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LISTE DES ABRÉVIATIONS, SIGLES ET ACRONYMES

ADN	Acide désoxyribonucléique
AKT	Protéine kinase B
APAF	Facteur d'activation de peptidase apoptotique
ARN	Acide ribonucléique
ARNm	Acide ribonucléique messager
ATP	Adénosine triphosphate
BID	Agoniste de mort interagissant avec le domaine BH3
CA-AKT	Protéine kinase B constitutivement active
CTC	Cellule tumorale circulante
Cyt *c*	Cytochrome c
DED	Domaine effecteur de mort
DR	Récepteur de mort
EGF	Facteur de croissance épidermique
EMT	Transition épithéliale-mésenchymateuse
ERK	Kinase régulée par un signal extracellulaire
FADD	Protéine associée au domaine de mort du récepteur Fas
FasR	Récepteur de mort
FasL	Ligand du récepteur Fas
FIs	Filaments intermédiaires
FLICE	Protéase inductrice de l'apoptose
FLIP	Protéine inhibitrice de FLICE
GAPDH	GIycéraldéhyde-3-phosphate déshydrogénase
GFAP	Protéine acide fibrillaire gliale
GTP	Guanosine triphosphate
HGF	Facteur de croissance des hépatocytes
IAP	Protéine inhibitrice de l'apoptose
ILK	Kinase liée aux intégrines

K8/18	Hétérodimère de kératines 8 et 18
MAPK	Protéine kinase activée par un mitogène
MEC	Matrice extracellulaire
MFs	Microfilaments d'actine
MMP	Métalloprotéases matricielles
mTORC2	Complexe 2 de la cible mammalienne de la rapamycine
MTs	Microtubules
NF-κB	Facteur nucléaire kappa B
PARP	PolyADP-ribose polymérase
PDGF	Facteur de croissance dérivé des plaquettes
PDK	Kinase dépendante des phosphoinositides
PH	Domaine d'homologie avec la plecstrine
PIP2	Phosphatidylinositol (4, 5)-biphosphate
PIP3	Phosphatidylinositol (3, 4, 5)-triphosphate
PI3K	Phosphatidylinositol 3-kinase
PKC	Protéine kinase C
PTEN	Phosphatase et homologue de la tensine
RTK	Récepteurs à activité tyrosine kinase
shRNA	Petit ARN interférent en épingle à cheveux (small hairpin RNA)
siRNA	Petit ARN interférent (small interfering RNA)
TGF-β	Facteur de croissance transformant beta
TNF	Facteur de nécrose tumorale
TNFR	Récepteur du TNF
TRADD	Protéine associée au domaine de mort du récepteur TNF
TRAIL	Ligand des récepteurs de mort de la famille du TNF
VEGF	Facteur de croissance de l'endothélium vasculaire
XIAP	Protéine inhibitrice de l'apoptose liée au chromosome X
18S rRNA	Sous-unité 18S de l'ARN ribosomal

CHAPITRE I

INTRODUCTION

1.1 La progression tumorale

Le processus cancéreux est caractérisé par une prolifération anormale et incontrôlée de cellules ayant subi des altérations génétiques (mutations, délétions, amplifications génomiques) ou épigénétiques (méthylation de gènes, acétylation des histones) conduisant à leur transformation (Shen et al. 2013). Ces anomalies peuvent être spontanées ou héréditaires et vont cibler des gènes essentiels dans l'homéostasie cellulaire : les oncogènes, qui stimulent la progression tumorale (par exemple *PIK3CA*, *MYC*, *KRAS* et *ERBB2*) et les gènes suppresseurs de tumeurs, qui freinent cette progression (par exemple PTEN et p53) (Shortt et al. 2012). Ces modifications génétiques vont conférer aux cellules cancéreuses un avantage sélectif menant à l'apparition d'une tumeur, suite à l'acquisition de diverses propriétés ayant pour conséquences leur immortalisation et la perte d'homéostasie (Figure 1.1). L'homéostasie se définit par l'état d'équilibre entre la prolifération et la mort cellulaire, ce qui assure le maintien de la taille et de la fonctionnalité d'un organe. Au sein d'une tumeur, les cellules cancéreuses perdent la sensibilité aux signaux qui maintiennent l'homéostasie. En effet, ces dernières deviennent incapables d'initier leur propre mort (apoptose) ou de répondre aux signaux extérieurs qui la déclenchent suite à l'inactivation des mécanismes moléculaires régulant l'apoptose. De plus, les cellules cancéreuses sont capables de proliférer en absence de stimulation suite à l'activation constitutive des voies de signalisation régulant ce processus, contrairement aux cellules normales qui ne se divisent que lorsqu'elles reçoivent un signal particulier tel que la présence de facteur de croissance. Les cellules tumorales sont aussi capables de stimuler la formation de nouveaux vaisseaux sanguins indispensables à la croissance de la tumeur, dont les besoins en oxygène sont importants. Enfin, l'acquisition d'un potentiel invasif par les cellules cancéreuses initie la formation de métastases du cancer (Hanahan et al. 2011).

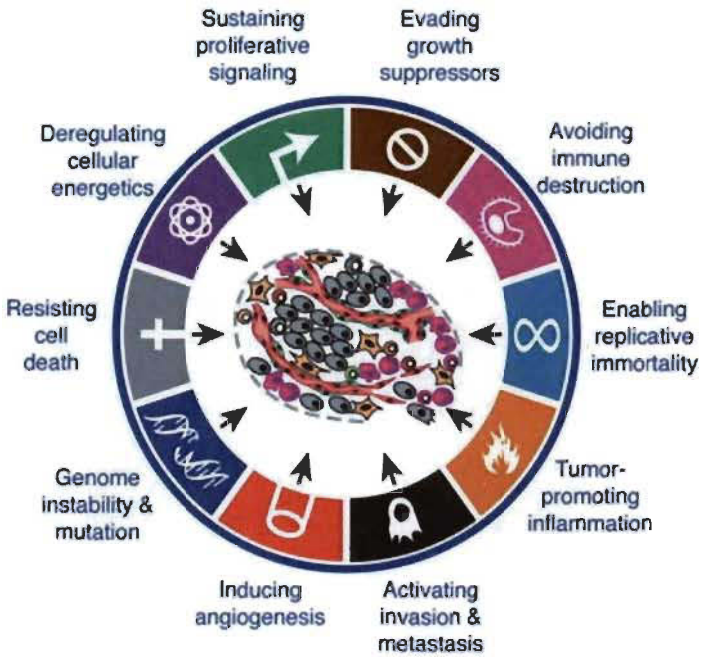


Figure 1.1 **Les dix propriétés du cancer.**
Ces processus distincts et complémentaires, qui favorisent la croissance tumorale et la formation des métastases, constituent une base solide pour la compréhension de la biologie du cancer et l'élaboration de stratégies thérapeutiques (Hanahan et al. 2011).

En absence de traitement, le cancer se développe d'abord de manière locale. Il provoque dans ce cas une compression des organes voisins, voire un envahissement et une destruction des tissus adjacents. À un stade avancé, le cancer envahit les ganglions lymphatiques et la circulation sanguine et se propage à distance. L'évolution dépend du type du cancer et de sa prise en charge : certains ne font que très peu de métastases et sont sensibles aux traitements permettant dans la grande majorité des cas une rémission complète et prolongée. D'autres sont difficilement maîtrisables et peuvent entraîner le décès à court terme.

Le traitement repose principalement sur l'exérèse chirurgicale de la tumeur, quand cela est possible, sur la radiothérapie et la chimiothérapie. Certains cancers peuvent bénéficier également d'un traitement hormonal visant à empêcher l'action proliférative des hormones sur la tumeur, ou immunologique consistant à augmenter la défense immunitaire contre la tumeur.

La chimiothérapie consiste en l'utilisation de substances chimiques capables d'interférer avec le processus de division cellulaire, la mitose. La chimiothérapie est donc particulièrement efficace sur les cellules qui se divisent activement telles que les cellules cancéreuses. Cependant, d'autres cellules à division rapide sont aussi affectées par le traitement, telles les cellules du bulbe pileux, les cellules intestinales et les cellules sanguines, expliquant les effets secondaires couramment rencontrés comme la perte des cheveux. Les cellules cancéreuses peuvent ne pas être sensibles à un ou à plusieurs agents chimiothérapeutiques. On dit alors qu'elles sont chimiorésistantes.

1.1.1 La chimiorésistance

La chimiorésistance est un problème majeur dans le traitement des cancers. Les tumeurs peuvent présenter une résistance intrinsèque ou acquise qui implique plusieurs mécanismes (Figure 1.2).

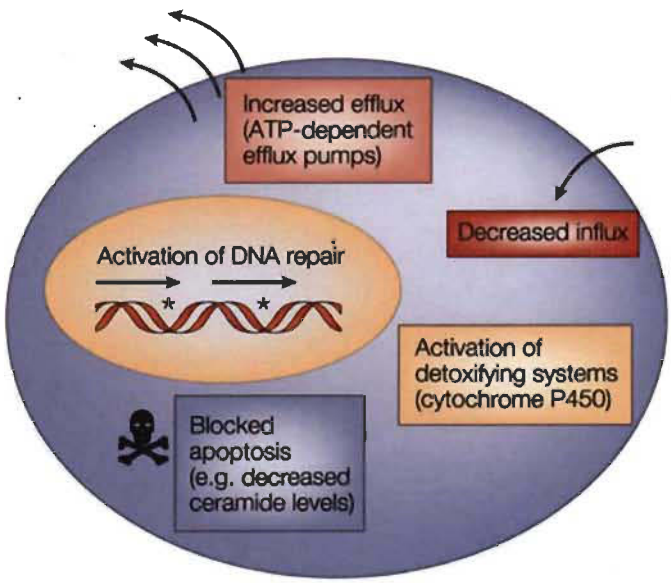


Figure 1.2 **Mécanismes cellulaires responsables de la chimiorésistance.** (Gottesman et al. 2002).

L'entrée de la drogue à l'intérieur de la cellule peut être diminuée et sa sortie facilitée. Parmi les nombreuses protéines membranaires responsables de ce type de résistance, on retrouve notamment les transporteurs ABC (ou transporteurs à *ATP*

binding cassette) capables d'assurer le transport de médicaments et, par là-même, de jouer un rôle dans la biodistribution de ces molécules, affectant ainsi la réponse thérapeutique (Wu et al. 2011).

Différents enzymes impliqués dans le métabolisme des drogues peuvent aussi induire une chimiorésistance. L'inactivation enzymatique diminue la quantité de drogue pouvant se lier à sa molécule cible intracellulaire. Parmi ces enzymes on peut citer la dihydropyrimidine déshydrogénase (DDP), la famille des glutathion S-transférases (GSH) et les métallothionéines (Longley et al. 2005).

La réponse cellulaire suite à un dommage au niveau de l'ADN est de réparer l'altération ou d'enclencher le processus apoptotique. Par conséquent, cette réponse a un impact direct sur la chimiorésistance des tumeurs. La réparation par excision de nucléotides ou NER (pour *nucleotide excision repair*) est un des systèmes naturels permettant la réparation de l'ADN dégradé. Toutefois, ce processus est particulièrement actif dans certaines tumeurs, conférant ainsi une résistance à l'effet cytotoxique des agents intercalants (Furuta et al. 2002). La réparation des mésappariements ou MMR (pour *mismatch repair*) est un autre mécanisme essentiel pour maintenir l'intégrité de l'information génétique contenue dans le génome au cours des multiples divisions cellulaires. L'inactivation de ce processus engendre une chimiorésistance induite par l'incapacité de la cellule à détecter la présence de dommages à l'ADN et d'activer l'apoptose (Fink et al. 1998).

La résistance à un agent chimiothérapeutique est souvent multifactorielle. Par exemple, l'activité du cisplatine peut être diminuée à la fois par son accumulation réduite dans le cytoplasme, par son inhibition suite à l'activité enzymatique des glutathion S-tranférases et par la réparation des dommages à l'ADN qu'il provoque (Shen et al. 2012).

1.1.2 La formation des métastases

La principale cause de mortalité du cancer est la formation de tumeurs secondaires dans les organes vitaux, les *métastases*. Les cellules cancéreuses peuvent acquérir certaines propriétés qui leur permettent d'envahir les tissus voisins, gagnant ainsi les canaux lymphatiques et les vaisseaux sanguins, par l'intermédiaire desquels elles peuvent être transportées dans d'autres organes.

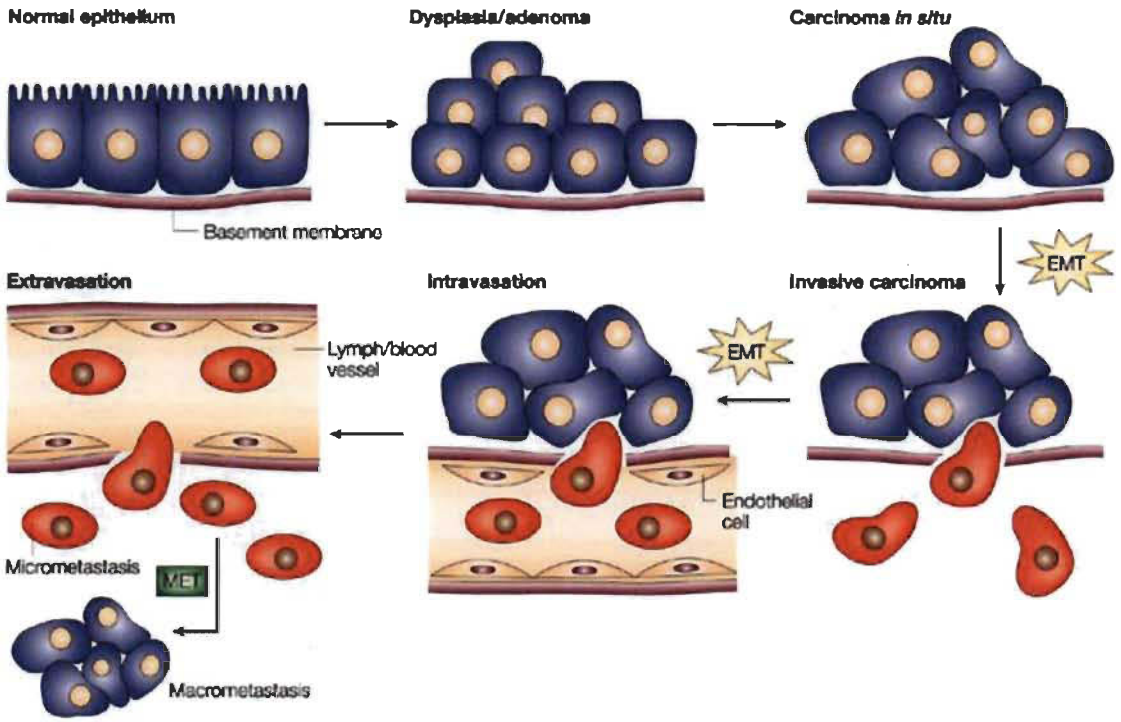


Figure 1.3 **Étapes de la formation des métastases.**
(Thiery 2002).

Chaque étape du processus métastatique représente un obstacle que seul un petit nombre de cellules cancéreuses réussiront à franchir. Les cellules de la tumeur primaire doivent d'abord envahir la matrice extracellulaire (MEC) et les différentes couches de cellules stromales du tissu environnant. La dégradation de la MEC est principalement effectuée par des enzymes appelées métalloprotéases matricielles (MMP) dont l'activité est anormalement élevée dans les cellules tumorales (Kessenbrock et al. 2010). L'interaction des cellules tumorales avec les cellules stromales (fibroblastes, adipocytes,

macrophages et autres cellules immunitaires) favorise leur prolifération et leur migration via la transduction de signaux initiés par des molécules d'adhésion telles que les intégrines (Joyce et al. 2009; Desgrosellier et al. 2010).

Les cellules tumorales doivent ensuite franchir la paroi des vaisseaux sanguins formée de cellules endothéliales et de péricytes. Cette étape, appelée intravasation, peut être facilitée par la sécrétion du facteur de croissance de l'endothélium vasculaire (VEGF) par les cellules cancéreuses elles-mêmes. Ce facteur stimule la formation de nouveaux vaisseaux sanguins à l'intérieur du microenvironnement tumoral via un processus appelé néo-vascularisation. Contrairement aux vaisseaux sanguins présents dans les tissus normaux, la néo-vascularisation produite par les cellules tumorales est fragile. La faible cohésion entre les cellules endothéliales adjacentes qui tapissent le vaisseau sanguin et l'absence d'une couverture de péricytes facilitent donc l'intravasation des cellules cancéreuses (Carmeliet et al. 2011).

Une fois la barrière endothéliale franchie, les cellules tumorales peuvent diffuser largement à travers la circulation veineuse et artérielle. Les récents progrès technologiques ont facilité la détection de cellules tumorales circulantes (CTC) dans le sang des patients (Nagrath et al. 2007). Les CTC doivent toutefois survivre à une variété de stress afin de parvenir à des organes éloignés. Par exemple, l'adhésion des cellules épithéliales à la MEC via les intégrines est normalement essentielle à la survie de la cellule. En l'absence d'un tel ancrage, les cellules épithéliales subissent l'anoïkis, une forme de mort cellulaire déclenchée par la perte d'ancrage au substrat (Guo et al. 2004). Les CTC doivent donc développer un mécanisme de résistance à l'anoïkis. De plus, les CTC doivent résister aux agressions du système immunitaire inné, régies par les cellules tueuses naturelles (ou cellules NK, pour *natural killer*). Une fois introduites dans la vascularisation d'organes éloignés, les CTC peuvent initier la croissance d'une tumeur secondaire suite à leur extravasation dans le parenchyme. Dans la grande majorité des cas, les cellules tumorales disséminées entrent dans un état de latence sous forme de micrométastases. Ces cellules dormantes sont insensibles à la chimiothérapie traditionnelle qui vise les cellules à division rapide. Elles sont donc responsables de

l'apparition de métastases tardives (quelques mois ou années après la tumeur primaire) (Chambers et al. 2002; Naumov et al. 2002). La formation de métastases nécessite que les CTC prolifèrent dans le microenvironnement étranger, générant ainsi des néoplasmes macroscopiques cliniquement décelables.

1.2 La transition épithéliale-mésenchymateuse dans la progression tumorale

Un des mécanismes cellulaires impliqués dans le processus métastatique est la transition épithéliale-mésenchymateuse (ou EMT, pour *epithelial-mesenchymal transition*) (Figure 1.3). Cette appellation fait référence aux deux types cellulaires, épithélial (parenchyme) et stromal (mésenchyme), dont les caractéristiques morphologiques et fonctionnelles sont distinctes (Figure 1.4). Les cellules épithéliales sont étroitement associées entre elles et reposent sur une membrane basale, ce qui leur confère une polarité apico-basale nécessaire à leur fonction. Les cellules mésenchymateuses n'ont pas de contact entre elles et possèdent une polarité directionnelle favorisant leur potentiel migratoire.

L'EMT consiste donc en une série d'événements morphogénétiques au cours desquels la polarité apico-basale est perdue, les jonctions intercellulaires sont altérées, le cytosquelette et la MEC sont modifiés et la transcription de gènes favorisant la motilité est induite (Kalluri et al. 2009). La cohésion intercellulaire est rompue suite à la diminution entre autres de l'expression de l'E-cadhérine et de ZO-1, formant les jonctions adhérentes et serrées respectivement. La réorganisation du cytosquelette se manifeste par la diminution ou la perte de l'expression des kératines en faveur de la vimentine et par la polymérisation de l'actine à la périphérie de la cellule. La laminine, constituant essentiel de la membrane basale, fait place à la fibronectine qui favorise l'adhésion des cellules à la MEC. La sécrétion de MMP est enclenchée afin de dégrader le tissu environnant. Enfin, une multitude d'activateurs ou de répresseurs de la transcription tels que Snail, Slug et Twist interviennent pour favoriser le phénotype mésenchymateux (Yang et al. 2004; Medici et al. 2008; Kalluri et al. 2009). L'EMT illustre de façon remarquable la plasticité phénotypique des cellules épithéliales.

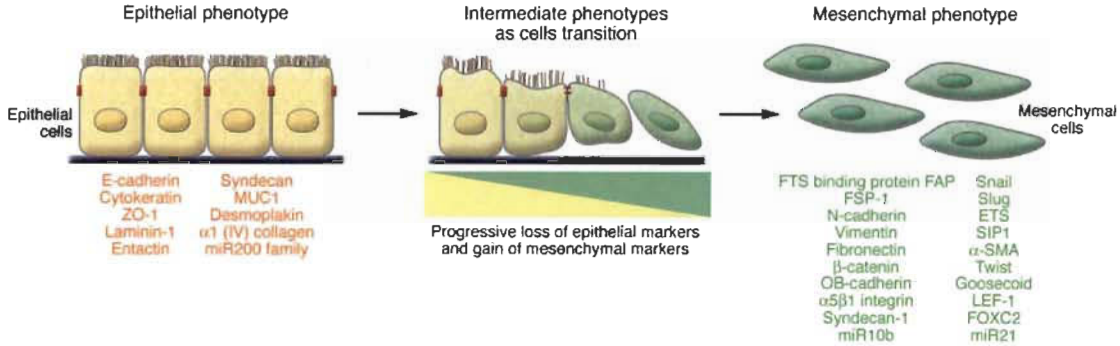


Figure 1.4 **Les différents marqueurs de l'EMT.**
Une EMT implique une transition fonctionnelle des cellules épithéliales polarisées en cellules mésenchymateuses motiles et sécrétrices de composantes de la matrice extracellulaire (Kalluri et al. 2009).

Ce sont les travaux de Garry Greenburg et Elizabeth Hay dans les années 1980 qui ont révélé l'existence de la transition entre ces deux phénotypes au cours du développement embryonnaire (Greenburg et al. 1982). En effet, ce processus est essentiel au cours du développement précoce de l'embryon. Il permet entre autres la formation des trois feuillets primordiaux durant la gastrulation (Larsen 2003). Les cellules épiblastiques perdent leur organisation et migrent comme des cellules mésenchymateuses afin de mettre en place les différents tissus et organes de l'embryon (Nakaya et al. 2009) (Figure 1.5, *A*). Ce mésenchyme primaire peut être à nouveau amené à former un épithélium secondaire par un processus de transition inverse (MET, pour *mesenchymal-epithelial transition*). La plasticité phénotypique de l'EMT s'observe tout au long du développement fœtal, dès l'implantation de l'embryon jusqu'à la formation des valves cardiaques en passant par le développement de la crête neurale (Kalluri et al. 2009).

L'EMT est aussi observée lors de la fibrose des tissus tels que les reins, le foie, le poumon et l'intestin (Kim et al. 2006; Zeisberg et al. 2007; Zeisberg et al. 2007). La fibrose des tissus est médiée par des cellules inflammatoires et des fibroblastes qui libèrent une variété de signaux inflammatoires ainsi que des composants de la MEC. Les premières observations d'une EMT dans ce contexte pathologique ont été faites chez des souris transgéniques exprimant des gènes rapporteurs sous l'action de promoteurs spécifiques à l'épithélium. Ces études ont fourni une preuve directe que les cellules épithéliales, par l'intermédiaire de l'EMT, peuvent être à l'origine des fibroblastes

retrouvés au site d'inflammation (Strutz et al. 1995; Okada et al. 1997; Rastaldi et al. 2002). L'identification de cellules hybrides, exprimant à la fois des marqueurs épithéliaux et mésenchymateux, a permis de constater que, sous la contrainte inflammatoire, les cellules épithéliales peuvent acquérir des propriétés mésenchymateuses à des degrés divers, introduisant ainsi la notion de « EMT intermédiaire ». Finalement, ces cellules vont quitter l'épithélium en traversant la membrane basale sous-jacente et vont s'accumuler dans le tissu interstitiel où elles auront complètement perdu leur phénotype épithélial pour exercer leur rôle de fibroblastes (Figure 1.5, *B*).

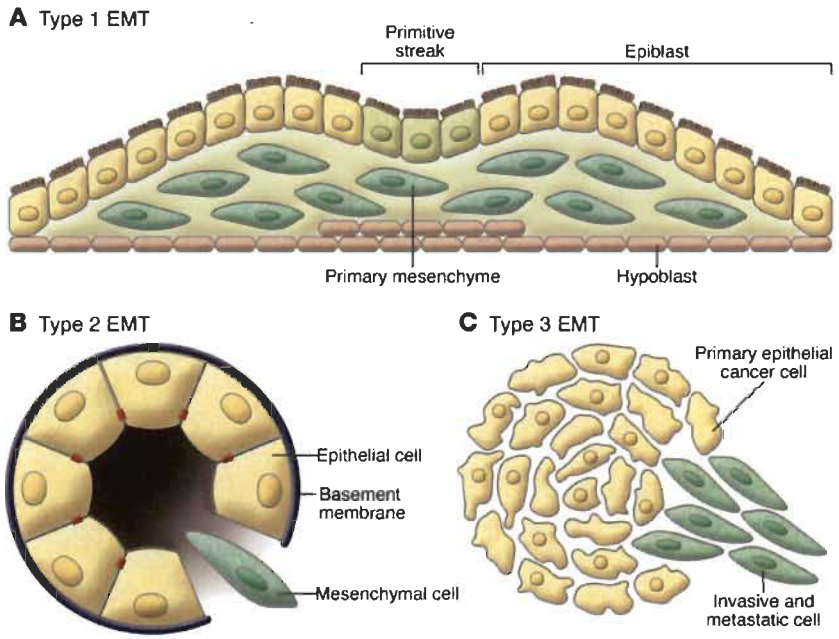


Figure 1.5 **Les différents types d'EMT.**
A, l'induction du mésoderme commence dans une zone spécifique de l'ectoderme primitif appelée la ligne primitive (primitive streak). *B*, l'EMT contribue à l'accumulation de composantes extracellulaire dans le tissu conjonctif. *C*, le potentiel invasif des cellules épithéliales cancéreuses est favorisé par l'EMT (Kalluri et al. 2009).

Il a fallu beaucoup de temps pour que l'EMT soit reconnue comme un mécanisme potentiel de progression du carcinome (Figure 1.5, *C*). L'une des principales raisons est que l'EMT ne peut être suivie dans le temps et l'espace au sein des tumeurs humaines. La grande diversité de types cellulaires qui est observée dans une tumeur empêche les pathologistes de reconnaître l'EMT sans ambiguïté. Le stroma tumoral contient des cellules endothéliales et des péricytes, qui permettent la vascularisation de la tumeur, des

fibroblastes ainsi que diverses cellules immunitaires telles que des macrophages, des neutrophiles, des mastocytes et des cellules myéloïdes suppressives (Joyce et al. 2009). Malgré le manque d'évidences cliniques, les mécanismes génétiques et épigénétiques qui sous-tendent l'acquisition du phénotype invasif et la dissémination subséquente des cellules tumorales ont fait l'objet de nombreuses études au cours de la dernière décennie. Bon nombre d'entre elles ont proposé que l'EMT représente un processus essentiel pour la progression maligne du cancer (Thiery 2002). Les cellules cancéreuses de type mésenchymateux sont généralement détectées en périphérie de la tumeur primaire et finissent par entrer dans les étapes ultérieures de la cascade de l'invasion métastatique, soit l'intravasation, le transport à travers la circulation sanguine ou lymphatique, l'extravasation, la formation de micrométastases et finalement la colonisation de l'organe secondaire (Figure 1.6). La formation de tumeurs secondaires nécessite que les cellules métastatiques subissent une transformation phénotypique inverse telle qu'observée au cours de l'embryogenèse, une MET, afin de s'intégrer à l'épithélium colonisé. En effet, d'un point de vue histo-pathologique, les tumeurs secondaires ressemblent à la tumeur primaire d'où elles sont issues.

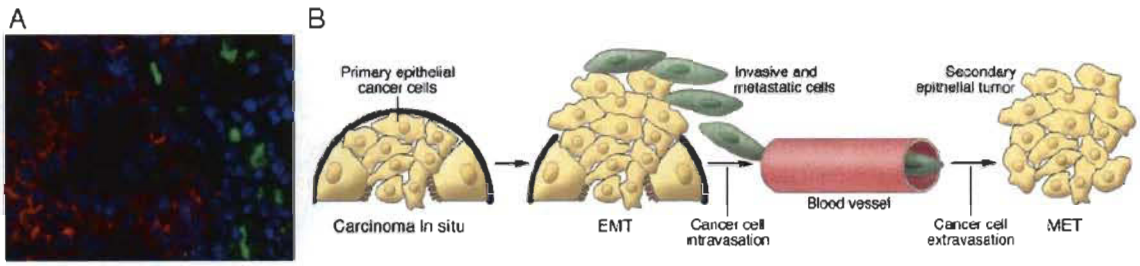


Figure 1.6 **Rôle de l'EMT dans la progression tumorale.**
A, La présence de macrophages en périphérie d'un carcinome pancréatique (vert) est associée à une perte d'expression de la E-cadhérine par les cellules épithéliales cancéreuses avoisinantes (Joyce et al. 2009), *B* Étapes de la métastasie (Kalluri et al. 2009).

La liste des signaux cellulaires qui contribuent à une EMT des cellules cancéreuses ne cesse de croître. Une hypothèse actuellement partagée par de nombreux chercheurs propose que les altérations génétiques et épigénétiques subies par les cellules cancéreuses au cours de la tumorigénèse les rendent propices à l'intégration des signaux provenant du microenvironnement tumoral. Ce sont des facteurs de croissance (notamment HGF, EGF, PDGF et TGF-β) et des composants de la matrice

extracellulaire (fibronectine, collagène et hyaluronane) qui, en se liant à des récepteurs spécifiques de la membrane cellulaire, engendrent différentes cascades de signalisation intracellulaire (Figure 1.7).

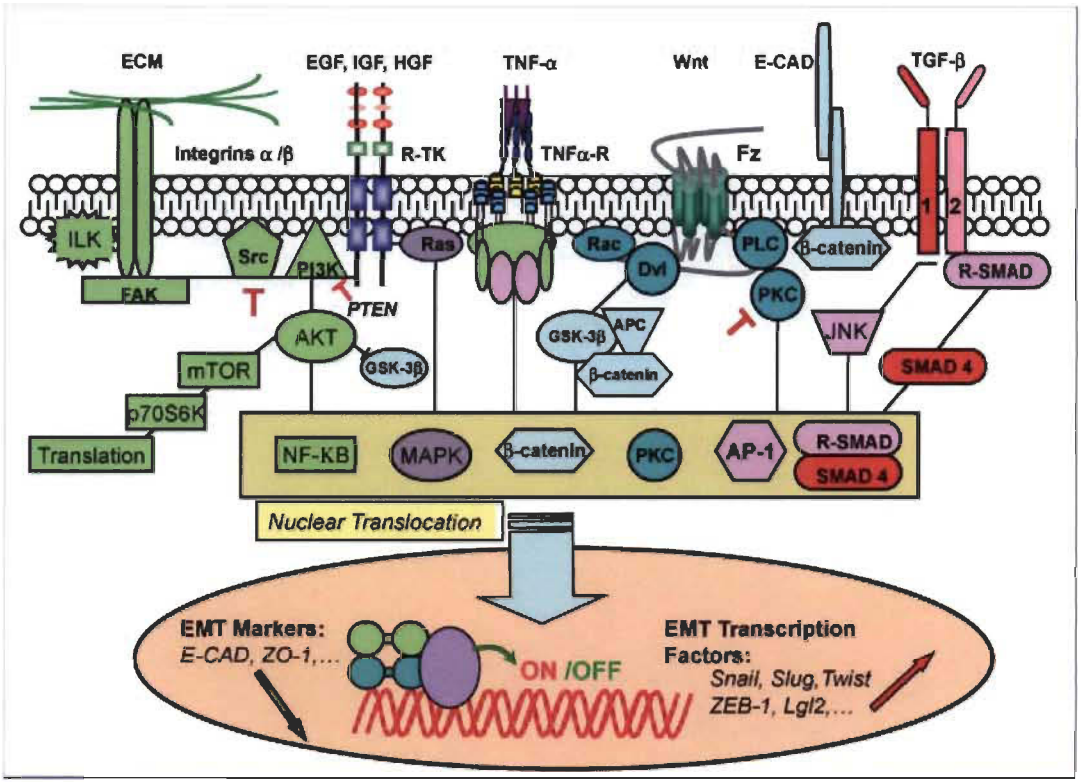


Figure 1.7 **Voies de signalisation impliquées dans l'EMT.**
(Sabbah et al. 2008).

Une des principales voies de signalisation qui favorise l'EMT est celle médiée par la famille de protéines kinases Akt. La première démonstration directe du rôle d'Akt dans l'EMT a été réalisée à l'aide d'un modèle de cellules cancéreuses qui, suite à l'expression ectopique de la kinase Akt active, ont subi une EMT caractérisée par la diminution de l'E-cadhérine et l'expression de la vimentine (Grille et al. 2003). Les cytokines TGF-β et TNF-α induisent l'EMT en recrutant de nombreux effecteurs dont Akt (Bakin et al. 2000; Lamouille et al. 2011; Wang et al. 2013). En effet, Akt permet la stabilisation de Snail (Bachelder et al. 2005) et collabore avec ce facteur de transcription pour inhiber l'expression de l'E-cadhérine (Villagrasa et al. 2012). Akt permet aussi la translocation nucléaire des facteurs de transcription NF-κB, médiateur de l'expression des MMP et de la vimentine (Min et al. 2008), et β-caténine qui induit l'expression de

Slug (Conacci-Sorrell et al. 2003). D'autres voies de signalisation permettent aussi l'activation de ces facteurs nucléaires, notamment celle régulée par les protéines SMAD en réponse au facteur de croissance TGF-β (Katsuno et al. 2013). La transduction de ces différents signaux s'achève par l'acquisition des caractéristiques morphologiques de la cellule mésenchymateuse qui nécessite l'altération des jonctions intercellulaires, la modification de la MEC et la réorganisation du cytosquelette d'actine et de filaments intermédiaires.

1.3 Le cytosquelette de filaments intermédiaires

Le cytosquelette consiste en un ensemble de plusieurs réseaux filamenteux qui s'étendent de la membrane plasmique à la membrane nucléaire, jusqu'à l'intérieur du noyau. Il permet l'ancrage de la cellule avec les cellules environnantes et la matrice extracellulaire, il maintient l'intégrité structurelle des cellules, joue un rôle dans la motilité, la réplication, l'apoptose, la différenciation et la signalisation cellulaire. Le cytosquelette est constitué de trois structures filamenteuses qui forment un réseau complexe à l'intérieur du cytosol: les microfilaments d'actine (MFs), les microtubules (MTs) et les filaments intermédiaires (FIs).

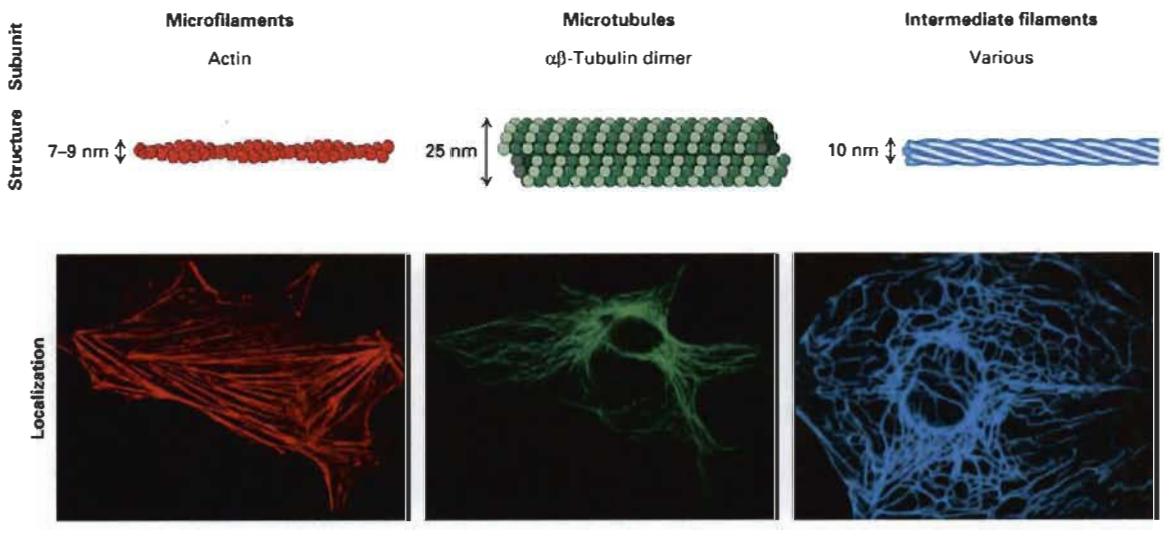


Figure 1.8 **Les trois réseaux de filaments formant le cytosquelette.**
(Lodish et al. 2012).

Les MFs sont des polymères d'actine dont l'assemblage nécessite la présence d'ATP. Les filaments d'actine sont polarisés et peuvent être organisés en un réseau plus ou moins dense suivant le type de cellule et la fonction qu'ils occupent. Ils jouent un rôle essentiel dans le maintien de la structure cellulaire en organisant la membrane plasmique et les jonctions intercellulaires. Les MFs sont connus pour jouer un rôle dans le mouvement des cellules et la contraction musculaire (Lodish et al. 2012).

Les MTs sont formés suite à la polymérisation de la tubuline alpha et bêta en présence de GTP. En raison de leur structure rigide, les MTs représentent un support mécanique considérable pour la cellule. De plus, ils sont impliqués dans la ségrégation des chromosomes au cours de la mitose, le maintien de l'organisation interne de la cellule et le transport vésiculaire (Lodish et al. 2012).

Contrairement aux MTs et aux MFs, le rôle des FIs dans les différentes fonctions cellulaires n'est pas encore entièrement compris. L'étude des FIs s'est avérée plus laborieuse étant donné la grande diversité de protéines incluses dans cette famille. En effet, les protéines de FIs sont codées par 67 gènes différents dont l'expression dépend du tissu et du stade de différenciation (Chang et al. 2004). Cependant, les protéines de FIs présentent toutes une structure similaire permettant ainsi la formation de filaments qui se ressemblent. Le domaine central de la protéine est constitué de quatre hélices alpha assemblées en spirale. Le corps du filament est formé de plusieurs segments (1A, 1B, 2A, 2B) séparés par de courtes régions intercalaires (L1, L12, L2) (Figure 1.9). La présence de certaines séquences très conservées au niveau de ce domaine central suggère qu'il est impliqué dans l'assemblage du filament. Les extrémités amino- et carboxy-terminales sont constituées de domaines hétérogènes. Elles varient en longueur et en séquence selon le type de filament et elles sont sujettes à des modifications post-traductionnelles. Il apparaît donc que les extrémités du filament participent à la régulation de la fonction des FIs (Omary et al. 2006). Suivant le type de FIs, ces monomères s'associent parallèlement en homodimères ou en hétérodimères. Par la suite, l'agrégation antiparallèle de deux dimères identiques forme un tétramère non polarisé. L'absence de polarité du filament intermédiaire le distingue des autres éléments du

cytosquelette. Le tétramère constitue l'unité de base pour l'assemblage du filament intermédiaire.

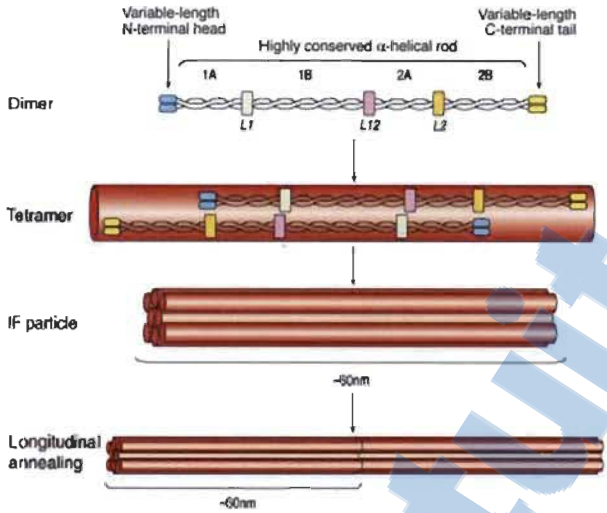


Figure 1.9 **Structure des protéines de filaments intermédiaires.**
Adapté de (Godsel et al. 2008).

Au fil des recherches concernant les protéines de FIs, il est devenu évident que différents types cellulaires possèdent différents types de protéines du cytosquelette de FIs. Par exemple, les cellules épithéliales contiennent principalement des kératines, les cellules musculaires expriment la desmine, la vimentine est spécifique aux cellules mésenchymateuses et les neurones contiennent des neurofilaments. Toutefois, certains types de cellules peuvent exprimer plusieurs protéines de FIs en fonction du stade de différenciation. Par exemple, la vimentine est détectée en début de développement musculaire, mais est absente des cellules musculaires. Les cellules cancéreuses peuvent aussi exprimer plusieurs types de FIs tel que le démontre la figure suivante.

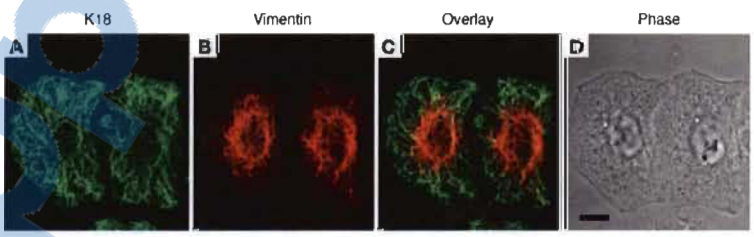


Figure 1.10 **Co-expression de deux types de filaments intermédiaires dans une même cellule.**
Marquage par immunofluorescence de la kératine 18 (*A*) et de la vimentine (*B*) chez des cellules d'un carcinome bronchiolo-alvéolaire. (*C*) Superposition des images *A* et *B*. (*D*) Contraste de phase. (Eriksson et al. 2009).

La classification des FIs en fonction de leur expression spécifique dans les différents types cellulaires étant donc très complexe, ce sont plutôt les similitudes dans la séquence du domaine central des protéines qui a permis d'élaborer six grands types de FIs (Omary 2009).

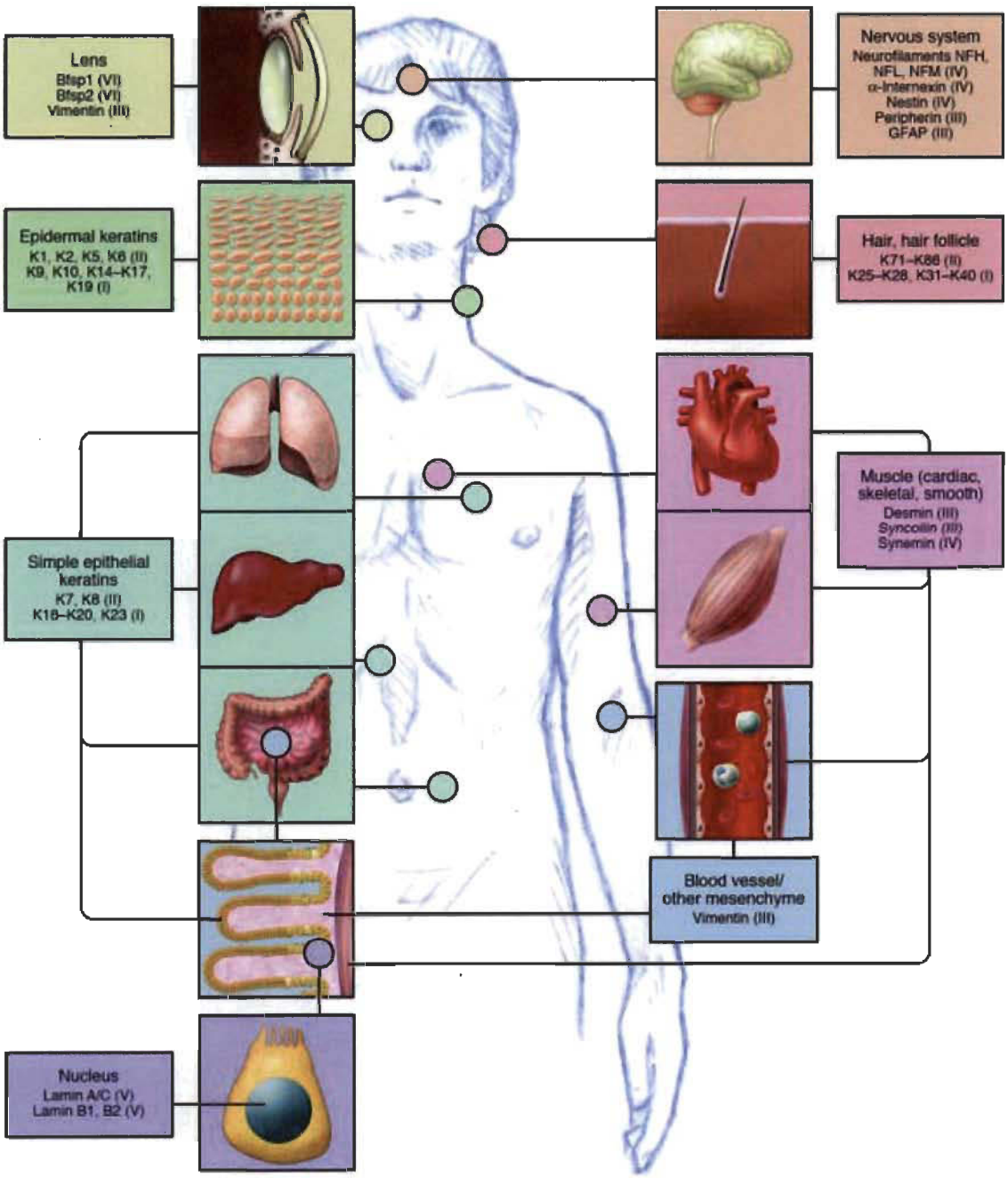


Figure 1.11 **Distribution des protéines de filaments intermédiaires dans les différents tissus.** (Omary 2009).

Selon cette classification, les FIs de type I et II regroupent les kératines qui constituent la plus grande famille de protéines de FIs. On dénombre actuellement 40 kératines différentes, excluant les kératines dites dures qui forment les cheveux et les ongles. Exprimées dans les cellules épithéliales, les kératines s'associent en hétérodimères constitués obligatoirement d'une kératine acide de type I (K9, K10, K12 à K20, K23 et K24) et d'une kératine neutre-basique de type II (K1 à K8, K76 à K80). Le type III se compose de la desmine et de la syncoïline exprimées par les cellules musculaires, de la périphérine retrouvée dans les neurones, de la protéine acide fibrillaire gliale (GFAP) exprimée par les cellules gliales et de la vimentine spécifique aux cellules du mésenchyme. Le type IV est constitué des neurofilaments (NF-L, -M et –H) et l'α-internexine exprimés par les neurones, de la nestine présente chez les cellules pluripotentes et de la synémine exprimée par les cellules musculaires. Contrairement aux kératines, les FIs de type III et IV peuvent former des homodimères ou différentes combinaisons d'hétérodimères. Les lamines (type A, B et C) constituent le type V et forment le squelette nucléaire. Le type VI regroupe les protéines orphelines BFSP1 et BFSP2, pour *beaded filament structural protein*, dont la structure est unique et l'expression limitée au cristallin de l'œil.

Les FIs ont d'abord été considérés comme une structure relativement statique du cytosquelette, dû à leurs remarquables propriétés viscoélastiques (flexibilité et extensibilité) et leur insolubilité dans des conditions où les MFs et les MTs sont dénaturés (Wagner et al. 2007; Herrmann et al. 2009). De plus, leur fonction mécanique a été mise en évidence par l'importante fragilité cellulaire observée dans diverses maladies associées aux FIs telles que l'épidermolyse bulleuse simple et la sclérose latérale amyotrophique (Eriksson et al. 2009). Cependant, cette vision purement structurelle du rôle des FIs est remise en question du fait que l'organisation du réseau de FIs est un processus très dynamique. En effet, la formation des filaments peut être initiée par des protéines de FIs nouvellement synthétisées ou par l'assemblage de celles déjà présentes au sein d'un compartiment soluble (Windoffer et al. 2011). Il est maintenant bien connu que la phosphorylation des FIs régule leur assemblage en modifiant l'équilibre entre le bassin soluble et polymérisé (Ku et al. 1996; Sahlgren et al. 2001; Eriksson et al. 2004) (Figure 1.12). D'autres modifications post-traductionnelles peuvent

également avoir un impact sur la dynamique de l'assemblage des FIs (Chou et al. 1993; Rogel et al. 2010; Snider et al. 2011).

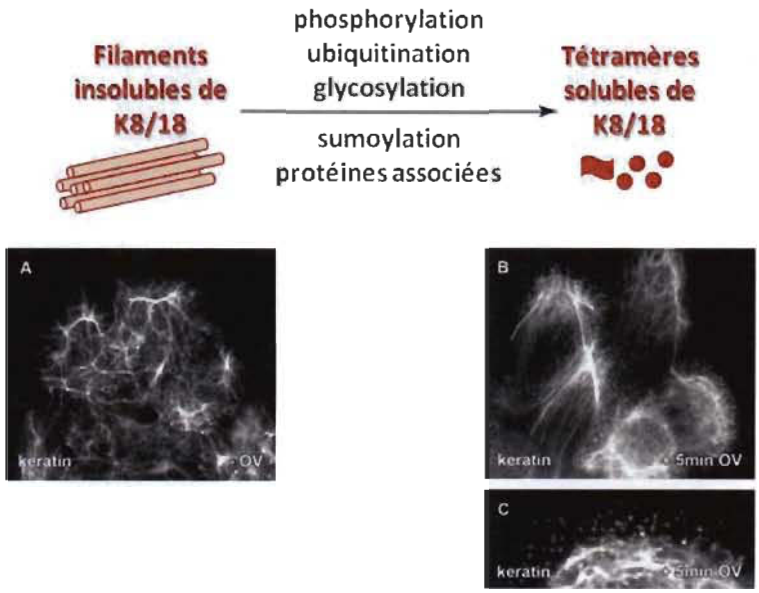


Figure 1.12 **Dynamique de l'assemblage des filaments intermédiaires.**
Plusieurs modifications post-traductionnelles au niveau des kératines peuvent influencer la polymérisation des filaments. Le traitement des cellules avec un inhibiteur de phosphatase, l'orthovanadate de sodium (OV), entraîne une réorganisation du cytosquelette en sous-unités solubles de filaments de kératines. Adapté de (Woll et al. 2007; Godsel et al. 2008).

Cet échange continuel entre les filaments et les particules solubles permet au cytosquelette épithélial de rester dynamique tout en maintenant l'intégrité structurale, ce qui nous autorise à penser que les FIs peuvent influencer l'homéostasie épithéliale. En effet, les nombreuses études réalisées au cours des dernières décennies ont révélé que le réseau de FIs, particulièrement les kératines de l'épithélium, constitue une importante plate-forme pour les différentes molécules de signalisation cellulaire.

1.3.1 Les kératines de l'épithélium

1.3.1.1 L'expression des kératines est spécifique au type d'épithélium et à son stade de différenciation

Un épithélium est formé de cellules juxtaposées et reliées entre elles par des systèmes de jonctions et séparées du tissu conjonctif sous-jacent par une lame basale.

Les jonctions intercellulaires permettent notamment la cohésion des cellules entre elles ainsi que l'adhésion à la MEC. Trois systèmes de jonctions intercellulaires permettent le maintien de la polarité apico-basale des cellules épithéliales ; les jonctions serrées, les jonctions adhérentes et les desmosomes. De concert avec les cytosquelettes d'actine et de filaments intermédiaires, ces systèmes de jonctions contribuent à l'organisation asymétrique de la membrane plasmique et du cytoplasme, permettant ainsi des échanges entre l'intérieur et l'extérieur de la cellule tels que le transport du glucose dans les entérocytes (Lodish et al. 2012).

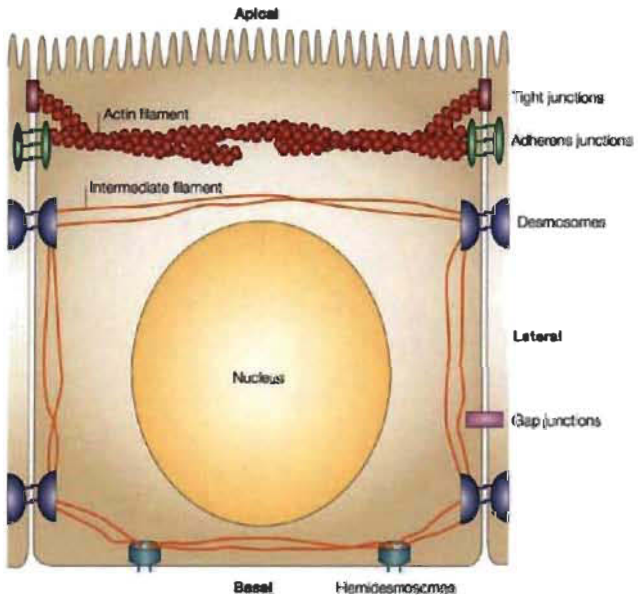


Figure 1.13 **Représentation schématique d'une cellule épithéliale intestinale.**
Les différents types de jonctions cellule-cellule et cellule-matrice extracellulaire sont représentés. Les jonctions serrées (tight junctions) et les jonctions adhérentes (adherens junctions) sont liées au cytosquelette d'actine alors que les desmosomes et hémidesmosomes sont liés aux filaments intermédiaires (Matter et al. 2003).

L'épithélium constitue d'abord une barrière contre le milieu extérieur en fournissant une protection mécanique (cohésion intercellulaire) et chimique (mucus). Il permet aussi un échange bidirectionnel sélectif (absorption et sécrétion) entre la lumière et le tissu grâce aux structures apicales telles que les cils vibratiles qui tapissent les organes respiratoires et les microvillosités intestinales. Enfin, l'épithélium assure le renouvellement du tissu grâce à la présence d'une population de cellules souches résidentes. Les cellules souches adultes ou somatiques sont caractérisées par leur grand

potentiel de prolifération et leur capacité à se différencier vers les types cellulaires épithéliaux du tissu dans lequel elles résident. Les cellules souches épithéliales peuvent prendre la forme de cellules isolées s'intercalant entre les cellules matures d'un épithélium simple. Elles sont alors toujours à proximité de la lame basale. Dans d'autres cas, les cellules souches forment une assise de cellules basales et participent alors à un épithélium stratifié (ex. : épiderme). Enfin, les cellules souches peuvent se regrouper sous forme de zones germinatives à partir desquelles les cellules filles migrent et renouvellent des régions de l'épithélium situées à distance de la zone germinative (ex. : épithélium intestinal). Dans tous les cas, les cellules souches épithéliales entretiennent des interactions étroites avec la lame basale, permettant le contrôle de la prolifération et de la différenciation des celles-ci.

L'épithélium simple est la première structure reconnaissable au moment de l'embryogenèse et forme le trophectoderme, qui est à l'origine des annexes embryonnaires constituant l'interface entre l'embryon et l'environnement maternel. Les kératines 8 et 18 (K8/18) sont les premières protéines de FIs exprimées par les cellules trophoblastiques. Leur expression est maintenue tout au long de la différenciation des cellules épithéliales simples telles que les hépatocytes, les cellules acineuses pancréatiques, celles des îlots de Langerhans et du tubule proximal du rein (Figure 1.14) (Moll et al. 2008). Les K8/18 sont aussi retrouvées dans certains épithéliums pseudo-stratifiés (ex. : voies respiratoires) et complexes (ex. : glandes mammaires), ainsi que dans l'urothélium. Lors de la différenciation des épithéliums simples, les cellules épithéliales peuvent acquérir les K7, K19 et K20. Les K7 et K19 se retrouvent au niveau de plusieurs glandes et épithéliums internes, particulièrement dans les cellules canalaires. La K19 est aussi présente dans les épithéliums simples en cours de différenciation, au niveau du follicule pileux et des glandes mammaires (Moll et al. 2008). La K20 s'exprime dans les cellules gastro-intestinales, l'urothélium et les cellules endocrines. Les K5 et K14 constituent la principale paire de kératines exprimées par les kératinocytes de l'épithélium stratifié et sont aussi présentes dans les cellules basales et myoépithéliales des épithéliums glandulaires, conjointement avec la K17. La K15 est aussi retrouvée dans les cellules basales des épithéliums stratifiés et glandulaires,

précisément dans les cellules souches somatiques de l'épiderme et des glandes mammaires. Les K6, K16 et K17 sont présentes aussi bien dans les épithéliums kératinisés (ex. : épiderme, ongles) que non-kératinisés (ex. : œsophage). Leur expression est induite lorsque l'épithélium entre dans une phase d'hyperprolifération, par exemple lors de la cicatrisation d'une plaie cutanée. Les K1/10 sont exprimées par les kératinocytes de l'épithélium stratifié kératinisé, alors que les K3/12 et K4/13 sont spécifiques à la cornée de l'œil et aux muqueuses respectivement. Les couches épineuses et granulaires de l'épiderme expriment aussi un autre type de kératine de type II, la K2, alors que la K9 est spécifique à l'épiderme palmo-plantaire (Moll et al. 2008).

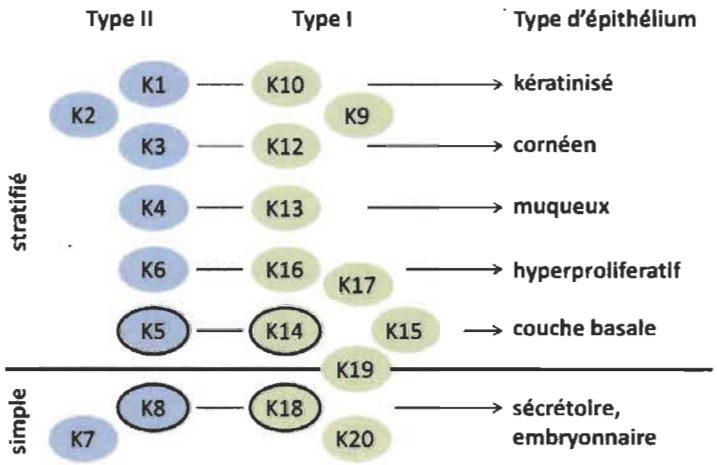


Figure 1.14 **Les hétérodimères de kératines sont exprimés de façon spécifique selon le type d'épithélium et son stade de différenciation.**
Les kératines encerclées représentent les premières paires exprimées dans chacun des types d'épithéliums. Adapté de (Lane et al. 1990).

L'expression des kératines est spécifique au type de tissu, mais aussi à son stade de différenciation. Ce processus est particulièrement visible au niveau des glandes mammaires et dans les différentes couches de l'épithélium stratifié (Figure 1.15). En effet, les deux couches de cellules épithéliales formant l'acinus de la glande mammaire se distinguent par leur expression spécifique en kératines. Les cellules myoépithéliales expriment les K5/14 et K17. Leur activité contractile et paracrine contribuent aux propriétés sécrétrices des cellules luminales, qui expriment principalement les K8/18 (Karantza 2011). Au cours de la différenciation progressive de l'épiderme, les cellules

de la couche basale se divisent activement et expriment principalement les K5/14. Lorsque le processus de différenciation est initié, les cellules basales cessent de proliférer et migrent vers les couches supra-basales, où elles expriment les K1/10 de façon prédominante. Lors d'une blessure au niveau de l'épiderme, il y a induction de l'expression des K6, K16 et K17 dans les couches supra-basales au détriment des K1/10 (Porter et al. 2003).

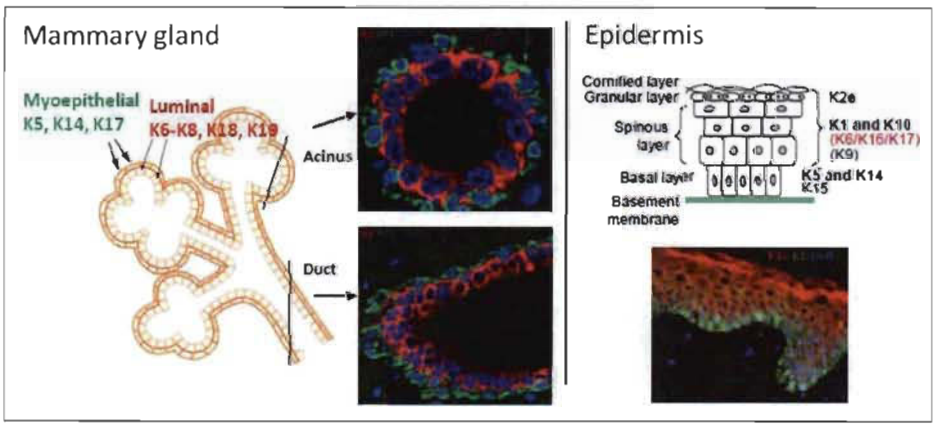


Figure 1.15 **Expression spécifique des kératines selon le type de tissu et le stade de différenciation.**
(Porter et al. 2003; Karantza 2011).

1.3.1.2 Les kératines préservent l'intégrité physique de la cellule

Les kératines jouent un rôle déterminant dans le maintien de l'intégrité cellulaire en protégeant les cellules contre les stress mécaniques et chimiques (Coulombe et al. 2002). Ce phénomène a été particulièrement bien caractérisé au niveau de la peau, de la cornée et du foie, suite à la découverte de mutations prédisposant à la fragilité du tissu et grâce au développement de souris déficientes ou mutantes en kératines. Par exemple, des mutations au niveau des K5 ou K14 exprimées par les cellules basales de l'épiderme fragilisent la peau et induisent l'apparition de vésicules épidermiques caractéristiques de l'épidermolyse bulleuse simple (Coulombe et al. 1991; Lane et al. 1992). D'ailleurs, les souris déficientes en K5 présentent aussi ces vésicules épidermiques et ne survivent que quelques heures après la naissance (Peters et al. 2001). Les souris n'exprimant pas la K14 montrent un phénotype comparable, mais de moindre gravité, entraînant la mort 4-5 jours après la naissance (Lloyd et al. 1995).

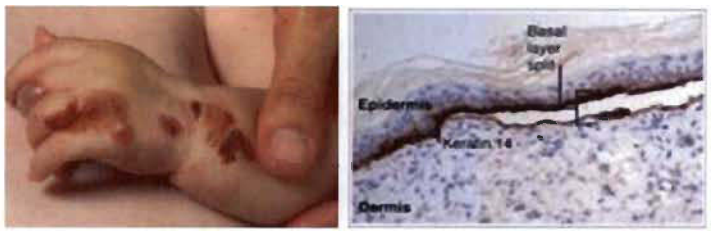


Figure 1.16 **Une mutation au niveau des kératines induit n phénotype de fragilité épithéliale importante.**
L'épidermolyse bulleuse simple est une maladie qui affecte la peau en créant des lésions cutanées sous forme de bulles, causée par des mutations au niveau des kératines 5 ou 14 de l'épiderme. Adapté de (McLean et al. 2011; Haines et al. 2012).

De façon similaire, des mutations au niveau des K3 ou K12 entraînent une importante fragilité de la cornée qui est à l'origine de la dystrophie de Messman (Irvine et al. 1997) et l'absence de K12 cause l'érosion de la cornée chez la souris (Kao et al. 1996). Les mutations des K8 et K18 prédisposent aux maladies du foie chroniques et aiguës (Ku et al. 1997; Ku et al. 2001; Ku et al. 2003). Les souris déficientes en K8 présentent un phénotype différent selon leur patrimoine génétique : les embryons de souris C57BL/6 subissent un retard de croissance et 94 % d'entre eux meurent entre 11 et 13 jours de gestation suite à une importante hémorragie du foie (Baribault et al. 1993), alors que 50 % des embryons de souris FVB/N vont survivre en présentant toutefois une prédisposition significative aux maladies du foie (Baribault et al. 1994). Les souris déficientes en K18 sont viables, bien qu'elles manifestent une plus grande susceptibilité aux maladies du foie telles que le diabète insulino-dépendant et l'hépatite chronique. Les pathologies qu'elles développent sont moins importantes en comparaison aux souris déficientes en K8, ce qui suggère que les kératines de type I et II ont des fonctions différentes, mais complémentaires au sein de l'épithélium (Magin et al. 1998). Les modèles de souris transgéniques ont aussi grandement contribué à la compréhension du rôle des K8/18 dans le maintien de l'intégrité cellulaire. Les souris surexprimant une K18 humaine mutée sur un site hautement conservé et riche en mutations dans diverses maladies de la peau (Arg89→Cys) présentent une grande fragilité des hépatocytes et développent une hépatite chronique (Ku et al. 1995). Ces souris sont aussi plus susceptibles à différents stress tels que l'exposition à diverses drogues hépatotoxiques, une hépatectomie partielle et une perfusion du foie (Ku et al. 1999; Zatloukal et al.

2000). Une mutation de la K8 retrouvée dans plusieurs maladies du foie (Gly61→Cys) fragilise aussi les hépatocytes à diverses drogues hépatotoxiques (Ku et al. 2006).

1.3.1.3 Le rôle des kératines dans l'homéostasie cellulaire

En plus de leur rôle dans le maintien de l'intégrité cellulaire contre différents stress, les kératines ont été plus récemment identifiées comme des régulateurs importants de la signalisation intracellulaire impliquée dans la prolifération et l'apoptose des cellules épithéliales. Ces processus fondamentaux maintiennent l'homéostasie cellulaire, c'est-à-dire la capacité à conserver l'équilibre de fonctionnement en dépit des contraintes extérieures, et ce, dans des conditions physiologiques et pathologiques. Toute perturbation dans cet équilibre a pour conséquence la prolifération incontrôlée ou la mort cellulaire. Plusieurs voies de signalisation sont connues pour réguler ces processus dont certaines impliquent la réorganisation du cytosquelette. Alors que le rôle des MTs dans l'anaphase et la réorganisation des MFs au cours de la cytocinèse sont bien établis, la participation des FIs dans la division cellulaire commence à être mise en lumière. Le cycle cellulaire est une séquence ordonnée d'événements régulés par des complexes kinasiques dépendants des cyclines (CDK) et menant ultimement à la division de la cellule-mère en deux cellules-filles identiques (Figure 1.17). Ainsi, le bon déroulement du cycle cellulaire est déterminé par l'activation appropriée de ces kinases. Il a été montré que la K8 réprime la progression du cycle cellulaire. En effet, la synthèse d'ADN est fortement augmentée dans les hépatocytes issus de souris déficientes en K8 comparativement aux hépatocytes de souris de type sauvage (Galarneau et al. 2007). Ainsi, l'absence de K8 favorise l'entrée en phase S du cycle cellulaire caractérisée par l'augmentation des niveaux de cycline A (Galarneau et al. 2007). À l'inverse, la K18 favorise le cycle cellulaire en interagissant avec les protéines de la famille 14-3-3 (Liao et al. 1996; Ku et al. 1998). Dans les cellules quiescentes, la progression du cycle cellulaire est inhibée par les protéines 14-3-3 qui séquestrent la phosphatase Cdc25 dans le cytoplasme, l'empêchant ainsi d'activer la kinase mitotique CDK1 au noyau (Tzivion et al. 2001). En phase de synthèse d'ADN (phase S du cycle) et lors de la mitose (phase M) des hépatocytes, la phosphorylation de la K18 augmente de manière

significative (Omary et al. 1998). La réorganisation du cytosquelette en particules solubles qui résulte de cette modification post-traductionnelle favorise l'interaction de la K18 avec les protéines 14-3-3 (Ku et al. 2002), alors incapables de séquestrer la phosphatase Cdc25. Il est intéressant de remarquer que le rôle des kératines dans la prolifération semble intimement relié au stade de différenciation des cellules. Par exemple, la division cellulaire des kératinocytes HaCaT est favorisée par la K14, alors qu'elle est inhibée par la K10. En effet, une étude récente a démontré que l'absence de K14 ralentit la division cellulaire et induit la différenciation de ces cellules basales, qui expriment alors la K1 caractéristique des couches différenciées de l'épiderme (Alam et al. 2011). À l'inverse, l'expression ectopique de la K10 provoque l'arrêt du cycle cellulaire en diminuant les niveaux de cycline D1 et en séquestrant les kinases Akt et PKCζ (Paramio et al. 2001). Ces études révèlent que les kératines peuvent moduler les différentes étapes du cycle cellulaire, principalement par la séquestration des molécules impliquées dans ce processus.

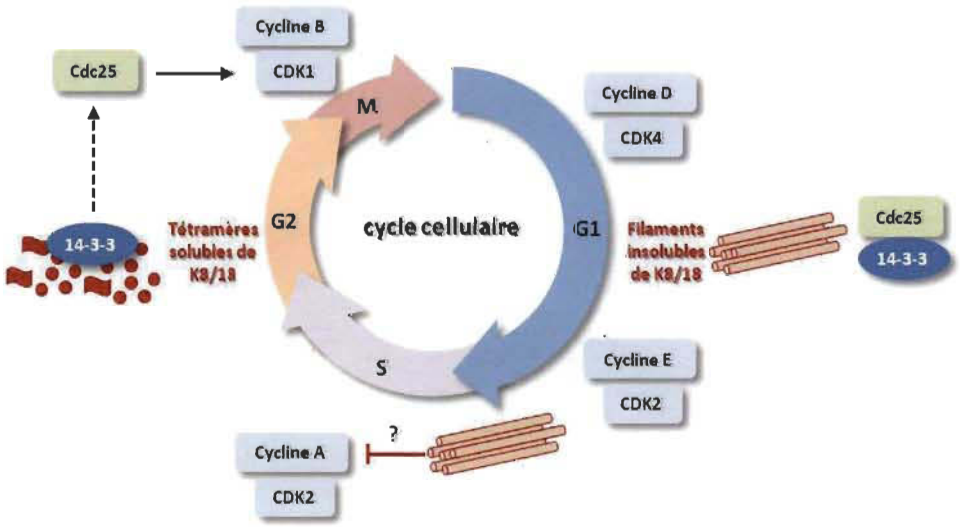


Figure 1.17 **Rôle des kératines 8 et 18 dans la progression du cycle cellulaire.**
La phosphorylation des kératines au cours de la mitose favorise l'interaction de la K18 avec les protéines 14-3-3, alors incapables de séquestrer la phosphatase Cdc25 dans le cytoplasme. La translocation nucléaire de Cdc25 permet l'activation de la kinase mitotique CDK1 et donc la progression du cycle cellulaire. Adapté de (Lodish et al. 2012).

Le processus de mort cellulaire programmée, ou apoptose, peut être initié en réponse à différents signaux externes (cytokines, absence de facteurs de croissance,

rupture de l'attachement à la matrice) ou internes (dommages à l'ADN, stress oxydatif, arrêt de cycle cellulaire). La liaison des cytokines de la famille des facteurs de nécrose tumorale (TNF) à leurs récepteurs enclenche une cascade de signalisation intracellulaire menant à l'apoptose. Par exemple, le ligand Fas (FasL) se lie au récepteur Fas (FasR), permettant le recrutement d'une molécule adaptatrice appelée FADD (pour *Fas Associated Death Domain protein*) nécessaire à la cascade d'activation de molécules apoptotiques. La cytokine TNF interagit avec le récepteur TNFR, qui s'associe alors avec TRADD (pour *TNF Receptor Associated Death Domain protein*). De la même façon, le ligand TRAIL (pour *TNF-Related Apoptosis-Inducing Ligand*) se lie aux récepteurs DR4 et DR5 (Locksley et al. 2001). La voie intrinsèque de l'apoptose est médiée par divers stimuli qui convergent à la mitochondrie où la libération du cytochrome C initie la signalisation apoptotique (Jin et al. 2005). Les deux voies d'apoptose impliquent l'activation d'une série de protéases, les *caspases*, qui sont responsables du clivage et de la dégradation subséquente de nombreuses protéines essentielles au métabolisme cellulaire (Figure 1.18). Si les caspases étaient actives dès leur synthèse dans les cellules, elles entraîneraient un phénomène apoptotique non régulé. Elles sont donc présentes dans le cytoplasme sous forme de proenzymes inactives appelées procaspases. Une fois clivées, les caspases vont pouvoir à leur tour activer d'autres procaspases et ainsi développer une activation en cascade. La cellule en apoptose va subir des changements morphologiques caractéristiques tels que la condensation du noyau, la fragmentation de l'ADN et la formation de corps apoptotiques qui seront phagocytés par les cellules environnantes (Fuchs et al. 2011).

À un stade précoce de l'apoptose, avant même l'activation des caspases et la fragmentation de la chromatine, les protéines DEDD (pour *Death Effector Domain containing DNA binding protein*) dirigent la procaspase 3 vers les filaments de kératine (Lee et al. 2002). La caspase 9 est également associée aux filaments de kératine dans les cellules épithéliales en apoptose (Dinsdale et al. 2004). Ces observations ont permis d'élaborer un modèle selon lequel les filaments de K8/18 constituent un échafaudage pour l'accumulation et l'activation des procaspases (Dinsdale et al. 2004). En effet, l'inhibition des protéines DEDD réduit l'activation de la caspase 3 (Lee et al. 2002). De

plus, la sensibilité des cellules épithéliales à l'apoptose dépend de la capacité des protéines DEDD à s'associer aux filaments de K8/18 (Schutte et al. 2006). Ainsi, le réseau de kératine régule la machinerie apoptotique et constitue une plate-forme pour l'activation des caspases.

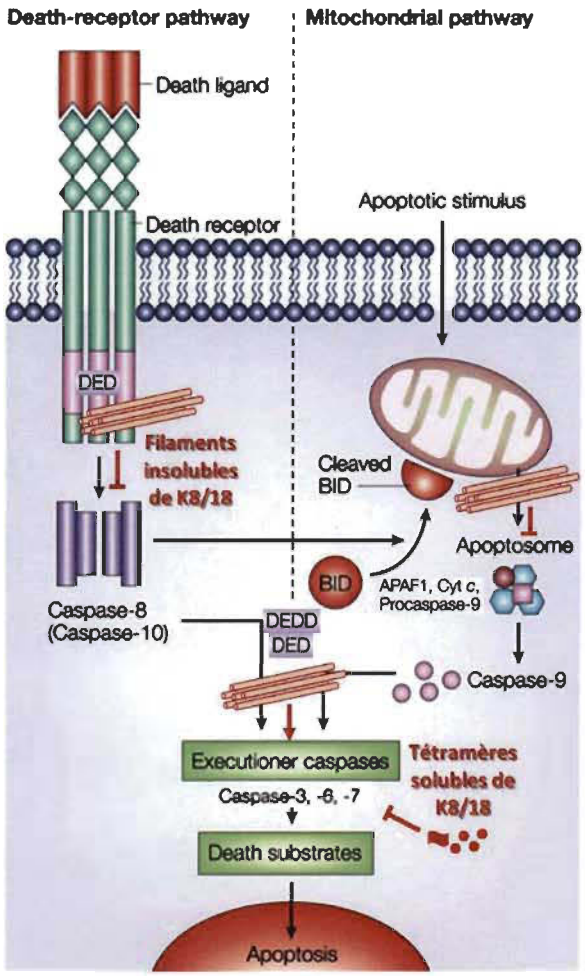


Figure 1.18 **Rôle des kératines 8 et 18 dans la signalisation apoptotique.**
Le cytosquelette de kératines 8 et 18 constitue une plate-forme pour l'activation des caspases en phase précoce de l'apoptose. Une fois le processus apoptotique enclenché, les K8/18 participent à la résistance des cellules à l'apoptose en séquestrant certains médiateurs de la signalisation apoptotique extrinsèque (death-receptor pathway) tels que les protéines adaptatrices des récepteurs de mort (DED) ou en limitant le relargage du cytochrome *c* (Cyt *c*) des mitochondries, caractéristique de la voie intrinsèque (mitochondrial pathway). Adapté de (Igney et al. 2002).

Parmi les premières cibles du clivage par les caspases se retrouvent les kératines de type I (Caulin et al. 1997) (Figure 1.19). La K18 est d'abord clivée dans la région carboxy-terminale (Asp397) par les caspases 3, 7 et 9 (Caulin et al. 1997; Leers et al.

1999; Schutte et al. 2004). Ce premier clivage n'affecte pas l'organisation filamenteuse des kératines, mais libère un fragment carboxy-terminal qui est rapidement transporté vers le noyau afin d'empêcher la condensation de la chromatine par la topoisomérase. Ceci a pour conséquence de préserver l'activité transcriptionnelle nécessaire durant les premiers stades de l'apoptose (Schutte et al. 2009). De plus, ce clivage génère un nouvel épitope couramment utilisé en tant que marqueur de l'activité de la caspase 3 (Leers et al. 1999).

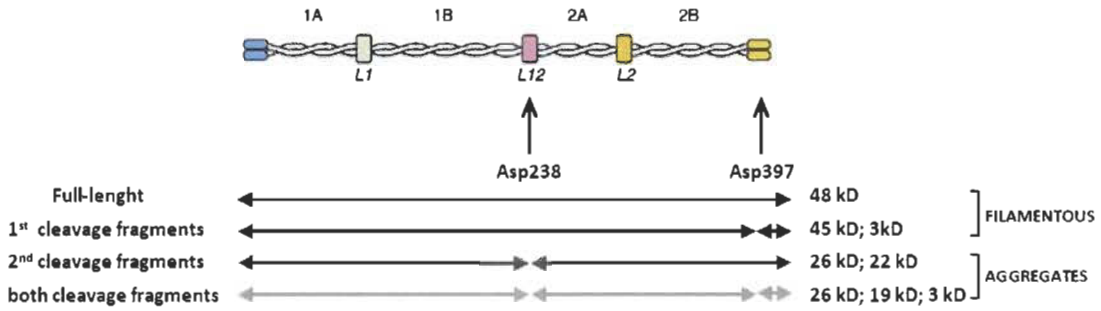


Figure 1.19 **Clivage des kératines de type I par les caspases.**
(Fortier et al. 2012).

Un deuxième site de clivage par les caspases a été identifié dans la région intercalaire L12 (Asp238) (Caulin et al. 1997; Omary et al. 1997). Cet événement induit une réorganisation du cytosquelette de kératines sous forme d'agrégats qui coïncide avec la perte de contacts intercellulaires et le détachement des cellules de leurs substrats (Schutte et al. 2004). Ces inclusions cytoplasmiques de K8/18 peuvent également contenir plusieurs facteurs pro-apoptotiques tel que les caspases 3 et 9, les protéines DEDD, la protéine adaptatrice TRADD et différentes protéines de choc thermique (Nakamichi et al. 2002; Dinsdale et al. 2004; Schutte et al. 2006). Ces facteurs pro-apoptotiques restent séquestrés dans ces inclusions de kératines (Dinsdale et al. 2004), suggérant une régulation séquentielle de l'apoptose induite par les K8/18 : au stade précoce de l'apoptose, le recrutement des procaspases 3 et 9 par les protéines DEDD au niveau des filaments de kératines augmente la disponibilité locale des caspases, ce qui sensibilise les cellules à l'apoptose (Schutte et al. 2006). Plus tard dans le processus, les inclusions cytoplasmiques de kératines, induites par le clivage de la K18 par les caspases, séquestrent ces protéines pro-apoptotiques potentiellement

nuisibles pour les cellules avoisinantes (Dinsdale et al. 2004). Ainsi, les filaments de kératines peuvent à la fois contrôler les étapes clés de la cascade des caspases afin d'assurer le bon déroulement de l'apoptose en plus de minimiser les effets de ce processus sur les cellules adjacentes.

Le fait que les kératines de type I sont des cibles des caspases nous autorise à penser qu'elles jouent un rôle anti-apoptotique. En effet, plusieurs études ont démontré que les K8/18 confèrent une résistance des cellules épithéliales en culture à l'apoptose induite par les cytokines FasL et TNF, en limitant le recrutement des récepteurs Fas à la membrane (Gilbert et al. 2001) et en séquestrant certains médiateurs de la signalisation apoptotique tels que le TNFR et la protéine adaptatrice TRADD (Caulin et al. 2000). Des études *in vivo* ont également confirmé que le cytosquelette de K8/18 protège les cellules épithéliales contre la mort cellulaire induite par Fas en comparant le foie des souris normales avec des souris transgéniques exprimant une K18 mutée (Ku et al. 2003) ou une K8 mutée sur un important site de phosphorylation, la sérine 73 (Ku et al. 2006). Les auteurs ont proposé que la K8 protège les tissus en absorbant la phosphorylation excessive induite par les kinases activées en réponse au stress, préservant ainsi les autres substrats de la dégradation (Ku et al. 2006). Les kératines interfèrent aussi avec la signalisation apoptotique intrinsèque en préservant l'intégrité des mitochondries en réponse au stress oxydatif. En effet, la distribution intracellulaire et la taille des mitochondries sont altérées dans les hépatocytes de souris déficientes en K8 (Tao et al. 2009). Ces observations corrèlent avec une diminution du contenu en cytochrome C et une augmentation de la perméabilité mitochondriale ce qui, par conséquent, sensibilise les cellules aux dommages oxydatifs. De façon similaire, on observe une accumulation de dérivés réactifs de l'oxygène dans les hépatocytes de souris transgéniques exprimant une K18 mutée (Arg89→Cys) (Zhou et al. 2005). Ces résultats suggèrent fortement que le réseau de K8/18 joue un rôle modérateur dans la signalisation apoptotique, en aval des récepteurs de mort cellulaire et de l'intégrité fonctionnelle des mitochondries.

Désormais, la protection de l'épithélium est bien reconnue comme une fonction fondamentale des kératines. Le cytosquelette de K8/18 est une structure dynamique qui

maintient l'intégrité physique et régule le fragile équilibre entre la prolifération et l'apoptose des cellules épithéliales. Lorsque cet équilibre est rompu en faveur de la prolifération cellulaire incontrôlée, il y a formation de tumeur. L'expression des kératines est maintenue lors de la tumorigénèse, suggérant qu'elles puissent aussi jouer un rôle dans le contexte pathologique du cancer en modulant la signalisation cellulaire oncogénique.

1.3.2 Les kératines dans le cancer

1.3.2.1 Intérêt diagnostique et pronostique des kératines

L'adénocarcinome est un cancer épithélial provenant du tissu glandulaire et il constitue la majorité des tumeurs malignes pouvant survenir dans différents organes. Il représente aussi la variété tumorale la plus difficile à rattacher à son site d'origine, car ces tumeurs affichent un aspect indifférencié. La capacité à identifier l'origine de l'adénocarcinome et ses métastases est donc essentielle pour l'élaboration d'une stratégie thérapeutique appropriée. L'utilisation des kératines en tant que marqueur diagnostique est devenue un outil indispensable, particulièrement dans les cas plus complexes pour lesquels les examens cliniques, radiologiques et endoscopiques ne permettent pas l'identification du cancer primitif. La plupart des adénocarcinomes expriment les kératines K8, K18 et K19, tandis que les K7 et K20 sont exprimées de façon variable (Moll et al. 2008) (Figure 1.20). La K20 est typique du tractus gastro-intestinal, offrant ainsi aux anticorps dirigés contre la K20 une valeur prédictive acceptable pour différencier par exemple un adénocarcinome primaire mucineux ovarien de métastases ovariennes provenant d'un cancer colorectal (Hernandez et al. 2005; Shin et al. 2010).

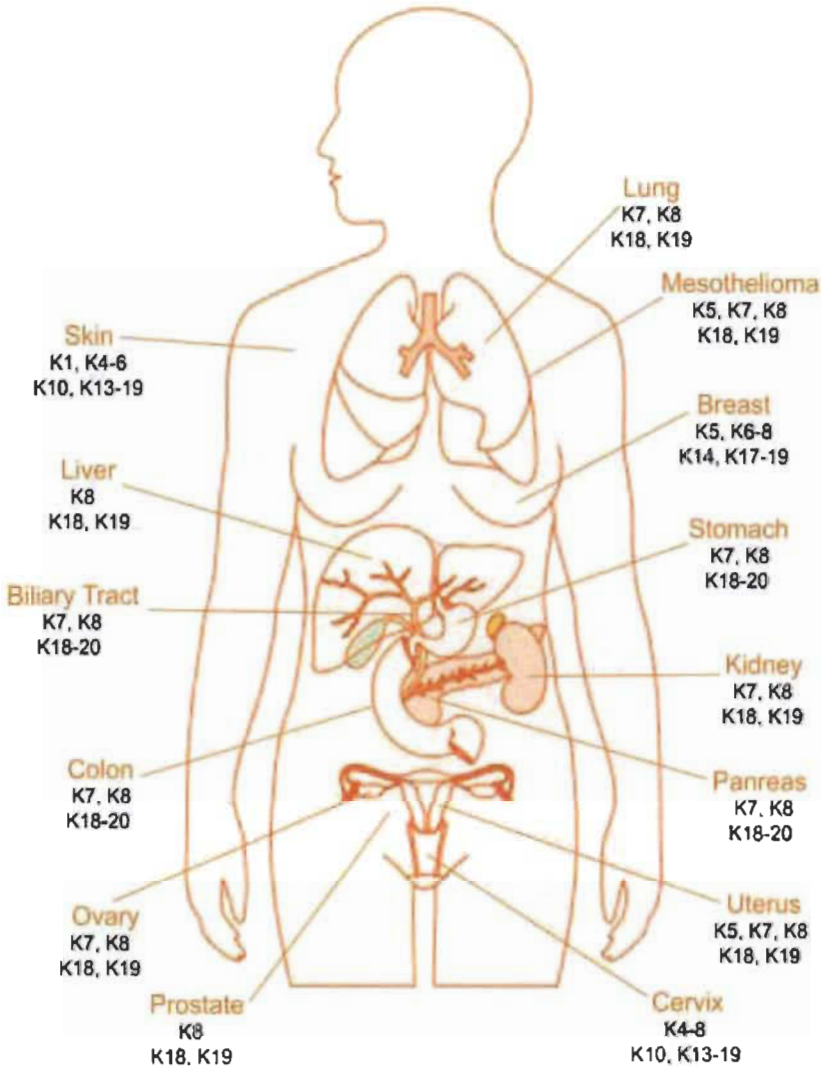


Figure 1.20 **Exemples de kératines couramment utilisées dans le diagnostic de plusieurs carcinomes humains.**
(Karantza 2011).

En plus de leur rôle bien établi en tant que marqueurs de diagnostic dans le cancer, les kératines sont également des indicateurs pronostiques dans une variété de tumeurs épithéliales malignes (Tableau 1.1). Par exemple, une expression diminuée de K8 et K20 dans le cancer colorectal est indicatif d'une agressivité tumorale élevée et d'une diminution de la survie des patients (Knosel et al. 2006). De plus, l'expression persistante du fragment de K18 (Asp397) dans le sérum de patients ayant subi l'ablation d'une tumeur colorectale est associée avec un risque de récidive dans les 3 ans (Ausch et al. 2009). Des niveaux élevés de ce fragment avant le traitement du cancer du poumon

suggèrent également une survie plus courte des patients (Ulukaya et al. 2007). De même, chez les patients ayant un cholangiocarcinome intrahépatique, un taux sérique élevé du fragment de K19 augmente le risque de récidive et diminue le taux de survie (Uenishi et al. 2008). L'expression de la K20 par les cellules tumorales pancréatiques, des K10 et K19 dans les carcinomes hépatocellulaires et de la K17 dans les tumeurs du sein, de l'ovaire et de l'estomac indiquent un mauvais pronostic (van de Rijn et al. 2002; Matros et al. 2006; Schmitz-Winnenthal et al. 2006; Yang et al. 2008; Ide et al. 2012; Wang et al. 2013). L'expression de kératines par les cellules tumorales disséminées dans la moelle osseuse de patients atteints de cancer de la prostate est un facteur de risque pour le développement de métastases (Weckermann et al. 2009). La détection de K19 dans les cellules tumorales circulantes (CTC) de patientes atteintes d'un cancer du sein est associée à un risque plus élevé de récidive, dû à la chimiorésistance du cancer (Xenidis et al. 2009). Inversement, les analyses d'expression génique ont indiqué que la K18 est souvent diminuée dans le cancer du sein métastatique (Hedenfalk et al. 2001; Zajchowski et al. 2001) et est en corrélation avec un stade tumoral avancé, la présence de micrométastases osseuses et un taux de survie très faible (Woelfle et al. 2003; Woelfle et al. 2004). En effet, la présence de K8 et K18 ubiquitinées et dégradées dans les cellules cancéreuses du sein détermine l'agressivité de la tumeur (Iwaya et al. 2003).

Tableau 1.1
L'expression anormale des kératines est indicatrice de l'évolution du cancer

TYPE DE CANCER	MAUVAIS PRONOSTIQUE
Carcinome colorectal	↓ K8/K20 Fragments de K18 dans le sérum
Carcinome pancréatique	↑ K20
Carcinome hépatocellulaire	↑ K10/K19
Cancer du sein	↑ K17 K19 dans les CTCs ↓ K18
Cancer de l'ovaire	↑ K17
Cancer de l'estomac	↑ K17
Cancer du poumon	Fragments de K18 dans le sérum
Cholangiocarcinome	Fragments de K19 dans le sérum

Adapté de (Karantza 2011).

1.3.2.2 Le rôle des kératines dans la tumorigénèse

Le rôle émergent des kératines dans la physiologie des cellules normales et dans le pronostic de certains cancers nous autorise à penser qu'elles peuvent être directement impliquées dans la tumorigénèse épithéliale. Bien que la plupart des souris transgéniques ou déficientes en kératines ne démontrent aucun phénotype tumoral apparent, l'absence de K8 chez les souris FVB/N provoque une hyperplasie colorectale (Baribault et al. 1994) et accélère l'apparition de tumeurs mammaires induite par l'oncogène MMTV-PyMT (Baribault et al. 1997). À l'inverse, l'expression ectopique de la K8 dans la peau des souris provoque une hyperplasie épidermique et favorise la progression maligne de tumeurs bénignes (Casanova et al. 2004). Dans le but de comprendre le rôle moléculaire des kératines dans la tumorigénèse et la progression cancéreuse, plusieurs études ont tenté de caractériser leur fonction dans le potentiel invasif *in vitro* des cellules cancéreuses et dans la formation des métastases *in vivo*. Les premières études à ce sujet ont montré que la surexpression des K8/18 dans des fibroblastes de souris et des cellules de mélanome humain augmente l'invasion de ces cellules à travers un mélange gélatineux de protéines de la matrice extracellulaire, le Matrigel (Chu et al. 1993; Chu et al. 1996). Inversement, la surexpression de K8 ou K18 dans des cellules de cancer du sein métastatique MDA-MB-231 (Buhler et al. 2005) et MDA-MB-435 (Iyer et al. 2013) entraîne une réduction dramatique du potentiel invasif et métastatique (Figure 1.21).

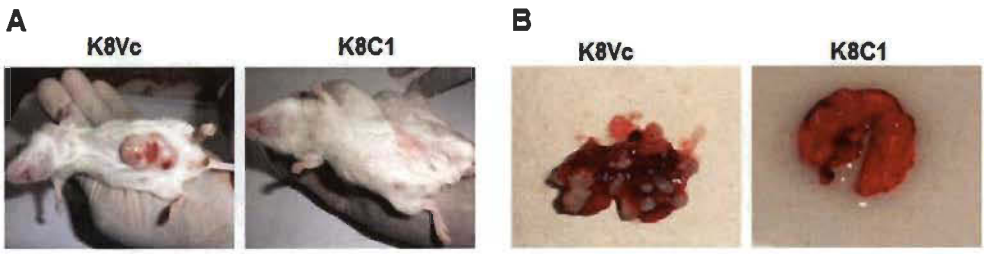


Figure 1.21 **La réexpression de la K8 par les cellules cancéreuses du sein bloque la formation de tumeur et de métastases chez la souris.**
Tumeurs mammaires (*A*) et métastases aux poumons (*B*) chez la souris nue suite à l'injection orthotopique de cellules cancéreuses du sein MDA-MB-435 contrôle (K8Vc) ou exprimant la K8 (K8C1) (Iyer et al. 2013).

La divergence des effets de l'expression ectopique des K8/18 a soulevé l'hypothèse selon laquelle l'expression anormale des kératines, en regard du type

d'épithélium et du stade de différenciation, favorise l'agressivité du cancer et son potentiel métastatique, alors que la réexpression des kératines présentes dans le tissu d'origine de la tumeur ralentit la progression du cancer (Vaidya et al. 2007).

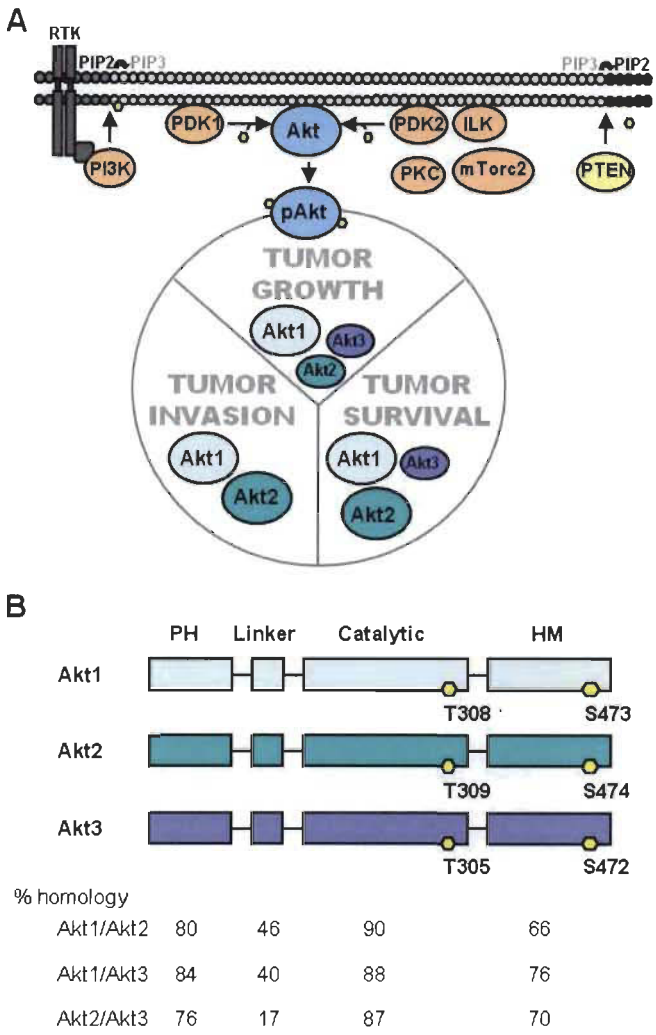
Les kératines maintiennent l'intégrité épithéliale en partie grâce à leur interaction avec les desmosomes et les hémidesmosomes (Kouklis et al. 1994; Green et al. 2007). Par cette interaction, les kératines empêchent le remodelage des jonctions intercellulaires qui est nécessaire à la motilité des cellules. En effet, l'absence de la K8 modifie la distribution des desmosomes au niveau de la membrane des hépatocytes de souris (Toivola et al. 2001; Loranger et al. 2006), des kératinocytes de souris (Seltmann et al. 2012), de l'épithélium embryonnaire de souris (Vijayaraj et al. 2009) et de cellules épithéliales humaines cancéreuses (MCF-7, HeLa et Panc-1) (Long et al. 2006). Par conséquent, les cellules cancéreuses humaines déficientes en K8 migrent plus rapidement (Long et al. 2006). Outre l'absence des kératines elles-mêmes, la réorganisation péri-nucléaire du réseau de filaments de K8/18 facilite aussi la motilité et l'élasticité cellulaires (Beil et al. 2003). Cette réorganisation résulte généralement de modifications post-traductionnelles au niveau des K8/18, particulièrement de la phosphorylation de la sérine 431 de la K8 (Busch et al. 2012).

Le rôle protecteur des kératines contre l'apoptose des cellules épithéliales peut s'avérer un obstacle dans un contexte de chimiothérapie. En effet, la surexpression des K8/18 confère une résistance des fibroblastes à de nombreuses drogues telles que la mitoxantrone, la doxorubicine et la vincristine (Bauman et al. 1994; Anderson et al. 1996). La sensibilité au cisplatine des cancers du col de l'utérus (Sullivan et al. 2010) et du nasopharynx (Wang et al. 2008) peut être favorisée suite à l'inhibition de l'expression des K8/18 par les cellules tumorales. Il a été suggéré que la chimiorésistance induite par la K8 implique son rôle dans l'adhésion des cellules à la MEC (Liu et al. 2008).

1.4 La signalisation intracellulaire oncogénique régulée par Akt

La famille de sérine-thréonine kinases Akt, aussi appelées protéine kinases B (PKB), joue un rôle central dans l'homéostasie cellulaire en régulant le métabolisme du glucose, la croissance cellulaire et la synthèse des protéines, la survie des cellules et leur capacité migratoire. En tant que médiateur central de nombreuses voies de signalisation favorisant la prolifération cellulaire, Akt constitue un proto-oncogène redoutable (Altomare et al. 2005). Cette famille de kinases regroupe trois protéines très similaires en séquence, mais qui sont codées par des gènes distincts: Akt1/PKBα, Akt2/PKBβ et Akt3/PKBγ, situés respectivement sur les chromosomes 14, 19 et 1 (Hanada et al. 2004) (Figure 1.22).

L'activation des différents isoformes d'Akt nécessite d'abord la liaison d'un récepteur à activité tyrosine kinase (RTK) avec son ligand (incluant IGF-1, EGF et l'insuline), ce qui permet la dimérisation du RTK et le déclenchement de son activité de kinase. L'auto-phosphorylation du RTK crée un site de fixation pour la phosphatidylinositol 3-kinase (PI3K), une enzyme qui transfère un phosphate de l'ATP sur le carbone 3' de l'inositol des phosphatidylinositides membranaires (PtdIns). La génération des PtdIns3P, PtdIns (3,4) P2 et PtdIns (3,4,5) P3 (PIP3) permet le recrutement membranaire de protéines possédant des domaines spécifiques, tel que le domaine PH (pour *plecstrin homology*) présent dans la séquence d'Akt. C'est à la membrane plasmique que se produit l'activation d'Akt, suite à sa phosphorylation sur un résidu thréonine du domaine catalytique (Thr308 pour Akt1, Thr309 pour Akt2 et Thr305 pour Akt3) par la kinase PDK1 (Alessi et al. 1997) et sur un résidu sérine du domaine C-terminal (Ser473 pour Akt1, Ser474 pour Akt2 et Ser472 pour Akt3) à la fois par auto-phosphorylation et par l'action d'autres kinases telles que mTORC2, ILK et PKC (Lynch et al. 1999; Persad et al. 2001; Partovian et al. 2004; Sarbassov et al. 2005). Akt est ensuite relocalisé, par un mécanisme inconnu, dans différents compartiments intracellulaires où ses substrats sont situés. La production de PIP3 et l'activation d'Akt sont inhibés par la phosphatase PTEN.



% homology

Akt1/Akt2	80	46	90	66
Akt1/Akt3	84	40	88	76
Akt2/Akt3	76	17	87	70

Figure 1.22 **Activation et fonction des différents isoformes d'Akt dans le cancer.**
A) Les trois isoformes d'Akt sont impliqués dans la croissance tumorale, l'invasion de cellules cancéreuses et la chimiorésistance à des degrés divers. L'importance relative de chaque isoformes dans ces processus est représentée par la taille du schéma. B) Les isoformes d'Akt sont composés d'un domaine PH, d'un domaine de liaison, d'un domaine catalytique central et d'un domaine régulateur hydrophobe. Le pourcentage d'homologie de séquence et les sites de phosphorylation spécifiques aux trois isoformes sont indiqués. (Fortier et al. 2011).

Jusqu'à tout récemment, les nombreuses études concernant Akt négligeaient la spécificité fonctionnelle de chacun des isoformes pour analyser l'activité globale d'Akt. Il est maintenant évident qu'une meilleure compréhension des mécanismes moléculaires régulés par cette famille d'oncogènes nécessite l'étude de l'importance relative de chacun des isoformes dans la tumorigénèse et la progression tumorale. Les fonctions physiologiques spécifiques des isoformes d'Akt ont été caractérisées grâce aux modèles

de souris déficientes en Akt1, Akt2 ou Akt3. Bien que les trois phénotypes se soient avérés viables, les souris ont présenté plusieurs anomalies spécifiques. Les souris déficientes en Akt1 ont montré un retard de croissance, une diminution de la taille de tous les organes ainsi qu'une augmentation de l'apoptose et de la mortalité néonatale (Chen et al. 2001; Cho et al. 2001; Yang et al. 2003). Les souris déficientes en Akt2 ont développé une résistance à l'insuline ainsi qu'un léger retard de croissance (Cho et al. 2001; Garofalo et al. 2003). L'absence d'Akt3 chez la souris a entraîné une diminution sélective de la taille du cerveau (Easton et al. 2005; Tschopp et al. 2005). Les phénotypes relativement subtils et la viabilité des souris dépourvues d'un seul isoforme d'Akt suggère une certaine redondance fonctionnelle entre les trois protéines. Des souris déficientes en deux isoformes d'Akt ont donc été générées. Les souris déficientes en Akt1 et Akt2 sont décédées peu après la naissance, et les nouveau-nés ont montré un développement altéré de la peau et des os, une atrophie sévère des muscles squelettiques et une adipogénèse réduite (Peng et al. 2003). La double délétion des isoformes Akt1 et Akt3 a résulté en une létalité embryonnaire due à de graves anomalies au niveau des systèmes cardiovasculaire et nerveux (Yang et al. 2005). Au contraire, les souris dépourvues d'Akt2 et Akt3 étaient viables malgré une taille corporelle réduite et une incapacité à métaboliser le glucose (Dummler et al. 2006). La viabilité de ce dernier phénotype suggère que la présence de l'isoforme Akt1 est suffisante pour effectuer toutes les fonctions essentielles à la survie postnatale. Mais qu'en est-il du rôle de chacun des isoformes dans la tumorigénèse et la progression tumorale? Étant donné que l'inhibition d'Akt interfère avec plusieurs processus physiologiques chez les cellules cancéreuses comme chez les cellules saines (Tan et al. 2010), il est impératif d'élaborer des stratégies thérapeutiques permettant de cibler spécifiquement les cellules cancéreuses ou l'isoforme d'Akt responsable du cancer afin de minimiser les effets secondaires. La spécificité fonctionnelle des isoformes d'Akt dépend tout d'abord de leur expression relative dans les différents tissus et cancers. Il est primordial d'identifier l'isoforme dominant dans la tumeur afin de justifier son ciblage par un inhibiteur pharmacologique. De plus, les avancées en recherche nous ont révélé l'existence d'une spécificité des isoformes envers leurs substrats. Ainsi, les trois kinases Akt n'ont pas nécessairement la même influence sur un même substrat. Enfin, nous savons aujourd'hui

que chaque isoforme possède une distribution intracellulaire distincte dépendamment du contexte cellulaire. Par conséquent, le potentiel d'activité de kinase est spécifique à chaque Akt en fonction de leur disponibilité dans la cellule (Fortier et al. 2011).

1.4.1 L'activité spécifique des isoformes d'Akt dans les tumeurs

Il est maintenant bien établi que l'activité anormalement soutenue d'Akt joue un rôle majeur dans la tumorigénèse et la progression tumorale d'une grande variété de cancers. Les trois isoformes d'Akt sont présents dans la plupart des tissus, mais leur niveau d'expression est variable. Par exemple, les niveaux d'expression d'Akt1 sont généralement plus élevés que ceux d'Akt2, excepté dans les tissus sensibles à l'insuline comme le foie, le muscle squelettique et le tissu adipeux (Altomare et al. 1998; Zinda et al. 2001). Akt3 est beaucoup moins présent, en particulier dans les tissus intestinaux et les muscles. Toutefois, on retrouve une forte expression d'Akt3 dans le cerveau, les testicules, les poumons et les reins (Brodbeck et al. 1999). Plusieurs études ont identifié une activité accrue ou constitutive de l'un des isoformes d'Akt dans différents cancers. Environ 40 % des cancers du sein et de l'ovaire, plus de 50 % des cancers de la prostate et environ 20 % des adénocarcinomes gastriques montrent une augmentation de l'expression et de l'activité d'Akt1 (Sun et al. 2001). Une activité élevée de la kinase Akt2 a été démontrée dans 25 % des cancers du sein (Sun et al. 2001) et dans environ 30 % des cancers de l'ovaire (Yuan et al. 2000), la majorité des cas étant des tumeurs ayant un stade avancé. Une proportion similaire de carcinomes pancréatiques ont montré une activité d'Akt2 nettement plus élevée comparativement aux tumeurs pancréatiques bénignes et au tissu normal (Altomare et al. 2002). Enfin, une expression élevée d'Akt2 a été observée dans près de 40 % des carcinomes hépatocellulaires (Xu et al. 2004). Akt3 est le principal isoforme actif dans environ 60 % des mélanomes de stade avancé alors que cette kinase est absente des mélanocytes normaux (Stahl et al. 2004). Une augmentation de l'activité d'Akt3 a également été rapportée dans les cancers hormonaux-indépendants du sein et de la prostate (Nakatani et al. 1999). Il est intéressant de noter que l'activation constitutive des isoformes d'Akt semble être davantage associée à la progression tumorale plutôt qu'à son initiation. Par exemple,

dans différents types de tumeurs, l'expression et/ou l'activité kinase d'Akt2 augmente avec le grade de malignité du cancer (Yuan et al. 2000; Sun et al. 2001; Altomare et al. 2002; Mure et al. 2010). La même corrélation est observée pour Akt3 dans les mélanomes métastatiques (Stahl et al. 2004).

Les causes de l'hyperactivation des isoformes d'Akt dans les tumeurs sont multiples. La phosphorylation constitutive d'Akt dans les cancers peut être expliquée par une mutation du gène, une amplification de sa transcription ou une stabilité accrue de l'ARN messager. Des mutations au niveau des gènes *AKT1*, *AKT2* et *AKT3* sont rarement observées dans les cancers humains (Soung et al. 2006). À ce jour, une seule mutation sur *AKT1* a été décrite, Glu17→Lys, située dans le domaine PH (Carpten et al. 2007). Cette mutation favorise la liaison d'Akt1 à la membrane plasmique et stimule donc son activation. Bien que cette mutation ait été signalée comme étant responsable de la tumorigénèse de divers cancers (sein, côlon, ovaires, poumon, endomètre, leucémie et rétinoblastome) (Carpten et al. 2007; Kim et al. 2008; Malanga et al. 2008; Cohen et al. 2009; Cohen et al. 2010), elle demeure exceptionnelle et contribue faiblement à la fréquente hyperactivation d'Akt1 observée dans de nombreux cancer. Des mutations sur les gènes *AKT2* et *AKT3* sont également très rares (Parsons et al. 2005; Davies et al. 2008; Dutt et al. 2009). L'analyse de plusieurs types de tumeurs a indiqué que l'amplification des gènes *AKT* et l'accumulation de leurs ARN messagers ne se produisent que dans un petit nombre de cellules cancéreuses et n'est pas un événement fréquent dans la tumorigénèse. En effet, l'amplification du gène *AKT1* a été initialement détectée dans un seul cas parmi 255 tumeurs humaines analysées (Staal 1987). Une étude plus récente a aussi révélé l'amplification et la surexpression d'*AKT1* dans un seul cas de gliosarcome parmi 103 tumeurs analysées (Knobbe et al. 2003). L'amplification d'*AKT2* est relativement plus fréquente, particulièrement dans les cancers ovariens, pancréatiques et mammaires (Cheng et al. 1992; Bellacosa et al. 1995; Cheng et al. 1996). Très peu d'anomalies concernant le gène *AKT3* ont été rapportées dans la littérature. Toutefois, une étude récente a clairement démontré une amplification de l'ARN messager dans certains cancers du sein et de la prostate (Nakatani et al. 1999; Kirkegaard et al. 2010). Dans les mélanomes, l'activité accrue d'Akt3 est la

conséquence de l'augmentation du nombre de copies du gène *AKT3* et de l'inactivation du suppresseur de tumeur PTEN (Stahl et al. 2004).

Ainsi, la mutation des gènes *AKT* n'est pas considérée comme un événement majeur dans le développement du cancer. En effet, la cause la plus fréquente de l'hyperactivation de ces kinases est l'altération d'éléments en amont de la signalisation intracellulaire d'Akt. Il s'agit notamment de l'amplification d'oncogènes tels que PI3K et Ras, la perte de gènes suppresseurs de tumeurs comme PTEN et l'activation aberrante ou la surexpression des récepteurs de facteurs de croissance tels que EGFR (pour *epidermal growth factor receptor*) et HER2 (pour *human epidermal growth factor receptor 2*) (Altomare et al. 2005; Berenjeno et al. 2009).

1.4.2 Le rôle des isoformes d'Akt dans la croissance tumorale

Plusieurs études ont tenté d'identifier les mécanismes par lesquels les isoformes d'Akt régulent la prolifération cellulaire de façon spécifique dans les différents types cellulaires. L'analyse des fibroblastes d'embryon de souris dépourvues d'Akt1 ou d'Akt2 a montré que l'absence d'Akt1 entraîne un retard dans le cycle cellulaire en diminuant l'accumulation de la cycline D1, alors que la déplétion d'Akt2 n'affecte pas la prolifération cellulaire (Yun et al. 2009). La substitution du domaine PH de la kinase Akt1 par celui de la kinase Akt2 a démontré que la spécificité fonctionnelle d'Akt1 dans la prolifération était due à son domaine PH (Yun et al. 2009). L'inhibition d'Akt1, par interférence avec son ARN (siRNA), s'est également avérée suffisante pour diminuer la prolifération des cellules d'un cancer ovarien en diminuant l'expression des cyclines D et E (Meng et al. 2006). Cependant, la spécificité du rôle d'Akt1 dans la régulation du cycle cellulaire a été remise en question par une autre équipe de chercheurs révélant que cette fonction était spécifique à l'isoforme Akt2 dans le même modèle cellulaire (Noske et al. 2007).

Pour ajouter à la complexité du rôle de chacun des isoformes d'Akt dans la croissance tumorale, une étude récente a démontré que l'activité des isoformes Akt1 et

Akt2 est nécessaire pour la prolifération *in vitro* et *in vivo* des cellules cancéreuses du côlon HCT116 et DLD1 (Ericson et al. 2010). L'effet synergique des isoformes d'Akt a également été observé dans différentes lignées cellulaires de gliomes malins. En effet, la diminution de la prolifération cellulaire a été plus importante suite à l'inhibition simultanée d'Akt2 et d'Akt3 comparativement à l'inhibition individuelle de chacun des isoformes (Mure et al. 2010).

L'activité d'Akt3 régule aussi la prolifération des cellules qui expriment majoritairement cet isoforme telles que les cellules de mélanomes (Stahl et al. 2004; Madhunapantula et al. 2007). De façon similaire, l'hyperactivité d'Akt3 qui a été rapportée dans les cancers du sein hormonaux-indépendants suggère que cette kinase puisse contribuer à l'agressivité du carcinome. En effet, la surexpression d'Akt3 dans la lignée cellulaire cancéreuse MCF-7 abolit la sensibilité de ces cellules aux œstrogènes et favorise leur croissance *in vivo* en absence de l'hormone (Faridi et al. 2003).

L'importance relative des isoformes d'Akt dans la prolifération cellulaire est spécifique au type cellulaire (Koseoglu et al. 2007). L'importance de cibler le bon isoforme est bien illustrée par cette étude révélant que l'inhibition d'Akt1 ou d'Akt2 peut avoir un effet inverse sur les tumeurs. En utilisant un modèle de cancer du sein chez la souris, les chercheurs ont montré que l'absence d'Akt1 inhibe la formation de tumeurs alors que l'absence d'Akt2 accélère leur apparition (Maroulakou et al. 2007). Par conséquent, afin d'obtenir l'effet le plus spécifique sur l'inhibition de la croissance tumorale, il est nécessaire de déterminer à l'avance quel isoforme est responsable de la croissance des cellules tumorales.

1.4.3 Le rôle des isoformes d'Akt dans la chimiorésistance

Akt1 joue un rôle essentiel dans la survie cellulaire et la chimiorésistance des cellules cancéreuses du poumon. La surexpression d'Akt1 dans ce type de cellules tumorales est suffisante pour leur conférer une résistance au cisplatine (Liu et al. 2007; Lee et al. 2008). En outre, l'inhibition d'Akt2 et d'Akt3 n'affecte pas le taux de mort

cellulaire induite par le cisplatine dans ces cellules (Lee et al. 2008). Cependant, dans les cellules cancéreuses utérines, la surexpression de l'isoforme Akt1 n'influence pas l'apoptose induite par le cisplatine, mais est suffisante pour rendre les cellules résistantes au taxol (Gagnon et al. 2008).

Akt2 participe généralement à la chimiorésistance des cellules cancéreuses dépendantes des hormones. Par exemple, cet isoforme confère une résistance au paclitaxel chez les cellules cancéreuses du sein MCF-7 (Cheng et al. 2007) et au cisplatine chez les cellules cancéreuses de l'endomètre (Girouard et al. 2013). L'activité d'Akt2 neutralise également l'apoptose induite par le docétaxel dans les cellules cancéreuses de l'ovaire et du sein par l'augmentation des niveaux de survivine (Xing et al. 2008; Weng et al. 2009). Cette protéine de survie fait partie d'une famille d'inhibiteurs de l'apoptose, appelés les IAP (pour *inhibitor of apoptosis proteins*), qui bloquent le clivage et l'activation des caspases et qui inclut les IAP-1, IAP-2 et XIAP (Schimmer et al. 2005). Leur expression dans les cellules cancéreuses est associée à la résistance à divers agents pro-apoptotiques comme TGF-β (Van Themsche et al. 2007), TNF-a (Van Themsche et al. 2008), TRAIL (Amantana et al. 2004; Gill et al. 2009) et à divers agents chimiothérapeutiques tels que la doxorubicine et le paclitaxel (Hu et al. 2003; Amantana et al. 2004; Gagnon et al. 2008; Cheng et al. 2010). Certaines études ont montré qu'Akt2 peut réguler les niveaux des IAP dans les cellules cancéreuses (Gagnon et al. 2003; Srinivasan et al. 2009). Par exemple, l'expression d'Akt2 bloque la diminution de XIAP induite par le cisplatine et donc l'apoptose dans les cellules cancéreuses de l'ovaire A2780 (Fraser et al. 2003). En effet, Akt2 peut phosphoryler XIAP afin de le protéger de la dégradation en réponse au cisplatine (Dan et al. 2004).

Akt3 peut également affecter la chimiorésistance des cellules cancéreuses qui l'expriment. Dans des lignées cellulaires de mélanome, la réduction de l'expression d'Akt3 augmente le clivage de la caspase 3 induit par la staurosporine (Madhunapantula et al. 2007). De façon similaire, les cellules cancéreuses du sein MCF-7 exprimant Akt3 se sont avérées résistantes à la mort cellulaire induite par le tamoxifène (Faridi et al. 2003).

Les isoformes d'Akt ont un impact différent sur la résistance des cellules à un agent chimiothérapeutique et l'activité d'un isoforme ne confère pas la même résistance aux différentes drogues. La chimiorésistance est un phénomène multifactoriel qui implique plusieurs voies de signalisation en aval de l'activation d'Akt. La spécificité de substrat pour chacun des isoformes joue donc un rôle majeur dans ce contexte.

1.4.4 Le rôle des isoformes d'Akt dans la migration cellulaire

1.4.4.1 Akt1 favorise le potentiel invasif

La migration et l'invasion cellulaire sont la conséquence d'une perte de la polarité apico-basale, d'une réorganisation du cytosquelette, de la dégradation de la MEC et de l'expression de gènes favorisant l'invasion cellulaire. Il est maintenant bien établi qu'Akt1 favorise l'invasion des fibroblastes et des cellules endothéliales (Higuchi et al. 2001; Zhou et al. 2003; Ackah et al. 2005). La spécificité du rôle des isoformes Akt1 et Akt2 dans la migration a été mise en évidence suite à l'étude des fibroblastes de souris déficientes en chacun de ces isoformes. Alors que l'absence d'Akt2 accélère la migration des cellules à travers la MEC, la délétion d'Akt1 ralentit ce processus comparativement aux fibroblastes de type sauvage (Zhou et al. 2006). Le potentiel invasif des fibroblastes dépourvus d'Akt2 est associé avec une augmentation de l'activation des protéines Pak1 et Rac. Ces deux protéines entraînent la réorganisation du cytosquelette d'actine et la formation de lamellipodes, étapes clés de la migration cellulaire. L'effet inhibiteur d'Akt2 sur la migration a été confirmé en montrant que, bien que les deux isoformes Akt1 et Akt2 soient capables de former un complexe avec Pak1, seul Akt1 peut l'activer. L'activité kinase des différents isoformes d'Akt peut donc être variable pour un même substrat. Akt1 favorise également l'invasion des fibroblastes HT1080 via la production des MMP induites par le facteur de transcription NF-κB (Kim et al. 2001). Cependant, l'expression d'Akt1 n'affecte pas les propriétés d'adhérence de ces fibroblastes entre eux ou avec la MEC. Certaines études ont révélé qu'Akt1 peut également induire la motilité des cellules cancéreuses d'origine épithéliales. Tel qu'observé dans les lignées cellulaires de fibroblastes, l'activation d'Akt1 dans les cellules épithéliales mammaires de souris augmente l'activité de la MMP-2, favorisant

ainsi le potentiel invasif (Park et al. 2001). En outre, l'absence d'Akt1 réduit les métastases pulmonaires de cancers du sein provoqués par l'oncogène ErbB2 chez la souris via l'activation du suppresseur de tumeur TSC2 (Ju et al. 2007). De même, l'inhibition d'Akt1 dans la lignée cellulaire de cancer ovarien OVCAR-3 est suffisante pour réduire la migration et l'invasion cellulaire (Meng et al. 2006).

1.4.4.2 Akt2 facilite aussi la motilité cellulaire

Akt2 peut également stimuler la migration et l'invasion de cellules cancéreuses. Une tendance semble se dégager des études *in vitro* selon laquelle Akt1 favorise généralement la migration des fibroblastes et des cellules endothéliales, alors qu'Akt2 serait responsable de ce phénomène dans les cellules épithéliales. De plus, il est intéressant de remarquer que dans les cellules où un isoforme stimule la motilité, un autre isoforme va généralement jouer un rôle limité, voire opposé. L'équipe du Dr Toker a montré que l'activité d'Akt1 dans le cancer du sein limite la migration cellulaire alors qu'Akt2 favorise ce phénotype (Chin et al. 2011). En effet, Akt1 diminue l'activité du facteur de transcription pro-migratoire NFAT (Yoeli-Lerner et al. 2005) et le remodelage de l'actine en maintenant le cytosquelette intact via la protéine palladine (Chin et al. 2010). De façon similaire, l'inhibition d'Akt1 favorise la migration et l'EMT des cellules épithéliales mammaires MCF10A via l'activation des kinases ERK, alors que l'inhibition d'Akt2 supprime cette EMT (Irie et al. 2005). L'absence d'Akt2 diminue aussi la migration induite par l'EGF des cellules MDA-MB-231, T47D, et MCF7 du cancer du sein (Wang et al. 2008). De plus, les cellules MDA-MB-231 dépourvues d'Akt2 forment beaucoup moins de métastases aux poumons de la souris (Wang et al. 2008). Les métastases de tumeurs provoquées par l'oncogène ErbB2 chez la souris sont aussi favorisées en présence d'Akt2, alors que l'activation d'Akt1 diminue les lésions métastatiques (Hutchinson et al. 2004; Dillon et al. 2009). Différents rôles pour les isoformes d'Akt ont également été observés dans certaines lignées des cancers du poumon et du côlon. L'inhibition d'Akt2 diminue grandement la migration et l'invasion de ces cellules, alors que les isoformes Akt1 et Akt3 ont très peu d'effet sur ces processus (Sithanandam et al. 2005; Rychahou et al. 2008). En utilisant une

approche opposée où l'expression ectopique de chaque isoforme d'Akt est induite, il a été démontré que seul Akt2 favorise la migration et l'invasion des cellules cancéreuses du sein et de l'ovaire, et ce, en augmentant l'expression des intégrines β1 (Arboleda et al. 2003). Conformément à ces résultats, d'autres chercheurs ont observé que le facteur de transcription Twist régulant l'EMT était capable d'induire spécifiquement l'expression d'Akt2 dans les cellules cancéreuses du sein (Cheng et al. 2007). De façon similaire, la kinase ILK associée aux intégrines active préférentiellement Akt2, qui induit à son tour l'activation de mTOR aboutissant à l'expression de la MMP-2 (Fan et al. 2009). Il apparaît donc qu'en dépit de leur grande homologie de séquence, Akt1 et Akt2 peuvent être régulés différemment par les kinases en amont et que leur action sur les substrats en aval peut aussi être distincte, voire opposée.

1.4.4.3 Les isoformes d'Akt peuvent jouer des rôles opposés dans la progression tumorale

La différence dans les fonctions exercées par les trois isoformes d'Akt peut être liée à leur expression et leur activation spécifiques dans les différents tissus, à la diversité de leurs substrats ou à leur localisation intracellulaire distincte (Figure 1.23). L'abondance et l'activité relatives des isoformes peuvent être dynamiques et changer en fonction des différents contextes cellulaires. Par exemple, Akt3 est l'isoforme prédominant dans la majorité des mélanomes. Il participe donc à la croissance et la progression tumorales (Stahl et al. 2004; Madhunapantula et al. 2007). Par contre, le potentiel invasif des lignées cellulaires de cancer du sein peut dépendre non pas de l'expression et l'activité des isoformes Akt1 et Akt2, mais de l'équilibre entre les deux et entre leur signalisation spécifique en aval (Yoeli-Lerner et al. 2005; Iliopoulos et al. 2009). En outre, les isoformes peuvent interagir avec des effecteurs distincts, comme TSC2 (Liu et al. 2006; Ju et al. 2007), NFAT (Yoeli-Lerner et al. 2005) et palladine (Chin et al. 2010) qui sont spécifiquement liés à l'activité d'Akt1. De plus, les isoformes d'Akt peuvent avoir un impact différent sur leurs substrats communs. Par exemple, Akt1 active Pak1, alors qu'Akt2 inhibe son activité kinase (Zhou et al. 2006). Enfin, la localisation intracellulaire distincte des isoformes influence certainement l'accès aux substrats et donc leur activité kinase. Une étude exhaustive de plusieurs lignées

cellulaires a permis de déterminer qu'Akt1 se situe généralement dans le cytoplasme, Akt2 se retrouve en majorité au niveau des organelles telles que les mitochondries et l'appareil de Golgi et Akt3 est présent dans le noyau de tous les types cellulaires analysés (Santi et al. 2010).

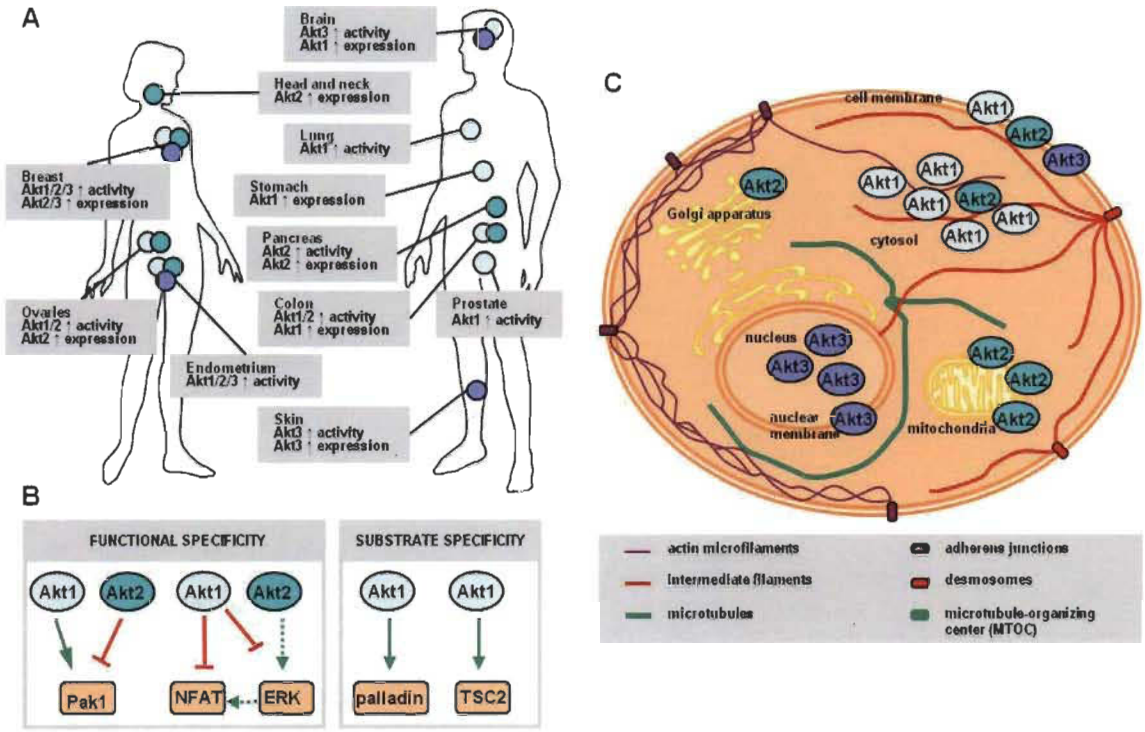


Figure 1.23 **Mécanismes impliqués dans la spécificité fonctionnelle des isoformes d'Akt au cours de la progression tumorale.**
La différence dans les fonctions exercées par les trois isoformes d'Akt peut être liée à leur expression et leur activation spécifiques dans les différents tissus (*A*), à la diversité de leurs substrats (*B*) ou à leur localisation intracellulaire distincte (*C*) (Fortier et al. 2011).

1.4.4.4 Le cytosquelette de filaments intermédiaires influence la localisation d'Akt

Il y a des preuves croissantes que la localisation distincte des isoformes d'Akt résulte de leur interaction avec le cytosquelette de FIs, ce dernier pouvant séquestrer ou libérer les kinases Akt selon le contexte cellulaire. En effet, il a été montré que le réseau de filaments de K10 séquestre Akt, ce qui empêche sa translocation membranaire et son activation. Les auteurs de l'étude ont suggéré que la K10 inhibe ainsi la prolifération des kératinocytes via la séquestration d'Akt (Paramio et al. 2001). Dans un contexte de

migration cellulaire, les filaments de kératines qui sont liés aux intégrines β4 des hémidesmosomes sont nécessaires à la transmission du signal vers Akt (Kippenberger et al. 2010). De plus en plus d'études soutiennent l'hypothèse selon laquelle les kératines jouent un rôle dans la régulation et la localisation des isoformes d'Akt, en séquestrant ou en agissant comme un échafaudage pour ces kinases.

La spécificité fonctionnelle des isoformes d'Akt dans la progression du cancer souligne la nécessité d'une meilleure analyse de leur profil d'expression et de leur spécificité de substrats. Dans certains cas, l'isoforme prédominant est le plus susceptible d'être impliqué dans la promotion de la croissance tumorale, la chimiorésistance et la migration cellulaire. Dans d'autres cas, un isoforme peut agir en synergie ou au contraire bloquer la signalisation médiée par un autre. Il est donc crucial de déterminer quel(s) isoforme(s) est(sont) responsable(s) de la progression d'une tumeur afin de développer une stratégie thérapeutique appropriée.

1.5 Problématique et objectifs de recherche

La formation de métastases est la principale cause de décès chez les patients atteints de cancer. Une des approches thérapeutiques envisagées par les chercheurs au cours des dernières années consiste à empêcher la dissémination des cellules tumorales afin de réduire le risque de récurrence de la maladie. Cette stratégie repose essentiellement sur la précocité du diagnostic et sur le ciblage de régulateurs moléculaires de l'EMT. Les tests de détection des cellules tumorales circulantes (CTC) reposent en grande partie sur l'expression des kératines dans le sérum des patients. Or, les cellules cancéreuses perdent cette expression au cours de l'EMT. Les analyses actuelles sous-estiment donc la présence des CTC, ce qui affecte directement le pronostic de la maladie et la stratégie de traitement (Bonnomet et al. 2011; Gradilone et al. 2011; Lecharpentier et al. 2011; Joosse et al. 2012). De plus, il est d'un urgent besoin de mieux comprendre les mécanismes moléculaires impliqués dans la régulation de l'EMT afin d'identifier de nouvelles cibles thérapeutiques pour bloquer la transformation maligne du cancer (Reka et al. 2011).

Le rôle des kératines dans le maintien de l'intégrité épithéliale et l'homéostasie cellulaire soulève l'hypothèse selon laquelle la préservation ou la réexpression des kératines par les cellules tumorales pourrait réprimer l'EMT et ultimement le potentiel métastatique de la tumeur.

Cette étude a pour objectif de déterminer le rôle des kératines dans la transformation maligne des cellules épithéliales cancéreuses en réponse aux oncogènes Akt, afin d'évaluer leur potentiel thérapeutique dans l'inhibition de l'EMT.

Plus spécifiquement, nos objectifs sont :

1. De déterminer l'impact des oncogènes Akt, importants médiateurs de l'EMT, sur l'expression des filaments intermédiaires chez différentes lignées de cellules épithéliales cancéreuses.

2. De caractériser les mécanismes moléculaires régulés par le cytosquelette de kératines dans la progression tumorale et l'EMT médiées par les oncogènes Akt chez différentes lignées de cellules épithéliales cancéreuses.

CHAPITRE II

AKT ISOFORMS REGULATE INTERMEDIATE FILAMENT PROTEIN LEVELS IN EPITHELIAL CARCINOMA CELLS

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RÉSUMÉ

Les filaments intermédiaires (FIs) sont un des composants majeurs du cytosquelette des cellules. Les FIs regroupent un grand nombre de protéines aux propriétés distinctes et dont la distribution est spécifique à différents tissus. Ce sont les *kératines* qui constituent le réseau de FIs dans les cellules épithéliales. Au cours de la tumorigénèse, l'expression des kératines 8 et 18 (K8/18) est maintenue. Toutefois, à un stade avancé du cancer, les cellules peuvent exprimer un autre type de FIs, la *vimentine*, normalement exprimée dans les cellules mésenchymateuses. Cette co-expression est associée à une augmentation du potentiel invasif/métastatique des cellules. La famille d'oncogènes Akt joue un rôle central dans la prolifération et la motilité cellulaire. On dénombre trois isoformes différents (Akt1, Akt2 et Akt3) dont les rôles spécifiques dans l'invasion et la métastasie sont peu définis. Notre hypothèse suggère que certains isoformes d'Akt pourraient augmenter le potentiel invasif/métastatique des cellules cancéreuses en régulant l'expression des FIs, soit les kératines et la vimentine. Nos résultats montrent que la surexpression de l'isoforme Akt1 augmente les niveaux protéiques des K8/18 alors que l'isoforme Akt2 induit l'expression de la K18 et de la vimentine chez les cellules épithéliales cancéreuses. Inversement, l'inhibition de l'activité des isoformes Akt1 et Akt2 par interférence à l'ARN diminue les niveaux de K8/18. Pour examiner la régulation de l'expression des FIs dans des conditions plus physiologiques, nous avons exposé les cellules au TGF-β1 et à l'insuline, connus pour induire l'activation d'Akt. Nous avons démontré que le TGF-β1 induit l'expression de la vimentine de façon dépendante de l'activité d'Akt. À notre connaissance, ces résultats représentent la première indication que les oncogènes Akt régulent l'expression des FIs. Ces résultats soutiennent l'hypothèse selon laquelle l'expression des FIs joue un rôle actif dans la progression du cancer.

Akt isoforms regulate intermediate filament protein levels in epithelial carcinoma cells

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Abbreviations: IFs, intermediate filaments, K8/18, keratins 8 and 18; vim, vimentin; PI3K, phosphatidylinositol 3-kinase; CA-Akt1, constitutively activated Akt1; TGF-β1, transforming growth factor β1; shRNA, short hairpin RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate buffered saline; CHX, cycloheximide.

ABSTRACT

Keratin 8 and 18 are simple epithelial intermediate filament (IF) proteins, whose expression is differentiation- and tissue-specific, and is maintained during tumorigenesis. Vimentin IF is often co-expressed with keratins in cancer cells. Recently, IF have been proposed to be involved in signaling pathways regulating cell growth, death and motility. The PI3K/Akt pathway plays a pivotal role in these processes. Thus, we investigated the role of Akt (1 and 2) in regulating IF expression in different epithelial cancer cell lines. Over-expression of Akt1 increases K8/18 proteins. Akt2 up-regulates K18 and vimentin expression by an increased mRNA stability. To our knowledge, these results represent the first indication that Akt isoforms regulate IF expression and support the hypothesis that IFs are involved in PI3K/Akt pathway.

INTRODUCTION

Intermediate filaments (IFs) constitute an extensive cytoskeletal network whose proteins constituents are encoded by a large family of genes that are expressed in a tissue- and differentiation state-specific manner [1]. Keratins are the major IFs proteins expressed in epithelial cells and are associated in obligate heteropolymers. The keratins 8 and 18 (K8/18) are typically co-expressed and constitute the primary keratin pair in simple epithelial cells. As part of the cytoskeleton, IFs are important in providing mechanical stability and integrity of cells and tissues submitted to mechanical and toxic stress. Moreover, recent studies have shown that IFs are involved in signalling pathways which regulate epithelial cell growth, resistance to apoptosis and motility [1].

K8/18 are expressed in most epithelial tumors (carcinomas) [2]. Moreover, vimentin, a mesenchymal-specific IFs protein, is often co-expressed with keratins in late-stage epithelial cancer cells presenting a dedifferentiated phenotype [3-5]. To understand the significance of IFs protein increase expression in cells, different researchers have addressed the question as to whether IFs expression affects tumor fate and behavior. It was reported that K18 over-expression correlates with reduced invasive/metastatic potential and tumorigenicity in a human breast cancer [6-7], whereas an enhanced migratory and invasive potential is observed in mouse fibroblasts [8]. The co-expression of keratins with vimentin is associated with an increased metastatic potential [9] and down-regulation of vimentin expression resulted in impaired invasion of colon and breast cancer cell lines [10]. For this reason, it is generally thought that the balance between keratins and vimentin expression in carcinoma cells would dictate cellular invasiveness and metastatic potential. However, the molecular mechanisms regulated by IFs as well as the regulation of their own expression pattern in a context of normal and cancer cells remain poorly understood.

Similar to IFs, PI3K/Akt signaling pathway plays a pivotal role in cell proliferation, survival and migration. There are three isoforms of Akt. Amplification of Akt1 and/or Akt2 isoform is frequently found in human cancers [11] and, increasing number of studies demonstrate isoforms-specific functions for Akt kinases in carcinoma

cells [12-13]. In breast cancer cells for example, it has been shown that overexpression of Akt1 decreases cellular invasiveness [14] whereas overexpression of Akt2, to the contrary, increases cellular invasiveness [15]. Akt3 has a minimal induction effect on these tumors [16]. Downstream targets of Akt isoforms which mediate their role in cancer progression, however, are only partially elucidated.

Akt is known to regulate cell proliferation [17] and there is increasing evidences that keratins could play a role in cell growth, protein synthesis and cell cycle by regulating key signaling molecules, like 14-3-3 proteins and proteins of the Akt-mTor pathway [17-18]. Another function of Akt is to prevent apoptosis in both intrinsic and extrinsic pathway [13-19]: IFs have been shown to attenuate pro-apoptotic signals such as TNFα and Fas by scaffolding and organizing death effector proteins [20-23]. Moreover, Akt is an important modulator of cell motility and invasion [24] and IFs are involved in cell migration and wound healing through their interaction with adhesion proteins [25-27]. All these findings suggest that IFs could be associated with Akt-regulated cellular processes.

In the present study, we have investigated whether Akt1 and/or Akt2 regulate the expression and organization of K8/18 and vimentin in different epithelial cancer cell lines.

MATERIALS AND METHODS

Cell lines and reagents

Human endometrial carcinoma KLE cell line, human cervical carcinoma HeLa cell line and human hepatocellular carcinoma HepG2 cell line were purchased from ATCC. Cells were maintained in growth medium supplemented with serum and 50 μg/ml gentamycin. Akt1, Akt2, pAkt (Ser-473) and pGSK3β (Ser-9) antibodies were purchased from Cell Signaling Technology (Beverly, MA). K8/18 antibodies were a generous gift from Dr M. Bishr Omary (University of Michigan, MI). Vimentin and GAPDH

antibodies were purchased from Abcam (Cambridge, MA). LY294002 was from Cell Signaling Technology. Cycloheximide and β-actin were from Sigma (St-Louis, MO). TGF-β1 was purchased from Calbiochem (San Diego, CA).

Transfection with short hairpin RNAs (shRNAs) and plasmids

Cells were transfected as described before [28] with Akt1, Akt2 or control (scrambled) shRNAs (SABiosciences, Frederick, MD), or constitutively active Akt1 vector (CA-Akt1), constitutively active Akt2 vector (CA-Akt2) or empty pcDNA3.1 vector [13].

Western blots and qRT-PCR

Total cellular proteins and mRNA were extracted as described [28]. Quantitative real-time PCR was performed with LightCycler® 480 (Roche, Laval, Canada) using K8 sense primer 5'-agggctgaccgacgagat-3' and antisense 5'-caccacagatgtgtccgaga-3'; K18 sense primer 5'-tgatgacaccaatatcacacga-3' and antisense 5'-ggcttgtaggccttttacttcc-3'; and vimentin sense primer 5'-aaagtgtggctgccaagaac-3' and antisense 5'-agcctcagagaggtcagcaa-3'. Samples were analyzed in triplicates from three independent experiments. Relative quantification of expression was calculated by the comparative $2^{-\Delta\Delta CT}$ method [29] with GAPDH and TBP as reference genes.

Immunofluorescence

Cells were grown on glass coverslips, fixed with 4% paraformaldehyde for 10 min, washed twice in PBS and permeabilized for 10 min in citrate solution (0.1% sodium citrate, 0.1%Triton X-100 in PBS) at room temperature. Triple immunofluorescence staining was performed as described before [28].

Statistical analyses

The data were subjected to one-way ANOVA analysis of variance (PRISM software version 3.03; GraphPad, San Diego, CA). Differences between experimental groups were determined by the Tukey's test. Statistical significance was accepted when $p < 0.05$.

RESULTS

Akt1 and Akt2 isoforms differentially-regulates IF proteins levels in epithelial carcinoma cells

To determine whether Akt isoforms can affect IFs expression in epithelial carcinoma cells, we used three epithelial carcinoma cell lines whose various differentiation state and p-Akt levels constitute an interesting experimental model: two p-Akt-negative cell lines, HepG2 and HeLa cells, obtained respectively from well and moderately-differentiated epithelial carcinoma, and one p-Akt-positive and poorly-differentiated cell line, KLE cells.

We have investigated the role of Akt1 and Akt2 isoforms in regulating the expression of IFs proteins in p-Akt-negative HeLa and HepG2 cells. Cells were transfected with constitutively active Akt (CA-Akt) constructs inducing the expression of myristoylated Akt proteins, which are constitutively phosphorylated/activated in the cells [13]. Increased phosphorylation of Akt proteins as well as one of their substrate GSK3β (Fig. 1, A and B) confirms that CA-Akt proteins are active in our experimental conditions.

Our results show that over-expression of CA-Akt1 isoform in p-Akt-negative HeLa and HepG2 cells increases K8 and 18 protein levels in the two cell lines. Vimentin, which is only detectable in HeLa cells, is not significantly affected (Fig. 1A). On the other hand, expression of CA-Akt2 isoform up-regulates K18 protein level in the two cell lines, while K8 is not affected. Interestingly, unlike CA-Akt1, overexpression of

CA-Akt2 isoform significantly up-regulates vimentin protein levels (Fig. 1B). These results suggest that activation of Akt1 and Akt2 regulates IFs protein expression and that the regulation is Akt is isoform-specific.

We also examined whether increase in K8/18 was regulated at transcriptional or posttranscriptional level. We found that transfection of HepG2 and HeLa cells with CA-Akt1 and CA-Akt2 had no effect on K8, K18 or vimentin mRNAs (Fig. 1C). Thus, the regulation of IFs protein levels by Akt1 and Akt2 activation occurs at the posttranscriptional level.

In the opposite manner, we have analysed the role of Akt1 and Akt2 isoforms in regulating the expression of IFs in p-Akt-positive KLE cells using shRNA. Knockdown of Akt1 decreases K8 protein level, whereas K18 and vimentin expression were not affected (Fig. 1D). This could be explained by the presence of active Akt2 isoform in KLE cells, which could maintain K18 and vimentin at a high level. For instance, Akt2 knockdown significantly decreases K18 as well as K8 protein levels (Fig. 1E). Knockdown of Akt1 or Akt2 individually in KLE cells was not sufficient to decreased vimentin protein levels (Fig. 1D). Altogether, these results reveal that Akt regulates IFs protein levels in carcinoma cells, in an isoform-specific manner.

To determine whether the increase in IFs proteins following Akt overexpression requires new protein, the effect of cycloheximide, a protein synthesis inhibitor, on Akt2-induced IFs expression was studied in HeLa cells. The induction of IFs proteins by Akt2 was reduced following cycloheximide treatment (Fig. 2), suggesting that new protein synthesis is required for the Akt2-induced IFs up-regulation. This suggests that Akt2 could modulate the stability of IFs mRNA and leads to the increased IFs expression.

Akt2 isoforms induces a reorganization of cytoplasmic IFs network in epithelial carcinoma cells

Epifluorescence microscopy using triple staining for pAkt, keratins/vimentin and nuclei shows that transfection of CA-Akt2 induces modifications of keratins and

vimentin networks. In CA-Akt2-transfected HeLa cells, pAkt shows a diffuse cytoplasmic staining with a prominent localization at the cell membrane (Fig. 3). In these cells, keratins and vimentin form a dense juxtanuclear cluster with little fibrillar extension which is not observed in non transfected cells. These results indicate that IFs network organization is modulated by Akt2 activation. Akt is known to remodel cytoskeleton by its interaction with actin in a cell migration context [30]. We performed a rhodamin-phalloidin staining to determine if Akt2-induced IFs reorganization could induce actin network remodelling. We observed that over-expression of Akt2 in HeLa cells induced actin cytoskeleton cortical remodelling, which is characterized by a denser actin network at the cell periphery. Taken together these results reveal that Akt2 regulates IFs and actin networks organization in carcinoma cells.

IFs are up-regulated by TGF-b1 and insulin in PI3K-dependent manner in epithelial carcinoma cells

To examine the regulation of IFs protein expression under a more physiological condition, we treated p-Akt-positive KLE cells with TGF-β1 or insulin, which are known to induce Akt activation [31, 32]. Exposure of the cells to TGF-β1 (Fig. 4A) or insulin (Fig.4B) increased the levels of Akt phosphorylation but did not modulate the levels of K8/18. However, vimentin protein level is significantly up-regulated (Fig. 4A). Treatment with PI3K inhibitor LY294002 blocked the TGF-β1-induced up-regulation of vimentin. These findings show that activation of PI3K/Akt pathway following treatment with physiological concentration of TGF-β1 is associated with upregulation of vimentin, in agreement with our findings that overexpression of constitutively active Akt proteins (Akt2 in this case) increases vimentin protein levels (Fig. 1B).

DISCUSSION

IFs protein expression pattern is commonly used as diagnostic marker in tumor pathology [2]. In fact, epithelial tumors maintain the keratin expression patterns of their respective cell type of origin and co-expression of vimentin is considered to be a marker

of de-differentiation (epithelial to mesenchymal transition) and invasiveness. In recent years, different studies have shown that IFs should not be considered only as markers proteins but also as regulators of differentiation and that they might play an active role in malignant transformation.

In this work, we investigated whether Akt isoforms could regulates IFs expression in epithelial carcinoma cells. Our results show that Akt1 up-regulates both K8 and K18 protein levels, whereas Akt2 increases K18 and vimentin expression, while K8 expression is not significantly modulated. These results suggest that IFs regulation mediated by Akt is isoform-specific. Although our results show that the increase in IFs protein does not result from increased transcription, it required new protein synthesis. Indeed, cycloheximide treatment inhibits Akt2-induced IFs up-regulation. We suggest that Akt could modulates mRNA stability and leads to the up-regulation of IFs proteins. Some studies have already shown that keratins protein synthesis could be regulated at a posttranscriptional level, through a stabilization of mRNA transcripts [33-35]. It has been proposed that some physiological block in translation prevents proteins synthesis until required during specific cell states and we can extrapolate this hypothesis to our model in that Akt activation could reverse the blocking of IFs translation in epithelial carcinoma cells [36-38].

As obligate heteropolymers, K8/18 are known to be associated and expressed in 1:1 ratio [1]. However, imbalance in K8/18 ratio has been shown to occur in different pathological conditions. For instance, K8 over-expression is associated with IFs aggregation and Mallory-Denk body formation in mice hepatocytes [39]. In vitro K18 over-expression has also been shown to induce IFs aggregation [40]. However, the functional significance of modifications in K8/18 ratio in cells is not known. Our results show that Akt2 induces an increase of K18 protein level, while K8 were not significantly modulated. The imbalance of K8/18 expression in Akt2-transfected cells could be related to vimentin, which are also up-regulated by Akt2 in HeLa cells. It was already shown that K18 could possibly interact with vimentin to form mixed filaments [41]. Another attractive explanation is that K18 could be unpolymerized either in insoluble

small aggregates [40] or in filamentous soluble pool, which could be undegraded due to their association with other proteins. All these possibilities need further investigation to be elucidated.

In conclusion, these results represent the first indication that Akt could regulate IFs protein levels in epithelial cancer cells. The physiological significance of differentially IFs up-regulation by Akt1 and Akt2 isoforms is not elucidated yet but this work suggest that IFs proteins are directly involved in PI3K/Akt signaling pathway.

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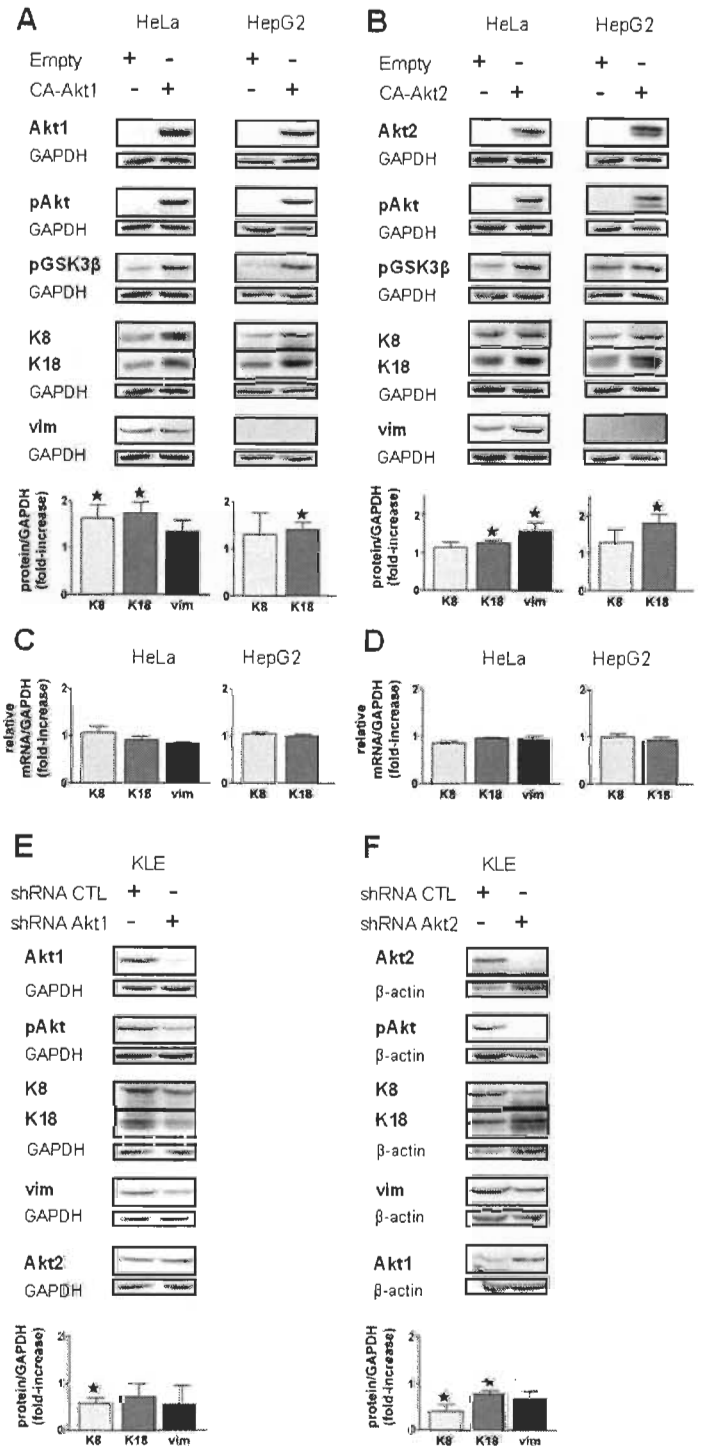

Figure 1. Intermediate filaments are differentially regulated by Akt1 and Akt2 isoforms in epithelial carcinoma cells. Protein levels of Akt1 or Akt2, pAkt, pGSK3, K8/18 and vimentin were analyzed by Western blot in HeLa and HepG2 cells transfected with control (empty) vector or with CA-Akt1 vector (***A***) or CA-Akt2 vector (***B***). mRNA levels of K8, K18 and vimentin were analyzed by quantitative real-time RT-PCR in HeLa and HepG2 cells transfected with empty vector or with CA-Akt1 vector (***C***) or CA-Akt2 vector (***D***). Protein levels of Akt1, Akt2, pAkt, K8/18 and vimentin were analyzed by Western blot in KLE cells transfected with control (scrambled) shRNA or with Akt1 shRNA (***C***) or Akt2 shRNA (***D***). GAPDH or β-actin was used as a loading control. ★indicates a p value of <0.05.

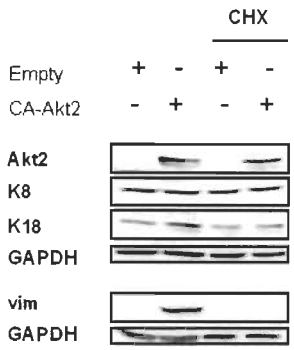

Figure 2. Akt2-induced intermediate filaments up-regulation requires new protein synthesis. HeLa cells were transfected with control (empty) vector or CA-Akt2 vector and treated with CHX (20 μg/mL) or vehicle for 24h. Protein levels of Akt2, K8, K18 and vimentin were analyzed by Western blot. GAPDH was used as a loading control.

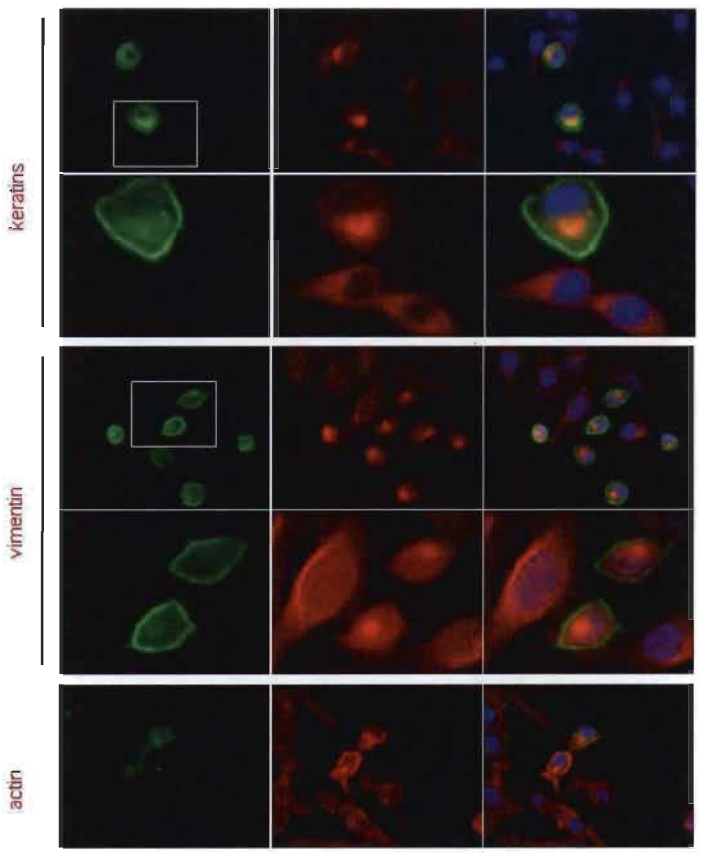

Figure 3. Intermediate filaments cytoskeletal network is reorganized by Akt2 isoform in HeLa cells. HeLa cells were transfected with CA-Akt2 vector and triple immunofluorescence staining of Akt2 (*green*), keratins/vimentin/actin (*red*) and nuclei (*blue*) was performed.

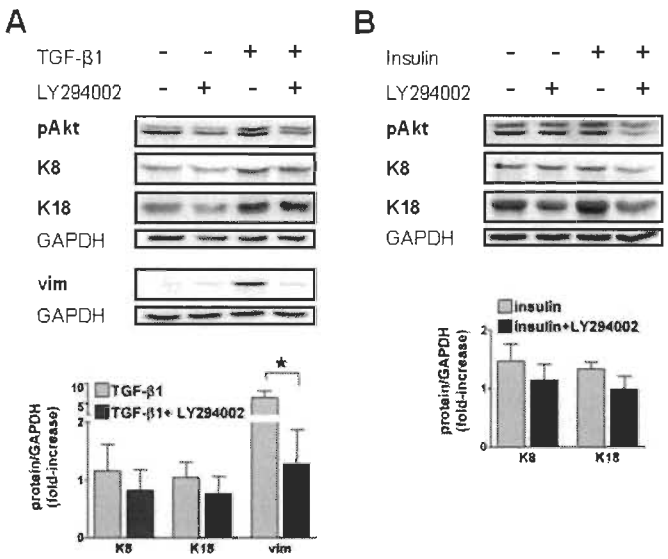

Figure 4. TGF-β1- and insulin-stimulated Akt modulates intermediate filaments expression via PI3K. KLE cells were treated for 24h with TGF-β1 (10 nM) (***A***) or insulin (200 nM) (***B***) in presence or not of PI3K inhibitor pre-treatment (LY294002 30 μM for 1h). Protein levels of pAkt, K8, K18 and vimentin were analyzed by Western blot. GAPDH was used as a loading control. ★indicates a *p* value of <0.05.

CHAPITRE III

KERATIN 8 AND 18 LOSS IN EPITHELIAL CANCER CELLS INCREASES COLECTIVE CELL MIGRATION AND CISPLATIN SENSITIVITY THROUGH CLAUDIN1 UP-REGULATION

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RÉSUMÉ

Dans les étapes précoces de la formation des métastases, les cellules de carcinome vont entrer dans un processus de dédifférenciation appelé « transition épithéliale-mésenchymateuse » (EMT). Parmi tous les changements morphogénétiques associés à cette transition, la diminution ou la perte de l'expression des kératines est considéré comme une caractéristique histologique majeure. Ce sont les kératines qui constituent le cytosquelette de filaments intermédiaires (FIs) dans les cellules épithéliales. Au cours de la tumorigénèse, l'expression des kératines 8 et 18 (K8/18) est maintenue jusqu'à ce que la tumeur devienne invasive. Au cours des dernières années, plusieurs études ont suggéré que les kératines puissent réguler la signalisation des cellules cancéreuses. Dans la présente étude, nous avons cherché à savoir si l'expression de K8/18 joue un rôle actif dans l'EMT. Par la technique d'interférence à l'ARN, nous avons inhibé de façon stable l'expression des K8/18 dans deux lignées cellulaires de carcinome, afin d'imiter la perte des kératines au cours de l'EMT. À l'aide de tests *in vitro* mesurant la motilité et l'invasion des cellules à travers le matrigel, nous avons observé que l'absence des K8/18 augmente la migration collective, le potentiel invasif et la chimio-sensitivité des cellules cancéreuses épithéliales, et ce, sans induire l'EMT. De plus, nous avons identifié la protéine de jonction serrée claudine-1 en tant que régulateur de ces processus. En effet, la claudine-1 est fortement augmentée dans les cellules déficientes en K8/18 et facilite la localisation membranaire des récepteurs de mort cellulaire Fas de même que l'activation des oncogènes Akt1 et Akt3. La claudine-1 favorise également la translocation nucléaire du facteur de transcription NF-κB d'une manière dépendante de la PI3K. L'activité transcriptionnelle de NF-κB dans les cellules déficientes en K8/18 induit l'expression des MMP et de la claudine-1 elle-même. Pour la première fois, nous avons démontré que NF-κB est un activateur de la transcription de la claudine-1. À la lumière de ces résultats, nous proposons une alternative à l'acquisition du potentiel invasif qui est indépendante de l'EMT : la perte des K8/18 favorise la migration collective des cellules cancéreuses épithéliales en facilitant la migration directionnelle et en augmentant la cohésion intercellulaire. À notre connaissance, ces résultats représentent la première indication que les K8/18 peuvent influencer le phénotype des cellules épithéliales cancéreuses à un niveau transcriptionnel et supportent l'hypothèse selon laquelle les kératines ne sont pas seulement des marqueurs de différenciation, mais aussi des régulateurs importants de la progression du cancer.

Keratin 8 and 18 loss in epithelial cancer cells increases collective cell migration and cisplatin sensitivity through claudin1 up-regulation*

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Abbreviations: EMT, epithelial-mesenchymal transition; MMP, metalloproteinase; IF, intermediate filaments; K8/18, keratin 8 and 18; PIP2, phosphatidylinositol 2-phosphate; PIP3, phosphatidylinositol 3-phosphate; PTEN, phosphatase and tensin homolog deleted on chromosome ten; PDK1, phosphoinositide-dependent kinase-1; PARP, *poly* (*ADP-ribose*) *polymerase*; IGF-1, insulin-like growth factor-1; NF-κB, nuclear factor of kappa light polypeptide gene enhancer in B-cells; IκBα, NF-κB inhibitor alpha; TJ, tight junction; CLDN1, claudin1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; XIAP, X-linked inhibitor of apoptosis protein; sFLIP, short variant of FLICE-like inhibitory protein; FasR, Fas receptor.

ABSTRACT

Keratins 8 and 18 (K8/18) are simple epithelial-specific intermediate filament proteins. Keratins are essential for tissues integrity and are involved in intracellular signaling pathways which regulate cell response to injuries, cell growth and death. K8/18 expression are maintained during tumorigenesis, hence their use as diagnostic marker in tumor pathology. In recent years, studies provided evidence that keratins should not be considered only as markers but also as regulators of cancer cell signaling. The loss of K8/18 expression during epithelial-mesenchymal transition (EMT) is associated with metastasis and chemoresistance. In the present study, we investigated whether K8/18 expression play an active role in EMT. We show that K8/18 stable knockdown using shRNA increases collective migration and invasiveness of epithelial cancer cells without modulating EMT markers. K8/18-depleted cells show PI3K/Akt/NF-κB hyperactivation and increased MMP2 and MMP9 expression. K8/18 deletion also increases cisplatin-induced apoptosis. Increased FasR membrane targeting suggest that apoptosis is enhanced via the extrinsic pathway. Interestingly, we identified the tight junction protein claudin1 as a regulator of these processes. This is the first indication that modulation of K8/18 expression can influence the phenotype of epithelial cancer cells at a transcriptional level and support the hypothesis that keratins play an active role in cancer progression.

INTRODUCTION

Metastasis, the foremost cause of mortality in cancer patients, is a coordinated biological process involving multiple intracellular signalling pathways. In the early steps of metastasis, carcinoma cells undergo through a 'dedifferentiation' process, also called 'epithelial-mesenchymal transition' (EMT), which is characterized by loss of cell polarity, alteration of cell junctions and reorganization of cytoskeletal components. Activation of several transcription factors also promotes the degradation of extracellular matrix through expression of metalloproteinases (MMPs) and increases resistance to apoptosis. All together these changes enhance invasiveness and chemoresistance, making EMT process a hallmark of tumor progression [1]. Among all these phenotypic changes, the reduction or loss of keratin proteins is often considered as a histological and biochemical feature for tumor cells that are going through an EMT [2]. Keratins are epithelial-specific intermediate filament (IF) proteins, which are expressed in a tissue- and differentiation state-specific manner. Keratins 8 and 18 (K8/18) are typically co-expressed as the primary keratin pair in simple epithelial cells and their expression is maintained during malignant transformation until the tumor become invasive. Their specific re-expression in tissue metastasis explain their use as diagnostic marker in determining tumor origin [3]. As part of the cytoskeleton, keratins are important for the mechanical stability and integrity of epithelial cells and tissues. Keratin filaments form a complex network that extends from the periphery of the nucleus to the plasma membrane where they associate with desmosomes and hemidesmosomes. IF proteins are not only structural and are now considered as regulatory proteins (for a review see [4]). For instance, they regulate organelles positioning and protein targeting [5]. Moreover, a number of keratins are involved in intracellular signaling pathways which regulate response to injuries [6-7], protein synthesis [8-9], cell cycle [8,10-12], cell death [13-16] and cancer progression [17-19]. Thus, a concept emerges from these different studies that IF should not be considered only as marker proteins in tumor cells but also as regulators of cancer cell signaling. For instance, our previous work highlighted a link between the oncogenic Akt isoforms and IF proteins expression [20] and PI3K/Akt pathway has been shown to play a major role in tumorigenesis, chemoresistance and EMT [21-24]. Class I PI3K are heterodimeric protein composed of a regulatory subunit

(p85) and a catalytic subunit (p110). Activated PI3K phosphorylates phosphoinositides at the position 3 of the inositol ring to generate the lipid second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which promotes Akt activation [25]. This process is antagonized by the tumor suppressor phosphatase and tensin homologue PTEN that hydrolyzes the PIP3 to generate phosphatidylinositol (4,5)-bisphosphate (PIP2) [26]. In epithelial polarized cells, PIP3 is specifically localized at the basolateral plasma membrane [27] whereas PTEN localizes to the apical plasma membrane where it enhances PIP2 levels [28]. Considering that the leading edge of migrating cells has a similar composition as the basolateral surface of epithelia, PI3K/PTEN have been proposed to maintain the phosphoinositides gradient that establish front-to-back polarity in invasive cancer cells [29-30].

In the present study, we investigated the role of K8/18 loss in EMT. Because we have previously shown that Akt isoforms induce reorganization of IF network [20], we analyzed the role of K8/18 network in the PI3K/Akt signaling in a context of cell motility, invasion and cisplatin-induced apoptosis.

EXPERIMENTAL PROCEDURES

Cell culture, transfection and reagents

Human endometrial carcinoma KLE, human hepatocellular carcinoma HepG2 and human cervical carcinoma HeLa cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were maintained in DMEM (without HEPES for KLE) supplemented with 10% FBS (KLE and HepG2) or 2% BGS (HeLa) (Thermo Fisher Scientific, Waltham, MA, USA) and 50 μg/ml gentamycin (Sigma, Oakville, ON, Canada). K8/18 stable knockdown was achieved using SureSilencing pre-designed shRNA sequences against keratin 8, keratin 18 or scrambled negative control (NC) (Qiagen, Mississauga, ON, Canada). The cells were transfected with Fugene6 according to manufacturer's protocol (Promega, Madison, WI, USA) and G418 was applied for isolating resistant clones (Invivogen, San Diego, CA, USA). Transient gene silencing

were performed using 100nM of srambled negative control (NC) siRNA, claudin1 siRNA (oligo#1 ID138564; oligo#2 ID 29051 from Ambion, Life Technologies, Burlington, ON, Canada) or NF-κBp65 siRNA (Cell Signaling Technology, Danvers, MA, USA) delivered in cells using TransIT-TKO reagent following supplier's instruction (Mirus Bio LLC, Madison, WI, USA). Transient transfection of claudin1 full length cDNA was carried out using the pCMV-SPORT6 vector (Thermo Fisher Scientific). Modulation of PI3K/Akt signaling pathway was done with 200 ng/mL of recombinant IGF-1 (Sigma), 5-50 μM LY294002 or 0.2-1 μM Wortmannin (Cell Signaling Technology). Cisplatin was purchased from Sigma. Cell proliferation was assessed by MTT assay as described in [31] using Cell proliferation kit I (Roche Diagnostics, Laval, QC, Canada).

Subcellular fractionation

Subcellular Protein Fractionation Kit (Thermo Fisher Scientific) was used according to manufacturer's instructions.

Immunoblotting and immunoprecipitation

Cell lysis, electrophoresis and western blot were processed as previously described [31]. All of the antibodies were from Cell Signaling Technology except for K8 (Troma1, Developmental Studies Hybridoma Bank, University of Iowa, IA, USA) and K18 (L2A1) [32] which was a generous gift from Dr. M. Bishr Omary (University of Michigan, MI, USA), Akt3 (Millipore, Billerica, MA, USA), horseradish-peroxidase (HRP)-conjugated β-actin (Sigma), β-tubulin (Abcam Inc, Cambridge, MA, USA) and HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Bar Harbor, ME, USA). Akt isoforms, PI3Kp85 and claudin1 were immunoprecipitated following a procedure previously described [33].

Immunofluorescence

Cells were fixed with 4% paraformaldehyde and prepared as described [31], using rhodamine phalloidin conjugate (Life Technologies), mouse monoclonal anti-β-tubulin clone TUB2.1 (Sigma), rabbit monoclonal anti-NF-κBp65 (D14E12) (Cell Signaling Technology), rabbit anti-claudin1 antibody (Life Technologies), mouse monoclonal anti-phospho-Akt (Ser473) clone 6F5 (Millipore) or isotypic control IgG (Santa Cruz Biotechnology). Cells were viewed under a Carl Zeiss Axio observer Z1 microscope. Normalization of the claudin1 staining at the leading edge was measured as previously described [34]. Fluorescence intensities (FI) of every pixel within three individual lines drawn across the cell membrane were divided by their average cytoplasmic FI using ImageJ software.

Wound-healing assay

To evaluate cell motility, cells were grown to near confluency and a wound was created with the blunt end of a tip. Images were captured at fixed timepoint between 0h and 48h post-wounding and the pictures were analyzed using ImageJ software. The results were plotted as a percentage of wound closure compared to the area of the initial wound. Each experiment was performed in duplicates and repeated three times.

Matrigel invasion assay

Invasive properties of cells were measured using 2mg/mL of Matrigel-coated Transwell inserts (BD Biosciences, Mississauga, ON, Canada). Invasive cells that had adhered to the porous insert were fixed in methanol and nuclear staining was performed with Hoechst dye. Total fluorescence of each insert was quantified by densitometric analysis. Each experiment was performed in duplicates and repeated three times.

PI3K assay

PI3K activity was assessed using a commercially available PI3-kinase ELISA kit (K-1000s, Echelon Biosciences, Salt Lake City, UT, USA). The cell lysates were precleared, immunoprecipitated with PI3Kp85 antibody and incubated with diC8-PIP2 substrate and reaction buffer. The amount of PIP3 formed from PIP2 by PI3K activity was detected using a competitive ELISA. Absorbance of the samples was measured at 450 nm and the PIP3 was quantified by comparison with a PIP3 standard curve carried out in parallel with the experimental samples and plotted on a log scale.

Conventional RT-PCR

Total RNA extraction, cDNA synthesis and PCR reactions were performed as previously described [31]. The conditions for PCR reactions are listed in Table 1. PCR products were analysed by electrophoresis on 1-2% agarose gels in TBE buffer.

Quantitative real-time RT-PCR

RNA was extracted using RNeasy Mini Kit (Qiagen). Total RNA (1μg) was subjected to reverse transcription using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). All samples were subjected to real time PCR analysis with SYBR Green Brilliant III Master Mix using an Mx3000P system (Agilent Technologies, Mississauga, ON, Canada). For each gene target, a standard curve was generated to determine the efficiency of the reaction and Pfaffl analysis method was used to measure the relative quantity of gene expression [35]. Each real-time PCR was performed in duplicates from at least three independent experiments. Human *CLDN1* was amplified using sense primer 5'-ccctatgaccccagtcaatg-3' and antisense primer 5'-acctcccagaaggcagaga-3'. For MMPs, expression was determined using sense primer 5'-atgccgcctttaactggag-3' and antisense primer 5'-aagaagtagctgtgaccgcc-3' (*MMP2*), sense primer 5'-gcactgcaggatgtcatagg-3' and antisense primer 5'-acgacgtcttccagtaccga-3' (*MMP9*). *18S rRNA* was used as reference genes based on its stable expression in all cell clones.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was done using EZ-ChIP kit (Millipore) as per manufacturer's protocol. One-tenth of sonicated (10 x 12-second pulse) and precleared chromatin from 2.5 x 10^7 HepG2 shK8/18 or HeLa cells was used for each ChIP. Immunoprecipitations were performed at 4°C overnight with 1 μg of NF-κBp65 antibody (Cell Signaling), RNA polymerase II antibody (Millipore) or normal IgG (Santa Cruz Biotechnology). The supernatant from the above reaction lacking the primary antibody was saved as total input of chromatin and was processed in the same way as the eluted immunoprecipitates, starting with the cross-linking reversal step. Inputs and immunoprecipitates were amplified using *CLDN1* promoter-specific primers under conditions listed in Table 1. *COX2* and *GAPDH* promoter-specific primers served as positive and negative controls respectively (Table 1). PCR products were analyzed by electrophoresis on 2% agarose gels in TBE buffer.

Luciferase reporter assay

Cells were transfected with NF-κB-Luc reporter plasmid (pGL4.32) and TK-hRLuc (pGL4.74) in a 10:1 ratio. After 24h, the cells were transfected with NC or claudin1 siRNA for 24h followed by the Dual-Luciferase Reporter assay (Promega). Each experiment was repeated three times.

Determination of apoptosis level

The induction of apoptosis was determined by counting the apoptotic cells (irregular Hoechst nuclear staining with multiple bright specks of chromatin fragmentation and condensation) stained with Hoechst 33258 dye (Sigma) and by flow cytometer analysis of Annexin V-PI staining as previously described [36].

Statistical analysis

Experiments were repeated three times. Statistical analyses were carried out with GraphPad (La Jolla, CA, USA) PRISM software, version 3.03. Differences between experimental groups were determined using the Student's t test. Statistical significance was accepted when P value was <0.05.

RESULTS

Keratin 8 and 18 knockdown increases epithelial cancer cell motility and invasion without modulating EMT markers

The conversion of epithelial cell into mesenchymal cell involves a change in the composition of IF proteins in that epithelial cells lose the expression of keratins and take on the expression of vimentin, a mesenchymal-specific IF protein [2]. In order to better understand the role of keratin cytoskeleton in EMT, we used RNA interference approach targeted against K8/18 to mimic keratin loss during EMT process. We used two epithelial carcinoma cell lines whose various differentiation states constitute an interesting experimental model: the HepG2 cell line obtained from well-differentiated carcinoma (K8/18+; vimentin-) and the KLE cell line from a poorly-differentiated carcinoma (K8/18+; vimentin+). To generate effective monoclonal population of cells deficient in K8/18, we used short hairpin RNA constructs (shRNA). We monitored the knockdown efficiency by analyzing K8 and K18 protein levels. We observed a decrease of 80% in KLE cells and more than 90% in HepG2 cells when compared to negative control cells (shNC) expressing scrambled shRNA (Fig. 1a). Moreover, specificity of the knockdown was validated by analyzing the expression levels of other keratin proteins normally expressed in KLE cell line. Keratin 7 and keratin 19 were not affected by K8/18 knockdown (Fig. 1a). As keratins loss is associated with EMT, firstly we determined whether K8/18 depletion induced expression of EMT markers. Western blot analysis showed that E-cadherin, N-cadherin and vimentin levels were not modified in K8/18 knockdown cells (Fig. 1b). Moreover, there were no detectable levels of Snail and Slug transcription factors in both cell lines (data not shown). Immunofluorescence

staining with anti-phalloidin and anti-β-tubulin antibodies also demonstrated that K8/18 knockdown did not affect cell morphology or cytoskeletal organization of actin and microtubules networks (Fig. 1c). However, using *in vitro* wound healing and transwell invasion assays, we observed that K8/18 knockdown directly affects the motility and invasiveness of cancer cells. Indeed, K8/18-deficient cells close the wound two to three times faster than the control cells (Fig. 1d). Moreover, K8/18 depletion significantly increased cell invasion through matrigel of KLE (2.1-fold ±0.12, $p<0.0002$) and HepG2 cells (1.95-fold ±0.28, $p<0.0247$) (Fig. 1e). The increase in cell invasion was not due to an enhanced proliferation since results from MTT assays revealed that stable knockdown of K8/18 had no effect or rather decreased cell viability (Fig. 1f).

Keratin 8 and 18 knockdown improves PI3K/Akt activation in epithelial cancer cells

PI3K/Akt pathway plays a pivotal role in cancer cell motility and invasion. We proceeded to an analysis of the expression levels of proteins involved in this pathway. KLE cells constitutively express the three Akt isoforms in their activated/phosphorylated form making this cell line a very useful tool for the present study [33]. The antibody against phospho-Akt recognizes two distinct bands on the blot; the upper band corresponds to phosphorylated Akt1 and Akt3 and the lower band corresponds to phosphorylated Akt2. This interpretation comes from a previous study in which we have characterized the antibody against Akt phosphorylated on serine 473 (#9271, Cell Signaling Technology) [33]. HepG2 cells also express Akt isoforms, but stimulation with IGF-1 is necessary to induce their activation/phosphorylation. Our results show that K8/18 depletion modestly affects Akt isoforms protein levels. Western blot analysis reveals that Akt1 level is similar whereas Akt2 and Akt3 isoforms slightly decreased in KLE cells without K8/18 compared to the control cells (Fig. 2a). In HepG2shK8/18 cells, Akt1 and Akt3 are slightly increased compared to control cells while Akt2 level is not affected by K8/18 knockdown (Fig. 2b). Interestingly, K8/18 depletion induces a 2.8-fold increase (±0.48, $p<0.02$) of Akt1 and/or Akt3 phosphorylation (upper band) in KLE cells (Fig. 2a and c). Similarly, after 15 minutes of IGF-1 stimulation, phospho-Akt level is 1.9-fold increased (±0.18, $p<0.0159$) in HepG2shK8/18 cells compared to

control cells (Fig. 2b and c). In order to determine which Akt isoform is activated in K8/18-knockdown cells, we immunoprecipitated each Akt isoform from shNC and shK8/18 cell lysates, and we assessed their phosphorylation state by phospho-Akt western blot (Fig. 2d). Akt3 is predominantly activated in KLEshK8/18 cells (3.25-fold ±0.52, $p<0.049$) compared to the control cells. The Akt1 isoform is slightly more phosphorylated in K8/18-depleted cells (1.51-fold ±0.025, $p<0.0065$) whereas the Akt2 isoform does not show any difference in its activation state (Fig. 2e). After stimulation with IGF-1, HepG2shK8/18 also show an increase in Akt1 (1.427 ±0.1334, $p<0.0397$) and Akt3 (1.496 ±0.069, $p<0.0021$) phosphorylation compared to the control cells (Fig. 2d and e) while Akt2 activation is decreased (0.3839 ±0.07, $p<0.0123$). PTEN, which is a negative regulator of Akt phosphorylation, is decreased in KLEshK8/18 cells while K8/18 knockdown does not affect PTEN level in HepG2 cells (Fig. 2a and b). No significant changes are observed on the total level of the PI3K p85 and p110a subunits in both cell lines (Fig. 2a and b).

Membrane recruitment is an essential step in Akt signaling since its activating kinase PDK1 is linked to the PIP3 in the plasma membrane [37-38]. Therefore, we performed biochemical subcellular fractionation of cytosolic (C), membrane (M) and nuclear (N) proteins from control and shK8/18 cell lysates to find out whether the keratin cytoskeleton could modulate the intracellular localization of PI3K/Akt pathway mediators. Western blots of β-tubulin (cytosolic), E-cadherin (membrane) and poly (ADP-ribose) polymerase (PARP) (nucleus) were used to validate the low cross contamination among fractions. The level of phospho-Akt (upper band) is significantly increased in both cytosolic and membrane fractions of shK8/18 cells when compared to the control in both cell lines (Fig. 2f-h). According to our immunoprecipitation results, Akt1 and Akt3 isoforms are significantly increased, predominantly in the membrane pool, whereas Akt2 does not change (KLE) or rather decrease (HepG2). The densitometric analysis also show a significant decrease of PTEN at the membrane of KLEshK8/18 cells which is consistent with the diminution of PTEN total level (Fig. 2a). In the opposite, the amount of PTEN in the membrane fraction of HepG2shK8/18 cells is increased compared to the control cells. The concomitant localization of PI3K/Akt and

PTEN in the membrane extract supports the concept of a polarized delivery of intracellular signaling during cell migration. PI3Kp85 level is increased in cytoplasm and membrane of KLEshK8/18 clone whereas in HepG2shK8/18 cells, there is an up-regulation of p110a subunit at the membrane. Interestingly, we can detect expression of some mediators of the PI3K/Akt pathway in the nuclear fraction, particularly in HepG2 cells. Nuclear pool of Akt3 is increased in K8/18-depleted cells as well as phospho-Akt and PI3Kp85 to a lesser extent. The distribution of Akt isoforms in distinct subcellular location has also been described by others [39]. All together these results suggest that the absence of K8/18 cytoskeleton facilitates and/or extends the activation of Akt1 and Akt3 isoforms by modulating their subcellular localization.

To determine whether PI3K/Akt activation is involved in motility of K8/18-knockdown cells, we used pharmacological PI3K inhibitors and performed wound healing and invasion assays. As shown by dose-response results, treatment of KLEshK8/18 and IGF-1-treated HepG2shK8/18 cells with 0.5-1 μM Wortmannin or 10 μM LY294002 reduces Akt phosphorylation, (Fig. 2i, supplemental Fig. S1a), particularly of Akt1/3 isoforms (upper band, Fig. 2i). However, only Wortmannin treatment does not affect cell proliferation for all the duration of experiments (Fig. 2k, supplemental Fig. S1c). Using these conditions, we observed that PI3K inhibition by both drugs significantly decreases cell motility (Fig. 2j, supplemental Fig. S1b) and invasion through matrigel (Fig. 2l, supplemental Fig. S1d).

Keratin 8 and 18 knockdown increases NF-κB transcriptional activity through PI3K pathway

Several studies have indicated that activity of the transcription factor NF-κB is involved in both promoting and maintaining an invasive phenotype [40]. Western blot analysis showed a 3.4-fold (±0.18, $p<0.0008$) and a 3.1-fold increase (±0.07, $p<0.0011$) of IκBa phosphorylation in K8/18-depleted KLE and HepG2 cells respectively (Fig. 3a and b). IκBa binds to and inhibits the transcriptional activity of NF-κB. Once phosphorylated, IκBa is degraded and NF-κB is free to translocate from cytoplasm to nucleus, where it binds to its response element on gene promoter regions and regulates a

wide spectrum of gene expression. Thus, we verified the intracellular localisation of NF-κBp65 by subcellular fractionation (Fig. 3c) and immunofluorescence staining (Fig. 3d). Both techniques revealed that there is an increase in nuclear NF-κBp65. Moreover, by using a luciferase reporter construct that contains five copies of an NF-κB response element, we found that there is a twofold increase in the NF-κBp65 transcriptional activity in K8/18-knockdown cells (2.6±0.52, $p<0.012$ for KLE and 2.0±0.048, $p<0.0001$ for HepG2) (Fig. 3e).

PI3K/Akt pathway can induce IκBa degradation through phosphorylation of IKK kinases. Since we have previously shown that PI3K/Akt signaling is activated in our model, we used PI3K inhibitor LY294002 to determine whether IκBa phosphorylation is dependent of PI3K activity. In both cell lines, inhibition of PI3K decreases IκBa phosphorylation to the basal level observed in control cells. These results suggest that K8/18 knockdown promote PI3K/Akt/NFκB signaling pathway activation (Fig. 3f).

Keratin 8 and 18 knockdown increases expression of the tight junction protein claudin1 through NF-κB transcriptional activity

During EMT, cell junctions are altered either by a decrease in expression or a mislocalization of their components. We investigated expression and distribution of three major cell junction proteins, claudin1, E-cadherin and plakoglobin that are part of the tight junctions (TJ), adherent junctions and desmosomes respectively. Whereas no change was observed in the protein level of E-cadherin and plakoglobin, the K8/18 knockdown induced a significant 3-fold increase of claudin1 protein level in both cell lines (3.3±0.51, $p<0.0012$ for KLE and 3.4±0.37, $p<0.0033$ for HepG2) (Fig. 4a and b). Quantitative real-time PCR analysis showed that claudin1 gene expression was also significantly increase in cells without K8/18 cytoskeleton (2.35-fold±0.23, $p<0.0004$ for KLE and 1.87-fold±0.12, $p<0.0023$ for HepG2) (Fig. 4b). Given the increased NF-κBp65 transcriptional activity in K8/18-deficient cells, we examined whether this transcription factor could regulate claudin1 expression. For this purpose, we used small interfering RNA (siRNA) targeted against NF-κBp65 which was decreased by 65% and 78% in KLE and HepG2shK8/18 cells respectively. As shown in Fig. 4c and d,

NF-κBp65 silencing significantly decreases claudin1 protein (KLE $p<0.0412$; HepG2 $p<0.0184$) and gene (KLE $p<0.0059$; HepG2 $p<0.0005$) expression in both cell lines compared to cells transfected with negative control (scrambled) siRNA (siNC). Based upon these findings, we further investigated if NF-κBp65 could directly bind the claudin1 promoter *in vivo* by performing ChIP analysis (Fig. 4e). We have immunoprecipitated NF-κBp65 cross-linked with sheared DNA from three different lysates of HepG2shK8/18 cells and one from HeLa cells. Then, we have performed PCR in different regions of the claudin1 promoter (site *a* and site *b*). Our results reveal that NF-κBp65 is linked to the *CLDN1* promoter (site *b*, -333 to -183bp relative to the translation start site) in all the samples. Binding of NF-κBp65 to *COX2* promoter was used as a positive control for NF-κBp65 DNA binding. To validate the assay, simultaneous ChIP was performed with an antibody to RNA polymerase II and DNA enrichment was monitored for the *GAPDH* gene. We confirm these results by finding the well-known NF-κB DNA binding motif [41] (5'-GGGRNNYYCC-3' where R correspond to a purine, Y represents a pyrimidine, and N can be any base) in the site *b* region (-310 to -301bp). For the first time, we identify NF-κBp65 as a functional regulator of claudin1 transcription which strongly supports the role of claudin1 in cancer cell invasion and tumor progression.

To determine whether the increase in claudin1 expression could influence its intracellular localization, we performed as described above a subcellular fractionation (Fig. 4f) and confirmed the results by immunofluorescence staining of claudin1 protein in KLE and HepG2 clones (Fig. 4g). Both techniques allowed us to assert that claudin1 is present in the three cellular fractions and that the distribution is not changed between control and K8/18-knockdown cells. However, a two and three-fold increase of claudin1 was respectively detected in the membrane and the nuclear fraction in shK8/18 cells compared to control cells (Fig. 4f). Biochemical fractionation demonstrates that claudin1 localization is mostly at the membrane in both cell lines, regardless of whether there is a K8/18 cytoskeleton. Since K8/18-knockdown cells show increased motility, we analyzed claudin1 localization in the context of wound healing by immunofluorescence staining. In subconfluent sheet of cells, claudin1 shows an extensive plasma membrane staining.

Interestingly, the migrating cells at the leading edge show a more scattered membrane staining (Fig. 4g, arrows) and an intense cytoplasmic/nuclear localization of claudin1 (Fig. 4g, unfilled arrows). The following cells demonstrate continuous membrane and moderate nuclear staining. To exclude the possibility that claudin1 staining at the leading edge was due to the thickening of the cell membrane, we compared claudin1 fluorescence intensity at the edge with E-cadherin and actin staining as controls. Cytoplasmic fluorescence was adjusted to the same level for the different staining. The ratio of the claudin1 signal at the edge versus the average cytoplasm signal was higher than that of E-cadherin or actin fluorescence, suggesting that the thickening of the cell at the leading edge contributed little to the claudin1 signal (Fig. 4h). Some studies have already shown that claudin1 expression is not exclusively localized at the TJ, but can be distributed continuously along the plasma membrane [42-43] as well as in the cytoplasm [44-45] and in the nucleus [45-46]. Our data indicate that the cellular localization of claudin1 depends on the cell location relative to the wound suggesting a role in cell motility.

Claudin1 is involved in cell motility and invasion of K8/18-deficient cells

Given the considerable increase in the expression level of claudin1, we evaluated if this protein could play a role in motility and invasion potential of K8/18-deficient cells. To this end, we used two different siRNA targeted against claudin1 (siCLDN1-1 and siCLDN1-2) which was decreased by 71% and 75% in KLEshK8/18 (Fig. 4i) and 58% and 53% in HepG2shK8/18 cells (supplemental Fig. S2a). Both cell clones transfected with siCLDN1-1 or siCLDN1-2 are unable to close the wound after 48 hours whereas cells transfected with negative control (scrambled) siRNA (siNC) show almost complete wound healing after the same period of time (Fig. 4k and supplemental Fig. S2c). Claudin1 is also involved in invasion potential of K8/18-depleted cells. Indeed, the results of invasion assays show that claudin1 inhibition by both oligonucleotides decreases the number of K8/18-knockdown cells able to pass through matrigel ($p<0.0004$) (Fig. 4l and supplemental Fig. S2d). However, we have to consider the 20% decrease in KLEshK8/18 cell proliferation induced by claudin1 silencing as

shown by MTT assay (Fig. 4j), while no effect on HepG2 cell viability was observed after siCLDN1 transfection (supplemental Fig. S2b). Overall, our results indicate that the absence of K8/18 cytoskeleton enhances epithelial cancer cell motility and invasion through an up-regulation of claudin1 expression and its localization at the cell membrane and the nucleus.

Claudin1 increases PI3K activity and phosphorylated Akt localization at the plasma membrane in K8/18-knockdown cells

As PI3K/Akt pathway is well known to regulate cell motility and invasion and that claudin1 is involved in these processes in our model, we wanted to determine if claudin1 could play a role in Akt hyperactivation observed in cells lacking K8/18. Western blot analysis of the membrane fraction of siCLDN1-1 or siCLDN1-2 transfected KLEshK8/18 and HepG2shK8/18 cells showed that claudin1 inhibition reduced membrane localization of phospho-Akt (upper band), Akt1 and Akt3 at basal level in KLE cells (Fig. 5a-b). These results are consistent with our preceding immunoprecipitation results in that Akt1 and Akt3 are the predominant activated isoforms in shK8/18 cells (Fig. 2e). Membrane targeting of PI3Kp85 is also decreased by the claudin1 silencing in KLE clones (Fig. 5a-b). However, claudin1 inhibition does not restore PTEN membrane localization (Fig. 5a-b). Thus, the role of claudin1 in the signaling axis PI3K/Akt/PTEN seems to be more related to the PI3K/Akt. The effect of claudin1 inhibition is quite similar in HepG2shK8/18 cells (Fig. 5c) although the Akt phosphorylation is decreased in a lesser extent. The lower efficiency of the siRNA transfection in HepG2 cells compared to KLE cells could explain the slight effect of siCLDN1 on cell motility, invasion and Akt phosphorylation observed in HepG2 cells (supplemental Fig. S2).

Since claudin1 depletion affects phospho-Akt membrane localization, we analyzed if there was a relationship between the localisation of the two proteins during cell migration. We performed a double immunofluorescence staining of claudin1 and phospho-Akt in K8/18-depleted HepG2 cells during wound healing. Interestingly, the cells at the front of migration show intense accumulation of phospho-Akt at specific

regions of the leading edge membrane alike the claudin1 staining (Fig. 5d, arrows). Akt is known to localize at the moving front of the plasma membrane where it promotes lamellipodia formation [47-48]. The recruitment of claudin1 and phospho-Akt at the leading edge is a specific event since there is no staining for phospho-Akt at cell-cell junctions (Fig. 5d, unfilled arrows). To determine whether the concomitant membrane targeting of claudin1 and Akt could be a common feature in cell migration, we performed the double staining in subconfluent IGF-1-stimulated HeLa cells transfected with claudin1. As we observed in HepG2shK8/18 cells, claudin1 localizes uniformly along the membrane with some punctuate aggregates which are also intensively stained for phospho-Akt (Fig. 5e, arrows). Interestingly, in comparison to claudin1-expressing HeLa cells, non-transfected cells show a diffuse cytoplasmic phospho-Akt staining (Fig. 5e, unfilled arrows). Based on these observations, we hypothesize that the enhanced membrane localization of claudin1 by K8/18 knockdown could increase Akt membrane recruitment in specific membrane domain and promote its activation.

Previous studies have shown that cell junction components could modulate PI3K/Akt signaling. For instance, occludin, a TJ partner of claudin1, has been shown to regulate cell migration by PI3K recruitment at the leading edge [34]. E-cadherin also recruits PI3K during adherens junction formation [49-50]. Thus, it was tempting to speculate that claudin1 could increase phospho-Akt membrane recruitment by regulating PI3K activity. The effect of claudin1 silencing on PI3K activity was determined with a kinase assay and detected by an ELISA for PIP3 product. Our data show that K8/18 knockdown significantly increases PI3K kinase activity in a claudin1-dependent manner in both cell lines (Fig. 5f). The PI3Kp85 immunoprecipitates used for the kinase assay were blotted to validate the experiment and to find out protein interaction. The decrease of Akt phosphorylation in claudin1-depleted cells correlates with PIP3 levels detected. As expected, PI3Kp110α subunit pulled down with PI3Kp85, but neither claudin1 nor E-cadherin interacts with the kinase. Immunoprecipitation of claudin1 confirmed that no interaction occurs between claudin1 and PI3K/Akt mediators in our models (Fig. 5g). Overall, these results highlight a role for claudin1 in the regulation of PI3K activity

which is in agreement with the improved Akt activation observed in K8/18-knockdown cells.

Claudin1 modulates invasiveness of K8/18-knockdown cells by increasing NF-κB-induced MMP2 and MMP9 expression

Claudin1 is known to promote MMP2 activation by recruiting MT1-MMP at the cell surface and increasing their protein expression [44, 51, and 52]. Since NF-κB can directly bind the *MMP9* promoter [53] and activate *MMP2* [54] through MT1-MMP expression [55], we investigated if the enhanced NF-κB transcriptional activity observed in K8/18-depleted cells could modulate cell invasiveness through MMP2 and MMP9 expression. We first validated that claudin1 expression is involved in NF-κB nuclear translocation and transcriptional activity of K8/18-knockdown cells. Indeed, claudin1 silencing decreases the level of nuclear NF-κBp65 in shK8/18 cells (Fig. 6a) and therefore decreased its DNA binding (Fig. 6b). Then, we examined whether claudin1 and NF-κBp65 could effectively modulate the expression of the gelatinases MMP2 and MMP9 in our model. We performed quantitative real-time PCR in HepG2shNC and HepG2shK8/18 transfected with negative control (NC) siRNA, claudin1 siRNA or NF-κBp65 siRNA. The results are shown as a fold-change from shNC cells (Fig. 6c). Knockdown of K8/18 induce a 2.6-fold increase in MMP2 (±0.45, $p<0.0269$) and a 3.9-fold increase in MMP9 (±0.41, $p<0.002$) expression. Both claudin1 and NF-κBp65 siRNAs significantly block these raises and reduce *MMP2* and *MMP9* expression close to the level of shNC cells. These results demonstrate that K8/18 depletion promote MMPs expression through claudin1/NF-κBp65 activity. Since we previously identify NF-κBp65 as a transcriptional regulator of claudin1 expression (Fig. 4c-e), altogether these results suggest a feedback loop process which could explain the overexpression of claudin1 in K8/18-knockdown cells.

K8/18 knockdown increases cisplatin sensitivity

Emerging evidences suggest a role for EMT in chemotherapeutic drugs resistance [56]. Thus, we examine the effect of K8/18 knockdown in cisplatin-induced apoptosis.

KLE and HepG2 clones were exposed to cisplatin (10μM) for 24h before analysis. K8/18 loss highly increased apoptosis in response to cisplatin as shown by the Annexin-V/propidium iodide staining (Fig. 7a-b), the percentage of apoptotic cells determined by irregular Hoechst 33258 dye staining (Fig. 7c) and the increase of pro-apoptotic caspase 3, 8, 9 and PARP cleavages (Fig. 7d). On the other way, anti-apoptotic proteins like X-linked inhibitor of apoptosis protein (XIAP) and phospho-Akt are decreased in KLEshK8/18 cells exposed to cisplatin compared to control cells under the same treatment (Fig. 7d). Similarly, XIAP is only decreased in HepG2shK8/18 cells treated with cisplatin. However, in absence of serum starvation and IGF-1 stimulation we can only detect a very weak signal of phospho-Akt in HepG2 cells. Interestingly, short variant of FLICE-like inhibitory protein (sFLIP), which is an inhibitor of Fas death receptor signaling, is highly reduced in both K8/18-depleted cell lines under control condition and cisplatin treatment (Fig. 7d). This raises the possibility that cisplatin-mediated apoptosis is enhanced by the Fas death receptor pathway in K8/18-knockdown cells. Thus, we investigated whether K8/18 loss affects the level and the localization of the Fas receptor (FasR). Western blot and PCR analysis show that there is an increase in the protein level of FasR between KLE shNC and shK8/18 cells (Fig. 7e). Subcellular fractionation of untreated KLE clones shows a highly significant increase of FasR at the membrane of K8/18-depleted cells (3.16-fold±0.4, $p<0.0009$) as well as in the cytosol to a lesser extent (1.62-fold±0.12, $p<0.0012$) (Fig. 7f). Importantly, in K8/18-knockdown cells claudin1 silencing reduces FasR membrane level exclusively (1.46-fold±0.23, $p<0.0261$) since siCLDN1 does not change cytoplasmic FasR level (Fig. 7f). These results indicate that K8/18 loss sensitizes cells to apoptosis by down-regulating anti-apoptotic protein sFLIP and by increasing FasR at the membrane through a claudin1-dependent mechanism. Altogether these data confirm that K8/18 loss affects epithelial cancer cell response to cisplatin in a different way than an EMT.

DISCUSSION

EMT is a phenotypic transformation of epithelial cancer cells that promotes their dedifferentiation and metastatic behavior. When tumor cells undergo EMT, the

expression of keratin is down-regulated and replaced by the expression of vimentin, a mesenchymal IF protein. Although change in IF protein composition is a useful biomarker of EMT, the role of keratin network in tumor progression only begins to be unraveled. For instance, studies have suggested that keratins contribute to the maintenance of epithelial phenotype and regression of malignancy. For example, forced expression of keratin 18 in a metastatic breast cancer cell line reduced vimentin expression level, invasiveness *in vitro* and metastatic spread in mice [18]. In a similar way, stable silencing of vimentin in squamous carcinoma cells results in re-emergence of keratins and concomitant reduction in cell invasiveness and tumorigenic potential [57]. Conversely, suppression of both K8/18 in non-small cell lung cancer cell lines increased their invasiveness [58]. In the present study, we have demonstrated that K8/18 constitutes a signaling platform capable of moderating invasion and cell death in tumor cells.

Our study establishes that K8/18 knockdown significantly increases epithelial cancer cell motility and invasiveness. This is associated with the overexpression of TJ protein claudin1 that seems to be a key step in these processes. In normal epithelium, TJ maintain cell polarity by preventing the free diffusion of membrane lipids and proteins between apical and basolateral cellular pole and by regulating the paracellular permeability [59]. Many tumors exhibit overexpressed TJ proteins, resulting in a deficient epithelial barrier function that increases access of nutrients and growth factors to cancer cells. Overexpression of claudin1 protein in cancer cell lines is associated with increased invasiveness and metastatic behavior [44, 46, 51, 52, 60-62]. Moreover, some cancerous tissues and cell lines show mislocalized TJ proteins, either in cytoplasm or in nucleus, suggesting that they can have more dynamic functions than simply be constituents of TJ [63]. However, the molecular mechanisms involved in overexpression and/or mislocalization of claudin1 in cancer are poorly understood. PKC and PKA activity were shown to modulate claudin1 expression and intracellular localization respectively [42, 44 and 45]. Endocytic recycling is also known to regulate internalization of TJ proteins [64]. Our present study uncovers a functional role for NF-κBp65 in transcriptional regulation of claudin1 gene. Our ChIP assays in

HepG2shK8/18 and HeLa cells demonstrate that it is a common transcriptional regulator which directly binds the *CLDN1* promoter independently of K8/18 expression. As nuclear translocation and transcriptional activity of NF-κBp65 is increased by K8/18 loss, our findings provide the first evidence that claudin1 is a target of NF-κB promoting cell motility and invasion. According to the EMT process, the keratin loss in our model supports the invasive behavior of cancer cells. A recent study also shows that depletion of keratins enhances keratinocytes migration through hemidesmosomes scattering along the basement membrane [65]. In a similar way, we observed that in K8/18-knockdown cells claudin1 is localized specifically at the leading edge of migrating cells. Indeed, front cells show an increase of claudin1 in the cytoplasm and the nucleus with some punctuate accumulation at the plasma membrane. Follower cells, meanwhile, show an increase of claudin1 at the membrane suggesting improved cell-cell cohesion in the rear moving sheet. Thus, keratin network seems to be essential to prevent the aberrant expression and localization of cell junctions and thus to restrain cell motility. Moreover, claudin1 internalization is known to occur in moving cells during wound-healing [66]. This process could explain the predominant cytoplasmic/nuclear localization of claudin1 that we observed in front migrating K8/18-knockdown cells. However, further investigations are required to determine whether K8/18 loss improves claudin1 internalization. Despite the fact that there is a clear correlation between altered cell junctions and invasive behavior, the relationship between claudin1 and signaling pathways that promote cell invasion is beginning to be understood. Extensive studies have correlated claudin1 overexpression with MT1-MMP level and MMP2 activity [44, 51 and 52] as well as with β-catenin/Lef transcriptional activity [46]. However, the significance of claudin1 nuclear localization in cell invasion is still ambiguous. For example, forced expression of claudin1 in the nucleus did not increase the invasion potential of melanoma cells compared to cells displaying a more cytoplasmic pattern [45]. On the other way, transcriptional regulation seems to be the major mechanism by which claudin1 promote colon cancer cell invasion by repressing E-cadherin via ZEB1 activity [67]. The results that we presented in this study establish a functional role for claudin1 in PI3K/Akt/NF-κB pathway. We show that both membrane and nuclear claudin1 enhance this intracellular signaling cascade to promote motility and

invasiveness in K8/18-depleted epithelial cancer cell. K8/18 knockdown increases Akt1 and Akt3 membrane targeting and activation. The predominant activity of Akt1 and Akt3 in cell motility and invasion is in agreement with our recent study showing that Akt2 blocks cell motility of endometrial carcinoma cells while Akt1 and Akt3 increase wound closure [68]. Since claudin1 silencing decreases the presence of phospho-Akt1 and Akt3 at the membrane, we proposed that the localized accumulation of claudin1 generates specific membrane regions suitable for PI3K/Akt activation. Indeed, we demonstrate that claudin1 increases PI3K activity and PIP3 production in K8/18-depleted cells which supports the improved Akt activation observed in these cells. However, we were unable to detect any interaction previously shown by others [34, 49 and 50]. Characterization of the link between claudin1 and PI3K activity will need further investigations. The absence of keratin cytoskeleton also induces an increase of claudin1 in the nucleus. We found that in K8/18-depleted cells, NF-κBp65 transcriptional activity was increased and induced *MMP2* and *MMP9* expression. It was already shown that K8/18 moderate TNFα-mediated NF-κB activity thus providing resistance to the apoptotic effect of TNFα [15]. Our data show that the presence of claudin1 is partially involved in the NF-κBp65 DNA binding and that claudin1 silencing strongly reduces *MMP2* and *MMP9* expression. The decrease in *MMP2* and *MMP9* is equivalent to that observed after NF-κB silencing. These results strongly suggest that nuclear claudin1 plays a role in MMPs transcription. Since NF-κB regulates claudin1 transcription, these data reveal the existence of a positive feedback loop that could amplify cell motility and invasion.

Our current work also shows that K8/18 knockdown significantly increases epithelial cancer cell sensitivity to cisplatin. Keratins are well known to provide resistance to stress-induced apoptosis in hepatocytes [13-14]. Recent reports also suggested that K8/18 could mediate cisplatin resistance in different cancer cell lines [69-70]. Here we show that K8/18 depletion increases cisplatin-induced apoptosis by increasing the level of cleaved caspases 3, 8 and 9 and their target PARP. Although K8/18 knockdown improve Akt1 and Akt3 activation, the cells without K8/18 are more sensitive to the drug. In fact, cisplatin treatment induces a decrease of Akt

phosphorylation and XIAP levels in K8/18-depleted cells only. Previous studies have already shown that cisplatin decreases Akt phosphorylation and XIAP levels in chemosensitive but not in chemoresistant cell lines [33, 71 and 72]. The present results suggest that K8/18 knockdown engages similar signaling processes to those involved in chemosensitivity. Moreover, our results show that K8/18 loss increases the membrane targeting of the FasR and decreases the level of the inhibitory protein sFLIP. The role of keratin cytoskeleton in the Fas death receptor pathway has already been shown in hepatocytes [14, 73]. Interestingly, our results reveal that claudin1 is involved in the enhanced localization of FasR at the membrane. A recent report demonstrated that claudin mislocalization promote the activation of extrinsic apoptotic pathway through a direct interaction with components of the death-inducing signaling complex [74]. Altogether, these results suggest a dual role for claudin1 in invasiveness and sensitivity to apoptosis of K8/18-depleted cells.

Cancer cells can migrate as single cells when undergoing EMT or can move collectively as an epithelial sheet. Collective migration requires maintenance of cell-cell contacts to provide pushing forces at the rear and forcing protrusions to occur at the front, thus establishing a collective front-back polarity and directionality [75]. Our results are in agreement with this concept in that at the leading edge of the migrating sheets, K8/18 loss improves PI3K/Akt activation involved in the direction of the movement [76] and local matrix degradation [47, 77] through NF-κB activity. In the following rows, K8/18 knockdown increases TJ protein claudin1 at the cell membrane which strengthens cell cohesion [78]. This led to our proposal of a model in which K8/18 loss promotes collective migration of epithelial cancer cell in a different way than an EMT, by increasing directional migration and intercellular cohesion (Fig. 8). Moreover, we show that K8/18 knockdown does not induce morphological changes nor resistance to apoptosis associated with an EMT. Our results are consistent with the increasing evidences that cancer cells can become invasive without undergoing an EMT [79-81].

In summary, our current findings demonstrate a functional role for the K8/18 cytoskeleton in the restraint of epithelial cancer cell motility, invasion and cisplatin sensitivity. K8/18 loss enhances *CLDN1* expression through NF-κB that activates the PI3K/Akt/NF-κB pathway and increases FasR membrane targeting. To our knowledge, this is the first study revealing that claudin1 is a target of NF-κB to promote cell motility and invasion. Our study strongly indicates that in cancer, keratins should not be considered only as markers of differentiation but also as key regulators in cancer progression.

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TABLE 1

Primers for conventional PCR amplification

Gene	Forward primer	Reverse primer	T_m	Cycles	Product size (bp)
KRT19	tttgagacggaacaggctct	gccatgacctcatattggct	58	35	275
KRT7	caggatgtggtggaggactt	ttgctcatgtaggcagcatc	58	35	116
β-actin	cctccctggagaagagcta	acgtcacacttcatgatgga	60	25	348
18S rRNA	tggtcgctcgctcctctccc	cagcgcccgtcggcatgtat	60	25	70
CLDN1 site *a*	tagtatccagactccagcgc	cgagaatgaagcccaacagc	61	40	244
CLDN1 site *b*	gtgagccgccctgaaacc	ggcgctggagtctggatac	61	40	150
COX2	ggcaaagactgcgaagaaga	gggtaggctttgctgtctga	58	40	426
GAPDH	tactagcggttttacgggcg	tcgaacaggaggagcagagagcga	59	40	166

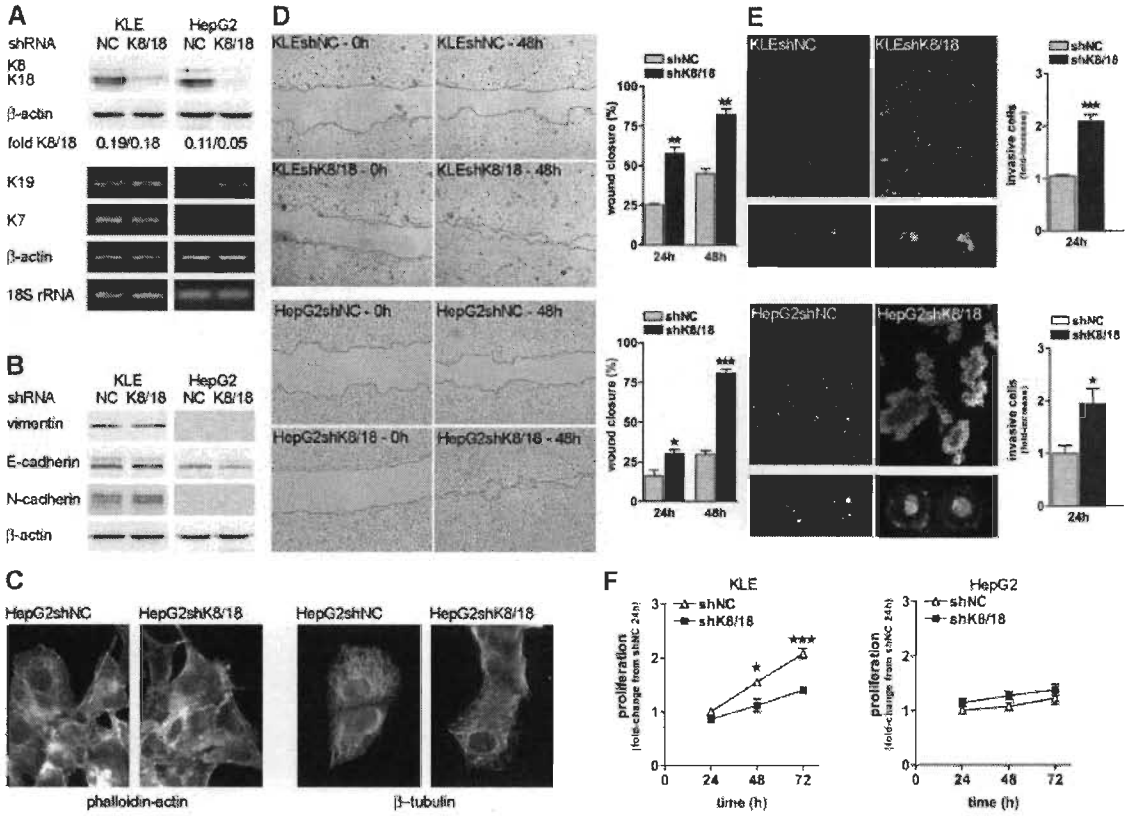

FIGURE 1. K8/18 loss promotes cancer cell motility and invasion without modulating epithelial phenotype. *A*, KLE and HepG2 cells were stably transfected with scrambled negative control (shNC) or keratin 8 and 18 (shK8/18) shRNA (as described in Experimental procedures) and K8/18 levels were analyzed by western blot analysis. β-actin levels were included as loading control. Silencing specificity was monitored by analysis of keratin 19 (K19) and keratin 7 (K7) mRNA levels. β-actin and 18S rRNA were used as reference genes. K8/18 knockdown does not affect EMT markers expression as shown by western blot of vimentin, E-cadherin and N-cadherin (*B*) nor the organization of other cytoskeletal networks (*C*) as shown by immunfluorescence staining of phalloidin-actin and β-tubulin in HepG2 cells stably transfected with NC or K8/18 shRNA. *D*, Wound healing assay. Stable shNC and shK8/18 KLE and HepG2 clones grown to subconfluence were scraped with the blunt end of a tip to make cell-free area (0h). The area was measured 24h and 48h after wounding and results are plotted as a percentage of wound closure (0h=100%). *E*, Matrigel invasion assay. Invasive shNC and shK8/18 KLE and HepG2 cells that had adhered to the transwell insert were fixed and stained with Hoechst dye. Results showed representative pictures of five randomly selected fields in the insert (magnification X100) as well as the total fluorescence of the insert (2 transwells/experiments). Quantification of total fluorescence of each insert was shown as fold-change from shNC densitometric values. *F*, Proliferation of KLE and HepG2 clones was assessed using MTT proliferation assay. The results are presented as fold-change from absorbance values of shNC clones grown 24h. Error bars, S.E. All experiments were performed three times in duplicate. (*$p<0.05$; **$p<0.005$, ***$p<0.0005$).

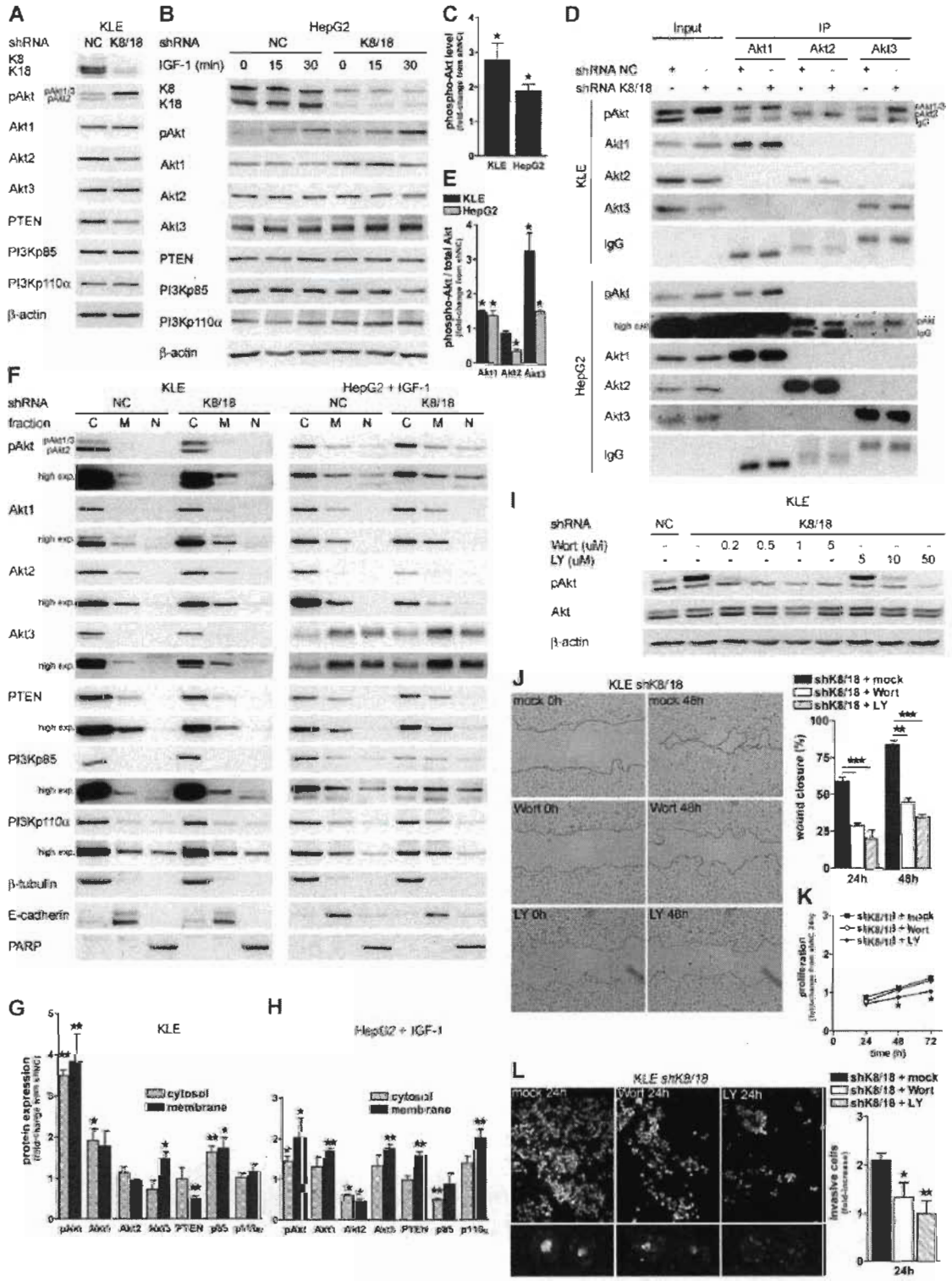

FIGURE 2. K8/18 knockdown improves Akt1 and Akt3 membrane targeting and phosphorylation. Western blot analysis of PI3K/Akt pathway mediators in KLE shNC and shK8/18 clones (*A*) and HepG2 shNC and shK8/18 clones treated with 200ng/mL IGF-1 for 0, 15 and 30min after 24h serum-starvation (*B*). β-actin levels were included as loading control. *C*, Phospho-Akt levels are shown as fold-change from densitometric values of KLEshNC clone (upper band on pAkt blot) and HepG2shNC clone treated with IGF-1 for 15min. *D*, The phosphorylation state of each Akt isoforms was monitored in shNC and shK8/18

cells by immunoprecipitation (IP) using anti-Akt1, anti-Akt2 or anti-Akt3 antibodies followed by western blot analysis with anti-phospho-Akt antibody. Protein content of whole cell lysates is shown (input). Quantification was determined by densitometric ratio of phospho-Akt on total Akt for each isoforms IP and is plotted as fold-change from shNC values (*E*). *F*, The impact of K8/18 knockdown on subcellular localization of PI3K/Akt pathway mediators in KLE and IGF-1-treated HepG2 cells was determined using cytosolic (C)/membrane (M)/nuclear (N) fractionation followed by western blot analysis. β-tubulin, E-cadherin and poly(ADP-ribose) polymerase-2 (PARP) were used as controls for the purity of C, M and N fractions respectively. Quantification of protein levels in C and M fractions of KLE shK8/18 (*G*) and IGF-1-treated HepG2 shK8/18 cells (*H*) is determined by densitometric analysis and is shown as fold-change from shNC for each fraction. *I*, Dose-response of KLE cells to PI3K inhibitors exposure (1h) and the effect on cell proliferation (K), motility (*J*) and invasion through matrigel (*L*). Results are shown as described in Figure 1. Data represent means of three independent experiments. Error bars, S.E. (*$p<0.05$; **$p<0.005$, ***$p<0.0005$).

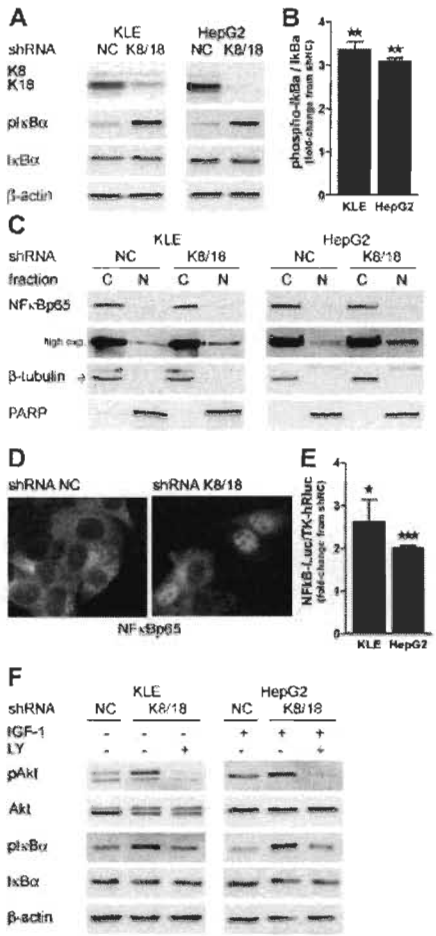

FIGURE 3. K8/18 knockdown increases NF-κBp65 transcriptional activity through PI3K. Loss of K8/18 increases IκBα phosphorylation as shown by western blot analysis (*A*) and densitometric quantification (*B*). β-actin levels were shown as loading control. The impact of K8/18 depletion on nuclear localization of NF-κBp65 was determined by cytosolic (C)/nuclear (N) fractionation (*C*) and immunofluorescence staining (*D*). β-tubulin (C) and poly(ADP-ribose) polymerase-2 (PARP) (N) were used to validate the low cross contamination among fractions. *E*, NF-κB DNA binding was monitored by dual luciferase reporter assay. KLE and HepG2 clones were co-transfected with NF-κB-Luc reporter plasmid and TK-hRLuc for 24h then reporter activity was measured and normalized on the Renilla luciferase activity used as an internal control. Each experiment was repeated three times in duplicate. *F*, Inhibition of PI3K activity using LY294002 (50μM, 1h treatment) reduced the phosphorylation of IκBα as shown by western blot analysis. Data represent means of three independent experiments. Error bars, S.E. (*p<0.05; **p<0.005, ***p<0.0005).

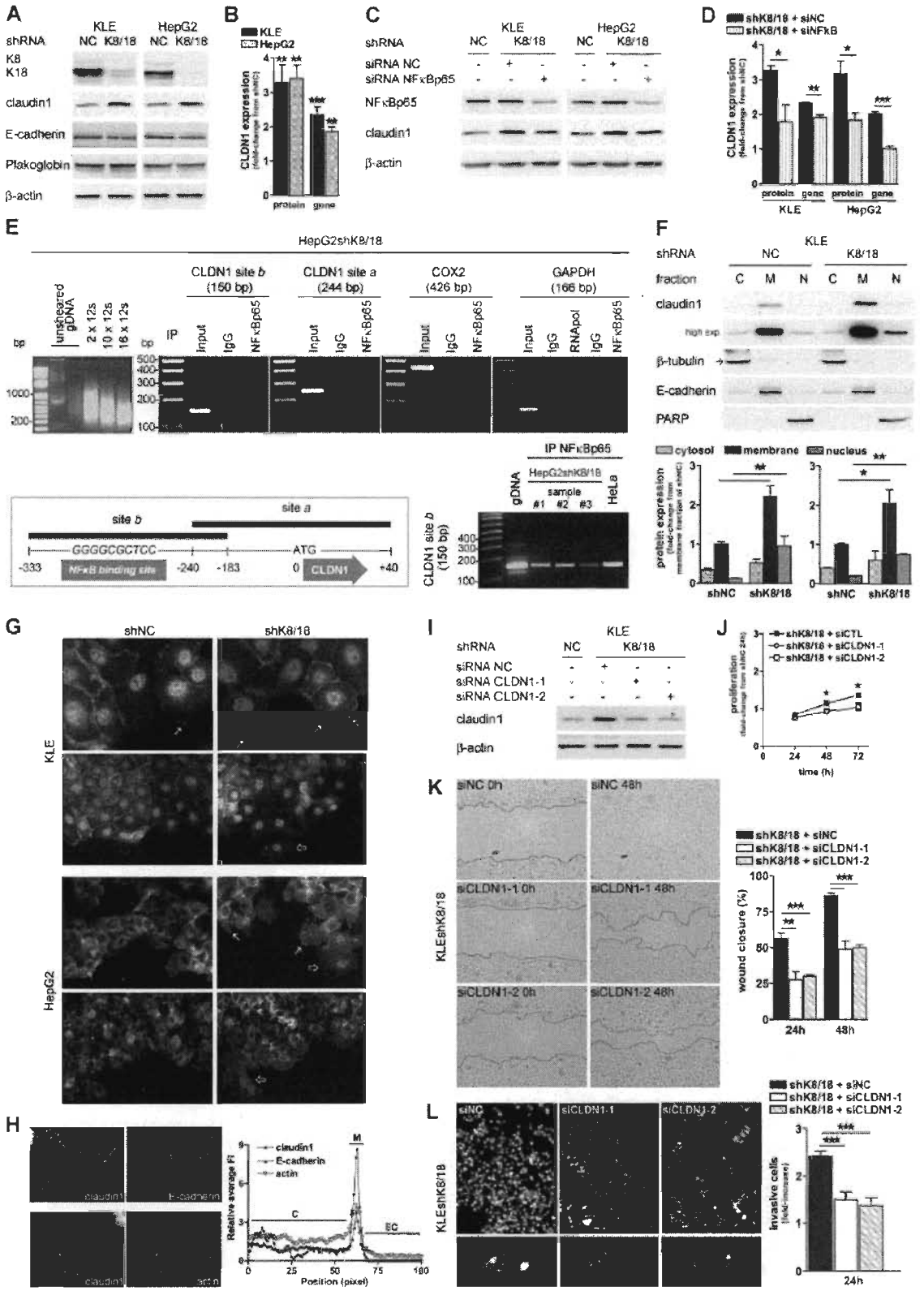

FIGURE 4. Claudin1 is up-regulated in K8/18-depleted cells and is involved in cell motility and invasiveness. K8/18 knockdown increases claudin1 protein (*A,B*) and gene (*B*) expression (qPCR) in KLE

and HepG2 cells but does not modulate other cell junction components as shown by western blot analysis of E-cadherin and plakoglobin. NF-κBp65 silencing decreases claudin1 protein (*C, D*) and gene (*D*) expression in both cell lines. *E*, In vivo binding of NF-κBp65 to the CLDN1 promoter. Average size of cross-linked DNA after shearing: the optimal sonication condition was 12 sec for 10 times. PCR amplifications of CLDN1 (site *a* and *b*), Cox2 and GAPDH promoters resulting from ChIP assay with IgG (negative control), NF-κBp65 antibody, RNA polymerase II antibody (negative and positive control) or input DNA (pre-cleared chromatin, no antibody) in HepG2shK8/18 cells. Schematic representation of NF-κBp65 binding site in the CLDN1 promoter (-310 to -301 bp). PCR amplifications of site *b* (-333 to -183 bp) in CLDN1 promoter resulting from ChIP assay with NF-κBp65 antibody in three different lysates of HepG2shK8/18 cells and one from HeLa cells. *F*, The effect of K8/18 loss on cellular localization of claudin1 was determined using cytosolic (C)/membrane (M)/nuclear (N) fractionation (*C, D*) followed by western blot analysis. β-tubulin, E-cadherin and poly(ADP-ribose) polymerase-2 (PARP) were used as controls for the purity of C, M and N fractions respectively. Quantification of claudin1 levels in each fraction of KLE shK8/18 and IGF-1-treated HepG2 shK8/18 cells (*D*) is determined by densitometric analysis and is shown as fold-change from shNC of membrane fraction. *G*, Claudin1 localization at the leading edge membrane (arrows) and in cytosol/nucleus of migrating cells (unfilled arrows) was compared between shNC and shK8/18 cells in both cell lines using immunofluorescence. Magnification: X400 and X200. *H*, KLEshK8/18 cells were immunostained for claudin1 with E-cadherin or phalloidin-actin after wounding. Relative average of fluorescence intensities (FI; y axis) at the indicated lines (distance along the line from inside to outside of the cell; x axis). FI are defined as (fluorescence intensities)/(mean cytoplasmic fluorescence intensities). C: cytoplasmic region, M: membrane region, EC: extracellular region. The role of claudin1 in cell motility and invasion was determined by transfection of KLEshK8/18 cells with control siRNA (siNC) or with two claudin1 siRNA (siCLDN1-1 and siCLDN1-2) for 24h (*I*) followed by proliferation (*J*), wound healing (*K*) and matrigel invasion assays (*L*). Results are shown as described in Figure 1. Data represent means of three independent experiments. Error bars, S.E. (*$p<0.05$; **$p<0.005$, ***$p<0.0005$).

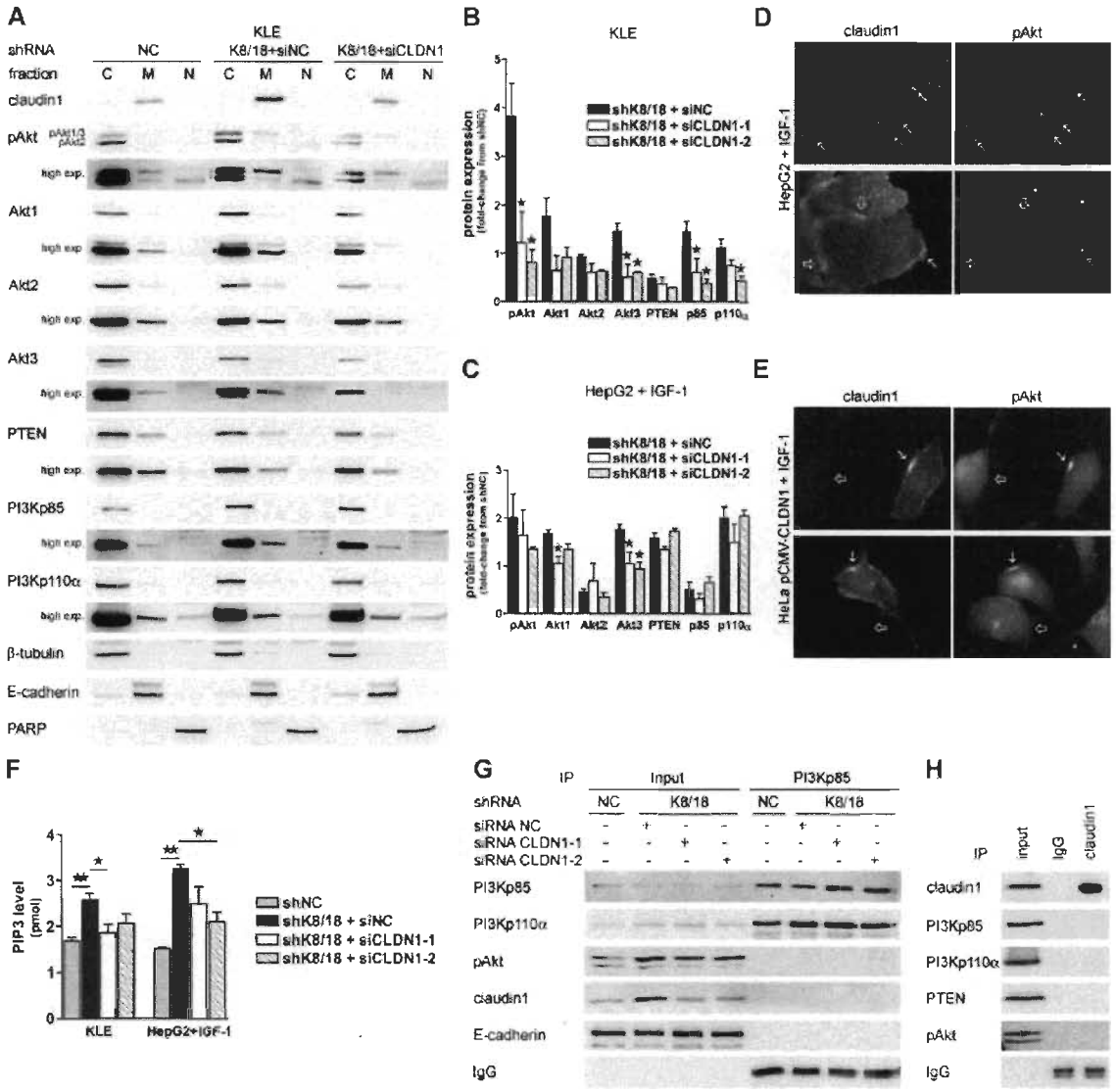

FIGURE 5. Claudin1 regulates membrane localization and activity of PI3K/Akt pathway mediators. *A*, The effect of claudin1 silencing on subcellular localization of PI3K/Akt pathway mediators in KLE cells was determined using cytosolic (C)/membrane (M)/nuclear (N) fractionation followed by western blot analysis. β-tubulin, E-cadherin and poly(ADP-ribose) polymerase-2 (PARP) were used to validate low cross contamination among fractions. Quantification of protein levels in membrane fraction of KLE shK8/18 (*B*) and IGF-1-treated HepG2 shK8/18 cells (*C*) transfected with control siRNA (siNC) or two claudin1 siRNA (siCLDN1-1 and siCLDN1-2) is determined by densitometric analysis and is shown as fold-change from shNC. Double immunofluorescence staining for claudin1 and phospho-Akt in IGF-1-treated HepG2 shK8/18 cells (*D*) and HeLa cells transfected with pCMV-CLDN1 vector (*E*) showed that both proteins are localized in same regions of the plasma membrane of front migrating cells (*D* and *E*, arrows) while no phospho-Akt staining is detected in cell-cell junction regions (*D*, unfilled arrows). Non-transfected HeLa cells showed a diffuse cytoplasmic staining for phospho-Akt (*E*, unfilled arrows). Magnification: X200 and X1000. *F*, The effect of claudin1 silencing on PI3K activity was measured as PIP3 generated from immunoprecipitated PI3Kp85 in a kinase assay and assessed in a competitive ELISA. Western blot analyses of PI3Kp85 (*G*) and claudin1 (*H*) immunoprecipitates. Data represent means of three independent experiments. Error bars, S.E. (*p<0.05; **p<0.005).

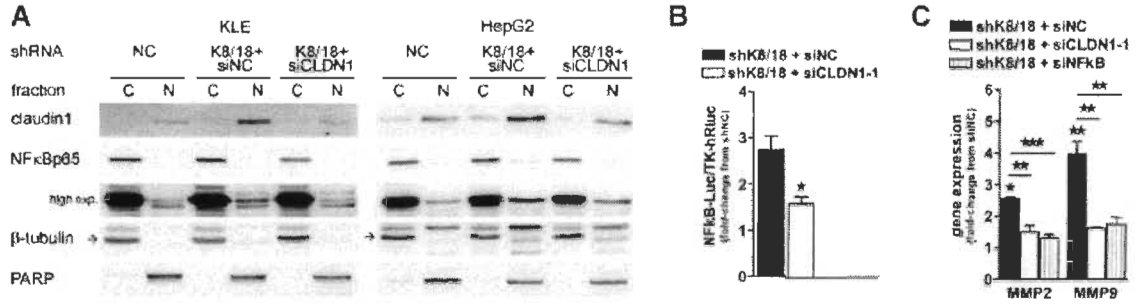

FIGURE 6. Claudin1 regulates NF-κBp65 transcriptional activity and MMPs expression. *A*, The effect of claudin1 silencing on subcellular localization of NF-κBp65 in KLE and IGF-1-treated HepG2 cells transfected with control siRNA (siNC) or claudin1 siRNA (siCLDN1-1) was determined using cytosolic (C)/nuclear (N) fractionation followed by western blot analysis. β-tubulin and poly(ADP-ribose) polymerase-2 (PARP) were used to validate low cross contamination among fractions. *B*, NF-κB DNA binding was monitored by dual luciferase reporter assay. KLE shK8/18 cells were co-transfected with NF-κB-Luc reporter plasmid and TK-hRLuc for 24h, followed by transfection with control siRNA (siNC) or claudin1 siRNA (siCLDN1-1) for 24h. Reporter activity was measured and normalized on the Renilla luciferase activity used as an internal control. Each experiment was repeated three times in duplicate. The role of claudin1 and NF-κBp65 on MMPs expression was determined by transfection of HepG2shK8/18 cells with control siRNA (siNC), claudin1 siRNA (siCLDN1-1) or NF-κBp65 siRNA (siNFkB) for 24h followed by qPCR analysis of MMP2 and MMP9 transcripts (*C*). Results are shown as fold-change from shNC. Data represent means of three independent experiments performed in duplicate. Error bars, S.E. (*p<0.05; **p<0.005, ***p<0.0005).

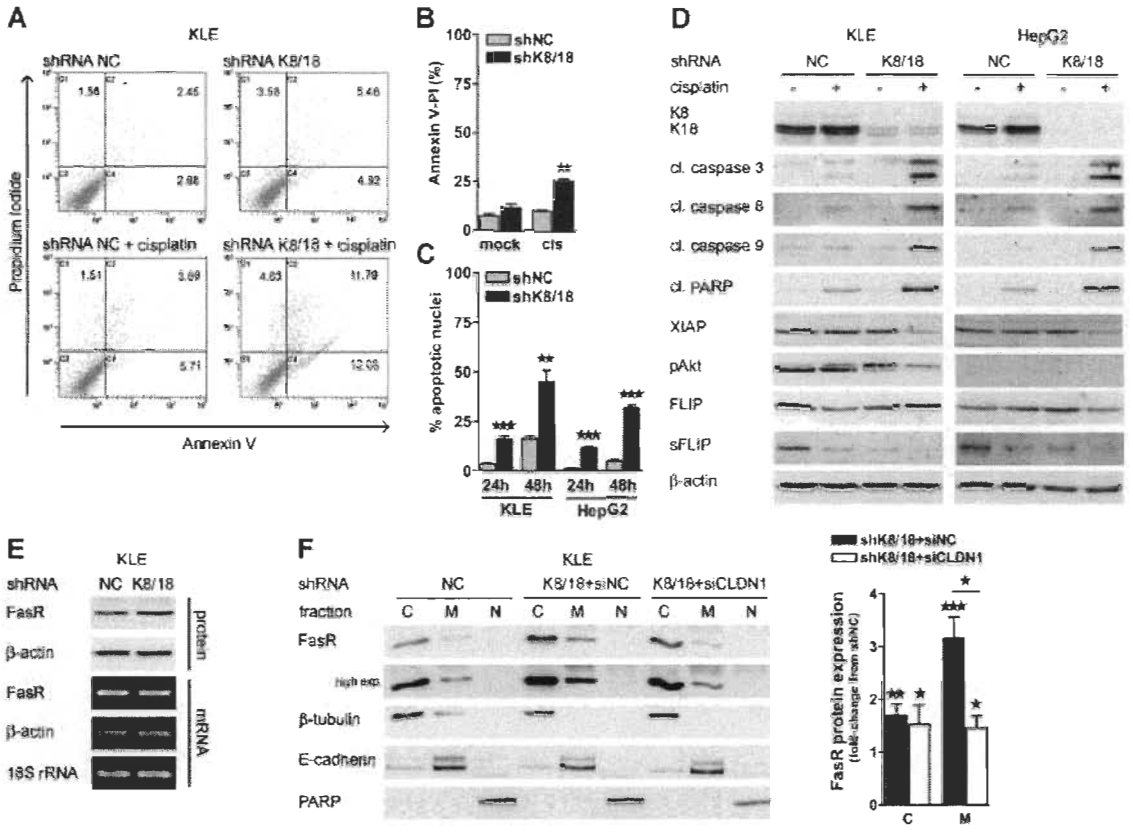

FIGURE 7. K8/18 loss sensitizes cells to cisplatin-induced apoptosis. Control (shNC) and K8/18-knockdown (shK8/18) cells were treated with mock or cisplatin (10μM) for 24h then collected for flow cytometry using Alexa Fluor 488-annexin V/propidium iodide staining (*A*, *B*), for apoptotic cells counting using Hoechst staining (*C*) and for Western blot analysis (*D*). C1, C2, C3, and C4 represent quadrants for dead, late apoptotic, viable and early apoptotic cells respectively, and percentage of cells in each quadrants is indicated (*A*). Panel *A* shows representative results of three independent experiments which are plotted in *B*. (*C*) Histogram showing percentage of apoptotic cells in different groups using Hoechst staining. The impact of K8/18 knockdown on Fas receptor (FasR) expression (*E*) and localization (*F*) was determined by Western blot and conventional PCR (*E*) and by cytosolic (C)/membrane (M)/nuclear (N) fractionation followed by Western blot analysis (*F*). β-actin and 18S rRNA were used as loading controls. β-tubulin, E-cadherin and poly(ADP-ribose) polymerase-2 (PARP) were used to validate low cross contamination among fractions. Quantification of FasR levels in cytosolic and membrane fractions of KLEshK8/18 transfected with control siRNA (siNC) or claudin1 siRNA (siCLDN1-1) is determined by densitometric analysis and is shown as fold-change from shNC. Data represent means of three independent experiments. Error bars, S.E. (*p<0.05; **p<0.005, ***p<0.0005).

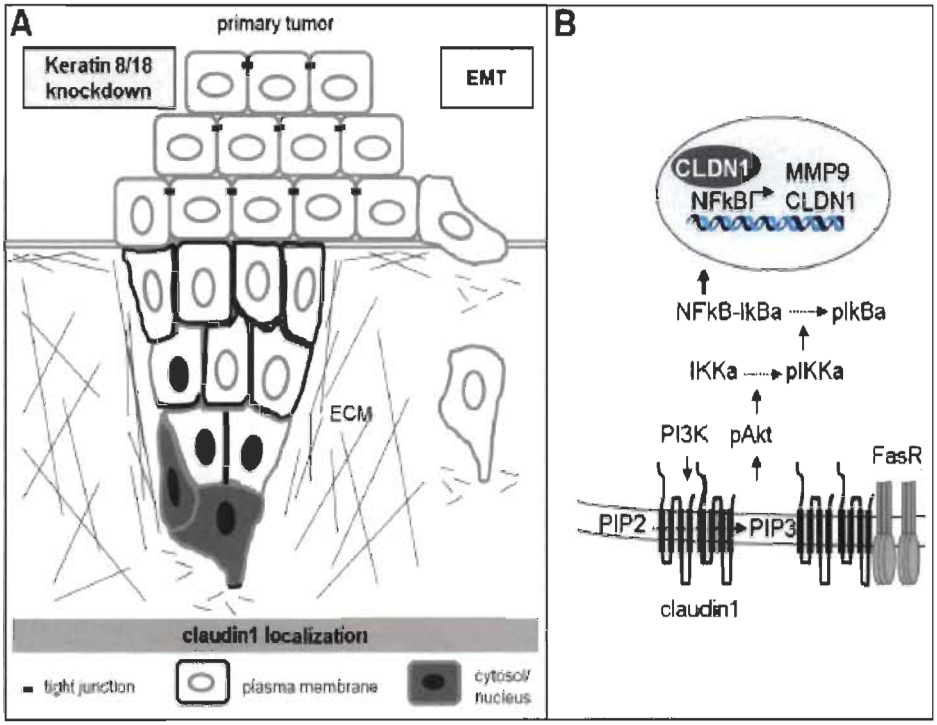

FIGURE 8. Hypothetical model of epithelial cancer cell invasiveness and sensitivity mediated by K8/18 loss. K8/18 knockdown in epithelial cancer cells promotes collective migration and sensitivity to apoptosis in a different way than epithelial-mesenchymal transition. *A*, At the leading edge of the migrating sheets, K8/18 loss improves PI3K/Akt/NF-κB/MMP activation through an increase of claudin1 in specific regions of the plasma membrane and in the cytosol/nucleus thus promoting the direction of the movement and local matrix degradation. In the following rows, K8/18 loss promotes cohesion between neighboring cells by increasing claudin1 at the plasma membrane. *B*, Localized accumulation of claudin1 at the plasma membrane generates specific membrane regions suitable for PI3K/Akt activation which led to an increase of NF-κB nuclear translocation and transcriptional activity. NF-κB induces MMP2 and MMP9 expression, as well as claudin1 expression, revealing a positive feed-back process. K8/18 loss also increases FasR membrane targeting in a claudin1-dependent manner thus enhancing cisplatin-induced apoptosis by the extrinsic pathway.

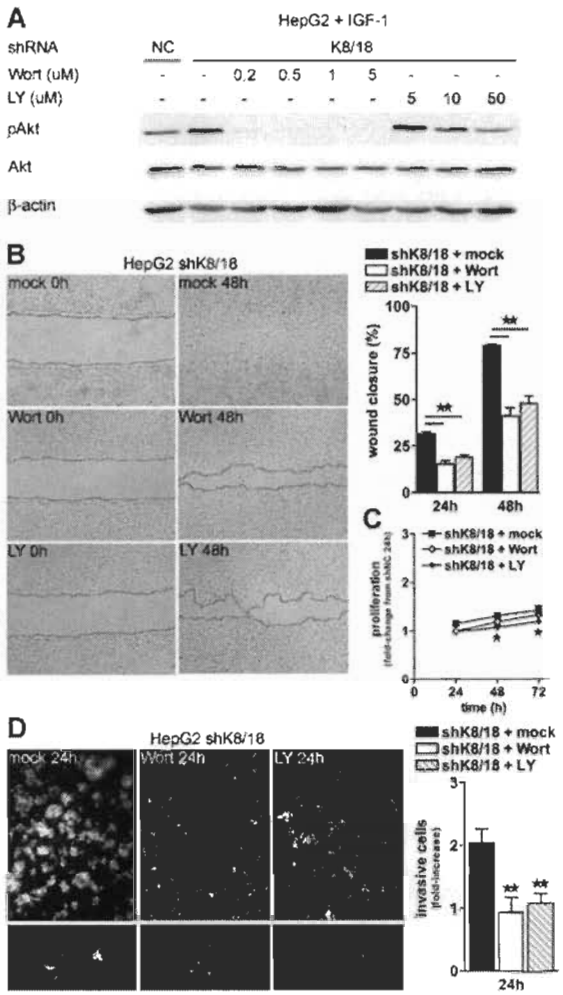

FIGURE S1. PI3K/Akt is involved in cell motility and invasion of K8/18-knockdown HepG2 cells. *A*, dose-response of HepG2 cells to PI3K inhibitors exposure (1h) and the effect on cell proliferation (*C*), motility (*B*), and invasion through matrigel (*D*). Results are shown as described in Fig. 1. Data represent means of three independent experiments. Error bars, S.E. (*p<0.05; **p<0.005).

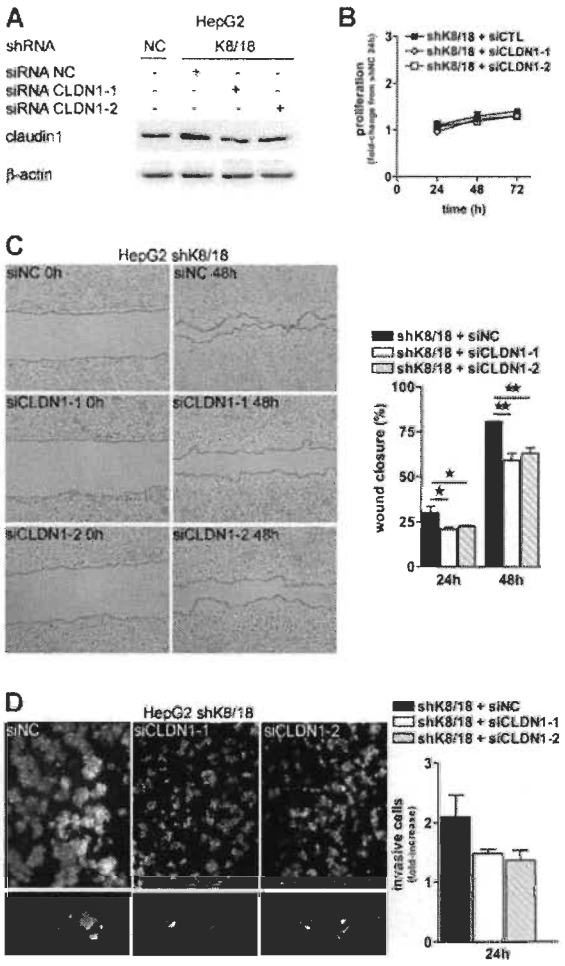

FIGURE S2. Claudin1 is involved in cell motility and invasion of K8/18-knockdown HepG2 cells. The role of claudin1 in cell motility and invasion was determined by transfection of HepG2shK8/18 cells with control siRNA (siNC) or with two claudin1 siRNA (siCLDN1-1 and siCLDN1-2) for 24h (*A*) followed by proliferation (*B*), wound healing (*C*) and matrigel invasion assays (*D*). Results are shown as described in Fig. 1. Data represent means of three independent experiments. Error bars, S.E. (*$p<0.05$; **$p<0.005$).

CHAPITRE IV

CONCLUSION ET PERSPECTIVES

Nous sommes tous concernés de près ou de loin par ce fléau qu'est le cancer. Les principales causes de mortalité de cette affliction sont la résistance des cellules cancéreuses aux traitements de chimiothérapie et la formation de tumeurs secondaires dans les organes vitaux, les métastases. La présence de cellules cancéreuses dans le sang des patients atteints de cancer est un facteur prédictif de la progression métastatique et de la récurrence de la maladie. À un stade avancé de la maladie, l'expression des kératines et autres marqueurs épithéliaux est considérablement réduite au profit des marqueurs mésenchymateux. Cette EMT constitue une étape clé dans la formation des métastases et l'inhibition de cette conversion phénotypique représente une stratégie thérapeutique prometteuse. Or, la plupart des stratégies actuelles de détection des CTC utilisent les kératines comme marqueurs, ce qui exclut une proportion considérable de cellules métastatiques (Gradilone et al. 2011). De plus, il est urgent d'identifier de nouvelles cibles pour maintenir ou favoriser la différenciation des cellules épithéliales cancéreuses (Reka et al. 2011).

Les kératines sont depuis longtemps utilisées comme marqueurs pour identifier l'épithélium d'origine d'un carcinome. Étant donné les preuves croissantes du rôle des protéines de FIs dans la signalisation intracellulaire, nous avons entrepris d'étudier la relation moléculaire entre les FIs et la signalisation oncogénique des isoformes d'Akt, en particulier au cours de l'EMT. Notre hypothèse selon laquelle l'induction du potentiel invasif et de l'EMT par certains isoformes d'Akt puisse impliquer le cytosquelette de FIs a fait l'objet de deux études différentes quant à leur modèle expérimental. Nous avons tout d'abord observé la dynamique des FIs suite à l'expression ectopique des isoformes d'Akt dans deux lignées cellulaires de carcinome humain. Par la suite, nous avons étudié l'effet de l'absence des K8/18 dans la signalisation endogène médiée par PI3K/Akt chez les mêmes lignées cellulaires. Bien que ces deux approches expérimentales soient

différentes, les observations que nous avons faites concernant l'implication des FIs dans l'activité oncogénique des isoformes d'Akt sont similaires.

4.1 L'agrégation péri-nucléaire ou l'absence complète de kératines induit un phénotype invasif et chimiosensible

La surexpression de l'oncogène Akt1 ou Akt2 provoque une importante réorganisation péri-nucléaire du réseau de FIs dans les cellules épithéliales cancéreuses suite à une augmentation de l'expression protéique des K8/18 ou K18/vimentine respectivement (Fortier et al. 2010). Une altération dans les niveaux protéiques des K8/18 est connue pour induire la formation d'agrégats de kératines dans les hépatocytes de souris et en culture *in vitro* (Nakamichi et al. 2002; Nakamichi et al. 2005). De plus, les modifications post-traductionnelles, telle que la phosphorylation, altèrent la formation des filaments et augmente la solubilité des kératines (Omary et al. 1998). Dans notre modèle, la surexpression des isoformes d'Akt augmente aussi la phosphorylation de la K8. L'agrégation des protéines de FIs qui en résulte est associée à une augmentation du potentiel invasif des cellules (Figure 4.1, résultats non publiés).

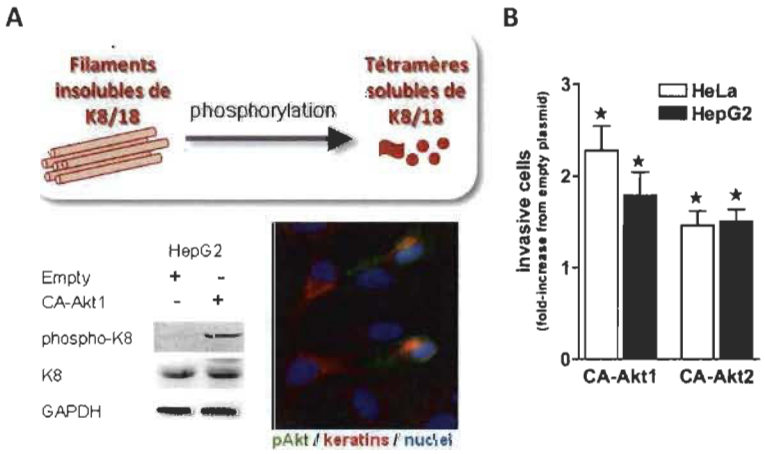

Figure 4.1 **L'agrégation péri-nucléaire des kératines induite par les isoformes d'Akt est associée à un potentiel invasif accru.**
A, La phosphorylation de la K8 (phospho-K8) augmente sa solubilité et favorise la formation d'agrégats péri-nucléaires tel que démontré par immunofluorescence. *B*, Cette réorganisation du cytosquelette de FIs est associée avec une augmentation du potentiel invasif des cellules HeLa et HepG2 qui expriment les isoformes Akt1 ou Akt2. Empty : vecteur contrôle, CA-Akt1 : constitutively active Akt1. (Résultats non publiés)

Il en va de même pour la résistance à l'apoptose. Des observations préliminaires nous ont révélé que la sensibilité des cellules épithéliales cancéreuses au cisplatine était associée au clivage de la K18 (Asp238) et à la présence d'agrégats de kératines (Figure 4.2, résultats non publiés). De façon similaire, les cellules déficientes en K8/18 montrent aussi une plus grande sensibilité à cette drogue (Fortier et al. 2013). Il est connu que les caspases peuvent être séquestrées par les kératines au cours de l'apoptose, protégeant ainsi les autres substrats de la dégradation (Dinsdale et al. 2004).

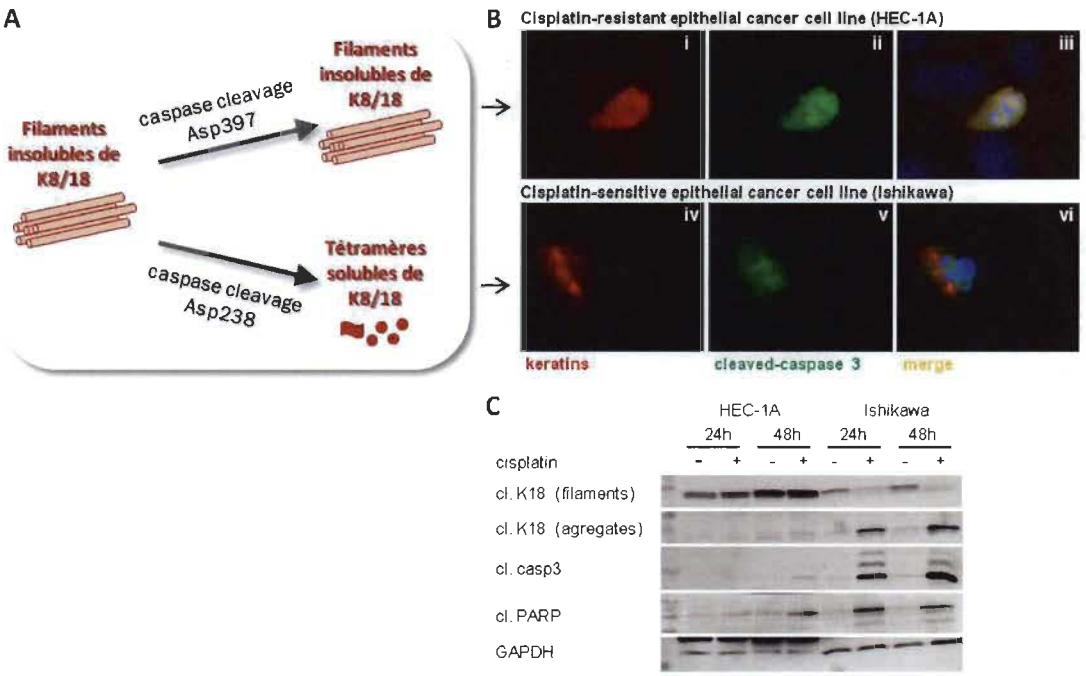

Figure 4.2 **La sensibilité au cisplatine est associée à la présence d'agrégats de fragments de kératines chez les cellules épithéliales cancéreuses.**
Le marquage par immunofluorescence montre une co-localisation des filaments de kératines avec la caspase 3 clivée seulement dans les cellules résistantes au cisplatine, suggérant que les filaments de kératines protègent contre l'apoptose en séquestrant les caspases (*B*, i-iii). Dans les cellules sensibles à la drogue, il y a formation d'agrégats de kératines qui ne co-localisent pas avec la caspase 3 clivée (*B*, iv-vi). *C*, les analyses par Western blot confirment que l'activation de la caspase 3 est associée à la présence des fragments Asp238 de la K18 à l'origine des agrégats de kératines. cl.K18 : kératine 18 clivée, cl. casp3 : caspase 3 clivée, cl. PARP : PARP clivée. (Résultats non publiés)

Ces deux modèles d'études nous ont donc permis de constater que l'agrégation péri-nucléaire des protéines de FIs induit un phénotype similaire à l'absence complète de filaments de kératines dans la cellule (Figure 4.3). Dans les deux cas, on observe une augmentation de la motilité cellulaire en réponse aux kinases Akt et une sensibilité

accrue au cisplatine. Il est intéressant de remarquer que l'agrégation péri-nucléaire et l'absence complète des kératines ont pour conséquence commune la perte de contact entre le cytosquelette de FIs et la membrane plasmique. Cette analyse suggère que le phénotype observé dans nos deux modèles relève de la perte de l'intégrité membranaire. En effet, la perte de contact entre le cytosquelette de FIs et la membrane influence nécessairement les jonctions intercellulaires et le trafic membranaire. L'absence de cytosquelette de kératines altère la distribution membranaire des desmosomes (Loranger et al. 2006), des hémidesmosomes (Seltmann et al. 2012) et des transporteurs du glucose GLUT1 et GLUT3 (Vijayaraj et al. 2009). Les kératines sont aussi impliquées dans le transport vésiculaire en régulant la protéine chaperonne Hsc70 (Planko et al. 2007) et l'annexine II (Chung et al. 2012). De façon similaire, notre étude a révélé que l'absence des K8/18 altère l'expression et la localisation intracellulaire de la protéine de jonction serrée claudine-1, favorisant ainsi la motilité cellulaire et l'invasion (Fortier et al. 2013).

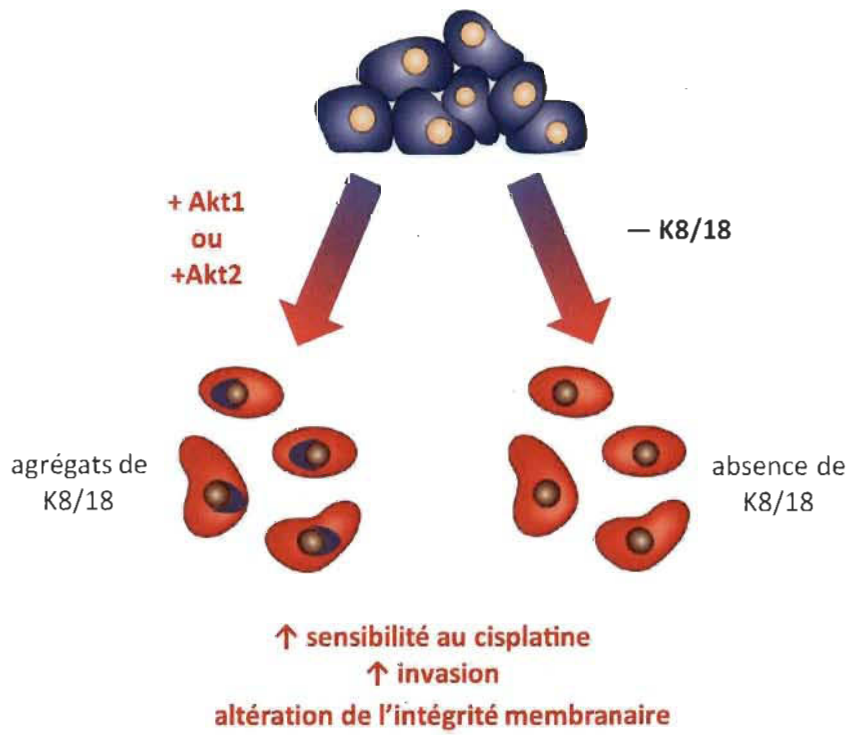

Figure 4.3 **L'agrégation péri-nucléaire des kératines phénocopie la délétion complète du cytosquelette de K8/18 dans les cellules épithéliales cancéreuses.**
Adapté de (Thiery 2002).

4.2 Les kératines répriment le potentiel invasif en préservant les niveaux d'expression et la localisation membranaire de la claudine-1

La protéine claudine-1 constitue les jonctions serrées de l'épithélium avec l'occludine et les JAM (pour *junctional adhesion molecules*) (Steed et al. 2010).

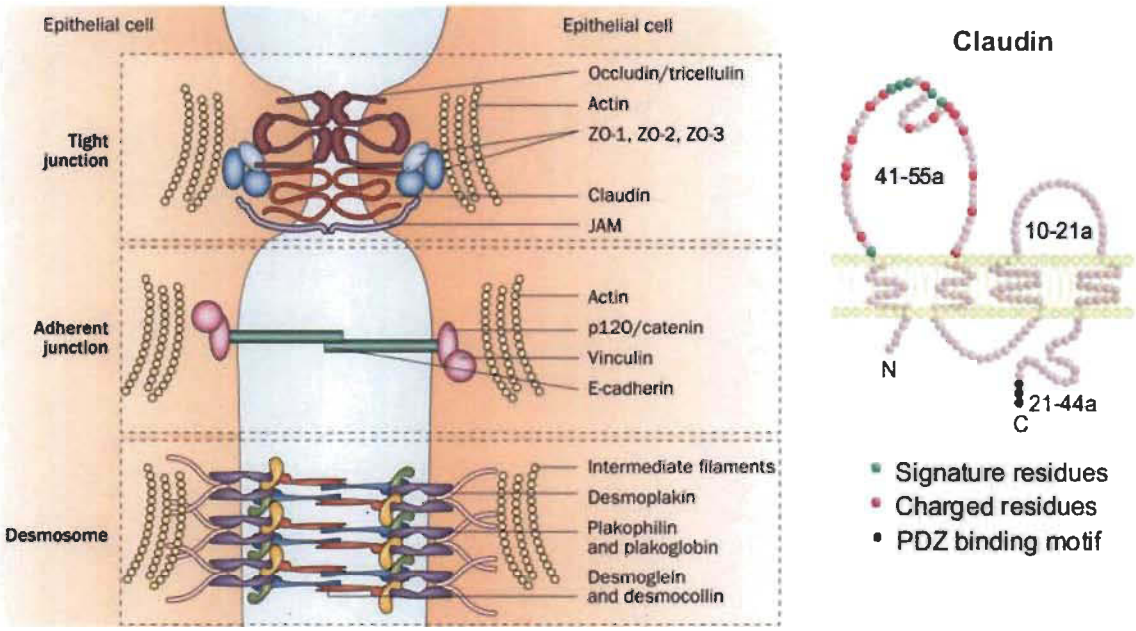

Figure 4.4 **Les claudines des jonctions serrées de l'épithélium.** (Gonzalez-Mariscal et al. 2007; Neunlist et al. 2013).

Depuis sa caractérisation en 1998, on retrouve désormais plus de 24 claudines différentes exprimées de façon spécifique dans les différents tissus (Furuse et al. 1998; Tsukita et al. 2008). Cette protéine transmembranaire participe au maintien de la polarité cellulaire en empêchant la libre diffusion des lipides et des protéines entre les domaines apical et baso-latéral. La claudine-1 possède des domaines de liaison PDZ qui favorisent l'interaction avec différentes protéines de jonctions et de signalisation cellulaire. Elle est aussi constituée de deux domaines extracellulaires qui définissent la perméabilité paracellulaire de l'épithélium. D'ailleurs, une altération dans l'expression et/ou la localisation de la claudine-1 est associée à plusieurs maladies inflammatoires de l'intestin (Bertiaux-Vandaele et al. 2011; Poritz et al. 2011). On retrouve aussi une expression altérée de la claudine-1 dans plusieurs cancers (Tableau 4.1). Dans la majorité des cas, l'augmentation de son expression est associée au potentiel invasif et

métastatique des cellules cancéreuses (Dhawan et al. 2005; Oku et al. 2006; Leotlela et al. 2007; Dos Reis et al. 2008; Yoon et al. 2010). La localisation aberrante de la claudine-1 dans le cytoplasme ou le noyau cellulaire est aussi impliquée dans la transformation maligne (Dhawan et al. 2005; French et al. 2009).

Tableau 4.1
Expression de la claudine-1 dans différents cancers

Types de cancer	Expression
Carcinome du sein	↓
Carcinome colorectal	↑
Carcinome endométrial	↑
Adénocarcinome gastrique	↑
Carcinome hépatocellulaire	↑
Carcinome épithélial ovarien	↑
Carcinome pancréatique	↑
Mélanome	↑

Adapté de (Singh et al. 2010).

Les cellules épithéliales cancéreuses déficientes en K8/18 montrent une augmentation de l'expression de la claudine-1 (Fortier et al. 2013). Pour la première fois, nous avons démontré que le facteur de transcription NF-κB est un activateur direct de la transcription du gène de la claudine-1. En effet, l'absence de kératines induit la translocation nucléaire de ce facteur et favorise par conséquent sa liaison à l'ADN. Il a déjà été rapporté que les K8/18 freinent l'activité transcriptionnelle de NF-κB en séquestrant certains médiateurs de la signalisation médiée par le TNF (Caulin et al. 2000; Inada et al. 2001). Dans notre modèle, la translocation nucléaire de NF-κB implique l'activité de la PI3K. Toutefois, des analyses préliminaires de co-immunoprécipitation montrent une interaction entre la K18 et NF-κB (Figure 4.5), suggérant que les kératines puissent séquestrer ce facteur de transcription dans le cytoplasme.

Les cellules déficientes en K8/18 montrent aussi une présence accrue de la claudine-1 dans le noyau, suggérant que l'absence de cytosquelette de kératines favorise

son internalisation. Le recyclage de la claudine-1 est un processus modulé par différents effecteurs du trafic membranaire tels que la myosine (Gonzalez-Mariscal et al. 2008), le complexe ESCRT (pour *endosomal sorting complex required for transport*) (Dukes et al. 2011) et la cathepsine L (Boudreau et al. 2007). Il est intéressant de noter que l'internalisation des claudines est particulièrement facilitée lors de la migration cellulaire (Matsuda et al. 2004).

Des études subséquentes seront nécessaires pour déterminer si le cytosquelette de K8/18 peut réguler l'endocytose de la claudine-1 et ainsi favoriser la motilité et l'invasion cellulaire. Étant donné que le cytosquelette de FIs n'est pas connu pour interagir avec les jonctions serrées, il serait important de procéder à une analyse exhaustive de l'expression et de la localisation des autres composants de ces jonctions afin de déterminer si l'absence de kératines affecte spécifiquement la claudine-1.

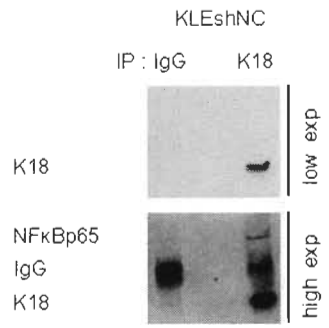

Figure 4.5 **Co-immunoprécipitation du facteur de transcription NF-κB avec la K18.**
IP IgG : immunoprécipitation d'immunoglobulines (contrôle isotypique), IP K18 : immunoprécipitation de la K18, exp : exposure. (Résultats non publiés).

4.3 Les kératines participent à la spécificité fonctionnelle des isoformes d'Akt

Considérant l'importance de la spécificité fonctionnelle des isoformes d'Akt dans la progression tumorale (section 1.4), nous avons analysé l'expression et l'activation de chacun d'eux dans les cellules déficientes en K8/18. Notre étude a révélé que le phénotype invasif est induit par l'activation des isoformes Akt1 et Akt3, alors que l'expression ou l'activation d'Akt2 est diminuée dans les cellules sans K8/18 (Fortier et al. 2013). D'ailleurs, le fait qu'Akt2 soit diminué pourrait expliquer l'absence

d'augmentation de la vimentine dans les cellules invasives étant donné son rôle dans l'expression de cette protéine de FIs (Fortier et al. 2010). Ainsi, les kératines freinent le potentiel invasif des cellules épithéliales cancéreuses en régulant de façon distincte les isoformes d'Akt. Toutefois, la compréhension des mécanismes moléculaires sous-jacents nécessitent une investigation plus approfondie. Nous avons montré que l'activité de la PI3K est augmentée dans les cellules sans K8/18 par un mécanisme dépendent de la claudine-1 (Fortier et al. 2013). Étant donné que nos résultats d'immunoprécipitation n'ont démontré aucune association directe entre la claudine-1 et la PI3K, il serait intéressant d'investiguer le rôle de la claudine-1 dans la densité membranaire des RTK, dont l'activation permet le recrutement de la PI3K au niveau de la membrane. En effet, nous avons démontré dans cette étude que la claudine-1 régule la densité membranaire des récepteurs de mort cellulaire Fas, ce qui nous autorise à penser qu'elle puisse aussi influencer les RTK. De plus, la localisation membranaire des isoformes Akt1 et Akt3 est particulièrement favorisée dans les cellules déficientes en K8/18, contrairement à Akt2. Bien que la claudine-1 soit impliquée dans ce processus, nos résultats actuels ne nous permettent pas d'identifier les mécanismes responsables de cette spécificité. Le recrutement des kinases Akt à la membrane plasmique nécessite la liaison du domaine PH d'Akt avec les phosphoinositides membranaires. La localisation membranaire spécifique des isoformes Akt1 et Akt3 pourrait s'expliquer par la grande homologie de séquence du domaine PH entre ces deux isoformes. L'utilisation de kinases Akt tronquées de différentes régions du domaine PH pourrait permettre de déterminer l'existence d'un site de liaison aux phosphoinositides membranaires commun chez Akt1 et Akt3 mais absent de la séquence protéique d'Akt2.

Bien que l'absence de kératines favorise l'activation des isoformes Akt1 et Akt3, les cellules déficientes en K8/18 sont plus sensibles au cisplatine (Fortier et al. 2013). Nous avons démontré que cette sensibilité à la drogue est associée à une augmentation de la densité membranaire des récepteurs Fas qui est régulée par la claudine-1. Toutefois, nous avons aussi observé que l'exposition au cisplatine entraîne une diminution des niveaux protéiques de l'inhibiteur d'apoptose XIAP. Il a déjà été démontré que l'isoforme Akt2 induit une résistance des cellules cancéreuses au

cisplatine en augmentant les niveaux d'expression protéique de XIAP (Fraser et al. 2003). Cela suggère que la diminution de l'expression ou de l'activation d'Akt2, observée dans les cellules sans K8/18, puisse être associée à l'induction de l'apoptose par le cisplatine. La réexpression ectopique d'Akt2 dans ces cellules permettrait d'investiguer le rôle spécifique de cet isoforme dans la régulation de XIAP et la réponse au cisplatine.

Nos observations concernant les rôles spécifiques des isoformes d'Akt sont en accord avec une récente étude effectuée dans notre laboratoire démontrant que la seule présence des isoformes Akt1 et Akt3 suffit à augmenter la motilité cellulaire alors qu'Akt2 est davantage impliqué dans la résistance au cisplatine (Girouard et al. 2013).

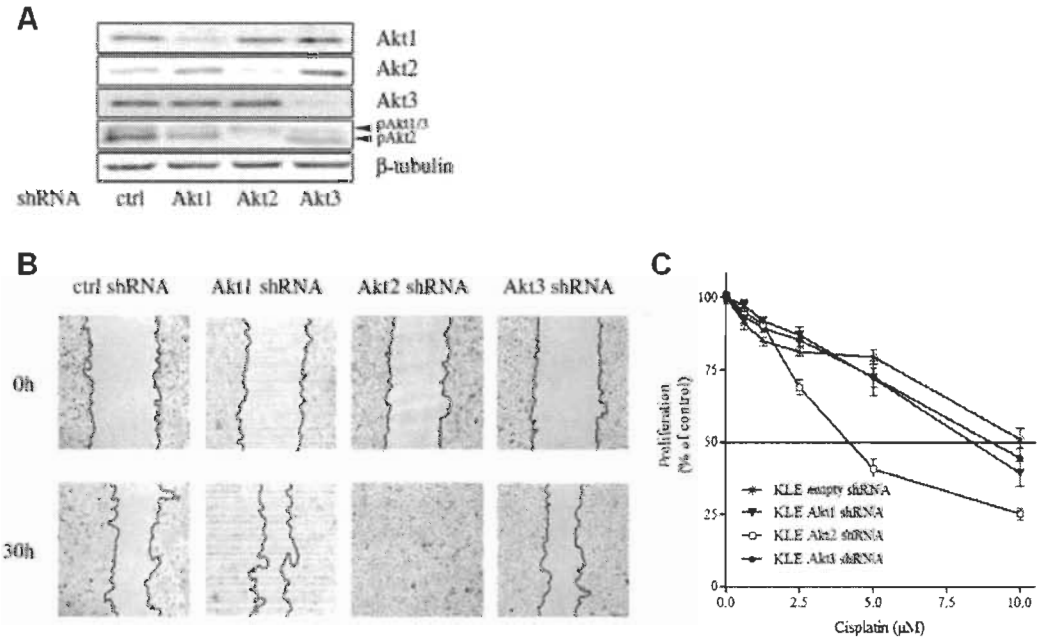

Figure 4.6 **Rôles des isoformes d'Akt dans la motilité et la réponse au cisplatine des cellules KLE.**
Les cellules KLE déficientes en Akt2 (Akt2 shRNA), mais qui expriment toujours les isoformes Akt1 et Akt3 (*A*), sont les plus motiles (*B*) et les plus sensibles (*C*) (Girouard et al. 2013).

4.4 Les cellules épithéliales cancéreuses peuvent devenir invasives suite à la perte des K8/18 sans subir une EMT

Notre étude a révélé que les marqueurs de l'EMT ne sont pas modulés dans les cellules épithéliales cancéreuses suite à la perte des K8/18 (Fortier et al. 2013). Or, le facteur de transcription NF-κB, qui est particulièrement actif dans les cellules déficientes en kératines, est bien connu pour induire la transcription de nombreux gènes favorisant l'EMT dont Snail (Julien et al. 2007). Des analyses préliminaires montrent que l'expression de Snail n'est pas augmentée chez les cellules déficientes kératines. En dépit de la présence accrue du facteur NF-κB au noyau des cellules déficientes en K8/18, les niveaux de la protéine Snail diminuent légèrement (Figure 4.7).

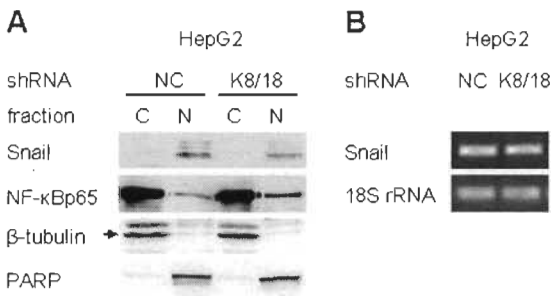

Figure 4.7 **L'absence des K8/18 n'augmente pas les niveaux d'expression de Snail.**
A, Fraction cytosolique (C) et nucléaire (N) des cellules HepG2 contrôles (shRNA NC) et sans kératines (shRNA K8/18). *B*, Niveaux d'ARNm détectés par PCR. shRNA NC : contrôle négatif de l'interférence à l'ARN, shRNA K8/18 : interférence à l'ARN des kératines 8 et 18, C : fraction cytosolique, N : fraction nucléaire. (Résultats non publiés).

Il a été récemment démontré qu'Akt2 interagit avec le facteur de transcription Snail pour inhiber l'expression de l'E-cadhérine et induire l'EMT (Villagrasa et al. 2012). La diminution de l'expression ou de l'activation d'Akt2 dans les cellules déficientes en K8/18 pourrait limiter l'activité de Snail dans l'induction de l'EMT. De façon similaire, la contribution d'Akt2 pourrait être nécessaire à l'augmentation de la vimentine par le facteur NF-κB (Fortier et al. 2010), autre caractéristique importante de l'EMT.

Notre étude nous a donc permis d'élaborer un modèle selon lequel l'absence des kératines favorise la migration collective des cellules cancéreuses par un mécanisme

polarisé et indépendant de l'EMT (Figure 4.8). La perte des K8/18 augmente l'expression de la claudine-1 via l'activité de NF-κB et favorise sa localisation nucléaire. La claudine-1 permet aux cellules cancéreuses situées au front de migration d'envahir la MEC par l'activation des isoformes Akt1 et Akt3 et renforce la cohésion entre les cellules cancéreuses qui suivent à l'arrière.

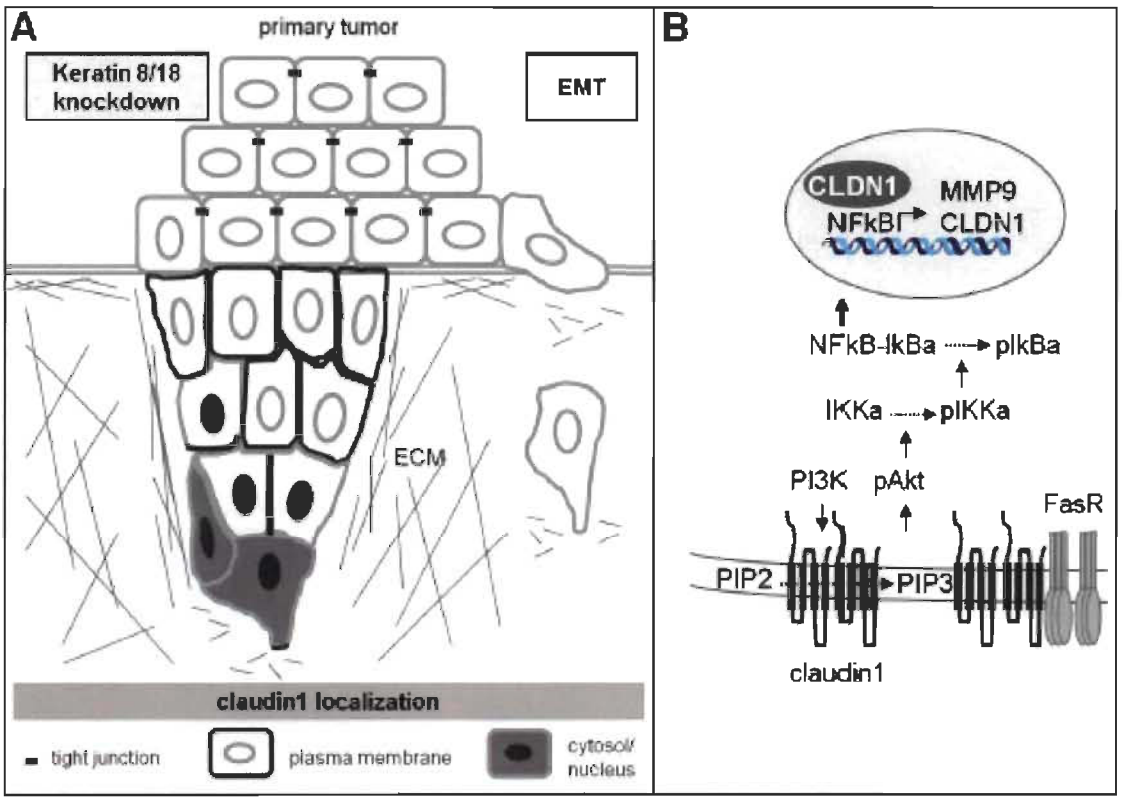

Figure 4.8 **Modèle hypothétique du processus de migration collective induit par l'absence du cytosquelette de kératines.**
(Fortier et al. 2013).

Ce processus alternatif d'invasion cellulaire peut avoir un impact majeur sur l'efficacité de la détection des CTC dans le diagnostic et le pronostic de cancer. Les tests de détection des CTC reposent principalement sur l'expression des kératines dans le sérum. Cependant, notre étude a démontré que les cellules cancéreuses peuvent devenir invasives suite à la perte de l'expression des kératines. Ces résultats soutiennent donc que l'utilisation des kératines en tant que marqueurs pour la dissémination des cellules cancéreuses sous-estime leur présence dans la circulation sanguine.

Un autre aspect du phénotype induit par l'absence des K8/18 confirme sa disparité avec l'EMT : la perte des kératines sensibilise les cellules au cisplatine. Or, L'EMT est généralement associée à la chimiorésistance des cellules tumorales (Singh et al. 2010). Nos résultats sont en accord avec la fonction fondamentale des kératines de maintenir l'intégrité de l'épithélium (chapitre I). De plus, nous avons démontré que la claudine-1 favorise la localisation membranaire du récepteur Fas et contribue ainsi à l'activation de l'apoptose par la voie extrinsèque en réponse au cisplatine (Fortier et al. 2013).

4.5 Le maintien ou la réexpression des kératines : une stratégie thérapeutique pour empêcher la progression tumorale

Le maintien du phénotype épithélial des cellules tumorales constitue une approche thérapeutique intéressante pour diminuer les risques de récidives du cancer. Les travaux de cette thèse ont pour objectif de déterminer le rôle des kératines dans l'EMT médiée par les oncogènes Akt, afin d'évaluer leur potentiel thérapeutique dans la sauvegarde du phénotype épithélial. Nos travaux démontrent que la perte des K8/18 facilite la migration collective et augmente la sensibilité des cellules épithéliales cancéreuses, mais n'est pas suffisante à l'induction d'une EMT. Par conséquent, la préservation ou la réexpression des kératines par les cellules tumorales peut réprimer leur potentiel invasif, mais risque aussi de les rendre chimiorésistantes.

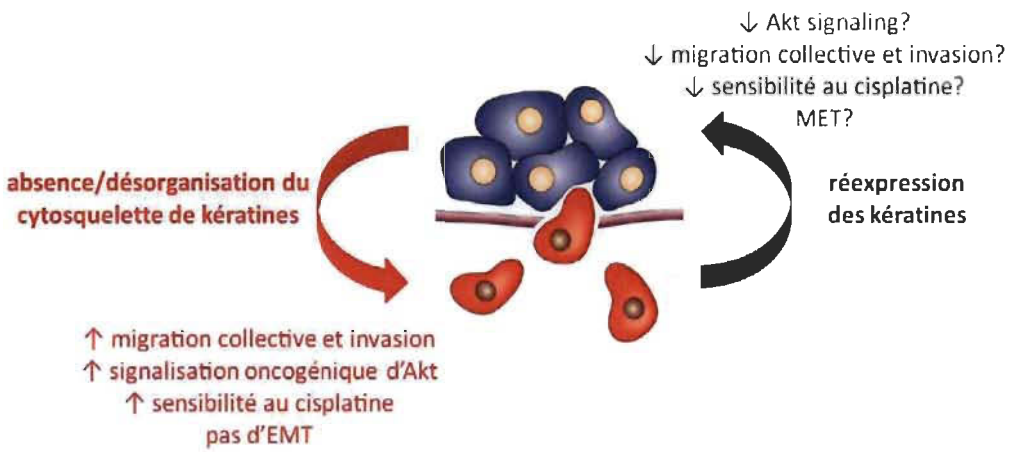

Figure 4.9 **La réexpression des kératines par les cellules tumorales peut réprimer leur potentiel invasif mais risque aussi de les rendre chimiorésistantes.**
Adapté de (Thiery 2002).

Nos résultats démontrent aussi que le phénotype épithélial n'est pas altéré en absence des kératines, suggérant que l'EMT est un processus indépendant de l'expression des kératines. Des études subséquentes seront nécessaires afin de déterminer si la réexpression des kératines pourrait renverser une EMT induite par des facteurs de transcription tels que Snail. De plus, bien que certaines cellules cancéreuses aient conservé leur phénotype épithélial, on ne peut exclure la possibilité que les altérations oncogéniques présentes dans ces cellules puissent influencer l'EMT. L'utilisation de cellules non-tumorigéniques exprimant ou non les K8/18 permettrait d'investiguer le rôle des kératines dans l'EMT induite de façon contrôlée, soit par la surexpression des isoformes d'Akt, du facteur de transcription Snail, ou par l'exposition prolongée des cellules au TGF-β.

De plus, l'étude du rôle des kératines dans le potentiel métastatique des cellules épithéliales cancéreuses nécessiterait une étude *in vivo*. Notre modèle expérimental *in vitro* a révélé que l'absence des kératines favorise d'une part la migration collective des cellules cancéreuses adhérées sur un support rigide (verre ou plastique) et, d'autre part, leur capacité à envahir une matrice gélatineuse reconstituant la membrane basale de l'épithélium. Ces processus constituent les premières étapes de la formation des métastases. Cependant, une fois la membrane basale franchie, les cellules cancéreuses doivent entrer dans la circulation sanguine et survivre à différents stress tels que l'anoïkis et les défenses du système immunitaire inné (section 1.1.2 du chapitre I). L'injection chez la souris de cellules épithéliales cancéreuses exprimant ou non les K8/18 permettrait de démontrer le rôle des kératines dans la formation de métastases. Deux approches expérimentales sont couramment utilisées. L'injection de cellules cancéreuses dans la circulation systémique (veine de la queue de l'animal) entraîne dans la majorité des cas la formation de tumeurs au niveau des poumons, reproduisant ainsi le cheminement intravasculaire des cellules jusqu'à l'organe distant. L'injection orthotopique de cellules cancéreuses (au niveau du tissu d'origine de la tumeur) induit la formation d'une tumeur primaire et de ses métastases subséquentes. Ce modèle est davantage fidèle au processus métastatique chez l'humain (Khanna et al. 2005). De plus, ces modèles de transplantation de tumeurs offrent une grande flexibilité pour les

stratégies d'imagerie actuelles. Les cellules tumorales peuvent être génétiquement modifiées pour devenir fluorescentes ou luminescentes, permettant ainsi d'étudier l'évolution de ces cellules cancéreuses dans l'animal entier.

L'objectif de cette thèse est de déterminer le rôle des kératines dans la transformation maligne des cellules épithéliales cancéreuses en réponse aux oncogènes Akt, afin d'évaluer leur potentiel thérapeutique dans l'inhibition de l'EMT. Nous avons démontré l'existence d'une relation isoforme-spécifique entre la signalisation oncogénique des isoformes d'Akt et le cytosquelette de filaments intermédiaires. Nous avons aussi révélé un rôle pour les kératines 8 et 18 dans la répression du potentiel invasif et la chimiorésistance des cellules épithéliales cancéreuses via la modulation d'une protéine de jonction cellulaire, la claudine-1. De plus, nous avons démontré pour la première fois que le facteur de transcription NF-κB, important médiateur de l'invasion cellulaire, est un activateur direct de la transcription de la claudine-1. Toutefois, l'absence du cytosquelette de K8/18 ne modifie pas le phénotype épithélial et n'induit pas d'EMT, révélant l'existence d'un processus de migration collective indépendant de l'EMT. Enfin, cette étude a permis de mieux comprendre les mécanismes moléculaires régulés par le cytosquelette de FIs dans la signalisation oncogénique des isoformes d'Akt. Désormais, les kératines ne devront pas être seulement considérées comme des marqueurs de l'épithélium, mais aussi comme des régulateurs de la progression tumorale.

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ANNEXE A

FUNCTIONAL SPECIFICITY OF AKT ISOFORMS IN CANCER PROGRESSION

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Review

Functional specificity of Akt isoforms in cancer progression

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Abstract

Akt/PKB kinases are central mediators of cell homeostasis. There are three highly homologous Akt isoforms, Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ. Hyperactivation of Akt signaling is a key node in the progression of a variety of human cancer, by modulating tumor growth, chemoresistance and cancer cell migration, invasion and metastasis. It is now clear that, to understand the mechanisms on how Akt affects specific cancer cells, it is necessary to consider the relative importance of each of the three Akt isoforms in the altered cells. Akt1 is involved in tumor growth, cancer cell invasion and chemoresistance and is the predominant altered isoform found in various carcinomas. Akt2 is related to cancer cell invasion, metastasis and survival more than tumor induction. Most of the Akt2 alterations are observed in breast, ovarian, pancreatic and colorectal carcinomas. As Akt3 expression is limited to some tissues, its implication in tumor growth and resistance to drugs mostly occurs in melanomas, gliomas and some breast carcinomas. To explain how Akt isoforms can play different or even opposed roles, three mechanisms have been proposed: tissue-specificity expression/activation of Akt isoforms, distinct effect on same substrate as well as specific localization through the cytoskeleton network. It is becoming clear that to develop an effective anticancer Akt inhibitor drug, it is necessary to target the specific Akt isoform which promotes the progression of the specific tumor.

Keywords: Akt/PKB isoforms; chemoresistance; intermediate filaments cytoskeleton; invasion; tumor growth.

Introduction

Cancer development results from the acquired capacity of normal cells to escape from their tightly regulated tissue-specific microenvironment signaling pathway and proliferate without control. The serine-threonine kinase Akt or protein kinase B (Akt/PkB) family is a member of the oncogene families involved in tumorigenesis. The Akt signaling pathway plays a central role in cell fate by linking together normal cellular physiological processes such as glucose metabolism, cell growth, protein synthesis, cell cycle control, cell survival and apoptosis, cell migration and angiogenesis. As a fundamental mediator of these crucial signaling cascades, alterations in Akt activity, by a gain of function, results in cellular perturbations that are the hallmark of cancer [reviewed in (1–3)].

Akt represent a family of three proteins that are encoded by distinct genes: Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ located, respectively, on chromosomes 14, 19 and 1 in human. Akt isoforms are highly related homologous proteins (4) (Figure 1). Following receptor tyrosine kinase activation, phospatidylinositol-3 kinase (PI3K) phosphorylates phosphoinositides (PtdIns) at the 3-position of the inositol ring, generating PtdIns3P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (PIP3), which specifically associates with the pleckstrin homology (PH) domains of several proteins, including Akt. Recruitment of Akt to the membrane by PIP3 allows the full activation of the kinase through its phosphorylation on threonine residue in the catalytic domain (T308 on Akt1, T309 on Akt2 and T305 on Akt3) by 3-phosphoinositide-dependent kinase 1 (PDK1) (5) and on serine residue in the C-terminal domain (S473 on Akt1, S474 on Akt2 and S472 on Akt3) by both autophosphorylation and phosphorylation by other kinases, such as PDK2, mammalian target of the rapamycin complex 2 (mTORc2), integrin-linked kinase (ILK) and protein kinase C (5–10). In a later step, active Akt is translocated, through an unknown mechanism, to different subcellular compartments such as mitochondria and nucleus where its substrates are located. This process and the production of PIP3 are negatively regulated by the lipid protein phosphatase tensin homolog deleted on chromosome 10 (PTEN) (Figure 1).

Many studies completed so far have examined Akt by using a general approach, without focusing on functional specificity of each isoforms. It is now clear that to understand the mechanisms on how Akt affects specific cancer cells, it is necessary to consider the relative importance of each of the three Akt isoforms in the altered cells. The specific physiological functions of the individual Akt isoforms were well demonstrated on studying mice deficient in Akt1, Akt2 or Akt3. All three phenotypes were viable but presented specific deficiencies. Disruption of Akt1 showed growth retardation with proportional decrease in the sizes of all organs, increased apoptosis and neonatal mortality (11–13).

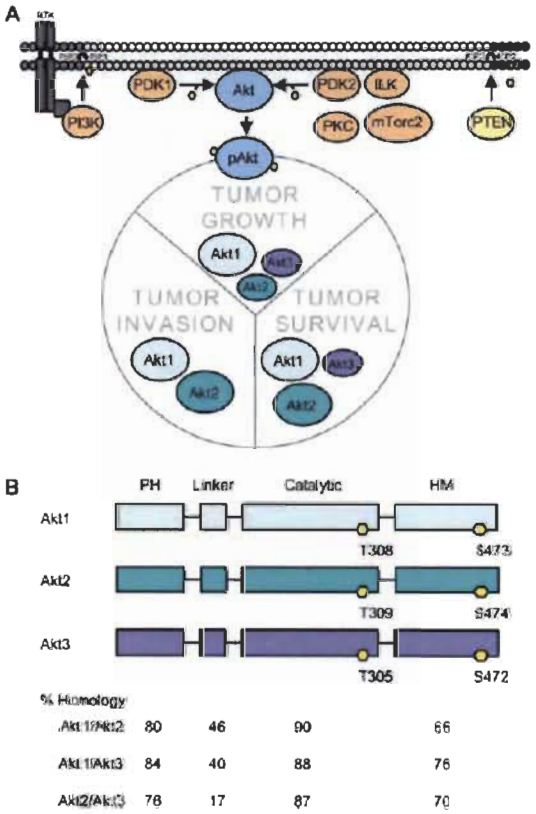

Figure 1 (A) Stimulation of receptor tyrosine kinase (RTK) recruits phosphatidylinositol-3 kinase (PI3K) to the cell membrane to phosphorylate phosphoinositide diphosphates (PIP2) to phosphoinositide triphosphates (PIP3), a process that is reversed by the phosphatase and tensin homolog (PTEN). PIP3 allows the recruitment of Akt to the cell membrane where it is phosphorylated on the threonine 308 (T308) by 3-phosphoinositide-dependent kinase 1 (PDK1) and on the serine 473 (S473) by other kinases such as 3-phosphoinositide-dependent kinase 2 (PDK2), integrin-linked kinase (ILK), protein kinase C (PKC) and mammalian target of the rapamycin complex 2 (mTORc2). After full activation, Akt translocates to cytosol and subcellular compartments to phosphorylate different substrates which are involved in tumor growth, cancer cell invasion and chemoresistance. Importance of each Akt isoforms in these processes is represented by the size of the draw. (B) Akt isoforms sequence composed of a conserved N-terminal pleckstrin homology (PH) domain, a linker domain, a central catalytic domain and a C-terminal regulatory hydrophobic motif (HM) domain, with percentage of homology and specific phosphorylation sites.

Akt2 knockout mice exhibited a diabetes-like syndrome with insulin resistance and, depending on the genetic background, mild growth retardation (14, 15). The absence of Akt3 in mice led to a selective decrease in brain size, without increased perinatal mortality, growth retardation or altered glucose metabolism (16, 17). The relatively subtle phenotypes of mice lacking individual Akt isoforms as well as the viability of the animals suggested that for many functions, Akt isoforms are able to compensate for each other. To address the issue of isoform redundancy, mice with combined Akt deficiencies have been generated. Mice lacking both Akt1 and Akt2 isoforms died shortly after birth, and the newborns displayed impaired skin and bone development, severe skeletal muscle atrophy and reduced adipogenesis (18). Akt1/Akt3 double-knockout induced embryonic lethality from severe developmental defects in the cardiovascular and nervous systems (19). Interestingly, mice lacking Akt2 and Akt3 were viable despite reduced body size and impaired glucose metabolism (20). The viability of this phenotype suggests that Akt1 isoform is sufficient to perform all essential Akt functions in postnatal survival.

In the present review, we will highlight the current knowledge about the roles of the different Akt isoforms in tumor growth, chemoresistance and cancer cell migration, invasion and metastasis. For instance, from an analysis of the literature, there is a trend showing that Akt isoforms specificity in definite cancer could be explained by differential relative expression/activation of all isoforms, specific substrates and subcellular distribution. There is increasing evidence that the distinct localization of Akt isoforms is controlled by their interaction with the cytoskeleton network. In accordance with others, our findings also suggest that the cytoskeleton could play a role as a regulatory element by sequestering and liberating Akt proteins depending on the cellular context.

Alterations of Akt isoforms activities in tumor

Increasing evidence suggests that hyperactivation of Akt signaling plays a central role in a variety of human cancers. Most tissue expresses the three Akt isoforms but the level of their expression is variable. For instance, Akt1 level is generally higher than Akt2 except in insulin-responsive tissues including liver, skeletal muscle and adipose tissue (21, 22). Akt3 is usually expressed at the lowest level especially in intestinal and muscle tissue. Its highest levels are found in brain, testes, lungs and kidneys (13, 23). Multiple reports describe an increased or constitutive activity of one of the three Akt isoforms in different cancers (Figure 2A). Indeed, approximately 40% of breast and ovarian cancers, more than 50% of prostate carcinomas and approximately 20% of gastric adenocarcinomas exhibited increased Akt1 expression and kinase activity (24). Elevated kinase activity of Akt2 has been demonstrated in 25% of primary breast carcinomas (25) and in approximately 30% of ovarian cancer specimens (26), the majority of cases being late-stage tumors. A similar ratio of pancreatic carcinomas showed markedly elevated levels of Akt2 activity compared with normal and benign pancreatic tumors (27). Elevated expression of Akt2 protein was reported in nearly 40% of hepatocellular carcinomas (28). Akt3 is the predominant active Akt isoform in approximately 60% of advanced-stage melanomas compared with melanocytes or early-stage lesions that have relatively low levels of Akt activity (29). Increased Akt3 activity has also been reported in estrogen receptor-deficient breast cancer and

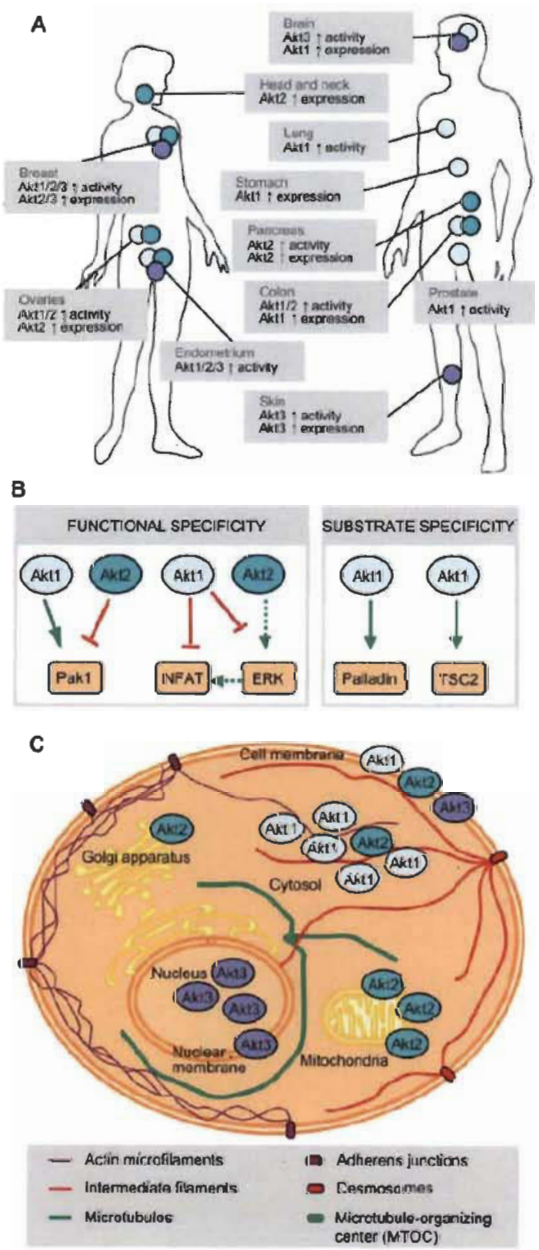

Figure 2 Mechanisms involved in the functional specificity of Akt isoforms during carcinoma progression.
(A) Tissue-specific alterations of Akt isoform expression and activity in different types of carcinomas. (B) Highlight of functional specificity of Akt isoforms on common substrates and distinct interaction of Akt isoforms with substrates. (C) Schematic representation of specific subcellular localization of Akt isoforms.

androgen-insensitive prostate cancer cell lines (30). Gliomas, bowel tumors, lung cancer and the majority of primary human lymphomas are also linked to Akt hyperactivity (31). Interestingly, Akt isoforms constitutive activation appears to be associated with tumor progression rather than initiation. For instance, in different types of tumors, Akt2 kinase activity and/or expression level are increased with the pathological grade of malignancy (25–27, 32). The same correlation is observed for Akt3 in advanced-stage of metastatic melanomas (29).

The causes of Akt isoforms hyperactivation in tumors are multiple. Constitutive phosphorylation of Akt in cancers can be explained by gene mutation, amplification and/or mRNA overexpression. However, the most common aberrant Akt activation occurs through numerous mechanisms that affect elements upstream of Akt. These include amplification of oncogenes such as PI3K and Ras, loss of tumor suppressor genes such as PTEN, NF1 (neurofibromin) and threonine kinase LKB1, and aberrant activation or overexpression of growth factor receptors such as the epidermal growth factor receptor (2).

Many cancers arise from mutations in regulatory proteins that confer growth advantage. Although Akt proteins are key regulatory proteins in the pathogenesis of many cancers, mutations of Akt genes are rarely observed in human cancers (33). To date, one mutation on Akt1 has been described (34). This mutation results in a substitution of a glutamic acid to a lysine at amino acid 17 (E17K) in the PH domain of Akt1. This mutation activates Akt1, by favoring its binding to the plasma membrane stimulating its activation. Even though the E17K mutation activates Akt1 pathway, and has been reported to be responsible for the development of various cancers (breast, colorectal, ovarian, lung, endometrial cancer, leukemia and retinoblastoma) (34–38), the E17K mutation is rare and accounts for a very small percentage of increased activation of Akt1 signaling pathway in cancer. As observed for Akt1, mutations on Akt2 and Akt3 are also rare (39–41). In conclusion, to date, mutations of Akt genes are not considered as a major event in cancer development and other mechanisms need to be taken into account to explain Akt misregulation in cancer.

Analysis of multiple tumor types has indicated that amplification of Akt genes and increase Akt mRNA expression occurs only in a small number of cancer cells and is not a frequent event in tumorigenesis. Indeed, Akt1 gene amplification was initially detected in only a single over five gastric carcinomas and one over 255 cases of different human tumors (42). Moreover, a recent investigation revealed amplification and overexpression of Akt1 in a single case of glioSarcoma in a series of 103 cases (43). Amplification of Akt2 is relatively more frequent than amplification of other isoforms and was found in a subset of 16 over 32 ovarian, 7 over 72 pancreatic and 3 over 106 breast carcinomas (44–46). Little has been reported on Akt3 amplification except for a recent study that clearly demonstrated using fluorescence *in situ* hybridization that Akt3 was amplified in a significant subset of estrogen receptor-positive breast carcinomas, whereas Akt1 and Akt2 were shown to be more likely to be deleted (47). Overexpression of Akt3 mRNA has also been shown to be increased in hormone-independent breast and prostate carcinomas (30). In melanomas, elevated Akt3

signaling occurs as a result of a combination of increased Akt3 gene copy number and PTEN loss (29).

Akt isoforms in tumor growth

Akt isoforms are important regulators of cell proliferation. For instance, although mutant mice lacking any one of the three Akt isoforms presented minor deficits (11–17), double mutants Akt1$^{-/-}$ Akt2$^{-/-}$ or Akt1$^{-/-}$ Akt3$^{-/-}$ and Akt2$^{-/-}$ Akt3$^{-/-}$ presented growth defects (18–20). Interestingly, the different mutants showed organ-specific deficiency (18–20). The analysis of these mice clearly demonstrated that *in vivo*, in a normal physiological situation, Akt isoforms are involved in cell proliferation and that their role is isoform-specific and tissue-specific.

In several recent studies, researchers have attempted to dissect the mechanisms by which specific isoforms of Akt are regulating cell proliferation in different cell types. For instance, the analysis of mouse embryo fibroblasts lacking Akt1 showed that the absence of Akt1 reduced cell proliferation and caused a delay in G1/S transition by decreasing accumulation of cyclin D1 (48). Embryonic fibroblasts lacking Akt2 showed normal proliferation and G1/S transition (48). The importance of Akt1 PH domain in regulating Akt1 effect on cell proliferation was shown by swapping Akt1 PH domain with that of Akt2. This substitution had a similar effect as Akt1 deletion. Finally, normal regulation of cell proliferation and G1/S transition time was recovered by reintroducing in the Akt1-deficient cells Akt1 or a chimeric protein containing the PH domain of Akt1 (48). Thus, the PH domain of Akt1 provides full kinase activity and is necessary for the G1/S transition. Moreover, this set of experiments demonstrated that Akt isoforms do not have identical function in cells. Downregulation of Akt1 by small interfering RNA (siRNA) in an ovarian cancer cell line has also been shown to be sufficient to interfere with cell proliferation. In these cells, downregulation of Akt1 was shown to affect cell cycle regulatory proteins (49). For instance, the reduction in the amount of Akt1 induced a decrease in the expression of cyclin D1, cyclin-dependant kinase 4, cyclin E, p21 and in phosphorylated retinoblastoma, and in this way reduced cell proliferation (49). The effect was similar to that of inhibiting PI3K by siRNA (49). However, the specificity of Akt1 isoform inhibition on abrogation of cell cycle in ovarian cancer cells is not that clear because downregulation of Akt2 was shown by other researchers to have a similar effect (50). The use of stable knockdown (49) instead of transient siRNA transfections (50) could explain the discrepancy between the two studies. Akt1 was shown to be required for anchorage-independent cell growth in a subset of cancer cell types: colon cancer cell line (HCT-15), lung carcinoma cell line (H460), pancreatic cancer cell line (MiaPaCa-2), cervix adenocarcinoma cell line (HeLa) and a fibrosarcoma cell line (HT1080) (51).

Adding to the complexity in understanding the role of individual Akt isoforms in tumor growth, the regulation of cell growth in cancer cells could involve two Akt isoforms. For instance, Ericson et al. (52) demonstrated that both Akt1 and Akt2 were required for *in vitro* and *in vivo* growth of human colon cancer cell lines HCT116 and DLD1. They showed that knockout of either Akt1 or Akt2 in these cells had minimum effects on cell growth or downstream signaling, whereas double-knockout (Akt1 and Akt2) resulted in markedly reduced proliferation *in vitro* when growth factors were limiting. Indeed, the role of Akt1 and Akt2 in tumor growth was evident only in more challenging microenvironments, when cells were triggered by starvation or in experimental metastasis in mice (52). Thus, this particular set of experiments shows that increases in activation of Akt1 together with Akt2 provide cells with a growth advantage that is necessary for tumor growth. The synergistic effect of Akt isoforms has also been observed in different malignant glioma cell lines. In these cells, the knockdown of Akt2 and Akt3 resulted in a stronger cell growth inhibition than knockdown of individual isoforms (32). These results suggest that Akt2 and Akt3 isoforms can play redundant functions in the regulation of cell growth.

Akt3-specific activity in cell growth has been less studied than Akt1 and Akt2. However, Akt3 is the most active Akt isoform in 70% of cases of advance-stage melanoma. The reduction in these cells of Akt3, but not Akt2, activity is done by using siRNA diminished anchorage-independent cell growth (53) and increased apoptosis in tumors growing in nude mice. Thus, Akt3 activity can be linked to cellular growth related to tumorigenicity in advance-stage melanoma (29, 53). As for melanoma, the elevated Akt3 activity that has been reported in estrogen receptor-deficient breast cancer suggests that Akt3 could contribute to the aggressiveness of breast carcinomas. In the hormone-responsive breast cancer cell line MCF7, expression of active Akt3 abolishes the hormone-responsiveness and the MCF7-Akt3 cells grow in an estrogen-independent manner. Moreover, Akt3-expressing cells were found to produce tumors in mice in the absence of estradiol (54).

In conclusion, there is accumulating evidence that the relative importance of Akt isoforms in cell growth is cell line-specific (55). Although in some cases the predominant active isoform is the one which is involved in promoting cell growth, it was also demonstrated that Akt isoforms could compensate for others in signal transduction. Thus, only combined knockdown led to a reduction in the phosphorylation level of the substrates and cell survival. Therefore, to obtain the most specific effect on tumor cell growth inhibition, it is necessary to predetermine which Akt isoform is the most responsible for tumor cell growth and inhibit this specific isoform. The importance of selecting the proper Akt isoform to inhibit cell growth is exemplified by a study from Maroulakou et al. (56), who showed that inhibition of Akt1 and Akt2 can have opposite effects on tumors. For instance, using a mouse breast cancer model, Maroulakou et al. showed that the ablation of Akt1 or Akt2 inhibited or accelerated PyMT- and Neu-driven tumor formation, respectively (56). An antisense oligonucleotide against Akt1 which shows growth inhibitory activity in several cancer cell lines and inhibits the tumor formation in two different xenograft mice models is currently used in clinical trials (57).

Akt isoforms in chemoresistance

Deregulations in the apoptotic and/or survival pathways are responsible for resistance to chemotherapeutic agent-induced apoptosis. Akt is well known to play a major role in cell survival, a function shared by all three Akt isoforms.

Akt1 forced expression in human lung cancer cells was sufficient to render the cells resistant to cisplatin. Similarly, Akt1 inhibition by its dominant negative mutant (58) or by siRNA targeting Akt1 (59) reversed the cisplatin-resistant phenotype through the inhibition of mTOR signaling and the MEK/ERK pathway, respectively. Analysis of Akt2 and Akt3 mRNA levels showed that they were not induced in cisplatin-resistant human non-small-cell lung cancer (NSCLC) cells A549 (58). Moreover, Akt2 siRNA or Akt3 siRNA transfection only marginally affected cisplatin-induced NSCLC cell death compared with cisplatin treatment alone (59). These results imply that Akt1 plays an essential role in cell survival and chemosensitivity in lung cancer cells. However, in uterine cancer cells, overexpression of constitutively active Akt1 isoform failed to protect cells from cisplatin-induced apoptosis, but was sufficient to decrease taxol-induced apoptosis (60). Therefore, simultaneous overexpression of multiple Akt isoforms is necessary to confer resistance to cisplatin in these cells (61).

Akt2 could confer cancer cells resistance to paclitaxel. In fact, Akt2 has been shown to mediate the resistance to paclitaxel of the breast cancer cells overexpressing the prosurvival transcription factor Twist. Knockdown of Akt2 significantly reduced the prosurvival effect of Twist on MCF7-Twist cells treated with paclitaxel (62). Akt2 activity can also counteract docetaxel-induced apoptosis in ovarian and breast cancer cells by regulating survivin levels (63, 64). The inhibitor of apoptosis proteins (IAPs) such as survivin and XIAP block the execution phase of apoptosis (65). Their expression in cancer cells is associated with resistance to various pro-apoptotic agents such as TGF-β (66) and TNF-α (67), TRAIL (68, 69) and to chemotherapeutic drugs (60, 69–71). Some studies have shown that Akt and particularly Akt2 regulate IAP levels in cancer cells (72, 73). For instance, expression of a constitutively active Akt2 prevented cisplatin-mediated downregulation of XIAP and apoptosis in A2780 ovarian cancer cells (74). Indeed, Dan and coworkers (75) demonstrated that Akt1 and Akt2 phosphorylate XIAP and protect it from ubiquitination and degradation in response to cisplatin. XIAP downregulation by siRNA largely abrogated constitutively active Akt2 protection of the cells from cisplatin-induced apoptosis (75). Moreover, Akt2 has been shown to render A2780 ovarian cancer cells resistant to cisplatin-induced apoptosis through regulation of the ASK1/JNK/p38 pathway (76). In fact, Akt2 inhibits JNK/p38 and Bax activation through phosphorylation of ASK1 and thus plays an important role in chemoresistance.

Akt3 can also affect resistance to chemotherapeutic agents. For instance, in melanoma cell lines, reduced Akt3 protein expression by siRNA increased cleaved caspase-3 levels following staurosporine exposure (53). Elevated Akt3 activity has been reported in estrogen receptor-deficient breast cancer tumors. A model of estrogen-responsive MCF7 cells expressing Akt3 has demonstrated a role for this isoform in estrogen receptor function. Indeed, the MCF7-Akt3 cells were found to be resistant to tamoxifen-induced cell death (54).

Akt isoforms have a distinct impact on cellular resistance to a given drug and Akt activity does not confer equal resistance to the different chemotherapeutic agents. Regarding the regulation of cell growth, studies demonstrate that in some cases the expression pattern of Akt isoforms could indicate which isoform is responsible for drug resistance. Chemoresistance is a multifactorial phenomenon which implies several signaling pathways downstream of Akt activation, which could account for Akt isoform-specific roles in chemoresistance.

Akt isoforms in cell migration, invasion and metastasis

Akt isoforms have been shown to play a role in cell migration, invasion and metastasis of many cancer cells. Regarding growth control, a specific Akt isoform can have different effects on these cellular processes in different cell types. Cell migration and invasion are generally regulated by activating proteins involved in the modulation of actin cytoskeleton organization, extracellular matrix (ECM) degradation, cellular interaction with the ECM, expression of proinvasive genes and establishment of cellular polarity.

Akt1 as an enhancer of cell invasion

In fibroblasts and endothelial cells, Akt1 has repeatedly been found to promote invasion (77–79). The differences between the effect of Akt1 and Akt2 activity in fibroblast migration was highlighted by Zhou et al. (78) who reported that Akt2 knockout mouse embryo fibroblasts migrated through ECM faster than wild-type cells. Moreover, Akt1 knockout cells migrated slower than wild-type cells. Consistently, Akt2 knockout cells had elevated levels of activated p21-activated kinase (Pak1) and Rac. These two proteins are implicated in a molecular cascade that leads to the rearrangement of actin cytoskeleton, the regulation of lamellipodium extension dynamic, as a cell spreading in response to adhesion. The inhibitory effect of Akt2 on migration was confirmed by showing that although both Akt2 and Akt1 formed a complex with Pak1, only Akt2 inhibited Pak1 (80). This suggests the existence of a different underlying mechanism that gives rise to the two isoform-specific phenotypes. Phosphorylation of the actin binding protein girdin by Akt1 also enhances fibroblast migration by promoting actin stress fibers and lamellipodia formation (81). Taken together, these results indicate that Akt1 and Akt2 play opposite roles in the regulation of Rac/Pak signaling in fibroblasts (80). Akt1 has also been shown to promote invasion of fibrosarcoma cell line HT1080 (82). In this case, increased cell migration was associated with ECM degradation through metalloproteinase-9 (MMP-9) production, in a manner that was highly dependent on Akt1 kinase activity and membrane-translocation ability. The increase in MMP-9 production was mediated by

Akt1 activation of nuclear factor-κB transcriptional activity. However, Akt1 did not affect the cell-cell or cell-matrix adhesion properties of HT1080 (82). Although it is well established that Akt1 promote fibroblasts and endothelial cell migration, some studies have shown that Akt1 can also induce motility in epithelial cancer cells. Regarding fibroblast cell lines, Akt1 activation in mouse mammary epithelial cells enhances MMP-2 activity, thereby increasing invasion potential (83). Moreover, Akt1 deficiency in Neu/Her2/erbB2-induced mammary epithelial cancer (MEC) cells was found to reduce lung metastases. The *in vivo* proinvasive role of Akt1 was mediated through the tumor suppressor tuberous sclerosis complex 2 (TSC2) phosphorylation (84). Indeed, Akt1 mediated MEC migration through paracrine signaling via induction of promigratory membrane-anchored chemokine CXCL16 and chemoattractant MIP1γ. Thus, Akt1 governs invasion of breast cancer originally induced by Neu/Her2/erbB2 oncogene by controlling cell polarity, migration and directionality. Similarly, Akt1 downregulation by siRNA in ovarian cancer cell lines is sufficient to reduce cell migration and invasion (49).

Akt2 as an enhancer of cell invasion

Although there is clear evidence that Akt1 stimulates migration in some cancer cells, Akt2 also stimulates migration in other cell types. Even though Akt1 governs migration of some breast and ovarian cancer cells, a trend seems to emerge from *in vitro* studies that Akt1 generally stimulates migration and invasion of fibroblasts and endothelial cells, whereas Akt2 promotes the same phenomenon in epithelial cells. Interestingly, in cells where one isoform stimulates motility, the other isoform usually has a limited or even opposite role.

The Toker group has shown that active Akt1 limits breast cancer cell migration. Indeed, Akt1 decreased the promigratory activity of the transcription factor NFAT (85) and actin remodeling by its association with Akt1-phosphorylated palladin (86). In a similar way, silencing Akt1 expression increased migration and epithelial-mesenchymal transition (EMT) of the breast MCF10A cells overexpressing IGF-I receptor via the ERK pathway. Interestingly, downregulation of Akt2 suppressed the EMT-like morphological conversion induced by Akt1 downregulation and inhibited migration in EGF-stimulated cells (87). Consistent with these findings, some studies have shown that reduction of Akt2 expression by siRNA inhibited EGF-induced chemotaxis of MDA-MB-231, T47D and MCF7 breast cancer cells (88) and glioblastoma cells (89). Expression of a wild-type Akt2 in Akt2 siRNA-transfected cells rescued migration properties of the cells. Indeed, Akt2-depleted MDA-MB-231 cells showed a marked reduction in metastasis to mouse lungs, demonstrating the biological relevancy of Akt2 activity in cancer metastasis *in vivo* (88). In glioma cells, Akt2 phosphorylates girdin and ACAP1 which promotes integrin recycling and increases MMP-9 expression (89). Regulation of girdin by Akt1 and subsequent lamellipodia formation has already been shown to occur in fibroblast migration (81). Different roles for Akt isoforms are also observed in A549 lung cancer cells. For instance, Akt2 siRNA remarkably downregulated migration and invasion, whereas Akt1 and Akt3 siRNA had little effect on these cellular events (90). Using an opposite approach where ectopic expression of Akt isoforms was achieved, it was demonstrated that in human breast and ovarian cancer cells Akt2, but not Akt1 or Akt3, increased migration and invasion by upregulating β1-integrins and subsequently rendering the cells more metastatic (91). Consistent with these results, Cheng et al. observed that Twist-mediated induction of Akt2 expression, but not Akt1, promoted migration/invasion of breast cancer cells (62). Likewise, ILK preferentially mediated phosphorylation of Akt2 which induced mTOR activation that resulted in MMP-2 expression and promoted endothelial cell wound-induced or chemotactic migration (92). Suppression of Akt2 expression in highly metastatic colorectal carcinoma cells inhibited their ability to metastasize in an experimental liver metastasis model. Akt1 overexpression did not restore metastatic potential of colorectal carcinoma cells with downregulated Akt2, suggesting nonredundant roles for both isoforms in these cells (93). Another study explained the Akt1 inhibiting role in cell motility by the phosphorylation of TSC2 and its targeting for degradation (94). For instance, proteasome-dependent degradation of TSC2 in mammary epithelial cell lines leads to reduced Rho-GTPase activity, decreases actin stress fibers and focal adhesions, and reduces motility and invasion (94). By contrast, *in vivo* experiments demonstrated that Akt1-induced phosphorylation of TSC2 leads to its activation rather than its degradation. This promoted cell migration through the signaling cascade of Rho-GTPase/actin remodeling (84). Discrepancy between *in vitro* and *in vivo* roles of Akt1 in cell migration and invasion could be due to a TSC2 stabilization mechanism that prevents its ubiquitin-mediated degradation (95, 96). Iliopoulos et al. (97) showed that the opposing roles of Akt1 and Akt2 on the migratory and invasive phenotype in breast cancer cell lines and primary tumors were due to the differential effects of Akt1 and Akt2 on the abundance of the tumor suppressor miR-200 microRNA family. Thus, a decreased ratio of Akt1 to Akt2 is crucial for the downregulation of microRNA gene expression and the induction of invasiveness (97). Maroulakou et al. (56) reported the specificity of Akt isoforms by focusing on the effects of their ablation on mice mammary oncogenesis driven by polyoma virus middle T antigen (PyMT) and Neu/Her2/erbB2 oncogene (56). Whereas $Akt1^{-/-}$PyMT and $Akt2^{-/-}$PyMT tumors were both invasive, $Akt1^{-/-}$Neu-induced tumors exhibited the highest degree of invasiveness. However, invasiveness did not always correlate with metastasis in that $Akt1^{-/-}$PyMT and $Akt2^{-/-}$PyMT tumors were significantly less metastatic than wild-type PyMT tumors. The Muller group (98) has reported that crossing MMTV/activated Akt1 transgenic mice with an activated Neu strain resulted in fewer metastatic lesions than the activated Neu strain alone. A subsequent study showed an increase in the incidence of pulmonary metastases following coexpression of Akt2 with the activated oncogene Neu in the mammary glands of transgenic mice. Thus, Akt1 and Akt2 might have opposite roles in tumor metastasis formation induced by the oncogene Neu (99).

How Akt isoforms could play opposite roles in cell migration?

It is now clear that Akt isoforms could play different roles in migration, invasion and metastasis. The different properties of the three highly homologous Akt isoforms could be related to tissue-specific expression/activation of all isoforms, distinct effectors or substrate specificity and/or differential compartmentalization and localization of the isoforms (Figure 2).

The relative abundance or activation of Akt isoforms could be dynamic and change depending on different cellular contexts. For instance, Akt3 is the predominant active isoform in the majority of advanced-stage melanomas and thus is involved in tumor growth, survival and metastasis (29, 53). Also, the invasive or metastatic potential of breast cancer cell lines could depend not on the expression and activity of Akt but on the balance between Akt1 and Akt2 and their specific downstream signaling (85, 97).

Moreover, the specificities of closely related Akt isoforms in cell invasion and metastasis involve their interaction with distinct effectors, such as TSC2/RhoGTPase (84, 94), NFAT (85) and palladin (86), which are specifically related to Akt1 isoform activity. Despite common preferred substrate motifs, Akt isoforms could have different impacts on the same substrate. For instance, Akt1 activates Pak1, whereas Akt2 inhibits its kinase activity (80).

Together with the two precedent mechanisms, the distinct subcellular localization of Akt isoforms could explain their differential behavior. Santi and Lee (100) have demonstrated, by doing an exhaustive immunocytochemistry analyses in a large range of cell types, that each Akt isoform is present, in most cases, at a unique subcellular location. According to the currently accepted model, activation of Akt is mediated by its recruitment and phosphorylation at the plasma membrane, in response to PI3K activation. Akt activation is followed by its release in the cytosol to accomplish its kinase function. As expected, active Akt1 and Akt2 were mainly localized in the cytoplasm. However, Akt2 showed some cluster staining around the nucleus, which colocalized with mitochondria and to a lesser extent with the Golgi apparatus. Akt3 mainly localized in the nucleus and nuclear membrane. Moreover, because mitochondrial Akt2 and nuclear Akt3 were activated, it is possible that Akt2 and Akt3 did not need to reach the plasma membrane to be phosphorylated (100). The formation of a stable fluorescent complex of Akt1 and PDK1 fused proteins demonstrated that Akt1 T308 phosphorylation was independent of PtdIns (101). With regard to the common functions mediated by Akt isoforms, Santi and Lee asked the question whether Akt isoform localization could be altered by the ablation of another one. Single as well as double isoform knockdown did not alter the subcellular localization of the remaining isoform. These data are consistent with the concept that all three Akt isoforms have their own unique functions that are not shared by other isoforms.

Akt isoforms moving through cytoskeleton

There is increasing evidence that the cytoskeleton network could modulate the localization of Akt isoforms. There are three types of cytoskeletal filamentous structures: actin microfilaments, microtubules and intermediate filaments (IFs). The cytoskeletal components constitute an extensive filamentous network which interacts with the different cellular organelles, adhesion complexes and non-cytoskeletal proteins (e.g., kinases, phosphatases, etc.) and participates to maintain cellular homeostasis by organizing the cytoplasm of the cell. A wide variety of cellular processes including movement of mitochondria, cell adhesion and cell migration are dependent on the dynamic of the cytoskeleton. Akt has been shown to associate with actin, tubulin and IFs (102). Indeed, it was shown in cultured human HaCaT keratinocytes that Akt was sequestered by the IF protein keratin 10, which impaired its translocation to the cell membrane and its activation. The authors reasoned that keratin 10 could directly inhibit cell proliferation via sequestration of Akt (103). We also observed that phosphorylated Akt colocalized

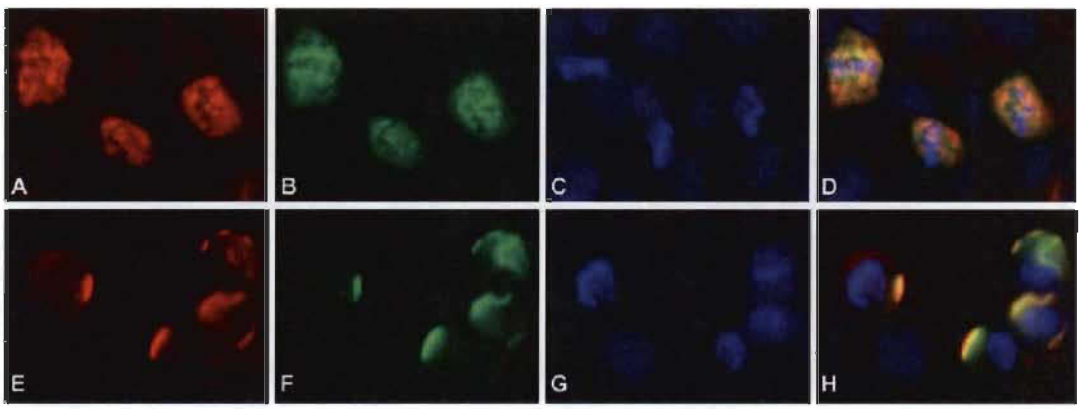

Figure 3 Colocalization of phosphorylated Akt with cytoskeletal intermediate filaments.
Immunofluorescence staining of keratin 18 (A, E), phosphorylated Akt (T308) (B, F), nuclei with Hoechst dye (C, G) and merge (D, H) in hepatocellular carcinoma HepG2 cells during mitosis (A–D) and upon toxic stress (E–H).

with keratin IFs in HepG2 cells during mitosis and upon toxic stress (Figure 3) (104). In the context of cell migration, keratin IFs which are linked to the β4 integrin has been shown to be necessary to convey signals from the β4 integrin to Akt (105). Thus, the cytoskeletal network could act as a scaffolding platform to convey signals up to Akt kinases. Other studies have demonstrated a similar role for another IF protein, vimentin, in the sequestration of cell migration mediators (106). RhoA-binding kinase α, implicated in the reorganization of actin filaments, has been shown to be associated with the vimentin IF network in HeLa cells. Upon vimentin IF collapse during the formation of actin stress fibers and focal adhesion complexes, RhoA-binding kinase α translocate to the cell periphery (107). In the same cells, we demonstrated that Akt2 overexpression induced a perinuclear collapse of vimentin network which was associated with an increase in cell invasion (108). Other studies have reported associations between vimentin perinuclear collapse and enhanced cell migration and invasion (109–111) and proposed that vimentin collapse contributed to the mechanisms of cell movement by the liberation of vimentin IF-sequestered proteins. All these studies support the hypothesis that the cytoskeleton plays a role in the regulation and the localization of Akt isoforms by sequestering or acting as a scaffold for the kinase and in this way modulates downstream signaling involved in cell migration and invasion.

Highlights

We have summarized recent advances in the functional specificity of Akt isoforms in cancer progression. This review highlights the need for a better understanding of tumor expression profiles of Akt isoforms, substrate specificity and interaction partners or scaffolds. In some cases, the most active isoform is the one being considered as the most susceptible to be involved in promoting tumor growth, chemoresistance and migration, invasion and metastasis. However, in other cases, Akt isoforms could compensate for others in signaling. Thus, targeting multiple isoforms might be necessary to inhibit cancer progression. It is crucial to determine which isoform(s) is(are) accountable for each tumor to develop the proper treatment. The non-universality of the Akt signaling pathway is an emerging topic in the development of specific drug targeting in cancer research.

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ANNEXE B

SIMPLE EPITHELIAL KERATINS K8 AND K18 : FROM STRUCTURAL TO REGULATORY PROTEIN

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Chapter 1

SIMPLE EPITHELIAL KERATINS K8 AND K18: FROM STRUCTURAL TO REGULATORY PROTEIN

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ABSTRACT

K8 and K18 (K8/18) are the major heteropolymeric intermediate filaments (IFs) present in simple layer epithelia. In hepatocytes, keratin filaments form an extensive cytoplasmic network that is denser at the cell periphery and around bile canaliculi. Keratin filaments are attached to the plasma membrane via desmosomes. K8/18 IFs have long been linked to human chronic liver diseases. In fact, modifications in keratin IFs network organization and the formation, in hepatocytes, of K8/18 containing aggregates, named Mallory-Denk bodies (MDBs), are characteristic of alcoholic and non-alcoholic steatohepatitis, copper metabolism diseases such as Wilson disease and Indian childhood cirrhosis and hepatocellular carcinoma. The formation of MDBs is the consequence of an increase of K8/18 mRNA and proteins, alterations in K8/18 post-translational modifications such as phosphorylation on multiple sites, transglutaminase

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mediated keratin crosslinking and a defect in K8/18 degradation by ubiquitin-proteasome pathway. The use of transgenic mouse models has allowed unravelling the significance of these changes in K8/18 dynamic in hepatocytes and revealed a function for keratins in protecting hepatocytes against mechanical and non-mechanical stresses. For instance, mice expressing an ectopic human K14, a mutated human K18 (Arg89→Cys) or K18 (Ser52→Ala) or K18-Gly(-). K8 (Ser73→Ala) or K8 deficient mice are more susceptible to various mechanical and toxic injuries. The recent observation of the existence of K8 and K18 mutations in cases of cryptogenic and non-cryptogenic forms or human liver disease is in total agreement with a role for keratin in maintaining cellular integrity under various threatening conditions. In a pursuit for understanding the molecular mechanism by which keratins could accomplish their protective role in cells, researchers have investigated the relationship between K8/18 and different regulatory pathways. There is now evidence that keratins are involved in signalling pathways regulating apoptosis, cell growth, and motility of various simple epithelial cells. It is noteworthy that expression of keratins is maintained during malignant transformation of simple epithelial cells. The PI3K/Akt pathway plays a pivotal role in apoptosis, cell growth, and motility and over-expression of the active form of Akt1 and Akt2 increase K8/18 protein levels suggesting that IFs are involved in PI3K/Akt pathway. The direct binding of K8 to Akt1 suggests that K8/18 IFs might provide a scaffold for Akt1 in cells. K8/18 interacts directly with other molecules involved in the apoptotic signalling pathway such as tumor necrosis factor receptor (TNFR), TNFR1-associated death domain protein (TRADD), Mrj co-chaperon, with Hsp/c70 and caspase 3. K8/18 also binds to key regulatory proteins of mitosis and cell proliferation such as 14-3-3, Cdc25 and Raf-1 kinase. Therefore keratins which were considered as only structural in the early 90s are now considered as key regulatory elements in modulating multiple signalling pathways.

INTRODUCTION

Intermediate filaments (IFs) are with microtubules and actin microfilaments the major cytoskeletal components of most mammalian cells. They also constitute an important part of the nucleoskeleton through the nuclear lamina. However, contrary to microtubules and actin microfilaments, which have known specific functions, IFs functions as a whole are still not fully understood. Even though more needs to be known about microtubules and microfilaments some of their functions are well documented in comparison to what we understand of IFs functions. For instance, microtubules are directly involved in intracellular transport of vesicles, granules, organelles like mitochondria, and chromosomes, while actin microfilaments are known to play a role, in establishment and

maintenance of cell membrane organization, cell junctions and cell shape, in cell motility and muscular contractions [1].

IF proteins, encoded by 70 genes in the human genome [2-4], all present similar structures composed of a central α-helical core domain flanked by variable extensible head and tail regions [5]. This large family of proteins has been divided into six groups according to their sequences similarities, gene structure and tissue distribution [6-8]. For instance, type I and II are respectively the acidic and neutral basic keratins expressed in epithelial cells; type III is composed of vimentin, desmin, and GFAP respectively expressed in mesenchymal, muscular and glial cells; type IV includes the nervous system associated nestin and synemin and the neurofilaments (NF-L, -M, and –H); type V, the lamins (A, B and C) constitute an important part of the nuclear skeleton; finally, type VI comprises filensin and phakinin expressed in the eye lens, tanabin expressed in the growth cones of embryonic vertebrate neurons, transitin and nestin are present in myogenic and neurogenic cells, and synemin is found in undifferentiated and mature muscle cells of mammals [9]. As Professor Traub wrote in 1995: 'The expression of a large number of cell type-specific and developmentally regulated subunit proteins is believed to provide multicellular organisms with different IF systems capable of differential interactions with the various substructures and components of their multiple, differentiated cells'[10]. This statement has served to justify a large number of proposals for having grant money to pursue studies on IFs.

Present in epithelial cells, keratins of type I and type II are expressed by 54 different genes and represent the largest family of IF proteins [11]. The two keratin types are coordinately expressed in pairs in various epithelia [12-14] and this differential gene expression is linked to epithelial cell lineage and differentiation (e.g. K18 and 14 of type I interact respectively with type II, K8 and K5 in liver and epidermal basal keratinocytes) [15]. More than two keratins can associate to form a filament but the ratio of type I and type II is obligatory 1:1. Type I and type II keratins form obligate heterodimers (i.e. at least one type I and one type II keratins) that assemble through a coiled coil interaction of the central α-helical rod domain of the proteins which are flanked by the largely variable flexible N- and C-terminus. Because of their variability from one protein to the other, the variable N- and C-terminus which are subjected to post-translational modifications such as phosphorylation and glycosylation are assumed to play a central role in regulating the functions of specific keratin combinations. The 10-12 nm filaments form by the association of the rod domains and gather to form a complex network in cells (Figure 1) [7, 16-18]. It is important to note that IFs and keratin IFs were for a long time considered to be relatively stable structures with

little turnover activity [19]. This interpretation was due to the apparent stability of the IFs when isolated. This myth slowed down the beginning of the study on keratin assembly and disassembly which was initiated by Professors R.D. Goldman and E. Fuchs [20-22].

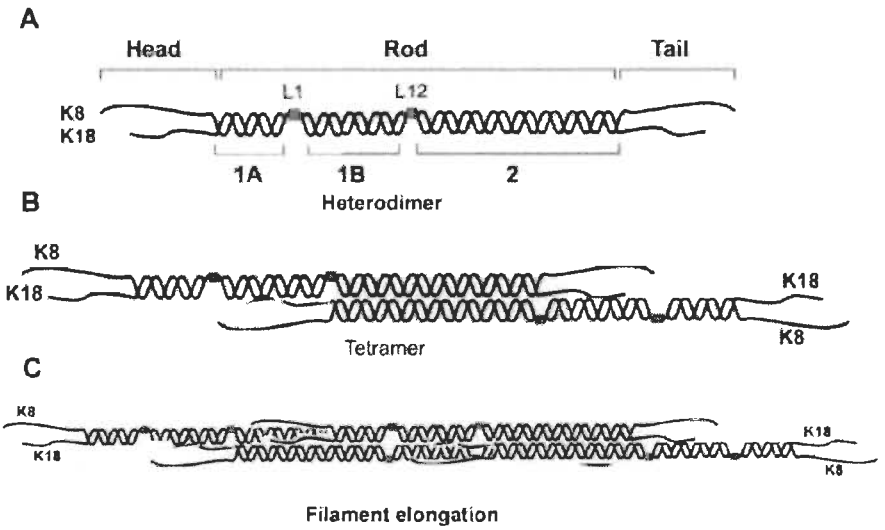

Figure 1. Schematic representation of K8/18 filaments formation. A) K8/18 heterodimers showing the non-covalent interaction of the alpha helical rod domain interrupted by linker 1 and linker 2. The head and the tail are non-alpha helical most variable and flexible domain of keratins. B) Lateral association of heterodimer form the 10-12 nm filaments. C) Elongation occurs by the association of the 1A domain of a tetramer with the coil 2 domain of a second tetramer. The head and the tail of keratins are subjected to post-translational modifications such as phosphorylation and glycosylation [23-25].

The aim of the present chapter is to review the progress that has been made over the recent years in our understanding of simple epithelial keratins. The chapter will be divided into three major sections: 1) study of liver K8/18 in health and disease, 2) K8/18 mutation and liver disease 3) involvement of K8/18 in major cellular signalling pathways.

1. Liver K8/18 in Health and Disease

In single layer epithelia (simple epithelia) present in digestive organs, K8 is expressed with different levels of K7, K18, K19 and K20 depending on the cell type and the organ (Table 1).

Table 1. Keratin expression in digestive organs

Organ	Epithelial cell type	Keratins
Liver	Hepatocytes	K8/K18
	Hepatobiliary ductal cells	K7/K8/K18/K19/K20 [26, 27]
	Oval cells	K7/K8/K18/K19 [26, 27]
Pancreas	Acinar cells	K7/K8/K18/K19/K20 [27] (cancer K23) [28]
	Ductal cells	K7/K8/K18/K19/K20 [27]
Intestine	Enterocytes small intestine	K8/K18/K19/K20 [27]
	Enterocytes colon	K8/K18/K19/K20 [27]
	Goblet cells	K8/K18/K19/K20* [27, 29]
Gallblader		K7/K8/K18/K19 [27]

K20*: presence of multiple phosphorylation
K20: Low level of expression

As depicted in Table 1, hepatocytes are unique among simple epithelia since they express solely K8 /18 in a 1:1 stoichiometric ratio. They have been known to be linked to liver diseases for a long time [30-32]. For instance, alcoholic hyaline bodies described for the first time by Professor F.B. Mallory in 1911 [33] and currently named Mallory Denk bodies (MDBs) [34] have been shown by Professor H. Denk and colleague in 1979 to be associated with modifications in keratin organization [32]. During the same period, Professor S.W. French's team performed biochemical analysis of MDBs to investigate the possibility that MDBs could be composed of IFs [35]. Professor H. Denk and Professor S.W. French are the pioneers in the field of intermediate filaments and MDBs, they have shaped the field and, with their team, they brought major contributions to our current understanding of the composition and mechanism of formation of MDBs.

1.1 Composition of MDBs

MDBs are now recognized to be common to many chronic human liver diseases such as alcoholic and non-alcoholic steatohepatitis, copper metabolism

diseases such as Wilson disease and Indian childhood cirrhosis, and hepatocellular carcinoma (reviewed in [34]). The existence of animal models for MDB formation has facilitated the study of their composition and mechanism of formation. Indeed three models that are chronic feeding of mice with a diet containing griseofulvin (GF) or 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) or dieldrin are well recognized for the induction of MDBs in hepatocytes. The formed MDBs are morphologically and biochemically very similar to human MDBs [34, 36-39]. Different studies have shown that MDBs are composed of native K8/18, post-translationally modified K8 and K18 as well as bile ductular epithelial keratins. They also contain stress proteins and chaperone, kinases and protein related to proteasome system of protein degradation. In non-diseased hepatocytes, K8/18 forms in hepatocytes a complex cytoplasmic network that is denser at the cell membrane (Fig. 2A). Chronic treatment of mice (4 months) with a diet containing 2.5% GF induces the formation in hepatocytes of keratin containing aggregates MDBs (Fig. 2B) [40]. These MDBs are morphologically similar to human MDBs (Fig. 2C). It is important to note that in diseased livers, all hepatocytes do not contain MDBs and that keratin IFs appear normal in hepatocytes in parts of the liver (Fig. 2D).

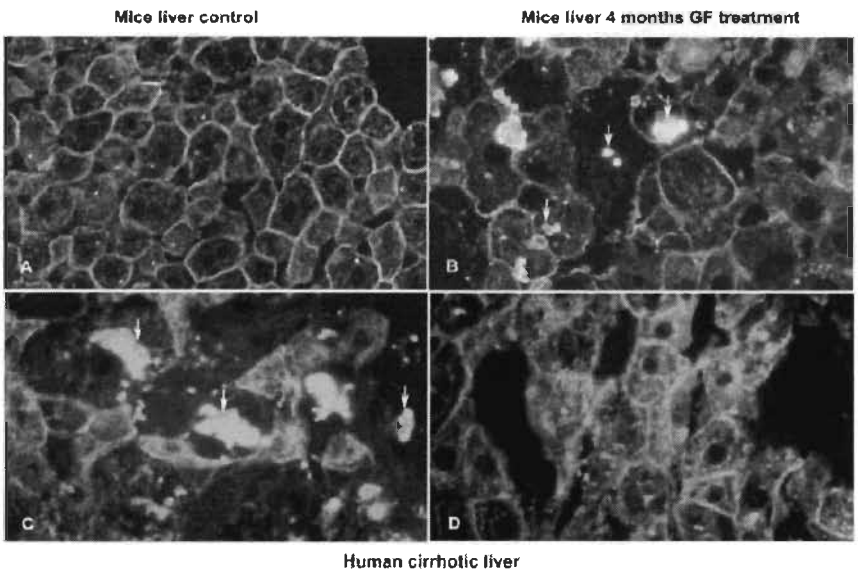

Figure 2. Immunofluorescence staining of keratin IFs in mice and human livers. A and B are respectively liver sections from control and 4 months GF-treated mice. C and D are sections from human cirrhotic liver. Arrows show MDBs. The liver has a heterogeneous response. Note that in D there is no MDB.

The major constituents of MDBs are native K8/18 and post-translationally modified K8/18 by cross-linking and phosphorylation. Early biochemical analysis showed that an important portion of MDBs is insoluble which suggested that MDBs contained cross-linked proteins [41-43]. Further analysis demonstrated that in MDBs some keratins are ubiquitinated or transaminated by tissue transglutaminase giving rise to high molecular weight keratins [44-48]. Related to ubiquitinated keratins and the ubiquitin-proteasome system, valosin-containing protein (VCP), NEDD8 and p62 molecular chaperone, are also present in variable amounts in MDBs [49-51]. Pressure tuning infrared spectroscopy showed the molecular structure of IFs in MDB containing liver to be largely modified [52]. Phosphorylation is one of the most studied keratin post-translational modification and is believed to modulate their function. By using antibodies against specific phosphorylation sites on K8 and K18 (K8 Ser79; K8 Ser436; K18 Ser33), it was shown that hyperphosphorylation of keratins is also an important post-translational modification that affects keratins in MDBs [53-57]. The presence on MDBs of phospho-p38 MAPK and phosphothreonine as detected by immunostaining suggests that these kinases are playing a role in the phosphorylation of keratins present in MDBs [58, 59]. The significance of keratin hyperphosphorylation is not totally understood. K8 Ser79 has been proposed to function as a phosphate sponge for stress activated kinases in the liver undergoing chronic stress [60]. However, this interpretation is difficult to generalize since, while all cells in the livers are subjected to chronic stress, only a small number of cells and MDBs contain K8 pSer79 [57]. Moreover the cells containing K8 pSer79 are in close association with apoptotic cells suggesting that in chronic liver disease K8 pSer79 is related to apoptosis [57]. While the major constituents of MDBs are K8/18, other keratins, proteins such as chaperones stress proteins, proteins related to protein turnover in cells and protein kinases are also present in the aggregates [34]. By immunostaining, K7, K19 and K20, which are not detectable in hepatocytes from normal liver, are sometimes present in MDBs [61-64]. This indicates that chronic diseases related to MDB formation are associated with changes in keratin gene expression toward a pattern of expression similar to biliary tract epithelia.

Non-keratin components of MDBs are proteins from the Heat stress protein family (Hsp). The presence of Hsp70 was first described by Omar et al. in 1990 [65]. Further immunocytochemical analyses confirmed the colocalization of Hsp70, Hsp90 and Hsp25 with MDBs [55, 59, 66]. Moreover, an increase in Hsps expression is observed in livers treated with GF [55]. Since Hsp70 has been shown to directly interact with K8 in normal and stressed cellular conditions [67, 68], one can conclude that Hsp70 tentatively attempts to rescue the

conformational change of K8 in diseased conditions. The same interpretation can be proposed for the other chaperone Hsp proteins. Conversely, other studies have shown a decrease in Hsp70 in MDB formation in DDC livers [69]. There is accumulating data concerning the composition of MDBs. However, since the composition varies depending on the hepatotoxic agent used, more need to be done to determine the relative importance of each component. The aim of the next section is to put some light into the mechanism of MDB formation in hepatocytes.

1.2 Molecular Mechanism of MDB Formation

The formation of MDBs is without a doubt the consequence of chronic-liver stress. In humans, the stress is caused by long term alcohol abuse or copper metabolism dysfunction. In the animal model used for the study of MDBs, chronic feeding of mice with diets containing hepatotoxic agents like GF, DDC or dieldrin induces their formation [32, 34, 36-38]. The existence of these animal models has made possible the study of the changes in cellular and molecular events that precede MDB formation and that could on the long term lead to the development of MDBs. A second approach to studying the molecular mechanism of MDB formation is the analysis of livers during the re-induction of MDB development after a recovery period [34, 70]. Feeding mice with a diet containing GF or DDC for 3 to 4 months (induction of MDB formation) followed by 1 month drug withdrawal (drug-primed mice) and 7 days of drug refeeding induce an increase, in the liver, of MDB formation. The number of MDBs is more numerous than after the 3 to 4 months pre-treatment [70].

Even though researchers are using the same model for their studies, important discrepancies exist in the results and interpretations about the mechanism of MDB formation. One important factor that was neglected for a long time, but is now considered as being crucial for the different analyses, is the genetic background of the animal used [55, 71]. The genetic background component can also explain why some humans can abuse of alcohol for their whole life and develop no liver disease while others after a short period of alcohol abuse develop hepatitis and cirrhosis.

In humans, MDBs are an important cellular sign of a chronic progressive liver injury. This is related in mice to a 3 to 4 month period with drugs such as GF and DDC (Fig 2B) [36]. Changes in K8/18 protein and mRNA expression are observed as early as one day after the initiation of the treatment [40, 72]. Both K8/18 protein and mRNA levels are higher than control value from day one up to the end of treatment i.e. after MDBs have formed in hepatocytes [40, 55, 72]. As

shown in figure 3. double labelling for the detection of keratin IFs by immunofluorescence and K8 mRNA by in situ hybridisation. shows that mostly all hepatocytes present an increased in K8 mRNA content in 2 weeks and 4 months of GF treatment (Fig 3D). The increase in K8/18 mRNA is also observed in MDB containing cells (Fig 3E. F).

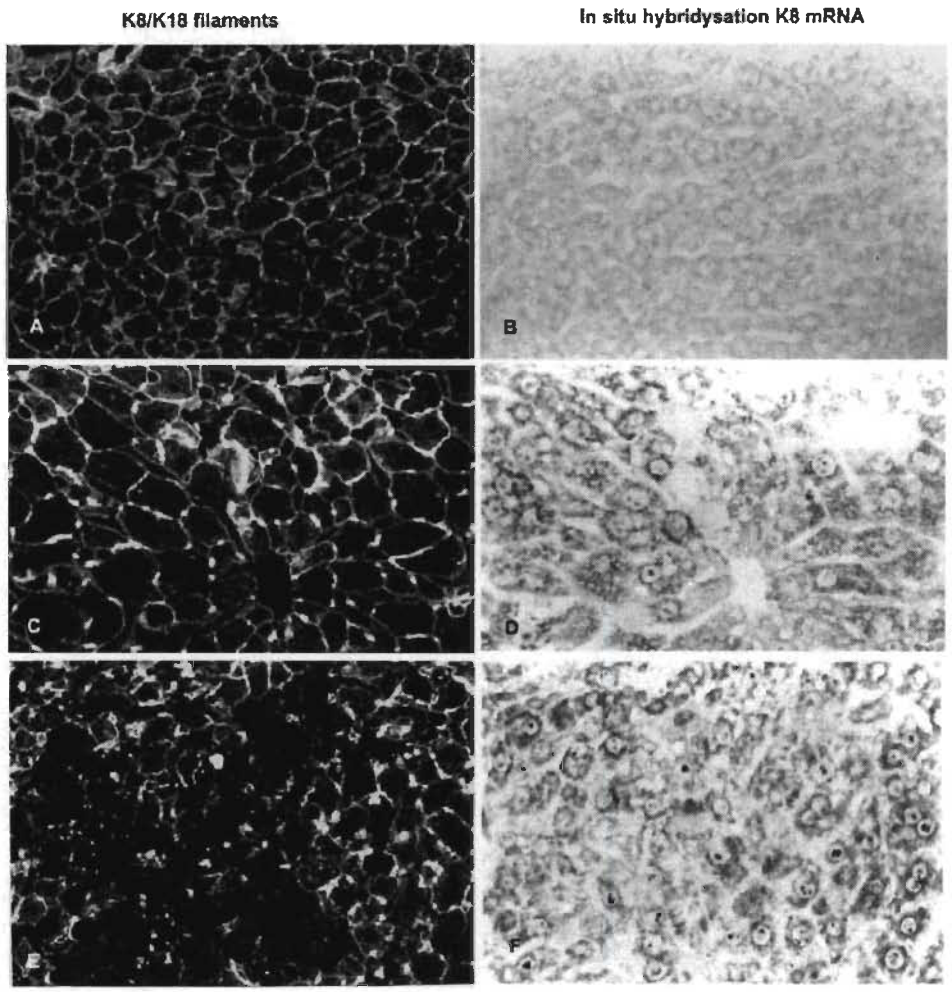

Figure 3. Double staining of keratin IFs (A. C. E) and K8 mRNA (in situ hybridization) (B. D. F) in normal (A. B). 2 weeks GF (C. D) and 4 months GF (E. F). These results show that K8 mRNA level is very low in hepatocytes of control liver and that an increase is observed before the formation of MDBs in hepatocytes (D. F).

By studying the effect of GF treatment of C3H mice liver. our laboratory showed that both keratins K8 and K18 as well as their respective mRNAs

increased in a parallel manner suggesting that the two genes were regulated in a coordinated manner and that the regulation was post-transcriptional [40]. Other authors have suggested that MDB formation depends on an increase in K8/18 protein levels associated with an imbalance between K8/K18 ratio K8 > K18 (reviewed in [34, 38]). The general interpretation is that K8/18 increase expression is necessary for the response of cells to the oxidative stress that is generated by the GF treatment [50, 52]. This interpretation is supported by the results obtained using genetically modified mice that either expressed no K8 ($K8^{-/-}$) no K18 ($K18^{-/-}$) or heterozygous mice $K8^{+/-}$ or $K18^{+/-}$ [73-75]. For instance, FVB/n mice that do not express K8 are extremely sensitive to GF or DDC treatment [76-78] while heterozygous mice are more sensitive than the wild type animal (Table 2). Morphological analysis of the livers from these animals is consistent with the result presented in Table 2. Haematoxylin/eosin staining of the liver following different period of GF treatment shows that the liver of K8 deficient mice are more susceptible to the treatment than K8 heterogeneous and wild type mice (Figure 4). Immunofluorescence staining of IFs and actin shows that livers containing less keratins are more prone to be affected by GF treatment than wild type animals (Figure 5). The extreme sensitivity of K8-null mice livers to GF indicates that the early modifications in keratin organization and dynamics are important phenomena in the response of hepatocytes to GF intoxication and that increases in K8/K18 levels represent a survival reaction. Thus, keratins play an active rather than a passive role in protecting hepatocytes from the GF- and DDC-induced toxic stress [74, 76, 77]. Moreover, the two alleles of the K8 gene are necessary for proper protection of hepatocytes against the stress induced by GF intoxication.

Table 2. Rate of mortality - weeks of GF treatment

Time with GF	FVB/n +/+	FVB/n +/-	FVB/n -/-
1 week	0	0.04	0.4
2 weeks	0	0.05	1
3 weeks	0	0.076	
4 weeks	0	0.4	
5 weeks	0	0.25	
6 weeks	0	0.33	

Mice from each group were given GF for different periods of time. FVB/n K8-/- are significantly more susceptible to GF treatment than FVB/n K8+/-, which are significantly more susceptible to GF treatment than control mice.

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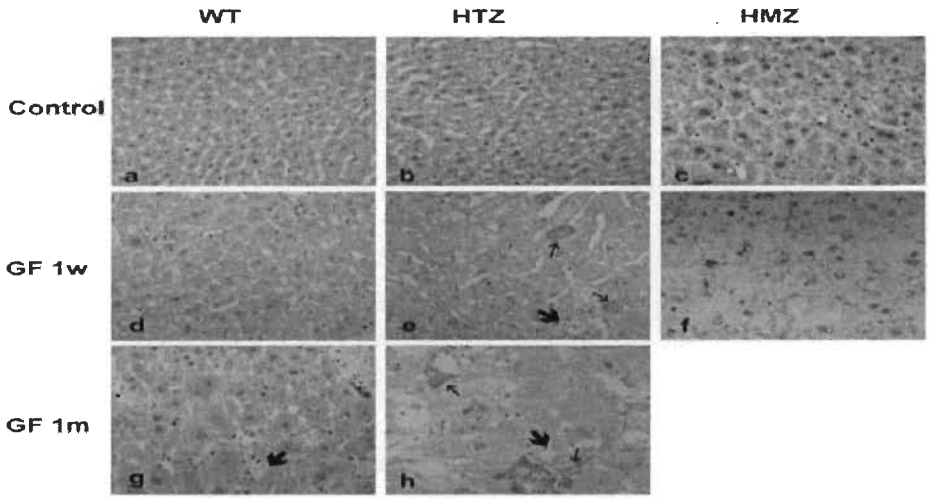

Figure 4. Histology analysis of GF-treated wild-type mice (WT), K8 heterozygous mice (HTZ) and K8-null mice (HMZ) livers. Hematoxylin/eosin staining of WT livers (a.d.g). HTZ livers (b.e.h) and HMZ livers (c.f). Mice were on the control diet (a.b.c) or treated with GF for 7 days (d.e.f) and 28 days (g.h). Large arrows in e and h highlight necrotic cells. Small arrow in g shows bile steatosis. Arrowheads in e, g and h show vacuolation of hepatocytes.

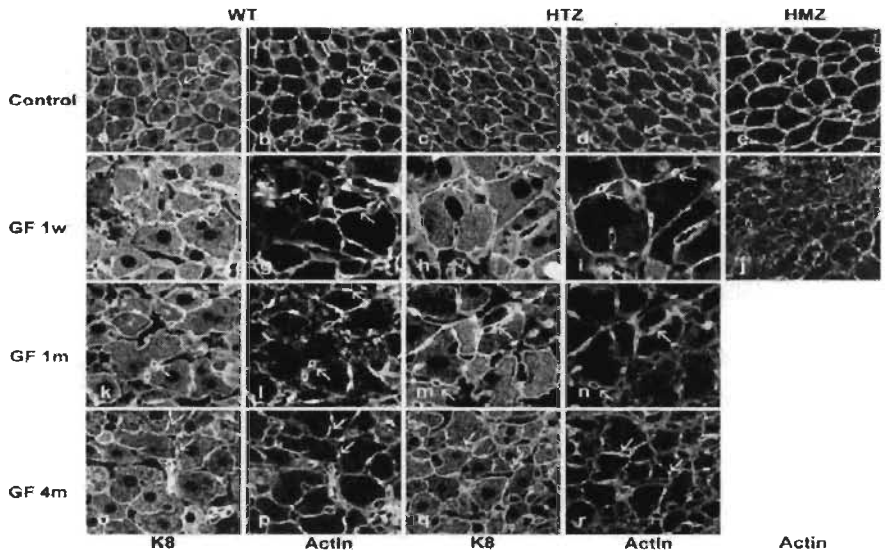

Figure 5. K8 and actin filaments organization in hepatocytes of GF-treated wild-type (WT) and K8-heterozygous (HTZ) mice. Immunofluorescence staining of K8 in WT livers (a, f, k, o) and HTZ livers (c, h, m, q). Mice were on the control diet (a-e) or treated with GF for 1 week (f-j), 1 month (k-n) and 4 months (o-r). Arrows point to bile canaliculi.

Phosphorylation is the major post-translational modification that affects keratins and is increased during the exposition of hepatocyte to oxidative stress. It is noteworthy that keratin phosphorylation sites are located in the head and the tail of the protein (Figure 1). As mentioned above, analysis of K8/18 phosphorylation with specific phospho-epitope antibodies showed that K8 and K18 phosphorylation increased during stress. In a recent study we have shown that the known K8/18 phosphorylation sites (K8 Ser23, K8 Ser73, K8 Ser431, K18 Ser33 and K18 Ser52) could not explain the number of isoforms observed by performing 2D gels on GF-treated livers. For instance, an increase in phosphorylation is observed on K8 Ser73, K8 Ser431 and K18 Ser33. However, K8 showed 2 phospho-isoforms in control and 7 and 6 phospho-epitopes after respectively two weeks and five months of GF treatment [57]. K18 has 2 phospho-isoforms in control liver and 3 in treated livers. The number of isoforms that we detect by 2D gels is compatible with the number predicted (http://www.cbs.dtu.dk/services/NetPhos/). This increase in keratin phosphorylation is an indication that K8/18 phosphorylation correlates with disease progression. In recent studies, using gene-targeted mice mutated on keratin phosphorylation sites, Professor M.B. Omary's laboratory demonstrated that these mice presented remarkably fragile hepatocytes (reviewed in [23]). They proposed that K8 Ser73 serves as a phosphate sponge and in that way protects cells from the numerous stress protein kinases that are activated in diseased cells [60]. This proposed function for phosphorylation is mostly based on K8 Ser73 phosphorylation, which is increased upon stress, and does not take into account the significance of the increased phosphorylation of other sites. We believe that further analysis of keratin phosphorylation sites is necessary to fully understand the significance of these post-translational modifications during the cellular response to stress. As an example, it is possible that phophosite specific combinations be modulated during stress response and creates a cellular environment that protects cells from stress. This interpretation is in agreement with the fact that post-translational modifications occur on the head and the tail of the protein, affect the conformational reorganization of these portions of the filaments and modify their interaction with cellular components involved in cell protection against stress.

The failure of the ubiquitin-proteasome pathway to properly degrade proteins is an important phenomenon in the development of MDBs [79]. Ubiquitination of keratins in a phosphorylation modulated fashion is the normal mechanism for regulating keratin turnover in cells [80]. The presence of UBB^{+1}, a frame shift mutant of ubiquitin that cannot target protein to the proteasome, in GF- or DDC-treated hepatocytes would inhibit the degradation of keratins by the ubiquitin-

proteasome pathway and induce their aggregation [79, 81, 82]. Valosin-containing protein (VCP), a protein related to proteasome inhibition, has been observed to directly bind polyubiquitinated proteins [83] and is present in MDBs [50]. The inhibition of proteasome activity by VCP is most likely playing a role in keratin aggregation and MDB formation in hepatocytes [50]. The defect in Hsps chaperoning capacity could also play a key role in the process of MDBs. The presence of Hsps on MDB suggests that the oxidative stress caused by the toxic agent alters keratin structure [52, 59]. Since Hsps levels increase during the long process of MDB formation, its chaperone capacity might help to maintain keratin structure for a certain period of treatment [69]. However, on the long term the chaperoning capacity may be reduced, leading to misfolded proteins aggregation.

During the last five years, Professor S.W. French and collegues focused their effort to determine the signalling pathways activated in hepatocytes which lead to the development of MDB in hepatocytes (ref 85-95). For this purpose they utilised the primed mice model in which MDB form in 7 days [70]. They postulate that rapid MDB formation in these mice is an epigenetic phenomenon [84]. Analysis of proteasomes in these animals show that the 26S proteasome formation shifts to an immunoproteasome formation, which cannot degrade ubiquitinated keratins. This change would be the consequence of an IFNγ- and TNFα-mediated pro-inflammatory response. Drug toxicity induces these mechanisms and the activation of the signalling pathway can be prevented by methyl donors SAMe and betaine [85-88]. More needs to be done to understand the mechanism of MDB formation and especially to characterize the signalling pathways, which induce the different phenomenon that are observed during the development of MDBs. The understanding of the signalling pathways could serve to develop molecules that could maintain the positive aspect of the hepatocyte response to oxidative stress while ending the processes of liver degeneration.

2. From K8/18 Genetically Modified Mice to Human Liver Disease

The first breakthrough in the search for keratin IFs functions come from studies on transgenic mice lacking or expressing mutated epidermal keratins by Coulombe et al. [89-91]. These mice display blistering skin disease and led to the identification of keratins as the cause of human skin diseases. From these initial studies, it was clear that IFs functions take part of their senses in the context of whole tissues and that their alteration is deleterious. As for epidermal keratins,

the use of gene-targeting has been useful to unravel roles for IFs in hepatocytes. For instance, mice expressing in hepatocytes an ectopic human K14 [92] presented an increased susceptibility to hepatotoxic agent. In an effort to understand the role of site specific function, mice carrying mutated keratin were generated. Mutation on IFs highly conserved site (human K18 Arg89→Cys) [93-95] or phosphorylation sites (K18 Ser52→Ala, K8 Ser73→Ala) [60, 96] all presented increased sensitivity of hepatocytes to mechanical and toxic stress. Increased sensitivity to hepatotoxic agent was also observed in K8 [76, 78, 97] and K18 deficient mice [98]. Importantly, glyscosylation which is another post-translational modification that affect keratin 8 and 18 protect also epithelial tissue from injuries [99].

Analysis of humans for the presence of mutations on K8/K18 has shown that K8 and K18 may present mutations. However, keratin mutations in humans are not found at the beginning or end of the rod domain where epidermal keratin mutations concentrate. The mutated keratins found in humans are not necessarily deleterious for the liver but are overrepresented in human suffering from cryptogenic and non-cryptogenic forms of human liver disease [23, 100-103]. These findings are in agreement with the results deducted from studying K8/18 mice livers and indicate that K8/18 play a significant role in maintaining hepatocytes integrity upon stress in humans. Since mutation at the beginning and end of the rod domain which are hot spot mutation sites in epidermal keratins that such mutations must be lethal. Moreover, absence of K8 or 18 is most likely lethal too. Mutations that would disrupt formation of filamentous K8/18 filaments would also be lethal or cause serious liver dysfunction [104]. These results all converge to the general interpretation that as observed in mice K8/18 are proteins that play essentials survival functions in human hepatocytes.

3. K8/18 IN CELLULAR SIGNALLING

Multiple signalling pathways regulate cell homeostasis, survival and death. During the last decade, several studies reveal that the protective role of K8/18 against stress and injuries may be related to their numerous interactions with cell signalling mediators. The next section summarizes the involvement of K8/18 in major cell signalling events accountable for cell fate.

15

3.1- K8/18 in Cell Division and Cell Death Homeostasis

Cell division and cell death are fundamental processes that together maintain tissue/organ homeostasis in both physiological and pathological conditions. Disturbances in this equilibrium underlie uncontrolled proliferation or death of cell population. Multiple signalling pathways have been identified to initiate and regulate these processes and during the last years, increasing evidences suggest a role for keratins IFs in the regulation of cell cycle and apoptosis.

Cell cycle is an ordered sequence of events largely controlled by cyclin-dependent kinases that leads to cell duplication and division. Thus, proper progression through the cell cycle is governed by activation of the appropriate cyclin-dependent kinase at the right time. In this regard, K8 has been shown to moderate cell cycle progression since K8-null hepatocytes enter more efficiently into S phase [105]. K8 loss promotes EGF- and insulin-stimulated cycle progression through a specific increase of cyclin A level [105]. Moreover, K18 can associate with and sequester the ERα-target/coactivator gene LRP16, thus attenuating the oestrogen-stimulated cell cycle progression of MCF-7 breast cancer cells [106]. On the opposite, K18 promotes cell cycle by interacting with 14-3-3 family proteins in a phosphorylation-dependent manner [107, 108]. In resting cells, 14-3-3 sequesters the cell cycle phosphatase Cdc25 in the cytoplasm and attenuates its nuclear import [109]. During hepatocytes mitosis, phosphorylation of K18 on Ser33 increases significantly with consequent sequestration of cytoplasmic and nuclear 14-3-3 by keratins [110]. These results suggest that keratins may sequester 14-3-3 during cell cycle progression and disrupt 14-3-3-Cdc25 complexes, thus promoting the cell cycle-dependent Cdc25 nuclear translocation and its function [109]. Thereby, these studies revealed that K8/18 could modulate different steps of cell cycle mainly by sequestering key regulators.

Cells undergo apoptosis through two major pathways, namely the extrinsic pathway (death receptor pathway) or the intrinsic pathway (the mitochondrial pathway). The extrinsic pathway is activated by the tumor necrosis factor (TNF) family and their receptors (TNFR). For example, FasL binds to FasR, TNF binds to TNFRI and TRAIL binds to DR4 and DR5 [111]. The intrinsic pathway is mediated by diverse apoptotic stimuli (DNA damage, high level of ROS, UV or ionizing radiation, growth factor withdraw and anoikis), which converge at the mitochondria where the release of cytochrome *c* initiates the apoptotic signalling [112]. Both pathways involve the activation of a cascade of "caspase" proteases that cleave regulatory and structural molecules leading to the death of the cell. At an early stage of apoptosis, preceding caspase activation and nuclear changes, the

death effector domain containing DNA binding protein (DEDD) directs procaspase 3 to keratin filaments in epithelial cells under apoptosis induced by either the death-receptor or mitochondrial dependent pathway [113]. Furthermore, the active caspase 9 is similarly concentrated on keratin fibrils [114]. These results support a model in which keratin K8/18 filaments provide a scaffold for accumulation and auto-activation of procaspase 9, which in turn cleaves procaspase 3 that is within close proximity. The active caspase 3 can in turn activate more procaspase 9, thereby facilitating a caspase amplification loop [114]. In line with this model, knockdown of DEDD or expression of its keratin-targeting-defective mutant inhibits activation of caspase 3 [113]. Furthermore, the ability of DEDD to associate with K8/18 filamentous network strongly correlates with an increased sensitivity to apoptosis [115]. Thus, the keratin network regulates apoptotic machinery and confers a caspase- activation/amplification platform.

Amongst the primary caspase-targets in epithelial cells are found keratins type I family (Figure 6) [116]. K18 is first cleaved at Asp397 site in the COOH terminal (tail) domain by caspase 3, 7 and 9 [116-118]. This initial K18 cleavage does not affect filamentous organization to a great extent, but liberates a carboxy-terminal fragment that is rapidly translocated to the nucleus to interfere with topoisomerase I-mediated chromatin condensation during apoptosis. This leads to preservation of transcriptional activity required during early stages of programmed cell death [119]. Moreover, cleavage at Asp397 generates a neoepitope reactive with the commercially available antibody M30 that can be used to monitor caspase 3 activation [118]. A second caspase cleavage site was identified in the conserved L1-2 linker region at Asp238 site [116, 120]. This event is responsible for the collapse of the keratin cytoskeleton into punctuate aggregates and coincides with loss of intracellular contacts and detachment of cells from their substrates [117]. These K8/18 cytoplasmic inclusions, which exhibited the same immunohistochemical and morphological features as MDBs, also contain several pro-apoptotic factors, such as caspase 3 and 9, DEDD, tumor necrosis factor receptor type-1-associated death domain protein (TRADD) and heat-shock proteins [114, 115, 121]. Many of their constituents, including active caspases, remain sequestered within these inclusions, even after detergent treatment and isolation [114]. As the apoptosis program proceeds, it is currently unclear whether active caspases are released from these inclusions to accomplish the cleavage of other cellular substrates. However, a recent study shows that the smallest subunit of eukaryotic translation initiation factor 3 complexes, eIF3k, also localizes to keratins network where it promotes the release of active caspase 3 from the K8/18 inclusions during apoptosis [122]. A sequential process

regulated by K8/18 emerges from all these observations. At early stage of apoptosis, effective recruitment of procaspase 3 and 9 at the keratin filamentous scaffold through DEDD leads to an increased local availability of caspases, which renders cells more apoptosis-prone [115]. Later in the process, the collapse of keratin filaments induced by the caspase cleavage of K18 sequesters all these pro-apoptotic proteins that could injure neighboring cells [114]. Thus, keratin filaments may both control key stages of the caspase cascade to facilitate an ordered cell death process and minimize the effects of this process on adjacent cells.

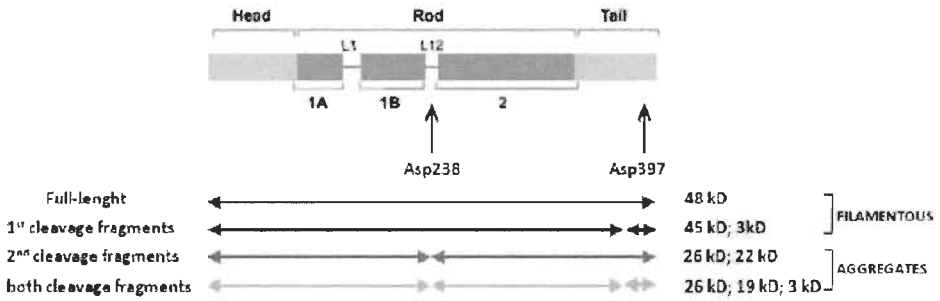

Figure 6. Shematic representation of K18 caspase-cleavage sites during apoptosis.

The fact that keratins of type I are a target of caspases suggest an anti-apoptotic role for these IFs proteins. Indeed, several studies demonstrate that K8/18 provide resistance to extrinsic and intrinsic pathways. Normal and malignant epithelial cells deficient in K8 and/or K18 are more sensitive to FasL and TNF-induced death [123, 124]. First, K8 moderates FasR targeting to the hepatocytes membrane in a microtubule-dependent manner [124]. Moreover, K8 and K18 both bind the cytoplasmic domain of TNFR2 [123] and K18 sequesters the adaptor protein TRADD [125]. These interaction moderate Jun NH(2)-terminal kinase (JNK) intracellular signalling and NFkappaB activation in simple epithelial cells [123]. *In vivo* study also shows that disrupted K8/18 filament network by mutant overexpression predispose mice hepatocytes to Fas- but not TNF-mediated apoptotic injury [60, 94]. K8 mutation on a phosphorylation site (Ser73) increases nonkeratin pro-apoptotic substrate phosphorylation by stress-activated kinases, thus predisposes to Fas-induced liver injury in transgenic mice [60]. The authors suggest that K8 can protect tissue from injury by serving as a phosphate sponge for stress-activated kinases [60]. All these studies provide evidence that moderation of death receptor downstream signalling may be a fundamental function of K8 and K18, particularly in liver regeneration.

K8/18 also provide resistance to intrinsic apoptosis pathway by a direct or indirect effect on mitochondria. Knockdown of K18 as well as its disassociation from Pirh2, a novel interacting protein which maintains keratin filaments organization, affected mitochondrial localization through a microtubule-mediated mechanism and led to nuclear clustering of mitochondria aggregates, thus to enhance UV-induced apoptosis in human lung cancer cell lines [126]. Absence of K8 also induced an irregular mitochondrial distribution pattern in hepatocytes from K8-null mice [127], that correlates with a significant reduction of mitochondrial size. These morphological modifications result in a functional decrease in cytochrome *c* content, an increased mitochondrial permeability and a higher sensitivity to oxidative injury [127]. K18 point mutation (Arg89→Cys) in transgenic mice modulates several oxidative stress-related genes and protein oxidation by-products to prime hepatocytes to oxidative injury [95]. Conversely, K8-knockout mouse hepatocytes and K8-knockdown H4-II-E-C3 rat hepatoma cells are more resistant to ROS-mediated cell death by altering PKCδ activity at mitochondria [128]. The authors propose that keratin loss in hepatic cells displaces PKCδ away from its death-related mitochondrial target, whereas mutated K8/K18 promote PKCδ mediation of mitochondria-dependent cell death in response to excess ROS [128]. Nonetheless, these findings establish a link between K8/18 filament network and mitochondrial functional integrity in intrinsic apoptotic signalling.

3.2- K8/18 in Cell Motility

First evidences linking keratin IFs expression with cell motility, invasion and metastasis come from observations that metastatic cancer cells presenting a dedifferentiated phenotype co-expressed keratins and vimentin, a mesenchymal-specific IF, whereas the poorly metastatic counterpart expressed only one type of the IFs proteins [129-131]. At this time, several studies indicate a role for keratin IFs in modulating cell migration. Mouse L fibroblasts and melanoma cells overexpressing human K8/18 filamentous network showed higher invasiveness through matrigel matrix than cells transfected with K8 or K18 alone [132, 133]. This migratory activity was directly correlated with the spreading ability of the cells on the same substrate, in which the K8/18 transfectants maintain a round morphology for a longer duration [132]. This study suggested that keratins may play a role in migration, by influencing cell shape. On the contrary, overexpression of K18 in MDA-MB-231 metastatic breast cancer cell line causes a dramatic reduction of the invasive and metastatic potential [134]. These

differential effects of ectopic K8/18 expression raise the hypothesis that the role of IFs in these processes could depend the epithelium type and differentiation state. It is well known now that cell motility, invasion and metastasis are closely related to the cell differentiation state in regard to epithelial-mesenchymal phenotype transition (EMT), that constitute a hallmark of malignant transformation [135]. In this context, keratins should not be considered merely as markers but also as regulators of differentiation in that inappropriate IFs expression strongly correlates with altered differentiation, invasiveness and metastatic potential. However, the molecular mechanisms regulated by K8/18 in cell motility and invasion remains elusive.

Given the extensive K8/18 filamentous network into the cell, IFs can act as signal transducers from the extracellular matrix to the nucleus. For instance, keratin IFs are connected to cell-cell and cell-matrix junctions by linking to desmosomes and hemidesmosomes respectively [136, 137]. By this interaction, K8/18 maintain epithelial cell junction integrity, which prevents desmosomes remodelling required for cell motility. Indeed, K8 loss leads to alterations in desmosomes distribution at the surface membrane of mouse hepatocytes [138, 139], mouse embryonic epithelia [140] and human epithelial cancer cell lines MCF-7, HeLa and Panc-1 [141]. Interestingly, vimentin-positive HeLa and Panc-1 cells fail to target desmoplakin, a desmosome component, at cell borders [141]. A previous study has already shown that desmoplakin associates more strongly with keratins than vimentin network [137]. As a consequence of reduced cell-cell contacts, knockdown of K8 with siRNA results in accelerated wound closure in HeLa and Panc-1 cell lines and in appearance of cells with a mesenchymal, irregularly spread morphology [141]. Thus, K8 is required for the maintenance of epithelial integrity during migration. Moreover, K8/18 complete filamentous network seems to be essential to exert the moderating role in cell motility since perinuclear reorganization of K8/18 network by sphingosylphosphorylcholine increases cellular elasticity and augments migration through limited-sized pores [142]. We also demonstrated that perinuclear reorganization of K8/18 network occurs in HeLa and HepG2 epithelial cancer cells overexpressing constitutively active Akt isoforms [143], and that is associated with increased K8/18 protein levels [143] and invasion through matrigel (Fortier et al., unpublished results). Disruption of K8/18 filamentous network in hepatocytes by transfection of mutant K18 or by proteasome inhibition also affects localization of desmoplakin, zonula occludens-1, beta-catenin and 14-3-3-zeta, which are relocated to keratin inclusions [144]. On the other hand, K8 has been identifed as a binding protein for plasminogen and urokinase-type plasminogen activator expressed on the external surfaces of hepatocytes and breast carcinoma cells [145, 146]. The authors

propose that the small fraction of total cellular K8, which is expressed on the outer cell surface, may promote cellular invasiveness by enhancing proteinase activation in the pericellular spaces [147].

Keratins are regarded as relatively stable cytoskeleton components that are important for epithelial flexibility to mechanical stress [148]. However, there is increasing evidence that they are highly dynamic. Indeed, keratins may also be important in migration by influencing cell shape, intercellular junctions and epithelial sheet integrity during collective cell migration. A key step during metastatic process is the invasion of cancer cells through the basal membrane and the endothelial layer, which requires both migration and cellular elasticity. Thus, keratins loss or network reorganization in perinuclear compartment could indeed facilitate cell migration and resilience.

Conclusion

Keratin IFs constitute a major cytoskeletal component of simple epithelial cells. Previously considered merely as a structural scaffold, keratin network is now known to notably regulate cell survival and motility. Since the hepatocytes only express K8/18, first evidence for their cell protective role came from liver diseases, which display characteristic keratin aggregates called Mallory Denk bodies. Over the years, the accurate analyses of these inclusions revealed that keratin network reorganization involves their post-translational modifications and their association with cell signalling proteins. Indeed, K8/K18 mutations or deficiencies in transgenic mice show that keratins play a pivotal role in hepatocytes resistance to mechanical and toxic stress. Today, the K8/18 function in cell survival is extended to other epithelial tissue and carcinomas. Increasing findings of keratin associated proteins define more precisely the role of K8/18 in cell homeostasis as a signalling platform.

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