2006; Jeyapalan et al., 2007; Krishnamurthy et al., 2004; Melk et al., 2004; Minamino et al., 2004; Ressler et al., 2006), 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 (Collado et al., 2005), benign prostatic hyperplasia (Choi et al., 2000), papillary hyperplasia (Yamaguchi et al., 2009), and metaplastic and dysplastic epithelium of the upper gastrointestinal tract (Going et al., 2002) supports this possibility. It is to be noticed that senescent cells were demonstrated to be apoptosis-resistant (Chaturvedi et al., 1999; Seluanov et al., 2001; Spaulding et al., 1999; Wang, 1995; Wang, 1997), which 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 (Hippert et al., 2006). 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* (Aita et al., 1999), its partner *UVRAG* (Liang et al., 2006), as well as *atg2B*, *atg5* and *atg9B* (Kang et al., 2009), which 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 *beclin1*+/- mutant mice were shown to spontaneously develop with a high incidence when became aged preneoplastic lesions in mammary glands and lung and malignant carcinomas of the lung and liver as well as lymphomas (Qu et al., 2003; Yue et al., 2003), suggesting that, in accordance with our present *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 (Karantza-Wadsworth et al., 2007). Therefore, a moderate macroautophagic activity may promote tumorigenesis not only by permitting autophagic cell death escape, but also by favoring DNA damage accumulation.

In conclusion, we propose that some of the senescent epithelial cells which accumulate in aged tissues could be the main source for the generation of sporadic carcinomas, which incidence indeed dramatically increases with age.

Acknowledgements

This work was supported by the Centre National de la Recherche Scientifique, the Université Lille 1, the Ligue contre le Cancer, the Association pour la Recherche sur le Cancer, and the Institut Pasteur de Lille. E.D. has a fellowship from the Institut Pasteur de Lille and the Région Nord/Pas-de-Calais. We thank the Microscopy-Imaging-Cytometry Facility of the Lille Pasteur Campus (MICPaL Facility) for access to instruments and technical advices; Arnau Augert from the Institut de Biologie de Lille for critical discussions.

References

Aita, V. M., Liang, X. H., Murty, V. V., Pincus, D. L., Yu, W., Cayanis, E., Kalachikov, S., Gilliam, T. C., and Levine, B. (1999). Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21. Genomics *59*, 59-65.

Allsopp, R. C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E. V., Futcher, A. B., Greider, C. W., and Harley, C. B. (1992). Telomere length predicts replicative capacity of human fibroblasts. Proc Natl Acad Sci U S A *89*, 10114-10118.

Arthur, C. R., Gupton, J. T., Kellogg, G. E., Yeudall, W. A., Cabot, M. C., Newsham, I. F., and Gewirtz, D. A. (2007). Autophagic cell death, polyploidy and senescence induced in breast tumor cells by the substituted pyrrole JG-03-14, a novel microtubule poison. Biochem Pharmacol.

Atwood, A. A., and Sealy, L. (2010). Regulation of C/EBPbeta1 by Ras in mammary epithelial cells and the role of C/EBPbeta1 in oncogene-induced senescence. Oncogene.

Ben-Porath, I., and Weinberg, R. A. (2005). The signals and pathways activating cellular senescence. Int J Biochem Cell Biol *37*, 961-976.

Benvenuti, S., Cramer, R., Quinn, C. C., Bruce, J., Zvelebil, M., Corless, S., Bond, J., Yang, A., Hockfield, S., Burlingame, A. L.*, et al.* (2002). Differential proteome analysis of replicative senescence in rat embryo fibroblasts. Mol Cell Proteomics *1*, 280-292.

Bernard, D., Gosselin, K., Monte, D., Vercamer, C., Bouali, F., Pourtier, A., Vandenbunder, B., and Abbadie, C. (2004). Involvement of Rel/nuclear factor-kappaB transcription factors in keratinocyte senescence. Cancer Res *64*, 472-481.

Bernard, D., Quatannens, B., Begue, A., Vandenbunder, B., and Abbadie, C. (2001a). Antiproliferative and antiapoptotic effects of crel may occur within the same cells via the up-

regulation of manganese superoxide dismutase. Cancer Res *61*, 2656-2664.

Bernard, D., Slomianny, C., Vandenbunder, B., and Abbadie, C. (2001b). cRel induces mitochondrial alterations in correlation with proliferation arrest. Free Radic Biol Med *31*, 943-953.

Blommaart, E. F., Krause, U., Schellens, J. P., Vreeling-Sindelarova, H., and Meijer, A. J. (1997). The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. Eur J Biochem *243*, 240-246.

Bowman, E. J., Siebers, A., and Altendorf, K. (1988). Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. Proc Natl Acad Sci U S A *85*, 7972-7976.

Boyce, S. T., and Ham, R. G. (1983). Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. J Invest Dermatol *81*, 33s-40s.

Cadet, J., Douki, T., and Ravanat, J. L. (2010). Oxidatively generated base damage to cellular DNA. Free Radic Biol Med *49*, 9-21.

Chaturvedi, V., Qin, J. Z., Denning, M. F., Choubey, D., Diaz, M. O., and Nickoloff, B. J. (1999). Apoptosis in proliferating, senescent, and immortalized keratinocytes. J Biol Chem *274*, 23358-23367.

Chen, J. H., Hales, C. N., and Ozanne, S. E. (2007). DNA damage, cellular senescence and organismal ageing: causal or correlative? Nucleic Acids Res *35*, 7417-7428.

Choi, J., Shendrik, I., Peacocke, M., Peehl, D., Buttyan, R., Ikeguchi, E. F., Katz, A. E., and Benson, M. C. (2000). Expression of senescence-associated beta-galactosidase in enlarged prostates from men with benign prostatic hyperplasia. Urology *56*, 160-166.

Collado, M., Gil, J., Efeyan, A., Guerra, C., Schuhmacher, A. J., Barradas, M., Benguria, A., Zaballos, A., Flores, J. M., Barbacid, M.*, et al.* (2005). Tumour biology: senescence in

premalignant tumours. Nature *436*, 642.

Coppe, J. P., Desprez, P. Y., Krtolica, A., and Campisi, J. (2010). The senescence-associated secretory phenotype: the dark side of tumor suppression. Annu Rev Pathol *5*, 99-118.

Cristofalo, V. J., and Kritchevsky, D. (1969). Cell size and nucleic acid content in the diploid human cell line WI-38 during aging. Med Exp Int J Exp Med *19*, 313-320.

d'Adda di Fagagna, F., Reaper, P. M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N. P., and Jackson, S. P. (2003). A DNA damage checkpoint response in telomere-initiated senescence. Nature *426*, 194-198.

Deruy, E., Gosselin, K., Vercamer, C., Martien, S., Bouali, F., Slomianny, C., Bertout, J., Bernard, D., Pourtier, A., and Abbadie, C. (2010). MnSOD upregulation induces autophagic programmed cell death in senescent keratinocytes. PLoS One *5*, e12712.

Di, X., Gennings, C., Bear, H. D., Graham, L. J., Sheth, C. M., White, K. L., Jr., and Gewirtz, D. A. Influence of the phosphodiesterase-5 inhibitor, sildenafil, on sensitivity to chemotherapy in breast tumor cells. Breast Cancer Res Treat *124*, 349-360.

Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O., and et al. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci U S A *92*, 9363-9367.

Ding, W. X., and Yin, X. M. (2008). Sorting, recognition and activation of the misfolded protein degradation pathways through macroautophagy and the proteasome. Autophagy *4*, 141-150.

Djordjevic, V. B. (2004). Free radicals in cell biology. Int Rev Cytol *237*, 57-89.

Eskelinen, E. L. (2008). New insights into the mechanisms of macroautophagy in mammalian cells. Int Rev Cell Mol Biol *266*, 207-247.

Fimia, G. M., Stoykova, A., Romagnoli, A., Giunta, L., Di Bartolomeo, S., Nardacci, R.,

Corazzari, M., Fuoco, C., Ucar, A., Schwartz, P.*, et al.* (2007). Ambra1 regulates autophagy and development of the nervous system. Nature *447*, 1121-1125.

Furuya, N., Yu, J., Byfield, M., Pattingre, S., and Levine, B. (2005). The evolutionarily conserved domain of Beclin 1 is required for Vps34 binding, autophagy and tumor suppressor function. Autophagy *1*, 46-52.

Gerland, L. M., Genestier, L., Peyrol, S., Michallet, M. C., Hayette, S., Urbanowicz, I., Ffrench, P., Magaud, J. P., and Ffrench, M. (2004). Autolysosomes accumulate during in vitro CD8+ T-lymphocyte aging and may participate in induced death sensitization of senescent cells. Exp Gerontol *39*, 789-800.

Gerland, L. M., Peyrol, S., Lallemand, C., Branche, R., Magaud, J. P., and Ffrench, M. (2003). Association of increased autophagic inclusions labeled for beta-galactosidase with fibroblastic aging. Exp Gerontol *38*, 887-895.

Glick, D., Barth, S., and Macleod, K. F. (2010). Autophagy: cellular and molecular mechanisms. J Pathol *221*, 3-12.

Going, J. J., Stuart, R. C., Downie, M., Fletcher-Monaghan, A. J., and Keith, W. N. (2002). 'Senescence-associated' beta-galactosidase activity in the upper gastrointestinal tract. J Pathol *196*, 394-400.

Gosselin, K., Deruy, E., Martien, S., Vercamer, C., Bouali, F., Dujardin, T., Slomianny, C., Houel-Renault, L., Chelli, F., De Launoit, Y., and Abbadie, C. (2009a). Senescent keratinocytes die by autophagic programmed cell death. Am J Pathol *174*, 423-435.

Gosselin, K., Martien, S., Pourtier, A., Vercamer, C., Ostoich, P., Morat, L., Sabatier, L., Duprez, L., T'Kint de Roodenbeke, C., Gilson, E.*, et al.* (2009b). Senescence-associated oxidative DNA damage promotes the generation of neoplastic cells. Cancer Res *69*, 7917-7925.

Greenberg, S. B., Grove, G. L., and Cristofalo, V. J. (1977). Cell size in aging monolayer

cultures. In Vitro *13*, 297-300.

Hayashi-Nishino, M., Fujita, N., Noda, T., Yamaguchi, A., Yoshimori, T., and Yamamoto, A. Electron tomography reveals the endoplasmic reticulum as a membrane source for autophagosome formation. Autophagy *6*.

Hayashi-Nishino, M., Fujita, N., Noda, T., Yamaguchi, A., Yoshimori, T., and Yamamoto, A. (2009). A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation. Nat Cell Biol *11*, 1433-1437.

Hayflick, L. (1965). The Limited in Vitro Lifetime of Human Diploid Cell Strains. Exp Cell Res *37*, 614-636.

Herbig, U., Ferreira, M., Condel, L., Carey, D., and Sedivy, J. M. (2006). Cellular senescence in aging primates. Science *311*, 1257.

Hippert, M. M., O'Toole, P. S., and Thorburn, A. (2006). Autophagy in cancer: good, bad, or both? Cancer Res *66*, 9349-9351.

Itakura, E., Kishi, C., Inoue, K., and Mizushima, N. (2008). Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. Mol Biol Cell *19*, 5360-5372.

Jeyapalan, J. C., Ferreira, M., Sedivy, J. M., and Herbig, U. (2007). Accumulation of senescent cells in mitotic tissue of aging primates. Mech Ageing Dev *128*, 36-44.

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., and Yoshimori, T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. Embo J *19*, 5720-5728.

Kabeya, Y., Mizushima, N., Yamamoto, A., Oshitani-Okamoto, S., Ohsumi, Y., and Yoshimori, T. (2004). LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. J Cell Sci *117*, 2805-2812.

Kang, M. R., Kim, M. S., Oh, J. E., Kim, Y. R., Song, S. Y., Kim, S. S., Ahn, C. H., Yoo, N. J.,

and Lee, S. H. (2009). Frameshift mutations of autophagy-related genes ATG2B, ATG5, ATG9B and ATG12 in gastric and colorectal cancers with microsatellite instability. J Pathol *217*, 702-706.

Karantza-Wadsworth, V., Patel, S., Kravchuk, O., Chen, G., Mathew, R., Jin, S., and White, E. (2007). Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. Genes Dev *21*, 1621-1635.

Klionsky, D. J., Cregg, J. M., Dunn, W. A., Jr., Emr, S. D., Sakai, Y., Sandoval, I. V., Sibirny, A., Subramani, S., Thumm, M., Veenhuis, M., and Ohsumi, Y. (2003). A unified nomenclature for yeast autophagy-related genes. Dev Cell *5*, 539-545.

Krishnamurthy, J., Torrice, C., Ramsey, M. R., Kovalev, G. I., Al-Regaiey, K., Su, L., and Sharpless, N. E. (2004). Ink4a/Arf expression is a biomarker of aging. J Clin Invest *114*, 1299-1307.

Kruk, P. A., Rampino, N. J., and Bohr, V. A. (1995). DNA damage and repair in telomeres: relation to aging. Proc Natl Acad Sci U S A *92*, 258-262.

Kuilman, T., and Peeper, D. S. (2009). Senescence-messaging secretome: SMS-ing cellular stress. Nat Rev Cancer *9*, 81-94.

Lee, A. C., Fenster, B. E., Ito, H., Takeda, K., Bae, N. S., Hirai, T., Yu, Z. X., Ferrans, V. J., Howard, B. H., and Finkel, T. (1999). Ras proteins induce senescence by altering the intracellular levels of reactive oxygen species. J Biol Chem *274*, 7936-7940.

Liang, C., Feng, P., Ku, B., Dotan, I., Canaani, D., Oh, B. H., and Jung, J. U. (2006). Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. Nat Cell Biol *8*, 688-699.

Liang, X. H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H., and Levine, B. (1999). Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature *402*, 672-676.

Lu, A. L., Li, X., Gu, Y., Wright, P. M., and Chang, D. Y. (2001). Repair of oxidative DNA damage: mechanisms and functions. Cell Biochem Biophys *35*, 141-170.

Melk, A., Schmidt, B. M., Takeuchi, O., Sawitzki, B., Rayner, D. C., and Halloran, P. F. (2004). Expression of p16INK4a and other cell cycle regulator and senescence associated genes in aging human kidney. Kidney Int *65*, 510-520.

Minamino, T., Miyauchi, H., Yoshida, T., Tateno, K., Kunieda, T., and Komuro, I. (2004). Vascular cell senescence and vascular aging. J Mol Cell Cardiol *36*, 175-183.

Mizushima, N., Kuma, A., Kobayashi, Y., Yamamoto, A., Matsubae, M., Takao, T., Natsume, T., Ohsumi, Y., and Yoshimori, T. (2003). Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate. J Cell Sci *116*, 1679-1688.

Mizushima, N., Levine, B., Cuervo, A. M., and Klionsky, D. J. (2008). Autophagy fights disease through cellular self-digestion. Nature *451*, 1069-1075.

Mizushima, N., Sugita, H., Yoshimori, T., and Ohsumi, Y. (1998). A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy. J Biol Chem *273*, 33889-33892.

Narita, M., Nunez, S., Heard, E., Lin, A. W., Hearn, S. A., Spector, D. L., Hannon, G. J., and Lowe, S. W. (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell *113*, 703-716.

Pearson, M., Carbone, R., Sebastiani, C., Cioce, M., Fagioli, M., Saito, S., Higashimoto, Y., Appella, E., Minucci, S., Pandolfi, P. P., and Pelicci, P. G. (2000). PML regulates p53 acetylation and premature senescence induced by oncogenic Ras. Nature *406*, 207-210.

Qu, X., Yu, J., Bhagat, G., Furuya, N., Hibshoosh, H., Troxel, A., Rosen, J., Eskelinen, E. L., Mizushima, N., Ohsumi, Y., *et al.* (2003). Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J Clin Invest *112*, 1809-1820.

Ressler, S., Bartkova, J., Niederegger, H., Bartek, J., Scharffetter-Kochanek, K., Jansen-Durr, P., and Wlaschek, M. (2006). p16INK4A is a robust in vivo biomarker of cellular aging in human skin. Aging Cell *5*, 379-389.

Romanov, S. R., Kozakiewicz, B. K., Holst, C. R., Stampfer, M. R., Haupt, L. M., and Tlsty, T. D. (2001). Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes. Nature *409*, 633-637.

Sasaki, M., Miyakoshi, M., Sato, Y., and Nakanuma, Y. (2010). Autophagy mediates the process of cellular senescence characterizing bile duct damages in primary biliary cirrhosis. Lab Invest *90*, 835-843.

Sedelnikova, O. A., Horikawa, I., Zimonjic, D. B., Popescu, N. C., Bonner, W. M., and Barrett, J. C. (2004). Senescing human cells and ageing mice accumulate DNA lesions with unrepairable double-strand breaks. Nat Cell Biol *6*, 168-170.

Seluanov, A., Gorbunova, V., Falcovitz, A., Sigal, A., Milyavsky, M., Zurer, I., Shohat, G., Goldfinger, N., and Rotter, V. (2001). Change of the death pathway in senescent human fibroblasts in response to DNA damage is caused by an inability to stabilize p53. Mol Cell Biol *21*, 1552-1564.

Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell *88*, 593-602.

Shelton, D. N., Chang, E., Whittier, P. S., Choi, D., and Funk, W. D. (1999). Microarray analysis of replicative senescence. Curr Biol *9*, 939-945.

Sohal, R. S., and Dubey, A. (1994). Mitochondrial oxidative damage, hydrogen peroxide release, and aging. Free Radic Biol Med *16*, 621-626.

Sou, Y. S., Tanida, I., Komatsu, M., Ueno, T., and Kominami, E. (2006). Phosphatidylserine in addition to phosphatidylethanolamine is an in vitro target of the mammalian Atg8 modifiers,

LC3, GABARAP, and GATE-16. J Biol Chem *281*, 3017-3024.

Spaulding, C., Guo, W., and Effros, R. B. (1999). Resistance to apoptosis in human CD8+ T cells that reach replicative senescence after multiple rounds of antigen-specific proliferation. Exp Gerontol *34*, 633-644.

Takahashi, Y., Coppola, D., Matsushita, N., Cualing, H. D., Sun, M., Sato, Y., Liang, C., Jung, J. U., Cheng, J. Q., Mule, J. J.*, et al.* (2007). Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. Nat Cell Biol *9*, 1142-1151.

Toussaint, O., Medrano, E. E., and von Zglinicki, T. (2000). Cellular and molecular mechanisms of stress-induced premature senescence (SIPS) of human diploid fibroblasts and melanocytes. Exp Gerontol *35*, 927-945.

Wang, E. (1995). Senescent human fibroblasts resist programmed cell death, and failure to suppress bcl2 is involved. Cancer Res *55*, 2284-2292.

Wang, E. (1997). Regulation of apoptosis resistance and ontogeny of age-dependent diseases. Exp Gerontol *32*, 471-484.

White, E., and DiPaola, R. S. (2009). The double-edged sword of autophagy modulation in cancer. Clin Cancer Res *15*, 5308-5316.

Yamaguchi, J., Sasaki, M., Harada, K., Zen, Y., Sato, Y., Ikeda, H., Itatsu, K., Yokoyama, Y., Ando, H., Ohta, T.*, et al.* (2009). Papillary hyperplasia of the gallbladder in pancreaticobiliary maljunction represents a senescence-related lesion induced by lysolecithin. Lab Invest *89*, 1018-1031.

Yamamoto, A., Tagawa, Y., Yoshimori, T., Moriyama, Y., Masaki, R., and Tashiro, Y. (1998). Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. Cell Struct Funct *23*, 33-42.

Young, A. R., Narita, M., Ferreira, M., Kirschner, K., Sadaie, M., Darot, J. F., Tavare, S.,

Arakawa, S., Shimizu, S., and Watt, F. M. (2009). Autophagy mediates the mitotic senescence transition. Genes Dev *23*, 798-803.

Yue, Z., Jin, S., Yang, C., Levine, A. J., and Heintz, N. (2003). Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc Natl Acad Sci U S A *100*, 15077-15082.

Zhan, H., Suzuki, T., Aizawa, K., Miyagawa, K., and Nagai, R. (2010). Ataxia telangiectasia mutated (ATM)-mediated DNA damage response in oxidative stress-induced vascular endothelial cell senescence. J Biol Chem *285*, 29662-29670.

Zhu, J., Woods, D., McMahon, M., and Bishop, J. M. (1998). Senescence of human fibroblasts induced by oncogenic Raf. Genes Dev *12*, 2997-3007.

Legends

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

(A) Growth curve of NHEKs showing the 3 phases of the culture: the exponential growth phase, the senescence plateau, and the post-senescence (PS) emergent phase. (B) Cells were taken at the exponential growth phase and at the beginning of PS emergence and analyzed by flow cytometry according to forward scatter factor, indicative of cell size. At the beginning of the PS emergence phase, the population was composed of both large cells, the senescent cells (s), and smaller ones, the PS emergent cells (pse). Senescent and PS emergent cells were then sorted by according to their forward scatter factor value. Proteins were extracted, separated in SDS-PAGE and expression profiles of Beclin-1, LC3 II, LAMP-1 and PCNA were analyzed by western blot. (C) LAMP-1 immunofluorescence assays were performed at the beginning of the PS emergence phase. Bar represents 20µm.

Supplemental figure 1: Identification of LC3II

Senescent and PS emergent NHEKs were sorted by FACS as in Fig. 1. Proteins from these cells and from young Normal Human Dermal Fibroblasts (NHDFs) were extracted, separated in SDS-PAGE and expression profiles of LC3 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 2: Lowering macroautophagy in H_2O_2-induced premature senescent NHEKs favors PS emergence

H_2O_2-induced premature senescent NHEKs were transfected with siRNAs targeting Atg5 or control siRNAs at different concentrations. (A) 72 hrs post-transfection, the efficiency of siRNAs was controlled by western blot with an anti-Atg5 antibody that 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 cells and pse a PS emergent cell. Bar represents 10µm (C) PS emerging clones were manually counted under microscopic examination and the emergence frequency was calculated by reporting the number of clones on the number of initially seeded cells. 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.

Figure 3: Lowering macroautophagy during normal and H_2O_2-induced senescence favors or limits PS emergence depending on the inhibitor concentration

(A) Cells were taken at the beginning of the senescence plateau and analyzed by flow cytometry according to forward scatter factor. As already described, the 15% of the cells with the highest forward scatter factor value corresponds to dying senescent cells (D). The 15% of the cells with just below values correspond to fully senescent but still alive cells (S) (Gosselin et al., 2009a). This last subpopulation was sorted, seeded at a low density and treated with 3-methyladenine (3MA) or its diluent H_2O, or with Bafilomycin A1 or its diluent DMSO. PS emerging clones were manually counted under microscopic examination after fixation and

purple violet coloration, and the emergence frequency was calculated by reporting the number of clones on the number of initially seeded cells. (B) NHEK at the growth phase were treated by 50 µM H_2O_2 during 48hrs, hence inducing premature senescence of the all population. The H_2O_2-induced senescent NHEKs were then seeded at low density, treated with autophagic inhibitors and the emergence frequency evaluated as above. The counts 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.

Figure 4: The probability of senescent cells to generate PS emergent clones is linked to their macroautophagic activity level

(A) NHEKs at the senescence plateau were stained with Lysotracker and analyzed by flow cytometry. Alive (S) and dying (D) senescent NHEKs were sorted firstly using forward scatter (upper panel) are secondly using the Lysotracker intensity (lower panels). (B) The resulting S1, S2, D1 and D2 subpopulations were seeded at a low density and monitored for emergence. PS emerging clones were manually counted under microscopic examination and the emergence frequency was calculated by reporting the number of clones on the number of initially seeded cells. The counts were performed in 4 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 of S1 on S2 and D1 on D2. P values were calculated using both student and Wilcoxon tests.

Supplemental figure 2: Autophagic activity and steady state levels of ROS in

exponentially growing versus senescent NHEKs

NHEKs from 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. (B) Lysotracker and (C) H2-DCFDA staining profiles comparison between exponentially growing NHEKs and R1 senescent NHEKs (values extracted using the WinMDI 2.9 software).

Figure 5: Levels of autophagic activity are directly correlated with levels of H_2O_2

NHEKs at the senescent plateau were stained with Lysotracker and H2-DCFDA and analyzed by flow cytometry. The four S1, S2, D1 and D2 subpopulations were determined according to their forward scatter factor. Their Lysotracker (rhodamine) and H2-DCFDA (GFP) staining intensities were then extracted using the WinMDI 2.9 software. The peak values of the H2-DCFDA staining intensity of each subpopulation are given.

Discussion générale

La sénescence des kératinocytes correspond à un état de stase et de mort cellulaire impliquant la macroautophagie

La sénescence des kératinocytes résulte de l'augmentation de l'activité des facteurs de transcription NFkappaB et en aval, de l'augmentation d'expression de la MnSOD, sans corégulation des autres enzymes antioxydantes, conduisant à la surproduction de H_2O_2. Au cours de cet état d'arrêt de croissance, les cellules présentent tous les critères morphologiques de sénescence classiquement décrits, augmentation de leur taille, de leur étalement sur le support, et d'un certain nombre de critères moléculaires comme une augmentation de l'activité SA.beta-galactosidase ainsi que de l'expression de la protéine $p21^{CIP1/WAF1}$. Mis à part ce dernier facteur que nous ne détectons qu'une fois la sénescence pleinement établie, nous n'avons jamais observé d'augmentation nette d'expression d'autres protéines susceptibles d'être à l'origine de l'arrêt du cycle cellulaire, notamment $p16^{INK4a}$. De plus, en l'absence de cassures double-brin et d'un raccourcissement des télomères, la voie DDR n'est pas non plus activée dans les kératinocytes sénescents (données non publiées du laboratoire). Une question reste donc encore en suspens : Quels sont les mécanismes, activés en réponse au stress oxydant, qui conduisent à l'arrêt du cycle cellulaire associé à la sénescence des kératinocytes ?

Les récents travaux de Young *et al* [398,399] montrent que l'autophagie joue un rôle central dans l'induction de la sénescence par l'oncogène *ras*. Dans cette étude, ils mettent en évidence que l'activation de la macroautophagie est nécessaire à l'établissement de la sénescence, en amont de l'activation de p16. Ces travaux ont été récemment confirmés *in vitro* et *in vivo* dans des cellules épithéliales de canaux biliaires issus de patients sains ou développant une cirrhose [400]. Les auteurs montrent notamment que les cellules issues de prélèvements de tissus malades co-expriment LC3B avec les marqueurs de sénescence $p16^{INK4a}$ et $p21^{Cip1/Waf1}$ et que l'inhibition de la macroautophagie repousse la survenue de la sénescence. Il apparaît donc dans ces études que l'activation de la macroautophagie pourrait être une nouvelle voie d'induction de l'arrêt dans le cycle cellulaire associé à la sénescence.

Les travaux que nous avons réalisés ont mis en évidence que la macroautophagie est également associée à la sénescence des kératinocytes et que de plus, elle induit la mort des cellules. Les différentes analyses par microscopie électronique que nous avons réalisées nous ont permis de voir que l'accumulation de vésicules macroautophagiques débute durant la phase de croissance exponentielle, bien avant l'induction de la sénescence. La macroautophagie pourrait donc avoir une importance fondamentale dans l'induction de l'arrêt dans le cycle cellulaire associé à la sénescence des kératinocytes, en plus d'être responsable de leur mort, mais ce point reste à démontrer.

Le stress oxydant est le déterminant clé du devenir des kératinocytes

Les travaux réalisés dans cette étude mettent en évidence un rôle central du stress oxydant dans le devenir des kératinocytes. La sénescence des kératinocytes est en effet essentiellement causée par la surexpression de la MnSOD en aval de la voie NFkappaB. L'augmentation d'expression de cette enzyme de détoxification du $O_2°^-$, sans augmentation d'expression des autres enzymes de réduction des ROS, conduit à la surproduction de peroxyde d'hydrogène responsable, *via* l'accumulation de dommages oxydants, de l'entrée des cellules en sénescence. L'endommagement des cellules est également à l'origine de l'accroissement de l'activité autophagique des kératinocytes à un niveau tel qu'elle entraînera, à terme, la mort cellulaire. Le stress oxydant est donc le principal inducteur de la sénescence et de la mort qui lui est associée.

De manière intéressante, Le stress oxydant est également la cause principale de l'émergence post-sénescence. En effet, la réduction du stress oxydant lors de la sénescence par traitement avec des agents antioxydants comme la catalase réduit fortement la probabilité qu'une cellule sénescente génère des cellules émergentes.

Le stress oxydant a donc un double effet sur le devenir des kératinocytes en induisant la sénescence et la mort, ou au contraire, en en favorisant l'échappement afin de promouvoir la survie et l'émergence néoplasique. Il est donc crucial de déterminer les paramètres différentiels qui influencent le devenir des kératinocytes sénescents vers l'une ou l'autre des finalités. De manière intéressante, nous avons mis en évidence que

l'autophagie est activée en réponse aux dommages oxydants qui altèrent la cellule. Il est donc possible qu'un trop fort endommagement cellulaire soit responsable d'une trop forte activation de la macroautophagie engendrant une dégradation massive des constituants cellulaires vitaux entrainant au final la mort cellulaire. Dans le cas de l'émergence post-sénescence, nous avons mis en évidence que les cellules progénitrices de cellules émergentes subissent un stress oxydant modéré en comparaison du reste des cellules sénescentes. Ce stress oxydant moindre pourrait conduire à un endommagement moindre, supportable par les différents systèmes de dégradation que sont le protéasome, la CMA et la macroautophagie. Cela créerait un contexte permissif dans lequel les ROS, *via* leurs propriétés mutagènes, pourraient induire la génération des cellules néoplasiques. Nous avons en effet mis en évidence la présence de microdélétions dans les kératinocytes émergents. Même si nous n'en avons pas la preuve directe, il est très possible que ces altérations du génome soient la conséquence des dommages oxydants affectant le noyau que nous avons mis en évidence dans les kératinocytes sénescents, notamment les cassures simple-brin et/ou les ponts AIP.

Le devenir des kératinocytes apparaît donc comme la résultante d'un ratio entre dommages oxydants délétères entrainant la mort des cellules et dommages oxydants mutagènes favorisant la transformation des cellules.

La sénescence, un mécanisme à la fois suppresseur et promoteur de tumeur

Malgré l'arrêt dans le cycle cellulaire, l'accumulation de dommages oxydants et l'induction de la mort par autophagie, certains kératinocytes sénescents sont capables de générer des cellules néoplasiques. La sénescence des kératinocytes a donc deux finalités antagonistes : soit elle conduit à la mort cellulaire, soit elle génère des cellules néoplasiques.

Dans le premier cas, la sénescence des kératinocytes a bien un effet suppresseur de tumeur. Le stress oxydant inducteur de la sénescence et de la mort par macroautophagie résulte de l'activité des membres de la famille des facteurs NFkappaB,

facteurs de transcription impliqués dans de nombreuses signalisations et généralement considérés comme des oncogènes (revue par [401]). Dans cet esprit, on peut alors assimiler la sénescence des kératinocytes à une OIS. Comme pour la sénescence induite par *ras*, la sénescence des kératinocytes passe donc par la production de ROS et potentiellement aussi par un stress réplicatif. En effet, nous avons montré qu'un des dommages oxydants majeurs des cellules sénescentes sont des ponts AIP détectés dans les noyaux. Ces ponts AIP affectant la chromatine pourraient conduire à un blocage des fourches de réplication et induire un stress réplicatif responsable de l'entrée en sénescence des cellules. Les ponts AIP pourraient également bloquer complètement la réplication et la transcription et conduire à la mort des cellules.

Dans le second cas, en générant des cellules néoplasiques, la sénescence a un effet promoteur de tumeur. En effet, non seulement l'existence d'une émergence montre que la sénescence n'est pas un processus suffisant pour bloquer la transformation, mais le fait que les cellules émergentes soient générées par des cellules sénescentes montre que la sénescence pourrait être préparatoire à l'émergence néoplasique. Le phénomène d'émergence post-sénescence n'est pas spécifique aux kératinocytes et a également été décrit pour les cellules épithéliales mammaires [245]. Dans ce type cellulaire aussi, la sénescence et l'émergence qui s'en suit peuvent être repoussées par traitement des cellules avec la catalase (données non publiées du laboratoire), suggèrant que, dans les cellules épithéliales, le stress oxydant puisse être au centre des mécanismes de sénescence et d'émergence, probablement *via* les dommages cellulaires et mutagènes générés par les ROS. Il est intéressant de constater que ce processus d'émergence post-sénescence n'a jamais été observé pour les fibroblastes sénescents humains [245,402], ce qui suggère que la sénescence des cellules épithéliales serait différente de la sénescence des fibroblastes, type cellulaire le plus utilisé jusqu'ici dans les études du rôle suppresseur de tumeur de la sénescence.

L'effet promoteur de tumeur de la sénescence peut également mettre en jeu un mécanisme paracrine des fibroblastes sénescents sur les cellules épithéliales *via* un sécrétome particulier, proche de celui des CAFs [240-244]. Ce mécanisme fait l'objet d'un axe de recherche développé par un autre doctorant de l'équipe. Il montre notamment

que le sécrétome des fibroblastes sénescents de derme est proche de celui de CAFs et qu'il accroit l'état de transformation des kératinocytes émergents post-sénescence. Les fibroblastes sénescents ont donc des propriétés tumorigènes indirectes en favorisant le développement cancéreux de leur partenaire épithélial. De manière intéressante, nous avons montré que les hyperplasies et carcinomes cutanés induits par les kératinocytes émergents n'apparaissent dans les souris qu'après un long délai post-injection, c'est-à-dire dans des souris alors âgées d'environ 9 mois. Ce laps de temps avant le développement tumoral pourrait être le reflet qu'un environnement vieillissant est plus favorable à la croissance tumorale qu'un environnement jeune.

Toutes ces données réunies mettent en évidence que la sénescence a des effets suppresseur et promoteur de tumeur reposant sur des mécanismes intrinsèques aux cellules épithéliales dans lesquelles le stress oxydant joue un rôle majeur, et/ou sur des mécanismes paracrines liés au vieillissement du microenvironnement des cellules épithéliales. La mise en lumière de ces mécanismes permet alors de mieux comprendre les données épidémiologiques qui montrent que l'incidence des cancers épithéliaux, et non des sarcomes, augmente avec l'âge.

Concernant la macroautophagie, il est intéressant de constater que l'expression d'un certain nombre d'Atg est souvent réduite dans les cancers. Une perte d'hétérozygotie du chromosome 17 (portant *atg6/beclin1*) est par exemple souvent observée dans les carcinomes de l'ovaire [403-406], du sein [407,408] ou de la prostate [409]. Il en est de même pour d'autres *atg* impliqués dans l'activation de l'autophagie comme *uvrag* ou *bif1*, souvent décrits mutés ou délétés dans les cancers [410,411], attestant d'un rôle suppresseur de tumeur de la macroautophagie (Tableau 1) [299]. Cependant, nos travaux associés à ceux d'autres équipes modulent cette conclusion. En effet, comme décrit précédemment, l'accumulation de dommages oxydants associés à la sénescence active une autophagie massive qui entraînera la mort des NHEKs sénescents. Nous montrons ici que les cellules qui émergent du plateau de sénescence échappent à la mort par autophagie, mais nous montrons aussi qu'une inhibition très forte de l'activité macroautophagique à la sénescence réduit la probabilité qu'une cellule génère des cellules émergentes. Ces résultats peuvent être mis en parallèle de ceux obtenus par

Yue *et al* dans lesquels ils mettent en évidence que la réduction de l'activité macroautophagique dans des souris hétérozygotes pour Atg6/Beclin1 induit spontanément la formation de tumeurs, mais que les *knock-out* homozygotes sont létaux [389]. Ils définissent alors Atg6/Beclin1 comme un gène suppresseur de tumeur haploinsuffisant et suggèrent qu'une activation limitée de la macroautophagie est insuffisante pour protéger les cellules de la transformation [389]. Associés aux nôtres, ces résultats suggèrent que l'expression monoallélique de Atg6/Beclin1 pourrait non seulement être inefficace pour empêcher la tumorigenèse, mais de surcroit être bénéfique à la transformation des cellules. Il est d'ailleurs intéressant de constater qu'une perte biallélique de Atg6/Beclin1 dans les tumeurs n'a jamais été décrite. Par conséquent, l'autophagie pourrait être un processus promoteur ou suppresseur de tumeur dépendamment de son niveau d'activation.

Dans un contexte clinique, les cancers sont traités par des chimiothérapies visant, pour certaines, à induire la mort cellulaire *via* la production de ROS [412]. Il a souvent été rapporté que la sénescence pouvait être activée au sein d'une tumeur après traitement [413]. Jusqu'ici, on estimait que ces cellules seraient éliminées par l'organisme ou à défaut n'évolueraient plus puisque considérées comme bloquées irréversiblement en phase G1 du cycle cellulaire. Or, nos travaux [402] associés à ceux de l'équipe de Tlsty [245] ont mis en évidence que la sénescence des kératinocytes comme celle des cellules épithéliales mammaires ne constitue pas un blocage suffisant pour empêcher l'évolution néoplasique des cellules, voire même la favorise. Activée en réponse à la production de ROS induite par les traitements, la sénescence deviendrait un moyen pour quelques cellules de résister à la chimiothérapie, d'autant que les cellules sénescentes sont généralement résistantes à l'apoptose [16-20]. Des cellules pourraient ensuite réémerger à partir des cellules cancéreuses sénescentes, ce qui entraînerait la récidive du cancer. Une macroautophagie modérée leur permettrait alors de contrôler leurs niveaux de dommages oxydants. Ainsi, ces processus associés que sont la sénescence et la macroautophagie pourraient être deux acteurs majeurs de la dormance tumorale et de la récidive de la maladie.

Références bibliographiques

1. Hayflick L: The Limited in Vitro Lifetime of Human Diploid Cell Strains. Exp Cell Res 1965, 37:614-636
2. Hayflick L, Moorhead PS: The serial cultivation of human diploid cell strains. Exp Cell Res 1961, 25:585-621
3. Rheinwald JG, Green H: Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 1975, 6:331-343
4. Bierman EL: The effect of donor age on the in vitro life span of cultured human arterial smooth-muscle cells. In Vitro 1978, 14:951-955
5. Mueller SN, Rosen EM, Levine EM: Cellular senescence in a cloned strain of bovine fetal aortic endothelial cells. Science 1980, 207:889-891
6. Tice RR, Schneider EL, Kram D, Thorne P: Cytokinetic analysis of the impaired proliferative response of peripheral lymphocytes from aged humans to phytohemagglutinin. J Exp Med 1979, 149:1029-1041
7. Cristofalo VJ, Kritchevsky D: Cell size and nucleic acid content in the diploid human cell line WI-38 during aging. Med Exp Int J Exp Med 1969, 19:313-320
8. Greenberg SB, Grove GL, Cristofalo VJ: Cell size in aging monolayer cultures. In Vitro 1977, 13:297-300
9. Narita M, Nunez S, Heard E, Lin AW, Hearn SA, Spector DL, Hannon GJ, Lowe SW: Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell 2003, 113:703-716
10. Galloway SM, Buckton KE: Aneuploidy and ageing: chromosome studies on a random sample of the population using G-banding. Cytogenet Cell Genet 1978, 20:78-95
11. Matsumura T: Multinucleation and polyploidization of aging human cells in culture. Adv Exp Med Biol 1980, 129:31-38
12. Cristofalo VJ, Kabakjian J: Lysosomal enzymes and aging in vitro: subcellular enzyme distribution and effect of hydrocortisone on cell life-span. Mech Ageing Dev 1975, 4:19-28
13. Maciera-Coelho A, Garcia-Giralt E, Adrian M: Changes in lysosomal associated structures in human fibroblasts kept in resting phase. Proc Soc Exp Biol Med 1971, 138:712-718
14. Shelton DN, Chang E, Whittier PS, Choi D, Funk WD: Microarray analysis of replicative senescence. Curr Biol 1999, 9:939-945
15. Benvenuti S, Cramer R, Quinn CC, Bruce J, Zvelebil M, Corless S, Bond J, Yang A, Hockfield S, Burlingame AL, Waterfield MD, Jat PS: Differential proteome analysis of replicative senescence in rat embryo fibroblasts. Mol Cell Proteomics 2002, 1:280-292
16. Wang E: Senescent human fibroblasts resist programmed cell death, and failure to suppress bcl2 is involved. Cancer Res 1995, 55:2284-2292
17. Wang E: Regulation of apoptosis resistance and ontogeny of age-dependent diseases. Exp Gerontol 1997, 32:471-484

18. Chaturvedi V, Qin JZ, Denning MF, Choubey D, Diaz MO, Nickoloff BJ: Apoptosis in proliferating, senescent, and immortalized keratinocytes. J Biol Chem 1999, 274:23358-23367
19. Spaulding C, Guo W, Effros RB: Resistance to apoptosis in human CD8+ T cells that reach replicative senescence after multiple rounds of antigen-specific proliferation. Exp Gerontol 1999, 34:633-644
20. Seluanov A, Gorbunova V, Falcovitz A, Sigal A, Milyavsky M, Zurer I, Shohat G, Goldfinger N, Rotter V: Change of the death pathway in senescent human fibroblasts in response to DNA damage is caused by an inability to stabilize p53. Mol Cell Biol 2001, 21:1552-1564
21. Terman A, Gustafsson B, Brunk UT: The lysosomal-mitochondrial axis theory of postmitotic aging and cell death. Chem Biol Interact 2006, 163:29-37
22. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, et al.: A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci U S A 1995, 92:9363-9367
23. Lee BY, Han JA, Im JS, Morrone A, Johung K, Goodwin EC, Kleijer WJ, DiMaio D, Hwang ES: Senescence-associated beta-galactosidase is lysosomal beta-galactosidase. Aging Cell 2006, 5:187-195
24. Dirac AM, Bernards R: Reversal of senescence in mouse fibroblasts through lentiviral suppression of p53. J Biol Chem 2003, 278:11731-11734
25. Dannenberg JH, van Rossum A, Schuijff L, te Riele H: Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. Genes Dev 2000, 14:3051-3064
26. Sage J, Mulligan GJ, Attardi LD, Miller A, Chen S, Williams B, Theodorou E, Jacks T: Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. Genes Dev 2000, 14:3037-3050
27. Sage J, Miller AL, Perez-Mancera PA, Wysocki JM, Jacks T: Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. Nature 2003, 424:223-228
28. Bischof O, Dejean A, Pineau P: [A review of cellular senescence: friend or foe of tumorigenesis?]. Med Sci (Paris) 2009, 25:153-160
29. Kulju KS, Lehman JM: Increased p53 protein associated with aging in human diploid fibroblasts. Exp Cell Res 1995, 217:336-345
30. Brown JP, Wei W, Sedivy JM: Bypass of senescence after disruption of p21CIP1/WAF1 gene in normal diploid human fibroblasts. Science 1997, 277:831-834
31. Wei W, Herbig U, Wei S, Dutriaux A, Sedivy JM: Loss of retinoblastoma but not p16 function allows bypass of replicative senescence in human fibroblasts. EMBO Rep 2003, 4:1061-1066
32. Pantoja C, Serrano M: Murine fibroblasts lacking p21 undergo senescence and are resistant to transformation by oncogenic Ras. Oncogene 1999, 18:4974-4982
33. Dulic V, Beney GE, Frebourg G, Drullinger LF, Stein GH: Uncoupling between phenotypic senescence and cell cycle arrest in aging p21-deficient fibroblasts. Mol Cell Biol 2000, 20:6741-6754

34. Shay JW, Pereira-Smith OM, Wright WE: A role for both RB and p53 in the regulation of human cellular senescence. Exp Cell Res 1991, 196:33-39
35. Smogorzewska A, de Lange T: Different telomere damage signaling pathways in human and mouse cells. Embo J 2002, 21:4338-4348
36. Beausejour CM, Krtolica A, Galimi F, Narita M, Lowe SW, Yaswen P, Campisi J: Reversal of human cellular senescence: roles of the p53 and p16 pathways. Embo J 2003, 22:4212-4222
37. Lowe SW, Sherr CJ: Tumor suppression by Ink4a-Arf: progress and puzzles. Curr Opin Genet Dev 2003, 13:77-83
38. Alcorta DA, Xiong Y, Phelps D, Hannon G, Beach D, Barrett JC: Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. Proc Natl Acad Sci U S A 1996, 93:13742-13747
39. Palmero I, McConnell B, Parry D, Brookes S, Hara E, Bates S, Jat P, Peters G: Accumulation of p16INK4a in mouse fibroblasts as a function of replicative senescence and not of retinoblastoma gene status. Oncogene 1997, 15:495-503
40. Sherr CJ: The INK4a/ARF network in tumour suppression. Nat Rev Mol Cell Biol 2001, 2:731-737
41. Brookes S, Rowe J, Gutierrez Del Arroyo A, Bond J, Peters G: Contribution of p16(INK4a) to replicative senescence of human fibroblasts. Exp Cell Res 2004, 298:549-559
42. Foster SA, Wong DJ, Barrett MT, Galloway DA: Inactivation of p16 in human mammary epithelial cells by CpG island methylation. Mol Cell Biol 1998, 18:1793-1801
43. Kiyono T, Foster SA, Koop JI, McDougall JK, Galloway DA, Klingelhutz AJ: Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. Nature 1998, 396:84-88
44. Rheinwald JG, Hahn WC, Ramsey MR, Wu JY, Guo Z, Tsao H, De Luca M, Catricala C, O'Toole KM: A two-stage, p16(INK4A)- and p53-dependent keratinocyte senescence mechanism that limits replicative potential independent of telomere status. Mol Cell Biol 2002, 22:5157-5172
45. Sharpless NE, Bardeesy N, Lee KH, Carrasco D, Castrillon DH, Aguirre AJ, Wu EA, Horner JW, DePinho RA: Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. Nature 2001, 413:86-91
46. Brenner AJ, Stampfer MR, Aldaz CM: Increased p16 expression with first senescence arrest in human mammary epithelial cells and extended growth capacity with p16 inactivation. Oncogene 1998, 17:199-205
47. Dickson MA, Hahn WC, Ino Y, Ronfard V, Wu JY, Weinberg RA, Louis DN, Li FP, Rheinwald JG: Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. Mol Cell Biol 2000, 20:1436-1447
48. Jarrard DF, Sarkar S, Shi Y, Yeager TR, Magrane G, Kinoshita H, Nassif N, Meisner L, Newton MA, Waldman FM, Reznikoff CA: p16/pRb pathway alterations are required for bypassing senescence in human prostate epithelial cells. Cancer Res 1999, 59:2957-2964

49. Puthenveettil JA, Burger MS, Reznikoff CA: Replicative senescence in human uroepithelial cells. Adv Exp Med Biol 1999, 462:83-91
50. Sandhu C, Peehl DM, Slingerland J: p16INK4A mediates cyclin dependent kinase 4 and 6 inhibition in senescent prostatic epithelial cells. Cancer Res 2000, 60:2616-2622
51. Ben-Porath I, Weinberg RA: The signals and pathways activating cellular senescence. Int J Biochem Cell Biol 2005, 37:961-976
52. Blackburn EH: Switching and signaling at the telomere. Cell 2001, 106:661-673
53. Callen E, Surralles J: Telomere dysfunction in genome instability syndromes. Mutat Res 2004, 567:85-104
54. Moyzis RK, Buckingham JM, Cram LS, Dani M, Deaven LL, Jones MD, Meyne J, Ratliff RL, Wu JR: A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. Proc Natl Acad Sci U S A 1988, 85:6622-6626
55. de Lange T, Shiue L, Myers RM, Cox DR, Naylor SL, Killery AM, Varmus HE: Structure and variability of human chromosome ends. Mol Cell Biol 1990, 10:518-527
56. Henderson ER, Blackburn EH: An overhanging 3' terminus is a conserved feature of telomeres. Mol Cell Biol 1989, 9:345-348
57. Griffith JD, Comeau L, Rosenfield S, Stansel RM, Bianchi A, Moss H, de Lange T: Mammalian telomeres end in a large duplex loop. Cell 1999, 97:503-514
58. van Steensel B, Smogorzewska A, de Lange T: TRF2 protects human telomeres from end-to-end fusions. Cell 1998, 92:401-413
59. Cesare AJ, Reddel RR: Alternative lengthening of telomeres: models, mechanisms and implications. Nat Rev Genet 2010, 11:319-330
60. Gilson E, Geli V: How telomeres are replicated. Nat Rev Mol Cell Biol 2007, 8:825-838
61. Ye JZ, Donigian JR, van Overbeek M, Loayza D, Luo Y, Krutchinsky AN, Chait BT, de Lange T: TIN2 binds TRF1 and TRF2 simultaneously and stabilizes the TRF2 complex on telomeres. J Biol Chem 2004, 279:47264-47271
62. Liu D, O'Connor MS, Qin J, Songyang Z: Telosome, a mammalian telomere-associated complex formed by multiple telomeric proteins. J Biol Chem 2004, 279:51338-51342
63. Zhong Z, Shiue L, Kaplan S, de Lange T: A mammalian factor that binds telomeric TTAGGG repeats in vitro. Mol Cell Biol 1992, 12:4834-4843
64. Chong L, van Steensel B, Broccoli D, Erdjument-Bromage H, Hanish J, Tempst P, de Lange T: A human telomeric protein. Science 1995, 270:1663-1667
65. Bilaud T, Brun C, Ancelin K, Koering CE, Laroche T, Gilson E: Telomeric localization of TRF2, a novel human telobox protein. Nat Genet 1997, 17:236-239
66. Broccoli D, Smogorzewska A, Chong L, de Lange T: Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2. Nat Genet 1997, 17:231-235
67. Li B, Oestreich S, de Lange T: Identification of human Rap1: implications for telomere evolution. Cell 2000, 101:471-483
68. Kim SH, Kaminker P, Campisi J: TIN2, a new regulator of telomere length in human cells. Nat Genet 1999, 23:405-412

69. Houghtaling BR, Cuttonaro L, Chang W, Smith S: A dynamic molecular link between the telomere length regulator TRF1 and the chromosome end protector TRF2. Curr Biol 2004, 14:1621-1631
70. Baumann P, Cech TR: Pot1, the putative telomere end-binding protein in fission yeast and humans. Science 2001, 292:1171-1175
71. Blackburn EH: Telomere states and cell fates. Nature 2000, 408:53-56
72. Giraud-Panis MJ, Pisano S, Poulet A, Le Du MH, Gilson E: Structural identity of telomeric complexes. FEBS Lett 2010, 584:3785-3799
73. Cristofari G, Sikora K, Lingner J: Telomerase unplugged. ACS Chem Biol 2007, 2:155-158
74. Palm W, de Lange T: How shelterin protects mammalian telomeres. Annu Rev Genet 2008, 42:301-334
75. Georgin-Lavialle S, Aouba A, Lepelletier Y, Gabet AS, Hermine O: [Telomeres and telomerase: relevance and future prospects in systemic lupus erythematosus]. Rev Med Interne, 31:345-352
76. de Lange T: Shelterin: the protein complex that shapes and safeguards human telomeres. Genes Dev 2005, 19:2100-2110
77. Greider CW, Blackburn EH: Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell 1985, 43:405-413
78. Morin GB: The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell 1989, 59:521-529
79. Nakamura TM, Cech TR: Reversing time: origin of telomerase. Cell 1998, 92:587-590
80. Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu CP, Adams RR, Chang E, Allsopp RC, Yu J, et al.: The RNA component of human telomerase. Science 1995, 269:1236-1241
81. Okazaki R, Okazaki T, Sakabe K, Sugimoto K, Sugino A: Mechanism of DNA chain growth. I. Possible discontinuity and unusual secondary structure of newly synthesized chains. Proc Natl Acad Sci U S A 1968, 59:598-605
82. Harley CB, Futcher AB, Greider CW: Telomeres shorten during ageing of human fibroblasts. Nature 1990, 345:458-460
83. Shay JW, Wright WE: Hayflick, his limit, and cellular ageing. Nat Rev Mol Cell Biol 2000, 1:72-76
84. d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, Fiegler H, Carr P, Von Zglinicki T, Saretzki G, Carter NP, Jackson SP: A DNA damage checkpoint response in telomere-initiated senescence. Nature 2003, 426:194-198
85. Allsopp RC, Vaziri H, Patterson C, Goldstein S, Younglai EV, Futcher AB, Greider CW, Harley CB: Telomere length predicts replicative capacity of human fibroblasts. Proc Natl Acad Sci U S A 1992, 89:10114-10118
86. Harley CB: Telomere loss: mitotic clock or genetic time bomb? Mutat Res 1991, 256:271-282
87. Liu L, Bailey SM, Okuka M, Munoz P, Li C, Zhou L, Wu C, Czerwiec E, Sandler L, Seyfang A, Blasco MA, Keefe DL: Telomere lengthening early in development. Nat Cell Biol 2007, 9:1436-1441

88. Rufer N, Migliaccio M, Antonchuk J, Humphries RK, Roosnek E, Lansdorp PM: Transfer of the human telomerase reverse transcriptase (TERT) gene into T lymphocytes results in extension of replicative potential. Blood 2001, 98:597-603
89. Yang J, Chang E, Cherry AM, Bangs CD, Oei Y, Bodnar A, Bronstein A, Chiu CP, Herron GS: Human endothelial cell life extension by telomerase expression. J Biol Chem 1999, 274:26141-26148
90. Yudoh K, Matsuno H, Nakazawa F, Katayama R, Kimura T: Reconstituting telomerase activity using the telomerase catalytic subunit prevents the telomere shorting and replicative senescence in human osteoblasts. J Bone Miner Res 2001, 16:1453-1464
91. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE: Extension of life-span by introduction of telomerase into normal human cells. Science 1998, 279:349-352
92. Vaziri H, Benchimol S: Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. Curr Biol 1998, 8:279-282
93. Toouli CD, Huschtscha LI, Neumann AA, Noble JR, Colgin LM, Hukku B, Reddel RR: Comparison of human mammary epithelial cells immortalized by simian virus 40 T-Antigen or by the telomerase catalytic subunit. Oncogene 2002, 21:128-139
94. Harada H, Nakagawa H, Oyama K, Takaoka M, Andl CD, Jacobmeier B, von Werder A, Enders GH, Opitz OG, Rustgi AK: Telomerase induces immortalization of human esophageal keratinocytes without p16INK4a inactivation. Mol Cancer Res 2003, 1:729-738
95. Milyavsky M, Shats I, Erez N, Tang X, Senderovich S, Meerson A, Tabach Y, Goldfinger N, Ginsberg D, Harris CC, Rotter V: Prolonged culture of telomerase-immortalized human fibroblasts leads to a premalignant phenotype. Cancer Res 2003, 63:7147-7157
96. Pellegrini G, Dellambra E, Paterna P, Golisano O, Traverso CE, Rama P, Lacal P, De Luca M: Telomerase activity is sufficient to bypass replicative senescence in human limbal and conjunctival but not corneal keratinocytes. Eur J Cell Biol 2004, 83:691-700
97. d'Adda di Fagagna F: Living on a break: cellular senescence as a DNA-damage response. Nat Rev Cancer 2008, 8:512-522
98. Denchi EL, de Lange T: Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1. Nature 2007, 448:1068-1071
99. Klaunig JE, Kamendulis LM: The role of oxidative stress in carcinogenesis. Annu Rev Pharmacol Toxicol 2004, 44:239-267
100. Nordberg J, Arner ES: Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. Free Radic Biol Med 2001, 31:1287-1312
101. Djordjevic VB: Free radicals in cell biology. Int Rev Cytol 2004, 237:57-89
102. Lenaz G, Genova ML: Structure and organization of mitochondrial respiratory complexes: a new understanding of an old subject. Antioxid Redox Signal 2010, 12:961-1008
103. Kushnareva Y, Murphy AN, Andreyev A: Complex I-mediated reactive oxygen species generation: modulation by cytochrome c and NAD(P)+ oxidation-reduction state. Biochem J 2002, 368:545-553

104. Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ: Production of reactive oxygen species by mitochondria: central role of complex III. J Biol Chem 2003, 278:36027-36031
105. Bienert GP, Schjoerring JK, Jahn TP: Membrane transport of hydrogen peroxide. Biochim Biophys Acta 2006, 1758:994-1003
106. Thannickal VJ, Fanburg BL: Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol 2000, 279:L1005-1028
107. Bienert GP, Moller AL, Kristiansen KA, Schulz A, Moller IM, Schjoerring JK, Jahn TP: Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. J Biol Chem 2007, 282:1183-1192
108. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J: Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007, 39:44-84
109. Klaunig JE, Kamendulis LM, Hocevar BA: Oxidative stress and oxidative damage in carcinogenesis. Toxicol Pathol 2010, 38:96-109
110. Terman A, Kurz T, Navratil M, Arriaga EA, Brunk UT: Mitochondrial turnover and aging of long-lived postmitotic cells: the mitochondrial-lysosomal axis theory of aging. Antioxid Redox Signal 2010, 12:503-535
111. Schrader M, Fahimi HD: Peroxisomes and oxidative stress. Biochim Biophys Acta 2006, 1763:1755-1766
112. Griendling KK, Sorescu D, Ushio-Fukai M: NAD(P)H oxidase: role in cardiovascular biology and disease. Circ Res 2000, 86:494-501
113. Babior BM: NADPH oxidase: an update. Blood 1999, 93:1464-1476
114. Han D, Ybanez MD, Ahmadi S, Yeh K, Kaplowitz N: Redox regulation of tumor necrosis factor signaling. Antioxid Redox Signal 2009, 11:2245-2263
115. Balaban RS, Nemoto S, Finkel T: Mitochondria, oxidants, and aging. Cell 2005, 120:483-495
116. Fridovich I: Oxygen toxicity: a radical explanation. J Exp Biol 1998, 201:1203-1209
117. Mary J, Vougier S, Picot CR, Perichon M, Petropoulos I, Friguet B: Enzymatic reactions involved in the repair of oxidized proteins. Exp Gerontol 2004, 39:1117-1123
118. Cabreiro F, Picot CR, Friguet B, Petropoulos I: Methionine sulfoxide reductases: relevance to aging and protection against oxidative stress. Ann N Y Acad Sci 2006, 1067:37-44
119. Dunlop RA, Brunk UT, Rodgers KJ: Oxidized proteins: mechanisms of removal and consequences of accumulation. IUBMB Life 2009, 61:522-527
120. Holmgren A: Antioxidant function of thioredoxin and glutaredoxin systems. Antioxid Redox Signal 2000, 2:811-820
121. Kelly KA, Havrilla CM, Brady TC, Abramo KH, Levin ED: Oxidative stress in toxicology: established mammalian and emerging piscine model systems. Environ Health Perspect 1998, 106:375-384
122. Grimsrud PA, Xie H, Griffin TJ, Bernlohr DA: Oxidative stress and covalent modification of protein with bioactive aldehydes. J Biol Chem 2008, 283:21837-21841
123. Sayre LM, Smith MA, Perry G: Chemistry and biochemistry of oxidative stress in neurodegenerative disease. Curr Med Chem 2001, 8:721-738

124. Herrera E, Barbas C: Vitamin E: action, metabolism and perspectives. J Physiol Biochem 2001, 57:43-56
125. Traber MG, Atkinson J: Vitamin E, antioxidant and nothing more. Free Radic Biol Med 2007, 43:4-15
126. Esterbauer H, Schaur RJ, Zollner H: Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 1991, 11:81-128
127. Marnett LJ: Lipid peroxidation-DNA damage by malondialdehyde. Mutat Res 1999, 424:83-95
128. Schutt F, Bergmann M, Holz FG, Kopitz J: Proteins modified by malondialdehyde, 4-hydroxynonenal, or advanced glycation end products in lipofuscin of human retinal pigment epithelium. Invest Ophthalmol Vis Sci 2003, 44:3663-3668
129. Terman A, Brunk UT: Lipofuscin. Int J Biochem Cell Biol 2004, 36:1400-1404
130. Yin D: Biochemical basis of lipofuscin, ceroid, and age pigment-like fluorophores. Free Radic Biol Med 1996, 21:871-888
131. Lu AL, Li X, Gu Y, Wright PM, Chang DY: Repair of oxidative DNA damage: mechanisms and functions. Cell Biochem Biophys 2001, 35:141-170
132. Fraga CG, Shigenaga MK, Park JW, Degan P, Ames BN: Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. Proc Natl Acad Sci U S A 1990, 87:4533-4537
133. Cadet J, Douki T, Ravanat JL: Oxidatively generated base damage to cellular DNA. Free Radic Biol Med 2010, 49:9-21
134. Marnett LJ: Oxyradicals and DNA damage. Carcinogenesis 2000, 21:361-370
135. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M: Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 2006, 160:1-40
136. Shibutani S, Takeshita M, Grollman AP: Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. Nature 1991, 349:431-434
137. Cheng KC, Cahill DS, Kasai H, Nishimura S, Loeb LA: 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G----T and A----C substitutions. J Biol Chem 1992, 267:166-172
138. Prowse KR, Greider CW: Developmental and tissue-specific regulation of mouse telomerase and telomere length. Proc Natl Acad Sci U S A 1995, 92:4818-4822
139. Todaro GJ, Green H: Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J Cell Biol 1963, 17:299-313
140. Parrinello S, Samper E, Krtolica A, Goldstein J, Melov S, Campisi J: Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. Nat Cell Biol 2003, 5:741-747
141. Atamna H, Paler-Martinez A, Ames BN: N-t-butyl hydroxylamine, a hydrolysis product of alpha-phenyl-N-t-butyl nitrone, is more potent in delaying senescence in human lung fibroblasts. J Biol Chem 2000, 275:6741-6748
142. Bernard D, Gosselin K, Monte D, Vercamer C, Bouali F, Pourtier A, Vandenbunder B, Abbadie C: Involvement of Rel/nuclear factor-kappaB transcription factors in keratinocyte senescence. Cancer Res 2004, 64:472-481

143. von Zglinicki T, Saretzki G, Docke W, Lotze C: Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts: a model for senescence? Exp Cell Res 1995, 220:186-193
144. Chen Q, Ames BN: Senescence-like growth arrest induced by hydrogen peroxide in human diploid fibroblast F65 cells. Proc Natl Acad Sci U S A 1994, 91:4130-4134
145. Chen QM, Bartholomew JC, Campisi J, Acosta M, Reagan JD, Ames BN: Molecular analysis of H2O2-induced senescent-like growth arrest in normal human fibroblasts: p53 and Rb control G1 arrest but not cell replication. Biochem J 1998, 332 (Pt 1):43-50
146. Chen QM, Liu J, Merrett JB: Apoptosis or senescence-like growth arrest: influence of cell-cycle position, p53, p21 and bax in H2O2 response of normal human fibroblasts. Biochem J 2000, 347:543-551
147. Frippiat C, Dewelle J, Remacle J, Toussaint O: Signal transduction in H2O2-induced senescence-like phenotype in human diploid fibroblasts. Free Radic Biol Med 2002, 33:1334-1346
148. Volonte D, Galbiati F, Pestell RG, Lisanti MP: Cellular stress induces the tyrosine phosphorylation of caveolin-1 (Tyr(14)) via activation of p38 mitogen-activated protein kinase and c-Src kinase. Evidence for caveolae, the actin cytoskeleton, and focal adhesions as mechanical sensors of osmotic stress. J Biol Chem 2001, 276:8094-8103
149. Thannickal VJ, Fanburg BL: Activation of an H2O2-generating NADH oxidase in human lung fibroblasts by transforming growth factor beta 1. J Biol Chem 1995, 270:30334-30338
150. Kang MK, Kim RH, Shin KH, Zhong W, Faull KF, Park NH: Senescence-associated decline in the intranuclear accumulation of hOGG1-alpha and impaired 8-oxo-dG repair activity in senescing normal human oral keratinocytes in vivo. Exp Cell Res 2005, 310:186-195
151. Lee HC, Yin PH, Chi CW, Wei YH: Increase in mitochondrial mass in human fibroblasts under oxidative stress and during replicative cell senescence. J Biomed Sci 2002, 9:517-526
152. Bianchi A, Stansel RM, Fairall L, Griffith JD, Rhodes D, de Lange T: TRF1 binds a bipartite telomeric site with extreme spatial flexibility. Embo J 1999, 18:5735-5744
153. Court R, Chapman L, Fairall L, Rhodes D: How the human telomeric proteins TRF1 and TRF2 recognize telomeric DNA: a view from high-resolution crystal structures. EMBO Rep 2005, 6:39-45
154. Hanaoka S, Nagadoi A, Nishimura Y: Comparison between TRF2 and TRF1 of their telomeric DNA-bound structures and DNA-binding activities. Protein Sci 2005, 14:119-130
155. Opresko PL, Fan J, Danzy S, Wilson DM, 3rd, Bohr VA: Oxidative damage in telomeric DNA disrupts recognition by TRF1 and TRF2. Nucleic Acids Res 2005, 33:1230-1239
156. von Zglinicki T, Pilger R, Sitte N: Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts. Free Radic Biol Med 2000, 28:64-74

157. Oikawa S, Kawanishi S: Site-specific DNA damage at GGG sequence by oxidative stress may accelerate telomere shortening. FEBS Lett 1999, 453:365-368
158. Hanahan D, Weinberg RA: The hallmarks of cancer. Cell 2000, 100:57-70
159. Land H, Parada LF, Weinberg RA: Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 1983, 304:596-602
160. Franza BR, Jr., Maruyama K, Garrels JI, Ruley HE: In vitro establishment is not a sufficient prerequisite for transformation by activated ras oncogenes. Cell 1986, 44:409-418
161. Hydbring P, Bahram F, Su Y, Tronnersjo S, Hogstrand K, von der Lehr N, Sharifi HR, Lilischkis R, Hein N, Wu S, Vervoorts J, Henriksson M, Grandien A, Luscher B, Larsson LG: Phosphorylation by Cdk2 is required for Myc to repress Ras-induced senescence in cotransformation. Proc Natl Acad Sci U S A, 107:58-63
162. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW: Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 1997, 88:593-602
163. Zhu J, Woods D, McMahon M, Bishop JM: Senescence of human fibroblasts induced by oncogenic Raf. Genes Dev 1998, 12:2997-3007
164. Pearson M, Carbone R, Sebastiani C, Cioce M, Fagioli M, Saito S, Higashimoto Y, Appella E, Minucci S, Pandolfi PP, Pelicci PG: PML regulates p53 acetylation and premature senescence induced by oncogenic Ras. Nature 2000, 406:207-210
165. Atwood AA, Sealy L: Regulation of C/EBPbeta1 by Ras in mammary epithelial cells and the role of C/EBPbeta1 in oncogene-induced senescence. Oncogene 2010
166. Michaloglou C, Vredeveld LC, Soengas MS, Denoyelle C, Kuilman T, van der Horst CM, Majoor DM, Shay JW, Mooi WJ, Peeper DS: BRAFE600-associated senescence-like cell cycle arrest of human naevi. Nature 2005, 436:720-724
167. Cotter MA, Florell SR, Leachman SA, Grossman D: Absence of senescence-associated beta-galactosidase activity in human melanocytic nevi in vivo. J Invest Dermatol 2007, 127:2469-2471
168. Michaloglou C, Soengas MS, Mooi WJ, Peeper DS: Comment on "Absence of senescence-associated beta-galactosidase activity in human melanocytic nevi in vivo". J Invest Dermatol 2008, 128:1582-1583; author reply 1583-1584
169. Gray-Schopfer VC, Soo JK, Bennett DC: Comment on "Absence of senescence-associated beta-galactosidase activity in human melanocytic nevi in vivo". J Invest Dermatol 2008, 128:1581; author reply 1583-1584
170. Lazzerini Denchi E, Attwooll C, Pasini D, Helin K: Deregulated E2F activity induces hyperplasia and senescence-like features in the mouse pituitary gland. Mol Cell Biol 2005, 25:2660-2672
171. Kilbey A, Blyth K, Wotton S, Terry A, Jenkins A, Bell M, Hanlon L, Cameron ER, Neil JC: Runx2 disruption promotes immortalization and confers resistance to oncogene-induced senescence in primary murine fibroblasts. Cancer Res 2007, 67:11263-11271
172. Pan D, Zhu Q, Luo K: SnoN functions as a tumour suppressor by inducing premature senescence. Embo J 2009, 28:3500-3513

173. Mallette FA, Gaumont-Leclerc MF, Huot G, Ferbeyre G: Myc down-regulation as a mechanism to activate the Rb pathway in STAT5A-induced senescence. J Biol Chem 2007, 282:34938-34944
174. Irani K, Xia Y, Zweier JL, Sollott SJ, Der CJ, Fearon ER, Sundaresan M, Finkel T, Goldschmidt-Clermont PJ: Mitogenic signaling mediated by oxidants in Ras-transformed fibroblasts. Science 1997, 275:1649-1652
175. Sundaresan M, Yu ZX, Ferrans VJ, Sulciner DJ, Gutkind JS, Irani K, Goldschmidt-Clermont PJ, Finkel T: Regulation of reactive-oxygen-species generation in fibroblasts by Rac1. Biochem J 1996, 318 (Pt 2):379-382
176. Bernard D, Quatannens B, Begue A, Vandenbunder B, Abbadie C: Antiproliferative and antiapoptotic effects of crel may occur within the same cells via the up-regulation of manganese superoxide dismutase. Cancer Res 2001, 61:2656-2664
177. Bernard D, Slomianny C, Vandenbunder B, Abbadie C: cRel induces mitochondrial alterations in correlation with proliferation arrest. Free Radic Biol Med 2001, 31:943-953
178. Di Micco R, Fumagalli M, Cicalese A, Piccinin S, Gasparini P, Luise C, Schurra C, Garre M, Nuciforo PG, Bensimon A, Maestro R, Pelicci PG, d'Adda di Fagagna F: Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature 2006, 444:638-642
179. Wisdom R, Johnson RS, Moore C: c-Jun regulates cell cycle progression and apoptosis by distinct mechanisms. Embo J 1999, 18:188-197
180. Wu CH, van Riggelen J, Yetil A, Fan AC, Bachireddy P, Felsher DW: Cellular senescence is an important mechanism of tumor regression upon c-Myc inactivation. Proc Natl Acad Sci U S A 2007, 104:13028-13033
181. Jacobs JJ, Keblusek P, Robanus-Maandag E, Kristel P, Lingbeek M, Nederlof PM, van Welsem T, van de Vijver MJ, Koh EY, Daley GQ, van Lohuizen M: Senescence bypass screen identifies TBX2, which represses Cdkn2a (p19(ARF)) and is amplified in a subset of human breast cancers. Nat Genet 2000, 26:291-299
182. Shvarts A, Brummelkamp TR, Scheeren F, Koh E, Daley GQ, Spits H, Bernards R: A senescence rescue screen identifies BCL6 as an inhibitor of anti-proliferative p19(ARF)-p53 signaling. Genes Dev 2002, 16:681-686
183. Rowland BD, Bernards R, Peeper DS: The KLF4 tumour suppressor is a transcriptional repressor of p53 that acts as a context-dependent oncogene. Nat Cell Biol 2005, 7:1074-1082
184. MacLaren A, Black EJ, Clark W, Gillespie DA: c-Jun-deficient cells undergo premature senescence as a result of spontaneous DNA damage accumulation. Mol Cell Biol 2004, 24:9006-9018
185. Toussaint O, Medrano EE, von Zglinicki T: Cellular and molecular mechanisms of stress-induced premature senescence (SIPS) of human diploid fibroblasts and melanocytes. Exp Gerontol 2000, 35:927-945
186. Dierick JF, Eliaers F, Remacle J, Raes M, Fey SJ, Larsen PM, Toussaint O: Stress-induced premature senescence and replicative senescence are different phenotypes, proteomic evidence. Biochem Pharmacol 2002, 64:1011-1017
187. Yang ES, Xia F: BRCA1 16 years later: DNA damage-induced BRCA1 shuttling. Febs J 2010, 277:3079-3085

188. Linger RJ, Kruk PA: BRCA1 16 years later: risk-associated BRCA1 mutations and their functional implications. Febs J 2010, 277:3086-3096
189. Harman D: Aging: phenomena and theories. Ann N Y Acad Sci 1998, 854:1-7
190. Herbig U, Ferreira M, Condel L, Carey D, Sedivy JM: Cellular senescence in aging primates. Science 2006, 311:1257
191. Jeyapalan JC, Ferreira M, Sedivy JM, Herbig U: Accumulation of senescent cells in mitotic tissue of aging primates. Mech Ageing Dev 2007, 128:36-44
192. Ressler S, Bartkova J, Niederegger H, Bartek J, Scharffetter-Kochanek K, Jansen-Durr P, Wlaschek M: p16INK4A is a robust in vivo biomarker of cellular aging in human skin. Aging Cell 2006, 5:379-389
193. Minamino T, Miyauchi H, Yoshida T, Tateno K, Kunieda T, Komuro I: Vascular cell senescence and vascular aging. J Mol Cell Cardiol 2004, 36:175-183
194. Krishnamurthy J, Torrice C, Ramsey MR, Kovalev GI, Al-Regaiey K, Su L, Sharpless NE: Ink4a/Arf expression is a biomarker of aging. J Clin Invest 2004, 114:1299-1307
195. Melk A, Schmidt BM, Takeuchi O, Sawitzki B, Rayner DC, Halloran PF: Expression of p16INK4a and other cell cycle regulator and senescence associated genes in aging human kidney. Kidney Int 2004, 65:510-520
196. Guan JZ, Maeda T, Sugano M, Oyama J, Higuchi Y, Makino N: Change in the telomere length distribution with age in the Japanese population. Mol Cell Biochem 2007, 304:353-360
197. Kang MK, Swee J, Kim RH, Baluda MA, Park NH: The telomeric length and heterogeneity decrease with age in normal human oral keratinocytes. Mech Ageing Dev 2002, 123:585-592
198. Kveiborg M, Kassem M, Langdahl B, Eriksen EF, Clark BF, Rattan SI: Telomere shortening during aging of human osteoblasts in vitro and leukocytes in vivo: lack of excessive telomere loss in osteoporotic patients. Mech Ageing Dev 1999, 106:261-271
199. Aikata H, Takaishi H, Kawakami Y, Takahashi S, Kitamoto M, Nakanishi T, Nakamura Y, Shimamoto F, Kajiyama G, Ide T: Telomere reduction in human liver tissues with age and chronic inflammation. Exp Cell Res 2000, 256:578-582
200. Lindsey J, McGill NI, Lindsey LA, Green DK, Cooke HJ: In vivo loss of telomeric repeats with age in humans. Mutat Res 1991, 256:45-48
201. Cristofalo VJ, Allen RG, Pignolo RJ, Martin BG, Beck JC: Relationship between donor age and the replicative lifespan of human cells in culture: a reevaluation. Proc Natl Acad Sci U S A 1998, 95:10614-10619
202. Cristofalo VJ, Volker C, Francis MK, Tresini M: Age-dependent modifications of gene expression in human fibroblasts. Crit Rev Eukaryot Gene Expr 1998, 8:43-80
203. Blasco MA, Lee HW, Hande MP, Samper E, Lansdorp PM, DePinho RA, Greider CW: Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell 1997, 91:25-34
204. Lee HW, Blasco MA, Gottlieb GJ, Horner JW, 2nd, Greider CW, DePinho RA: Essential role of mouse telomerase in highly proliferative organs. Nature 1998, 392:569-574

205. Herrera E, Samper E, Martin-Caballero J, Flores JM, Lee HW, Blasco MA: Disease states associated with telomerase deficiency appear earlier in mice with short telomeres. Embo J 1999, 18:2950-2960
206. Rudolph KL, Chang S, Lee HW, Blasco M, Gottlieb GJ, Greider C, DePinho RA: Longevity, stress response, and cancer in aging telomerase-deficient mice. Cell 1999, 96:701-712
207. Sohal RS, Dubey A: Mitochondrial oxidative damage, hydrogen peroxide release, and aging. Free Radic Biol Med 1994, 16:621-626
208. Sohal RS, Sohal BH: Hydrogen peroxide release by mitochondria increases during aging. Mech Ageing Dev 1991, 57:187-202
209. Sohal RS, Arnold LA, Sohal BH: Age-related changes in antioxidant enzymes and prooxidant generation in tissues of the rat with special reference to parameters in two insect species. Free Radic Biol Med 1990, 9:495-500
210. Wei YH, Lee HC: Oxidative stress, mitochondrial DNA mutation, and impairment of antioxidant enzymes in aging. Exp Biol Med (Maywood) 2002, 227:671-682
211. Linnane AW, Marzuki S, Ozawa T, Tanaka M: Mitochondrial DNA mutations as an important contributor to ageing and degenerative diseases. Lancet 1989, 1:642-645
212. Wei YH: Mitochondrial DNA alterations as ageing-associated molecular events. Mutat Res 1992, 275:145-155
213. Sastre J, Pallardo FV, Vina J: The role of mitochondrial oxidative stress in aging. Free Radic Biol Med 2003, 35:1-8
214. Ishii N, Fujii M, Hartman PS, Tsuda M, Yasuda K, Senoo-Matsuda N, Yanase S, Ayusawa D, Suzuki K: A mutation in succinate dehydrogenase cytochrome b causes oxidative stress and ageing in nematodes. Nature 1998, 394:694-697
215. Melov S, Ravenscroft J, Malik S, Gill MS, Walker DW, Clayton PE, Wallace DC, Malfroy B, Doctrow SR, Lithgow GJ: Extension of life-span with superoxide dismutase/catalase mimetics. Science 2000, 289:1567-1569
216. Sun J, Tower J: FLP recombinase-mediated induction of Cu/Zn-superoxide dismutase transgene expression can extend the life span of adult Drosophila melanogaster flies. Mol Cell Biol 1999, 19:216-228
217. Parkes TL, Elia AJ, Dickinson D, Hilliker AJ, Phillips JP, Boulianne GL: Extension of Drosophila lifespan by overexpression of human SOD1 in motorneurons. Nat Genet 1998, 19:171-174
218. Sohal RS, Weindruch R: Oxidative stress, caloric restriction, and aging. Science 1996, 273:59-63
219. Lin YJ, Seroude L, Benzer S: Extended life-span and stress resistance in the Drosophila mutant methuselah. Science 1998, 282:943-946
220. Taub J, Lau JF, Ma C, Hahn JH, Hoque R, Rothblatt J, Chalfie M: A cytosolic catalase is needed to extend adult lifespan in C. elegans daf-C and clk-1 mutants. Nature 1999, 399:162-166
221. Honda Y, Honda S: The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans. Faseb J 1999, 13:1385-1393
222. Johnson TE, Henderson S, Murakami S, de Castro E, de Castro SH, Cypser J, Rikke B, Tedesco P, Link C: Longevity genes in the nematode Caenorhabditis elegans

also mediate increased resistance to stress and prevent disease. J Inherit Metab Dis 2002, 25:197-206
223. Hamilton ML, Van Remmen H, Drake JA, Yang H, Guo ZM, Kewitt K, Walter CA, Richardson A: Does oxidative damage to DNA increase with age? Proc Natl Acad Sci U S A 2001, 98:10469-10474
224. Bokov A, Chaudhuri A, Richardson A: The role of oxidative damage and stress in aging. Mech Ageing Dev 2004, 125:811-826
225. Sohal RS: Role of oxidative stress and protein oxidation in the aging process. Free Radic Biol Med 2002, 33:37-44
226. Moriwaki S, Ray S, Tarone RE, Kraemer KH, Grossman L: The effect of donor age on the processing of UV-damaged DNA by cultured human cells: reduced DNA repair capacity and increased DNA mutability. Mutat Res 1996, 364:117-123
227. Goukassian D, Gad F, Yaar M, Eller MS, Nehal US, Gilchrest BA: Mechanisms and implications of the age-associated decrease in DNA repair capacity. Faseb J 2000, 14:1325-1334
228. Rao KS: DNA repair in aging rat neurons. Neuroscience 2007, 145:1330-1340
229. Vyjayanti VN, Rao KS: DNA double strand break repair in brain: reduced NHEJ activity in aging rat neurons. Neurosci Lett 2006, 393:18-22
230. Lombard DB, Chua KF, Mostoslavsky R, Franco S, Gostissa M, Alt FW: DNA repair, genome stability, and aging. Cell 2005, 120:497-512
231. Meyer JN, Boyd WA, Azzam GA, Haugen AC, Freedman JH, Van Houten B: Decline of nucleotide excision repair capacity in aging Caenorhabditis elegans. Genome Biol 2007, 8:R70
232. Kyng KJ, Bohr VA: Gene expression and DNA repair in progeroid syndromes and human aging. Ageing Res Rev 2005, 4:579-602
233. Puzianowska-Kuznicka M, Kuznicki J: Genetic alterations in accelerated ageing syndromes. Do they play a role in natural ageing? Int J Biochem Cell Biol 2005, 37:947-960
234. Busciglio J, Yankner BA: Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons in vitro. Nature 1995, 378:776-779
235. Pallardo FV, Degan P, d'Ischia M, Kelly FJ, Zatterale A, Calzone R, Castello G, Fernandez-Delgado R, Dunster C, Lloret A, Manini P, Pisanti MA, Vuttariello E, Pagano G: Multiple evidence for an early age pro-oxidant state in Down Syndrome patients. Biogerontology 2006, 7:211-220
236. Campisi J: The biology of replicative senescence. Eur J Cancer 1997, 33:703-709
237. Tanaka H, Fujita N, Sugimoto R, Urawa N, Horiike S, Kobayashi Y, Iwasa M, Ma N, Kawanishi S, Watanabe S, Kaito M, Takei Y: Hepatic oxidative DNA damage is associated with increased risk for hepatocellular carcinoma in chronic hepatitis C. Br J Cancer 2008, 98:580-586
238. Miyake H, Hara I, Kamidono S, Eto H: Oxidative DNA damage in patients with prostate cancer and its response to treatment. J Urol 2004, 171:1533-1536
239. Diakowska D, Lewandowski A, Kopec W, Diakowski W, Chrzanowska T: Oxidative DNA damage and total antioxidant status in serum of patients with esophageal squamous cell carcinoma. Hepatogastroenterology 2007, 54:1701-1704
240. Coppe JP, Patil CK, Rodier F, Sun Y, Munoz DP, Goldstein J, Nelson PS, Desprez PY, Campisi J: Senescence-associated secretory phenotypes reveal cell-

nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. PLoS Biol 2008, 6:2853-2868
241. Bavik C, Coleman I, Dean JP, Knudsen B, Plymate S, Nelson PS: The gene expression program of prostate fibroblast senescence modulates neoplastic epithelial cell proliferation through paracrine mechanisms. Cancer Res 2006, 66:794-802
242. Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR: Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res 1999, 59:5002-5011
243. Kuilman T, Peeper DS: Senescence-messaging secretome: SMS-ing cellular stress. Nat Rev Cancer 2009, 9:81-94
244. Coppe JP, Desprez PY, Krtolica A, Campisi J: The senescence-associated secretory phenotype: the dark side of tumor suppression. Annu Rev Pathol 2010, 5:99-118
245. Romanov SR, Kozakiewicz BK, Holst CR, Stampfer MR, Haupt LM, Tlsty TD: Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes. Nature 2001, 409:633-637
246. Farout L, Friguet B: Proteasome function in aging and oxidative stress: implications in protein maintenance failure. Antioxid Redox Signal 2006, 8:205-216
247. Jung T, Bader N, Grune T: Oxidized proteins: intracellular distribution and recognition by the proteasome. Arch Biochem Biophys 2007, 462:231-237
248. Schrader EK, Harstad KG, Matouschek A: Targeting proteins for degradation. Nat Chem Biol 2009, 5:815-822
249. Grune T, Merker K, Sandig G, Davies KJ: Selective degradation of oxidatively modified protein substrates by the proteasome. Biochem Biophys Res Commun 2003, 305:709-718
250. Ngo JK, Davies KJ: Importance of the lon protease in mitochondrial maintenance and the significance of declining lon in aging. Ann N Y Acad Sci 2007, 1119:78-87
251. Yokota S, Dariush Fahimi H: Degradation of excess peroxisomes in mammalian liver cells by autophagy and other mechanisms. Histochem Cell Biol 2009, 131:455-458
252. Bulteau AL, Szweda LI, Friguet B: Mitochondrial protein oxidation and degradation in response to oxidative stress and aging. Exp Gerontol 2006, 41:653-657
253. De Duve C, Wattiaux R: Functions of lysosomes. Annu Rev Physiol 1966, 28:435-492
254. Todde V, Veenhuis M, van der Klei IJ: Autophagy: principles and significance in health and disease. Biochim Biophys Acta 2009, 1792:3-13
255. Mizushima N, Levine B, Cuervo AM, Klionsky DJ: Autophagy fights disease through cellular self-digestion. Nature 2008, 451:1069-1075
256. Chiang HL, Terlecky SR, Plant CP, Dice JF: A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. Science 1989, 246:382-385
257. Dice JF: Peptide sequences that target cytosolic proteins for lysosomal proteolysis. Trends Biochem Sci 1990, 15:305-309

258. Chiang HL, Dice JF: Peptide sequences that target proteins for enhanced degradation during serum withdrawal. J Biol Chem 1988, 263:6797-6805
259. Majeski AE, Dice JF: Mechanisms of chaperone-mediated autophagy. Int J Biochem Cell Biol 2004, 36:2435-2444
260. Kaushik S, Cuervo AM: Autophagy as a cell-repair mechanism: activation of chaperone-mediated autophagy during oxidative stress. Mol Aspects Med 2006, 27:444-454
261. Dice JF: Chaperone-mediated autophagy. Autophagy 2007, 3:295-299
262. Klionsky DJ: Autophagy: from phenomenology to molecular understanding in less than a decade. Nat Rev Mol Cell Biol 2007, 8:931-937
263. Suzuki K, Kirisako T, Kamada Y, Mizushima N, Noda T, Ohsumi Y: The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. Embo J 2001, 20:5971-5981
264. Kim J, Huang WP, Stromhaug PE, Klionsky DJ: Convergence of multiple autophagy and cytoplasm to vacuole targeting components to a perivacuolar membrane compartment prior to de novo vesicle formation. J Biol Chem 2002, 277:763-773
265. Axe EL, Walker SA, Manifava M, Chandra P, Roderick HL, Habermann A, Griffiths G, Ktistakis NT: Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. J Cell Biol 2008, 182:685-701
266. Hayashi-Nishino M, Fujita N, Noda T, Yamaguchi A, Yoshimori T, Yamamoto A: A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation. Nat Cell Biol 2009, 11:1433-1437
267. Eskelinen EL: Maturation of autophagic vacuoles in Mammalian cells. Autophagy 2005, 1:1-10
268. Mizushima N: Autophagy: process and function. Genes Dev 2007, 21:2861-2873
269. Nakatogawa H, Suzuki K, Kamada Y, Ohsumi Y: Dynamics and diversity in autophagy mechanisms: lessons from yeast. Nat Rev Mol Cell Biol 2009, 10:458-467
270. Mercer CA, Kaliappan A, Dennis PB: A novel, human Atg13 binding protein, Atg101, interacts with ULK1 and is essential for macroautophagy. Autophagy 2009, 5:649-662
271. Chan EY, Longatti A, McKnight NC, Tooze SA: Kinase-inactivated ULK proteins inhibit autophagy via their conserved C-terminal domains using an Atg13-independent mechanism. Mol Cell Biol 2009, 29:157-171
272. Chang YY, Neufeld TP: An Atg1/Atg13 complex with multiple roles in TOR-mediated autophagy regulation. Mol Biol Cell 2009, 20:2004-2014
273. Jung CH, Jun CB, Ro SH, Kim YM, Otto NM, Cao J, Kundu M, Kim DH: ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. Mol Biol Cell 2009, 20:1992-2003
274. Shaw RJ, Cantley LC: Ras, PI(3)K and mTOR signalling controls tumour cell growth. Nature 2006, 441:424-430
275. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B: Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 1999, 402:672-676

276. Karantza-Wadsworth V, Patel S, Kravchuk O, Chen G, Mathew R, Jin S, White E: Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. Genes Dev 2007, 21:1621-1635
277. Liang C, Feng P, Ku B, Dotan I, Canaani D, Oh BH, Jung JU: Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. Nat Cell Biol 2006, 8:688-699
278. Takahashi Y, Coppola D, Matsushita N, Cualing HD, Sun M, Sato Y, Liang C, Jung JU, Cheng JQ, Mule JJ, Pledger WJ, Wang HG: Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. Nat Cell Biol 2007, 9:1142-1151
279. Liang C, Lee JS, Inn KS, Gack MU, Li Q, Roberts EA, Vergne I, Deretic V, Feng P, Akazawa C, Jung JU: Beclin1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking. Nat Cell Biol 2008, 10:776-787
280. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y, Cattoretti G, Levine B: Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J Clin Invest 2003, 112:1809-1820
281. Hamacher-Brady A, Brady NR, Logue SE, Sayen MR, Jinno M, Kirshenbaum LA, Gottlieb RA, Gustafsson AB: Response to myocardial ischemia/reperfusion injury involves Bnip3 and autophagy. Cell Death Differ 2007, 14:146-157
282. Maiuri MC, Le Toumelin G, Criollo A, Rain JC, Gautier F, Juin P, Tasdemir E, Pierron G, Troulinaki K, Tavernarakis N, Hickman JA, Geneste O, Kroemer G: Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1. Embo J 2007, 26:2527-2539
283. Maiuri MC, Criollo A, Tasdemir E, Vicencio JM, Tajeddine N, Hickman JA, Geneste O, Kroemer G: BH3-only proteins and BH3 mimetics induce autophagy by competitively disrupting the interaction between Beclin 1 and Bcl-2/Bcl-X(L). Autophagy 2007, 3:374-376
284. Daido S, Kanzawa T, Yamamoto A, Takeuchi H, Kondo Y, Kondo S: Pivotal role of the cell death factor BNIP3 in ceramide-induced autophagic cell death in malignant glioma cells. Cancer Res 2004, 64:4286-4293
285. Abedin MJ, Wang D, McDonnell MA, Lehmann U, Kelekar A: Autophagy delays apoptotic death in breast cancer cells following DNA damage. Cell Death Differ 2007, 14:500-510
286. Marino G, Salvador-Montoliu N, Fueyo A, Knecht E, Mizushima N, Lopez-Otin C: Tissue-specific autophagy alterations and increased tumorigenesis in mice deficient in Atg4C/autophagin-3. J Biol Chem 2007, 282:18573-18583
287. Kang MR, Kim MS, Oh JE, Kim YR, Song SY, Kim SS, Ahn CH, Yoo NJ, Lee SH: Frameshift mutations of autophagy-related genes ATG2B, ATG5, ATG9B and ATG12 in gastric and colorectal cancers with microsatellite instability. J Pathol 2009, 217:702-706
288. Barrett JC, Hansoul S, Nicolae DL, Cho JH, Duerr RH, Rioux JD, Brant SR, Silverberg MS, Taylor KD, Barmada MM, Bitton A, Dassopoulos T, Datta LW, Green T, Griffiths AM, Kistner EO, Murtha MT, Regueiro MD, Rotter JI, Schumm LP, Steinhart AH, Targan SR, Xavier RJ, Libioulle C, Sandor C, Lathrop M, Belaiche J,

Dewit O, Gut I, Heath S, Laukens D, Mni M, Rutgeerts P, Van Gossum A, Zelenika D, Franchimont D, Hugot JP, de Vos M, Vermeire S, Louis E, Cardon LR, Anderson CA, Drummond H, Nimmo E, Ahmad T, Prescott NJ, Onnie CM, Fisher SA, Marchini J, Ghori J, Bumpstead S, Gwilliam R, Tremelling M, Deloukas P, Mansfield J, Jewell D, Satsangi J, Mathew CG, Parkes M, Georges M, Daly MJ: Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. Nat Genet 2008, 40:955-962

289. Massey DC, Parkes M: Genome-wide association scanning highlights two autophagy genes, ATG16L1 and IRGM, as being significantly associated with Crohn's disease. Autophagy 2007, 3:649-651
290. Saitoh T, Fujita N, Jang MH, Uematsu S, Yang BG, Satoh T, Omori H, Noda T, Yamamoto N, Komatsu M, Tanaka K, Kawai T, Tsujimura T, Takeuchi O, Yoshimori T, Akira S: Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. Nature 2008, 456:264-268
291. Kuballa P, Huett A, Rioux JD, Daly MJ, Xavier RJ: Impaired autophagy of an intracellular pathogen induced by a Crohn's disease associated ATG16L1 variant. PLoS One 2008, 3:e3391
292. Lee JS, Li Q, Lee JY, Lee SH, Jeong JH, Lee HR, Chang H, Zhou FC, Gao SJ, Liang C, Jung JU: FLIP-mediated autophagy regulation in cell death control. Nat Cell Biol 2009
293. Harrison B, Kraus M, Burch L, Stevens C, Craig A, Gordon-Weeks P, Hupp TR: DAPK-1 binding to a linear peptide motif in MAP1B stimulates autophagy and membrane blebbing. J Biol Chem 2008, 283:9999-10014
294. Schildhaus HU, Krockel I, Lippert H, Malfertheiner P, Roessner A, Schneider-Stock R: Promoter hypermethylation of p16INK4a, E-cadherin, O6-MGMT, DAPK and FHIT in adenocarcinomas of the esophagus, esophagogastric junction and proximal stomach. Int J Oncol 2005, 26:1493-1500
295. Zhang M, Chen L, Wang S, Wang T: Rab7: roles in membrane trafficking and disease. Biosci Rep 2009, 29:193-209
296. Chia WJ, Tang BL: Emerging roles for Rab family GTPases in human cancer. Biochim Biophys Acta 2009, 1795:110-116
297. Matsunaga K, Saitoh T, Tabata K, Omori H, Satoh T, Kurotori N, Maejima I, Shirahama-Noda K, Ichimura T, Isobe T, Akira S, Noda T, Yoshimori T: Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. Nat Cell Biol 2009, 11:385-396
298. Zhong Y, Wang QJ, Li X, Yan Y, Backer JM, Chait BT, Heintz N, Yue Z: Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1-phosphatidylinositol-3-kinase complex. Nat Cell Biol 2009, 11:468-476
299. Liang C, Jung JU: Autophagy genes as tumor suppressors. Curr Opin Cell Biol 2010, 22:226-233
300. Jung CH, Ro SH, Cao J, Otto NM, Kim DH: mTOR regulation of autophagy. FEBS Lett 2010, 584:1287-1295
301. Diaz-Troya S, Perez-Perez ME, Florencio FJ, Crespo JL: The role of TOR in autophagy regulation from yeast to plants and mammals. Autophagy 2008, 4:851-865

302. Ganley IG, Lam du H, Wang J, Ding X, Chen S, Jiang X: ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy. J Biol Chem 2009, 284:12297-12305
303. Hosokawa N, Hara T, Kaizuka T, Kishi C, Takamura A, Miura Y, Iemura S, Natsume T, Takehana K, Yamada N, Guan JL, Oshiro N, Mizushima N: Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. Mol Biol Cell 2009, 20:1981-1991
304. Mammucari C, Milan G, Romanello V, Masiero E, Rudolf R, Del Piccolo P, Burden SJ, Di Lisi R, Sandri C, Zhao J, Goldberg AL, Schiaffino S, Sandri M: FoxO3 controls autophagy in skeletal muscle in vivo. Cell Metab 2007, 6:458-471
305. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM: Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 2005, 307:1098-1101
306. Mehrpour M, Esclatine A, Beau I, Codogno P: Overview of macroautophagy regulation in mammalian cells. Cell Res 2010, 20:748-762
307. Backer JM: The regulation and function of Class III PI3Ks: novel roles for Vps34. Biochem J 2008, 410:1-17
308. Zeng X, Overmeyer JH, Maltese WA: Functional specificity of the mammalian Beclin-Vps34 PI 3-kinase complex in macroautophagy versus endocytosis and lysosomal enzyme trafficking. J Cell Sci 2006, 119:259-270
309. Geng J, Klionsky DJ: The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. 'Protein modifications: beyond the usual suspects' review series. EMBO Rep 2008, 9:859-864
310. Aita VM, Liang XH, Murty VV, Pincus DL, Yu W, Cayanis E, Kalachikov S, Gilliam TC, Levine B: Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21. Genomics 1999, 59:59-65
311. Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, Packer M, Schneider MD, Levine B: Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 2005, 122:927-939
312. Wei Y, Pattingre S, Sinha S, Bassik M, Levine B: JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. Mol Cell 2008, 30:678-688
313. Fimia GM, Stoykova A, Romagnoli A, Giunta L, Di Bartolomeo S, Nardacci R, Corazzari M, Fuoco C, Ucar A, Schwartz P, Gruss P, Piacentini M, Chowdhury K, Cecconi F: Ambra1 regulates autophagy and development of the nervous system. Nature 2007, 447:1121-1125
314. Mizushima N, Sugita H, Yoshimori T, Ohsumi Y: A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy. J Biol Chem 1998, 273:33889-33892
315. Mizushima N, Yoshimori T, Ohsumi Y: Role of the Apg12 conjugation system in mammalian autophagy. Int J Biochem Cell Biol 2003, 35:553-561
316. Mizushima N, Kuma A, Kobayashi Y, Yamamoto A, Matsubae M, Takao T, Natsume T, Ohsumi Y, Yoshimori T: Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate. J Cell Sci 2003, 116:1679-1688
317. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T: LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. Embo J 2000, 19:5720-5728

318. Abeliovich H, Dunn WA, Jr., Kim J, Klionsky DJ: Dissection of autophagosome biogenesis into distinct nucleation and expansion steps. J Cell Biol 2000, 151:1025-1034
319. Kuznetsov SA, Gelfand VI: 18 kDa microtubule-associated protein: identification as a new light chain (LC-3) of microtubule-associated protein 1 (MAP-1). FEBS Lett 1987, 212:145-148
320. Ichimura Y, Kirisako T, Takao T, Satomi Y, Shimonishi Y, Ishihara N, Mizushima N, Tanida I, Kominami E, Ohsumi M, Noda T, Ohsumi Y: A ubiquitin-like system mediates protein lipidation. Nature 2000, 408:488-492
321. Kabeya Y, Mizushima N, Yamamoto A, Oshitani-Okamoto S, Ohsumi Y, Yoshimori T: LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. J Cell Sci 2004, 117:2805-2812
322. Sou YS, Tanida I, Komatsu M, Ueno T, Kominami E: Phosphatidylserine in addition to phosphatidylethanolamine is an in vitro target of the mammalian Atg8 modifiers, LC3, GABARAP, and GATE-16. J Biol Chem 2006, 281:3017-3024
323. Tanida I, Tanida-Miyake E, Ueno T, Kominami E: The human homolog of Saccharomyces cerevisiae Apg7p is a Protein-activating enzyme for multiple substrates including human Apg12p, GATE-16, GABARAP, and MAP-LC3. J Biol Chem 2001, 276:1701-1706
324. Mousavi SA, Kjeken R, Berg TO, Seglen PO, Berg T, Brech A: Effects of inhibitors of the vacuolar proton pump on hepatic heterophagy and autophagy. Biochim Biophys Acta 2001, 1510:243-257
325. Jager S, Bucci C, Tanida I, Ueno T, Kominami E, Saftig P, Eskelinen EL: Role for Rab7 in maturation of late autophagic vacuoles. J Cell Sci 2004, 117:4837-4848
326. Tanaka Y, Guhde G, Suter A, Eskelinen EL, Hartmann D, Lullmann-Rauch R, Janssen PM, Blanz J, von Figura K, Saftig P: Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. Nature 2000, 406:902-906
327. Eskelinen EL, Schmidt CK, Neu S, Willenborg M, Fuertes G, Salvador N, Tanaka Y, Lullmann-Rauch R, Hartmann D, Heeren J, von Figura K, Knecht E, Saftig P: Disturbed cholesterol traffic but normal proteolytic function in LAMP-1/LAMP-2 double-deficient fibroblasts. Mol Biol Cell 2004, 15:3132-3145
328. Gutierrez MG, Munafo DB, Beron W, Colombo MI: Rab7 is required for the normal progression of the autophagic pathway in mammalian cells. J Cell Sci 2004, 117:2687-2697
329. Webb JL, Ravikumar B, Rubinsztein DC: Microtubule disruption inhibits autophagosome-lysosome fusion: implications for studying the roles of aggresomes in polyglutamine diseases. Int J Biochem Cell Biol 2004, 36:2541-2550
330. Aplin A, Jasionowski T, Tuttle DL, Lenk SE, Dunn WA, Jr.: Cytoskeletal elements are required for the formation and maturation of autophagic vacuoles. J Cell Physiol 1992, 152:458-466
331. Pankiv S, Alemu EA, Brech A, Bruun JA, Lamark T, Overvatn A, Bjorkoy G, Johansen T: FYCO1 is a Rab7 effector that binds to LC3 and PI3P to mediate microtubule plus end-directed vesicle transport. J Cell Biol 2010, 188:253-269
332. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, Overvatn A, Bjorkoy G, Johansen T: p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation

of ubiquitinated protein aggregates by autophagy. J Biol Chem 2007, 282:24131-24145
333. Komatsu M, Waguri S, Koike M, Sou YS, Ueno T, Hara T, Mizushima N, Iwata J, Ezaki J, Murata S, Hamazaki J, Nishito Y, Iemura S, Natsume T, Yanagawa T, Uwayama J, Warabi E, Yoshida H, Ishii T, Kobayashi A, Yamamoto M, Yue Z, Uchiyama Y, Kominami E, Tanaka K: Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. Cell 2007, 131:1149-1163
334. Beau I, Esclatine A, Codogno P: Lost to translation: when autophagy targets mature ribosomes. Trends Cell Biol 2008, 18:311-314
335. Gottlieb RA, Carreira RS: Autophagy in health and disease. 5. Mitophagy as a way of life. Am J Physiol Cell Physiol 2010, 299:C203-210
336. Kanki T, Wang K, Cao Y, Baba M, Klionsky DJ: Atg32 is a mitochondrial protein that confers selectivity during mitophagy. Dev Cell 2009, 17:98-109
337. Kanki T, Klionsky DJ: Atg32 is a tag for mitochondria degradation in yeast. Autophagy 2009, 5:1201-1202
338. Okamoto K, Kondo-Okamoto N, Ohsumi Y: Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy. Dev Cell 2009, 17:87-97
339. Carreira RS, Lee Y, Ghochani M, Gustafsson AB, Gottlieb RA: Cyclophilin D is required for mitochondrial removal by autophagy in cardiac cells. Autophagy 2010, 6
340. Elmore SP, Qian T, Grissom SF, Lemasters JJ: The mitochondrial permeability transition initiates autophagy in rat hepatocytes. Faseb J 2001, 15:2286-2287
341. Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, Ohsumi Y, Tokuhisa T, Mizushima N: The role of autophagy during the early neonatal starvation period. Nature 2004, 432:1032-1036
342. Stypmann J, Janssen PM, Prestle J, Engelen MA, Kogler H, Lullmann-Rauch R, Eckardt L, von Figura K, Landgrebe J, Mleczko A, Saftig P: LAMP-2 deficient mice show depressed cardiac contractile function without significant changes in calcium handling. Basic Res Cardiol 2006, 101:281-291
343. Nakai A, Yamaguchi O, Takeda T, Higuchi Y, Hikoso S, Taniike M, Omiya S, Mizote I, Matsumura Y, Asahi M, Nishida K, Hori M, Mizushima N, Otsu K: The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. Nat Med 2007, 13:619-624
344. Kim I, Rodriguez-Enriquez S, Lemasters JJ: Selective degradation of mitochondria by mitophagy. Arch Biochem Biophys 2007, 462:245-253
345. Klionsky DJ: Neurodegeneration: good riddance to bad rubbish. Nature 2006, 441:819-820
346. Komatsu M, Waguri S, Ueno T, Iwata J, Murata S, Tanida I, Ezaki J, Mizushima N, Ohsumi Y, Uchiyama Y, Kominami E, Tanaka K, Chiba T: Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J Cell Biol 2005, 169:425-434
347. Komatsu M, Ueno T, Waguri S, Uchiyama Y, Kominami E, Tanaka K: Constitutive autophagy: vital role in clearance of unfavorable proteins in neurons. Cell Death Differ 2007, 14:887-894

348. Ling YM, Shaw MH, Ayala C, Coppens I, Taylor GA, Ferguson DJ, Yap GS: Vacuolar and plasma membrane stripping and autophagic elimination of Toxoplasma gondii in primed effector macrophages. J Exp Med 2006, 203:2063-2071
349. Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V: Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell 2004, 119:753-766
350. Halonen SK: Role of autophagy in the host defense against Toxoplasma gondii in astrocytes. Autophagy 2009, 5:268-269
351. Kudchodkar SB, Levine B: Viruses and autophagy. Rev Med Virol 2009, 19:359-378
352. Clarke PG: Developmental cell death: morphological diversity and multiple mechanisms. Anat Embryol (Berl) 1990, 181:195-213
353. Schweichel JU, Merker HJ: The morphology of various types of cell death in prenatal tissues. Teratology 1973, 7:253-266
354. Jacobson MD, Weil M, Raff MC: Programmed cell death in animal development. Cell 1997, 88:347-354
355. Dawd DS, Hinchliffe JR: Cell death in the "opaque patch" in the central mesenchyme of the developing chick limb: a cytological, cytochemical and electron microscopic analysis. J Embryol Exp Morphol 1971, 26:401-424
356. Bursch W: The autophagosomal-lysosomal compartment in programmed cell death. Cell Death Differ 2001, 8:569-581
357. Bursch W, Ellinger A, Kienzl H, Torok L, Pandey S, Sikorska M, Walker R, Hermann RS: Active cell death induced by the anti-estrogens tamoxifen and ICI 164 384 in human mammary carcinoma cells (MCF-7) in culture: the role of autophagy. Carcinogenesis 1996, 17:1595-1607
358. Chi S, Kitanaka C, Noguchi K, Mochizuki T, Nagashima Y, Shirouzu M, Fujita H, Yoshida M, Chen W, Asai A, Himeno M, Yokoyama S, Kuchino Y: Oncogenic Ras triggers cell suicide through the activation of a caspase-independent cell death program in human cancer cells. Oncogene 1999, 18:2281-2290
359. Xue L, Fletcher GC, Tolkovsky AM: Autophagy is activated by apoptotic signalling in sympathetic neurons: an alternative mechanism of death execution. Mol Cell Neurosci 1999, 14:180-198
360. Kroemer G, Levine B: Autophagic cell death: the story of a misnomer. Nat Rev Mol Cell Biol 2008, 9:1004-1010
361. Lum JJ, Bauer DE, Kong M, Harris MH, Li C, Lindsten T, Thompson CB: Growth factor regulation of autophagy and cell survival in the absence of apoptosis. Cell 2005, 120:237-248
362. Shimizu S, Kanaseki T, Mizushima N, Mizuta T, Arakawa-Kobayashi S, Thompson CB, Tsujimoto Y: Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. Nat Cell Biol 2004, 6:1221-1228
363. Yu L, Wan F, Dutta S, Welsh S, Liu Z, Freundt E, Baehrecke EH, Lenardo M: Autophagic programmed cell death by selective catalase degradation. Proc Natl Acad Sci U S A 2006, 103:4952-4957
364. Xue L, Fletcher GC, Tolkovsky AM: Mitochondria are selectively eliminated from eukaryotic cells after blockade of caspases during apoptosis. Curr Biol 2001, 11:361-365

365. Inbal B, Bialik S, Sabanay I, Shani G, Kimchi A: DAP kinase and DRP-1 mediate membrane blebbing and the formation of autophagic vesicles during programmed cell death. J Cell Biol 2002, 157:455-468
366. Mills KR, Reginato M, Debnath J, Queenan B, Brugge JS: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is required for induction of autophagy during lumen formation in vitro. Proc Natl Acad Sci U S A 2004, 101:3438-3443
367. Thorburn J, Moore F, Rao A, Barclay WW, Thomas LR, Grant KW, Cramer SD, Thorburn A: Selective inactivation of a Fas-associated death domain protein (FADD)-dependent apoptosis and autophagy pathway in immortal epithelial cells. Mol Biol Cell 2005, 16:1189-1199
368. Scarlatti F, Bauvy C, Ventruti A, Sala G, Cluzeaud F, Vandewalle A, Ghidoni R, Codogno P: Ceramide-mediated macroautophagy involves inhibition of protein kinase B and up-regulation of beclin 1. J Biol Chem 2004, 279:18384-18391
369. Zhang XD, Wang Y, Wu JC, Lin F, Han R, Han F, Fukunaga K, Qin ZH: Down-regulation of Bcl-2 enhances autophagy activation and cell death induced by mitochondrial dysfunction in rat striatum. J Neurosci Res 2009, 87:3600-3610
370. Zhang J, Ney PA: Role of BNIP3 and NIX in cell death, autophagy, and mitophagy. Cell Death Differ 2009, 16:939-946
371. Yee KS, Wilkinson S, James J, Ryan KM, Vousden KH: PUMA- and Bax-induced autophagy contributes to apoptosis. Cell Death Differ 2009, 16:1135-1145
372. Maiuri MC, Tasdemir E, Criollo A, Morselli E, Vicencio JM, Carnuccio R, Kroemer G: Control of autophagy by oncogenes and tumor suppressor genes. Cell Death Differ 2009, 16:87-93
373. Lee CY, Baehrecke EH: Steroid regulation of autophagic programmed cell death during development. Development 2001, 128:1443-1455
374. Lee CY, Cooksey BA, Baehrecke EH: Steroid regulation of midgut cell death during Drosophila development. Dev Biol 2002, 250:101-111
375. Gorski SM, Chittaranjan S, Pleasance ED, Freeman JD, Anderson CL, Varhol RJ, Coughlin SM, Zuyderduyn SD, Jones SJ, Marra MA: A SAGE approach to discovery of genes involved in autophagic cell death. Curr Biol 2003, 13:358-363
376. Lee CY, Clough EA, Yellon P, Teslovich TM, Stephan DA, Baehrecke EH: Genome-wide analyses of steroid- and radiation-triggered programmed cell death in Drosophila. Curr Biol 2003, 13:350-357
377. Martin DN, Baehrecke EH: Caspases function in autophagic programmed cell death in Drosophila. Development 2004, 131:275-284
378. Kawahara A, Ohsawa Y, Matsumura H, Uchiyama Y, Nagata S: Caspase-independent cell killing by Fas-associated protein with death domain. J Cell Biol 1998, 143:1353-1360
379. Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, Tschopp J: Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 2000, 1:489-495
380. Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, Baehrecke EH, Lenardo MJ: Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. Science 2004, 304:1500-1502

381. Wei Y, Sinha S, Levine B: Dual role of JNK1-mediated phosphorylation of Bcl-2 in autophagy and apoptosis regulation. Autophagy 2008, 4:949-951
382. Zalckvar E, Berissi H, Mizrachy L, Idelchuk Y, Koren I, Eisenstein M, Sabanay H, Pinkas-Kramarski R, Kimchi A: DAP-kinase-mediated phosphorylation on the BH3 domain of beclin 1 promotes dissociation of beclin 1 from Bcl-XL and induction of autophagy. EMBO Rep 2009, 10:285-292
383. Betin VM, Lane JD: Caspase cleavage of Atg4D stimulates GABARAP-L1 processing and triggers mitochondrial targeting and apoptosis. J Cell Sci 2009, 122:2554-2566
384. Luo S, Rubinsztein DC: Apoptosis blocks Beclin 1-dependent autophagosome synthesis: an effect rescued by Bcl-xL. Cell Death Differ 2009, 17:268-277
385. Djavaheri-Mergny M, Maiuri MC, Kroemer G: Cross talk between apoptosis and autophagy by caspase-mediated cleavage of Beclin 1. Oncogene 2010, 29:1717-1719
386. Levine B, Kroemer G: Autophagy in the pathogenesis of disease. Cell 2008, 132:27-42
387. Mathew R, Karantza-Wadsworth V, White E: Role of autophagy in cancer. Nat Rev Cancer 2007, 7:961-967
388. Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, Mukherjee C, Shi Y, Gelinas C, Fan Y, Nelson DA, Jin S, White E: Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell 2006, 10:51-64
389. Yue Z, Jin S, Yang C, Levine AJ, Heintz N: Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc Natl Acad Sci U S A 2003, 100:15077-15082
390. Levine B, Sinha S, Kroemer G: Bcl-2 family members: dual regulators of apoptosis and autophagy. Autophagy 2008, 4:600-606
391. Guertin DA, Sabatini DM: Defining the role of mTOR in cancer. Cancer Cell 2007, 12:9-22
392. Mathew R, Kongara S, Beaudoin B, Karp CM, Bray K, Degenhardt K, Chen G, Jin S, White E: Autophagy suppresses tumor progression by limiting chromosomal instability. Genes Dev 2007, 21:1367-1381
393. Mathew R, Karp CM, Beaudoin B, Vuong N, Chen G, Chen HY, Bray K, Reddy A, Bhanot G, Gelinas C, Dipaola RS, Karantza-Wadsworth V, White E: Autophagy suppresses tumorigenesis through elimination of p62. Cell 2009, 137:1062-1075
394. Mizushima N: Methods for monitoring autophagy. Int J Biochem Cell Biol 2004, 36:2491-2502
395. Kimura S, Noda T, Yoshimori T: Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. Autophagy 2007, 3:452-460
396. Roberts P, Moshitch-Moshkovitz S, Kvam E, O'Toole E, Winey M, Goldfarb DS: Piecemeal microautophagy of nucleus in Saccharomyces cerevisiae. Mol Biol Cell 2003, 14:129-141
397. Kovacs AL, Rez G, Palfia Z, Kovacs J: Autophagy in the epithelial cells of murine seminal vesicle in vitro. Formation of large sheets of nascent isolation

membranes, sequestration of the nucleus and inhibition by wortmannin and 3-ethyladenine. Cell Tissue Res 2000, 302:253-261
398. Narita M, Young AR: Autophagy facilitates oncogene-induced senescence. Autophagy 2009, 5:1046-1047
399. Young AR, Narita M, Ferreira M, Kirschner K, Sadaie M, Darot JF, Tavare S, Arakawa S, Shimizu S, Watt FM: Autophagy mediates the mitotic senescence transition. Genes Dev 2009, 23:798-803
400. Sasaki M, Miyakoshi M, Sato Y, Nakanuma Y: Autophagy mediates the process of cellular senescence characterizing bile duct damages in primary biliary cirrhosis. Lab Invest 2010, 90:835-843
401. Luqman S, Pezzuto JM: NFkappaB: a promising target for natural products in cancer chemoprevention. Phytother Res 2010, 24:949-963
402. Gosselin K, Martien S, Pourtier A, Vercamer C, Ostoich P, Morat L, Sabatier L, Duprez L, T'Kint de Roodenbeke C, Gilson E, Malaquin N, Wernert N, Slijepcevic P, Ashtari M, Chelli F, Deruy E, Vandenbunder B, De Launoit Y, Abbadie C: Senescence-associated oxidative DNA damage promotes the generation of neoplastic cells. Cancer Res 2009, 69:7917-7925
403. Russell SE, Hickey GI, Lowry WS, White P, Atkinson RJ: Allele loss from chromosome 17 in ovarian cancer. Oncogene 1990, 5:1581-1583
404. Eccles DM, Russell SE, Haites NE, Atkinson R, Bell DW, Gruber L, Hickey I, Kelly K, Kitchener H, Leonard R, et al.: Early loss of heterozygosity on 17q in ovarian cancer. The Abe Ovarian Cancer Genetics Group. Oncogene 1992, 7:2069-2072
405. Cliby W, Ritland S, Hartmann L, Dodson M, Halling KC, Keeney G, Podratz KC, Jenkins RB: Human epithelial ovarian cancer allelotype. Cancer Res 1993, 53:2393-2398
406. Tangir J, Muto MG, Berkowitz RS, Welch WR, Bell DA, Mok SC: A 400 kb novel deletion unit centromeric to the BRCA1 gene in sporadic epithelial ovarian cancer. Oncogene 1996, 12:735-740
407. Futreal PA, Soderkvist P, Marks JR, Iglehart JD, Cochran C, Barrett JC, Wiseman RW: Detection of frequent allelic loss on proximal chromosome 17q in sporadic breast carcinoma using microsatellite length polymorphisms. Cancer Res 1992, 52:2624-2627
408. Saito H, Inazawa J, Saito S, Kasumi F, Koi S, Sagae S, Kudo R, Saito J, Noda K, Nakamura Y: Detailed deletion mapping of chromosome 17q in ovarian and breast cancers: 2-cM region on 17q21.3 often and commonly deleted in tumors. Cancer Res 1993, 53:3382-3385
409. Gao X, Zacharek A, Salkowski A, Grignon DJ, Sakr W, Porter AT, Honn KV: Loss of heterozygosity of the BRCA1 and other loci on chromosome 17q in human prostate cancer. Cancer Res 1995, 55:1002-1005
410. Ionov Y, Nowak N, Perucho M, Markowitz S, Cowell JK: Manipulation of nonsense mediated decay identifies gene mutations in colon cancer Cells with microsatellite instability. Oncogene 2004, 23:639-645
411. Lee WC, Balsara B, Liu Z, Jhanwar SC, Testa JR: Loss of heterozygosity analysis defines a critical region in chromosome 1p22 commonly deleted in human malignant mesothelioma. Cancer Res 1996, 56:4297-4301

412. Wang J, Yi J: Cancer cell killing via ROS: to increase or decrease, that is the question. Cancer Biol Ther 2008, 7:1875-1884
413. Collado M, Serrano M: Senescence in tumours: evidence from mice and humans. Nat Rev Cancer 2010, 10:51-57