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Intérêt de l'utilisation de stratégies anti-métaboliques pour le traitement du cancer du sein

Diana Farhat

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Intérêt de l'utilisation de stratégies anti-métaboliques pour le traitement du cancer du sein

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“Logic will get you from A to Z; imagination will get you everywhere”

Albert Einstein

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LISTE DES ABREVIATIONS

AIF : Apoptosis-inducing Factor	18
AMPK : AMP-activated Protein Kinase.....	17
bcl-2 : B-cell lymphoma 2	18
bcl-XL : B-cell leukemia XL	18
Bim : Proapoptotic Bcl-2 homology 3-only protein	18
cdk-cyclines : cyclin-dependent kinases/cyclines	46
CREB : , cAMP response element-binding protein.....	43
CSC : Cellules Souches Cancéreuses	22
EGF : Epidermal Growth Factor.....	41
EMT : Epithelial Mesenchymal Transition.....	17
ERK1/2 : Extracellular signal - Regulated Kinase	40
ER α : Estrogen Receptor alpha.....	35
FAK : Focal Adhesion Kinase	21
FSH : Follicle Stimulating Hormone	41
furin (PCSK3) : Proprotein Convertase Subtilisin/Kexin Type 3.....	57
GH : Growth Hormones.....	41
HER2 : Human Epidermal growth factor Receptor 2	35
INCA : Institut National du Cancer	36
IR : Insuline Receptor	51
IRS-1 : Insulin Receptor Substrate 1	42
LA : Lipoic Acid.....	15
MAPK : Mitogen-Activated Protein Kinase.....	40
Mcl-1 : Myeloid cell leukemia 1	18
MMP-2 : Matrix Metalloproteinase 2	21
MT1-MMP : Membrane-Type 1 Matrix Metalloproteinase	60
mTOR : Mammalian Target Of Rapamycin	17
NF- κ B: Nuclear Factor kappa-light-chain-enhancer of activated B cells.....	25
p53 : p53 Tumor Suppressor.....	41
PACE4 : Paired basic Amino acid Cleaving Enzyme 4	62
PARP : poly ADP-ribose polymerase.....	37
PC : Protein Convertase	62
PCSK2 : Proprotein Convertase, Subtilisin/Kexin Type 2	57
PCSK4 : Proprotein Convertase, Subtilisin/Kexin Type 4	57
PCSK5 : Proprotein Convertase, Subtilisin/Kexin Type 5	57
PCSK7 : Proprotein Convertase, Subtilisin/Kexin Type 7	57
PDGF : Platelet-Derived Growth Factor.....	41
PI3K : Phosphatidylinositol- 4,5-bisphosphate 3-kinase	17
PR : Progesterone Receptor	35
PSCK1 : Proprotein Convertase, Subtilisin/Kexin 1	57

PTP1B : protein tyrosine phosphatase 1B	17
PTP1Ba : Protein tyrosine phosphatases 1 B a	17
Raf : Rapidly Accelerated Fibrosarcoma	40
ROS : Reactive Oxygen Species	15
RTK : Récepteur Tyrosine Kinase	17
SMAD : Mothers against decapentaplegic homolog	21
TGF β : Transforming Growth Factor betta	19
TNBC : Triple Negative Breast Cancer	56
VEGF-C : Vascular endothelial growth factor C	60
VEGF-D : Vascular endothelial growth factor D	60
WT- p53 : Wild Type p53	18

Préface

Le cancer du sein est le deuxième cancer le plus répandu dans le monde et, de loin, le plus fréquent chez les femmes (25 % tous types de cancers confondus). Malgré les progrès thérapeutiques, les mécanismes de résistance restent la cause sous-jacente de la morbidité et de la mortalité. Il est bien connu que le métabolisme du glucose augmente dans la majorité des tumeurs afin d'assurer un statut redox compatible avec la survie cellulaire, conduisant à une résistance aux thérapies.

L'acide lipoïque (LA) est un antioxydant naturel et un cofacteur essentiel du métabolisme oxydatif *via* sa fonction dans les complexes de la pyruvate déshydrogénase et de l' α -cétol déshydrogénase. Ses effets thérapeutiques potentiels ont été bien documentés dans le traitement de pathologies associées au stress oxydant, telles que le diabète, l'athérosclérose, les maladies du foie et les maladies neurodégénératives. De plus, les chercheurs ont démontré ses effets anticancéreux dans divers cancers, mais ses mécanismes d'action ne sont pas entièrement compris.

Mon projet de thèse vise à évaluer l'effet inhibiteur de LA sur la prolifération de diverses lignées cellulaires du cancer du sein et d'étudier les mécanismes d'action susceptibles d'être impliqués dans ce processus.

Ce manuscrit expose dans un premier temps les données bibliographiques sur l'acide lipoïque et ces effets anti-cancéreux, puis sur le cancer du sein avec ses classifications, ses traitements, l'IGF-1R et son rôle dans les cancers, ensuite les proprotéines convertases seront abordées, notamment la furine avec ses substrats, ses effets physiologiques et son importance dans le cancer. Dans un second temps, les résultats obtenus durant ma thèse seront présentés sous forme de 2 articles avec à chaque fois une petite introduction et discussion. Dans un troisième temps, vous trouverez une discussion générale sur mes travaux et puis les conclusions et perspectives.

En annexe vous trouverez les 2 revues publiées durant ma thèse.

Chapitre I
Introduction

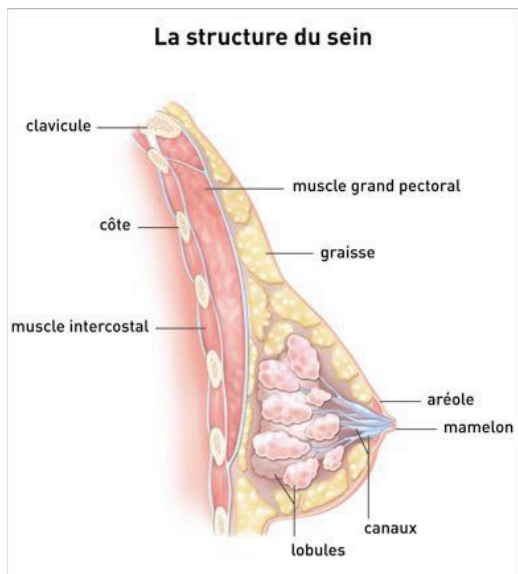
Introduction

I. Cancer du sein

Le cancer du sein est le cancer féminin le plus fréquent dans le monde. Il est caractérisé par une multiplication accrue et incontrôlée des cellules mammaires formant ainsi une tumeur. Il est très rare chez les hommes avec une fréquence inférieure à 1 %, reflétant généralement une prédisposition génétique (Institut National du Cancer, INCA : <http://www.e-cancer.fr>).

En raison de facteurs génétiques, du style de vie (alimentation, sédentarité, etc.) et de facteurs exogènes, son incidence augmente annuellement et demeure toujours un problème majeur de santé publique.

Figure 1 : Anatomie du sein (laboratoires-arion.fr).



1. Anatomie du sein

Le sein est un organe dont la structure reflète sa fonction spéciale : la lactation. Il est constitué de lobules responsables de la production de lait qui va être déversé dans des canaux galactophores l'emmenant au mamelon. La plupart des cancers du sein est originaire des cellules formant les lobules et les canaux (**figure 1**).

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2. Epidémiologie du cancer du sein

2.1. Incidence

L'incidence du cancer du sein augmente partout dans le monde. Il s'agit du cancer le plus fréquemment diagnostiqué chez les femmes dans 140 pays parmi les 184 existants. A l'échelle mondiale, 14 millions de nouveaux cas ont été diagnostiqués en 2012 (Organisation Mondiale de la Santé : OMS) (Institut National Du Cancer, INCa 2013).

- **En France :** 54 062 nouveaux cas de cancer du sein ont été dénombrés en 2015, accompagnés du décès de 11 913 patientes (INCa, 2015). L'incidence a augmenté entre 1990 et 2018 (+1,1 % par an en moyenne, passant de 72,8 nouveaux cas de cancer du sein pour 100 000 femmes) (santepubliquefrance.fr).

8 cas de ce cancer sur 10 touchent des femmes âgées d'au moins 50 ans. A noter que l'âge moyen lors du diagnostic est de 63 ans (INCa, 2013).

- **Au Liban :** 1 700 nouveaux cas de cancer du sein ont été dénombrés en 2009. Son incidence est de 197 pour 100 000 habitants et reste notamment moins élevée qu'en Europe. Ce cancer est le plus fréquent au Liban (22 % des cancers au Liban).

71 % des femmes âgées de plus de 40 ans n'ont jamais eu de mammographie, ce qui pose un problème majeur au Liban (MOH, NCR, 2007).

2.2. Facteurs de risques

Le cancer du sein est une maladie multifactorielle. En effet, de nombreuses études épidémiologiques et expérimentales permettent d'affirmer que la prédisposition génétique, l'exposition à des facteurs environnementaux et hormonaux et le mode de vie jouent un rôle important dans l'étiologie de cette maladie (Nkondjock et Ghadirian, 2005).

2.2.1. Facteurs génétiques

La prédisposition familiale est associée, de manière générale, à un risque accru de cancer du sein. Ce dernier augmente chez les jeunes femmes lorsque la maladie se développe chez une proche parente avant l'âge de 50 ans (Pharoah et al., 1997). Les études ont permis d'identifier un certain nombre de mutations génétiques favorisant la survenue et le développement du cancer du sein. Les

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mutations les plus fréquentes sont celles qui touchent les gènes BRCA1 (Breast Cancer 1) et BRCA2 (Breast Cancer 2). Ces derniers codent pour deux protéines qui sont impliquées dans les mécanismes de réparation des lésions de l'ADN. Porter la mutation sur l'un de ces gènes ne se traduit pas systématiquement par l'apparition d'un cancer mais augmente le risque de la survenue d'un cancer. A partir de 70 ans, le risque associé aux mutations citées avoisine les 87 % pour les femmes et 6 % pour les hommes (Ford et al., 1998; Wolpert et al., 2000).

2.2.2. Facteurs environnementaux et sanitaires

Les facteurs de risque externes sont liés à l'environnement (pollution, rayons UV en déclenchant l'apparition de mutations génétiques et de tumeurs) et aux modes de vie (alimentation, activités physiques, tabac, alcool, etc.). Alors que, les facteurs de risque internes reposent sur les caractéristiques physiologiques de l'individu (âge, poids, contraception). Ces facteurs peuvent augmenter le risque de cancer du sein en augmentant le taux d'œstrogène qui joue lui-même un rôle important dans le développement des cellules du cancer du sein (Rocheffort, 2017).

2.2.3. Facteurs hormonaux liés à la reproduction

Dans la majorité des cas, le cancer du sein est un cancer hormono-dépendant. Dans ce contexte, les cellules cancéreuses sont sensibles à certaines hormones sexuelles féminines (comme l'œstrogène et la progestérone) secrétées par les ovaires. Ces hormones sexuelles ont une action cancérigène si les cellules de la tumeur présentent à leur surface une quantité anormalement élevée de récepteurs spécifiques, ces cellules ont alors développé une hypersensibilité aux hormones. Lorsque l'hormone se fixe sur son récepteur, elle stimule leur division cellulaire favorisant le développement d'une tumeur (Khanfir et al., 2013). L'hormone n'a en revanche aucun effet sur les cellules cancéreuses dépourvues de récepteur spécifique. De ce fait, les expositions précoces (premières règles avant l'âge de 12 ans) et prolongées (ménopause tardive) comme la prise de contraceptif durant l'activité ovarienne, sont des facteurs qui augmentent le risque de cancer du sein (Glade et al., 1999).

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3. Classification du cancer du sein

Le cancer du sein englobe un groupe hétérogène de tumeurs ayant diverses caractéristiques morphologiques et biologiques (Rakha et Lee, 2011). La découverte d'un cancer du sein est souvent due à des symptômes cliniques (douleurs, masse palpable) et une série d'examen (mammographie, échographie puis biopsie) permettant de poser le diagnostic. Cependant, seul l'examen histologique de la biopsie peut confirmer avec certitude la présence d'un cancer infiltrant où les cellules passent la membrane basale pour envahir les ganglions puis d'autres tissus dans l'organisme pour former des métastases (**figure 2**). La gestion clinique actuelle du cancer du sein repose sur la disponibilité de solides facteurs pronostiques et prédictifs pour aider la prise de décision et adapter au mieux le choix du traitement vers la chirurgie, la chimiothérapie, l'hormonothérapie et/ou la radiothérapie. Ces critères morphologiques et biologiques sont associés à la précocité du diagnostic, au type histologique de la tumeur, à son examen anatomopathologique et à ses caractéristiques moléculaires. Ils sont brièvement exposés dans le paragraphe ci-dessous.

3.1. Critères histologiques

Le cancer du sein le plus fréquent est à 95 % un adénocarcinome qui se développe à partir des cellules épithéliales des canaux ou des lobules de la glande mammaire. Un adénocarcinome naissant à partir des cellules des canaux est dit adénocarcinome canalaire et il représente 75 % des cancers du sein et celui qui se développe à partir des lobules est dit adénocarcinome lobulaire, représentant seulement 10 % des cancers mammaires. D'autres types de cancers du sein moins fréquents peuvent aussi être observés : cancer inflammatoire du sein, cancer du sein triple négatif, cancer du sein de type basal et, très rarement, un sarcome (Bertucci et al., 2008).

La classification histologique permet de diviser les tumeurs en deux catégories : les adénocarcinomes *in situ* et les adénocarcinomes infiltrants. Lorsque les cellules cancéreuses restent à l'intérieur des canaux ou des lobules, ce sont des adénocarcinomes *in situ*. En revanche, quand la membrane basale entourant les canaux ou les lobules est franchie, c'est un adénocarcinome infiltrant. Ce dernier se propage généralement vers les ganglions lymphatiques et présente un pronostic moins favorable qu'un adénocarcinome *in situ* (Malhotra et al., 2010) (**figure 2**).

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Il existe d'autres types de cancers du sein plus rares, ayant des particularités histologiques spécifiques et des pronostics plus ou moins favorables. Notamment, les carcinomes médullaires, mucineux, tubuleux, papillaires et la maladie du Paget du mamelon.

3.2. Critères anatomopathologiques

Lorsque le diagnostic du cancer est confirmé, la stratégie thérapeutique est guidée par les facteurs pronostiques tels que le stade de cancer, la taille de la tumeur, le statut des récepteurs hormonaux etc. (Kollias et al., 1999). Le stade de la tumeur dépend de sa taille, de son caractère infiltrant, de l'envahissement ganglionnaire et de la présence ou non de métastases. Quant au grade de la tumeur, il évalue son agressivité et prend en considération la différenciation des cellules tumorales et leur vitesse de croissance.

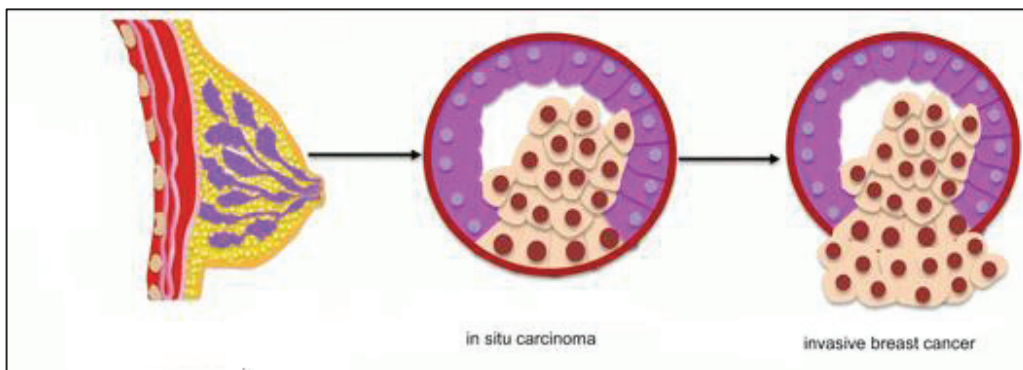


Figure 2 : Les adénocarcinomes (oncohemakey.com).

3.2.1. Le stade de la tumeur (classification TNM)

Le système de classification des différents stades du cancer du sein, appelé classification TNM, a été établi par l'Union Internationale Contre le Cancer (UICC) et l'American Joint Committee on Cancer (AJCC). La classification TNM («Tumor, Node, Metastasis») est basée sur l'analyse de la taille de la tumeur (T), la présence ou l'absence d'envahissement ganglionnaire (N) et la présence ou l'absence de métastase (M) (**tableau 1**). Ces critères permettent ensuite de subdiviser les tumeurs en 5 stades, soit le stade 0 où les cellules cancéreuses se trouvent seulement dans le canal ou le lobule dans lequel elles ont pris naissance et elles n'ont pas envahi le tissu mammaire voisin (tumeur non infiltrante) suivi du stade 1 où la tumeur mesure moins de 5 cm et le cancer ne s'est pas propagé à plus de 3 ganglions lymphatiques (cancer du sein précoce), stade 2 et 3 où la tumeur mesure plus de 5 cm et le cancer peut s'être propagé à la peau, aux muscles de la paroi thoracique

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ou à plus de 3 ganglions lymphatiques (Cancer du sein localement avancé) et stade 4 où le cancer s'est propagé à d'autres parties du corps (Cancer du sein métastatique) (**tableau 2**).

Tableau 1 : Description de la classification TNM (Tumor Node Metastasis).

T : envahissement tumoral
Tis : tumeur intra épithéliale, <i>in situ</i> (is) T1 : tumeur limitée à la muqueuse ou à la sous muqueuse T2a : tumeur étendue à la sous-séreuse T3 : tumeur envahissant la séreuse T4 : tumeur envahissant un organe de voisinage
N : envahissement ganglionnaire
N0 : pas d'envahissement ganglionnaire Nx : ganglions non évalués ou moins de 5 examinés N1 : 1 à 6 ganglions régionaux métastatiques N2 : 7 à 15 ganglions régionaux métastatiques N3 : plus de 15 ganglions régionaux métastatiques
M : dissémination métastatique
M0 : pas de métastase M1 : métastase à distance

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Tableau 2 : Les différents stades du cancer du sein selon “American Cancer Society” (2008).

Stade	Description
0	Les cellules cancéreuses sont localisées seulement dans la membrane d'un canal galactophore. Cancer in situ.
I	La taille de la tumeur cancéreuse est ≤ 2 cm. Pas de propagation à l'extérieur du sein.
II	La taille est comprise entre 2 cm et ≤ 5 cm. Propagation à quelques ganglions lymphatiques voisins.
III	La taille de la tumeur cancéreuse est ≥ 5 cm. Propagation à plusieurs ganglions lymphatiques.
IV	Le cancer s'est propagé à d'autres organes. Présence de métastases

3.2.2. Le grade histo-pronostique de Scarff, Bloom et Richardson (SBR)

Une autre classification est utilisée pour déterminer l'agressivité tumorale, il s'agit du grade histo-pronostique de Scarff Bloom et Richardson (SBR) (Bloom and Richardson, 1957). Trois critères sont pris en compte : l'architecture, l'atypie nucléaire et l'index mitotique. Elle permet de classer les tumeurs selon trois grades de I à III. Le grade I présente un pronostic favorable alors que le grade III possède le pronostic le plus défavorable (**tableau 3**).

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Tableau 3 : Grade SBR modifié par Elston et Ellis (1991).

Architecture tumorale:	
Proportion de canaux ou glandes dans la tumeur (en % de surface tumorale)	
>75 %: Tumeur bien différenciée	1
10-75%: Tumeur moyennement différenciée	2
<10 % : Tumeur peu différenciée	3
Atypies= Forme et taille du noyau (apprécié sur la population tumorale prédominante)	
Noyaux petits, réguliers, uniformes	1
Pléomorphisme modéré	2
Variations marquées de taille, de forme, avec nucléoles proéminents	3
Activité mitotique:	
Compter 10 champs au grossissement x400, valeurs définies pour un champ de 0.48mm de diamètre	
0 à 6 mitoses	1
7 à 12 mitoses	2
> 12 mitoses	3
TOTAL	
Grade I: Tumeurs bien différenciées, de bon pronostic	3, 4, 5
Grade II: Tumeurs moyennement différenciées, pronostic moyen	6,7
Grade III: Tumeurs peu différenciées, mauvais pronostic	8.9

3.3. Critères moléculaires

Les récents progrès dus aux approches génomiques ont permis d'utiliser des critères moléculaires pour classer le cancer du sein en trois principaux groupes (Yarbro et al., 2011). Ces critères se résument par la présence ou l'absence des facteurs pronostiques bien décrits comme le récepteur aux œstrogènes (ER), le récepteur à la progestérone (PR) et l'amplification de HER-2 (Human Epidermal Growth factor Receptor 2). Ils impactent la survie des patientes et conditionnent la prise en charge thérapeutique (**figure 3**).

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3.3.1. Les tumeurs lumineales A et B

Les tumeurs lumineales représentent environ 70 % des cancers du sein. Elles expriment le récepteur à l'œstrogène et sont dites «ER α +». Elles sont divisées en 2 sous types. Les lumineales A qui ont une forte expression de ER mais qui expriment faiblement des gènes de prolifération cellulaire, comme la cycline D1 et/ou le marqueur de prolifération Ki67 (Brenton et al., 2005). Elles sont de bon pronostic (Dai et al., 2015). Les lumineales B possèdent des similarités moléculaires avec les tumeurs lumineales A (ER α +/ PR +/ HER2 + ou ER α +/ PR+/ HER2-) mais elles ont une expression élevée du marqueur Ki67 (Dai et al., 2015). Leur pronostic est moins favorable que les lumineales A (**figure 3**).

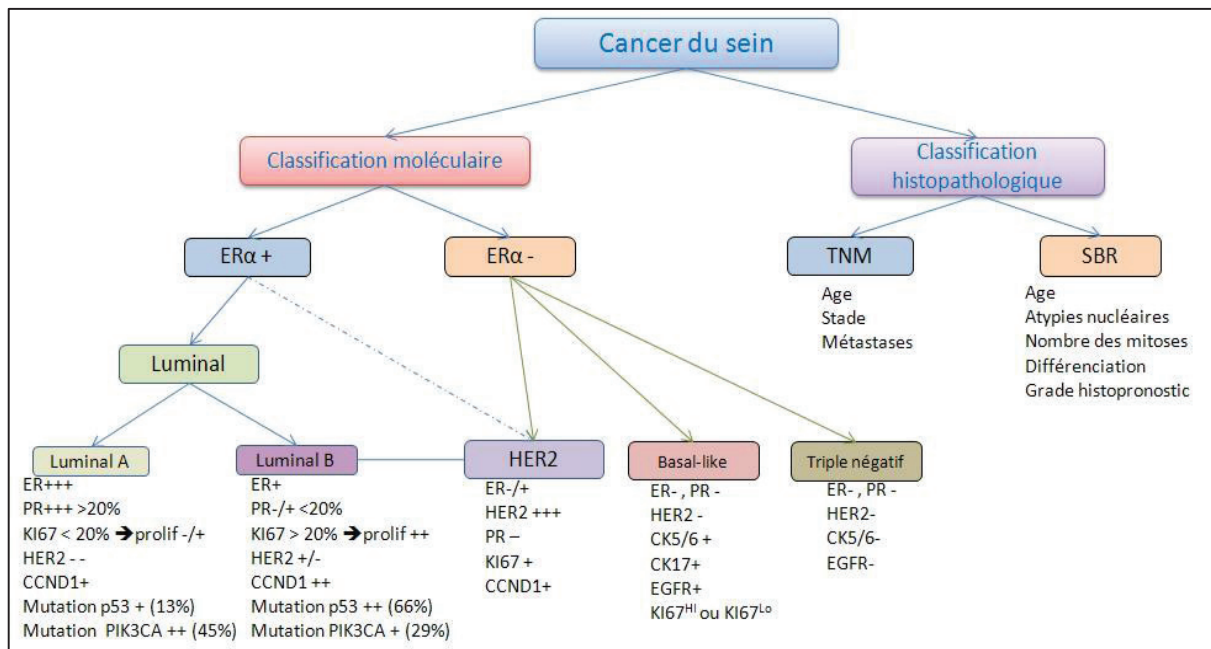


Figure 3 : Classification moléculaire ou histopathologique du cancer du sein. (CCND1 : Cycline D1 ; CK : cytokeratine).

3.3.2. Les tumeurs HER2+/ErbB2

L'amplification du gène *HER2*, responsable d'une croissance et d'une division incontrôlée des cellules mammaires, et/ou la surexpression de la protéine correspondante sont des marqueurs de mauvais pronostic pour la réponse du cancer du sein à la chimiothérapie. La surexpression protéique de HER2 est retrouvée dans environ 20 % des tumeurs mammaires invasives (Slamon

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et al., 1987). En parallèle, ces dernières expriment faiblement ER et PR et sont caractérisées par une prolifération cellulaire importante (Brenton et al., 2005; De et al., 2010) (**figure 3**).

3.3.3. Les tumeurs Basal-Like

Ces tumeurs sont constituées de cellules qui ressemblent aux cellules épithéliales basales des galactophores (Perou et al., 2000). Elles représentent entre 10 à 20 % des cancers du sein. Ce sous-type est caractérisé par l'absence de l'expression des gènes liés aux récepteurs hormonaux (ER- et PR-), une faible expression du gène *HER2* et une forte expression des gènes liés à la prolifération (Hu et al., 2006; Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2003). Le pronostic des tumeurs « basal-like » est mauvais malgré une bonne sensibilité à la chimiothérapie conventionnelle (Sorlie et al., 2001; Sorlie et al., 2003; Sotiriou et al., 2003) (**figure 3**).

4. Traitements

Le traitement du cancer du sein dépend du diagnostic établi. En général, une combinaison de plusieurs types de traitement est adoptée en fonction du diagnostic moléculaire. Dans ce contexte, il y a les traitements locorégionaux comme la chirurgie et la radiothérapie, qui concentrent leur action sur la zone de la tumeur et les traitements systémiques comme la chimiothérapie, l'hormonothérapie et les nouveaux traitements ciblés. Ils peuvent être utilisés soit seuls, soit, le plus souvent, en association.

4.1. La chimiothérapie

Ce traitement cible les cellules en division accrue dans l'organisme, notamment les cellules cancéreuses, pour réduire leur prolifération et induire leur apoptose, une mort génétiquement programmée. L'administration aux patientes se fait par voie intraveineuse ou orale. Les principaux agents utilisés en chimiothérapie sont les alkylants, les anthracyclines, les poisons du fuseau mitotique et les anti-métabolites. A titre d'exemple, depuis une trentaine d'années et jusqu'à présent, des agents appartenant à la famille des taxanes (poison du fuseau mitotique), tel que le paclitaxel (Taxol™) et le docétaxel (Taxotère™), sont utilisés en polychimiothérapie en combinaison avec le cyclophosphamide (INCA 2015).

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4.2. L'hormonothérapie

Certains cancers du sein, exprimant les récepteurs hormonaux ER α et/ou PR, sont qualifiés par leur hormono-sensibilité (60 à 70 % des cancers du sein). En effet, les hormones féminines, œstrogène (E2) et progestérone, naturellement produites par l'organisme, stimulent la prolifération des cellules cancéreuses. D'où le principe de l'hormonothérapie qui consiste à empêcher l'action stimulante de ces hormones sur les cellules cancéreuses. Des antagonistes des récepteurs aux œstrogènes comme le fulvestrant (Faslodex™) et le Tamoxifène (Nolvadex™), et des anti-aromatases qui inhibent la synthèse des œstrogènes, comme l'anastrozole (Arimidex™), sont utilisés dans le cadre de l'hormonothérapie et peuvent être associés à la chimiothérapie selon le stade de dissémination tumorale (atteinte de la chaîne des ganglions lymphatiques) (Lumachi et al., 2015). Comme les cancers du sein de type ER α + sont étroitement affectés par E2, alors ER α constitue un bon marqueur prédictif de la sensibilité des tumeurs aux hormones et de l'efficacité de l'hormonothérapie comme plan de traitement adopté (Lumachi et al., 2015).

4.3. La thérapie ciblée

La thérapie ciblée consiste à utiliser des molécules ciblant spécifiquement une voie de signalisation moléculaire. 20 % des tumeurs mammaires surexpriment la protéine HER2 impliquée dans la prolifération des cellules tumorales. Ceci a poussé les chercheurs à développer un anticorps monoclonal, le Trastuzumab (Herceptin), qui la cible (Menard et al., 2003). Son efficacité a été bien prouvée en clinique et il est toujours associé à la chimiothérapie (Piccart-Gebhart et al., 2005). Depuis, de nouveaux anticorps monoclonaux ont obtenus une autorisation de mise sur le marché (AMM). Notamment, les inhibiteurs des récepteurs à tyrosine kinase comme le Lapatinib (Tyverb) (Geyer et al., 2006) et l'Évérolimus (Afinitor) et le Bevacizumab (Avastin) qui se lie au Vascular Endothelial Growth Factor (VEGF) et bloque ainsi la néoangiogenèse (Salter and Miller, 2007; Liu et al., 2009). Quant aux tumeurs triples négatives, aucune thérapie ciblée n'est validée, mais de nombreux médicaments sont en cours de développement (Mirzania, 2016). Les auteurs ont montré que certaines nouvelles molécules ont un intérêt important pour les traitements du cancer du sein chez les patientes présentant des mutations de BRCA1, à citer les inhibiteurs des kinases de la famille Src, les inhibiteurs de PARP et les agents de platine (Mirzania, 2016). Cependant, différents mécanismes de résistance parviennent comme obstacle majeur limitant l'efficacité de

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ces traitements et ceci par l'activation, d'une part, des mécanismes de croissance indépendants des œstrogènes et d'autre part des voies de signalisation de survie cellulaire.

II. Voie de signalisation d'IGF1 et son implication dans les mécanismes de résistance à la chimiothérapie

1. 1. Aperçu Général

Dans cette voie de signalisation, l'insuline fut le premier membre découvert en 1921 et dont l'implication fut ultérieurement étudiée par Frédéric Banting (Tan and Merchant, 2017) dans le métabolisme de glucose et le traitement de diabète (Majumdar, 2001). Ensuite, les facteurs de croissance ressemblant à l'insuline, appelés IGFs (Insulin-like Growth Factors), furent découverts et caractérisés. Il s'agit des hormones peptidiques possédant une structure chimique semblable à celle de la pro-insuline. En effet, IGF-1 et IGF-2 présentent approximativement 50 % d'homologie avec l'insuline (Blundell et al., 1983; Rinderknecht et Humbel, 1978) (**figure 4**). La terminologie "insulin-like" est utilisée généralement pour désigner les facteurs qui peuvent stimuler l'absorption de glucose dans des cellules adipeuses et musculaires (Brahmkhatri et al., 2015).

Les IGFs sont des hormones de croissance naturelles jouant un rôle crucial dans la croissance, la morphogénèse et le développement. De plus, leur système de signalisation est un acteur important dans la physiopathologie de l'athérosclérose, le diabète, l'obésité et le cancer (Clemmons, 2007; Crudden, Girnita, 2015). Les IGFs contrôlent de nombreuses fonctions cellulaires telles que la croissance du cartilage de conjugaison des os longs, la croissance de tous les tissus, la filtration glomérulaire au niveau du rein etc, en interagissant avec des récepteurs membranaires spécifiques et en activant par la suite diverses cascades de signalisation intracellulaires. Les réponses cellulaires à l'insuline et aux IGFs sont induites principalement par le récepteur à l'insuline (IR) et le récepteur à l'IGF-1 (IGF-1R). A noter que le récepteur IGF-1R est un membre de la famille des récepteurs à tyrosine kinase.

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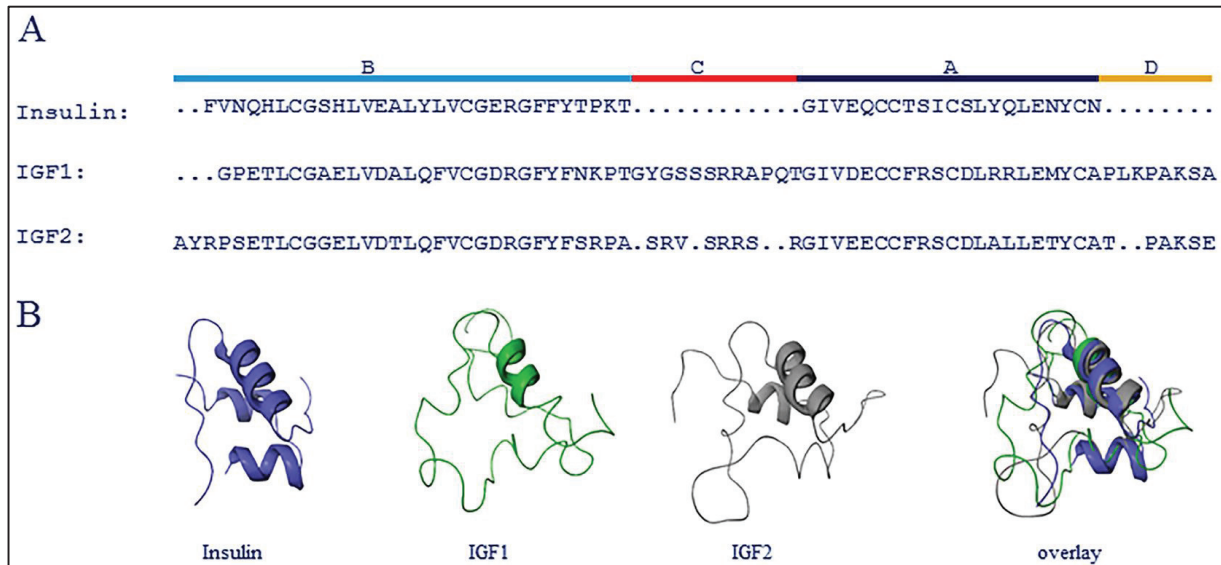


Figure 4 : Comparaison de la structure de l'insuline, de l'IGF-1 et de l'IGF-2. **(A)** Alignement de séquence d'insuline, d'IGF-1 et d'IGF-2. Les différentes régions protéiques sont indiquées en haut. **(B)** Comparaison de la structure de l'insuline (bleu), IGF-1 (vert) et IGF-2 (gris) (Andersen et al., 2017).

1. 2. Le récepteur IGF-1R

Au niveau cellulaire, les IGFs induisent diverses réponses cellulaires, notamment la prolifération, la différenciation, la migration et la survie des cellules, en se liant au récepteur à l'IGF-1, IGF-1R. Ce récepteur existe sous une forme inactive localisée dans le cytoplasme cellulaire, le pro-récepteur IGF-1R, pro-IGF-1R. Son recrutement à la membrane plasmique a lieu après le clivage par la furine de la forme pro-IGF-1R en IGF-1R actif constitué de sous-unités α et β , respectivement 135 kDa et 95 kDa (Massagué and Czech, 1982; Ward et al., 2001). Les deux sous-unités α forment le domaine de liaison au ligand (Garrett et al., 1998; McKern et al., 1997) et les deux sous-unités β contiennent les domaines tyrosine kinase transmembranaire et intracellulaire (Pautsch et al., 2001). Ces sous unités α et β sont liées par des ponts disulfures formant ainsi un récepteur fonctionnel (Adams et al., 2000). La liaison du ligand à son récepteur induit des changements au niveau de sa conformation facilitant ainsi son autophosphorylation conduisant à son activation (Hubbard and Till, 2000) (**figure 5**). En raison de leur importance fonctionnelle, les résidus tyrosine localisés dans les sous-unités β ont été le sujet de nombreuses études (Cianfarani et al., 2007; Favellyukis et al., 2001; Wu et al., 2008).

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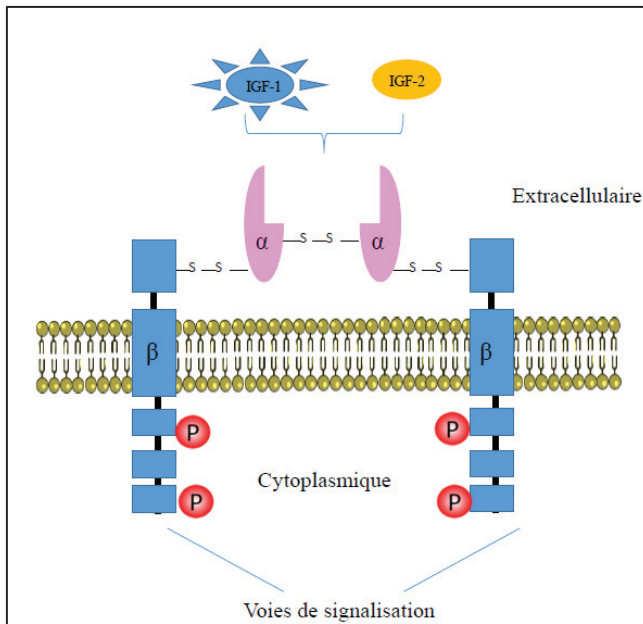


Figure 5 : Représentation schématique illustrant les différentes sous-unités α et β constituant le récepteur tyrosine kinase IGF-1R et les sites de phosphorylation.

La fixation du ligand sur les sous-unités α d'IGF-1R induit son autophosphorylation au niveau de trois résidus tyrosine (Y 1131, Y 1135 et Y 1136) situés dans la boucle d'activation aboutissant à la transphosphorylation des sous-unités β opposées (Hubbard et Till, 2000). Sous cette forme phosphorylée, IGF-1R active à son tour plusieurs cascades de signalisation, telles que les voies PI3K/Akt et Ras - Raf-MAPK-MEK-(ERK1/2) conduisant à la prolifération cellulaire, au transport du glucose, la synthèse des protéines, l'invasion, la migration, l'inhibition de l'apoptose etc. (Coolican et al. , 1997; Duan et al., 2000; Imai et Clemmons, 1999) (**figure 6**).

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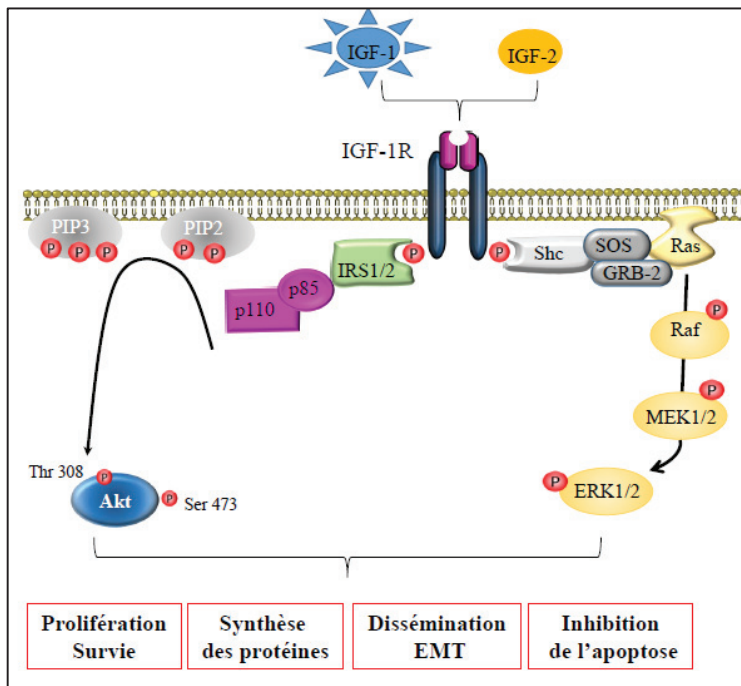


Figure 6 : Les facteurs de croissance stimulent IGF-1R et provoquent l'activation des voies de signalisation en aval, notamment PI3K/Akt et Ras-MEK-ERK, qui favorisent la prolifération, la survie cellulaire, les métastases et l'inhibition de l'apoptose.

Les hormones stéroïdes et les facteurs de croissance régulent l'expression d'IGF-1R (Sepp-Lorenzino, 1998; Stewart and Rotwein, 1996). Le faible taux d'expression d'IGF-1R provoque une accumulation de son ligand, IGF-1, qui, à son tour, va exercer un rétrocontrôle négatif inhibant ainsi l'expression d'IGF-1R (Hernández-Sánchez et al., 1997). Contrairement à l'effet des IGFs, d'autres facteurs de croissance, notamment le FGF, le PDGF et l'EGF, stimulent l'expression d'IGF-1R (Rosenthal et al., 1991; Rubini et al., 1994). De même, les œstrogènes, les glucocorticoïdes, Growth Hormone (GH), l'hormone folliculo-stimulante (FSH), l'hormone lutéinisante (LH) et les hormones thyroïdiennes stimulent l'expression d'IGF-1R (Katz et al., 1995; Werner et al., 1995a). D'autre part, les gènes suppresseurs de tumeurs, tels que la protéine p53 et la protéine de tumeur de Wilms (WT1), inhibent l'expression d'IGF-1R (Prisco et al., 1997; Werner et al., 1995b, 1995a).

L'IGF-1R est un composant clé assurant la médiation des fonctions d'IGFs *in vivo*. En raison de son expression ubiquitaire, presque tous les tissus sont affectés négativement par un manque de fonction d'IGF, y compris le muscle squelettique, les os, les poumons, la peau et le système

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nerveux (Allan et al., 2001; Bondy et al., 1990; Jones et Clemmons, 1995). Des études entamées sur des souris dépourvues du gène qui code pour l'IGF-1R fonctionnel montrent que ces souris présentent un retard de croissance *in utérin* significatif comparé aux souris sauvages et meurent après leur naissance d'une insuffisance respiratoire (Baker et al., 1993; Liu et al., 1993). Le knockout (KO) permet de caractériser davantage la fonction d'IGF-1R dans des tissus individuels et la fonction d'IGFs dans des tissus spécifiques. En effet, la perturbation sélective du gène codant pour IGF-1R dans les ostéoblastes de souris entraîne une diminution importante du volume osseux et une faible connectivité trabéculaire accompagnée également d'une diminution significative du taux de minéralisation (Zhang et al., 2002). La délétion d'IGF-1R dans les cellules β du pancréas entraîne des anomalies de la sécrétion d'insuline stimulée par le glucose et une altération de la tolérance au glucose (Kulkarni et al., 2002). Cependant, dans les adipocytes, elle n'affecte pas l'absorption de glucose stimulée par l'insuline mais induit une augmentation de la concentration d'IGF-1 dans la circulation et montre un effet systémique sur la croissance somatique (Klötting et al., 2008). De plus, l'inactivation d'IGF-1R dans le tissu adipeux entraîne une augmentation de la masse adipocytaire. L'inactivation d'IGF-1R dans le cerveau provoque une altération de la remyélinisation en réponse à une démyélinisation induite par un agent neurotoxique (Mason et al., 2003). A noter que la fonction d'IGF-1R *in vivo* est conservée au cours de l'évolution. En effet, Schlueter et al. ont montré que, même chez le zébrafish, IGF-1R est nécessaire à la viabilité de l'embryon et à une croissance normale, et plus particulièrement au développement des yeux, de l'oreille interne, du cœur et du muscle (Schlueter et al., 2006). Le KO d'IGF-1R dans le foie diminue sa capacité de régénération et révèle l'importance de la cascade de signalisation IGF-1R/IRS-1/ERK en tant que voie intracellulaire contrôlant la progression du cycle cellulaire dans le foie en régénération (Desbois-Mouthon et al., 2006). Des études ultérieures, utilisant des oligonucléotides antisens ou un dominant négatif, ont élucidé les mécanismes cellulaires de cette voie essentielle lors de l'embryogenèse des vertébrés. A l'échelle moléculaire, les observations ont montré que l'inhibition d'IGF-1R fait augmenter l'apoptose neuronale par l'activité accrue des caspases, protéines enzymatiques impliquées dans l'apoptose. Cet effet est atténué par la co-injection d'ARNm anti-apoptotique de la famille Bcl-2. L'étude du cycle cellulaire a démontré des défauts de progression des cellules au cours des phases du cycle cellulaire chez des embryons déficients en IGF-1R et ce indépendamment de l'apoptose (Schlueter et al., 2007).

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2. Transduction du signal d'IGF-1R et fonctions biologiques

Les voies de signalisation sont communes à de nombreux récepteurs de facteurs de croissance, y compris l'IR (Insulin Receptor). Toutefois, de légères différences dans le recrutement et l'activation des facteurs intracellulaires permettent d'identifier des effets spécifiques pour chaque récepteur (Belfiore and Frasca, 2008; Boucher et al., 2010; Frasca et al., 2008).

L'engagement des différentes cascades de signalisation en aval du récepteur est médié par l'interaction d'IGF-1R actif avec des protéines adaptatrices, notamment la protéine Shc et le substrat du récepteur de l'insuline IRS-1 (Dupont and LeRoith, 2001). Lors de la stimulation d'IGF-1R par IGF-1, la tyrosine, située au niveau de la face cytosolique de la membrane plasmique, est phosphorylée pour former un site «d'accueil» afin de recruter et phosphoryler la protéine adaptatrice Shc (Easton, 2006; Kurmasheva, 2006; Tartare-Deckert, 1995). Cette liaison induit la phosphorylation des tyrosines 239, 240 et 317 dans le domaine CH de Shc, entraînant son association avec la protéine Grb-2. L'ensemble de ces protéines forment un complexe avec SOS appelé p21^{ras} guanine nucleotide exchange factor (Sasaoka et al., 1996). La liaison Shc-Grb2 est médiée par le domaine SH2 de Grb2 qui présente une affinité pour les résidus tyrosine phosphorylés dans le domaine CH de Shc (Salcini et al., 1994) (**figure 7**).

La protéine Raf activée phosphoryle à son tour la protéine MEK, kinase à double spécificité phosphorylant des sérine/thréonine et/ou tyrosine. Elle active ensuite par phosphorylation les protéines de la famille ERKs (Extracellular signal-regulated kinases) qui sont des sérine/thréonine kinases, ERK-1 et ERK-2. Cette activation des protéines ERKs provoque la phosphorylation de substrats cytoplasmiques puis la translocation de ces derniers dans le noyau pour aboutir à l'activation de divers facteurs de transcription (c-Myc, ETS, CREB, AP1) contrôlant l'expression de nombreux gènes impliqués dans divers processus biologiques (**figure 7**).

Introduction

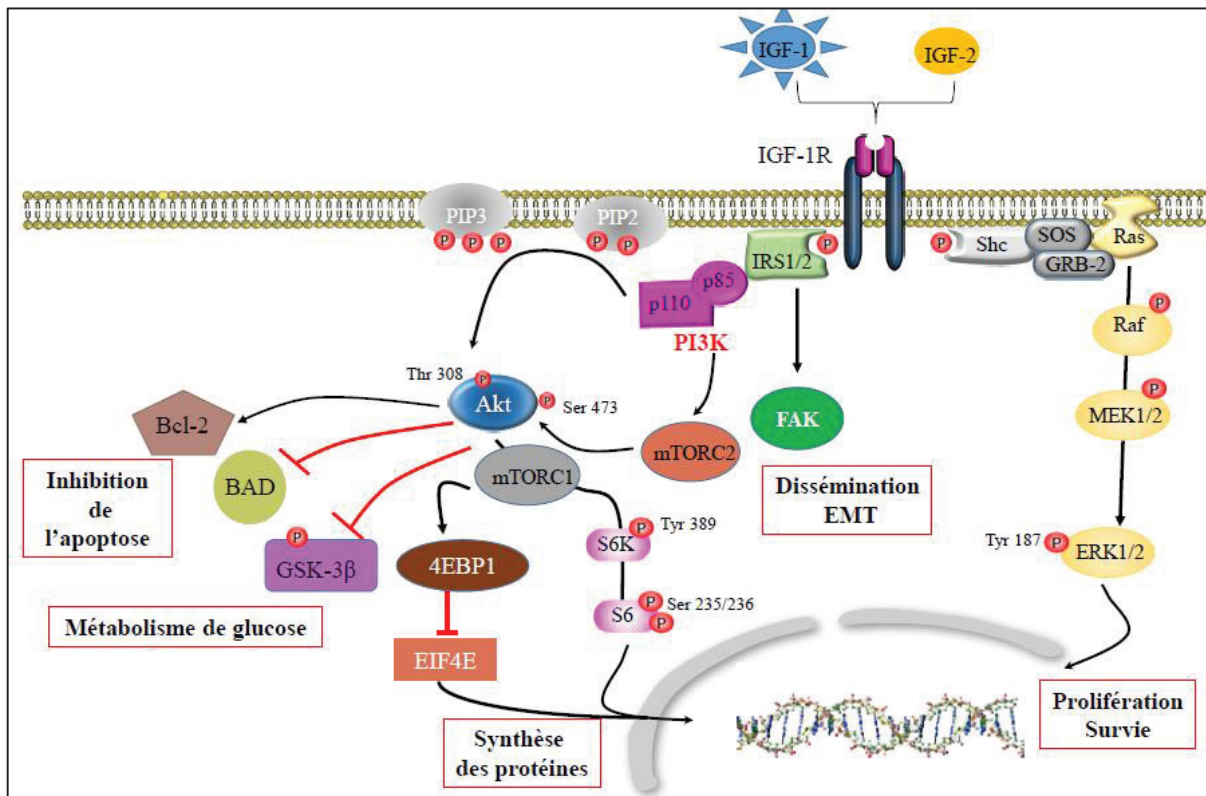


Figure 7 : Les voies de signalisation activées par IGF-1R. Les IGF-1/2 fonctionnent comme un ligand pour interagir avec le récepteur d'IGF-1, IGF-1R, situé dans la membrane cellulaire, ce qui conduit à l'autophosphorylation et au recrutement des protéines adaptatrices IRS-1/2 et Shc. L'interaction d'IRS-1 et IRS-2 avec IGF-1R induit l'activation de la phosphoinositide 3-kinase (PI3K) de classe I. PI3K convertit le PIP2 en PIP3, second messager lipidique. Akt est activée par PDK1 et par le complexe mTORC2 contenant mTOR, ce qui entraîne la phosphorylation au niveau de la thréonine 308 (Thr308) et de la sérine 473 (Ser473), respectivement. Akt activée régule ensuite les molécules de signalisation en aval qui inhibent le complexe mTORC1 et engendrent la phosphorylation de S6K1/2 et 4E-BP1, Bad et Bcl-2. Ces molécules en aval sont impliquées dans plusieurs processus cellulaires, notamment la synthèse des protéines, le métabolisme du glucose et la survie des cellules. En parallèle, l'activation de Shc induit l'activation de la voie Ras/MAP kinase, ce qui entraîne une prolifération cellulaire accrue.

De fait, cette cascade de signalisation aboutit à la génération d'effets prolifératifs et anti-apoptotiques dans de nombreux types cellulaires (Kolch, 2000). A titre d'exemple, ERK phosphoryle la thréonine 125 de la forme zymogène de la protéine pro-apoptotique caspase-9 et empêche par la suite le changement de sa conformation en caspase-9 active, améliorant ainsi la survie cellulaire (Allan et al., 2003) (**figure 8**). A noter que la protéine Grb2 peut également être recrutée par IRS-1, une autre protéine adaptatrice d'IGF-1R (Kim et al., 1998).

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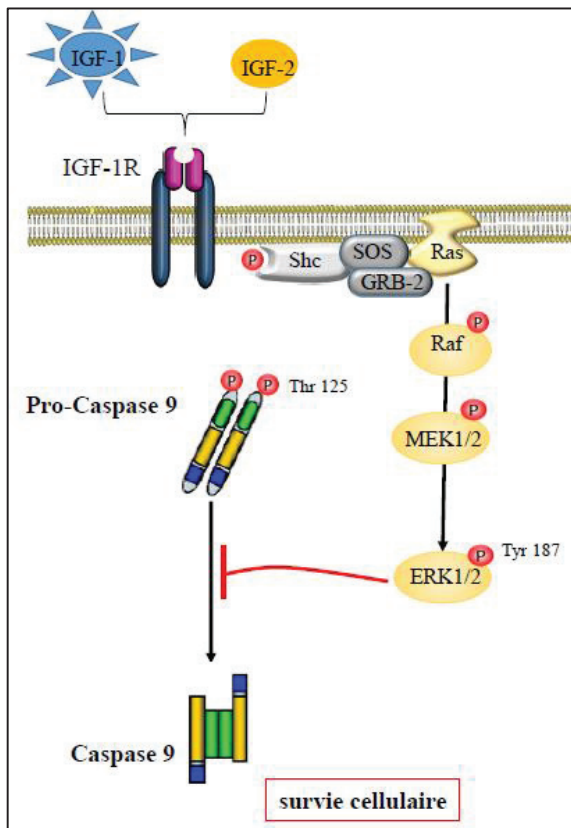


Figure 8 : Inhibition de la caspase-9 par la phosphorylation sur la Thr 125 par ERK/MAPK.

Outre la cascade de signalisation Ras/Raf/MEK/ERK, l'activation d'IGF-1R peut déclencher la voie PI3K/Akt à travers la protéine adaptatrice IRS-1. (Lavan, 1997; Miralpeix et al., 1992; Sun et al., 1991). Cette dernière est une protéine de grande taille (> 1 200 aa) composée d'un domaine PH (domaine d'homologie avec la pleckstrine) qui joue le rôle de module de liaison entre le récepteur, IGF-1R, et le domaine de liaison à la phosphotyrosine (PTB) de l'IRS-1 pour se fixer sur le site «d'accueil» de la tyrosine, Y 950, phosphorylée, ou pour les triplets Y 1131, Y 1135 et Y 1136 d'IGF-1R (Hennessy et al., 2005; Kooijman et al., 2006; Sun et al., 1991). L'IRS-1 est phosphorylée par IGF-1R au niveau de son extrémité carboxy-terminale, sur deux tyrosine, Y 612 et 632. Etant donné que l'IRS-1 agit en se liant à des molécules de transmission de signal en aval, elle recrute dans son motif de liaison, pYXXM, la sous-unité régulatrice p85 de la phosphoinositide 3-kinase (PI3K), qui active ensuite la sous-unité catalytique p110 (Copps and White, 2012). La PI3K ainsi activée phosphoryle le phosphatidylinositol-4,5-biphosphate, PIP2, lié à la membrane plasmique pour générer du phosphatidylinositol-3,4,5-triphosphate, PIP3, entraînant à son tour le recrutement de la 3-phosphatidylinosotide-dépendante kinase, PDK-1, au niveau membranaire (Hennessy et al., 2005). La PDK-1 active ensuite d'autres protéines kinases

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situées en aval, notamment, p70S6 (p70^{S6K1}), Protéine Kinase C (PKC) et Akt (Cianfarani et al., 2007b). A propos de la protéine kinase, Akt, elle va être phosphorylée par PDK-1 sur la thréonine 308, T308, pour être partiellement active. Pour obtenir une protéine Akt totalement active, elle doit subir une phosphorylation supplémentaire sur la sérine 473, S 473, processus qui est assuré par le complexe protéique mTORC2 (Mechanistic target of rapamycin, dénommé auparavant mammalian target of rapamycin) (Mora et al., 2004) (**figure 7**). mTORC1 qui est différent de mTORC2 est un autre acteur important de la voie PI3K/Akt. L'activation de mTORC1 se fait par action d'Akt sur le complexe protéique TSC2/TSC1 (Tuberous Sclerosis Complex) qui se comporte comme un suppresseur de tumeur (van Slegtenhorst et al., 1997). La protéine Akt activée va phosphoryler la protéine TSC2 altérant l'activité GTPasique du complexe TSC2/TSC1, ce qui permet à la petite protéine G, Rheb (ras homolog enriched in brain), de s'accumuler sous la forme Rheb-GTP et d'activer mTORC1 (Gao et al., 2002; Inoki et al., 2002). mTORC1 augmente la synthèse des protéines et favorise la croissance cellulaire et la progression du cycle cellulaire principalement par l'intermédiaire des effecteurs p70^{S6K1} et 4E-BP1 (**figure 7**) (Manning and Cantley, 2007).

La progression des cellules au cours des différentes phases du cycle cellulaire est due à l'activité de complexes cdk-cyclines (cyclin-dependent-kinases/cyclines). Il existe différentes cdks et cyclines durant les phases du cycle cellulaire. L'activation d'IGF-1R provoque une augmentation de l'expression des cyclines D1, A et B, impliquées, respectivement, lors des phases G1/S, S/G2 et M et de cdks, (Furlanetto, Harwell, & Frick, 1994) et pour revue (Samani et al., 2007). De même, IGF-1R activé peut diminuer la transcription d'inhibiteurs de cdk, cdkis, comme p27^{KIP1} (Coats et al., 1996). L'activation de la voie de signalisation PI3K/Akt par IGF-1R permet aux cellules de progresser au cours du cycle cellulaire en augmentant le niveau de cycline D1 *via* mTORC1 qui induit la traduction de cette cycline (Muise-Helmericks et al., 1998) L'activation d'IGF-1R permet aussi d'activer des mécanismes de réparation de l'ADN par l'enzyme Rad51, enzyme clé du processus de recombinaison homologue (Baumann and West, 1998) ; Trojanek et al., 2003). Par conséquent IGF-1R est un médiateur important pour maintenir l'intégrité du génome en réponse à IGF-1. De plus, IGF-1R est impliqué dans la protection de l'apoptose (Kulik et al., 1997). Il provoque, *via* la protéine Akt, la phosphorylation de la protéine pro-apoptotique Bad (Datta et al., 1997). Cette phosphorylation empêche l'interaction entre Bad et la protéine anti-apoptotique Bcl-xL, par conséquent Bcl-xL exerce son rôle de protecteur de mort cellulaire (Datta et al., 1997;

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Zha et al., 1996). L'activation d'Akt est aussi associée à l'inhibition de l'apoptose en phosphorylant d'autres cibles protéiques telles que la procaspase 9 (Kulik et al., 1997) et les protéines FOXO (Dijkers et al., 2002) et pour revue (Hennessy et al., 2005). En outre, la voie de signalisation PI3K/Akt augmente l'expression de protéines anti-apoptotiques, telles que Bcl-2, Bcl-xL (Minshall et al., 1997; Párrizas and LeRoith, 1997), l'activation de facteurs de transcription induisant la survie cellulaire, NF- κ B (par le biais de la phosphorylation d'IKK) et CREB (Girnita et al., 2014). En plus des effets sur la régulation de la prolifération et la survie, Akt contrôle le métabolisme cellulaire. L'inhibition de la glycogène synthase kinase-3 (GSK-3) par Akt entraîne une augmentation de la glycolyse (Manning et Cantley, 2007).

Les IGFs favorisent la migration et l'invasion cellulaire par les voies susmentionnées en augmentant l'expression de protéines impliquées dans l'adhésion cellulaire et le microenvironnement, tel que les MMPs (Matrix Metalloproteinases) et le VEGF (Zhang et al., 2002). IGF-1R est également impliqué dans la dégradation de la matrice extracellulaire et la vascularisation tumorale (Long et al., 1998).

3. Implication d'IGF-1R dans le cancer du sein

IGF-1 est un facteur mitogène essentiel à la formation de bourgeons terminaux et à la morphogenèse canalaire au cours du développement mammaire (Ruan et al., 1999). En outre, IGF-1 stimule la prolifération accrue des cellules épithéliales mammaires et leur survie favorisant ainsi la tumorigenèse mammaire (Tian et al., 2012). Pour cela, les mécanismes d'action et effets biologiques d'IGF-1R dans la prolifération, la survie, la résistance aux traitements et à la formation de métastases dans le cancer du sein a toujours été étudiée. Dans ce contexte, de nombreuses études ont investi d'une part la corrélation qui existe entre la surexpression d'IGF-1R et le développement des différents types de cancer y compris le cancer du sein (**figure 9**) et d'autre part le rôle de sa signalisation dans la promotion de la prolifération des lignées cancéreuses mammaires (Christopoulos et al., 2015; Gross and Yee, 2003a; Karey and Sirbasku, 1988a; Riss et al., 1988).

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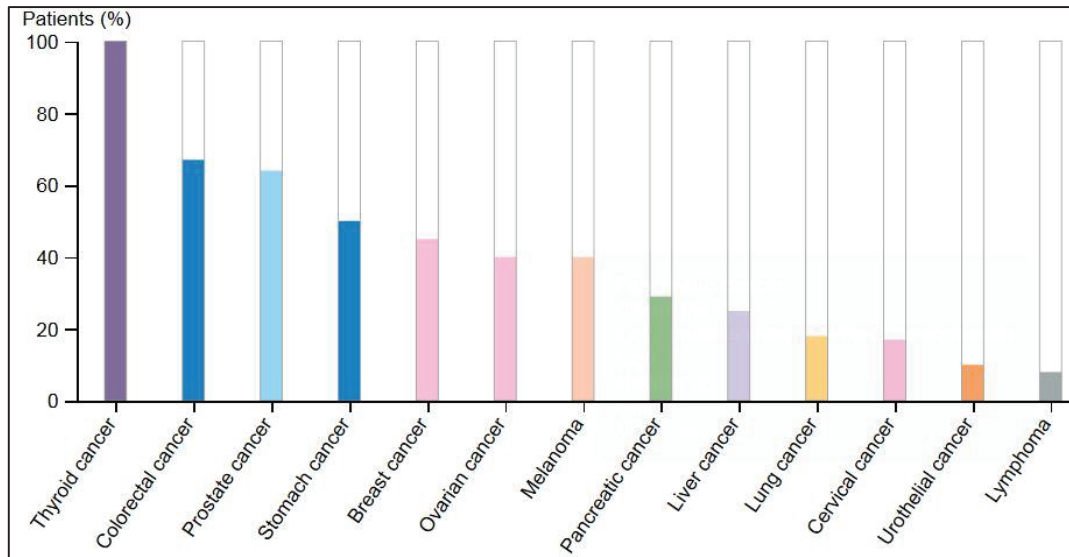


Figure 9 : Expression d'IGF-1R dans différents types de cancer (proteintlas.org).

Des modèles de xénogreffes tumorales mammaires ont montré que le système de signalisation d'IGF-1 joue un rôle important dans la stimulation de la croissance tumorale *via* des mécanismes paracrines et/ou autocrines (Karamouzis and Papavassiliou, 2012). De plus, des modifications génomiques du système IGF-1 sont relevées dans 15 % des cancers du sein comme le stipule « The Cancer Genome Atlas » (TCGA) (Réseau du génome du cancer, 2012). L'analyse du niveau d'ARNm a montré qu'il existe des altérations moléculaires au niveau des membres de la famille d'IGFs dans 45,3 % des cancers du sein. 9 % des tumeurs présentent IGF-1R surexprimé, ou muté ou amplifié et 50 % des tumeurs du sein expriment une forme constitutivement active d'IGF-1R (Farabaugh et al., 2015)

De nombreuses études sont menées pour déterminer la corrélation entre l'expression d'IGF-1R et les différents sous-types de cancer du sein. IGF-1R est plus fortement surexprimé dans les cancers mammaires de type luminal B que ceux exprimant HER2. Cette surexpression est corrélée à un meilleur pronostic, contrairement aux résultats des patients ayant un cancer de type luminal A qui ne montrent aucune corrélation spécifique (Kolacinska et al., 2012; Yerushalmi et al., 2012). Au contraire, la surexpression d'IGF-1R, trouvée dans 22 à 46 % des tumeurs triple négatives, est corrélée à une courte survie des patientes (Farabaugh et al., 2015). Dans le cas de cancer du sein précoce, l'expression d'IGF-1R est corrélée à des marqueurs de bon pronostic et est exprimé de manière différentielle avec un impact pronostique qui varie d'un sous-type à l'autre (Yerushalmi

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et al., 2012). En dépit de la surexpression d'IGF-1R dans les cancers du sein, l'expression de cette protéine ne peut pas être utilisée en tant que facteur pronostique car il n'existe pas de seuil défini pour cette surexpression (Karamouzis and Papavassiliou, 2012).

Il est rapporté dans la littérature que l'activité du promoteur du gène *IGF-1R* est contrôlée par BRCA1 (Abramovitch and Werner, 2003, 2003; Maor et al., 2000; Sarfstein et al., 2006). Dans ce contexte-là, il est observé dans les tumeurs déficientes en BRCA1, une surexpression d'IGF-1 et d'IGF-1R favorisant la survie cellulaire et inhibant l'apoptose. En outre, le ciblage de l'activité d'IGF-1R réduit la prolifération des cellules ayant BRCA1 muté. L'ensemble de ces résultats suggère que la signalisation d'IGF-1 est impliquée dans la prolifération et la survie des cellules cancéreuses présentant une mutation de BRCA1 (Motallebnezhad et al., 2016).

Toujours dans le cancer du sein, les effets anti-apoptotiques d'IGF-1 sont médiés par IGF-1R, responsable de l'induction de plusieurs voies de signalisation anti-apoptotiques, y inclus celle de PI3K/Akt, qui phosphoryle et inactive la protéine pro-apoptotique Bad (Motallebnezhad et al., 2016; Peruzzi et al., 1999; Prisco et al., 1999).

Le rôle de l'IGF-1R dans la résistance à l'apoptose a été démontré dans de nombreuses études. Le traitement des fibroblastes et des cellules cancéreuses du sein par IGF-1 donne lieu à un phénomène de résistance à la chimiothérapie et protège les cellules contre la mort induite par les radiations. La surexpression d'IGF-1R est aussi corrélée à la récurrence du cancer du sein après une radiothérapie et une tumorectomie (Gross et Yee, 2003 ; Bhattacharya et al., 2018).

Les métastases sont une cause majeure de décès chez les femmes atteintes de cancer du sein. Ainsi, l'identification des facteurs qui est impliquée est cruciale pour le développement de stratégies thérapeutiques. Des études antérieures ont montré que la signalisation d'IGF-1 intervient dans les métastases du cancer du sein en stimulant la migration de lignées cancéreuses mammaires par l'activation d'IGF-1R *in vitro* (Brodt et al., 2001; Karey and Sirbasku, 1988b; Riss et al., 1988; Samani et al., 2007; Gross et Yee, 2003). Il a été démontré que la suppression de l'expression d'IGF-1R entraîne l'inhibition de la formation de métastases, de l'invasion et de l'adhésion des cellules cancéreuses du sein (Elbaz et al., 2017; Li et al., 2017; Yan et al., 2017; Dunn et al., 1998). Sachdev et al. ont montré qu'un dominant négatif d'IGF-1R empêche la formation métastatique de cellules cancéreuses chez l'homme (Sachdev et al., 2004). Les voies de signalisation en aval d'IGF-1R contribuent aussi au développement de métastases (Wang et al., 2012c). De plus, Saldana et al.

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ont démontré que l'inhibition de la voie de signalisation d'IGF-1R entraîne une réduction des métastases du cancer du sein au niveau du cerveau (Saldana et al., 2013).

4. IGF-1R, cible potentielle pour le traitement du cancer du sein

IGF-1R est une cible thérapeutique bien décrite dans le cancer du sein et de nombreux essais cliniques portant sur ce récepteur ont été menés (Ekyalongo and Yee, 2017). Le ciblage du système d'IGF-1, en particulier l'IGF-1R, et l'inhibition de leurs voies de signalisation sont nécessaires pour inhiber la croissance tumorale et augmenter les possibilités d'interventions thérapeutiques. Le ciblage repose principalement sur quatre stratégies thérapeutiques : les anticorps monoclonaux (mAb) dirigés contre IGF-1/-2 et IGF-1R, les inhibiteurs de l'activité tyrosine kinase (TKIs) d'IGF-1R, les siRNA et l'IGF-1R tronqué (**figure 10**). Ces différentes stratégies sont abordées succinctement ci-dessous.

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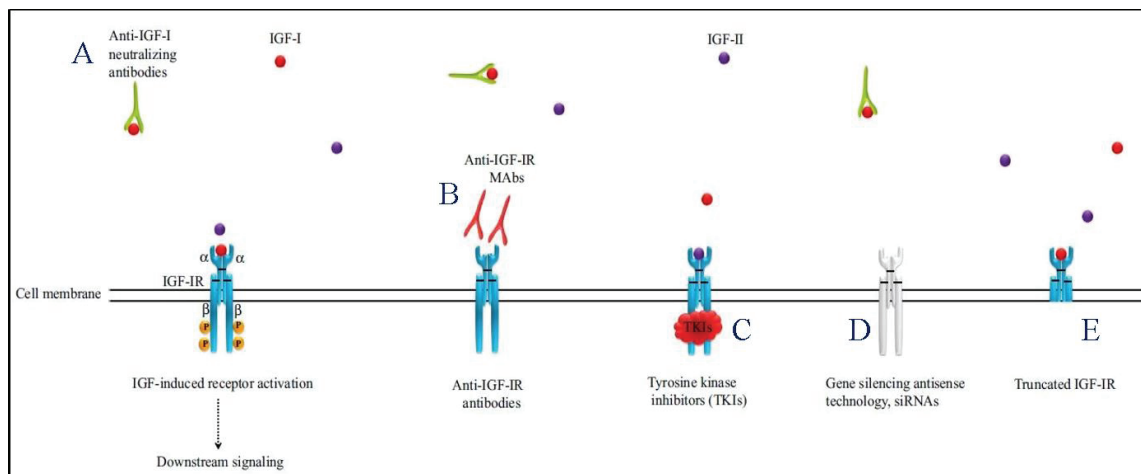


Figure 10 : Thérapies anticancéreuses ciblant IGF-IR. Représentation des différentes stratégies thérapeutiques ciblant IGF-IR dans le cancer; **A** : Anticorps neutralisant les ligands, **B** : anticorps anti-IGF-IR, **C** : inhibiteurs de l'activité de la tyrosine kinase, **D** : inhibition du gène et **E** : récepteur tronqué (Motallebnezhad et al., 2016).

5.1. Anticorps monoclonaux

La liaison des ligands IGFs à leur récepteur conduit à l'activation de ce dernier, ce qui déclenche les voies de signalisation prolifératives en aval augmentant ainsi l'identité tumorigène de la cellule. Donc, le blocage ou la réduction de la liaison ligand-récepteur et/ou le blocage de l'activation du récepteur peut jouer un rôle bénéfique dans le traitement du cancer. Dans ce cadre-là, les thérapies ciblées enregistrées par clinicaltrials.gov représentent 625 essais cliniques et, comme quelques traitements uniquement reposent sur l'anticorps neutralisant le ligand, seuls deux essais mettent en jeu un mAb contre IGF-1/2.

MEDI-573 est un double anticorps neutralisant IGF-1 et IGF-2. Il inhibe les voies de signalisation induites par les IGFs en empêchant leurs liaisons aux récepteurs IGF-1R et/ou IR (Gao et al., 2011). Des études ont combiné MEDI-573 avec d'autres inhibiteurs de signalisation, tels que les inhibiteurs de mTOR, et ont montré une diminution significative du taux de croissance tumorale (Iams and Lovly, 2015) (**figure 11**).

Un autre anticorps ciblant IGF1 et 2, appelé BI836845 ou Xentuzumab, est actuellement le sujet d'essais cliniques visant à étudier ses effets sur la croissance tumorale humaine. La phase I a montré une diminution de l'activation du récepteur hybride IGF-1R/IR et un faible taux de prolifération cellulaire. Ces résultats ont encouragé les essais cliniques à passer en phase II où le Xentuzumab fut associé à l'Évérolimus (inhibiteur de mTOR) et à l'Exémestane (Aromasine®)

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(inhibiteur de l'aromatase) chez des patients atteints d'un cancer métastatique (Ekyalongo et Yee, 2017; Ireland et al., 2018).

Outre les anticorps monoclonaux utilisés pour réduire les taux d'IGFs, des antagonistes de l'hormone GHRH (Growth Hormone-Releasing Hormone) comme JV-1-38 et des antagonistes de l'hormone de croissance GH, tel que le Pegvisomant (Somavert[®]), constituent également une stratégie supplémentaire pour obtenir le même effet anticancéreux (Motalebnezhad et al., 2016).

Des anticorps monoclonaux ciblant IGF-1R sont aussi produits afin de bloquer son activité et d'induire son internalisation puis sa dégradation ultérieure. De nombreux candidats présentent une activité préclinique encourageante, à savoir le Ganitumab, le Figitumab, le Dalotuzumab entre autres (Simpson & Bogenrieder, 2017). Bien que ces anticorps entraînent une inhibition d'IGF-1R et/ou du récepteur hybride IGF-1R/IR, ils n'inhibent pas toujours l'activation du récepteur IR sous l'effet de l'insuline ou d'IGF-I (Gao et al., 2011). Par conséquent, la signalisation physiopathologique d'IR persiste limitant la prise en charge de ce traitement. De plus, le traitement par les anticorps anti-IGF-1R entraîne une hyperglycémie et un syndrome métabolique, probablement dus à la perturbation de l'homéostasie d'IGF-1R et à l'élévation ultérieure des hormones de croissance. Ceci induit une résistance à l'insuline, d'où une augmentation du taux d'insuline et une activation potentielle de son récepteur (Ekyalongo and Yee, 2017). Les limites envisagées au cours de l'utilisation de ces anticorps révèlent la nécessité de suivre d'autres stratégies thérapeutiques. La **figure 11** montre toutes les thérapies combinatoires possibles utilisées ou pouvant être utilisées dans l'avenir pour cibler les voies de signalisation complexes d'IGF-1R.

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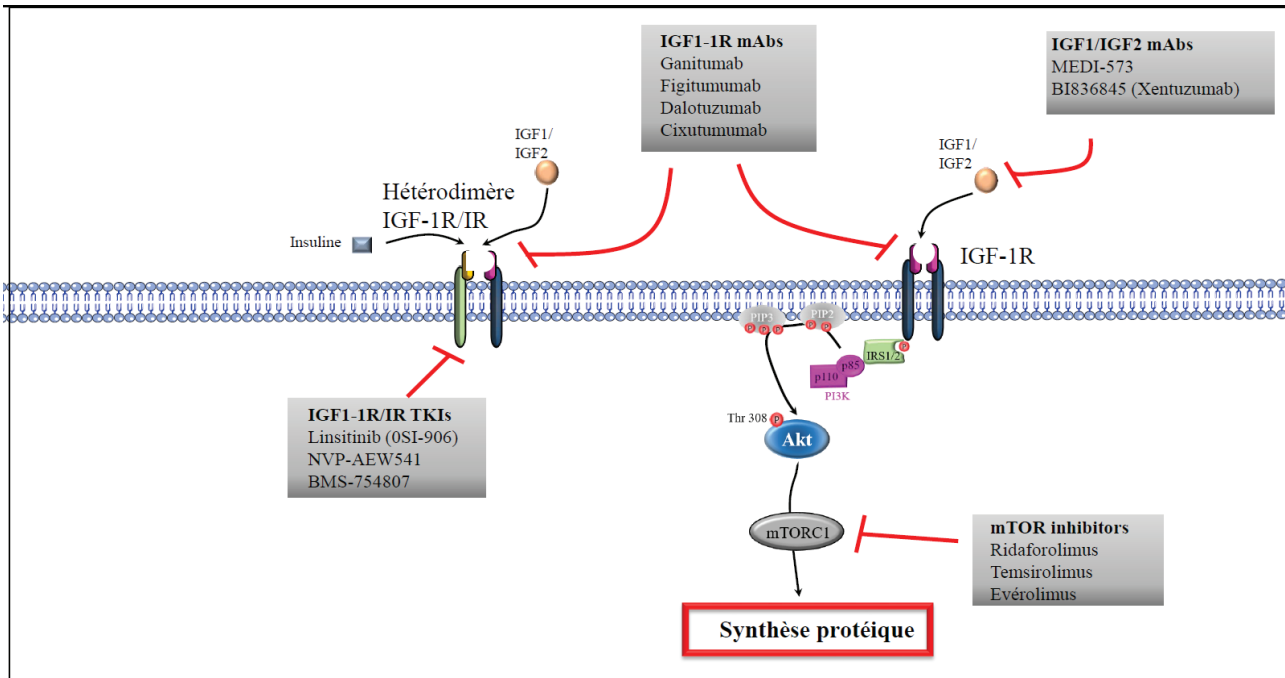


Figure 11 : Thérapie ciblée de l'expression et de l'activité d'IGF-1R dans le cancer du sein. Différents traitements anti-IGF-1R inhibent l'activité d'IGF-1R et ses cascades effectrices en aval, notamment les voies PI3K/Akt/mTOR/S6K et Ras/Raf/MEK/ERK ; d'après (Ochnik and Baxter, 2016)

5.2. Inhibiteurs tyrosine kinase (TKIs)

Cibler l'activité tyrosine kinase d'IGF-1R permet d'inhiber les voies de signalisation activées en aval de ce dernier. Les TKIs sont de petites molécules qui entrent en compétition avec l'ATP pour le site de liaison correspondant sur le domaine catalytique du récepteur. Il en résulte une inhibition de l'autophosphorylation du récepteur empêchant la propagation du signal prolifératif.

Plusieurs études ont montré que l'utilisation des TKIs inhibe efficacement la prolifération cellulaire et la croissance tumorale tout en renforçant l'induction de l'apoptose. En effet, des essais cliniques sont entamés pour étudier l'efficacité du Linsitinib (OSI-906) et du BMS-754807 chez l'homme (Carboni et al., 2009; Law et al., 2008; Wittman et al., 2009). Les inhibiteurs ciblant l'activité d'IGF-1R peuvent également cibler IR en raison de l'homologie entre les deux types de récepteurs. Ainsi, les TKIs comme le Linsitinib, le BMS-754807 et le KW-2450 sont appelés inhibiteurs doubles IGF-1R/IR (Simpson et al., 2017). Ekyalongo et Yee ont récemment décrit que ces inhibiteurs doubles ne sont pas développés plus en avant vers des essais cliniques de phase III, probablement en raison de leurs toxicités métaboliques et de leurs effets néfastes sur l'absorption

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du glucose sanguin (Ekyalongo and Yee, 2017). Le tableau suivant résume les problèmes rencontrés dans ces études (**tableau 4**).

Tableau 4 : Toxicités associées aux traitements anti-IGF-IR (-) : Absence

Compound type				
	Estimated enrolled patients	Metabolism and nutrition disorders (grade 3 and 4)	Hyperglycemia (grade 3 and 4)	Clinical trial evolution
<i>IGF-IR mAbs</i>				
Figitumumab (CP-751,871)	115	47.91%	52.08.48%	Terminated at Phase II
Cixutumumab (A12)	19	43.75%	56.24%	Terminated at Phase II
Dalotuzumab (MK0646)	11	(-)	(-)	Terminated at Phase II
AVE1642	18	(-)	(-)	Terminated at Phase II
<i>Non-ATP antagonist TKIs</i>				
Linsitinib (OSI-906)	11	70.58	29.41%	Terminated at Phase II

5.3. Inhibition du gène IGF-1R et du récepteur tronqué

En plus du ciblage au niveau d'IGF-1R, un ciblage au niveau de son ARNm est réalisé par différentes approches. De fait, l'inhibition de son expression et de sa fonction par les microARN et les siRNA est une autre approche pour cibler ce récepteur. Elle consiste à inhiber l'expression du gène en utilisant des petits ARN interférents (siRNA : small interfering RNA) sans affecter le récepteur IR. Ceci permet aux siRNA de bloquer la signalisation d'IGF-1 et d'améliorer par la suite la sensibilité des cellules tumorales de différents types de cancer à la radiothérapie et à la chimiothérapie, favorisant ainsi l'apoptose cellulaire et présentant une nouvelle voie thérapeutique (Macaulay, 2004; Pian et al., 2018; Riedemann and Macaulay, 2006). Durfort et al. ont montré dans des cancers mammaires que l'utilisation de siRNA ciblant IGF-1R inhibe la croissance tumorale, réduit la phosphorylation de protéines impliquées dans les voies de signalisation en aval, comme Akt et bloque les cellules en phase G1 du cycle cellulaire. De plus, l'utilisation de ces siRNA a conduit à une réduction du volume tumorale et une activation de la réponse immunitaire

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antitumorale dans un modèle murin greffée par des lignées du sein exprimant IGF-1R (Durfort et al., 2012). De même, le ciblage de l'expression d'IGF-1R par des micro-ARN spécifiques s'est montré efficace pour inhiber la croissance tumorale, les métastases et l'invasion (Shen et al., 2012; Wang et al., 2012a).

En outre, l'utilisation des dominants négatifs, tel que les IGF-1R tronqués, a montré une efficacité élevée pour cibler les récepteurs en question. Leur mécanisme d'action implique la séquestration du ligand et la formation de chimères inactives avec le récepteur endogène (Bähr and Groner, 2004; Brahmkhatri et al., 2015).

Généralement, l'efficacité clinique du ciblage d'IGF-1R dépend de plusieurs facteurs majeurs, comme le rôle d'IGF-1R lui-même dans les tumeurs, l'inhibition potentielle des siRNA et des thérapies antisens *in vivo* et l'activation des autres voies de signalisation dues à la perte d'IGF-1R (Macaulay, 2004).

5.4. Thérapie combinatoire

Les traitements ciblant IGF-1R seul dans le cancer du sein n'ont pas répondu aux attentes des chercheurs. Pour cette raison, de nombreux essais cliniques ont été récemment réalisés, associant les thérapies ciblées contre IGF-1R à d'autres chimiothérapies et aux anti-œstrogènes. Le rôle d'IGF-1R dans les mécanismes de résistance à la chimiothérapie étant relativement bien établi, alors la modulation de son expression au cours de la chimiothérapie semble être une stratégie pertinente dans le traitement du cancer du sein. En effet, en 2013, les essais de phase III multicentriques randomisés, NEOZOTAC, ont montré que les patientes atteintes d'un cancer de sein de type HER2 négatif présentent un meilleur résultat clinique lorsque l'expression d'IGF-1R est réduite pendant et après la chimiothérapie néo-adjuvante (De Groot et al., 2016). Dans le cas de lignées triple négatives (TNBC : Triple-Negative Breast Cancer), de nombreuses études précliniques ont montré que le ciblage simultané des axes IGF-1R et PI3K est capable de réduire la viabilité de nombreuses lignées TBNC. De plus l'utilisation d'inhibiteur double d'IGF-1R et d'IR (Linsitinib) montre une forte synergie avec des inhibiteurs de PI3K. Cette combinaison thérapeutique pourrait se révéler bénéfique pour les TNBC (de Lint et al., 2016; Saldana et al., 2013; Yang and Yee, 2012). De plus, cibler la voie mTOR (par le Ridaforolimus) en même temps

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qu'IGF-1R a également potentialisé l'activité anti-tumorale dans ces cellules (Di Cosimo 2015 ; Baselga et al., 2017).

Il convient encore de mentionner les effets de la combinaison des thérapies dirigées contre IGF-1R et les récepteurs à des facteurs de croissance, comme l'EGFR, le VEGFR et le HER2, en raison du « crosstalk » entre l'ensemble de ces récepteurs et leurs voies de signalisation induites. A titre d'exemple, une thérapie ciblant HER2 (le Lapatinib et le Nélatinib) combinée à un inhibiteur d'IGF-1R (NVP-AEW541) ou siRNA a permis de réduire la résistance des cellules et d'améliorer l'effet anti-tumoral du traitement dans le cas du cancer du sein de type HER2 + (Simpson et al., 2017). En outre, des études ont suggéré qu'en plus d'inhiber les voies PI3K/Akt/mTOR et Ras/Raf/ERK/MAPK, le co-ciblage d'IGF-1R et des CDK4/6 et S6K pourrait être utile pour atténuer les voies de signalisation prolifératives induites par IGF-1R (Ochnik and Baxter, 2016). L'ensemble des données exposées permet de conclure que le ciblage de l'axe IGF-1/IGF-1R est une stratégie thérapeutique attrayante pour tous les cancers exprimant ce récepteur et en particulier pour le cancer du sein, en raison de ses implications dans la progression de la maladie et la résistance aux traitements thérapeutiques. Cependant, le traitement ciblant IGF-1R seul présente des effets secondaires néfastes se manifestant par des maladies métaboliques et des toxicités, d'où la nécessité de combiner des inhibiteurs d'IGF-1R avec d'autres thérapies comme la chimiothérapie et/ou les anti-œstrogènes. Malheureusement, définir une stratégie thérapeutique appropriée pour co-cibler IGF-1R et d'autres composants de signalisation ne fonctionnent pas parfaitement. Cela est dû à la complexité de sa signalisation et le « crosstalk » avec les autres RTKs et leurs cascades de signalisation. En revanche, l'association d'anti-IGF-1R avec des inhibiteurs de la PI3K et d'autres chimiothérapies dans TNBC, ainsi que des anti-œstrogènes dans le cancer du sein sensible aux hormones, a donné des résultats prometteurs. Pour cette raison, nous pensons que le ciblage spécifique du récepteur pourrait avoir un effet bénéfique dans le cancer du sein fortement corrélé à son expression. Il est alors nécessaire de poursuivre les essais cliniques afin de définir une stratégie de ciblage appropriée afin d'atteindre les meilleurs résultats cliniques en fonction du type moléculaire du cancer du sein.

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III. Les proprotéines convertases (PCs)

Les proprotéines convertases (PCs) de la famille des subtilisines sont des endoprotéases à sérine dépendante du calcium. Elles jouent un rôle actif dans les modifications post-traductionnelles de nombreuses protéines inactives pour les rendre actives. Ces dernières peuvent avoir une importance primordiale dans le développement et la progression du cancer. Cette famille de protéases comprend 7 membres principales, PC1/3 (PSCK1), PC2 (PCSK2), furin (PCSK3), PC4 (PCSK4), PC5/6 (PCSK5), PACE4 (PCSK6), PC7/8 (PCSK7). La huitième proprotéine convertase, SKI-1 (également connue sous le nom de protéase de site 1 (S1P)), est une nouvelle subtilisinelike homologue à la pyrolysine et finalement la neuvième proprotéine convertase, PCSK9, était à l'origine connue sous le nom de convertase 1 (**figure 12**).

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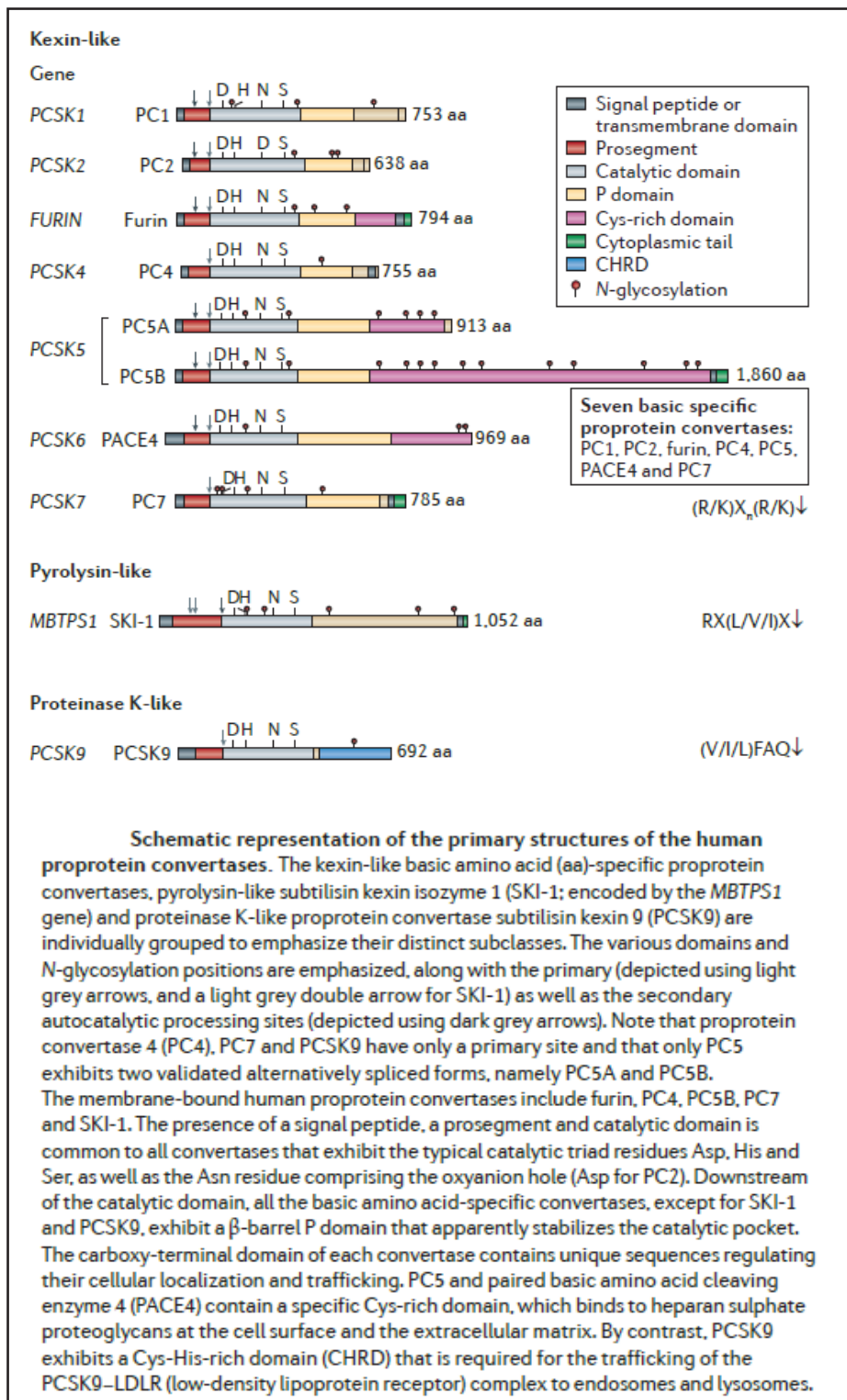


Figure 12 : Représentation schématique des structures primaires des proprotéines convertases humaines (Seidah and Prat, 2012).

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L'expression, la localisation intracellulaire et l'activité de la furine dans les cellules cancéreuses semblent être régulées par différents facteurs. A titre d'exemple, l'hypoxie a été rapportée induire la transcription de la furine (McMahon et al., 2005; Silvestri et al., 2008). De plus, dans diverses conditions, elle peut être régulée par d'autres facteurs, tels que le facteur de transformation transformant $\beta 1$ (TGF- $\beta 1$) (Blanchette et al., 1997, 2001), signal de l'hormone thyroïdienne (Chen et al., 2008) ou prostaglandine-endoperoxyde synthase 2 (PTGS2) (Brant and Leikauf, 2014).

La dérégulation de l'activité de ces enzymes est associée à de nombreuses pathologies telles que les endocrinopathies (Jackson et al., 1997), la maladie d'Alzheimer (Bennett et al., 2000) et le cancer (Bassi et al., 2001a, 2001b; Khatib et al., 2002). En outre, elles sont surexprimées dans de nombreuses tumeurs malignes tels que la peau, la tête et le cou, l'ovaire, la prostate, le colon etc. (Bassi et al., 2017).

1. La proprotéine convertase furine (PCSK3)

La furine est une endoprotéase à sérine dépendante du calcium et est exprimée de manière ubiquitaire chez tous les vertébrés et de nombreux invertébrés. Elle est exprimée sous forme de zymogène de 794 acides aminés qui va subir deux étapes de clivage auto-catalytique pour donner une enzyme totalement active (Anderson et al., 1997). La furine a été identifiée comme le premier membre de la famille PC il y a 29 ans (van de Ven et al., 1990) . En raison de son importance biologique, elle est également le membre le plus étudié de cette famille d'enzymes.

1. a. Sa localisation et ses substrats

La furine mature est majoritairement trouvée au niveau de l'appareil de Golgi, à la surface des cellules, ainsi que dans les endosomes où elle peut cliver ses substrats (Molloy et al., 1999). A partir de l'appareil de Golgi, elle peut atteindre les endosomes et arriver à la surface cellulaire (Bosshart et al., 1994).

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De nombreux facteurs de croissance impliqués dans la prolifération tumorale sont activés par l'action de la furine, notamment le PDGF-A et -B ainsi que le TGF- β (Dubois et al., 1995; Siegfried

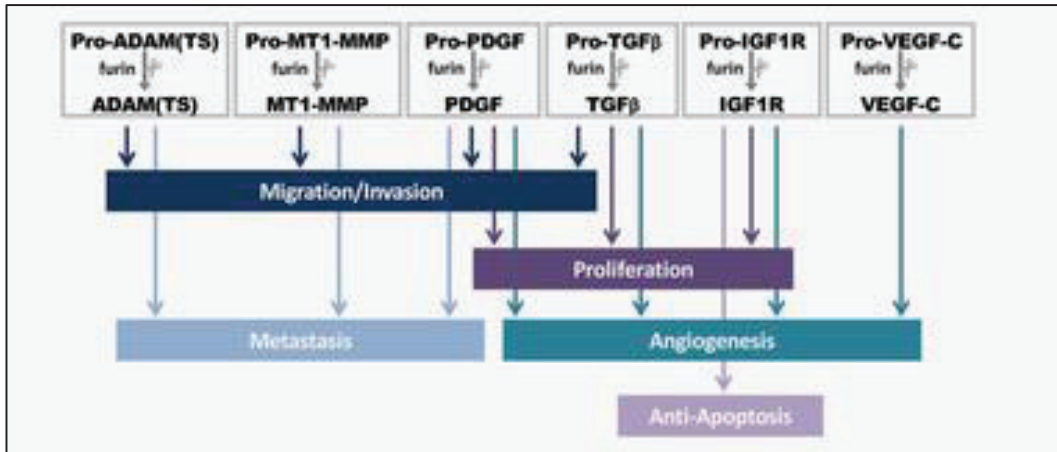


Figure 13 : Les substrats de furine et leur implication dans la progression tumorale. Représentation schématique de certaines protéines liées au cancer qui sont clivées par la furine et des PC similaires à la furine et leur pertinence dans des processus importants liés au cancer. ADAM, une désintégrine et une métalloprotéase; IGF-1R, récepteur du facteur de croissance 1 analogue à l'insuline; MT1-MMP, métalloprotéase matricielle à membrane de type 1; PDGF, facteur de croissance dérivé des plaquettes; TGF- β , facteur de croissance transformant β ; VEGF-C, facteur de croissance endothélial vasculaire C (Jaaks and Bernasconi, 2017).

et al., 2003, 2005). De plus, IGF-1R ainsi que ses ligands naturels IGF-1 et -2 sont clivés et activés pas la furine (Duguay et al., 1995; Khatib et al., 2001). En outre, une croissance tumorale efficace nécessite un apport suffisant en oxygène et en nutriments, soulignant l'importance d'une vascularisation adéquate. Dans ce contexte, VEGF-C ainsi que le VEGF-D sont clivés et activés par la furine pour stimuler la signalisation du VEGF et induire l'angiogenèse (Jaaks and Bernasconi, 2017; Joukov et al., 1997; McColl et al., 2007; Millauer et al., 1993)

Plusieurs autres substrats de furine pourraient jouer un rôle central dans la migration, la motilité et la formation de métastases des cellules cancéreuses. Dans ce contexte, la furine active la MT1-MMP de manière catalytique (Maquoi et al., 1998; Remacle et al., 2006; Yana and Weiss, 2000). La MT1-MMP est à son tour l'enzyme principale permettant d'activer la pro-MMP2 (Ohuchi et al., 1997; Sato et al., 1994) donnant lieu à une forme intermédiaire nécessitant un clivage supplémentaire intermoléculaire en MMP2 totalement active. D'autres études suggèrent que le traitement intracellulaire de la pro-MMP2 a lieu dans l'appareil de Golgi, le plus probablement par la furine (Cao et al., 2005). D'autres métalloprotéases MMPs impliquées dans la dégradation de la MEC sont synthétisées sous forme de zymogènes inactifs et semblent nécessiter

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un clivage par la furine pour obtenir leur pleine activité. Ceux-ci comprennent par exemple les MMPs d'adamalysine ADAM 12 et ADAM 17, également connues sous le nom de facteur de nécrose tumorale α convertase (TACE) (Loechel et al., 1998; Schlöndorff et al., 2000), et ADAMTS-1 (Kuno Terashima et al., 1999). On peut citer aussi le précurseur de l'hormone parathyroïdienne, précurseur du facteur de croissance TGF- β 1, proalbumine, pro-bêta-sécrétase, sous-unité bêta du facteur de croissance nerveuse (NGF) et facteur de von Willebrand (**figure 13**).

2. a. Importance de la furine dans les cancers

La tumorigénèse nécessite l'activité de nombreuses protéines qui participent au phénotype malin pour que la tumeur puisse proliférer, échapper à l'apoptose, assurer l'angiogenèse et activer des processus métastatiques (Hanahan and Weinberg 2011). La plupart de ces proprotéines convertases (PCs) sont surexprimées dans de nombreux types de cancers et lignées de cellules tumorales et ont une activité enzymatique importante favorisant le processus tumoral et ce en agissant à diverses étapes de ce processus, comme la prolifération, la migration etc. (**Tableau 5**) (Schlalken 1987, Mbikay 1997, Bassi et al., 2001a; Cheng et al., 1997; Couture et al., 2015). L'expression de la furine est associée à une forte agressivité dans plusieurs carcinomes. Notamment dans les cancers du poumon, un taux protéique élevé de furine a été observé et plus spécifiquement dans les carcinomes pulmonaires « non à petites cellules » (CBNPC). La furine a été proposée comme marqueur pour faire la distinction entre les CBNPC et les carcinomes du poumon à petites cellules (CPPC) (Schalken et al., 1987). Le faible niveau d'ARNm de furine dans les CPPC a été confirmé dans une étude de Mbikay et al. (Mbikay et al., 1997). Cependant, les deux études se limitent aux niveaux d'expression et ne commentent pas la relation fonctionnelle entre l'expression de la furine et l'état malin des types de cancer du poumon. Néanmoins, une autre étude utilisant un inhibiteur de furine, α 1-PDX, a montré que l'inhibition des PCs de type furine pourrait réduire la prolifération et la migration de cellules de de carcinome du poumon A549 (Ma et al., 2014). De plus dans des lignées de cancers gastriques, il a été démontré un lien entre l'expression de la furine et de PTH-rP (parathyroid hormone related peptide) et la croissance cellulaire. La furine est impliquée dans la production de la forme mature de PTH-rP (Nakajima et al., 2002). Cette forme mature a des effets biologiques quand elle est localisée dans le noyau. Elle confère une résistance à l'anoïkis, active la prolifération, la migration et l'invasion cellulaire dans

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diverses lignées tumorales (prostate, colon et sein) (Bhatia et al., 2009; Park and McCauley, 2012; Shen et al., 2004). Des niveaux élevés d'expressions d'ARNm et de protéine de la furine ont été trouvées dans des tumeurs de la tête et du cou. Ces niveaux sont corrélés avec l'expression élevée du taux de MT1-MMP mature et de l'agressivité des carcinomes épidermoïdes de la tête et du cou (HNSCC), mesurés par la présence de métastases chez les patients (Bassi et al., 2001a, 2001b). Des études complémentaires ont montré que l'inhibition de la furine par l' α 1-PDX diminue le potentiel invasif des lignées cellulaires HNSCC invasives *in vitro* et *in vivo* en réduisant la maturation de divers facteurs, tels que TGF- β , MT1-MMP et MMP2. De même, l'expression de la furine dans des carcinomes épidermoïdes de la langue est considérée comme un marqueur de la progression tumorale. De fait, dans les néoplasies préinvasives et invasives, une corrélation a été montrée entre une augmentation de l'expression de la furine et celle du VEGF (López de Cicco et al., 2004). La furine permet d'activer le VEGF favorisant ainsi la néoangiogénèse (McColl et al., 2007, Jaaks et al., 2016). Dans des lignées de cancer de l'ovaire, des taux élevés de furine ont également été trouvés. Cette surexpression semble liée à un mauvais pronostic chez les patientes (Page et al., 2007). De plus, il a été montré que l'expression simultanée de c-Myc, un régulateur clé de nombreuses voies oncogéniques et de la furine participent à accroître la prolifération cellulaire dans des lignées cancers de l'ovaire (Hasegawa-Minato et al., 2018). Cheng et al. ont étudié l'expression de six PCs dans des cellules cancéreuses du sein et ont indiqué que les taux d'ARNm de PC1/3, furine, PACE4 et PC7 étaient élevés dans la plupart des cas (Cheng et al., 1997). De plus, la surexpression exogène de furine augmente non seulement le taux de prolifération des cellules de la lignée de cancer du sein, MCF7, mais aussi la résistance de ces cellules au tamoxifène (Cheng et al., 2001). De même, Lapierre et al. ont utilisé l'expression exogène de la furine pour améliorer la connaissance de ses effets biologiques dans la lignée cancéreuse du sein, MDA-MB-231. Ils ont démontré que l'activité de la furine est corrélée avec le phénotype agressif et que son inhibition réduit la motilité, la migration et l'invasion cellulaire (Lapierre et al., 2007). Plus récemment, Li et al 2018, ont démontré que l'invasion et la formation de métastases de cellules de cancer mammaire sont augmentées lorsque la furine interagit avec la protéine Plac-1 (Placenta-specific-1) (Li et al., 2018). Cette dernière est retrouvée fortement exprimée dans de nombreux cancers (Devors 2013 ; Liu 2015).

Toutes ces données montrent que la furine intervient dans de nombreux processus tumoraux et que son inhibition semble être une stratégie pertinente dans le traitement des cancers.

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Tableau 5 : Les études effectuées sur les rôles de la furine dans différents type de cancer (Jaaks and Bernasconi, 2017).

Cancer type	Type of study
Carcinomas	
Lung carcinoma	Expression study
	Inhibition or silencing of furin
	Regulation mechanisms of furin expression
Head and neck squamous cell carcinoma	Expression study
	Inhibition or silencing of furin
	Furin overexpression
Oral squamous cell carcinoma	Expression study
	Inhibition or silencing of furin
Oesophagus squamous cell carcinoma	Expression study
Laryngeal cancer	Expression study
	Inhibition or silencing of furin
Breast cancer	Expression study
	Overexpression of furin
	Inhibition or silencing of furin
Endometrial cancer	Expression study
Cervical cancer	Expression study
	Regulation mechanisms of furin expression
Ovarian cancer	Expression study
	Inhibition or silencing of furin
Gastric cancer	Expression study
	Overexpression of furin
Colon cancer	Inhibition or silencing of furin
	Regulation mechanism of furin expression
Sarcomas	
Rhabdomyosarcoma	Expression study
	Overexpression, inhibition or silencing of furin
Osteosarcoma	Inhibition or silencing of furin
Fibrosarcoma	Inhibition or silencing of furin
Cancers of the brain and central nervous system	
Astrocytoma	Expression study
	Inhibition or silencing of furin
Glioma	Regulation mechanisms of furin expression
	Inhibition or silencing of furin
Other cancers	
Skin cancer	Transgenic mouse model

IV. L'acide lipoïque (LA)

L'acide lipoïque (acide 6,8-dithiooctanoïque), dénommé par la suite LA, a été identifié pour la première fois par Reed et ses collaborateurs et par Jukes et ses collaborateurs dans les années 50 (pour revue (Solmonson and DeBerardinis, 2018)). Ces dernières décennies, les propriétés chimiques et les fonctions biologiques de ce cofacteur unique ont été bien documentées. LA est un composé naturel présent dans toutes les cellules procaryotes et eucaryotes. Il est synthétisé en petites quantités par la plupart des organismes vivants y compris les êtres humains. Il peut se trouver dans tous les aliments surtout dans l'épinard, brocoli et dans les abats pour les produits d'origine animale (Durrani et al., 2010). La voie de biosynthèse de LA chez *E. coli* et *S. cerevisiae* a été bien étudiée (**figure 14**). Chez les mammifères, la biosynthèse de LA est réalisée par un orthologue de l'octanoyltransférase et un orthologue d'acide lipoïque synthase appelés LIPT2 et LIAS respectivement (Solmonson and DeBerardinis, 2018) (**figure 14**). Le taux plasmatique de LA a été mesuré entre 12,3 et 43,1 ng·mL⁻¹ (Oizumi and Hayakawa, 1989; Teichert and Preiss, 1992, 1995). LA joue un rôle essentiel au sein de l'organisme, c'est un cofacteur pour plusieurs enzymes mitochondriales (Ziegler et al., 1995) impliquées dans la catalyse de 5 réactions d'oxydo-réduction : une dans le système de clivage de la glycine (GCS) et quatre déshydrogénases, la pyruvate déshydrogénase (PDH), l' α -cétoglutarate déshydrogénase (KGDG), la cétoacide déshydrogénase à chaîne ramifiée (BCKDH) et le 2-oxoadipate déshydrogénase (OADH). La KGDH et la PDH sont impliquées dans le métabolisme énergétique alors que les trois autres enzymes sont impliquées dans le métabolisme des acides aminés (Mayr et al., 2014). Toutes ces enzymes sont composées de plusieurs sous-unités E1-3 et partagent une architecture commune. L'un des rôles les plus étudiés de LA est celui de cofacteur de la PDH impliquée dans la synthèse de l'ATP à partir du glucose en transformant le pyruvate en acétyl-CoA qui rentre ensuite dans le cycle de Krebs. En outre, en fonction de sa concentration intracellulaire, LA exerce des effets anti-ou pro-oxydants (Moini et al., 2002). Une Concentration élevée de LA révèle normalement une activité pro-oxydante. En revanche, une faible concentration de LA engendre un effet antioxydant. Dans ce contexte, plusieurs études ont distingué quatre propriétés anti-oxydantes de LA : (i) la capacité de chélation des métaux, comme Fe²⁺ et Cu²⁺, (ii) la capacité de réduction des espèces réactives de l'oxygène (ROS) par recyclage du glutathion oxydé (GSSG) en glutathion réduit (GSH), processus qui joue un rôle clé dans la régulation des ROS pour protéger les cellules

contre le stress oxydatif, (iii) la capacité de régénérer des antioxydants endogènes tels que la vitamine C et la vitamine E, (iv) son implication dans le système de réparation des dommages oxydatifs causés aux protéines en améliorant l'activité de la méthionine sulfoxyde réductase (PMSR) (Solmonson and DeBerardinis, 2018). Grâce à ses propriétés, LA est donc fréquemment utilisé pour le traitement de pathologies associées au stress oxydant, telles que le diabète, l'athérosclérose, les maladies du foie et les maladies neurodégénératives. Au-delà de son efficacité dans le traitement de ces maladies chroniques, les chercheurs ont démontré ses effets anticancéreux dans différents types de cancers.

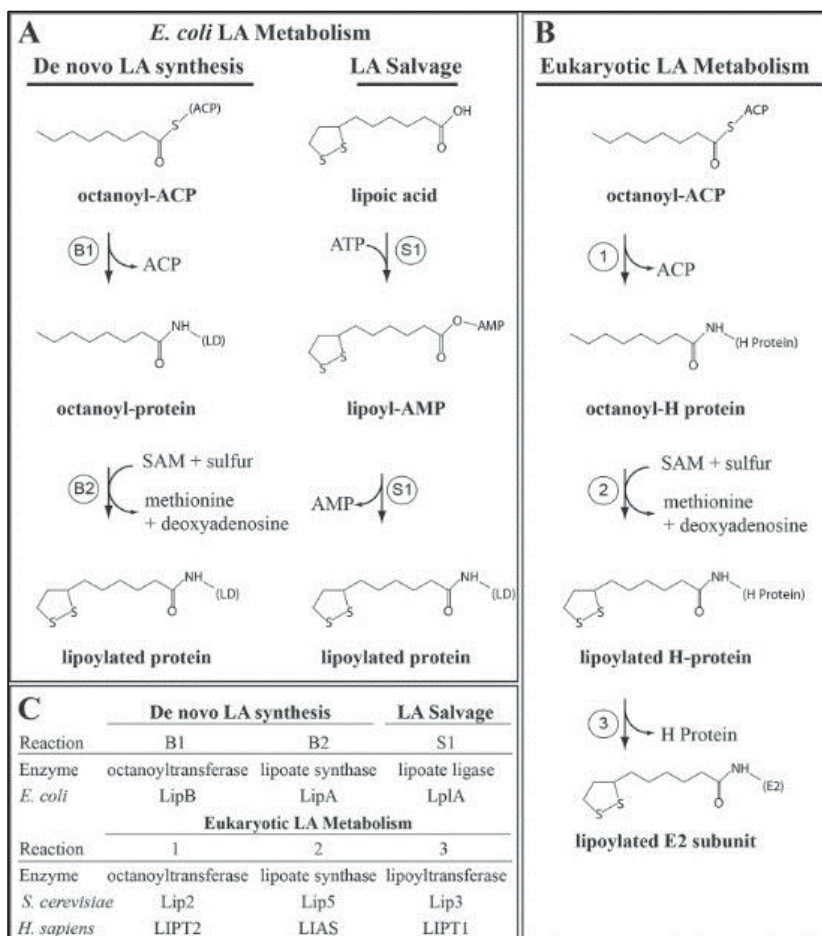


Figure 14 : Structures, enzymes et mécanismes réactionnels du métabolisme de l'acide lipoïque. **A**, Synthèse *de novo* de l'acide lipoïque et voies de récupération chez *E. coli*. **B**, voie métabolique de l'acide lipoïque chez *S. cerevisiae* et *Homo sapiens*. **C**, enzymes orthologues associées au métabolisme de l'acide lipoïque dans chaque organisme (Solmonson and DeBerardinis, 2018).

5. Acide lipoïque et cancer

Ses effets thérapeutiques potentiels ont été bien démontrés dans divers cancers y compris le cancer du sein. L'acide lipoïque agit en régulant plusieurs voies de signalisation impliquées dans la prolifération, l'invasion, la migration, l'EMT et l'apoptose.

a. Les effets de l'acide lipoïque sur la prolifération et l'apoptose

Des études précédentes ont montré que l'administration exogène de LA inhibe la prolifération dans plusieurs types de cancers : leucémie (Pack et al., 2002; Selvakumar and Hsieh, 2008a), de l'ovaire (Vig-Varga et al., 2006), du côlon (Kono et al., 2012), de neuroblastome (Feuerecker et al., 2012), du poumon (Yang et al., 2017) et du sein (Kuban-Jankowska et al., 2017; Na et al., 2009; Tripathy et al., 2018). Il est bien connu que la prolifération tumorale est due à la surexpression de différents récepteurs de tyrosine kinase (RTKs), conduisant à l'activation de voies de signalisation oncogéniques telles que PI3K/Akt, ERK et mTOR (Jeon et al., 2016; Tripathy et al., 2018). Il a été démontré que LA réduit la viabilité des cellules de cancer du sein en inhibant l'activité de 2 phosphatases, PTP1Ba et de SHP2, qui jouent un rôle clé dans la signalisation des récepteurs de tyrosine kinase (Tajan et al., 2015; Tonks, 2003). De plus, elles sont souvent surexprimées dans les cellules de cancer du sein, constituant ainsi des cibles potentielles pour les traitements anti-cancéreux (Kuban-Jankowska et al., 2017). De plus, LA induit dans de nombreux types de cancers l'expression protéique de 2 inhibiteurs du cycle cellulaire, p27^{kip1} et p21^{Cip1} conduisant à un arrêt du cycle cellulaire (Dozio et al., 2010). AMPK est un capteur majeur du niveau d'énergie cellulaire qui maintient l'homéostasie énergétique. Son activation par phosphorylation limite la progression tumorale en inhibant le complexe protéique mTOR qui stimule la synthèse des protéines et d'autres processus anaboliques nécessaires pour la croissance et la prolifération cellulaire et qui est activé par Akt. L'acide lipoïque induit l'activation d'AMPK, ce qui renforce l'inhibition de la voie Akt, réduisant ainsi la prolifération des cellules cancéreuses en agissant à 2

niveaux moléculaires distincts (Lee et al., 2005; Li et al., 2015b; Park et al., 2008, 2015) (figure 15).

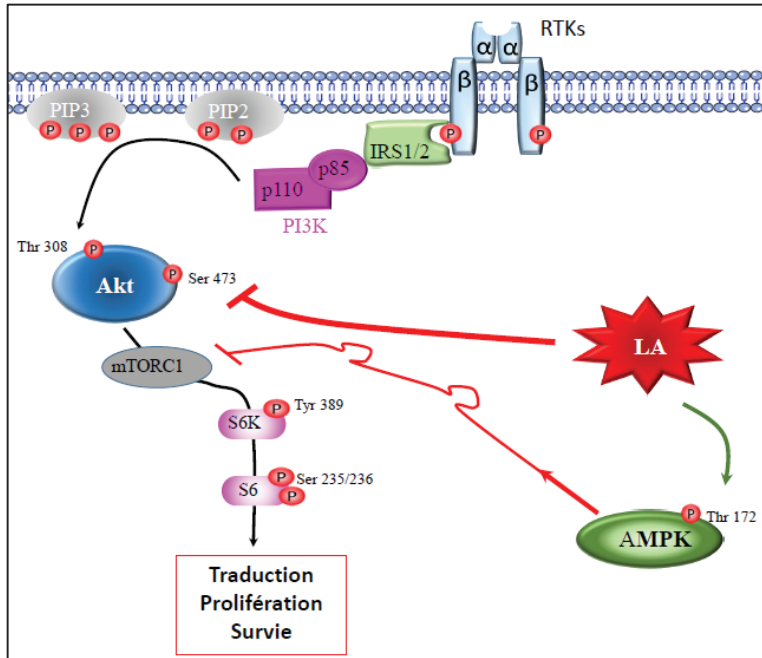


Figure 15 : L'acide lipotique (LA) réduit la prolifération cellulaire en agissant à deux niveaux moléculaires distincts. LA inhibe mTORC1 en activant d'une part AMPK et en inhibant d'autre part Akt.

Par ailleurs, LA induit l'apoptose dans différents types de cellules cancéreuses. Des données antérieures ont montré que LA génère des ROS, qui favorisent l'induction de l'apoptose des cellules cancéreuses en dérégulant le rapport entre les protéines anti-apoptotiques et pro-apoptotiques (Kafara et al., 2015a; Melli et al., 2008; Na et al., 2009). En effet, les protéines anti-apoptotiques Mcl-1, Bcl-2 et Bcl-XL, sont inhibées de manière concentration et temps dépendantes par LA dans plusieurs lignées cancéreuses : poumon, sein et ovaire (Dörsam and Fahrner, 2016). De plus, l'expression des protéines pro-apoptotiques, Bim et Noxa, est régulée positivement en réponse à LA provoquant une mort cellulaire par apoptose (Kafara et al., 2015a). Dans des lignées leucémiques et dans le cancer du sein, le rapport Bax/Bcl-2 (indice d'apoptose) augmente de manière significative après le traitement par LA avec une activité accrue de la caspase-3 (Na et al., 2009). Une autre étude réalisée sur des cellules cancéreuses hépatiques a également montré que LA induit la voie apoptotique intrinsèque par l'activation de caspase-9 et caspase-3 (Dörsam and Fahrner, 2016). Conformément à ces résultats, une étude précédente sur le

cancer du côlon a révélé que LA stabilise la protéine WT-p53 qui est un activateur de l'apoptose en induisant la transcription de gènes pro-apoptotiques, déclenchant ainsi la voie d'apoptose intrinsèque. Cependant, Dörsam et al ont démontré que LA a déclenché la mort cellulaire dans le cancer colorectal indépendamment de la stabilisation de p53 (Dörsam et al., 2015). En outre, des travaux antérieurs sur des cellules cancéreuses du poumon ont suggéré que LA induit la mort cellulaire associée à l'autophagie via AIF, facteur induisant l'apoptose (Choi et al., 2009). Toutes ces études révèlent que LA participe à l'induction de l'apoptose mais les mécanismes moléculaires ne sont pas élucidés (**figure 16**).

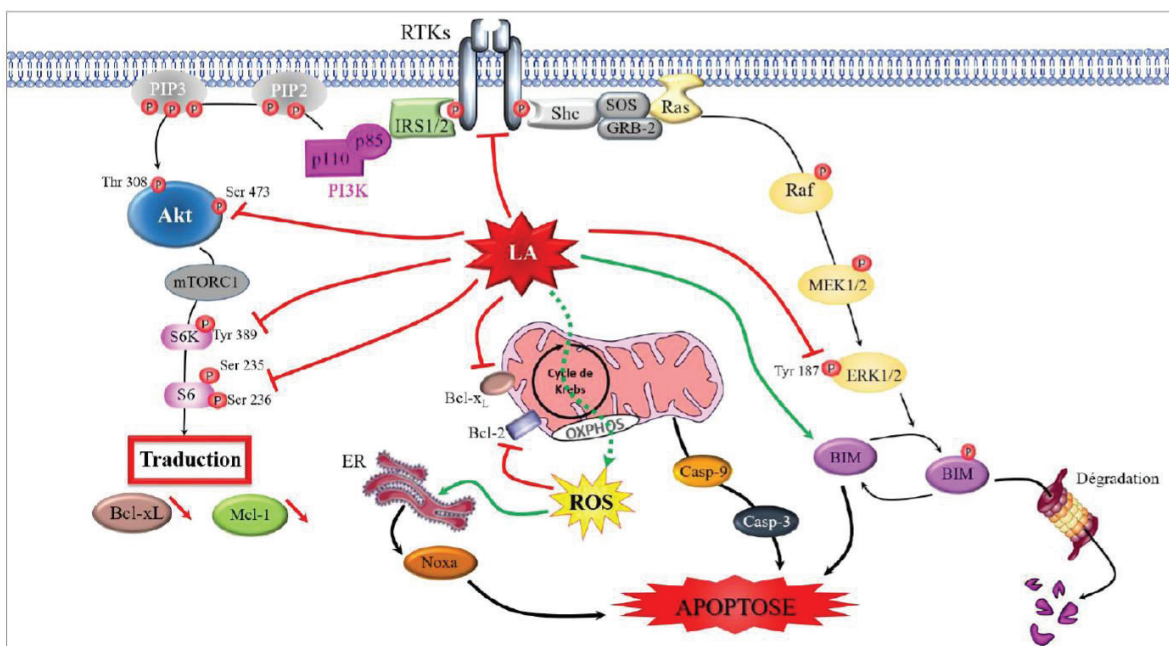


Figure 16 : Voies apoptotiques induites par LA.

1. b. L'impact de l'acide lipoïque sur les métastases, l'invasion, la migration et l'EMT

La formation de métastases est un processus complexe, la première étape critique étant l'invasion de cellules par des barrières extracellulaires et la pénétration de la membrane basale, suivie d'une infiltration du système vasculaire, d'une extravasation et d'une colonisation d'organes distaux. Ces événements sont régulés par deux voies cruciales induites par les 2 facteurs TGF- β et FAK (**figure 17**). Une fois que ces voies sont activées, des facteurs de transcription liés à la transition épithélio-mésenchymateuse (EMT), tels que la vimentine, Slug, Twist et Snail sont induits. Ces facteurs favorisent l'EMT en inhibant l'E-cadhérine et en déclenchant la surexpression de plusieurs marqueurs de l'EMT (**figure 18**).

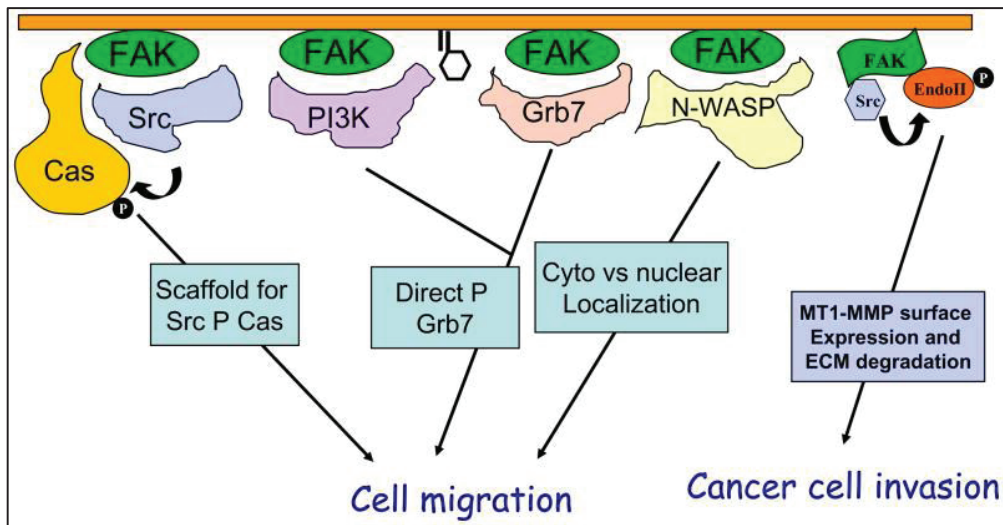


Figure 17 : Régulation de la migration cellulaire et de l'invasion par FAK via plusieurs voies de signalisation (Zhao and Guan, 2011).

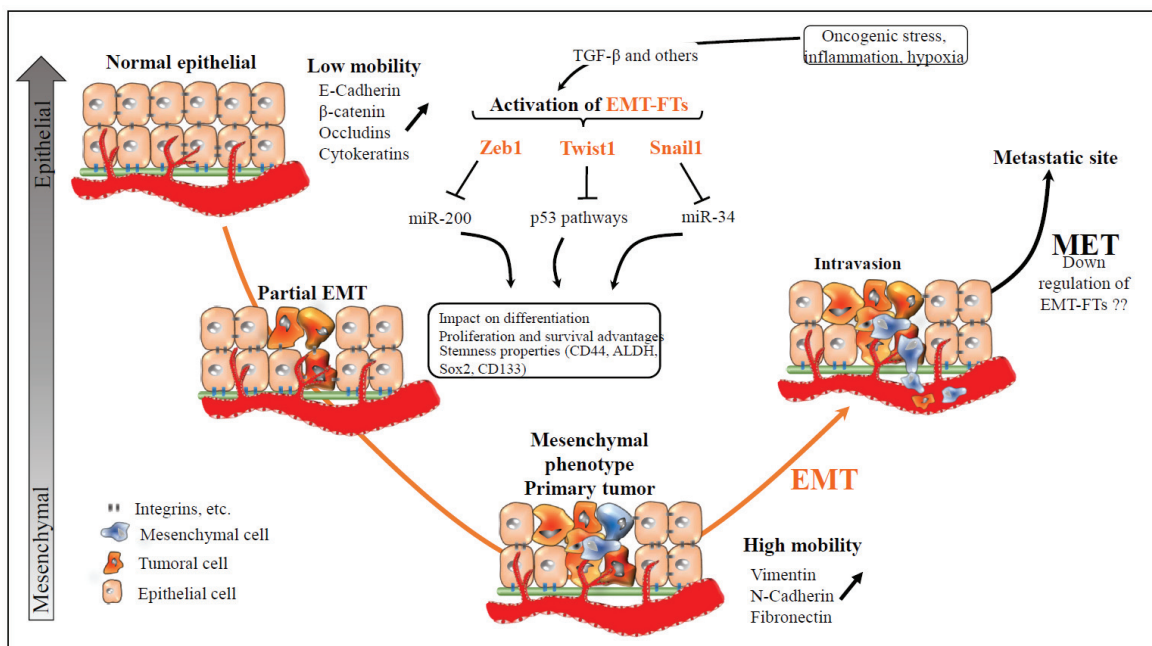


Figure 18 : Schéma des différentes étapes de l'EMT dans le cancer.

Dans deux lignées cellulaires de cancer du sein (ER α -), une étude récente a démontré que LA inhibe la migration cellulaire en réprimant les marqueurs EMT induits par le TGF- β tels que Snail, la vimentine et Zeb1 (Tripathy et al., 2018). L'expression de ces marqueurs est également réduite lors du traitement par LA dans des lignées cancéreuses du poumon (Phiboonchaiyanan and

Chanvorachote, 2017). De plus, la voie SMAD, connue pour être impliquée dans le processus de l'EMT après son activation par la signalisation du TGF- β , est inhibée par LA (Jeon et al., 2016). Ces résultats indiquent que LA supprime l'invasion et la migration en inhibant l'EMT. De plus, lors de l'invasion des cellules cancéreuses, de petites projections appelées « invadopodia » facilitent l'adhésion des cellules à la matrice extracellulaire (MEC) de manière dépendante de l'intégrine. Ces protéines de surface cellulaire sont connues pour activer la voie FAK. Le traitement par LA altère la formation de ces projections en raison de la régulation négative de l'expression de l'intégrine β 1 entraînant l'inhibition de l'invasion et de la migration des cellules cancéreuses (Yamasaki et al., 2014). L'invasion repose également sur l'expression des métalloprotéinases (MMPs), car ces enzymes clés, principalement MMP-2 et MMP-9, participent à la dégradation du collagène de type IV provoquant une dégradation de la MEC facilitant ainsi la migration cellulaire. Ces MMPs jouent un rôle crucial dans la dissémination et la malignité des cellules tumorales (Lee et al., 2010). De plus, il a été démontré que LA inhibe l'invasion en réduisant l'expression des ARNm de MMP-2 et -9 de manière dépendante de la concentration en LA dans des lignées cellulaires du cancer du sein (Lee et al., 2010). En accord avec ces résultats, LA inhibe également le processus de l'EMT en augmentant la E-cadhérine et en diminuant la N-cadhérine, la vimentine et la β -caténine activée (Phiboonchaiyanan and Chanvorachote, 2017). Toutes ces études démontrent que LA provoque une réduction de la dissémination cellulaire donc à la formation de métastases (**figure 19**).

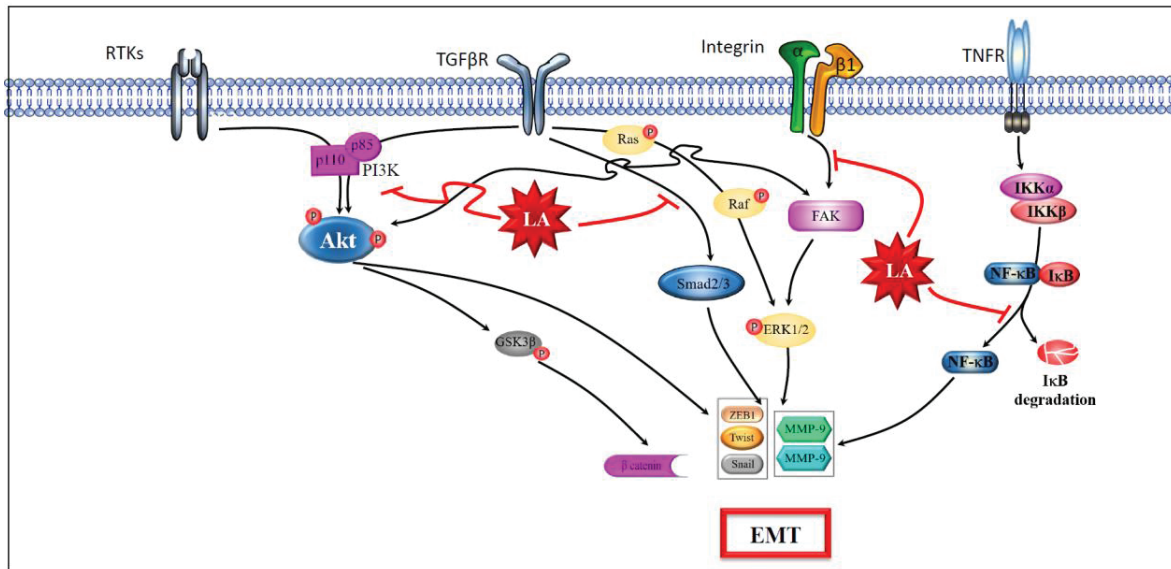


Figure 19 : Effets de LA sur l'expression des facteurs impliqués dans la dissémination cellulaire et la formation des métastases.

1. c. L'acide lipoïque réduit le caractère souche (souchitude) dans le cancer

Les cellules souches cancéreuses (CSC) sont des cellules immortelles rares capables de donner naissance à plusieurs types de cellules constituant la tumeur, en raison de leurs capacités d'auto-renouveau et de différenciation (Pattabiraman and Weinberg, 2014). Cette population est capable d'induire la prolifération cellulaire et la dissémination. Malgré les progrès réalisés dans les traitements ciblant le volume tumoral, les CSC pourraient rester indemnes, favorisant la récurrence du cancer et l'apparition de phénomènes de chimio- et hormono-résistance.

Des recherches antérieures ont établi que l'activation d'Akt par sa phosphorylation est cruciale pour le maintien des cellules souches. En effet, les cellules CD133-positives ont montré une augmentation du taux d'Akt phosphorylé (Wei et al., 2013), alors que l'inhibition de son activité entraînait une diminution des taux de protéines impliquées dans la stabilisation de caractère souche, telle que Oct-4 et ainsi une inhibition de la prolifération des CSC (Zhao et al., 2015). Il est évident que Oct-4 est stabilisée par la phosphorylation, p-Oct-4, induite par Akt et cette forme phosphorylée, a une localisation nucléaire prédominante favorisant la transcription de Nanog, un marqueur des CSC (Lin et al., 2012). Dans ce contexte, il a été démontré que LA induit une diminution de la phosphorylation d'Akt dans une lignée de cancer pulmonaire humain non à petites cellules, conduisant à une diminution d'expression des protéines Oct-4 et Nanog, cette dernière est

essentielle pour la prolifération, l'agressivité et la résistance de divers types de cellules cancéreuses (Phiboonchaiyanan and Chanvorachote, 2017; Samardzija et al., 2012; Sławek et al., 2016; Sun et al., 2015) (**figure 20**). Conformément au concept selon lequel Oct-4 est dégradé par le protéasome après ubiquitinylation (Shi and Jin, 2010), ils ont constaté que le traitement par LA conduit à la dégradation d'Oct-4 après fixation de molécules d'ubiquitine (Phiboonchaiyanan and Chanvorachote, 2017) et par conséquent à la perte du caractère de « souchitude ». De plus, ils ont démontré que LA induit également la dégradation protéosomale de la β -caténine, un autre régulateur qui favorise le phénotype souche et l'EMT. A noter que, la β -caténine est dégradée par GSK3 β (Verheyen and Gottardi, 2010). Cette dégradation peut être inhibée par la phosphorylation inhibitrice de GSK3 β sur la sérine 9 induite par Akt (De Sarno et al., 2002). Dans ce contexte, l'inhibition d'Akt par LA aboutit à la dégradation de la β -caténine par GSK3 β active (**figure 20**). Cette étude a démontré le rôle de LA dans la suppression des phénotypes du CSC et renforce son implication cruciale dans la lutte contre la chimiorésistance, la progression et les métastases induites par les CSC (**figure 20**).

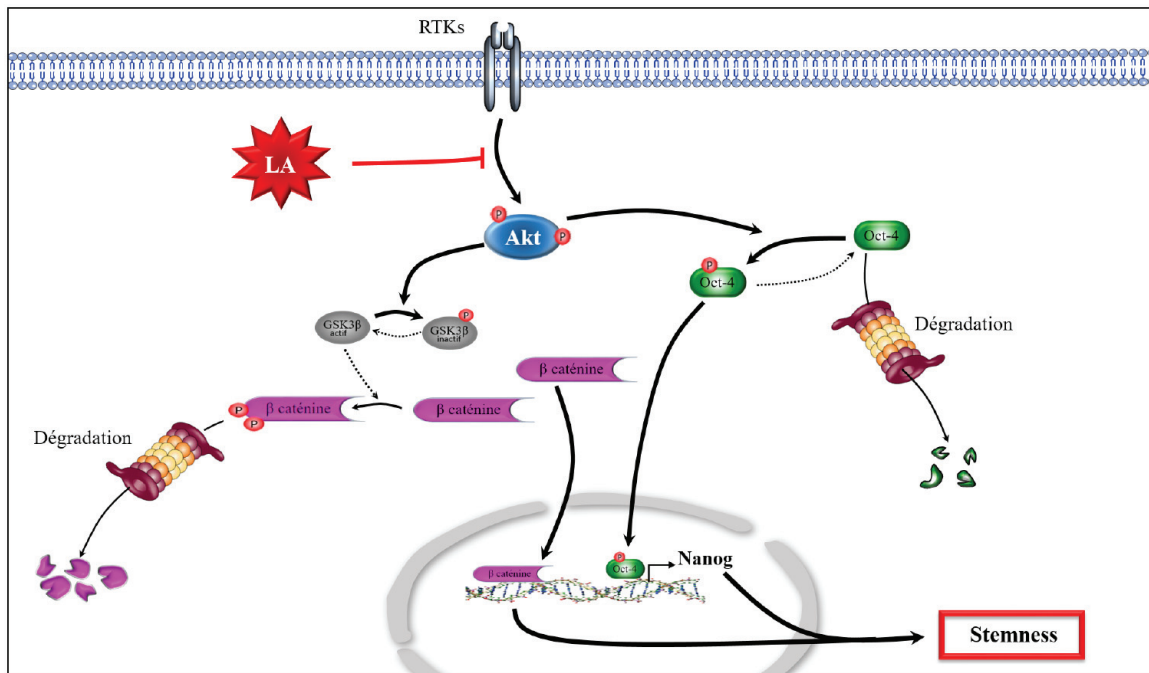


Figure 20 : Schéma regroupant les effets de LA sur l'inhibition du phénotype souche dans les cancers pulmonaires humains non à petites cellules. LA diminue la phosphorylation activatrice d'Akt réduisant l'expression de la β-caténine et d'Oct-4 et augmentant leur ubiquitinylation. Les faibles niveaux de β-caténine et d'Oct-4 réduisent le phénotype souche (d'après (Phiboonchaiyanan and Chanvorachote, 2017)).

La **figure 21** résume les effets anti-cancéreux de LA en agissant sur différentes voies de signalisation impliquées dans la tumorigénèse.

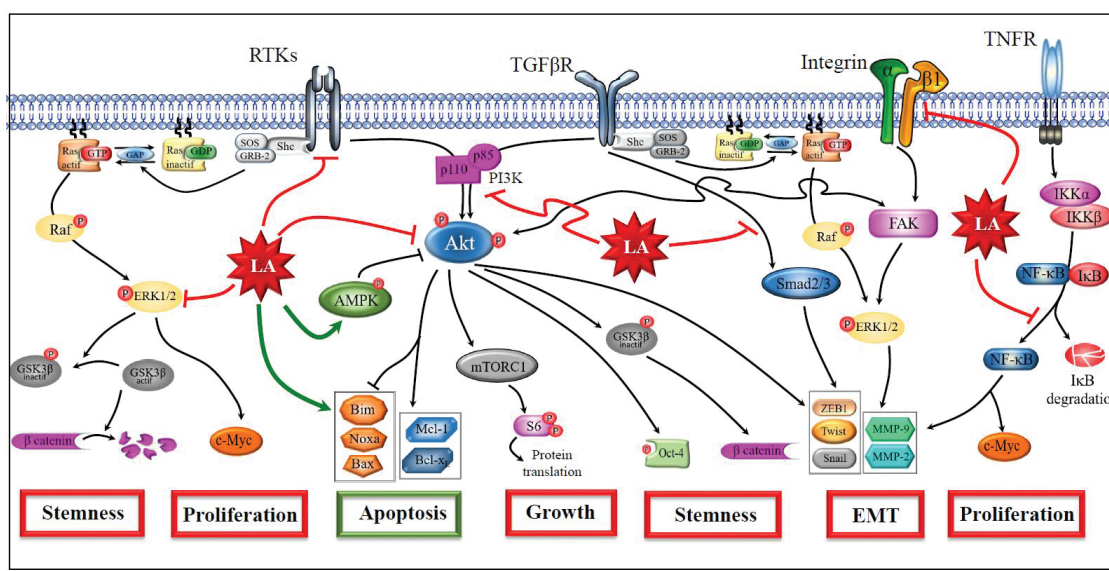


Figure 21 : Schéma récapitulatif des effets de l'acide lipoïque (LA) sur les différentes voies de signalisation impliquées dans la tumorigénèse (Farhat & Lincet, 2019).

2. e. L'acide lipoïque augmente l'efficacité de la chimiothérapie et prévient ses effets secondaires

Malgré les progrès des thérapies conventionnelles, les mécanismes de résistance conduisant à la récurrence des tumeurs et à l'apparition de tumeurs secondaires, demeurent les principales causes de la mortalité. Plusieurs études ont montré que LA améliore la sensibilité des cellules cancéreuses aux traitements conventionnelles. Notamment, LA augmente l'efficacité du paclitaxel dans les cellules cancéreuses du sein et du poumon en inhibant la voie de signalisation NF- κ B et des intégrines β 1 et β 3, respectivement (Li et al., 2015a; Puchsaka et al., 2016). Une autre étude a montré que LA augmente la cytotoxicité de deux agents anticancéreux possédant des mécanismes d'actions différents utilisés dans le cancer colorectal (5-fluorouracile, Témazolomide). De même, LA surmonte la résistance au géfitinib, inhibiteur de l'EGFR, en réduisant l'activation de ce récepteur dans les cellules cancéreuses du poumon (Michikoshi et al., 2013). Une étude récente a montré que l'administration de LA et de Docetaxel à l'aide de nanoparticules lipidiques augmente l'apoptose dans des cellules cancéreuses du sein (Kothari et al., 2019). En plus de son rôle potentiel dans l'augmentation de la cytotoxicité de la chimiothérapie, LA joue un rôle préventif contre les effets secondaires de la chimiothérapie en protégeant les patients contre la neuropathie, les dommages intestinaux et la diarrhée. Dans ce contexte, le prétraitement par LA avant l'administration de cisplatine peut permettre de surmonter les dommages induits par le cisplatine, tels que la néphrotoxicité, la neurotoxicité et l'ototoxicité, en restaurant le système redox (Farhat and Lincet BBA Reviews on Cancer, 2019 in Press).

1. f. Essais précliniques et cliniques

Au total, plus de 35 études cliniques ont démontré l'innocuité de LA chez l'Homme : plus de 1300 participants ont reçu de LA à des concentrations allant généralement de 600 mg à 2 400 mg/jour et sur des durées allant de quelques semaines à 24 mois (Cremer et al., 2006). De plus, la tolérance générale de LA s'est avérée très acceptable (94 % pour le groupe recevant 1 200 mg/jour contre 100 % pour le groupe placebo) (Cremer et al., 2006). Chez les souris, une étude a montré que LA n'a aucun effet toxique quel que soit leur sexe et cela après une administration orale pendant 4 semaines à des concentrations de 31,6 et 61,9 mg/kg (Cremer et al., 2006). Cependant, de fortes doses (121 mg/kg) présentent une toxicité avec une augmentation des enzymes hépatiques (ALAT and GLDH) chez les mâles et une formation de micro-granulomes dans le foie chez les deux sexes

(Cremer et al., 2006). Les études précliniques et cliniques ont été réalisées avec l'idée que LA diminuerait les effets toxiques de la chimiothérapie et augmenterait leurs efficacités. En effet, LA a été testé en association avec la chimiothérapie chez la souris et l'Homme. Dans certains cas, LA présente un effet antagoniste inhibant les effets anticancéreux de la chimiothérapie. Dans ce contexte, il a été observé *in vitro* dans la majorité des combinaisons de doxorubicine et LA que l'effet de LA sur la doxorubicine était antagoniste. L'effet synergique de la doxorubicine et de LA n'a été observé qu'aux concentrations relativement élevées des deux médicaments (Novotny et al., 2008). Cependant, l'utilisation de la doxorubicine et de LA *in vivo* a entraîné une augmentation de la survie des souris expérimentales. La combinaison d'une dose unique de 5 mg/kg de doxorubicine et de 16 mg/kg de LA a entraîné un effet super-additif sur la survie des souris porteuses de la leucémie (Novotny et al., 2008). D'autre part, une seule étude chez l'Homme a montré qu'un mode de vie sain avec l'administration de LA (300 à 600 mg 2 jours par semaine), et une dose faible de naltrexone (LDN) (4,5 mg au coucher) par voie intraveineuse pourrait prolonger la vie du patient atteint d'un cancer du pancréas pendant plusieurs années sans aucun effet toxique (Berkson et al., 2006).

V. Objectifs

Le cancer du sein est le cancer le plus courant chez les femmes. Malgré les progrès réalisés dans la stratification du risque et les options thérapeutiques, les taux de survie globale doivent encore être améliorés, en particulier chez les patientes atteintes de maladie métastatique. En outre, les stratégies de traitement multimodal classiques entraînent souvent des effets secondaires aigus et des différents mécanismes de résistance limitent l'efficacité de ces traitements. Par conséquent, l'identification et la caractérisation de nouveaux traitements sont essentielles pour augmenter l'efficacité des thérapies conventionnelles et surmonter les problèmes de la résistance. Comme décrit ci-dessus, l'acide lipoïque (LA) présente des activités anti-tumorales dans plusieurs modèles de cancers y compris le cancer du sein. Jusqu'à présent, ses mécanismes d'action restent non élucidés. Par conséquent des recherches supplémentaires sont nécessaires pour améliorer la compréhension de l'action de LA comme agent anti-prolifératif. Dans le cancer du sein, plusieurs études ont déjà démontré que LA inhibe la prolifération cellulaire en bloquant les voies de signalisation telles que PI3K/Akt et MAPK/ERK, la viabilité et la migration cellulaire sans comprendre réellement ses mécanismes d'action.

L'objectif principal de ma thèse était d'améliorer la compréhension des mécanismes par lesquels LA exerce ses effets biologiques dans des lignées cellulaires de cancer du sein.

- Dans un **premier temps**, nous avons validé les effets anti-prolifératifs de LA sur plusieurs lignées cancéreuses du sein exprimant ou non le récepteur à l'œstrogène, (ER α (+) et ER α (-)), puis montré que les voies de signalisation PI3K/Akt et MAPK/ERK sont inhibées après un traitement de nos lignées cellulaires par LA.
- Dans un **second temps**, nous avons émis l'hypothèse que LA inhibe la prolifération cellulaire en inhibant les récepteurs à tyrosine kinase (RTKs) car ces derniers activent les voies de signalisation prolifératives. Nous avons démontré pour la première fois que LA agit en amont de ces deux voies de signalisation en inhibant l'expression protéique de plusieurs RTKs : IGF-1R, EGFR et IR. Des études antérieures, décrites dans la partie bibliographique de ma thèse (Cf. Paragraphe III.3), ont montré que l'activation d'IGF-1R passe par une protéolyse assurée par la proprotéine convertase, furine, qui est surexprimée dans différents types de cancer y compris le cancer du sein (Cf. Paragraphe III.4).

- Dans un **troisième temps** et en se basant sur ces études antérieures, nous avons émis l'hypothèse que LA pourrait inhiber l'expression protéique d'IGF-1R en inhibant sa maturation *via* la réduction de l'expression et/ou de l'activité enzymatique de la furine. Nos résultats ont montré que LA inhibe l'expression protéique de la furine mais aussi son taux d'ARNm dans nos modèles cellulaires de cancer du sein empêchant ainsi le récepteur IGF-1R d'être recruté à la membrane plasmique pour jouer son rôle de RTK.

L'ensemble de ces résultats seront regroupés dans **un premier article** intitulé « *Lipoic acid decreases breast cancer cell proliferation by inhibiting IGF-1R via furin downregulation* » soumis à British Journal of Cancer, actuellement en révision.

Dans un **dernier temps**, nous avons poursuivi les recherches pour essayer d'élucider comment le traitement par LA de cellules de cancer du sein provoque une diminution d'expression de la furine. Une étude antérieure a démontré que l'expression de la furine est induite par le facteur de transcription CREB (Zhou et al., 2014), lui-même régulé par les ROS (Fu et al., 2019). Nous avons formulé l'hypothèse que LA pourrait inhiber la furine en régulant l'expression du facteur CREB *via* son rôle pro-oxydant. Nos résultats ont démontré que la présence du facteur de transcription CREB induit l'expression de la furine et que LA provoque une baisse d'expression de CREB. Cette baisse d'expression est en relation avec l'activité pro-oxydante de LA. Ces résultats ont donné lieu à un **second article** intitulé « *Lipoic acid-induced oxidative stress abrogates IGF-1R maturation by inhibiting CREB/furin axis in breast cancer cell lines* ». Cet article va être soumis prochainement sous forme de short communication à un journal à comité de lecture internationale.

Au final, les résultats obtenus durant ma thèse ont montré que LA induit une baisse du facteur de transcription CREB qui ne peut plus déclencher la transcription de la furine bloquant *in fine* la maturation du récepteur IGF-1R.

Chapitre II
Résultats

Résultats

1^{er} Article

Lipoic acid decreases breast cancer cell proliferation by inhibiting IGF-1R via furin downregulation

Diana Farhat^{1, 2, 3, 4}, Sophie Léon⁵, Sandra E. Ghayad⁶, Nicolas Gadot⁷, Philippe Icard^{8, 9}, Muriel Le Romancer^{1, 2, 3}, Nader Hussein⁴ and Hubert Lincet^{1, 2, 3, 10}

Objectif de l'étude

Notre présente étude vise à investir les mécanismes d'action de l'acide lipoïque (LA) par lesquels LA bloque la prolifération des cellules de cancer du sein afin de pouvoir identifier un nouveau traitement ou adjuvant permettant de contourner le problème de la résistance chez les patientes. En effet, des études précédentes ont montré les effets anti-cancéreux de LA dans de nombreux cancers sans élucider ses mécanismes d'action. Lorsque nous avons commencé ce travail, nous avons démontré l'effet anti-prolifératif de LA dans un panel de plusieurs lignées de cancers du sein (ER α (+) ou ER α (-)) et aussi dans des lignées de cellules tumorales humaines établies à partir de xénogreffes de cancer du sein humain (PDX : Patient-derived Tumor Xenografts), ce qui était en cohérence avec les études précédentes. Pour répondre à notre objectif, nous avons étudié l'effet de LA sur l'expression du récepteur tyrosine kinase, IGF-1R, qui est impliqué dans la tumorigénèse et la formation de métastases des cellules cancéreuses. Nous avons pu établir l'implication de LA dans l'inhibition de la maturation d'IGF-1R en réduisant l'expression de la proprotéine convertase, la furine. Les résultats obtenus nous ont amenés à élucider un nouveau mécanisme d'action de LA sur l'inhibition de la prolifération des modèles cellulaires de cancer mammaire.

Modèles d'étude

Les principaux modèles cellulaires étudiés sont des adénocarcinomes mammaires : la lignée humaine MCF7 de type ER α positif ER α (+) et MDA-MB-231 de type ER α négatif ER α (-). D'autres modèles mammaires ont été également utilisés comme les lignées T47D et Cama-1 qui sont ER α (+) et les lignées Skbr3, MDA-MB-568 qui sont ER α (-). Nous avons également utilisé des lignées cellulaires établies à partir des xénogreffes de cancer du sein humain (PDX), ces lignées ont été établies par le Dr. Marangoni. Il y a la lignée cellulaire HBCc-12A- qui est de type ER α (-) et la lignée HBCx-3 qui est ER α (+). Pour réaliser cette étude, diverses approches *in vitro* et

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ex vivo ont été utilisées pour nous permettre d'élucider le mécanisme d'action antiprolifératif de LA, parmi lesquelles IncuCyte Zoom, western blot, immunofluorescence, immunohistochimie, transfection de siRNA et RT-qPCR. Concernant l'approche *ex vivo*, des tumeurs fraîches de cancer du sein sont coupées à l'aide d'un vibratome en tranche de 250 μm puis exposées ou non à l'acide lipoïque pendant 48h. Après ce temps de traitement les tranches sont fixées puis une étude immunohistochimique est réalisée, pour déterminer l'indice de prolifération tumorale, Ki67, et aussi le pourcentage d'apoptose en détectant la PARP clivée.

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Lipoic acid decreases breast cancer cell proliferation by inhibiting IGF-1R via furin downregulation

Diana Farhat^{1, 2, 3, 4}, Sophie Léon⁵, Sandra E. Ghayad⁶, Nicolas Gadot⁷, Philippe Icard^{8, 9}, Muriel Le Romancer^{1, 2, 3}, Nader Hussein⁴ and Hubert Lincet^{1, 2, 3, 10, ✉}.

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Abstract

Background: Breast cancer is the second most common cancer in the world and, by far, the most frequent among women (25 % of all cancers). Despite advances in therapies, mechanisms of resistance remain the underlying cause of morbidity and mortality. It is well-known that glucose metabolism increases in a majority of tumors in order to ensure a redox status compatible with cellular survival, leading to resistance to therapies. Lipoic acid (LA) is a natural antioxidant and an essential cofactor in oxidative metabolism via its function in pyruvate dehydrogenase and α -keto dehydrogenase complexes. Its potential therapeutic effects have been well documented in various cancers, but its mechanisms of action (MOA) are not fully understood.

Methods: The aim of this study is to validate the inhibitory effect of LA on the proliferation of a various breast cancer cell lines and to investigate the MOA that may be involved in this process and we tested LA effects by an *ex vivo* studies on fresh human mammary tumor samples.

Results: We herein demonstrate that LA strongly inhibits the proliferation of several breast cancer cells independently of estrogen receptor α (ER α). Moreover, we found that LA treatment strongly inhibits Akt and ERK signaling. While searching for upstream dysregulations, we discovered the loss of expression of the tyrosine kinase receptor IGF-1R (Insulin Growth Factor-1 Receptor) upon exposure to LA. This decrease is due to the downregulation of the pro-protein convertase, furin, which is implicated in the maturation of a wide variety of proteins including IGF-1R. Moreover, *ex vivo* studies on fresh human mammary tumor samples showed that LA significantly decreases the expression of the proliferation marker Ki67, as well as IGF-1R.

Conclusion: LA exerts its anti-proliferative effect by inhibiting the maturation of IGF-1R via the downregulation of furin. Our findings suggest that LA may constitute an attractive molecule in combination with conventional therapies to improve the clinical outcome of patients.

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Introduction

Lipoic acid (LA) is a natural compound synthesized *de novo* in small amounts by plants and animals. It is an essential coenzyme for mitochondrial enzymes, such as pyruvate dehydrogenase and α -ketoglutarate dehydrogenase (Packer et al., 1997). Aside from its important role in mitochondrial energy metabolism, it is also known for its powerful antioxidant effect (Shay et al., 2009). The benefits of LA treatment have been described in the management of several non-cancerous pathologies including atherosclerosis, diabetes, and neurodegenerative disorders (de Oliveira et al., 2011). Beyond its efficacy in treating these chronic diseases, researchers have demonstrated its anti-proliferative effect on various cancers including breast cancer (Dozio et al., 2010b; Feuerecker et al., 2012; Kafara et al., 2015b; Selvakumar and Hsieh, 2008b; Wenzel et al., 2005a). Breast cancer is the second most common cancer in the world and, by far, the most frequent cancer among women (25 % of all cancers).

It is classified under several molecular subtypes according to the presence or absence of estrogen receptor α (ER α). This classification is important for disease prognosis, as well as for determining and providing an adequate therapy.

Despite advances in chemotherapy, hormonotherapy and targeted therapy in combination or not with surgery, mechanisms of resistance remain the underlying cause of morbidity and mortality (Tang et al., 2016). In breast cancer, it has been demonstrated that the expression of the Insulin-like Growth Factor-1 receptor (IGF-1R) increases in both ER-positive (ER+) and ER-negative (ER-) breast cancers and plays an oncogenic role (Law et al., 2008). Moreover, Many studies have shown the ability of IGF-1R to induce mammary tumor growth and metastases (Carboni et al., 2005; Kim et al., 2007; Kucab and Dunn, 2003). Activation of IGF-1R in turn activates multiple signal transduction cascades, including PI3K/Akt and MAPK/ERK pathways (Coolican et al., 1997; Duan et al., 2000; Imai and Clemmons, 1999), contributing to the promotion of breast cancer metastasis.

Indeed, both PI3K/Akt and MAPK/ERK pathways, have been shown to be involved in various processes such as cancer cell proliferation, migration, and metabolism (Asati et al., 2016). Once Akt is activated, it stimulates the mammalian target of the rapamycin complex (mTORC1), which is a key regulator of cellular growth. In addition, the mTOR pathway is indirectly downregulated by activated AMPK which is an energy sensor implicated in the regulation of

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protein and lipid metabolism in response to changes in fuel availability (Yu and Cui, 2016). Moreover, activation of AMPK is known to have anti-cancer effects in various cancer models, such as breast, colon and lung cancers (Kim and He, 2013). Activation of the pathways described above by IGF signaling requires the cleavage of pro-IGF-1R into its mature form. This maturation process is ensured by members of the pro-protein convertase family (PCs) including furin (PCSK3, PACE, SPC1). This latter is highly expressed in many types of cancers including breast cancer, and was shown to promote the cancer cell proliferation, migration and invasion (Yang et al., 2013).

In breast cancer, several studies demonstrated an anti-proliferative effect of LA (Dozio et al., 2010a; Feuerecker et al., 2012; Kuban-Jankowska et al., 2017; Lee et al., 2010; Li et al., 2015a; Na et al., 2009; Tripathy et al., 2018). However, the precise molecular mechanisms by which LA elicits its anti-cancer properties remain largely unknown yet. In the current study, we deciphered for the first time, how LA inhibits the proliferation of breast cancer cells. As such, we investigated the inhibitory role of LA on the main proliferative pathways and on the maturation of their upstream effectors, notably IGF-1R. Our *in vitro* results demonstrate that LA inhibits IGF-1R maturation and these results are supported by *ex vivo* findings.

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Materials and methods

Lipoic acid

Lipoic acid (LA) was purchased from Sigma (Sigma). A stock solution was prepared by dissolving LA in absolute ethanol and stocked at -20°C. LA was diluted in culture medium for *in vitro* and *ex vivo* experiments.

Cell lines, culture and siRNAs transfections

ER α ⁺ cells lines (MCF7, Cama 1) and ER α ⁻ cell lines (MDA-MB-231, Skbr3 and HBL100) were obtained from the ATCC. All of the cell lines were maintained at 37 °C in the appropriate medium supplemented with 10% fetal bovine serum, 1% non-essential amino acid and 2% penicillin/streptomycin. All cell lines were regularly tested for mycoplasma contamination. We also used frozen tumors from human breast cancer derived xenografts (PDX) from Dr. Marangoni. HBCc-12A- (ER α ⁻) and HBCx-3-expressing (ER α ⁺) cell lines were established from the human breast cancer xenografts as described by (Marangoni et al., 2007)

For knockdown experiments, *furin*-specific siRNAs from ThermoFisher scientific (ambion s9988 and s9987) siRNA furin 1: sens: 5'-GGGCCUUCAUGACAACUCATT-3'; anti sens: UGAGUUGUCAUGAAGGCCAG; siRNA furin 2: sens:GGUGGAAAAUGGACUGGCUTT; anti sens: AGCCAGUCCAUUUCCACCTT; the scramble siRNA (Eurogentec) (20 nM) were transfected into MCF-7 and MDA-MB-231 cells using the lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen).

Cell proliferation assay using IncuCyte Technology

Cell proliferation was assayed using an IncuCyte® ZOOM Live-Cell Analysis System (Essen Bioscience, Sartorius), by collecting real-time data of cell confluence. Cells were seeded onto 96-well plates (5,000 cells/well) and left to attach for 24 hrs. The cells were then treated with different concentrations of LA or absolute ethanol. Cell proliferation data were obtained by the

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cell confluence increment in each of the treatments and expressed as percentage relative to that of control cells. Each sample was tested in triplicate.

Western blot analysis

Cells were lysed using RIPA buffer (50 mM Tris HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 0.25% deoxycholate) supplemented with protease inhibitor tablets (Roche Molecular Biochemicals) and phosphatase inhibitors (1 mM NaF, 1 mM Na₃VO₄ and 1 mM beta-glycerophosphate). Cell lysates were cleared by centrifugation (13,000×g, 15 min, 4 °C) and protein concentration was determined by the Bradford method (Bio-Rad protein Assay). Proteins samples were separated on SDS-PAGE and membranes were incubated with primary antibodies overnight at 4°C on a shaker with the following primary antibodies: anti-IGF-1R (Cell Signaling), anti-IR-alpha (Cell Signaling), anti-EGFR (Cell Signaling), anti-furin (Proteintech), anti-Akt and anti-pAkt (Cell Signaling), anti-ERK and anti-pERK (Cell Signaling), anti-S6 and anti-pS6 (Cell Signaling), anti-S6K1 and anti-pS6K1 (Cell Signaling), anti-AMPK and anti-pAMPK (Cell Signaling), anti-Tubulin (Sigma). Membranes were washed with TBS-Tween and incubated with anti-rabbit or anti-mouse secondary antibodies. Detection was carried out using ECL Prime reagent (GE Healthcare).

RNA isolation, reverse transcription and quantitative real time polymerase chain reaction (RT-qPCR)

Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed to complementary DNA (cDNA) using SuperScript™ II Reverse Transcriptase (Life Technologies). The primers are listed as follows: *IGF-1R* Forward: 5'-TGTCCAGGCCAAAACAGGA-3' and Reverse: 5'-CGGGTTCACAGAGGCATACA-3'; *furin* Forward: 5'-TATGGCTACGGGCTTTTGG-3' and Reverse: 5'-TTCGCTGGTGTTTTCAATCTCT-3'; *28S* Forward: 5'-CGATCCATCATCCGCAATG-3' and Reverse: 5'-AGCCAAGCTCAGCGCAAC-3'. RT-qPCR was performed using the Bio-Rad CFX 96 Real-time PCR system (Bio-Rad) using SsoAdvanced™ Universal SYBR® Green Supermix according to the manufacturer's instructions. The mRNA level was normalized to 28S

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using livak's method ($2^{-\Delta\Delta Cq}$) method. RT-qPCR conditions were as follows: 1 activation step for 5 min at 94°C, 35 cycles of denaturing at 94°C for 15 s, primer annealing at 59°C for 45 s, and primer extension at 72°C for 2 min, and a final extension at 72°C for 5 min.

Immunofluorescence analysis

80,000 MCF7 cells were seeded onto glass coverslips and incubated with LA for several time points (24, 48 hrs). The cells were fixed in methanol for 2 min and washed once with cold PBS. We used rabbit anti-IGF-1R as primary antibody (Cell Signaling) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) as secondary antibody.

Ex vivo assay

Fresh human mammary samples were obtained from chemotherapy naïve patients with invasive carcinoma after surgical resection at the Centre Léon Bérard (CLB, Lyon, France). As required by the French Committee for the Protection of Persons, informed consent was obtained from patients to use their surgical specimens and clinicopathological data for research purposes, and the local ethics committee approved the protocols. Nine tumors were cut into thin slices of 250 μm using a vibratome (HM 650V Microm) and incubated for 48 hrs with or without 2 mM of LA (Table 1). Slices were then fixed in 4% paraformaldehyde and paraffin embedded. Sections (4 μm) were then cut for standard histological analysis assessed by hematoxylin phloxin saffron (HPS) staining and immunohistochemistry analysis using the Ki67 antibody (Cell Signaling), which is a marker of proliferation. The percentage of positive cells among 200 cancer cells was counted.

Immunohistochemistry analysis

Immunohistochemistry was performed on 4-mm-thick sections of formalin-fixed, paraffin-embedded and heat-treated (for antigen retrieval) tissues (DakoCytomation). Sections were stained with hematoxylin-eosin-safran and for immunohistochemistry detection, the primary antibodies used were anti-IGF-1R (Cell signaling), anti-Ki67 (Cell signaling). Diaminobenzidine

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was used as chromogen. Images were acquired using a Zeiss Axiovert. The whole slide was scanned automatically with the Histolab 6.2.0 MICROVISION Instrument system.

Statistical analysis

Data are presented as mean \pm SEM of three independent experiments. Unpaired two-tailed Student's t-test was used for statistical analysis. A *P*-value of < 0.05 was considered statistically significant. * *P* < 0.05 ; ** *P* < 0.01 ; *** *P* < 0.001 . The graph artwork was created using Microsoft office Excel 2010 and GraphPad prism 6.

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Results

LA inhibits the proliferation of breast cancer cells in an ER α -independent manner

To investigate the effect of lipoic acid (LA) on breast cancer cell proliferation, we treated the cells with MCF7 and MDA-MB-231 breast cancer cell lines were treated with different concentrations of LA and monitored their viability using the IncuCyte real-time imaging system. We first studied its role on ER α ⁺ cell lines (MCF7, Cama1 and HBCx-3). Lipoic acid treatment at 0.5 mM was able to reduce the proliferation of Cama1 cell line. However, this concentration of LA had little effect in MCF7 and HBCx-3 cell lines, while increasing concentrations totally inhibited cell proliferation (Figure 1, Supplementary Data, Figure 1). The same results were obtained for ER α ⁻ cell lines (MDA-MB-231, Skbr3 and HBCc-12A) (Figure 1, Supplementary Data, Figure 1). In contrast, LA did not inhibit the proliferation of non-malignant HBL 100 cells (Supplementary Data, Figure 1). These results demonstrate that LA inhibits the proliferation of cancer cells independently of their ER α status, but not that of non-tumorigenic cells. Owing to the similarities in the results obtained between cell lines, our subsequent experiments focused on an ER α ⁺ and an ER α ⁻ cell line, namely MCF7 and MDA-MB-231, respectively.

LA inhibits ERK and Akt pathways and activates the AMPK pathway in breast cancer cell lines in an ER α -independent manner

It is well known that ERK and Akt pathways play a central role in cell proliferation²⁸. We thus investigated the effect of LA on these two pathways. As shown in Figure 1C, in MCF7 cells, LA strongly reduced ERK phosphorylation at 1 mM and 2 mM at 48 hrs, while at 72 hrs, ERK phosphorylation was totally abolished as compared to control cells treated with 2 % of EtOH (NT). In MDA-MB-231 cells, LA blocked ERK phosphorylation only after 72 hrs of treatment and at 2 mM (Figure 1D). In addition, LA prevented Akt phosphorylation on Ser 473 in MCF7 cells at all treatment time periods and concentrations (Figure 1C). In MDA-MB-231 cells, 2 mM of LA also blocked Akt phosphorylation on Ser 473 from 48 hrs onwards (Figure 1D). As depicted in Figure 1C and D, LA treatment totally inhibited Akt-induced S6K phosphorylation, which is present downstream of the Akt signaling cascade. Moreover, LA partially inhibited p-ribosomal protein S6 (S6) phosphorylation which is downstream of S6K in both cell lines.

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We also evaluated whether LA activates AMPK signaling by phosphorylation of AMPK on Thr 172, because activated AMPK specifically downregulates the mTOR signaling pathway leading to the inhibition of cell proliferation. The level of AMPK phosphorylation increased in a time- and concentration-dependent manner in MCF7 cells (Supplementary Data, Figure 2 A). In MDA-MB-231 cells, LA increased AMPK phosphorylation only at 2 mM of LA at 48 hrs and 72 hrs (Supplementary Data, Figure 2 B). These results show that LA inhibits proliferation of breast cancer cells by simultaneously targeting ERK and Akt pathways. Moreover, these results show an activation of AMPK which activates the tuberous sclerosis (TSC) 1/2 complex which is a negative regulator of mTORC1 (Liang and Mills 2013).

LA suppresses IGF-1R expression at the protein level in breast cancer cells

In order to decipher how LA inhibits the PI3K/Akt and MAPK/ERK signaling pathways, as these pathways are major downstream pathways of several tyrosine kinase receptors involved in cell proliferation and survival, we studied the expression of IGF-1R, IR and EGFR upon LA treatment. As shown in Figure 2A, LA treatment of MCF7 cells, decreased the IGF-1R expression level in a time- and concentration-dependent manner. From 24 hrs onwards, at 2 mM of LA, we observed a slight decrease in the level of IGF-1R expression. Of note, at 72 hrs, no band was detected for the IGF-1R protein irrespective of the LA concentration used (1 or 2 mM). In MDA-MB-231 cells, 2 mM LA treatment resulted in a slight decrease in the level of the IGF-1R protein from 17 hrs onwards (Figure 2B). Forty-eight hours after LA treatment (1 or 2 mM), IGF-1R expression was almost lost. The effect of LA on the reduction of IGF-1R expression level was faster in the MCF7 cell line than in MDA-MB-231 cells. IGF-1R downregulation under LA treatment was also detected in other breast cancer cell lines (Supplementary Data, Figure 3). Interestingly, we found that LA also reduces the expression of other tyrosine kinase receptors, the IR and EGFR in a concentration- and time-dependent manner in both cell lines (Figure 2C and D).

Next, we wondered whether LA could impact IGF-1R transcription, by conducting RT-qPCR experiments. No decrease in *IGF-1R* mRNA was observed, irrespective of LA concentration and exposure (Supplementary Data, Figure 4). These data showed that this inhibitory effect did not occur at the transcriptional level but rather at the translational or post-translational levels.

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Our results showed that LA at 1 or 2 mM represses IGF-1R expression 48 hrs after exposure. We then evaluated whether this effect was reversible or not. As such, cells were treated with 2 mM of LA for 48 hrs and then incubated in LA-free medium for 24 or 48 hrs. In the two cell lines, we observed the reappearance of IGF-1R expression 24 hrs following LA withdrawal, and a continuous increase until 48 hrs (Figure 2E and F). Hence, these results reveal that LA induces a transient decrease in IGF-1R protein expression.

LA promotes the accumulation of pro-IGF-1R and reduces IGF-1R plasma membrane localization

Based on our previous results, we hypothesized that LA could have an effect on the post-translational processing of pro-IGF-1R into mature and active IGF-1R. Indeed, we observed an accumulation of the pro-IGF-1R in response to LA treatment from 48 hrs onwards in MCF7 and MDA-MB-231 cells, while mature IGF-1R was totally absent (Figure 3A and B). Similarly, the pro-IR form increased in these cell lines in response to LA treatment, whereas we observed a reduction in the mature IR form (Supplementary Data, Figure 5A and B). It is well-known that IGF-1R is localized at the level of the plasma membrane whereas pro-IGF-1R has a cytoplasmic localization²⁹. We then tested the localization of IGF-1R in the MCF7 cell line upon LA treatment by immunofluorescence confocal microscopy. Twenty-four hours after LA treatment, a partial loss of IGF-1R at the plasma membrane was detected in favor of the cytoplasmic localization, compared to control cells (figure 3C). In coherence with these results, we have demonstrated by western blot that LA (1 or 2 mM) treatment for only 24 hrs is not sufficient to abolish the total expression of processed IGF-1R and to induce a total shift to its uncleaved form (Data not shown). Thus, these results explain why there is still detectable IGF-1R at the plasma membrane after LA (1 or 2 mM) treatment for 24 hrs (figure 3C).

This loss of IGF-1R at the plasma membrane increased until 48 hrs and no plasma membrane expression was observed at this time (Figure 3C).

LA inhibits the maturation of pro-IGF-1R by targeting the pro-protein convertase furin

The pro-protein convertase, furin, catalyzes the endoproteolytic cleavage of the inactive pro-IGF-1R into its active form^{30,31}. Thus, we investigated whether LA treatment had an effect on its

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expression. Forty-eight hours after LA treatment, the expression of furin decreased in MCF7 and MDA-MB-231 cell lines (Figure 4A and B), in a time- and concentration-dependent manner. Indeed, 72 hrs after incubation in 2 mM of LA the expression of furin decreased notably in the MCF7 cell line compared to MDA-MB-231 cells (Figure 4A and B). We confirmed that LA downregulates the furin protein expression by validating the loss of maturation of other furin substrates such as Notch 3 (Supplementary Data, Figure 6).

We also examined the mRNA level of *furin* by RT-qPCR to determine whether this downregulation is due to an inhibition of the transcriptional activity in response to LA. Cell lines were treated with 1 or 2 mM of LA for 36 or 48 hrs. A significant reduction in *furin* mRNA was observed after treatment with LA compared to untreated MCF7 or MDA-MB-231 cells (Figure 4C and D). Next, we evaluated whether this effect was reversible. To achieve this, cells were treated with 2 mM of LA for 36 or 48 hrs and then incubated in LA-free medium for 24 hrs. As described in the Supplementary Data, Figure 7A and B, the level of *furin* mRNA increased in MCF7 and MDA-MB-231 cell lines following the removal of LA. Thus, our results show that LA acts on the maturation process of pro-IGF-1R and pro-Notch into active forms by reversibly inhibiting *furin* expression. In order to prove the implication of furin in IGF-1R and IR maturation in our cellular models, we transfected ER α ⁺ and ER α ⁻ breast cancer cell lines with furin or scramble siRNA for 48 hrs. We demonstrated that *furin* silencing leads to an accumulation of their pro-forms (Figure 4E and F). Indeed, our results showed that siRNA-mediated silencing of *furin* had a profound effect on the processing and maturation of IGF-1R and IR.

Preclinical evaluation of exposure to LA on human mammary tumors

Next, we tested the pre-clinical effects of LA on fresh human mammary tumor samples after surgical resection. In effect, we evaluated the response of nine breast tumors to LA using an *ex vivo* organotypic culture assay in which the tumor microenvironment is closely mimicked to evaluate its clinical potential. The tumors from patients with untreated breast cancer were included, five of which were positive for ER α and four negative for this receptor. IHC scores of positive cells for the proliferation marker Ki67 and the HPS staining were determined in the epithelial tumor cell compartments. Figure 5A, illustrates their IHC detection on a representative tumor sample treated or not with 2 mM of LA for 48 hrs. We observed a decrease in Ki67 in

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response to LA compared to the non-treated condition. Among the nine tumors, Ki67 staining displayed a mean score of 30% (ranging from 10 to 98%) in LA-treated tumors compared to 43% (ranging from 5 to 80%) in non-treated tumors (Figure 5B). Moreover, we detected the IGF-1R expression by IHC on these tumors samples. Only, three out of nine tumors samples expressed IGF-1R at the basal level. In these three cases, our results showed a repression of IGF-1R in response to LA compared to non-treated tumors or 2% absolute ethanol condition (Figure 5C). IGF-1R downregulation obtained by *ex vivo* approach corroborates our *in vitro* results.

Discussion

Lipoic acid (LA), an essential cofactor of a mitochondrial respiratory enzyme, exhibits anti-tumor activities in several cancer models such as thyroid, bladder, ovarian, colon, lung and breast cancer by acting on most of the signaling pathways implicated in proliferation, invasion, migration, EMT, stemness and apoptosis^{1,21,32-38 39 39}. Several studies demonstrated the beneficial effects of LA on some breast cancer cell lines by reducing the breast cancer cell viability, proliferation and metastasis process, and by promoting apoptosis^{5,21-24}. However, the mechanisms by which LA elicits its anti-cancer effects remain unclear. Herein, we demonstrate the inhibitory effect of LA on the proliferation of a large number of ER α ⁺ and ER α ⁻ breast cancer cell lines. Regardless of the ER α status, LA was able to inhibit crucial signaling pathways such as PI3K/Akt/mTOR and ERK in a time- and concentration-dependent manner. It is known that these pathways are directly implicated in cancer progression and especially cancer cell proliferation^{40,41}. Under our experimental conditions, we demonstrated that LA inhibits these pathways by suppressing the phosphorylation of Akt and ERK. Moreover, LA reinforces the Akt inhibition by activating AMPK through its phosphorylation. Accordingly, once AMPK is activated, it phosphorylates and thereby activates the TSC complex leading to mTOR inhibition⁴². Furthermore, we also showed that LA inhibits the downstream effectors of mTOR, S6K1 and S6, by reducing their phosphorylation in a time- and concentration-dependent manner. These latter were previously reported to be involved in the translation process in cancer cells⁴³. In line with these studies, our results suggest that LA could reduce the translation of proteins required for cancer cell growth and survival, such as cyclin, c-Myc and bcl-2^{44,45}.

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To gain further insight into these mechanisms, we studied the expression of the tyrosine kinase receptor, IGF-1R, which is an attractive therapeutic target in breast cancer, in response to LA treatment. Of note, the correlation between IGF-1R overexpression and cancer development, as well as the role of its downstream signaling cascade, such as activation of the PI3K/Akt pathway, in the promotion of proliferation of breast cancer cell lines have been reported in several studies⁴⁶⁻⁴⁸. Dunn et al. also demonstrated that suppression of IGF-1R expression leads to inhibition of metastasis, invasion, and adhesion of breast cancer cells⁵⁰. In this context, targeting IGF-1R appears to be a promising approach for inhibiting tumor growth and increasing therapeutic efficacy. Interestingly, our results showed that LA reduces IGF-1R expression from 24 hrs onwards and that this expression was abolished at 48 hrs upon treatment with 1 mM of LA in several ER α + and ER α - cell lines. Moreover, we demonstrated that this downregulation did not occur at the transcriptional level but rather at the post-translational level, since this dramatic decrease in IGF-1R was coupled with an accumulation of the immature (pro-IGF-1R) form upon LA treatment in a time- and concentration-dependent manner. These results were confirmed by immunofluorescence highlighting a change in the localization of IGF-1R from the plasma membrane to the cytoplasm. Herein, the cytoplasm localization of IGF-1R is an indication of its deactivation^{30,51}. However, it is not always the case. In this context, it has long been recognized that ligand binding at the plasma membrane induces internalization of activated IGF-1R⁵².

Furthermore, based on previous studies^{30,31}, IGF-1R maturation is processed by the pro-protein convertase furin. This enzyme is enriched in the Golgi and this is where cleavage of IGF-1R pro-receptor into an active form is thought to occur. Moreover, furin is also involved in processing of IGF 1/2⁵³. It is well-known that furin is overexpressed in cancer and it is considered as a tumor progression marker or as prognostic factor⁵⁴. For this reason, researchers were interested in determining its role in cancer progression and promotion. Accordingly, several studies have considered furin as an effective anti-cancer target⁵⁴⁻⁵⁶. Indeed, we herein demonstrated that LA reduces *furin* mRNA levels leading to a decrease in its protein expression.

In addition, IGF-1R is highly homologous to another tyrosine kinase receptor, namely the insulin receptor (IR)⁵⁷. Of note, numerous *in vitro* and *in vivo* studies have demonstrated the correlation between IR overexpression and malignancy in several cancer models including breast cancer⁵⁸. The two structurally different isoforms of IR, IR- α and IR- β , are overexpressed in many cancers⁵⁹. IR- α is the main isoform that plays an important role in cancer cell stemness, in tumor

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progression and in resistance to IGF-1R targeted therapies (for review,⁵⁹. For these reasons, we also studied the expression of IR upon LA treatment. Interestingly, our results showed that IR- α is abolished in favor of the immature form (pro-IR) upon LA treatment (1 and 2 mM) in our breast cancer cell lines. Collectively, our present results show that LA inhibits the IGF-1R and IR- α maturation by reducing furin expression. This work reveals the important role of LA in cancer treatment thanks to its dual inhibition of the IGF-1R/IR network which is a serious obstacle to treatment efficacy⁵⁶.

Moreover, our results have shown that LA reduces the EGFR expression with appearance of a doublet by western blots (Fig 3D). Of note, the EGFR ligands are expressed as transmembrane precursors. Their activation is realized by their cleavage by ADAM 17 which is a furin substrate. After cleavage, these ligands can bind and activate EGFR⁶⁰. Based on these data and our results, we can hypothesize that the downregulation of furin upon LA treatment could induce the accumulation of the pro-ADAM17 (inactive form), which then could inhibit the release of EGFR ligands and their binding on their receptor. These events could induce EGFR internalization and degradation.

The *ex vivo* model presented herein enabled us to replicate the microenvironment of intact breast tumor tissues. This approach maintains specific interactions between tumor cells and surrounding normal tissue components, and supplies a powerful and reproducible tool to study the response of fresh individual tumors to anti-cancer drugs. We tested a series of 9 human primary breast tumors in this *ex vivo* assay. Our results showed that 5 tumors revealed a significant decrease in Ki67 levels in response to LA. Moreover, among these 9 tumors, only 3 tumors expressed IGF-1R at the basal level, and this expression decreased upon LA treatment. Thus, these results confirm the beneficial effects of LA on breast cancer cells in their microenvironment.

Taken together, we herein show that LA inhibits the proliferation of various ER α + and ER α - breast cancer cell lines *in vitro* and *ex vivo* by inhibiting the maturation of IGF-1R (Figure 6). This inhibition is the consequence of the reduction of furin expression which is implicated in tumor aggressiveness. Nevertheless, the mechanism underlying LA downregulation of furin expression remains unclear. Though, LA could be considered as a potential agent for the treatment of cancer, possibly as a supplement to systemic anticancer treatments, further *in vivo* studies are needed to evaluate its efficacy in combination with conventional therapy.

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Ethical approval and consent to participate: For the BRC of CLB (n°BB-0033-00050) biological material collection and retention activity is declared to the Ministry of Research (DC-2008-99 and AC-2013-1871). Samples were used after patients provided written informed consent. This study was approved by the review boards of Centre Léon Bérard. This BRC quality certified according to AFNOR NFS96900 (N° 2009/35884.2) and ISO 9001 (Certification N° 2013/56348.2).

Consent to publish: The manuscript does not contain any individual person's data in any form.

Data availability: All data presented within the article and its supplementary information files are available upon request from the corresponding author.

Conflicts of interest: The authors declare no conflicts of interest.

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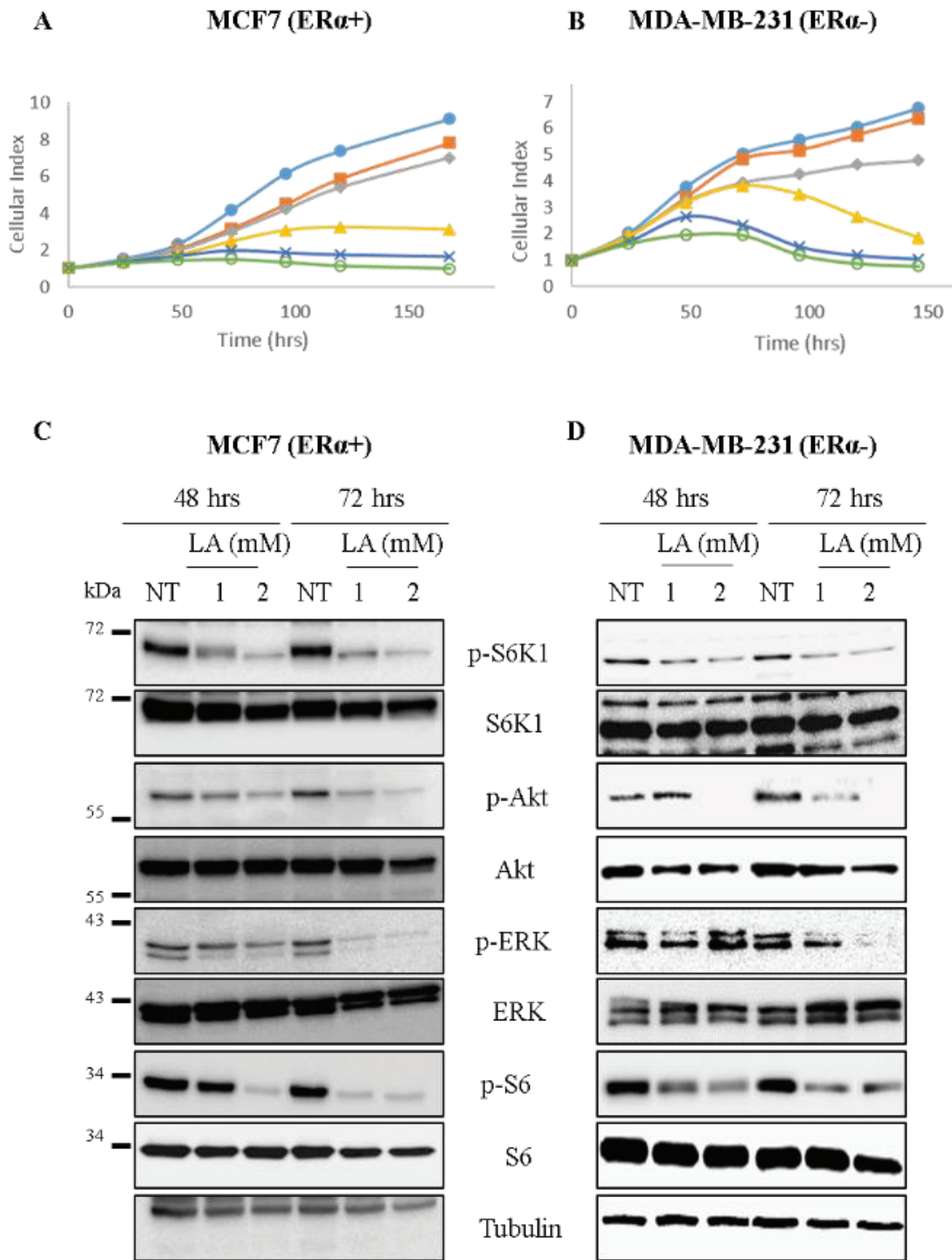


Figure 1 FARHAT et al.

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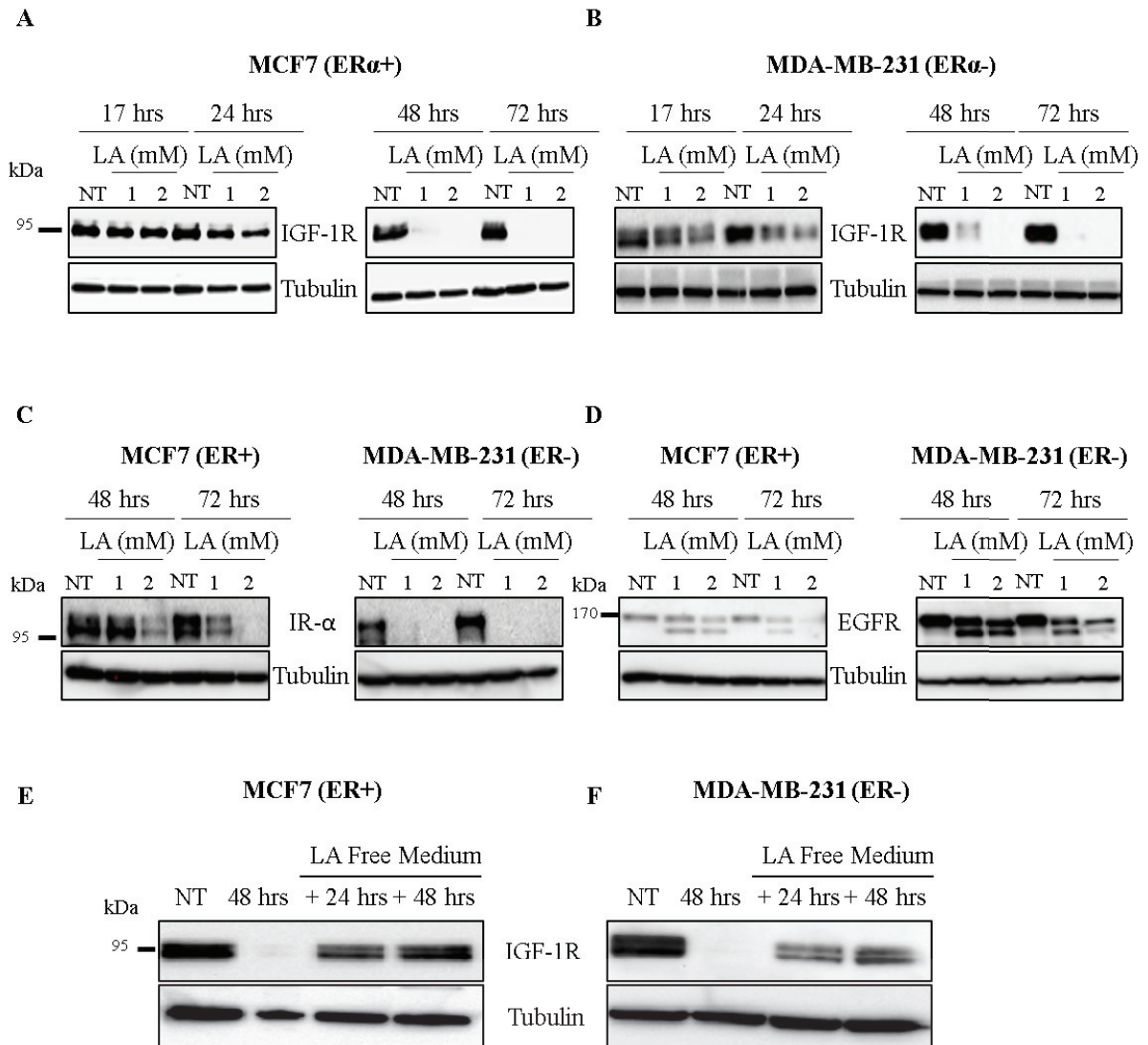


Figure 2: FARHAT et al.

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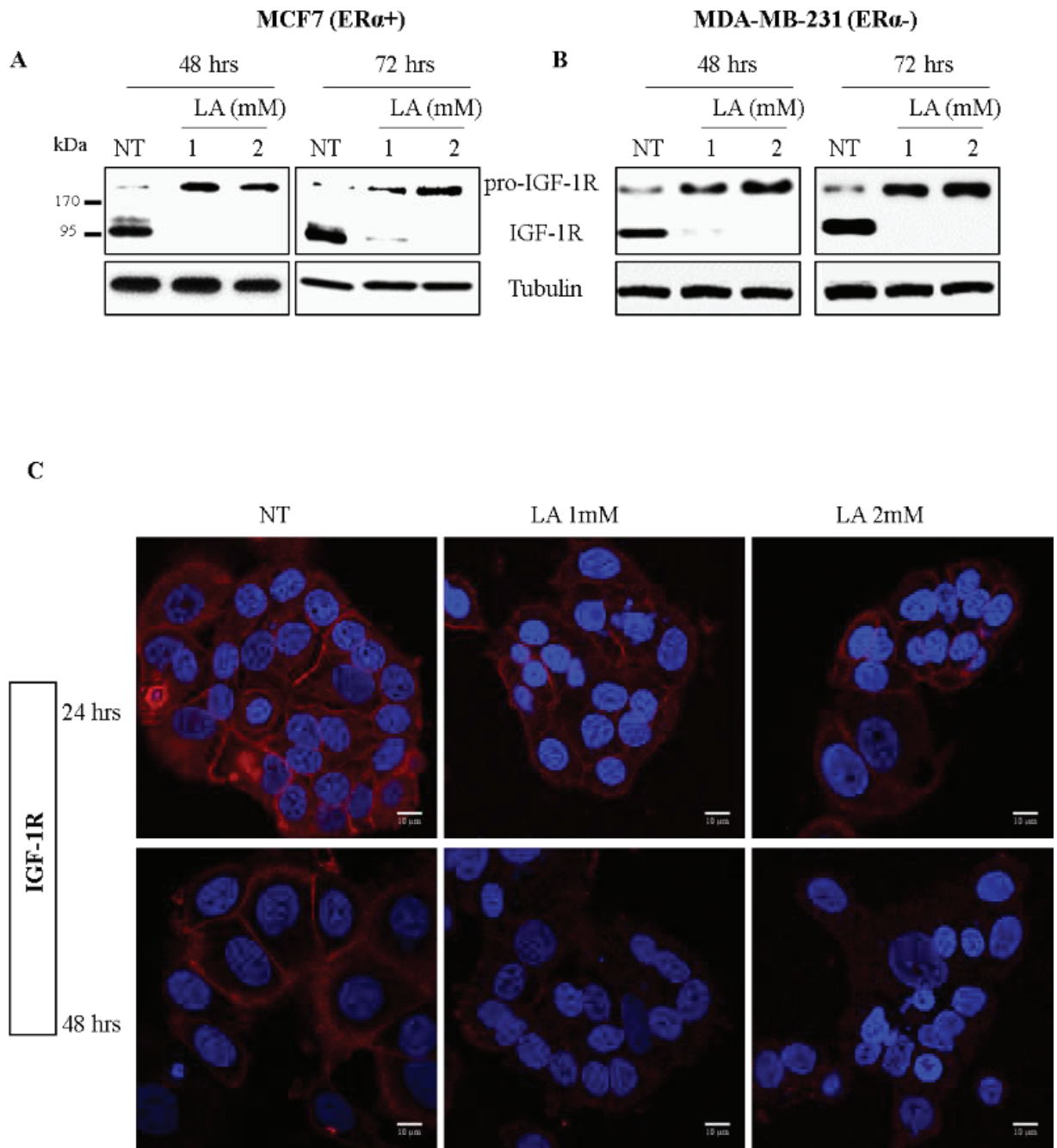


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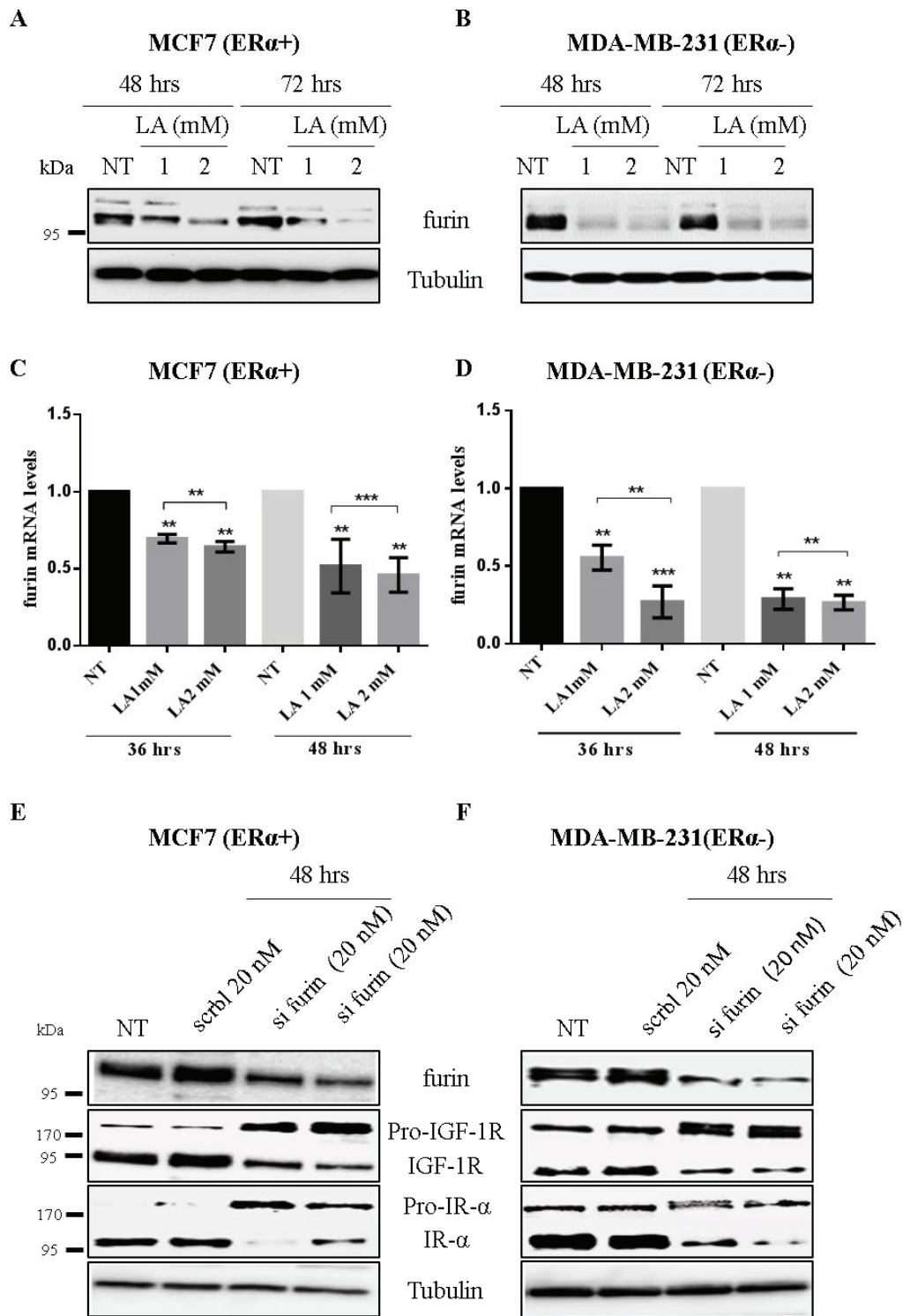


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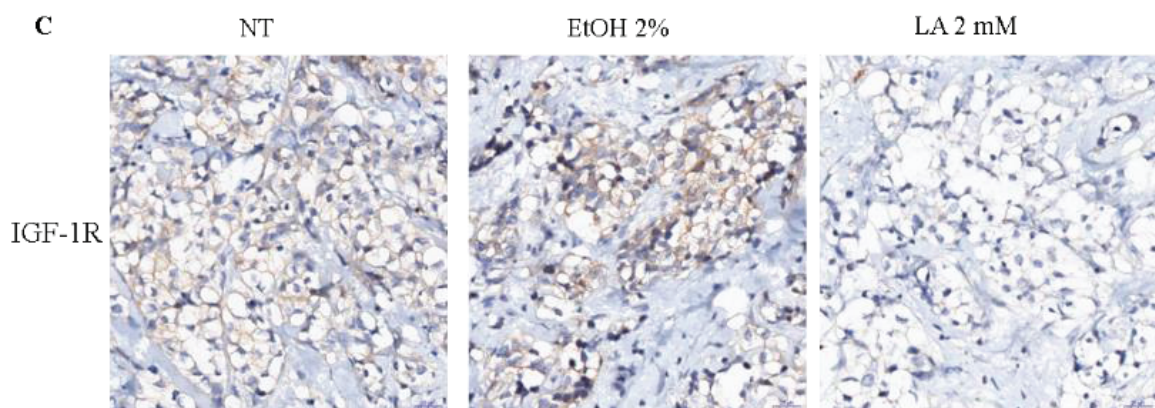
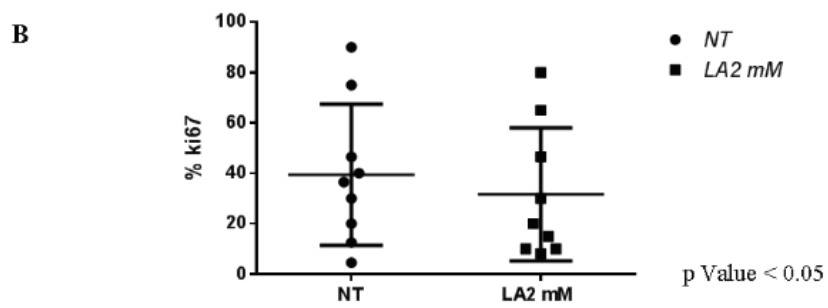
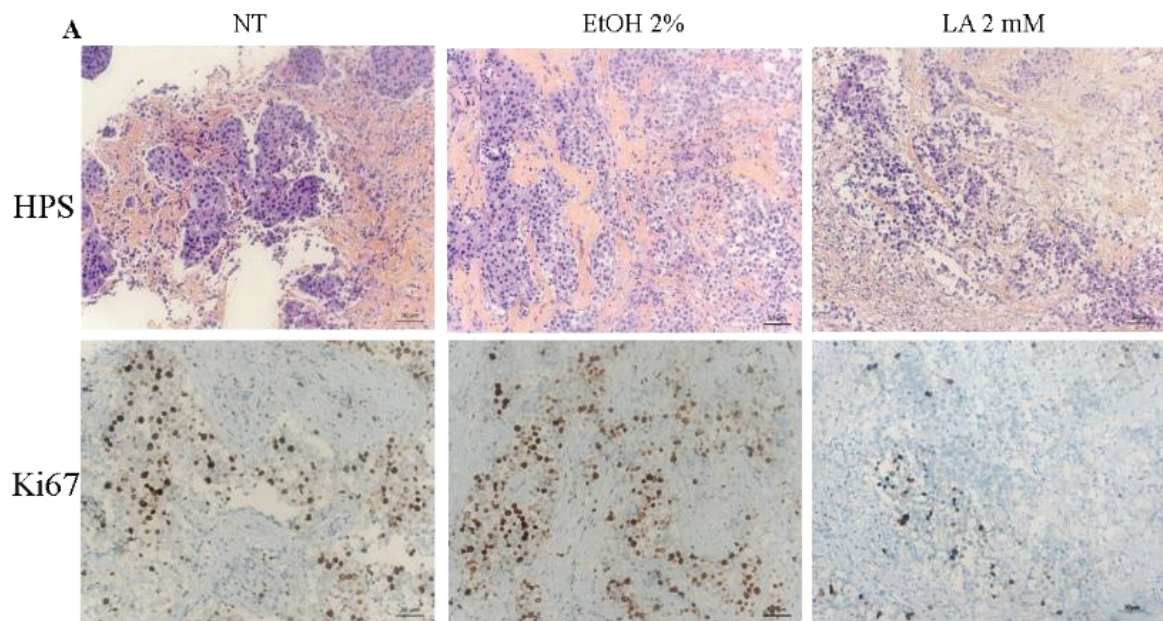


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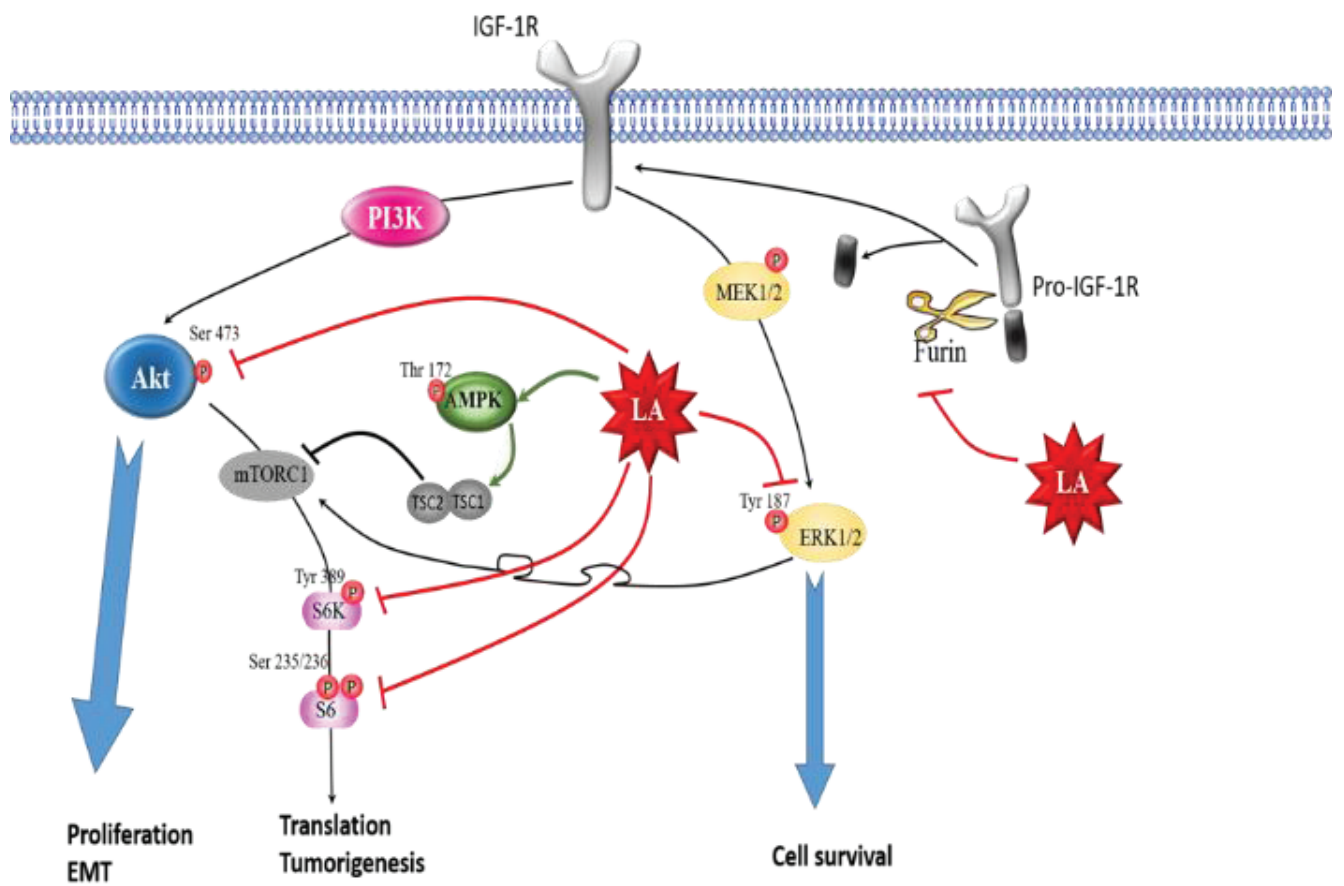


Figure 6: FARHAT et al.

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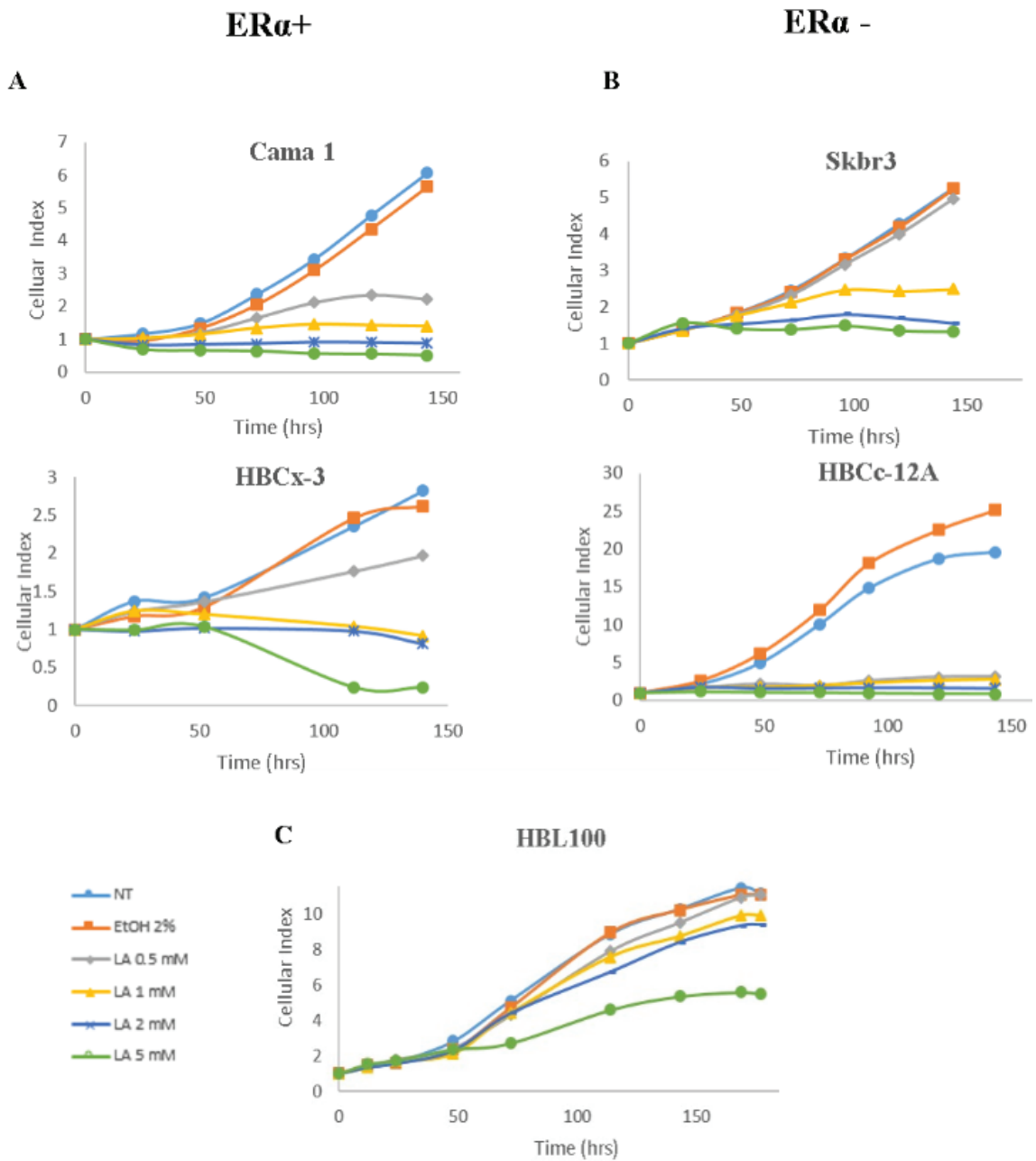


Figure 1 SD: FARHAT et al.

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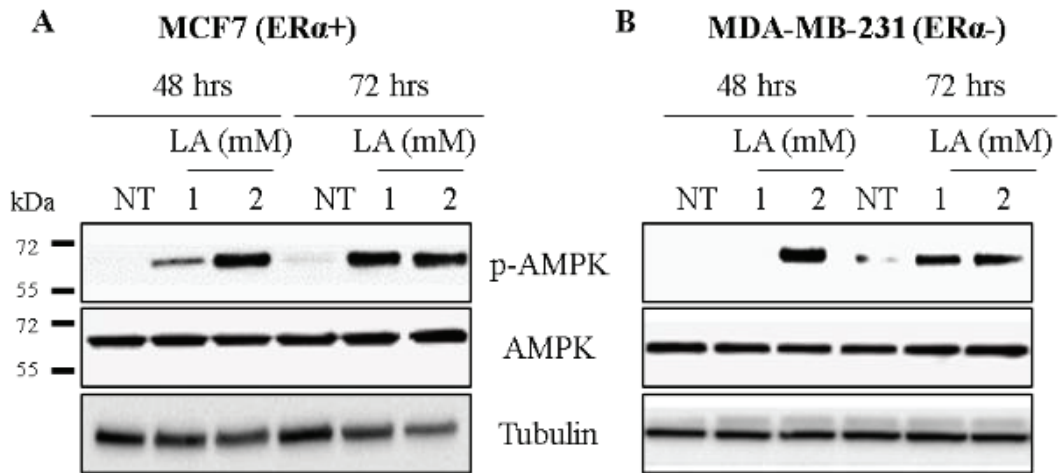


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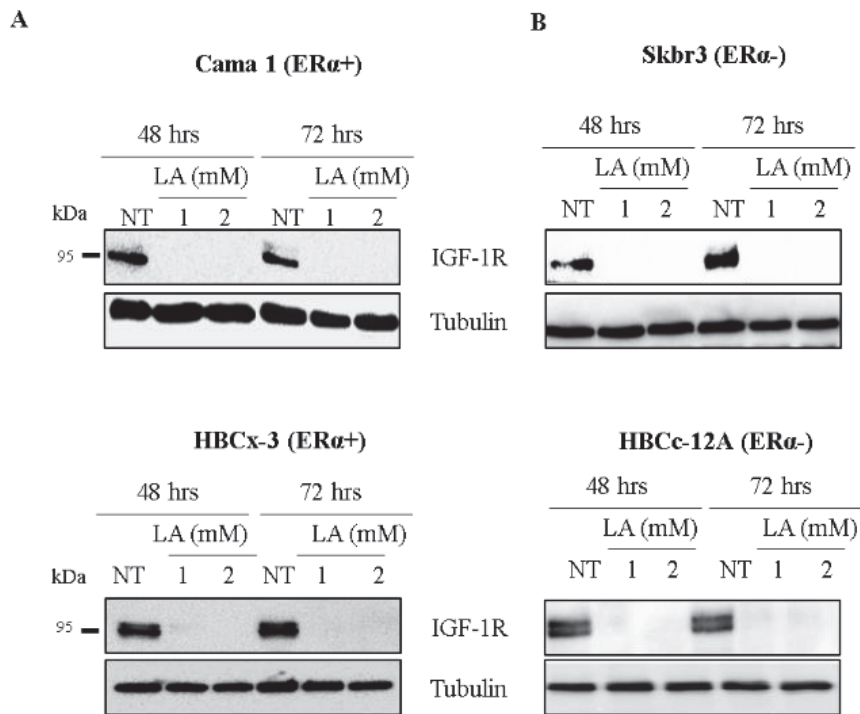


Figure 3 SD: FARHAT et al.

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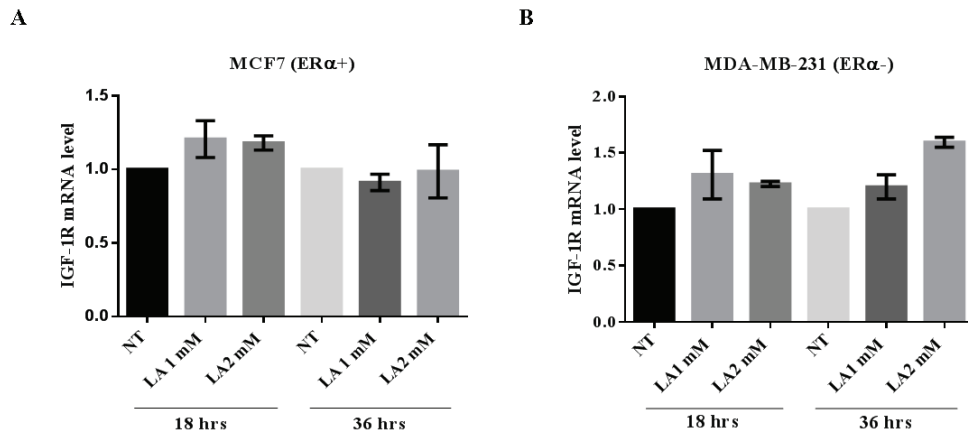


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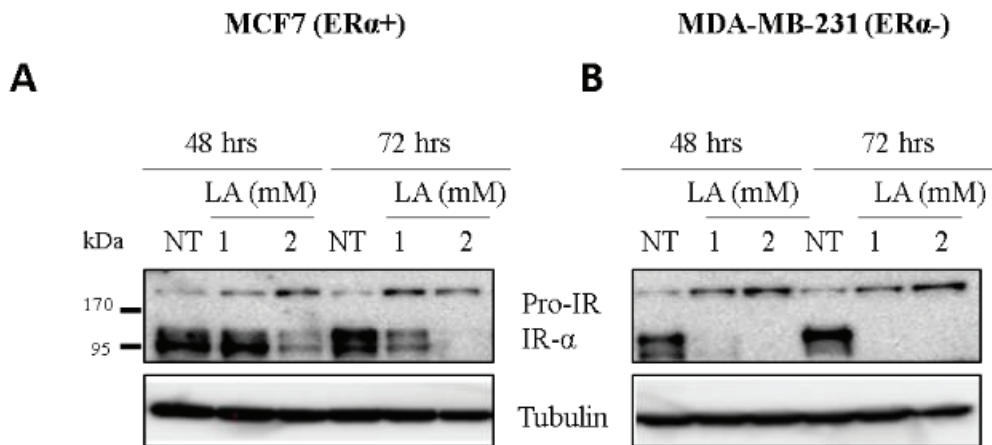


Figure 5 SD: FARHAT et al.

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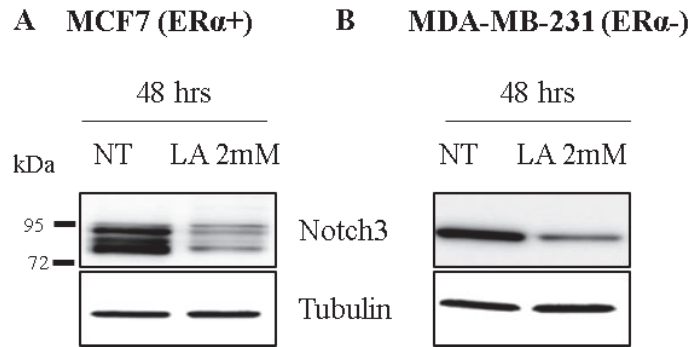


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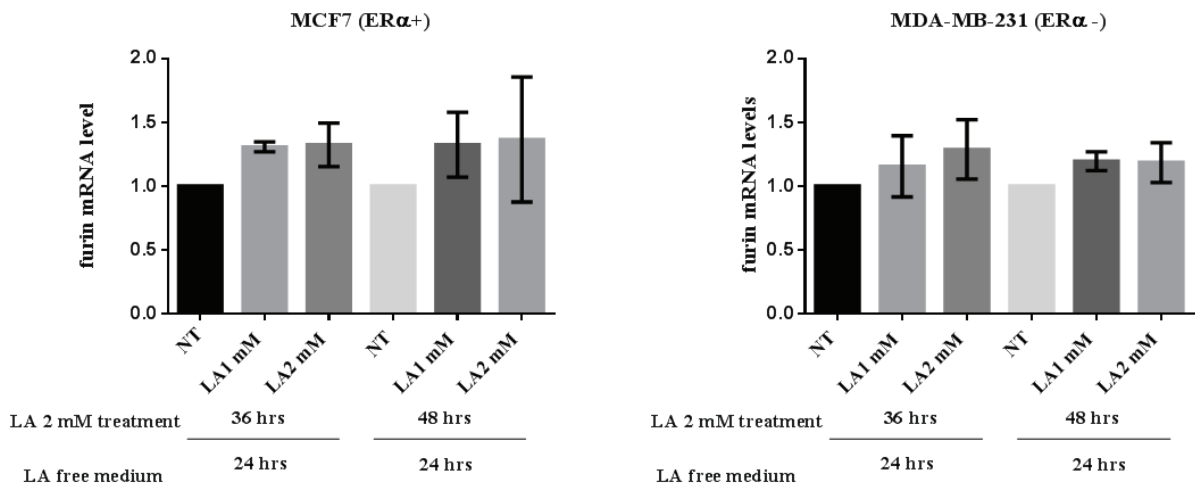


Figure 7 SD: FARHAT et al.

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Figure Legends

Figure 1: Dose-dependent effect of lipoic acid on the proliferation of ER α ⁺ and ER α ⁻ breast cancer cell lines. ER α ⁺ cell line MCF7 and ER α ⁻ cell line MDA-MB-231 were treated with LA at increasing concentrations (0.5, 1, 2 and 5 mM) followed by assessment of cell growth using the IncuCyte ZOOM technology. A: MCF7 cell line; B: MDA-MB-231 cell line. These experiments were performed in triplicate and repeated three independent times with similar results. Western blot analyses of LA (1 or 2 mM) reducing PI3K/Akt and ERK downstream effectors in C: MCF7 (ER α ⁺) and D: MDA-MB-231 (ER α ⁻) cell lines.

Figure 2: LA reduces the expression of several TKRs in MCF7 (ER α ⁺) and MDA-MB-231 (ER α ⁻) cell lines via a reversible mechanism. MCF7 and MDA-MB-231 breast cancer cell lines were treated with EtOH 2 % (NT) or with LA (1 or 2 mM) for 48 and 72 hrs. Effect of LA (1 or 2 mM) on IGF-1R level after 17, 24, 48 and 72 hrs of treatment was evaluated by western blotting in A: MCF7 (ER α ⁺) and B: MDA-MB-231 (ER α ⁻) cells lines. Effect of LA (1 or 2 mM) on C: IR- α level and D: EGFR level after 48 and 72 hrs of treatment was detected by western blotting in MCF7 (ER α ⁺) and MDA-MB-231 (ER α ⁻) cells lines. LA has a reversible effect on IGF-1R downregulation. IGF1R is re-expressed after 24 and 48 hrs in lipoic acid-free medium. IGF-1R protein level was detected by Western blot analysis in E: MCF7 (ER α ⁺) and F: MDA-MB-231 (ER α ⁻) cell lines treated with LA (2 mM) for 48 hrs followed by 24 or 48 hrs of incubation with LA-free media.

Figure 3: LA promotes pro-IGF-1R accumulation and reduces mature IGF-1R expression. MCF7 and MDA-MB-231 breast cancer cell lines were treated with EtOH 2 % (NT) or with LA (1 or 2 mM) for 24, 48 and 72 hrs. Pro-IGF-1R and mature IGF-1R expressions were detected by Western blot analysis after 48 and 72 hrs of LA treatment (1 or 2 mM) in A: MCF7 (ER α ⁺) and B: MDA-MB-231 (ER α ⁻) cell lines; C: IGF-1R protein expression and localization were evaluated by immunofluorescence in MCF7 cells treated with 1 or 2 mM of LA for 24 and 48 hrs, as indicated.

Figure 4: LA reduces furin mRNA and protein levels in breast cancer cell lines. furin silencing inhibits the maturation of IGF-1R and IR- α or furin silencing promotes the pro-IGF-1R and pro-IR accumulation and decreases the IGF-1R and IR- α expression in breast cancer cell lines. MCF7 and MDA-MB-231 breast cancer cell lines were treated with EtOH 2% (NT) or with LA

(1 or 2 mM) for 36, 48 and 72 hrs. The furin protein expression was detected by Western blot analysis after 48 and 72 hrs of LA treatment (1 or 2 mM) in A: MCF7 (ER α ⁺) and B: MDA-MB-231 (ER α ⁻) cell lines. The furin mRNA level was assessed by real-time quantitative reverse transcription PCR in C: MCF7 (ER α ⁺) and D: MDA-MB-231 (ER α ⁻) cell lines treated with LA (1 or 2 mM). Data are normalized against 28S mRNA level used as an endogenous control. Results are expressed relative to the levels in control cells set at one. This analysis was performed 3 times. Pro-IGF-1R, pro-IR, mature IGF-1R and mature IR- α expressions were detected by Western blot analysis in E: MCF7 (ER α ⁺) and F: MDA-MB-231 (ER α ⁻) transfected with two different furin siRNA or scramble siRNA for 48 hrs.

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Figure 5: LA decreases the expression of Ki67 and IGF-1R. IHC was conducted on 9 breast tumors. Paraffin sections were stained with standard hematoxylin-eosin-safran stains. **A:** Ki67 labeling was performed 48 hrs after LA (2 mM) or 2 % ethanol treatment in addition to the non-treated tumors. Nuclear localization was noted (thick black arrows). Harris' hematoxylin counterstain; **B:** Scatter plot showing the Ki67 staining mean score in LA-treated tumors compared to non-treated (NT) tumors; Unpaired two-tailed Student's t-test was used for statistical analysis with P value <0.05; **C:** IGF-1R labeling was done 48 hrs after LA (2 mM) or 2 % ethanol treatment in addition to the NT tumors.

Figure 6: Schematic representation of the antiproliferative role or anticancer effects of lipoic acid in breast cancer cells. Cellular proliferation, survival and EMT are controlled by the activation of ERK and PI3K/Akt pathways. LA prevents the phosphorylation of ERK, in particular by blocking the activation of c-Myc which is a centerpiece of the several processes implicated in cancer development. Moreover, LA inhibits the activation of Akt leading to the inhibition of mTOR effectors S6K1 and S6 which induce the translational process, thus LA reduces cellular proliferation and growth processes. On the other hand, LA activates the AMPK protein which negatively regulates the Akt pathway thereby reducing the translation of several proteins involved in the tumorigenesis process. Moreover, LA impedes the activation of ERK and PI3K/Akt pathways by the inhibition of some TKRs expression, such as IGF-1R, which is an upstream activator of these pathways, reinforcing its inhibition. IGF-1R maturation is inhibited by LA by downregulating the furin expression leading to a decrease of mature IGF-1R at the membrane level.

Supplementary data:

Figure 1: Dose-dependent effect of lipoic acid on the proliferation of ER α ⁺ and ER α ⁻ breast cancer cell lines. ER α ⁺ cell lines (Cama1 and HBCx-3), ER α ⁻ cell lines (Skbr3 and HBCc-12A) and nonmalignant cell line (HBL100) were treated with LA at increasing concentrations (0.5, 1, 2 and 5 mM) followed by assessment of cell growth using the IncuCyte ZOOM technology. **A:** ER α ⁺ cell lines; **B:** ER α ⁻ cell lines. LA did not reveal a cytotoxic effect in **C:** nonmalignant cell line treated with increasing concentrations of LA. These experiments were performed in triplicate and repeated three independent times with similar results.

Figure 2: Lipoic acid induces AMPK phosphorylation in **A:** MCF7 and **B:** MDA-MB-231 cell lines 48 hrs and 72 hrs after treatment.

Figure 3: LA inhibits the expression of IGF-1R in several ER α ⁺ and ER α ⁻ breast cancer cell lines. These cell lines were treated with EtOH 2 % (NT) or with LA (1 or 2 mM). Effect of LA (1 or 2 mM) on IGF-1R level after 48 and 72 hrs of treatment was evaluated by western blot analysis in **A:** ER α ⁺ cell lines (Cama1 and HBCx-3); **B:** ER α ⁻ cell lines (Skbr3 and HBCc-12A).

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Figure 4: LA does not regulate IGF-1R mRNA level. MCF7 and MDA-MB-231 breast cancer cell lines were treated with EtOH 2 % (NT) or with LA (1 or 2 mM) for 48 and 72 hrs. IGF-1R mRNA level was assessed by real-time quantitative reverse transcription PCR in **A**: MCF7 (ER α +) and **B**: MDA-MB-231 (ER α -) cell lines treated with LA (1 or 2 mM). Data are normalized against 28S mRNA level used as an endogenous control. Results are expressed relative to the levels in control cells set at one. This analysis was performed 3 times.

Figure 5: LA promotes the pro-IR accumulation and reduces the mature IR- α expression. Pro-IR and mature IR- α expressions were detected by Western blot analysis after 48 and 72 hrs of Ethanol (EtOH 2 %) treatment or LA treatment (1 or 2 mM) in **A**: MCF7 (ER α +) and **B**: MDA-MB-231 (ER α -) cell lines.

Figure 6: LA inhibits the Notch3 maturation in breast cancer cell lines. The mature Notch3 protein expression was detected by Western blot analysis after 48 hrs of Ethanol treatment (EtOH 2 %) or LA treatment (2 mM) in **A**: MCF7 (ER α +) and **B**: MDA-MB-231 (ER α -) cell lines.

Figure 7: LA has a reversible effect on furin downregulation. The furin mRNA level is re-expressed after 24 hrs in LA-free medium. furin mRNA level was assessed by real-time quantitative RT-PCR in **A**: MCF7 (ER α +) and **B**: MDA-MB-231 (ER α -) cell lines treated with LA (1 or 2 mM) for 36 or 48 hrs followed by 24 hrs of incubation with LA-free medium. Data are normalized against 28S mRNA level used as an endogenous control. Results are expressed relative to the level in control cells set at one. This analysis was performed 3 times.

Table legends

Table 1: Main clinical characteristics of 9 human breast cancer using for ex vivo assay.

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2^{ème} article

Lipoic acid-induced oxidative stress abrogates IGF-1R maturation by inhibiting CREB/furin axis in breast cancer cell lines.

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Objectif de l'étude

Dans le premier article, nous avons démontré que LA inhibe la maturation du récepteur de la tyrosine kinase IGF-1R en réduisant l'expression de la proprotéine convertase furine dans des lignées cellulaires de cancer du sein ER α (+) et ER α (-). Cependant, nous n'avons pas élucidé les mécanismes d'action de LA provoquant la réduction d'expression de la furine. Dans ce second article, nous démontrons que l'expression de l'ARNm et de la protéine de la furine est induite par le facteur de transcription CREB et que le rôle pro-oxydant de LA inhibe l'expression protéique du facteur CREB favorisant ainsi la baisse d'expression de la furine.

Modèles d'étude

Les modèles cellulaires étudiés lors de cette étude sont la lignée cellulaire humaine de cancer du sein, MCF7, provenant d'un adénocarcinome mammaire de type ER α (+) et MDA-MB-231 de type ER α (-). Pour réaliser ces expériences, diverses approches *in vitro* ont été mises en œuvre parmi lesquelles IncuCyte Zoom, western blot, transfection de siRNA, dosage des ROS et RT-qPCR.

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Abstract

The beneficial effects of lipoic acid (LA) in cancer treatment have been well documented in the last decade. Indeed, LA exerts crucial anti-proliferative effects by reducing breast cancer cell viability, cell cycle progression and the epithelial-to-mesenchymal transition (EMT). However, the mechanisms of action (MOA) underlying these anti-proliferative effects remain to be elucidated. Recently, we demonstrated that LA decreases breast cancer cell proliferation by inhibiting IGF-1R maturation *via* the downregulation of the proprotein convertase furin. The aim of the present study was to investigate the MOA by which LA inhibits furin expression in estrogen receptor α (ER α) (+) and (-) breast cancer cell lines. We unveil that LA exerts a pro-oxidant effect on these cell lines, the resulting reactive oxygen species (ROS) generated being responsible for the reduction in the expression of the major (CREB) protein. This transcription factor is overexpressed in many types of cancers and regulates the expression of furin in breast cancer cells independently of ER α , as evidenced herein by the inhibition of furin expression following CREB silencing. Consequently, our findings expose for the first time the complete MOA of LA *via* the CREB/furin axis leading to inhibition of breast cancer cell proliferation.

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Introduction

Lipoic acid (LA) is a naturally-occurring compound found in all prokaryotic and eukaryotic cells. It is synthesized in small quantities by most living organisms including humans. It plays a vital role within the body, where it functions as a cofactor of several mitochondrial enzymes implicated in energy metabolism, such as pyruvate dehydrogenase (PDG) and α -ketoglutarate dehydrogenase (KGDG) (Ziegler et al., 1995). In addition, depending on its intracellular concentration, LA exerts either anti- or pro-oxidant effects. Owing to its anti-oxidant properties, LA is frequently used for the treatment of pathologies associated with oxidative stress, such as diabetes, atherosclerosis, liver diseases and neurodegenerative diseases. Aside from its efficacy in treating these chronic diseases, researchers have demonstrated its anti-proliferative effects in different types of cancers including breast cancer. Consistently, we recently demonstrated that LA inhibits the maturation of IGF-1R, reducing the proliferation of several ER α + and ER α - breast cancer cell lines (Farhat et al., in press). Mechanistically, we showed that this inhibitory effect is due to the inhibition of the proprotein convertase furin, which is directly involved in IGF-1R maturation. Of note, furin is a calcium-dependent protease and ubiquitously expressed in all vertebrates and many invertebrates. Furin is the most extensively studied member of the proprotein convertase (PC) family, after being the first member identified 29 years ago (van de Ven et al., 1990). Furin catalyzes the endoproteolytic cleavage of the inactive pro-IGF-1R into its active form (Massagué and Czech, 1982; Ward et al., 2001), and its expression is associated with an enhanced aggressiveness in several carcinomas (Bassi et al., 2001a, 2001b; Bennett et al., 2000; Cheng et al., 2001). High levels of furin expression have been observed in several types of cancers such as lung, colon, cervical, head and neck and ovarian cancers (Jaaks and Bernasconi, 2017; Jaaks et al., 2016). In breast cancer, various studies have shown that the expression of furin is upregulated and is correlated with a poor prognosis (Jaaks and Bernasconi, 2017; Jaaks et al., 2016). Moreover, the exogenous overexpression of furin increases resistance of breast cancer cells to tamoxifen treatment (Jaaks and Bernasconi, 2017). These malignant effects of furin are correlated with its catalytic activity, cleaving and thus activating several precursors such as RTKs (IGF-1R and IR), growth factors (IGF1, IGF2, and TGF- β) and metalloproteases (MT1-MMP). Accordingly, silencing furin by siRNA or inhibiting its activity by α 1-PDX reduces the motility, migration and malignancy of breast cancer cell lines (Lapierre

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et al., 2007). In this study, we further document the role of furin in breast cancer by unveiling that the inhibition of IGF-1R maturation is due to the inhibition of the CREB/furin axis in response to LA treatment.

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Materials and methods

Lipoic acid

Lipoic acid (LA) was purchased from Sigma (Sigma). A stock solution was prepared by dissolving LA in absolute ethanol, which was then stored at -20°C. LA was diluted in culture medium for *in vitro* experiments.

Cell lines, culture conditions and siRNA transfections

The ER α (+) cell line MCF7 and ER α (-) cell line MDA-MB-231 were obtained from the ATCC. These cell lines were maintained at 37°C in the appropriate medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids and 2% penicillin/streptomycin. All cell lines were regularly tested for mycoplasma contamination.

For knockdown experiments, *CREB*-specific siRNAs from ThermoFisher scientific (ambion) sens: 5'-GGUGGAAA AUGGACUGGCUTT-3'; anti sens: 5'-AGCCAGUCCA UUUUCCACCTT-3'; the scramble siRNA (Eurogentec) (20 nM) were transfected into MCF-7 and MDA-MB-231 cells using the lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen).

Cell proliferation assay using the IncuCyte technology

Cell proliferation was assayed using an IncuCyte® ZOOM Live-Cell Analysis System (Essen Bioscience, Sartorius), by collecting real-time data of cell confluence. Cells were seeded onto 96-well plates (5,000 cells/well) and left to adhere for 24 hrs. The cells were then treated with different concentrations of LA or absolute ethanol. Cell proliferation data were obtained by the cell confluence increment in each treatment and expressed as a percentage relative to that of control cells. Each sample was tested in triplicate.

Western blot analysis

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Cells were lysed using RIPA buffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 % NP-40 and 0.25% deoxycholate) supplemented with protease inhibitor tablets (Roche Molecular Biochemicals) and phosphatase inhibitors (1 mM NaF, 1 mM Na₃VO₄ and 1 mM beta-glycerophosphate). Cell lysates were cleared by centrifugation (13,000×g, 15 min, 4°C) and protein concentration was determined by the Bradford method (Bio-Rad protein Assay). Protein samples were separated on SDS-PAGE and membranes were incubated with primary antibodies overnight at 4°C on a shaker with the following primary antibodies: anti-IGF-1R (Cell Signaling), anti-furin (Proteintech), anti-CREB (Cell signaling), anti-Tubulin (Sigma). Membranes were washed with TBS-Tween and incubated with anti-rabbit or anti-mouse secondary antibodies. Detection was carried out using ECL Prime reagent (GE Healthcare).

RNA isolation, reverse transcription and quantitative real time polymerase chain reaction (RT-qPCR)

Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed to complementary DNA (cDNA) using SuperScript™ II Reverse Transcriptase (Life Technologies). The primers are listed as follows: *furin* Forward: 5'-TATGGCTACGGGCTTTTGG -3' and Reverse: 5'-TTCGCTGGTGTTCATCAATCTCT -3'; *28S* Forward: 5'-CGATCCATCATCCGCAATG -3' and Reverse: 5'-AGCCAAGCTCAGCGCAAC -3'. RT-qPCR was performed using the Bio-Rad CFX 96 Real-time PCR system (Bio-Rad) with the SsoAdvanced™ Universal SYBR® Green Supermix according to the manufacturer's instructions. The mRNA level was normalized against the 28S using livak's ($2^{-(\Delta\Delta Cq)}$) method. RT-qPCR conditions were as follows: 1 activation step for 5 min at 94°C, 35 cycles of denaturing at 94°C for 15 s, primer annealing at 59°C for 45 s, and primer extension at 72°C for 2 min, and a final extension at 72°C for 5 min.

Analysis of reactive oxygen species (ROS) generation

MCF-7 and MDA-MB-231 Cells were treated with LA 2 mM alone, NAC 10 mM alone or in combination for 48 hrs. Cells were harvested and stained with 5 µM CellROX Green Reagent from Life Technologies for 30 min at 37 °C. The generation of ROS was evaluated using a

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fluorescent CellROX Green Reagent (Molecular probes), which binds to DNA upon oxidation, thereby turning the nuclei and mitochondria green. *Statistical analysis*

Data are presented as mean \pm SEM of three independent experiments. Unpaired two-tailed Student's t-test was used for statistical analysis. A *P*-value of < 0.05 was considered statistically significant. * *P* < 0.05 ; ** *P* < 0.01 ; *** *P* < 0.001 . The graph artwork was created using Microsoft office Excel 2010 and GraphPad prism 6.

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Results

LA inhibits the expression of the transcription factor CREB

Based on a previous study showing that furin expression is activated by the transcription factor CREB (Zhou et al., 2014), and having demonstrated that LA inhibits IGF-1R maturation *via* furin downregulation (Farhat et al. in press), we speculated that LA may impact the expression of CREB. In order to validate our hypothesis, we assessed the expression of CREB in MCF7 and MDA-MB-231 cell lines 18 and 24 hrs following LA treatment (at 1 mM or 2 mM). From 18 hrs onwards, we observed a decrease in the expression of total CREB in both cell lines at both LA concentrations (Figure 1 A and B).

CREB silencing reduces furin expression

In a previous publication, we demonstrated that LA inhibits the maturation of IGF-1R by targeting furin (Farhat et al. in press). In order to prove the implication of CREB in regulating furin expression in our cellular models, we transfected MCF7 (ER α +) and MDA-MB-231 (ER α -) breast cancer cell lines with CREB siRNA or scramble siRNA (20 nM) for 48 hrs. We found that *CREB* silencing led to a decrease in CREB expression at 48 hrs, which in turn resulted in a reduced expression of furin at the protein (Figure 1 C and D) and mRNA (Figure 1 E and F) levels.

LA inhibits CREB expression through its pro-oxidant effect

We also formerly reported that LA (0.5 mM and 1 mM) exerts its anticancer effects in the context of ovarian cancer by inducing the generation of ROS (Kafara et al., 2015a). In order to determine whether LA treatment generates ROS in our present models, we used the CellROX Green Reagent (Molecular probes). The generation of ROS was evaluated using a fluorescent confocal microscopy. LA treatment (2 mM) induced an increased ROS production by MCF7 and MDA-MB-231 cells after 48 hrs (Figure 2), whereas, pretreatment with 10 mM of the ROS scavenger, N-acetyl-L-cysteine (NAC), clearly decreased LA-induced ROS generation (Figure 2).

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It is worth noting that CREB expression is impaired by hydrogen peroxide H₂O₂ (Fu et al., 2019). We thus hypothesized that LA could inhibit CREB expression through its pro-oxidant activity. Our results confirmed once again the decrease in CREB protein expression in response to LA, leading to a reduction in the expression of furin (Figure 3A). Furthermore, the addition of NAC (10 mM) to LA (2 mM) abolished the inhibitory effect of LA on the CREB and furin proteins (Figure 3A), as well as on the mRNA level of furin (Figure 3B), indicating that this effect was ROS-mediated.

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Discussion

It is well established that lipoic acid (LA) exerts pro-apoptotic / anti-proliferative effects in different types of cancers including breast cancer. These beneficial effects are mostly due to its ability to inhibit cell cycle progression, and impair cell viability or EMT, *via* its impact on major proliferative pathways such as PI3K/Akt and MAPK/ERK. Moreover, LA reduces the metastatic process by targeting two crucial pathways, namely the transforming growth factor- β (TGF- β) and the focal adhesion kinase (FAK) pathways (Farhat and Lincet; Jeon et al., 2016; Kafara et al., 2015; Kono et al., 2012; Lee et al., 2010; Tripathy et al., 2018). Interestingly, the combination of LA and conventional chemotherapy, including paclitaxel, efficiently reduces their toxicity thus increasing their efficacy (Farhat and Lincet, BBA reviews on cancer, 2019 in press).

However, the mechanisms by which LA elicits its anti-proliferative effects remained understudied. Nevertheless, we recently showed that LA inhibits the proliferation of several ER α (+) and ER α (-) breast cancer cell lines by targeting the main proliferative pathways PI3K/Akt and MAPK/ERK. Indeed, LA treatment prevents ERK but also Akt phosphorylation (Farhat and Lincet, BBA reviews on cancer, 2019 in press). We demonstrated that this inhibitory effect of LA relies on inhibiting the maturation of the tyrosine kinase receptor IGF-1R, which is an upstream activator of these pathways as well as an attractive therapeutic target in breast cancer (Ireland et al., 2018b; Riedemann and Macaulay, 2006; Sachdev et al., 2004; Wang et al., 2012a). Of note, it is well-known that the IGF-1R is cleaved into its bioactive form by the proprotein convertase furin. Based on previous studies, furin is overexpressed in several carcinomas and is associated with tumor aggressiveness and poor prognosis. Moreover, we demonstrated that LA inhibits the maturation of IGF-1R by inhibiting the expression of furin (Farhat and Lincet, BBA reviews on cancer, 2019 in press). In the present study, we further decipher the MOA of LA that, in our ER α (+) and ER α (-) breast cancer cell line models, reduces CREB expression inducing a drop in furin protein and mRNA levels (figure 4). Of note, a previous study reported that the transcription factor CREB induced furin transcription (Yang et al., 2013). Similarly, in our models, furin mRNA expression might be controlled by CREB at the transcriptional level. Under our experimental conditions, we showed that LA treatment (1 and 2 mM) inhibits CREB expression in MCF7 and MDA-MB-231 cell lines regardless of the ER α status. Notably, several studies have suggested an oncogenic potential of CREB and it is thus considered as a promising target for cancer therapy (Steven and Seliger, 2016). A previous study

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also highlighted that the protein expression of CREB is inhibited by the generation of ROS (Fu et al., 2019). The anticancer effect of LA is therefore not only associated with its anti-oxidant properties, but with a pro-oxidant effect in several cancer cell lines (Choi et al., 2009; Mounjaroen et al., 2006; Na et al., 2009; Selvakumar and Hsieh, 2008b; Shi et al., 2008; Simbula et al., 2007b; Wenzel et al., 2005b). In this context, it has been demonstrated that ROS is involved in pro- and anti-tumorigenic signaling pathways according to their cellular levels. Increased ROS production is correlated with proliferation and/or cell survival through many pathways including PI3K/Akt, MAPK/ERK and inactivation of their downstream targets including Bad, Bax, Bim, Foxo and PTEN. However, toxic levels of ROS cause cell cycle arrest, senescence and cell death initiated by intrinsic apoptotic signaling in the mitochondria or extrinsic apoptotic signaling by death receptor pathways (Smyth and Bertram, 2019). Consistently, we demonstrated that LA inhibits CREB expression by its pro-oxidant role. Indeed, we first validated that LA treatment (1 or 2 mM) induces ROS generation in our models, we then demonstrated that this oxidative stress induced by LA is essential to inhibit CREB expression. Moreover, the reduction of furin expression is the consequence of the downregulation of CREB. As mentioned above, furin is involved in the maturation of various RTKs including IGF-1R (Jaaks and Bernasconi, 2017). Collectively, our results show that LA causes ROS production, reducing the expression of CREB, which in turn promotes the downregulation the furin expression. All of these events contribute to an inhibition of IGF-1R maturation. Our study has provided fundamental insights into LA mechanisms and suggest that CREB is a targetable mediator in response to LA exposure.

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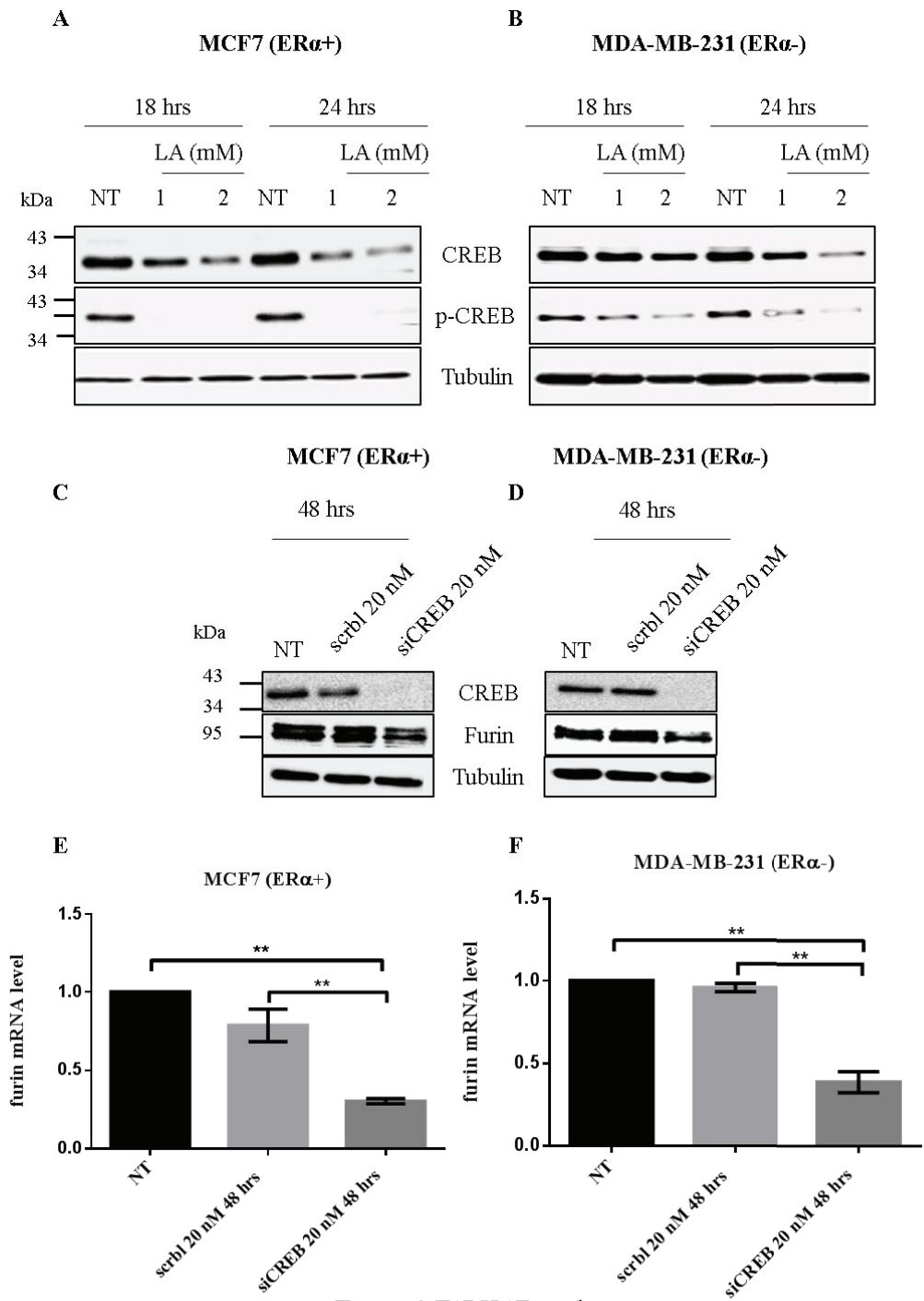


Figure 1 FARHAT et al.

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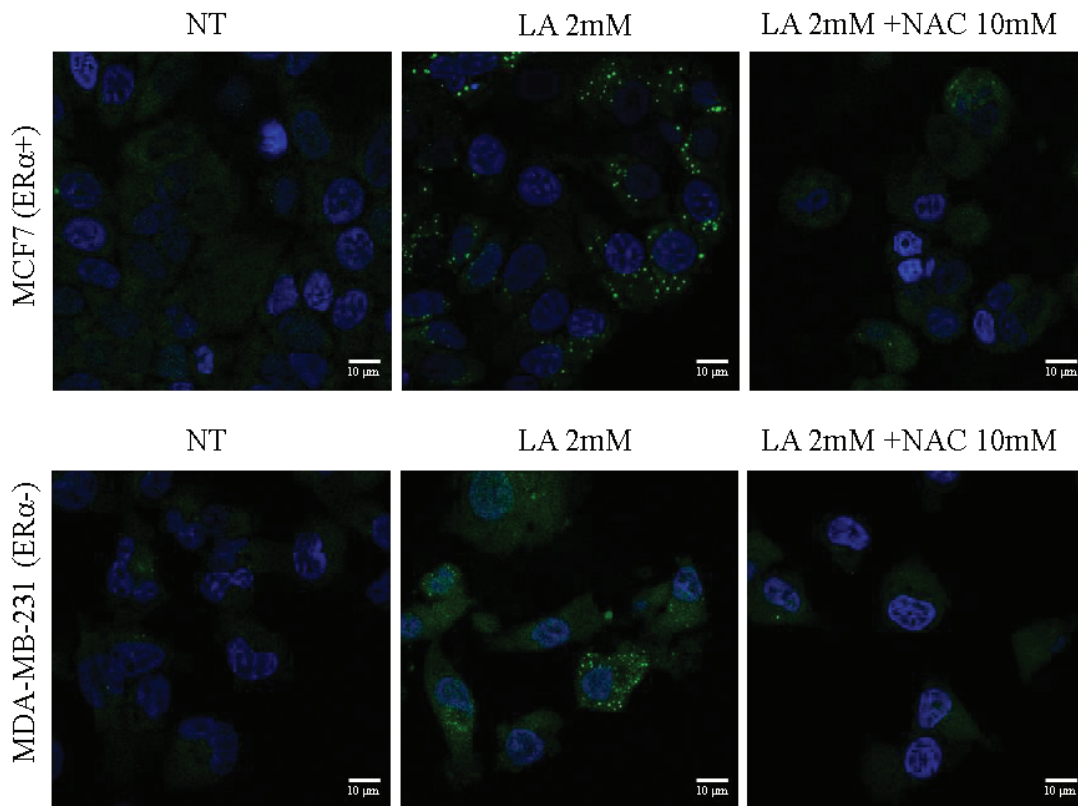


Figure 2 FARHAT et al.

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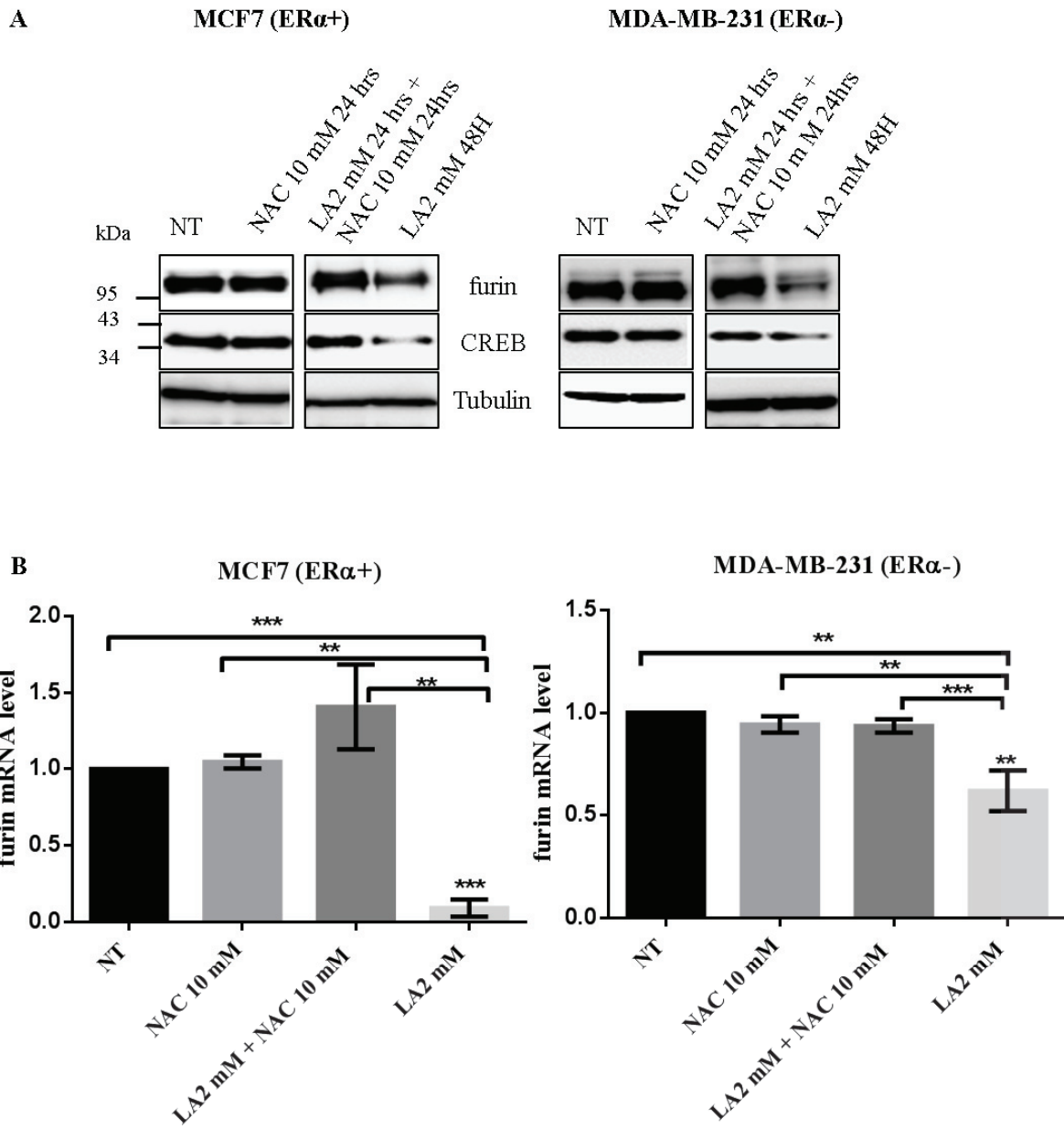


Figure 3 FARHAT et al.

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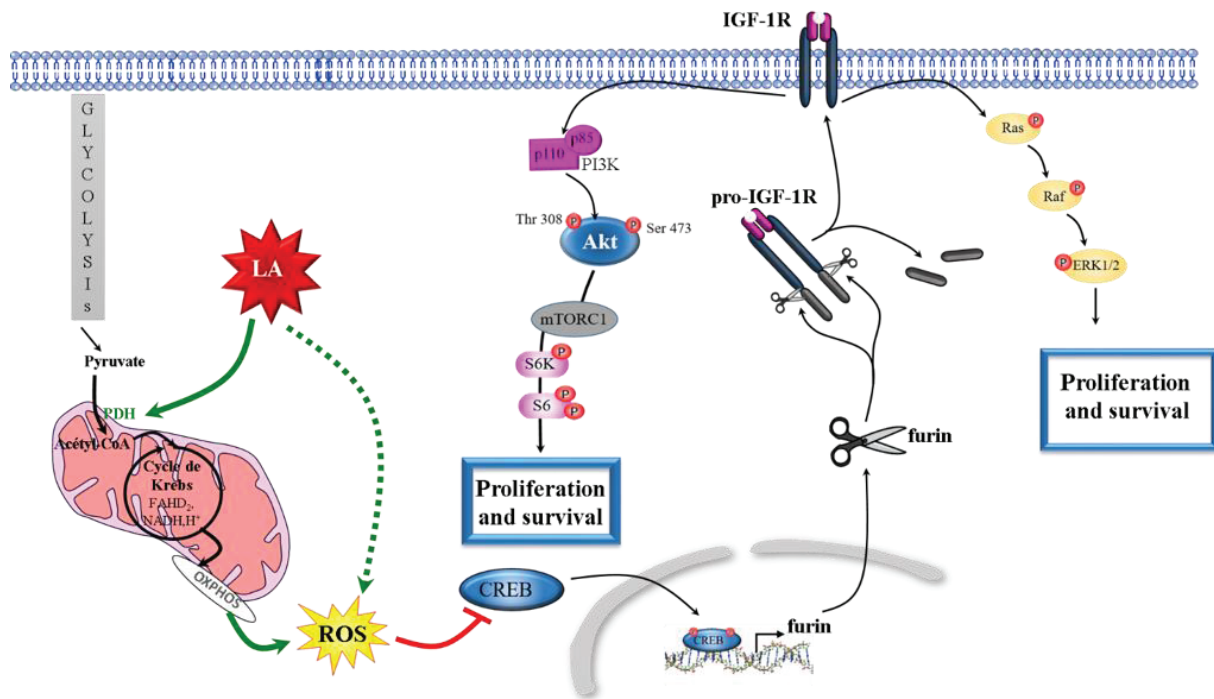


Figure 4 FARHAT et al.

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Legends of figures

Figure 1: Lipoic acid (LA) reduces the expression of the cyclic AMP response element binding (CREB) protein. MCF7 and MDA-MB-231 breast cancer cell lines were treated with LA (1 or 2 mM) for 18 and 24 hrs. Western blot analyses of LA (1 or 2 mM) reducing CREB and p-CREB expression in **A:** MCF7 (ER α +) and **B:** MDA-MB-231 (ER α -) cell lines. CREB silencing inhibits the protein and mRNA expression of furin in breast cancer cell lines. MCF7 and MDA-MB-231 breast cancer cell lines were treated with scrbl 20 nM or with siCREB 20 nM for 48 hrs. The furin protein level was detected by Western blot analysis **C:** MCF7 (ER α +) and **D:** MDA-MB-231 (ER α -) cell lines. The mRNA expression of furin was assessed by real-time quantitative reverse transcription PCR in **E:** MCF7 (ER α +) and **F:** MDA-MB-231 (ER α -) cell lines treated with scrbl or siCREB 20 nM. Data are normalized against 28S mRNA level used as an endogenous control. Results are expressed relative to the levels in control cells set at one. This analysis was performed in triplicate. Unpaired two-tailed Student's t-test was used for statistical analysis. A *P*-value of < 0.05 was considered statistically significant. * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001.

Figure 2: LA 2 mM plays a pro-oxidant role in breast cancer cell lines. Oxidative stress measured using CellROX green fluorescent dye by confocal microscopy (Zeiss). MCF7 and MDA-MB-231 breast cancer cell lines were treated with LA 2 mM for 24 hrs or with LA + NAC (N-acetyl-L-cysteine, a ROS scavenger) for 24 hrs.

Figure 3: ROS generation by LA reduces the CREB/furin axis. MCF7 and MDA-MB-231 breast cancer cell lines were treated with LA 2 mM alone, NAC 10 mM alone or in combination for 48 hrs. CREB and furin protein levels were detected by Western blot analysis **A:** MCF7 (ER α +) and **B:** MDA-MB-231 (ER α -) cell lines. The furin mRNA level was assessed by real-time quantitative reverse transcription PCR in **C:** MCF7 (ER α +) and **D:** MDA-MB-231 (ER α -) cell lines treated with LA 2 mM alone, NAC 10 mM alone or in combination for 48 hrs. Data are normalized against 28S mRNA level used as an endogenous control. Results are expressed relative to the levels in control cells set at one. This analysis was performed in triplicate. Unpaired two-tailed Student's t-test was used for statistical analysis. A *P*-value of < 0.05 was considered statistically significant. * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001.

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Figure 4: Schematic representation of the anti-proliferative role of lipoic acid in breast cancer cells. LA induces ROS generation in our breast cancer cell lines. ROS reduces the CREB protein levels, a transcription factor which induces furin expression. This reduction of CREB leads to the inhibition in furin expression. These events inhibit the maturation process of the RTKs IGF-1R thus inhibiting cell proliferation and survival.

Consent to publish: The manuscript does not contain any individual person's data in any form.

Data availability: All data presented within the article and its supplementary information files are available upon request from the corresponding author.

Conflicts of interest: The authors declare no conflicts of interest.

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Chapitre III
Discussion générale

Discussion

Le cancer du sein est l'un des cancers solides les plus répandus et agressifs chez les femmes. Il y a quelques années encore, le taux de survie globale à 5 ans de patientes atteintes de cancer du sein métastatique ne dépassait pas 23 % (American Cancer Society). Les études réalisées sur les effets de nouveaux traitements dans le cancer du sein ont été confrontés, dans la plupart des cas, à la mise en place de mécanismes de résistance limitant ainsi l'efficacité de ces traitements. Ces phénomènes de résistance sont considérés comme un problème majeur en cancérologie. Dans ce contexte, il est crucial de trouver de nouvelles stratégies afin de réduire la résistance aux thérapies conventionnelles et d'augmenter leur efficacité.

L'acide lipoïque (LA) est un cofacteur essentiel pour plusieurs enzymes impliquées dans le métabolisme biochimique. C'est un agent naturel qui est utilisé pour traiter différentes maladies neurodégénératives et métaboliques (Moura et al., 2015; Rochette et al., 2015; Shay et al., 2009). Durant la dernière décennie, l'intérêt de LA dans le traitement des cancers a été bien démontré. Plus précisément, dans le cancer du sein, LA joue un rôle anti-cancéreux primordial. En 2010, Lee *et al* ont démontré que LA inhibe la formation de métastases de la lignée MDA-MB-231 et que cette inhibition est probablement due à la diminution de l'activité et des niveaux d'expression des ARNm de MMP-2 et MMP-9 (Lee et al., 2010). Quelques années plus tard, Li *et al* ont étudié l'effet de LA en combinaison avec le paclitaxel sur la prolifération de la lignée MCF7. Les résultats ont montré que LA seul ou associé au paclitaxel inhibe l'expression du facteur de transcription NF- κ B et inhibe la prolifération de cellules de cancer mammaire (Li et al., 2015a). En 2017, une autre étude a été faite sur des lignées de cancer du sein et a montré que LA inhibe la viabilité et la prolifération de ces cellules en réduisant l'activité de PTP1B et SHP2, des protéines-tyrosine phosphatases qui ont des rôles clés dans des événements de signalisation dépendants de la phosphorylation de la tyrosine) (Tajan et al., 2015; Tonks, 2003).

De plus, des données récentes ont démontré que LA inhibe la migration et l'invasion de cellules tumorales de cancer du sein en inhibant les voies de signalisation ERK-1/-2 et Akt et TGF- β (Tripathy et al., 2018; Yamasaki et al., 2014). Cependant, les études antérieures étaient limitées sur quelques lignées cellulaires et dans un cadre descriptif seulement. Dans ce contexte, des études supplémentaires sont nécessaires afin d'élucider les mécanismes d'action qui déterminent dans quel contexte LA exerce ses effets anti-cancéreux. Dans la première étude réalisée durant ma thèse, nous avons démontré l'aptitude de LA à inhiber la prolifération de plusieurs lignées cellulaires de

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cancer du sein (ER α (+) et ER α (-)). Quel que soit le statut de ER (+ ou -), LA inhibe la prolifération des cellules des différentes lignées cellulaires et ce de manière concentration et temps dépendants. En outre, il est bien connu que la prolifération tumorale est induite par l'activation accrue de différentes voies de signalisation prolifératives comprenant les voies PI3K/Akt et MAPK/ERK (Yuan and Cantley, 2008). Dans ce contexte, il était intéressant d'étudier l'effet de LA sur ces voies de signalisation. Nos résultats ont montré que l'effet anti-prolifératif de LA est dû à l'inhibition de ces deux principales voies et à l'activation de l'AMPK, senseur métabolique qui active les voies cataboliques (glycolyse / β -oxydation des acides gras) et à l'inverse inhibe les voies anaboliques (synthèse des acides gras, des protéines et du cholestérol). Le traitement par des concentrations de 1 ou 2 mM de LA inhibe la phosphorylation activatrice des protéines Akt et ERK. Cette inhibition d'Akt empêche alors l'activation de mTOR et par conséquent de ces effecteurs situés plus en aval comme p70S6K1 et S6. D'autre part, LA induit la phosphorylation activatrice d'AMPK qui va, à son tour, inhiber l'activation d'Akt. De ce fait, LA semble bloquer l'activation d'Akt par 2 voies différentes. Ensuite, nous avons cherché à comprendre comment LA inhibe ces voies de signalisation, PI3K/Akt et MAPK/ERK. En amont de ces voies se trouvent des récepteurs à différents facteurs de croissance, comme IGF-1R, EGFR, IR. Ces récepteurs appartiennent à la famille des récepteurs à tyrosine kinase et ils sont impliqués dans de nombreux processus biologiques. Le récepteur, IGF-1R, est impliquée, entre autre, dans la prolifération, la survie cellulaire et la formation de métastases. De manière très intéressante, le niveau d'expression d'IGF1-R a été trouvé diminué de manière temps et concentration dépendant à LA dans nos modèles cellulaires. Nous avons cherché à savoir si LA agit au niveau de la transcription d'IGF-1R. Par RT-qPCR, aucune variation d'expression de l'ARNm d'IGF-1R a été observée quelle que soit la concentration de LA et du temps de traitement. Donc, nous avons cherché à déterminer si LA modifie le processus de maturation post-traductionnelle d'IGF-1R. Nos résultats ont démontré explicitement pour la première fois que LA réduit l'expression de la forme mature d'IGF-1R avec une perte de sa localisation membranaire. De façon concomitante à cette perte d'expression de la forme mature, nous avons noté une accumulation de la forme immature d'IGF-1R, pro-IGF-1R, en fonction du temps et de la concentration de LA. Cette forme immature a été retrouvée localisée dans le cytoplasme de la cellule où elle ne peut pas exercer son rôle d'activateur des voies de signalisation PI3K/Akt et MAPK/ERK. Nos travaux ont montré pour la première fois que LA

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bloque le processus de maturation d'IGF-1R entraînant ainsi une inhibition des voies de signalisation situées en aval.

La maturation d'IGF-1R nécessite un clivage protéolytique catalysé par la proprotéine convertase, furine. Il nous a semblé important d'étudier le niveau d'expression protéique et transcriptionnel de la furine en réponse à un traitement par LA. De manière très intéressante, nos résultats ont montré que LA réduit le niveau d'ARNm et l'expression protéique de la furine. LA agit par l'intermédiaire de molécules non encore élucidées sur la régulation transcriptionnelle du gène de la *furine*. Cette régulation transcriptionnelle de la furine repose sur la présence du facteur de transcription CREB. Ce facteur est activé par les voies de signalisation PI3K/Akt et MAPK/ERK. Par conséquent, nous voulons déterminer si le facteur CREB peut intervenir lors d'un traitement par LA sur l'inhibition de la transcription de furine. Nos résultats ont montré dans nos lignées cellulaires de cancer du sein que LA inhibe l'activation transcriptionnelle et l'expression protéique du facteur CREB de manière temps et concentration dépendant. Il est à noter qu'une étude antérieure a démontré que l'expression de CREB peut être inhibée par la génération de ROS (Özgen et al., 2009). Par ailleurs, il est connu que LA joue un rôle pléiotropique et paradoxal : il peut être soit pro- soit anti-oxydant. Ainsi de par son rôle pro-oxydant, LA pourrait inhiber l'expression de CREB. Ainsi, nos résultats ont révélé que LA induit des ROS et que cette induction permet de réprimer l'expression protéique du facteur de transcription CREB dans nos lignées de cancer du sein. Le co-traitement de cellules de cancer du sein par une molécule anti-oxydante et de LA diminue la production de ROS et s'accompagne d'une restauration du niveau d'expression protéique de CREB. **En conclusion**, LA produit des ROS qui vont diminuer le niveau d'ARNm et de protéine de CREB. Cette baisse d'expression protéique engendre une réduction de l'expression de la furine donc une réduction du processus de maturation d'IGF-1R. L'importance de notre étude réside dans le fait qu'elle est la première qui a déchiffré les mécanismes anti-prolifératifs de LA dans le cancer du sein.

L'acide lipoïque et les proprotéine convertases (PCSKs)

Carrier et al ont démontré que LA réduit l'expression d'un autre membre de la famille des proprotéines convertases, la PCSK9 qui est impliquée dans l'hypercholestérolémie et l'accumulation de graisse hépatique, par conséquent LA a un effet protecteur vis-à-vis de ces processus (Carrier et al., 2014). Cependant, aucune étude n'a démontré l'effet de LA sur les

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membres de la famille des proprotéines convertases dans les cancers. A noter que, ces enzymes, et plus particulièrement la furine, sont surexprimées dans de nombreux types de cancers. Ces surexpressions sont liées avec un mauvais pronostic. La furine est impliquée dans le clivage protéolytique d'IGF-1R et d'IR, mais aussi d'autres substrats comme le TGF- β . Une étude antérieure a démontré que LA est impliquée dans l'inhibition de la voie du TGF- β et dans la production de TGF- β mature dans des cellules de cancer de la thyroïde (Jeon et al., 2016). En se basant sur ces résultats et les nôtres, il semblerait logique de suggérer que LA inhibe la voie du TGF- β en réduisant l'expression de la furine.

L'acide lipoïque et les récepteurs à tyrosine kinase

Des études précédentes ont démontré l'effet de LA sur la phosphorylation du récepteur à tyrosine kinase EGFR et les conséquences de cette inhibition sur la prolifération des cellules cancéreuses du poumon (Yang et al., 2017). Cependant, aucune étude n'a démontré l'effet de LA sur le processus post-traductionnel des protéines et notamment des RTKs comme IGF-1R. Il est important de préciser que, la surexpression d'IGF-1R est associée avec un mauvais pronostic dans divers types de cancer y inclus le cancer du sein. Ils existent de nombreux traitements ciblant ce récepteur mais malheureusement des mécanismes de résistance (détaillés dans le paragraphe 3.4) limitent l'efficacité de ces traitements. Fait intéressant, nous avons montré que LA réduit non seulement l'expression de la forme mature d'IGF-1R mais aussi l'expression de la forme immature d'IR, surexprimée dans le cancer du sein (Chan et al., 2017) et corrélée avec la malignité dans plusieurs modèles de cancer, y compris le cancer du sein (Vella et al., 2018). Donc, cette double inhibition de la maturation d'IGF-1R et d'IR *via* l'inhibition de la furine en réponse à un traitement par LA pourrait réduire le problème de résistance aux inhibiteurs d'IGF-1R actuels.

L'acide lipoïque et l'inhibition de la prolifération tumorale par la suppression du facteur de transcription CREB

Concernant le facteur de transcription CREB, sa surexpression et son activation sont retrouvées dans de nombreux types de cancer comme le carcinome pulmonaire non à petites cellules (CNSCLC), le glioblastome, le carcinome mammaire et le mélanome (Steven and Seliger, 2016). De plus, sa surexpression est associée à une progression tumorale, une résistance à la chimiothérapie et une réduction de la survie des patients (Herkel et al., 2017; Rodríguez and Setaluri, 2014; Xie et al., 2015; Yu et al., 2017). Dans ce contexte, en plus de son rôle de facteur

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de transcription de la furine démontré par notre présente étude. Il a été montré que ce facteur CREB induit l'expression de nombreux gènes cibles qui sont impliqués dans diverses fonctions cellulaires, notamment le métabolisme (Steven and Seliger, 2016). De ce fait, l'activation de CREB pourrait favoriser l'effet Warburg, provoquant ainsi la déconnexion entre la glycolyse et le cycle de Krebs (Chen et al., 2017; Liu et al., 2014). Cela serait le reflet d'une adaptation métabolique de la cellule cancéreuse assurant l'obtention rapide d'ATP, la biosynthèse des macromolécules et la maintenance d'un statut redox compatible avec la survie cellulaire et l'émergence de phénomènes de résistance (Icard et al., 2018). Pour reconnecter la glycolyse au cycle de Krebs, l'utilisation de LA semble pertinente car c'est un des cofacteurs de la pyruvate déshydrogénase (PDH), enzyme mitochondriale qui transforme le pyruvate en acétyl-CoA (Mayr et al., 2014). Ainsi, LA pourrait agir à deux niveaux distincts, (1) sur l'inhibition du facteur CREB, comme nos travaux l'ont démontré, et (2) sur la reconnexion entre la glycolyse et le cycle de Krebs. La baisse d'expression de CREB pourrait réduire l'adaptation métabolique favorisant la tumorigénèse. De plus, comme mentionnée précédemment (Cf. Paragraphe I.1.a), différentes études ont démontré que LA inhibe la progression de cycle cellulaire et induit l'apoptose mais sans réellement expliquer les mécanismes d'action. Ce blocage du cycle cellulaire et l'entrée des cellules en apoptose en réponse à un traitement par LA pourraient s'expliquer par la baisse d'expression du facteur CREB, qui a été décrit comme impliqué dans la transcription de gènes contrôlant la progression du cycle cellulaire et dans l'inhibition de l'apoptose (Alper et al., 2001; Linnerth et al., 2005; Tan et al., 2012 et (pour revue (Steven and Seliger, 2016))).

Acide lipoiqne et autres inhibiteurs de furine

À notre connaissance, les seuls inhibiteurs spécifiques de la furine connus sont les « dromedary heavy-chain-derived nanobodies » qui peuvent inhiber spécifiquement la furine de manière non compétitive (Zhu et al., 2012). Malheureusement, cet inhibiteur n'est pas encore disponible pour un usage clinique. Cependant, ces inhibiteurs bloquent également d'autres protéines convertases. Ainsi, la question se pose de savoir quelle est l'importance de la spécificité vis-à-vis de la furine seule ? D'une part, un inhibiteur de convertases à action élargie pourrait présenter une puissance accrue dans l'inhibition de la maturation de substrats impliqués dans des processus biologiques essentiels.

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Dans notre présente étude, nos résultats démontrent que LA inhibe l'expression de la furine *via* la suppression du facteur de transcription CREB. À notre connaissance, parmi les membres de la famille des convertases, la furine est le seul gène cible régulé par CREB. Ainsi, LA pourrait agir comme un inhibiteur spécifique de la furine. D'autre part, l'efficacité d'inhibiteur de furine est limitée à sa capacité de cibler exclusivement une suractivité de la furine dans les tumeurs. Etant donné que LA ne montre aucune toxicité ou une réduction de la prolifération des cellules non tumorales, il pourrait être considéré comme un traitement sélectif. Par conséquent, l'administration de LA comme un inhibiteur de furine pourrait être une approche prometteuse pour limiter les effets secondaires potentiels causés par l'inhibition de l'activité de furine dans des tissus sains.

Comment équilibrer l'effet thérapeutique de LA ?

En se basant sur des études antérieures, LA joue un rôle paradoxal : (i) un rôle majeur comme un adjuvant anti-cancéreux et un agent chimio-préventif lorsqu'il est associé au bortézomib, inhibiteur du protéasome, par exemple, en réduisant la réponse au stress induite par l'inhibition du protéasome (Tibullo et al., 2017) (ii) un rôle mineur comme un antagoniste de certains médicaments anti-cancéreux utilisés en clinique, qui agissent en générant des radicaux libres (agents alkylants). Dans ce dernier exemple, l'administration de LA avec du cisplatine peut présenter un effet thérapeutique réduit, puisque les radicaux actifs formés par le cisplatine peuvent être éliminés par LA (Jeon et al., 2016). Par ailleurs, il a déjà été démontré que la toxicité de certains médicaments anti-cancéreux est liée à la formation de radicaux libres et peut être diminuée par LA (Jeon et al., 2016). Dans ce contexte, en ce qui concerne les différents mécanismes d'action des traitements anticancéreux, le co-traitement de LA pourrait être utile en combinaison avec certains traitements en réduisant la toxicité, l'inflammation et le stress oxydatif. Par contre, l'effet anti-oxydant de LA pourrait inhiber les effets anti-cancéreux de certains médicaments qui induisent un stress oxydatif pour exercer leur action d'anti-cancéreux.

En résumé, des études supplémentaires sur les mécanismes d'action de chaque médicament anti-cancéreux et des études toxicologiques sont nécessaires pour équilibrer l'effet thérapeutique et les effets secondaires toxiques de leur association avec LA. En se basant sur ce mécanisme d'action

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de LA et les mécanismes d'action des différents agents anti-cancéreux, on peut choisir la bonne combinaison afin d'essayer d'obtenir un effet synergique ou additif de LA. Néanmoins, l'impact des données de notre étude est limité par l'absence de validation sur des modèles *in vivo* en combinaison avec la chimiothérapie.

Conclusion générale et perspectives

Ce travail de thèse a étudié l'effet de l'acide lipoïque (LA) sur la prolifération de plusieurs lignées de cancer du sein. Nos résultats ont montré que LA inhibe la prolifération cellulaire en inhibant les voies de signalisation prolifératives PI3K/Akt et MAPK/ERK qui sont normalement fortement induites dans le cancer et ce quel que soit le statut d'ER. Nos résultats ont contribué à une meilleure compréhension des mécanismes d'action conduisant à cet effet anti-prolifératif de LA. En effet, nous avons mis en évidence la réduction de l'expression de la proprotéine convertase, furine, responsable de la maturation d'IGF-1R en réponse à LA aboutissant *in fine* à l'inhibition de la maturation de ce récepteur, IGF-1R, qui est un récepteur crucial en cancérologie. En outre, nous avons démontré que l'effet pro-oxydant de LA aboutit à la réduction de l'expression du facteur de transcription, CREB, qui est impliqué dans l'induction de l'expression de la furine. En conclusion, nous avons démontré pour la première fois comment LA agit pour aboutir à l'inhibition de la prolifération cellulaire dans des lignées de cancer du sein. Nous proposons le mécanisme d'action suivant : LA induit rapidement et brutalement des ROS par la phosphorylation oxydative qui inhibent l'expression du facteur de transcription CREB. Cette inhibition bloque alors l'activation transcriptionnelle de la furine, enzyme qui est cruciale pour permettre le passage de la pro-forme d'IGF-1R, qui est immature et non fonctionnelle, à la forme mature, IGF-1R qui est alors recrutée à la membrane plasmique (**figure 22**).

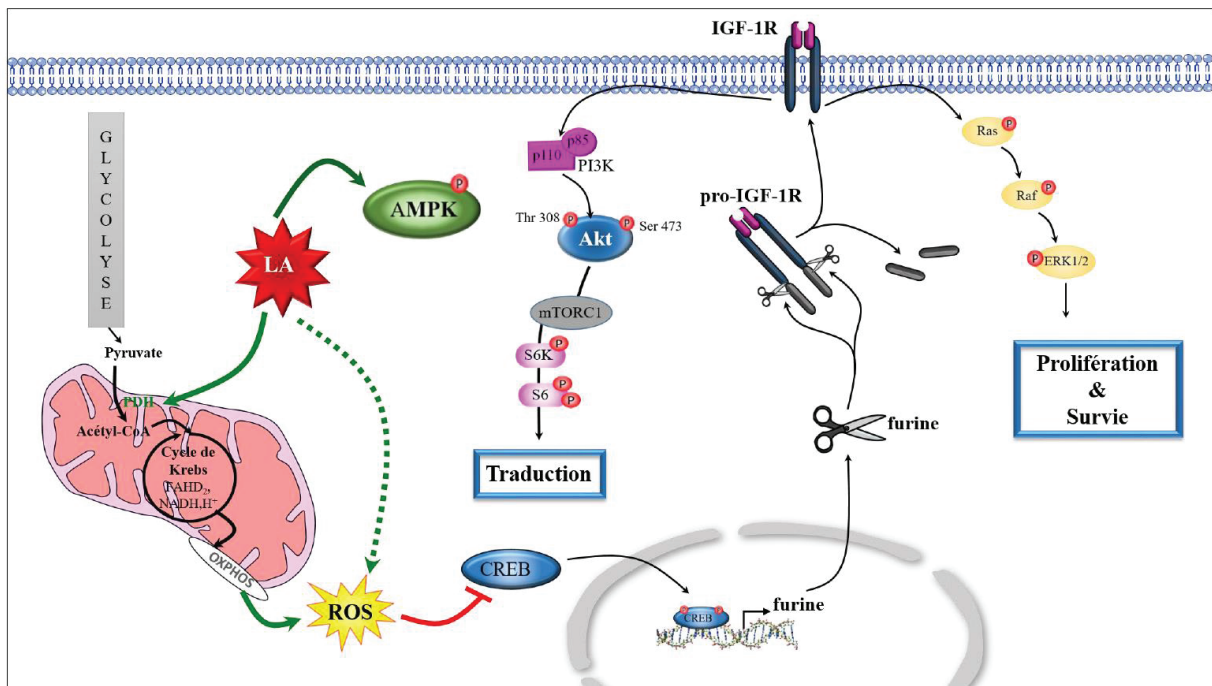


Figure 22 : Schéma de synthèse résumant les mécanismes d'action de LA sur l'inhibition de la prolifération et la survie cellulaire dans le cancer du sein.

Les résultats obtenus durant ma thèse laissent des perspectives de recherche fondamentale mais aussi avec une approche plus translationnelle. D'un point de vue **fondamentale**, il serait intéressant de démontrer que LA reconnecte la glycolyse au cycle de Krebs dans des cellules de cancer du sein en agissant sur la PDH et que la production massive de ROS générée par la phosphorylation oxydative en réponse à un traitement par LA impacte la prolifération cellulaire. Il faudrait aussi investiguer plus en profondeur les effets de la répression du facteur de transcription CREB par la production de ROS sur différents processus biologiques comme la transcription de gènes impliqués dans la progression du cycle cellulaire, l'invasion et la migration. En parallèle, il serait intéressant d'étudier une autre voie de signalisation, la voie du TGF- β . LA a été démontré inhiber cette voie de signalisation (Tripathy 2018 ; Liu et al., 2019). D'autre part, comme mentionné précédemment, le TGF- β induit la transcription de la furine (Blanchette et al., 1997, 2001), il y aurait alors la possibilité d'inhiber la transcription de la furine, par une autre voie que la voie des ROS, en inhibant le TGF- β .

De plus, nos résultats ont montré que LA induit l'activation d'AMPK par sa phosphorylation. Des études précédentes ont bien montré que la voie AMPK est activée suite à toute augmentation du rapport AMP/ATP intracellulaire témoignant d'un manque énergétique (Forcet et al., 2005) *via* le gène suppresseur de tumeur LKB1 (Hawley et al., 2003; Shaw et al., 2004a, 2004b). D'autre part, des études antérieures ont montré que LA pourrait réduire l'effet Warburg et ainsi la production d'ATP. En se basant sur ces études et nos résultats précédents, il serait intéressant d'élucider les mécanismes d'action par lesquels LA active AMPK. Est-ce que cette activation passe par LKB1? Pour répondre à cette question il serait intéressant d'étudier l'expression et l'activité de LKB1 en réponse à LA. De plus, pour valider clairement la sélectivité de LA sur la furine, il serait intéressant alors de tester ses effets sur les autres membres de la famille qui sont indispensables et nécessaires dans différents processus physiologiques favorisant la prolifération cellulaire. Nous avons montré que LA inhibe l'expression de la forme mature de différents récepteurs à tyrosine kinase, IGF-1R, EGFR et IR. Il serait intéressant d'étudier si LA pourrait diminuer l'expression d'un autre récepteur à tyrosine kinase présent dans 25 % des tumeurs mammaires, HER2/neu, récepteur favorisant la croissance cellulaire. Concernant les perspectives avec une approche **translationnelle**, il faudrait évaluer l'intérêt thérapeutique de combiner LA avec une thérapie conventionnelle (chimiothérapie et/ou hormonothérapie) pour mettre en évidence un effet anti-prolifératif additif et/ou synergique la chronologie d'administration des 2 *in vitro*. Des études *in vivo* ont montré un intérêt en oncologie. Dans le cas de résultats positifs, il faudra bien évidemment poursuivre les études par une approche *in vivo* pour démontrer que l'association LA et thérapie conventionnelle engendre une régression du volume tumoral.

ANNEXES

Review Number 1

**How the Warburg effect supports aggressiveness and drug
resistance of cancer cells?**

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How the Warburg effect supports aggressiveness and drug resistance of cancer cells?

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ABSTRACT

Cancer cells employ both conventional oxidative metabolism and glycolytic anaerobic metabolism. However, their proliferation is marked by a shift towards increasing glycolytic metabolism even in the presence of O₂ (Warburg effect). HIF1, a major hypoxia induced transcription factor, promotes a dissociation between glycolysis and the tricarboxylic acid cycle, a process limiting the efficient production of ATP and citrate which otherwise would arrest glycolysis. The Warburg effect also favors an intracellular alkaline pH which is a driving force in many aspects of cancer cell proliferation (enhancement of glycolysis and cell cycle progression) and of cancer aggressiveness (resistance to various processes including hypoxia, apoptosis, cytotoxic drugs and immune response). This metabolism leads to epigenetic and genetic alterations with the occurrence of multiple new cell phenotypes which enhance cancer cell growth and aggressiveness. In depth understanding of these metabolic changes in cancer cells may lead to the development of novel therapeutic strategies, which when combined with existing cancer treatments, might improve their effectiveness and/or overcome chemoresistance.

1. Introduction

In eukaryotic cells, cellular respiration is primarily aerobic, but in some instances, cells can produce energy anaerobically through glycolytic fermentation. These two processes share a common initial metabolic pathway called glycolysis which is regulated by phosphofruktokinase1 (PFK1) and which leads to the production of pyruvate from glucose. Under aerobic conditions, pyruvate enters the mitochondria and fuels the tricarboxylic acid (TCA) cycle which produces citrate, high levels of ATP and CO₂. In contrast, under anaerobic conditions, pyruvate remains in the cytosol and is converted into lactate and markedly smaller yield of ATP.

Otto Warburg first demonstrated that proliferating cancer cells

enhanced their glucose consumption and produced lactate, even under aerobic conditions (Warburg, 1930, 1956). This reprogrammed cellular metabolism is now recognized as a hallmark of cancer (Hanahan and Weinberg, 2011) and it is important to better understand its underlying molecular mechanisms for the development of new therapeutic interventions. In the current review, we attempt to explain why cancer cells reduce their oxidative metabolism in order to adapt their ability to survive. We also show how this metabolic shift towards increased glycolytic functioning sustains tumor cell proliferation and aggressiveness (resistance to various processes including hypoxia, apoptosis and immune response). As discussed in the current review, the Warburg effect also promotes many tumor characteristics that result in drug resistance including increased drug efflux and DNA damage repair, metabolic

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inactivation of drugs, epigenetic alterations, mutations in drug targets, activation of survival pathways and evasion of cell death (Holohan et al., 2013; Raz et al., 2014).

In the present review, we describe the many facets of the Warburg effect in cancer cells and discuss how a better understanding of this metabolic shift may facilitate the development of new anti-cancer drug treatments and strategies to overcome resistance to the treatments that are currently available.

2. The Warburg effect in cancer cells

2.1. The “Warburg effect” enhances glycolysis and glutaminolysis to sustain cellular proliferation

While normal cells oxidize glucose to provide ATP, cancer cells use a large fraction of their glucose to produce building blocks for cellular proliferation. For that purpose, they enhance their consumption of glucose and transform much of it into various molecules that sustain nucleotide and triglyceride biosynthesis (Assaraf, 2007; DeBerardinis and Thompson, 2012; Gonen and Assaraf, 2012; Raz et al., 2016; Vander Heiden et al., 2009). Furthermore, cancer cells consume glutamate derived from glutamine (*i.e.* glutaminolysis) to produce molecules such aspartate entering nucleotide biosynthesis and citrate furnishing acetyl-coA for fatty acid biosynthesis and protein acetylation (Cai et al., 2011; DeBerardinis and Cheng, 2010; Hatzivassiliou et al., 2005; Huang et al., 2014; Mates et al., 2009; Wellen et al., 2009; Zaidi et al., 2012). Accordingly, glucose transporters (Chen et al., 2017; Macheda et al., 2005; Yu et al., 2017), glycolytic enzymes (Madhukar et al., 2015) and glutaminase 1 (GLS1) (Diaz-Moralli et al., 2013) represent a substantial proportion of the cancer metabolic proteome and metabolome.

Nutrient availability (glucose, glutamine, fatty acids, etc.) and O₂ concentration play a major role in the marked cell growth differences that may occur between various regions of the same tumor (Nakazawa et al., 2016). The nutrients are transformed into “bricks” for anabolic synthesis and/or are degraded to produce ATP required for biosynthesis and/or cell survival. The crossroad between anabolic and catabolic pathways is mainly regulated by pyruvate kinase which is re-expressed in its embryonic form PKM2 (Mazurek, 2012; Mazurek et al., 2002). When PKM2 is inactivated, anabolic synthesis (and branched pathways in glycolysis) are promoted (Lepleux et al., 2012; Locasale et al., 2011; Yang and Vousden, 2016), whereas when PKM2 is active, phosphoenolpyruvate (PEP) is transformed into pyruvate, hence producing one molecule of ATP (Mazurek, 2012). Glycolysis can deliver ATP up to 100-fold more rapidly than mitochondria in cancer cells (Pfeiffer et al., 2001) and PKM2 adjusts the concentration of ATP into the range needed for cellular proliferation and cell survival. Of note, the PKM2 reaction can be bypassed by an alternative pathway not furnishing ATP (Vander Heiden et al., 2010), this bypass may be avoiding an excess of ATP production (Fig. 1).

2.2. The Warburg effect promotes a shift from oxidative to reductive metabolism

The increased consumption of glucose leads proliferating cells to reduce their oxidative metabolism, avoiding overproduction of ATP and citrate by mitochondria which would otherwise arrest glycolysis because these molecules downregulate PFK1 (Icard and Lincet, 2012; Lehninger, 1975).

The shift from oxidative to reductive metabolism (*i.e.* the fermentation of glucose into lactate) is linked to the inhibition of pyruvate dehydrogenase (PDH) by pyruvate dehydrogenase kinase 1 (PDK1) (Semenza, 2012; Papandreou et al., 2011; Semenza, 2013). PDH inhibition disconnects the TCA cycle from glycolysis and leads to a marked decrease in mitochondrial production of ATP and citrate. PDK1 is activated by hypoxia inducible factor-1 (HIF-1) and by two kinases

which translocate into mitochondria: Akt (Chae et al., 2016) and the multifunctional enzyme PGK1 (Li et al., 2016b). The disconnection of the TCA cycle from glycolysis is reinforced by the decreased expression of the mitochondrial pyruvate carrier (Schell et al., 2014). HIF-1 also transactivates lactate dehydrogenase 5 (LDH5) that transforms pyruvate into lactate, which is exported by monocarboxylate transporter 4 (MCT4) (Marin-Hernandez et al., 2009; Semenza, 2013).

The main activator of the Warburg effect is HIF-1, which selects cells that are able to survive when their oxygen supply is reduced (Rohwer and Cramer, 2011; Semenza, 2016). HIF-1 allows adaptation to hypoxia by increasing glucose transport, glycolysis and lactate production, whereas it can favor autophagy (Leung et al., 2017; Zhang et al., 2008). Importantly, HIF-1 can also be upregulated in the presence of O₂ (Dhup et al., 2012; Keith et al., 2012; Lee et al., 2015; Ryu et al., 2011).

The upregulation of HIF-1 results from several processes: a) inactivation of prolyl-hydroxylase (PHD) by hypoxia, lactate, fumarate, and succinate; PHD inactivation arrests HIF-1 degradation (Ryu et al., 2011; Selak et al., 2005; Snell et al., 2014); b) increased production of reactive oxygen species (ROS) stimulated by hypoxia (Chandel et al., 2000; Ryu et al., 2011); c) inactivation of the mitochondrial tumor suppressor deacetylase sirtuin3 (SIRT-3) due to the lack of mitochondrial NAD⁺, SIRT-3 promotes HIF-1 stabilization (Bell et al., 2011; Haigis et al., 2012); and d) upregulation of PKM2 which activates HIF-1 through the NF-kappaB pathway (Azoitei et al., 2016).

All these processes (activation and/or stabilization of HIF-1) create positive feedback loops which further drive glycolysis towards glucose consumption and lactate production.

It is noteworthy that the Warburg effect can be reinforced by the Crabtree effect which is the inhibition of OXPHOS when cancer cells are fed with large amounts of glucose (Crabtree, 1929; Diaz-Ruiz et al., 2008; Diaz-Ruiz et al., 2011; Redman et al., 2013; Smolkova et al., 2011). This effect should be related to the inactivation of complex IV of OXPHOS by fructose 1,6-biphosphate (F1,6-BP) (Diaz-Ruiz et al., 2008; Diaz-Ruiz et al., 2011).

2.3. The Warburg effect reduces the mitochondrial production of ATP, citrate, CO₂ and reactive oxygen species (ROS)

Excessive production of citrate and ATP by mitochondria is detrimental to cellular proliferation (Krupig et al., 2012; Lincet et al., 2013; Lu et al., 2011; Samudio et al., 2009; Zhang et al., 2009; Zhou et al., 2012). Thus, the Warburg effect may appear as a regulatory mechanism avoiding mitochondrial overproduction of ATP and citrate, two molecules which are known to arrest glycolysis at various levels (PFK1 and PK) (Lehninger, 1975). The upregulation of ATP citrate lyase (ACLY) (Hatzivassiliou et al., 2005; Pietrocola et al., 2015; Zaidi et al., 2012) participates in the maintenance of low concentrations of ATP and citrate, promoting tumor aggressiveness *i.e.* resistance to apoptosis and dedifferentiation (Hanai et al., 2013; Zhou et al., 2012); for review, see (Icard and Lincet, 2017) (Fig. 1). Accordingly, mitochondrial ATP production has been found to decrease to 50% in poorly differentiated cancer cells (Nakashima et al., 1984; Pedersen, 1978). The decreased production of ATP in cancer cells could be also related to the frequent damage of the respiratory chain, primarily occurring at the ATPase (complex V) (Cuezva et al., 2002; Garcia-Heredia and Carnero, 2015; Matoba et al., 2006).

Diminished oxidative metabolism also reduces the production of CO₂. ATP and CO₂ are the two major sources of H⁺ inside cells (Swietach et al., 2014) and their decrease promotes the establishment of an alkaline intracellular pH (pHi) in cancer cells (between 7.12 and 7.65), slightly elevated over that of normal cells (between 6.99 – 7.20) (Webb et al., 2011); for a recent review see (Alfarouk et al., 2014; White et al., 2017). It should be noted that pH is a logarithmic value, and a slight variation implies a potentially large decrease in the concentration of H⁺. Several membrane exchangers (in particular the

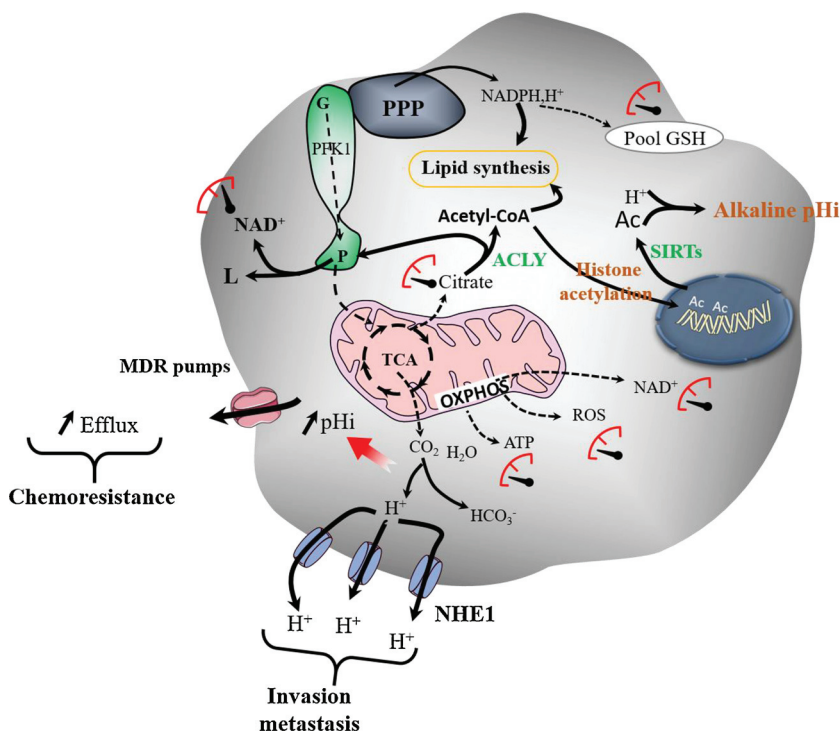


Fig. 1. The Warburg effect and its consequences.

The Warburg effect promotes a shift towards increasing in glycolytic metabolism even in the presence of O_2 . HIF-1 induces PDH inactivation (indirectly) leading to a disconnection between glycolysis and the TCA cycle. The down-regulation of mitochondrial activity prevents an overproduction of ATP and citrate which otherwise arrest glycolysis. Citrate is mainly consumed in the production of acetyl-CoA which sustains lipid biosynthesis and/or the acetylation of proteins such as histones. The production of ROS is modulated to remain in a concentration range compatible with cell proliferation. The decrease in ATP and CO_2 is associated with the increased activities of membrane pumps favoring an intracellular alkaline pH and an extracellular acidity. This pH condition is a driving force in many aspects of cancer cell proliferation (enhancement of glycolysis and cell cycle progression) and of tumor aggressiveness (invasion, resistance to hypoxia, apoptosis, cytotoxic drug resistance as well as resistance to the immune response).

Ac: Acetyl; ACLY: ATP Citrate Lyase; G: glucose; GSH: Glutathione; H^+ : proton; L: lactate; MDR: Multidrug Resistance; NAD^+ : Nicotinamide Adenine Dinucleotide; OXPHOS: Oxidative Phosphorylation; P: Pyruvate; PFK1: PhosphoFructoKinase1; PPP: Pentose Phosphate Pathway; ROS: Reactive Oxygen Species; SIRT6: Sirtuins; TCA: Tricarboxylic Acid Cycle

Na^+/H^+ exchanger 1 (NHE1)) are upregulated in tumor cells which expel H^+ into the extracellular milieu (for reviews, see (Porporato et al., 2011; Taylor et al., 2015; White et al., 2017)) (Fig. 1). The hydration of CO_2 , which is facilitated by carbonic anhydrase (CA), contributes to maintain alkaline pHi because HCO_3^- is recaptured by a sodium bicarbonate co-transporter while H^+ is excreted (Cardone et al., 2005; Parks et al., 2013).

Of note, deacetylation of histones helps to maintain an alkaline pHi, because acetate leaves the chromatin and neutralizes excessively high concentrations of H^+ when pHi goes below approximately 7.0 (Kurdistani, 2014; McBrien et al., 2013) (Fig. 1). Alkaline pHi is an essential condition for cell proliferation because it enhances the activity of PFK1 up to a 100-fold, while it reduces pyruvate oxidation (Relman, 1972; Trivedi and Danforth, 1966). Moreover, it helps the bypass of cell cycle checkpoints (Cardone et al., 2005; Chambard and Pouyssegur, 1986; Diaz-Moralli et al., 2013; Parks et al., 2011, 2013) and favors the initiation of mitosis and microtubule polymerization around the centrosome (Gagliardi and Shain, 2013). Alkaline pHi is also a factor of tumor aggressiveness because it limits apoptosis (Lagadic-Gossman et al., 2004) and inactivates proteins such as p53 (DiGiammarino et al., 2002), leading to the inhibition of TP53-inducible glycolysis and apoptosis regulator (TIGAR), a factor which normally keeps the glycolytic activator fructose-2,6-bisphosphate at a low level (Lee et al., 2014).

Multidrug resistance (MDR) mediated by the efflux transporter P-glycoprotein, could also rely on pH alterations (Omran et al., 2017; Thews et al., 2006) and for reviews (Li et al., 2016a; Wijdeven et al., 2016; Zhitomirsky and Assaraf, 2016). In cancer cells, the inversed pH gradient formed on each side of the cell membrane reduces the diffusion of weakly basic anti-cancer drugs into the cells; this includes the anthracyclines doxorubicin, daunorubicin and mitoxantrone (Webb et al., 2011; Zhitomirsky and Assaraf, 2016). Furthermore, the extracellular acidity may increase the functioning of efflux pumps which expel chemotherapeutic drugs outside the cell (Breedveld et al., 2007) (Fig. 1).

The Warburg effect down-regulates the activity of mitochondria, a process which limits the ROS production and maintains the levels of ROS in a concentration range that is not toxic or lethal for cancer cells

(Sullivan and Chandel, 2014; Traverso et al., 2013). This process sustains various phenomena such as inflammation, cell growth, epigenetic changes, genomic instability and DNA damage, epithelial to mesenchymal transition (EMT), angiogenesis and cancer cell dissemination (Assi, 2017; Bhat et al., 2017; Costa et al., 2014; Porporato and Sonveaux, 2015). Of note, superoxide anion O_2^- promotes intracellular alkalization (Ikebuchi et al., 1991; Shibnuma et al., 1988) and H_2O_2 activates HIF-1 (Chandel et al., 2000; Lopez-Lazaro, 2006; Ryu et al., 2011). The reduced activity of mitochondria also limits the production of H_2O in the cytosol: a slight osmotic swelling promotes cell proliferation and migration, whereas an excessive swelling can be destructive (Saadoun et al., 2005; Schreiber, 2005).

In summary, the Warburg effect limits the production of ATP, citrate, CO_2 , ROS, and H_2O in a concentration range compatible with cellular proliferation and cell survival. It further promotes a pH condition favoring growth, invasiveness and MDR (Fig. 1).

2.4. The Warburg effect impacts on the levels of nicotinamide cofactors

The Warburg effect impacts on the pool of the two crucial nicotinamide cofactors $NADPH$, H^+ and NAD^+ which are essential for cell metabolism and proliferation of cancer cells.

The $NADPH$, H^+ cofactor is regenerated by the oxidative part of the pentose phosphate pathway (PPP), by the malic enzyme (ME) and by dehydrogenases such as IDH (Wise et al., 2011). The transmembrane proteins $NADPH$ -oxidases (NOX), nucleotides and *de novo* lipid biosynthesis heavily consume $NADPH$, H^+ (Costa et al., 2014; Vander Heiden et al., 2009; Zamkova et al., 2013). This high consumption reduces availability of this cofactor for the reconstitution of reduced glutathione which protects cells against the toxic damage inflicted by ROS, especially those occurring during DNA replication (Sullivan and Chandel, 2014; Traverso et al., 2013). Thus, a lack of $NADPH$, H^+ favors genomic lesions and resistance to apoptosis because the redox cytochrome c remains inactivated (Vaughn and Deshmukh, 2008).

The pool of NAD^+ depends on both the biosynthesis of NAD^+ by nicotinamide phosphoribosyltransferase (NAMPT) (Galli et al., 2010; Imai and Yoshino, 2013; Kennedy et al., 2016) as well as on its consumption. The Warburg effect reduces the mitochondrial NAD^+ , a lack

that inactivates PDH, succinate dehydrogenase (SDH) and SIRT-3 and –5 functioning (Hallows et al., 2011; Nakagawa et al., 2009; Yang and Sauve, 2016), resulting in HIF-1 stabilization (Bell et al., 2011; Haigis et al., 2012). At the same time, the activities of cytosolic dehydrogenases regenerating NAD^+ such as malate dehydrogenase and LDH-5 are enhanced (for review, see (Galli et al., 2010)). NAD^+ is consumed in various processes, such as the non-oxidative part of glycolysis, pyrimidine synthesis, deacetylation of histone (Osborne et al., 2014), by the surface protein CD38 which functions as cyclic ADP ribose hydrolase (Escande et al., 2013), DNA repair supported by Poly (ADP-ribose) polymerase 1 (PARP1) (Sousa et al., 2012; Weaver and Yang, 2013), and the inactivation of caspases by SIRT1 (Canto et al., 2015; Hallows et al., 2006). These various production and consumption systems of NAD^+ promote the occurrence of different cell phenotypes. For example, a lack of NAD^+ (due to excessive consumption or insufficient recycling) may favor carcinogenesis by several processes, in particular the loss of circadian control and the inactivation of PARP1 (promoting DNA damage) and/or the SIRT1 (promoting mitochondria downregulation) (Altman, 2016; Nakahata et al., 2009).

2.5. The Warburg effect controls cellular proliferation and the energy state of the cell

In well-vascularized zones of tumors, cells are fed by high amounts of O_2 , glucose and nutrients. This condition allows the energy (ATP/ADP, $\text{NADH,H}^+/\text{NAD}^+$) and the proliferative state of the cell to be high (Icard et al., 2014). In contrast, limited blood supply restricts O_2 and nutrient availability, since without blood vessels the diffusion of these essential molecules is only possible for very short distances (Vaupel and Mayer, 2012). This limited blood supply reduces cellular proliferation but selects robust cells which are especially resistant to radiotherapy and cytotoxic drugs (Gonen and Assaraf, 2012; Raz et al., 2014; Rohwer and Cramer, 2011). A catabolic functioning sustains the survival of hypoxic cells with enhancement of the end part of glycolysis producing ATP and LDH-5 activity (Frezza et al., 2011) (Fig. 2).

Hypoxia affects cancer cells' ability to maintain citrate biosynthesis. This molecule can be produced by an alternative cytosolic reverse isocitrate dehydrogenase (IDH2) route (Metallo et al., 2011; Mullen et al., 2012; Wise et al., 2011), although the frequent mutation of IDH increases the formation of 2 hydroxyglutarate (2-HG), an oncometabolite which alters methylation of histones and DNA (Janke et al., 2017; Ma et al., 2015). Therefore, the net contribution of the IDH route to citrate production is unclear and probably low (Fan et al., 2013; Icard and Lincet, 2017). However, acetyl-coA can be directly synthesized from acetate by acetyl-CoA synthase (ACSS2) (Comerford et al., 2014; Kamphorst et al., 2014; Schug et al., 2016), and this reaction consumes ATP (like that of ACLY) (Fig. 2). In hypoxic cells, the decreased ATP/ADP ratio stimulates the AMP-activated protein kinase (AMPK) pathway which promotes autophagy and mitophagy (Laker et al., 2017) and for review, see (Herzig and Shaw, 2017). Autophagy is stimulated by GAPDH, an enzyme which also protects cells from caspase-independent cell death (CICD) (Jacquin et al., 2013) (Fig. 2). In this manner, malignant cells can use glycolysis and autophagy to withstand adverse environmental conditions. AMPK also promotes fatty acid β -oxidation (FAO) which confers upon cancer cells the possibility of survival and proliferation in tumor zones with low glucose concentrations (Biswas et al., 2012; Carracedo et al., 2013). FAO sustains the production of acetyl-coA and/or of ketone bodies such as β -hydroxybutyrate (β -OHB). The latter molecule can stimulate tumor growth as an oxidative energy source, but it may have an anti-cancer effect at high concentrations via inhibition of histone deacetylases (HDACS) (Branco et al., 2016; Donohoe et al., 2012; Rodrigues et al., 2017) (Fig. 2).

Finally, the establishment of distinct cellular energy states (reflected by the ratios of ATP/ADP, $\text{NADH,H}^+/\text{NAD}^+$) favors the development of heterogeneous cancer cell phenotypes in the same tumor. The plasticity of this metabolism allows cancer cells to adapt metabolic changes

occurring in their microenvironment.

2.6. The Warburg effect sustains abnormal branched pathways promoting tumor cell survival

The Warburg effect stimulates or downregulates several branched pathways of glycolysis or the TCA cycle. The inactivation of mitochondrial SIRT3 and SIRT5 inhibits ornithine transcarbamoylase (OTC) (Hallows et al., 2011; Nakagawa et al., 2009) and arginosuccinate synthase 1 (ASS1) (Bean et al., 2016). This inhibition of the urea cycle leads to the overproduction of NH_4^+ (Eng et al., 2010), nitric oxide (NO) (Delage et al., 2010; Wheatley and Campbell, 2003) and polyamines (Icard et al., 2014; Israel and Schwartz, 2011). All these products promote cancer cell proliferation via multiple mechanisms reviewed elsewhere (Soda, 2011).

Other branched pathways which are affected are: a) The hexosamine pathway which consumes glucose, glutamine and acetyl-CoA (Marshall et al., 1991); it produces uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) supporting protein folding and trafficking (Wellen et al., 2010; Wellen and Thompson, 2012); b) The methylglyoxal pathway, which is sustained by dihydroxyacetone phosphate (DHAP), produces D-lactate which can be also used as a fuel by mitochondria. Furthermore, methylglyoxal, a toxic molecule, is also involved in the MDR phenotype; specifically, methylglyoxalase overexpression was found to be associated with MDR (Thornalley and Rabbani, 2011); c) Triglyceride synthesis which is fed by glycerol phosphate (Pellon-Maison et al., 2014); *de novo* lipid biosynthesis is an essential feature for biomembrane formation (Kim and DeBerardinis, 2016); d) The heme biosynthesis pathway which is required for the formation of cytochrome entering the respiratory chain, is supported by glycine and succinyl-CoA (Hooda et al., 2013); e) The synthesis of abnormal collagen which supports the formation of extracellular matrix (ECM); it is sustained by proline and glutamine biosynthesis (Fang et al., 2014). Finally, all these branched pathways promote an abnormal metabolism supporting tumor cell proliferation and aggressiveness.

2.7. The Warburg effect impacts on the epigenome and genome

The Warburg effect provides various modes of histone acetylation/deacetylation, which as epigenetic remodeling processes, modify chromatin structure and function, consequently impacting gene expression. The acetylation state of histones results from an imbalance between the activities of various histone acetyl transferase (HATs) also dependent on ACLY activity (Hanai et al., 2013; Zaidi et al., 2012; Zhao et al., 2016) and the functioning of HDACs such as SIRT1 which is an NAD^+ -dependent deacetylase (Kleszcz et al., 2015).

The Warburg effect alters the methylation and acetylation state of DNA and thus its structure and function. It promotes the activation of the glycolytic branch serine-methionine pathway (Locasale et al., 2011; Yang and Vousden, 2016) which produces methyl groups for DNA and polyamines biosynthesis (Dai et al., 2017; Icard et al., 2014; Israel and Schwartz, 2011; Newman and Maddocks, 2017; Shen and Laird, 2013). This latter synthesis likely participates in the global hypomethylation state of the genome in cancer cells, a condition favoring genomic instability, chromosome breaks, translocations and allelic loss (Ehrlich, 2002; Funaki et al., 2015; Goelz et al., 1985; Plass et al., 2013; Shen and Laird, 2013).

It is noteworthy that the accumulation of various metabolites including succinate, fumarate, and 2-HG due to inactivation of the TCA cycle and/or to mutations in SDH, FH and IDH promotes methylation (Laurenti and Tennant, 2016). Indeed, these metabolites inhibit two families of demethylases: the α -ketoglutarate-dependent JmjC family and the FAD-dependent LSD family which exhibit tumor promotion although some studies have reported loss of function of these demethylases (Adam et al., 2014; Xiao et al., 2012). Finally, these oncometabolites likely participate in local hypermethylation of "CpG-

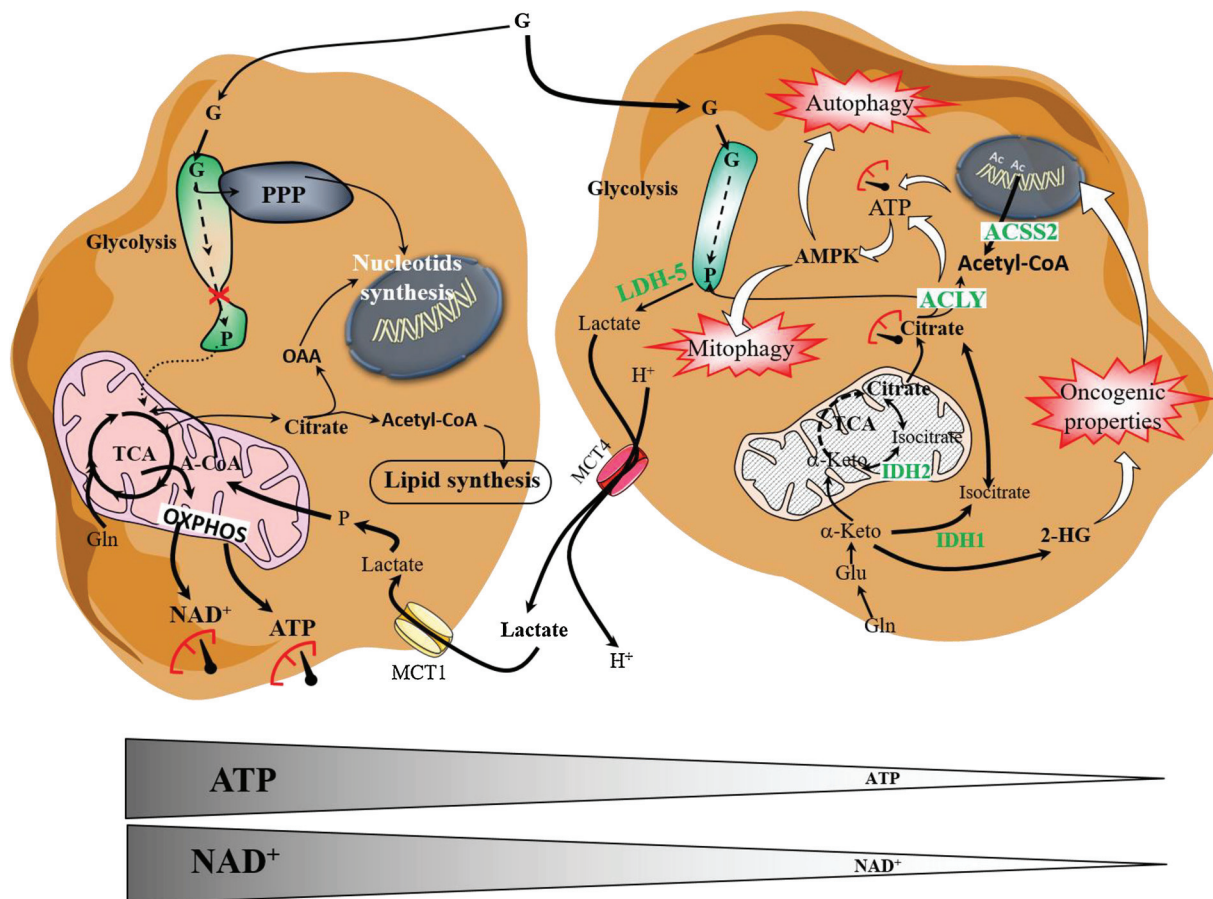


Fig. 2. The cooperation between cancer cells.

In rapidly dividing cancer cells (cell on the left), anabolic synthesis (such as that of nucleotides) is enhanced due to the accumulation of glycolysis intermediates upstream of PKM2 inactivation. This inactivation creates a “bottle neck” at the end of glycolysis which reduces pyruvate formation. However, pyruvate can emerge from alanine by transamination (not shown) or from lactate which is taken up from the microenvironment by MCT1. This pyruvate and α -keto (derived from glutamine transformation) can be used to sustain the mitochondrial production of ATP and citrate. The Warburg effect promotes the inactivation of mitochondria (in relation to PDK1 activation by HIF-1) (cell on the right). The increased activity of ACLY reduces the cytosolic level of citrate when an alternative reversed IDH pathway is unable to produce this molecule. However, acetyl-CoA can be produced directly from acetate by ACSS2 activity. Mutations in IDH1 and IDH2 can lead to the production of 2-HG which favors epigenetic and genetic alterations. The activation of PKM2 stimulates the functioning of the end part of glycolysis producing ATP while LDH-5 regenerates NAD⁺. When acetyl-coA, ATP and/or NAD⁺ production are not sufficient, cells slow down or arrest their division, whereas AMPK is activated. AMPK promotes cell survival and drug resistance through the activation of autophagy and mitophagy.

2-HG: 2-Hydroxyglutarate; Ac: Acetyl; ACLY: ATP-Citrate Lyase; A-CoA: Acetyl-CoA; ACSS2: Acetyl-CoA synthase 2; AMPK: AMP-activated Protein Kinase; G: Glucose; Gln: Glutamine; IDH: Isocitrate dehydrogenase; LDH-5: Lactate dehydrogenase-5; MCT 1 and 4: Mono Carboxylate Transporter 1 and 4; NAD⁺: Nicotinamide Adenine Dinucleotide; OAA: Oxaloacetate; OXPHOS: Oxidative phosphorylation; P: Pyruvate; PKM2: Pyruvate Kinase isoform M2; PPP: Pentose Phosphate Pathway; TCA: Tricarboxylic Acid Cycle.

islands”, a process favoring the inactivation of tumor suppressor genes (for recent reviews see (Plass et al., 2013; Shen and Laird, 2013; Smith et al., 2017)). Of note, the spontaneous deamination of methylated cytosine into thymine, results in endogenous point mutations (Hendrich et al., 1999) which are frequently observed in various cancers (Sjblom et al., 2006).

2.8. The Warburg effect sustains the establishment of a tumor ecosystem which inhibits the immune response

Metabolites and waste products excreted by cancer cells (L-lactate, CO₂, H⁺, NH₄⁺, NO, butyrate, polyamines, ROS, etc.) establish an acidic microenvironment which is compatible with cellular proliferation and dissemination, promoted through various processes such as decreased cell adherence (Stern et al., 2002), angiogenesis (Dhup et al., 2012; Ding et al., 2017) and EMT (Jiang et al., 2016; Liu et al., 2016; Zhao et al., 2017). ROS and HIF can promote a “reversed Warburg functioning” in cancer-associated fibroblasts (CAFs) which expel lactate via the monocarboxylate transporter 4 (MCT4) (Witkiewicz et al.,

2012). Lactate can be captured by cancer cells expressing MCT1 to produce pyruvate which fuels mitochondria (Sonveaux et al., 2012; Witkiewicz et al., 2012). Finally, the Warburg effect (regular or reversed) supports the development of an intricate ecosystem where CAFs produce energy-rich molecules (e.g. lactate and glutamine) for the development of cancer cells (Icard et al., 2014).

Cancer cells escape immune destruction by several processes: a) Lactate secretion inhibits immune response (Choi et al., 2013; Fischer et al., 2007), whereas the avidity of cancer cells for glucose inactivates effector T cells (Chang et al., 2015; Ho et al., 2015; Sukumar et al., 2013; Sukumar et al., 2015) and dendritic cells (DCs) (Kelly and O’Neill, 2015; Ryan and O’Neill, 2017). Deprived of glucose, tumor-infiltrating lymphocytes (TILs) cannot destroy cancer cells through acute inflammation and secretion of interferon γ , whereas programmed cell death ligand-1 (PD-L1) stimulates glycolysis and the Akt pathway in cancer cells (Sukumar et al., 2015).

The concentrations of succinate and citrate have an impact on the functioning of tumor-associated macrophages (TAMs) and DCs (Kelly and O’Neill, 2015; Palmieri et al., 2015). M1 macrophages, which have

a pro-inflammatory and anti-cancer role, rely on glycolysis and on an abnormal TCA cycle leading to accumulation of citrate and succinate (Kelly and O'Neill, 2015). Citrate accumulation increases the production of key mediators of acute inflammation (NO, ROS and prostaglandins), whereas succinate induces the production of IL-1 β , a central inflammatory molecule (Infantino et al., 2011) (for details see (Kelly and O'Neill, 2015)). In contrast, M2 macrophages which promote tumor progression and angiogenesis through secretion of TGF β , rely on OXPHOS and an intact TCA cycle (Fridman et al., 2012) and for recent review, see (Kelly and O'Neill, 2015).

3. Discussion

3.1. The Warburg effect promotes cancer cell proliferation, aggressiveness and drug resistance.

Well-oxygenated cells adapt (and reduce) their mitochondrial functioning to allow increased glycolysis and cellular proliferation, whereas hypoxic cells increasingly shift towards glycolysis. This capability confers adaptability to O₂ levels and nutrient availability (Vaupel and Mayer, 2012). Hypoxia reduces the rate of cellular proliferation and promotes the Warburg effect. Robust cells are selected and defeat cancer therapies which preferentially target rapidly proliferating cells (Leung et al., 2017; Lv et al., 2015; Rohwer and Cramer, 2011; Roizin-Towle and Hall, 1978; Tredan et al., 2007). HIF-1 is a key player in the Warburg effect which promotes a disconnection of the TCA cycle from glycolysis and induces a reductive glycolysis. The down-regulation of mitochondria and the upregulation of membrane exchangers maintain an alkaline pH inside cells which favors glycolysis and cell cycle progression (Cardone et al., 2005; Diaz-Moralli et al., 2013; White et al., 2017). The metabolic waste products and the metabolic exchanges promote an ecosystem which benefits cancer cell growth, invasion and dissemination (Icard et al., 2014). Importantly, the avidity of cancer cells for glucose inhibits the immune response (Choi et al., 2013; Fischer et al., 2007). Sukumar et al., 2015).

As discussed above, the Warburg effect promotes epigenetic and genetic changes leading to the coexistence of cells within the tumor with various genotypes, different patterns of cell proliferation, dedifferentiation, EMT, metastasis and drug resistance (Chen and Dent, 2014; Jiang et al., 2016; Liu et al., 2016; Meacham and Morrison, 2013; Pietrocola et al., 2015; Puisieux et al., 2014; Wijdeven et al., 2016; Zhitomirsky and Assaraf, 2016)). For example, the re-activation of the embryonic gene coding PKM2 is crucial for the initiation and maintenance of glycolysis and proliferation (Christofk et al., 2008; Mazurek et al., 2002) (for recent review, see (Dayton et al., 2016)). Furthermore, cancer stem cells (CSCs) which are inherently drug resistant (Holohan et al., 2013; Shervington and Lu, 2008) are often located in hypoxic niches and rely on glycolysis for cell survival (Varum et al., 2011; Xu et al., 2017). Interestingly, they may contain fewer mitochondria (Nesti et al., 2007) and harbor mitochondrial DNA mutations (Prigione et al., 2011).

The loss of mitochondria in poorly differentiated cancer cells (Elliott and Barnett, 2011; Pedersen, 1978) should be more extensively explored: it could be related to the low expression and/or the inactivation of PGC-1 α (Canto et al., 2015; LeBleu et al., 2014; Santidrian et al., 2013; Watkins et al., 2004) and/or to an excess of mitophagy (Schonenberger and Kovacs, 2015), a cellular "cleaning" process which is stimulated by GAPDH (Colell et al., 2007; Jacquin et al., 2013). Moreover, loss and/or downregulation of mitochondria reduces the production of citrate, which is likely associated with a state of resistance to cytotoxic drugs, dedifferentiation and robustness (Icard and Lincet, 2017; Zhang et al., 2009). Of note, increasing citrate concentration by inhibition of ACLY can reverse dedifferentiation and a CSCs phenotype (Hanai et al., 2013).

Nowadays, the prevailing model considers that mutations occurring at random in DNA of normal stem cells are the main origin of cancer

(Tomasetti and Vogelstein, 2015). However, it is not yet possible to definitively conclude whether or not these mutations drive the metabolism or are driven by it and what exact process drives cancer's genetic development? A new perspective could be that the alterations in mitochondria (dysfunction and mutations) drive a metabolism which promotes cell transformation and development (Seyfried, 2015).

3.2. The Warburg effect is not specific for cancer cells and escapes normal control mechanisms

The Warburg effect also operates in various non-transformed cells, supporting the rapid growth phases of immune cells (Sukumar et al., 2015; Wang et al., 1976), stem cells or embryonic cells (Cagnone and Sirard, 2016; Turner et al., 2014). However, in these non-cancer cells, the Warburg metabolism is responsive to stringent regulatory mechanisms such as p53 which leads to the building of well-organized structures in the embryo. By contrast, the unrestrained proliferation of cancer cells stimulated by the Warburg mechanism is not responsive to such regulation (Kawauchi et al., 2008; Schwartzberg-Bar-Yoseph et al., 2004; Zhang et al., 2011), and mutated proto-oncogenes (such as K-ras and c-Myc (Cairns, 2015; Dang, 2013; Ward and Thompson, 2012)) promote pathways such as the PI3K-AKT-mTOR pathway which sustain the Warburg effect (Gaglio et al., 2011; Gao et al., 2009; Kolch et al., 2015; Morrish et al., 2010; Smith et al., 2016). Of note, fructose-1,6-biphosphate (Fru1,6bisP) (Li et al., 2015) activates RAS likely through Cdc25 (Peeters et al., 2017). These proliferative pathways are not adequately counteracted by suppressive pathways such as the liver kinase B1-AMP-activated protein kinase (LKB1-AMPK) pathway which inhibits anabolic synthesis (Shackelford and Shaw, 2009). However, this pathway can maintain cancer cell survival by stimulating autophagy which can be a source of energy (Faubert et al., 2013; Kim and He, 2013). Therefore, the effect of LKB1 likely depends on the stage of tumorigenesis, the oncogenic background, and on the cellular energy state (for review, see (Li et al., 2015)).

3.3. Is the Warburg effect always reversible in cancer cells?

Cancer cells use the Warburg effect to ensure cell survival. However, they can undergo a reversed shift from reductive to oxidative metabolism, as demonstrated by inhibition of LDH-5 (Fantin et al., 2006). Furthermore, it has been shown that numerous cancer cells display an OXPHOS activity comparable to that of normal cells (Moreno-Sanchez et al., 2007; Zu and Guppy, 2004). Therefore, the Warburg effect could often be an adaptive metabolism (may be reinforced by the Crabtree effect) that takes place to avoid an arrest of glycolysis when cells are fed with high amounts of glucose. The capability of cancer cells to switch from oxidative to reductive metabolism (and back again) allows adaptation not only to O₂, but also to varying supplies of nutrients including glucose, glutamine, alanine, and fatty acids.

However, chronic hypoxia and repeated cell injuries (such as those provoked by ROS and cytotoxic drugs) select cell phenotypes with dysfunctional mitochondria (Cuezva et al., 2002; Garcia-Heredia and Carnero, 2015) which produce ROS molecules promoting mitochondrial mutations (Petros et al., 2005) and favoring several metastatic processes including EMT and cell migration (Kim and Cho, 2014; Penny et al., 2016; Radisky et al., 2005). Therefore, the Warburg effect is likely not always reversible and the heavy reliance on glycolysis could constitute the Achilles's heel of these robust tumor cells which are frequently resistant to current therapies (Leung et al., 2017; Lv et al., 2015; Rohwer and Cramer, 2011; Roizin-Towle and Hall, 1978; Tredan et al., 2007).

3.4. The inhibition of the Warburg effect arrests proliferation but can promote tumor cell migration

The reactivation of mitochondria promotes migration of cancer cells

(LeBleu et al., 2014; Porporato et al., 2014). In fact, brain metastases of breast cancers exhibit a dramatic increase in their oxidative metabolism in contrast to primary tumors or bone metastases (Chen, 2012). The inhibition of the Warburg effect arrests cell growth and leads to a switch from reductive to oxidative metabolism (Fantin et al., 2006; Lin et al., 2012; Papandreou et al., 2011). This switch is favored by the activation of PGC-1 α (Aquilano et al., 2010) which stimulates mitochondrial biogenesis and OXPHOS promoting cell mobility and metastases (Andrzejewski et al., 2017; Carracedo et al., 2012; Chen, 2012; Hooda et al., 2013; LeBleu et al., 2014). Factors other than PGC-1 α can promote OXPHOS activity such as: a) An increased expression of the TNF receptor-associated protein 1 (TRAP-1) (Yoshida et al., 2013); b) An enhanced activity of GAPDH which favors the preservation of intact mitochondria (Jacquin et al., 2013); and c) A reversed Warburg functioning in CAFs excreting lactate (Sonveaux et al., 2012; Witkiewicz et al., 2012).

The link between OXPHOS stimulation and tumor cell migration could be related to the overproduction of ROS (in particular superoxide) which results from the overload of the electron transport chain or its partial inhibition (Porporato et al., 2014; Porporato and Sonveaux, 2015).

4. Conclusion and future perspectives

The Warburg effect is a key hallmark of cancer which enables cancer cell survival, growth, and spread, and further favors tumor resistance to apoptosis and destruction by the immune system. In hypoxic tumor areas, it allows the survival of cells lacking an adequate supply of O₂; tumor areas receiving an adequate blood supply of O₂ and nutrients, allow for rapid tumor cell growth by selecting metabolic pathways that facilitate a balanced production of ATP, citrate, CO₂, H⁺, ROS and nicotinamide cofactors. This balanced production of ATP avoids many of the feedback constraints that normally limit tumor cell growth.

Furthermore, cancer cells are also endowed with the ability to switch on and off the Warburg metabolism depending on environmental requirements for cell survival, aggressive cell growth, or tumor cell migration. This flexibility equips cancer cells with many options for cell growth and survival in hostile microenvironments where they are under a continuous attack by the immune system and by cytotoxic activity of combination chemotherapy. Nevertheless, the reliance of these robust tumor cells on glycolysis, which are frequently resistant to current therapies, could constitute their Achilles' heel.

Profound understanding of the Warburg effect allowed us to propose and test the activity of new Warburg disruptive treatments which could be used on their own, or in conjunction with traditional cytotoxic therapies. To selectively target drug resistant cells which consume high amounts of glucose to sustain cell survival and cell proliferation and which down-regulate mitochondrial metabolism to avoid an arrest of glycolysis and an overproduction of toxic ROS. Hence, one may consider a strategy of both inhibiting glycolysis and stimulating restoration of mitochondrial functionality. The inhibition of glycolysis aims to decrease the energy and building blocks available for cell proliferation, while the stimulation of mitochondrial activity aims to increase the production of ROS, ATP and citrate and to decrease cellular alkaline pH. All of these processes lead to reduced cell growth and to the overcoming of drug resistance. Inhibition of glycolysis can be attempted by various molecules which have been recently listed and reviewed elsewhere (De Preter et al., 2016; Pietrocola and Kroemer, 2017; Yu et al., 2016). The reconnection of glycolysis to mitochondria could be attempted by inhibition of lactate production or its recycling back into mitochondria (Marchiq et al., 2015) and/or by increasing PDH activity with molecules such as lipoic acid (a cofactor of PDH) or dichloroacetate (DCA) (an inhibitor of PDK1) (Papandreou et al., 2011).

Targeting the "main street" could be at best associated with additional "orthogonal" strategies that also inhibit branched pathways, such as the serine-folate metabolism (Kremer et al., 2017; Lepleux et al.,

2012; Raz et al., 2016) and the methionine-polyamine biosynthetic pathway (Setty et al., 2016). It is noteworthy that the fraction of cancer cells displaying apparent normal mitochondrial OXPHOS, could be targeted by molecules which inhibit both glycolysis and OXPHOS, such as 3-bromopyruvate (Galina, 2014; Icard et al., 2014; Lis et al., 2016). Increasing citrate concentration inside tumor cells could be another strategy which aims at inhibiting energy production, while it may also increase the effectiveness of chemotherapeutic drugs as shown by citrate administration tested in various models *in vitro* and *in vivo* (Ren et al., 2017; Zhang et al., 2009). This strategy could be reinforced by inhibition of citrate transformation (ACLY) (Hanai et al., 2012; Khwairakpam et al., 2015; Pietrocola et al., 2015) and/or by inhibition of triglyceride biosynthesis (Hanai et al., 2012).

In order to optimize the effectiveness of chemotherapy, it could be interesting to modulate glucose intake during the course of drug administration. Glucose deprivation prior to and during chemotherapy, may increase cellular stress and thus could enhance the efficacy of cytotoxic drugs (Branco et al., 2016; Buono and Longo, 2018). Whereas, increased glucose uptake after chemotherapy may activate tumor-infiltrating lymphocytes (TILs) to aid in the destruction of residual cancer cells (Icard et al., 2017). Testing various of these strategies with current available therapies is certainly warranted.

Conflict of interest

Authors have no conflict of interest to disclose.

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Review Number 2

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Lipoic acid a multi-level molecular inhibitor of tumorigenesis

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Abstract

We discuss how lipoic acid (LA), a natural antioxidant, induces apoptosis and inhibits proliferation, EMT, metastasis and stemness of cancer cells. Furthermore, owing to its ability to reduce chemotherapy-induced side effects and chemoresistance, LA appears to be a promising compound for cancer treatment.

1. Introduction

Lipoic acid (LA) is a natural antioxidant compound which is present in all prokaryotic and eukaryotic cells. It is synthesized in small amounts by plants and animals, including humans. In human beings, LA acts as an essential enzyme-bound cofactor for 5 redox reactions in mitochondria: one in the glycine cleavage system (GCS) and four in the 2-ketoacid dehydrogenases such as pyruvate dehydrogenase (PDH), α -ketoglutarate dehydrogenase (KGDH), branched-chain ketoacid dehydrogenase (BCKDH), and 2-oxoadipate dehydrogenase (OADH) [2]. The KGDH and the PDH are involved in energy metabolism whereas the other three enzymes are involved in amino acid metabolism [3]. All these enzymes are composed of multiple subunits E1-3, and share a common architecture. One of the most studied roles of LA is as a cofactor of

PDH which is involved in ATP synthesis from glucose by transferring the acyl group to CoA, generating an acyl-CoA in Krebs cycle. Moreover, several studies have distinguished four antioxidant properties and aspects of LA: (i) the metal-chelating capacity such as Fe²⁺ and Cu²⁺, (ii) the reactive oxygen species (ROS)-scavenging capacity by recycling of oxidized glutathione (GSSG) and reduced glutathione (GSH), a process that plays a key role in ROS regulation to protect cells from oxidative stress, (iii) the capacity to regenerate endogenous antioxidants such as vitamin C and vitamin E, (iv) its involvement in repair oxidative damage to proteins by improving the activity of peptide methionine sulphoxide reductase (PMSR). Thus, due to these antioxidant properties, LA is frequently used as a treatment for oxidative stress-associated pathologies, like diabetes, atherosclerosis, liver and neurodegenerative diseases. Beyond its efficacy in treating these chronic diseases, researchers have demonstrated its positive effects in various types of cancers. In the current review we will summarize the anti-cancer properties of LA and highlight its effective role in reducing chemotherapy-induced side effects and in preventing chemoresistance.

2. The effects of lipoic acid on proliferation and apoptosis

Various studies have shown that the exogenous administration of LA inhibits proliferation in several type of cancers. It is well known that tumor proliferation is due to the overexpression of different receptors tyrosine kinase (RTKs), including the epidermal growth factor receptor (EGFR), leading to the activation of oncogenic signaling pathways such as PI3K/Akt, ERK and mTOR [4]. In this latter case, or Interestingly, the downstream effectors of these proliferative pathways are inhibited upon LA treatment in several types of cancers contributing to its antitumor activity by the restriction of cancer cell proliferation [5,6]. Taken together, LA plays a crucial anti-proliferative role by targeting EGFR signaling pathways. Further studies are necessary to unveil other RTKs affected by LA, such as the insulin-like growth factor receptor 1 (IGF1R) (Figure 1). Moreover, it has been demonstrated that LA targets some protein tyrosine phosphatases which are implicated in tumorigenesis. Indeed, in breast cancer cells, LA was shown to decrease their viability by reducing the activity PTP1B and SHP2 which are often overexpressed in breast cancer cells and present potential targets for anticancer therapy [7]. Moreover, LA fosters the upregulation of the cyclin-dependent kinase inhibitors p27^{kip1} and p21^{Cip1} in many types of cancers leading to

cell cycle arrest [8] (Figure 1). AMPK is a major sensor of energy level that maintains the energy homeostasis. Its activation by its phosphorylation has been described to restrict tumor progression by inhibiting the mTOR protein complex which is a downstream Akt effector. In addition to its inhibitory role, LA leads to strengthen the AMPK activation and then reinforces the inhibition of the Akt pathway thereby reducing cancer cell proliferation [9–12].

In addition to its anti-proliferative role, LA induces apoptosis of different types of cancer cells in a dose-dependent manner. Previous data have shown that LA is able to generate ROS, which promote apoptosis in a variety of cancer cell lines by deregulating the ratio between anti-apoptotic and pro-apoptotic proteins. Indeed, the mitochondrial anti-apoptotic proteins Mcl-1, bcl-2 and bcl-X_L, are downregulated in a concentration-dependent manner following LA treatment in several ovarian lung and breast cancer cell lines [13]. Contrariwise, the expression of the pro-apoptotic proteins Bim and Noxa is upregulated in response to LA. All these events lead to cell death [14] (Figure 1). In leukemia and breast cancer, the Bax/bcl2 ratio (apoptotic index) increases significantly after LA treatment with increased caspase-3 activity [15]. Another study performed in hepatoma cancer cells also showed that LA triggered the intrinsic apoptotic pathway by the activation of caspase-9 and caspase-3 [13]. In line with these findings, previous study has been done in colon cancer revealed that LA stabilizes the WTp53 protein by inhibiting the NF- κ B signaling (Figure 1). It is known that WTp53 is an activator of apoptosis by inducing the transcription of pro-apoptotic genes thereby triggering intrinsic apoptosis. However, Dörsam et al demonstrated that LA triggered cell death in colorectal cancer independently of p53 stabilization [16]. Furthermore, a previous work on lung cancer cells suggested that LA treatment provoked other cell death pathways by caspase-independent mechanisms mediated by the Apoptosis-Inducing Factor (AIF) leading to necrosis and autophagy-associated cell death [17].

3. The impact of lipoic acid on metastasis, invasion, migration and EMT

The metastasis cascade is a complex multi-step process, the first critical step being cell invasion through extracellular barriers and penetration of the basement membrane, followed by infiltration of the vasculature, extravasation and colonization of distal organs. These events are regulated by two crucial pathways, namely the transforming growth factor β (TGF β) and the focal adhesion kinase (FAK). Once these pathways are activated, epithelial-mesenchymal transition (EMT)-related transcription factors (TFs) such as vimentin, Slug, Twist and Snail are induced. These TFs

promote EMT by downregulating E-cadherin and by triggering the overexpression of several EMT markers.

In two breast cancer cell lines, namely MDA-MB-231 and 4T1, a recent study demonstrated that LA inhibits cell migration by repressing TGF β -induced EMT markers such as Snail, vimentin and ZEB1 [6]. The expression of these markers is also reduced upon LA treatment in other types of cancers [18] (Figure 1). Moreover, the Smad pathway, known to be implicated in the EMT process after its activation by TGF β signaling, is inhibited by LA [5]. Collectively, these results indicate that LA suppresses invasion and migration by inhibiting the EMT (Figure 1). In addition, during cancer cell invasion, small projections called invadopodia facilitate cell adhesion to the extracellular matrix (ECM) in an integrin-dependent manner. These cell surface proteins are known to activate the FAK pathway. LA treatment impairs the formation of these projections due to the downregulating of β 1-integrin expression, leading to the inhibition of cancer cell invasion and migration [19] (Figure 1). Invasion also relies on metalloproteinases (MMPs), since these key enzymes, mainly MMP-2 and MMP-9, degrade type IV collagen and play crucial roles in tumor cell invasion and malignancy [20], and once again LA was shown to inhibit invasion by reducing MMP-2 and MMP-9 mRNA expression in a dose-dependent manner in breast cancer cell lines [20] (Figure 1). Consistent with these findings, LA also inhibits the EMT process by increasing E-cadherin and decreasing of N-cadherin, vimentin and activated β -catenin.

4. Lipoic acid reduces stemness properties

Cancer stem cells (CSCs) are rare immortal cells able to give rise to multiple cell types constituting the tumor, due to their self-renewal and differentiation capacities. This population is able to induce cell proliferation and dissemination. Despite advances in therapies targeting the tumor bulk, CSCs could remain unaffected, promoting cancer relapse and therapeutic drug resistance. The CSC phenotype is maintained through the activation of the Akt pathway which is important for the stabilizing CSC regulatory proteins, such as Oct-4, which promotes the transcription of Nanog, a key protein sustaining stemness, aggressiveness and chemoresistance of various cancer cell types. A recent study revealed that LA negatively regulates CSC-like phenotypes of human non-small cell lung cancer-derived cells by suppressing the Akt signaling pathway, leading to the proteosomal degradation of Oct-4 [18]. This study demonstrated the role of LA in suppressing CSC

phenotypes and reinforces the crucial implication of LA in overcoming CSC-mediated chemoresistance, progression and metastasis (Figure 1).

5. Lipoic acid enhances the cytotoxicity of chemotherapy and prevents their side effects

Despite advances in conventional therapies, mechanisms of resistance including the generation of DNA damage and free radicals, leading to the growth of secondary tumors, remain the underlying causes of morbidity and mortality. Several studies have shown that LA sensitizes cancer cells to chemotherapeutic agents. Notably, LA increases the paclitaxel efficiency in breast and lung cancer cells by inhibiting NF- κ B signaling and integrin β 1 and β 3, respectively [21,22] (Figure 1). Similarly, a recent study has shown that co-loading LA and Docetaxel using solid lipid nanoparticles (DTX-ALA co-loaded SLN) increases apoptosis as compared to single drug-loaded SLNs (DTX -loaded SLN or ALA loaded SLN) in breast cancer cells [23]. Another study showed that LA potentiates the cytotoxicity of two anti-cancer agents used in colorectal cancer (5-fluorouracil, Temozolomide) which have different mechanisms of action. Likewise, LA overcomes gefitinib resistance by reducing the activation of growth factor receptors in non-small cell lung cancer cells [24]. Owing to these studies, it would be interesting to combine LA with anticancer drugs in order to improve their efficiency. In addition to its potential role in enhancing the cytotoxicity of chemotherapy, LA has a preventing role against the chemotherapy side effects by protecting patients against neuropathy, intestinal damage and diarrhea. In this context, pretreatment with LA before cisplatin administration can overcome cisplatin-induced damages such as nephrotoxicity, neurotoxicity and ototoxicity by restoring the redox system [25].

6. Preclinical and clinical trials

In total, more than 35 toxicological clinical studies support the safety of LA. In human, several studies monitored both efficacy and safety of LA treatment at dosage of 600 mg up to 2400 mg/day and for time periods ranging from weeks to 24 months. However, very high doses exhibit a toxicity with small changes of liver enzymes activities [26]. In mice, a 4-week oral study, at concentrations of 31.6 and 61.9 mg/kg body weight, LA did not produce any toxic effects in Wistar rats of either sex [26]. The preclinical and clinical studies were performed with the idea that LA would decrease the toxicities of chemotherapy and increase their efficiency. Indeed, LA was tested in combination with chemotherapy in mice and humans. In some cases, LA has an antagonist effect and it inhibits

the anti-cancer effects of chemotherapy. However, an additive effect was observed after the combination of 5mg/ kg of doxorubicin and 16 mg/kg of LA on survival of leukemia-bearing mice while synergistic effect was observed only at the high concentrations of both drugs. In this context, in order to avoid toxicity and improve efficacy, appropriate dose of LA is crucial in designing therapeutic protocols [27]. On the other hand, on single study has reported that the intravenous LA and low-dose naltrexone (LA-N) protocol with a healthy lifestyle could extend the life of pancreatic cancer patient for several years without any toxic effect [28]. Collectively, LA plays a paradoxical role: (i) a major role as an anticancer adjuvant and a chemo-preventive agent when it is combined with bortezomib for example, by reducing the stress response induced by proteasome inhibition [29], (ii) a secondary role as an antagonist of some clinically-used anticancer drugs that act through generating various radical species (i.e. alkylating agents). In this latter example, LA administration with alkylating agents such as cisplatin may demonstrate decreased therapeutic effect as their active radicals may be eliminated by LA [27]. On the other hand, it was already demonstrated that toxicity of some anticancer drugs is related to the formation of free radicals and may be decreased by LA. Taken together, regarding the different mechanisms of action of anti-cancer treatments, LA co-treatment could be useful in combination with some treatments by reducing toxicity, inflammation and oxidative stress. However, the antioxidant effect of LA could inhibit the anti-cancer effects of some drugs that need this oxidative stress in order to achieve its anti-cancer role. In summary, additional studies of mechanisms of action of each anti-cancer drug and toxicological studies are needed to balance the therapeutic effect and toxic side effects of their combination with LA.

7. Conclusions

Hence, LA exhibits anti-tumor activities in several cancer models, by impacting several hallmarks on most of the signaling pathways implicated in proliferation, invasion, migration, EMT, stemness and apoptosis. Its mechanisms of action are so far not completely elucidated, and further investigations are needed to improve our understanding of these underlying anti-cancer effects. Another ongoing challenge is to prove its effectiveness *in vivo* in combination with chemotherapeutic agents. These additional studies may provide crucial data for the design of

combinatorial LA therapies in order to increase its efficacy and to restore cancer cell chemosensitivity. LA could thus be a promising molecule to improve anti-cancer therapies.

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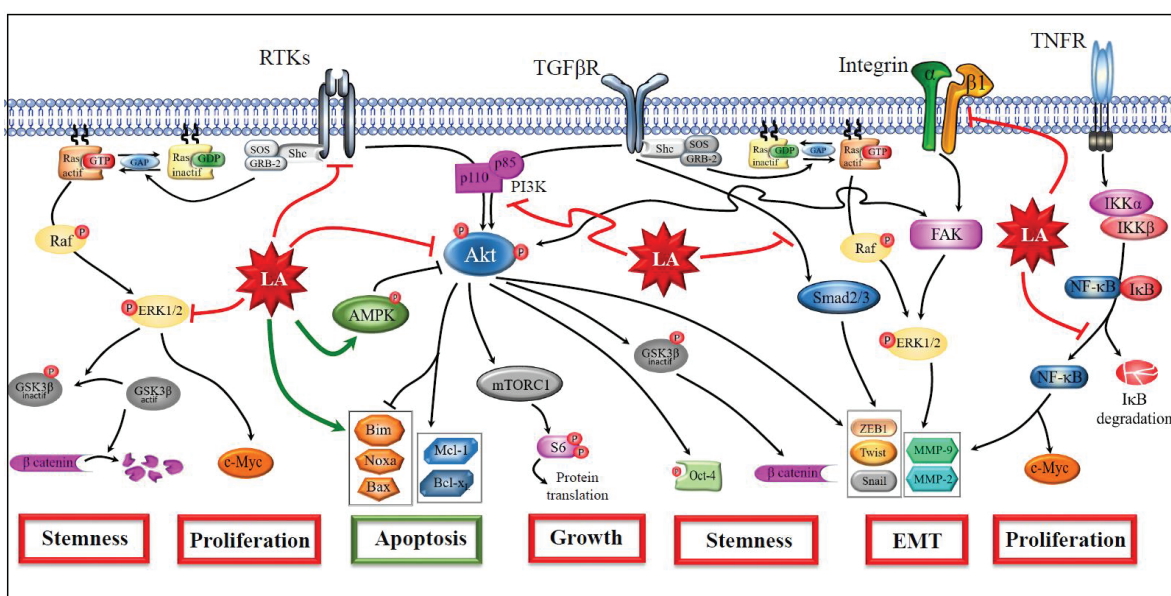


Figure 1 : Effects of lipoic acid (LA) on several signaling pathways implicated in tumorigenesis.

Stemness Upon activation of receptor tyrosine kinases (RTKs) or TGFβR, the GRB2 and SHC complex is recruited resulting in the recruitment of SOS to the plasma membrane where Ras is located. Therefore, activated SOS promotes the exchange of GDP for GTP on Ras generating an active GTP-bound Ras. Activated Ras leads to the activation by phosphorylation of the downstream effectors (raf, ERK 1/2) leading to the phosphorylation and inactivation of GSK3, resulting in the stabilization of β-catenin and activation of Oct-4 by phosphorylation. These events play a crucial role in stemness. LA inhibits the activation of Akt by reducing its phosphorylation, thus preventing the stemness process. **Cellular proliferation and growth** are controlled by the activation of ERK and PI3K/Akt pathways. LA prevents the phosphorylation of

ERK, in particular by blocking the activation of c-Myc which is a centerpiece of the several processes implicated in cancer development. Moreover, LA inhibits the activation of Akt leading to the inhibition of mTORC1 which induces the translational process, thus LA reduces cellular proliferation and growth. **Apoptosis** is controlled by two subtypes of proteins, pro- or anti-apoptotic proteins. Phosphorylation of Akt provokes the induction of anti-apoptotic proteins (e.g. Mcl-1, bcl-x_L) and the reduction of pro-apoptotic proteins (e.g. Bim, Nova, Bax). LA acts at three different levels: (i) it inhibits the Akt pathway causing a repression of anti-apoptotic proteins and an increase in pro-apoptotic proteins, (ii) it induces the transcription of pro-apoptotic proteins by the generation of ROS, and (iii) it activates the AMPK protein which negatively regulates the Akt pathway. Moreover, LA impedes the activation of PI3K as well as some RTKs, such as EGFR, which are upstream of the Akt or ERK pathways, reinforcing the inhibition of these pathways. In addition, LA suppresses the downstream oncogenic pathways activated by TNF α by inhibiting **NF- κ B signaling** which is a critical promoter of tumorigenesis. All of these events favor the inhibition of proliferation, growth and the induction of apoptosis. The **EMT process** leading to cell migration and invasion is controlled by several proteins (e.g. ZEB1) and enzymes (e.g. MMPs). LA inhibits FAK activation by downregulating β 1 and β 3 integrins expression. This action prevents ERK activation, and reduces the mRNA levels of MMP-9 and -2. In addition, LA inhibits the Smad signaling which is a main signal transducer for receptors of the transforming growth factor beta (TGF β) superfamily, which are essential for regulating growth and EMT. (Green arrows and frame reveal activation and red arrows and frames reveal inhibition).

AMPK: AMP-activated Protein Kinase; Akt: Protein Kinase B, Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma 2; c-Myc: Cellular Myelocytomatosis; ERK: Extracellular signal - Regulated Kinase; FAK: Focal Adhesion Kinase; GAP: GTPase-activating proteins; GDP: Guanosine diphosphate; GEF: guanine nucleotide exchange factors; GRB2: Growth factor receptor-bound protein 2; GSK3 β : Glycogen synthase kinase 3 beta; LA: Lipic Acid; Mcl-1: myeloid cell leukemia 1; MMP: Matrix Metalloproteinase Protein; mTORC1: mammalian Target Of Rapamycin Complex 1; Oct-4: Octamer-binding transcription factor 4; Pi: phosphate; PI3K: Phosphoinositide 3-kinase; RAS: rat sarcoma; RAS-GTP: rat sarcoma /Guanosine Triphosphate;

SHC: Squalene-Hopene Cyclase ; SOS: Son of sevenless homolog; S6: ribosomal protein S6;
TGFβR: Transforming Growth Factor Beta Receptor; RTKs: Receptors Tyrosine Kinase.

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