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The hypoxic tumor microenvironment regulates the LPA signaling axis to promote cancer cell invasion and metastasis

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Dedicated to the memory of my Grandmother (Dorothy Harper)

RÉSUMÉ

Le microenvironnement hypoxique tumoral régule la signalisation du LPA pour favoriser l'invasion des cellules cancéreuses et le développement des métastases

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Thèse présentée à la Faculté de médecine et des sciences de la santé en vue de l'obtention du diplôme de philosophiae doctor (Ph.D.) en immunologie, Faculté de médecine et des sciences de la santé, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4

Le développement des métastases est la cause principale de mortalité des patients atteints de cancer, mais demeure un obstacle majeur aux traitements. L'hypoxie, une caractéristique commune des tumeurs solides, est fortement impliquée dans l'invasion cellulaire et le développement des métastases, mais les mécanismes sous-jacents demeurent méconnus. La signalisation du LPA joue un rôle important au cours de la tumorigenèse et du développement des métastases, les membres de cette voie étant souvent régulés à la hausse dans les cellules tumorales. La signalisation du LPA a également été impliquée dans la production de structures de dégradation, les invadopodes, qui sont nécessaires à la formation de métastases. Des études récentes indiquent que la formation d'invadopodes est également induite par l'hypoxie. Par conséquent, nous avons voulu élucider l'influence du microenvironnement hypoxique sur l'axe de signalisation du LPA et si celui-ci joue un rôle dans la production d'invadopodes et la formation de métastases.

Nous avons découvert que le LPA1 est un récepteur utilisé de façon commune et majoritaire pour la production d'invadopodes induite par l'hypoxie, et ce, dans diverses lignées cellulaires cancéreuses. Nous avons démontré que l'hypoxie favorise la formation d'invadopodes en utilisant une voie de signalisation distincte qui implique une communication croisée entre le récepteur LPA1 et l'EGFR, médiée par la kinase Src, Dans ce contexte, l'inhibition combinée du LPA1 et de l'EGFR agit en synergie afin d'empêcher la formation de métastases spontanées. Étant donné que la toxicité et la résistance aux inhibiteurs de l'EGFR représentent un défi important pour les patients atteints de cancer, ce travail permet l'identification d'une cible potentielle, le LPA1, qui pourrait être inhibée conjointement avec l'EGFR dans le but d'améliorer la survie de ces patients. D'autres études sur la régulation hypoxique de l'axe de signalisation du LPA ont démontré que l'hypoxie peut contrôler les niveaux d'expression des enzymes impliqués dans la production (ATX) et la dégradation (LPP1 / LPP3) du LPA, des évènements qui conduisent à une production accrue d'invadopodes. L'hypoxie permet également de modifier la localisation de ces protéines, ce qui pourrait constituer un mécanisme additionnel de régulation de l'axe de signalisation du LPA en hypoxie.

Notre travail suggère que l'hypoxie est un régulateur important de l'axe de signalisation du LPA menant à l'invasion et à la formation de métastases. Par conséquent, les thérapies ciblant cet axe pourraient être bénéfiques pour contrer les effets néfastes de l'hypoxie tumorale sur la survie des patients atteints de cancer. De plus, un traitement combiné, ciblant le LPA1 et l'EGFR, pourrait être utile afin de réduire les effets secondaires et la résistance aux inhibiteurs de l'EGFR. Des études supplémentaires seront nécessaires afin de valider le potentiel thérapeutique de ce type de traitement.
Mots clés : [Autotaxin, LPA, Invadopodia, Hypoxia, Metastasis]

SUMMARY

The hypoxic tumor microenvironment regulates the LPA signaling axis to promote cancer cell invasion and metastasis

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Thesis presented at the Faculty of Medicine and Health Sciences for the obtention of Doctor degree diploma philosophiae doctor (Ph.D.) in Immunology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4

Metastasis is the leading cause of cancer patient mortality yet remains a major hurdle for treatment. Hypoxia, a common feature of solid tumors, has been critically involved in cell invasion and metastasis but the underlying mechanisms remain poorly understood. The LPA signaling axis plays an important role during tumorigenesis and metastasis, with members of this pathway often being upregulated in tumor cells. LPA signaling has also been implicated in production of the degradative structures invadopodia, which are known to be required for metastasis. Interestingly, formation of invadopodia can also be induced by hypoxia. Therefore, we endeavoured to elucidate the influence of the hypoxic tumor microenvironment on the LPA signaling axis and whether this could play a role in invadopodia production and metastasis.

We uncovered LPA_1 as a common and major receptor used for hypoxia-induced invadopodia production in various cancer cell lines. We demonstrated that hypoxia promotes invadopodia formation through a distinct signaling pathway that involves Src-mediated cross-communication between LPA_1 and EGFR, and that combined inhibition of LPA_1 and EGFR acts synergistically to impede spontaneous metastasis. Since EGFR inhibitor toxicity and resistance represents a current challenge for cancer patients, this work identifies a potential target, LPA_1 that could be inhibited in conjunction with EGFR to improve patient outcomes. Further studies into hypoxic regulation of the LPA signaling axis demonstrated that hypoxia can control the expression levels of LPA producing (ATX) and degrading (LPP1/LPP3) enzymes, events that lead to increased invadopodia production. Hypoxia was also found to alter the localization of these proteins, uncovering an additional mechanism of hypoxic regulation.

Our work suggests that hypoxia is a master regulator of the LPA signaling axis that leads to metastasis, therefore therapies targeting this axis could be beneficial to counteract the detrimental effects of tumor hypoxia on cancer patient survival. Furthermore, LPA_1-EGFR combination therapy could be a useful strategy to reduce EGFR inhibitor side effects and resistance and therefore warrants further studies to evaluate the potential of combination therapies in cancer patients.

Keywords: [Autotaxin, LPA, Invadopodia, Hypoxia, Metastasis]

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LIST OF ABBREVIATIONS

AC	adenylyl cyclase
ADAM	a disintegrin and metalloproteinase
ADF	actin depolymerizing factor
AP-1	activator protein 1
Arg	Abelson-related gene
Arp2/3	actin-related proteins
ATP	adenosine Triphosphate
ATX	autotaxin
Bcl-2	B-cell lymphoma 2
bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
BMDC	bone marrow-derived cells
Bmp-2	bone morphogenic protein 2
C1P	ceramide 1-phosphate
CAF	cancer-associated fibroblasts
CAIX	carbonic anhydrase 9
cAMP	cyclic adenosine 3',5'-monophosphate
CDK	cyclin-dependent kinase
cPA	cyclic phosphatidic acid
CREB:	cAMP response element-binding
CXCR	chemokine receptor
DAG	diacylglycerol
DDR1	Discoidin domain receptor family, member 1
DPP4	dipeptidyl dipeptidase IV
EBV	Epstein-Barr virus
ECM	extracellular matrix
Edg	endothelial differentiation gene
EGF	epidermal growth factor

EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
E.R	endoplasmic reticulum
ERK1/2	extracellular signal-regulated kinases
F-actin	filamentous actin
FAK	focal adhesion kinase
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine exchange factor
GLUT	Glucose transporter
GM1	monosialotetrahexosylganglioside
GPCR	G-protein coupled receptor
GPR	G-protein receptor
Grb2	Growth factor receptor-bound protein 2
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HB-EGF	heparin-binding EGF-like growth factor
HDAC	histone deacetylases
HGF	hepatocyte growth factor
HIF	Hypoxia-inducible factor
HUVECs	human umbilical vascular endothelial cells
IFN	interferon
IGFR1	insulin-like growth factor receptor 1
IL	interleukin
IP3	inositol triphosphate
IRS-1	insulin receptor substrate 1
ITGA	integrin subunit alpha
ITGB	integrin subunit beta
JNK	c-Jun N-terminal kinases
LDH	lactate dehydrogenase
LIMK	LIM motif-containing protein kinase

LOX	lysyl oxidase
LPA	lysophosphatidic acid
LPAR	lysophosphatidic receptor
LPAAT	lysophosphatidic acid acyl transferases
LPC	lysophosphatidyl choline
LPP	lipid phosphate phosphatase
LPS	lipopolysaccharide
lysoPLD	lysophospholipase D
MAG	monoacylglycerol
MAPK	mitogen activated protein kinase
MβCD	methyl-beta cyclodextrin
MCP-1	monocyte chemoattractant protein-1
MCT	monocarboxylate transporter
MMP	matrix metalloproteinase
MORFO	modulator of oligodendrocyte remodeling and focal adhesion organization
mRNA	messenger ribonucleic acid
MT1-MMP	membrane-type 1 MMP
NAC	N-acetyl-L-cysteine
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NFAT	nuclear factor of activated T-cells
NF-kB:	nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NHE	Na^+/H^+ exchanger
NHERF1/EBP50	Na^+-H^+exchanger regulatory factor/ ERM-binding protein 50
NPP2	nucleotide pyrophosphatase/phosphodiesterase 2
N-WASP	neuronal Wiskott-Aldrich syndrome protein
PA	phosphatidic acid
PAP	phosphatidic acid phosphatase
PAP2	type 2 PAP
PAI-1	type 1 plasminogen activator inhibitor

PDGF	platelet-derived growth factor
PFK	phosphofructokinase
PHD	prolyl hydroxylase domain protein
PI3K	phosphoinositide 3-kinases
PKC	protein kinase C
PLA1 or 2	phospholipase A1 or A2
PLC	phospholipase C
PLD	phospholipase D
PTP	protein tyrosine phosphatase
PX	phox homology
RGD	arginine-glycine-aspartic acid
RNA	ribonucleic acid
ROCK	rho-associated coiled-coil-forming protein kinase
ROR2	receptor tyrosine kinase-like orphan receptor 2
ROS	reactive oxygen species
RPLP0	ribosomal protein lateral stalk subunit P0
RT-PCR	reverse transcriptase polymerase chain reaction
RTK	receptor tyrosine kinase
S1P	sphingosine-1-phosphate
SDF-1	stromal cell-derived factor 1
SH2	src-homology 2 domain
SH3	src-homology 3 domain
SHIP	SH2 domain-containing inositol 5-phosphatase 2
SMB	somatomedin B domain
SOS	son of sevenless
shRNA	short hairpin RNA
SRA	steroid receptor RNA activator 1
SRE	serum response element
STED	Stimulated emission depletion
TAM	tumor-associated macrophage
TGF-β	transforming growth factor beta

TKS5/FISH	tyrosine kinase substrate 5/five SH3 domains
TLR	toll-like receptor
TNF- α	tumor necrosis factor-alpha
TrkA	tropomyosin receptor kinase A
tyr	tyrosine
uPA	urokinase-type plasminogen activator
uPAR	urokinase receptor
VATPase	vacuolar-type H^+-ATPase
VEGF	vascular endothelial growth factor
ZEB	zinc finger E-box binding homeobox

1. INTRODUCTION

Cancer is the deadliest disease for Canadians, accounting for 30% of deaths in Canada in 2012 (Canadian Cancer Society's Advisory Committee on Cancer Statistics, 2017). Over 90% of cancer patients end up dying from metastatic cancer rather than the growth of the primary tumor. However treatments specifically targeting metastasis are not in clinical use due, in part, to the complexity of this process (Gandalovičová et al., 2017). For cancer cells to metastasize they must first acquire migratory and invasive capabilities in order to leave the site of the primary tumor by degrading their surrounding extracellular matrix (ECM) and/or underlying basement membrane. The cancer cells then eventually enter into lymphatic or blood vessels, allowing them to travel to distant sites in the body. At these distant sites, cancer cells will need to leave the blood vessels to form micrometastasis and eventually full-blown macrometastasis at the new site (Steeg and Theodorescu, 2008). The acquisition of cellular invasive capabilities is therefore an essential step for metastasis, which allows cancer cells to degrade ECM and leave the primary tumor, as well as intravasate into, and extravasate out of blood vessels. A better understanding of the mechanisms underlying the invasion-metastasis cascade should lead to the development of novel targeted therapeutics to inhibit this deadly aspect of tumor progression.

1.1 The Tumor microenvironment promotes invasion and metastasis

The tumor microenvironment has become acknowledged as a major player promoting tumorigenesis. It has been increasingly recognized that tumor cell intrinsic properties, such as immortality and sustained proliferation, alone are not enough to drive tumor progression and metastasis. Rather, as tumor cells exist in complex tissue environments, the surrounding tumor microenvironment has an important role to play, interacting with, or being modified by, tumor cells to promote tumorigenesis. The tumor microenvironment consists of many factors such as stromal, immune, and endothelial cells, as well as non-cellular components such as ECM, growth factors and cytokines (Sounni and Noel, 2013). Sites of chronic inflammation are often associated with the development of cancer, reinforcing the importance of the microenvironment during cancer development (Balkwill and Mantovani, 2001). For example, liver cirrhosis is associated with increased incidence

of hepatocellular carcinoma (Sangiovanni et al., 2004) and inflammatory bowel disease with increased risk of colorectal cancer (Beaugerie et al., 2013). The tumor microenvironment consequently contains many inflammatory cells, for example tumor-associated macrophages (TAMs), which support tumor progression and drive tumor cell invasion by supplying promigratory factors like epidermal growth factor (EGF) and ECM degrading proteases (Condeelis and Segall, 2003; Qian and Pollard, 2010; Quail and Joyce, 2013; Wyckoff et al., 2007). Other immune cell types in the microenvironment, such as regulatory T cells, act as immunosuppressants contributing to tumor immune evasion (Whiteside et al., 2012). Another important tumor microenvironment cell type are the cancer-associated fibroblasts (CAFs), which have been shown to affect tumorigenesis and metastasis by providing a major source of secreted growth factors such as vascular endothelial growth factor (VEGF), Transforming growth factor-β (TGF-β) and hepatocyte growth factor (HGF), pro-inflammatory factors such as monocyte chemoattractant protein-1 (MCP-1) and Interleukin-1 (IL-1), and ECM degrading proteases such as matrix metalloproteinases (MMPs), particularly in breast cancer (Dumont et al., 2013; Kalluri and Zeisberg, 2006). Endothelial cells in the microenvironment can also aid tumor progression by forming new blood vessels to support the growth of the tumor (Du et al., 2008; Semenza, 2013; Weis and Cheresh, 2011). A major driver of angiogenesis is hypoxia, which is another important factor in the tumor microenvironment. Hypoxia promotes angiogenesis in part by inducing the expression of the major angiogenic factor, VEGF, to affect endothelial cells, pericytes and bone marrow-derived cells (BMDCs) to induce vessel growth (Chouaib et al., 2012; Du et al., 2008; Petrova et al., 2018; Semenza, 2013; Weis and Cheresh, 2011). Hypoxia in the tumor microenvironment can also induce the recruitment of immune cells, such as macrophages, through endothelin-2 and VEGF secretion from tumor cells. Hypoxia then promotes the switch of macrophages to a pro-tumorigenic phenotype through upregulation of genes affecting tumor growth, invasion, angiogenesis and immune evasion such as HGF, platelet-derived growth factor (PDGF), MMP7, VEGF, and tumor necrosis factor-α (TNF-α) (Chouaib et al., 2012; Lewis and Murdoch, 2005; Petrova et al., 2018). Hypoxic cancer cells also secrete paracrine signaling molecules like TGF-β and PDGF to promote the transformation of fibroblasts into CAFs, which subsequently secrete pro-tumorigenic factors like HGF and angiogenic factors such

as VEGF and angiopoietin (Murdoch et al., 2004; Petrova et al., 2018; Yan et al., 2015). In addition to these effects on the tumor cellular microenvironment, hypoxia has profound effects on the tumor cells themselves, which will be discussed in the following sections. It is therefore important to take into consideration the supportive role of the tumor microenvironment during cancer progression (Fang and Declerck, 2013).

1.1.1 Hypoxia in the tumor microenvironment

Hypoxia is a condition of low oxygen concentration, commonly found within solid tumors. While normal tissues have oxygen levels varying from 4% to 9.5%, depending on vascular networks and metabolic activity of the tissue in question, oxygen levels in various tumors have been shown to fall between 0.3% and 2% (Muz et al., 2015). However, the majority of these tumors have regions of hypoxia around 1% oxygen (Muz et al., 2015), which is the oxygen level commonly used in experimental settings to evaluate the effects of hypoxia. The level of oxygen within a tumor depends on many factors such as the initial oxygenation of the tissue as well as the size and stage of the tumor (Carreau et al., 2011; Höckel et al., 1991; Müller et al., 1998; Vaupel et al., 2007). Hypoxia first arises in solid tumors due to their rapid proliferation resulting in a high demand for oxygen and nutrients, to sustain their metabolic needs, that quickly exceeds the supply available from normal vasculature. The rapid tumor growth also results in increased distance between the cells and blood vessels further limiting access to oxygen and nutrients (Muz et al., 2015; Semenza, 2000; Thomlinson and Gray, 1955). This starts a vicious cycle as hypoxia then induces angiogenesis that is structurally and functionally abnormal, resulting in chaotic, immature and leaky blood vessels that are prone to collapse generating additional regions of hypoxia within the growing tumor mass (Vaupel and Harrison, 2004). The exposure to hypoxia within tumors may be acute or chronic. Acute hypoxia is a brief and abrupt exposure to low oxygen levels that could be due to a blood vessel occlusion and lasts several minutes. Acute hypoxia is therefore often reversible and may even be cycling, with several minutes of hypoxia followed by reoxygenation and then hypoxia again. *In vitro*, acute hypoxia is considered to be several minutes up to 72hr of hypoxic exposure, compared to chronic hypoxia, which is considered to be a few hours up to several weeks. Chronic hypoxia is observed more often in larger tumors where changes in blood flow and decreased oxygen availability cause a sustained lack of oxygen to the cells (Bayer and Vaupel, 2012; Vaupel

and Harrison, 2004). This tends to lead to more long-term effects on the cells, including increased DNA damage (Luoto et al., 2013).

1.1.2 Mechanisms of hypoxia-induced effects in cells

1.1.2.1 Gene expression

Hypoxia activates a diverse array of transcription factors to profoundly affect cellular gene expression promoting long-term cell survival and adaptation to hypoxic conditions. Some of the transcription factors activated in hypoxic cells are NF-κB, CREB, and AP-1, which regulate genes involved in cell proliferation, apoptosis, angiogenesis and inflammatory responses (Beitner-Johnson and Millhorn, 1998; Koong et al., 1994; Millhorn et al., 1997). For example, NF-κB reduces apoptosis through effects on Bcl-2 family members and induces angiogenesis by regulating the expression of important chemokines such as IL-8 (D'Ignazio and Rocha, 2016). However, many of the transcriptional responses to hypoxia are orchestrated by the hypoxia-inducible factors (HIFs), with HIF-1 being the most well known and studied. HIFs are heterodimers composed of a HIFα and HIFβ subunit that together bind to hypoxia-responsive elements in the promotor region of many genes. They are members of the basic helix-loop-helix (bHLH) family of transcription factors (Wang et al., 1995). These transcription factors are sensitive to hypoxia because the HIFα subunit is normally degraded under normoxic conditions due to the effects of oxygen-dependent prolyl hydroxylase domain proteins (PHDs) (Epstein et al., 2001). The HIF1α subunit is therefore stabilized under hypoxic conditions due to the inactivity of these PHD proteins (Schofield and Ratcliffe, 2004). HIF-1 regulates the transcription of 100s of genes affecting diverse physiological and pathological processes (Semenza, 2012). One of the most well-known effects of hypoxia is the induction of angiogenesis and HIF-1 can promote this effect through upregulation of pro-angiogenic factors such as VEGF and SDF-1 (Siemeister et al., 1996; Zagzag et al., 2005). Hypoxia is also known to alter cell metabolism by inducing anaerobic glycolysis. This process is also affected by HIF-1-mediated gene expression of GLUT-1 and GLUT-3, glucose transporters that help supply the hypoxic cells with sufficient glucose for energy production through glycolysis (Iliopoulos et al., 1996; Iyer et al., 1998). Hypoxia modulates apoptosis and cell survival through HIF-1 regulation of p53, TGF-β and bFGF (An et al., 1998c; Semenza, 2000).

Hypoxia is a strong inducer of EMT in part through HIF-dependent regulation of E-cadherin, Zeb1 and Zeb2, regulating EMT, adhesion and motility (Krishnamachary et al., 2006). Finally, HIF-1 induces CXCR4, CAIX, LOX, MMP2 and MMP9 expression promoting migration and invasion (Erler et al., 2006; Grabmaier et al., 2004; Semenza, 2012; Staller et al., 2003). Transcription factors of the HIF family can therefore drive many aspects of tumorigenesis increasing cell survival and angiogenesis while reducing cell-cell attachment allowing cancer cells to migrate and invade. These slower transcriptional responses however are not responsible for all of the effects of hypoxia, especially not the acute responses.

1.1.2.2 Metabolism and pH alterations

One of the major and immediate adaptations to hypoxia is a change in metabolism to anaerobic glycolysis. This results in production of lactic acid, which, along with a decrease in CO_2 dispersion, contributes to the acidification of the tumor microenvironment (Cassavaugh and Lounsbury, 2011). In hypoxic cells the pH gradient is altered (reversed) with an acidic extracellular environment and a more alkaline intracellular environment. This is due to the action of a variety of proton pumps and transporters that are responsible for regulating cellular pH, such as Na+/H+ exchangers (NHEs), vacuolar-type H+-ATPase (VATPases), monocarboxylate transporters (MCTs) and CAIX. NHE-1, which is found at the cell membrane, is responsible for expelling protons, by exchanging one intracellular proton for one extracellular sodium ion. VATPases, similarly remove protons from the cytosol, however they move these protons into intracellular vesicles such as endosomes and lysosomes. MCTs move lactic acid and protons, produced by glycolysis, out of the cell. Finally, CAIX has an extracellular catalytic domain that catalyses the hydration of carbon dioxide to bicarbonate and protons. Therefore, all of these proteins contribute to the reversed pH gradient found in cancer cells, by acidifying the extracellular environment, while increasing the pH in the cell cytosol and can in turn be regulated by hypoxia (Chiche et al., 2010). The reversed pH gradient in hypoxia has profound effects on the cell. First, increased intracellular pH promotes proliferation and cell survival (Pouysségur et al., 1985). Proliferation is promoted in part by the increased activity of CDK2 (cyclin-dependent kinase 2), a major driver of cell cycle progression, under alkaline conditions.

This is due to reduced expression of the protein kinase Wee1 in alkaline conditions, resulting in a reduction of the inhibitory phosphorylation of CDK2 (Putney and Barber, 2003). The increased cell survival is mediated in part by limiting apoptosis, as apoptosis is associated with a lower more acidic pH, which results in conformational changes in the pro-apoptotic BAX protein (Lagadic-Gossmann et al., 2004). Increased intracellular pH also further promotes glycolysis, which will result in increased lactic acid production thereby sustaining the acidic microenvironment (Kuwata et al., 1991). The activity of several enzymes important for glycolysis is controlled by pH, such as phosphofructokinase (PFK) and lactate dehydrogenase (LDH), which are more active at slightly alkaline pH (Chiche et al., 2010; Halprin and Ohkawara, 1966). Finally, the alkaline intracellular pH also facilitates migration, as many actin-binding proteins including cofilin (Pope et al., 2004), profilin (McLachlan et al., 2007), villin (Grey et al., 2006), and talin (Srivastava et al., 2008), are pH sensors. These proteins adapt to altered pH due to conserved histidines found within their structure. Histidines are the only amino acid with a pKa close to physiological pH and can therefore be protonated or deprotonated by changes in pH. This can result in conformational changes in the histidine containing protein, which can affect their activity or binding partners (Webb et al., 2011). Cofilin, for example, dissociates from membrane lipids at high pH with an associated increase in its activity (Frantz et al., 2008). Finally, the acidic extracellular environment induced under hypoxic conditions also greatly affects cells. Most notably, lower extracellular pH promotes degradation of the ECM essential for cancer cell invasion by activating many proteases, such as MMP-3, urokinase-type plasminogen activator (uPAR) and cathepsins. Low pH also facilitates the conversion of pro-MMPs to active MMPs and the secretion of certain proteases such as MMP-9 and cathepsin B and L (Rozhin et al., 1994; Stock and Schwab, 2009).

1.1.2.3 Endocytosis regulation

Many endocytosis-associated proteins, such as clathrin, Rab25 and caveolin 1, are deregulated in cancer cells, with an associated alteration in receptor trafficking, that has been implicated in malignant transformation (Mosesson et al., 2008). Recent evidence demonstrates that hypoxia regulates endocytosis in several ways in order to mediate cellular effects. For example, while hypoxia is known to upregulate signaling through

EGFR and other receptor tyrosine kinases (RTKs), most notably by increasing their expression, hypoxia was also shown to increase EGFR signaling by prolonging the half-life of EGFR (Wang and Ohh, 2010). Endocytosis involves early and late endosomal fusion events that are controlled by Rabs, a group of Ras-like small guanosine triphosphatases (GTPases) (Wang et al., 2009). Under hypoxic conditions there is a decrease in Rab5-mediated early endosome fusion due to HIF-dependent downregulation of rabaptin-5. The delay of early endosome fusion results in a delay in EGFR movement through the endocytic pathway and degradation. This retention of EGFR in internalized vesicles results in prolonged signaling. The authors suggest that the deceleration of the endocytic cycle in hypoxia could therefore affect many signaling events due to delayed endocytosis-mediated deactivation of receptors (Wang et al., 2009). Hypoxia may also affect integrin recycling as it stimulated Rab11 recycling of integrin α6β4 to the plasma membrane (Yoon et al., 2005). This was associated with increased invasion and migration by maintaining integrins at the leading edge of cells (Caswell et al., 2007). Acute hypoxia induces endocytosis of N, K-ATPase thereby inhibiting its activity. Endocytosis and internalization of N, K-ATPase has been associated with metastasis in several cancers (Dada et al., 2003). Hypoxia was also found to promote the relocalization of the proprotein convertase furin to the plasma membrane where it can process proproteins involved in tumorigenesis. Importantly, this relocalization was associated with increased cellular invasion (Arsenault et al., 2012). More recently hypoxia was found to change global protein endocytosis via caveolin-1-dependent mechanisms. Acute hypoxia inhibited global endocytosis in a HIF-1 independent manner (Bourseau-Guilmain et al., 2016). However certain proteins were found to have enhanced internalization under hypoxic conditions, including several RTKs such as EGFR, DDR1, IGFR1 and ROR2, and several integrins such as ITGA1, 2, or 3 and ITGB1 and 5 (Bourseau-Guilmain et al., 2016). Therefore, it seems that hypoxia can have differing effects on protein internalization and trafficking depending on the protein being studied so much remains to be discovered on this subject.

1.1.2.4 Activation of signaling pathways

Additionally, many important signaling pathways are activated in hypoxic cells, resulting in complex signaling networks that interact with each other to mediate the effects of hypoxia.

Two major pathways activated under hypoxic conditions are PI3K/AKT/mTOR and MAPK/ERK, which are the main pathways responsible for cell proliferation, survival, apoptosis, metabolism, migration and inflammation (Courtnay et al., 2015; Muz et al., 2015; Sanchez et al., 2012). For example, the PI3K/AKT pathway plays an important role in hypoxia-induced changes to metabolism by regulating glucose uptake (Courtnay et al., 2015). Hypoxia also induces activation of PI3K/AKT/mTOR cell survival pathways resulting in protection against apoptosis (Alvarez-Tejado et al., 2001). Interestingly, the mTOR pathway can also be activated independently of HIF-1 under hypoxia. In hypoxia, mTOR and its effectors were readily hypophosphorylated resulting in rapid inhibition of mRNA translation (Arsham et al., 2003). Activation of the MAPK/ERK pathway is probably most well-known for its major role in cell proliferation in part by increasing the expression of Myc and cyclin D (Zhang and Liu, 2002). The MAPK/ERK pathway was also found to be essential for hypoxia-induced effects on endothelial cells, potentially contributing to enhanced expression of PDGF, TGF-β and metalloproteinases by upregulating the gene expression of Egr-1 (Lo et al., 2001). The MAPK pathway is also a key regulator of hypoxia-induced effects on inflammation (Sanchez et al., 2012). These pathways can of course be activated independently of hypoxia by cytokines, chemokines and growth factors binding to RTKs, GPCRs and Toll-like receptors. Interestingly, activation of each of these pathways can in turn lead to activation of HIFs further complicating matters (Muz et al., 2015). For example, PI3K was shown to activate HIF under normoxic conditions (Agani and Jiang, 2013). On the other hand, ERK kinases activated in hypoxia were found to be involved in HIF activation in hypoxia by directly phosphorylating HIF (Minet et al., 2000).

1.1.3 Hypoxia as a driver of invasion and metastasis

As mentioned above, many of the genes regulated by hypoxia as well as other effects of hypoxia on the cells are implicated in various aspects of cancer progression. Hypoxic cells have repeatedly been demonstrated to be more aggressive, invasive and prone to recurrence (Hockel et al., 1996). For example, cervical carcinoma cells exposed to hypoxia had increased metastasis to lymph nodes in an orthotopic murine model (Cairns and Hill, 2004) and acute hypoxia was also found to increase lung metastasis of sarcoma cells in mice

(Cairns et al., 2001). Many studies have investigated the mechanisms responsible for hypoxia-induced cellular invasion and a multitude of pathways have been implicated so far, surely with many more to come, as hypoxia appears to be a master regulator of cell invasion through a diverse array of effects. Several HIF-dependent mechanisms of hypoxia-induced invasion have been delineated. These include increased melanoma invasion through PDGFR- and FAK-mediated activation of Src as well as increased ECM degradation via MT1-MMP and MMP-2 expression under hypoxia (Hanna et al., 2013). Another study found a role for the Rho-family activator β-Pix in matrix degradation dependent on HIF-1 (Md Hashim et al., 2013). Hypoxia, via HIF-1, was also found to promote metastasis by regulating the expression of Twist1, a major promotor of EMT (Yang et al., 2008). Other studies identified HIF-independent mechanisms of hypoxia-induced invasion, including many that depend on the altered pH observed in hypoxia. Hypoxia was found to induce cellular invasion through enhanced activity of HDAC6, via EGFR, promoting TGF-β signaling (Arsenault et al., 2013). Under hypoxia, HIF-independent activation of NHE-1 was found to induce cell invasion in fibrosarcoma cells (Lucien et al., 2011). Furthermore, NHE-1 was found to promote cell invasion by regulating cortactin-cofilin binding (Magalhaes et al., 2011) and through acidification of the extracellular space (Busco et al., 2010). Interestingly, in these reports, the increase in cellular invasion was associated with production of invadopodia, which are specialized cell structures required for cancer cell dissemination that will be discussed in the next section. All of this suggests that hypoxia is a tumor promoting force driving cancer cell invasion that we need to overcome in order to treat solid tumors and specifically block invasion and metastasis.

1.2 Invadopodia mediate cancer cell invasion

As previously discussed, cancer cells acquire invasive capabilities in order to metastasize and invadopodia have been shown to be the main structures mediating cancer cell invasion. Invadopodia have been implicated in many important steps of metastasis including crossing the basement membrane, invading through ECM and entering blood vessels (Chen, 1989; Kelly et al., 1998; Wolf and Friedl, 2009). Invadopodia can be defined as actin-rich protrusions formed at the ventral side of cells. These cell structures possess proteolytic

activity directly associated with sites of matrix degradation (Mueller and Chen, 1991). Electron microscopy studies have revealed that invadopodia structures consist of many protrusions, from 100 nm to several µm, extending from a deep invagination of approximately 8 µm in width and 2 µm in depth (Baldassarre et al., 2003; Chen, 1989). Invadopodia structures are stable with a long half-life of two hours or more and are found in proximity to the Golgi, which is orientated toward invadopodial protrusions, suggesting a possible relationship between membrane/protein transport and proteolytic activity (Baldassarre et al., 2003). The following sections will detail the stages of invadopodium production, including the important players involved, as well as the regulators of their formation and their *in vivo* relevance to metastasis.

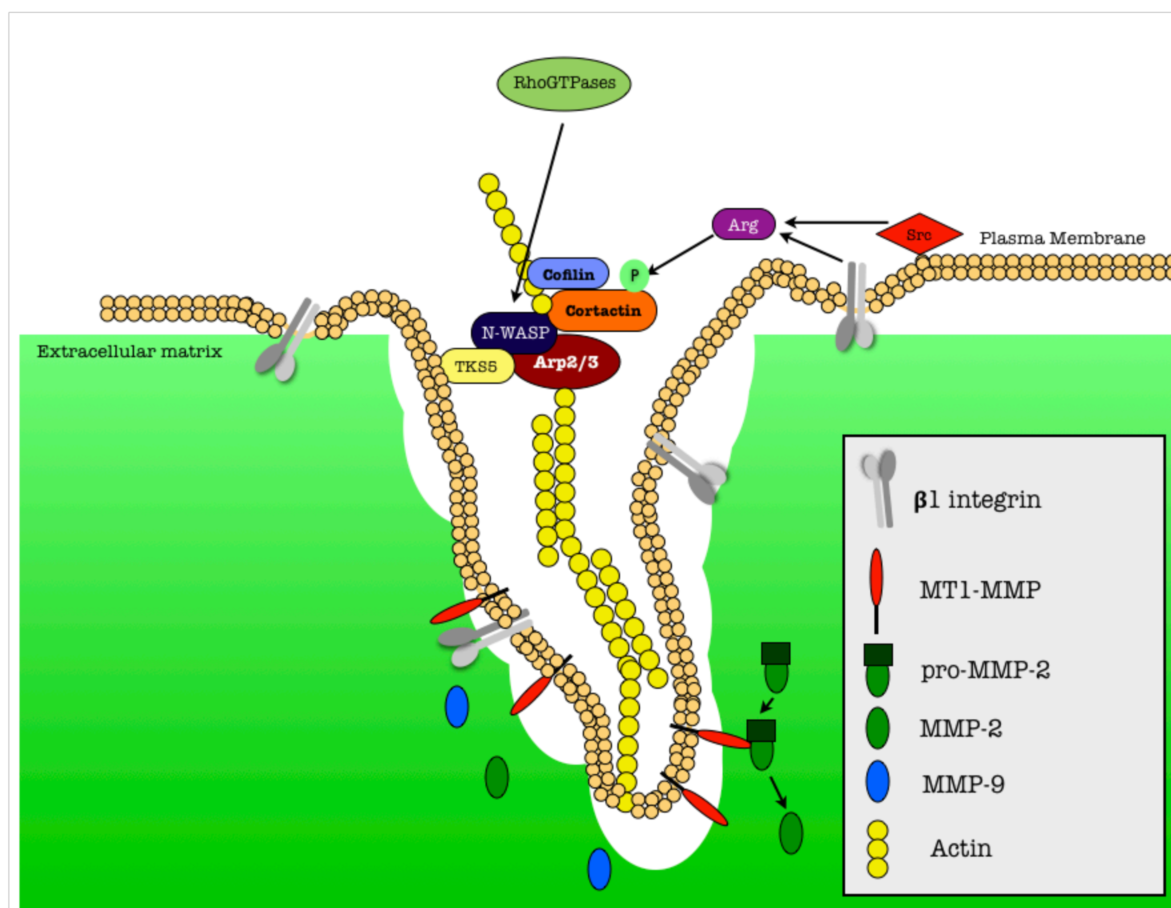

Figure 1 Schematic representation of an invadopodium

The core structure of an invadopodium is composed of actin and cortactin along with the actin regulatory proteins Arp2/3, N-WASP and cofilin. Upstream signals from RhoGTPases activate N-WASP to promote actin polymerization. Recruitement of Tks5 to the core structure during the stabalization phase tethers these proteins to the plasma membrane. During the maturation phase 1 integrins and Src activate Arg, which

phosphorylates cortactin, further promoting actin polymerization. MT1-MMP is recruited to the plasma membrane and activates a cascade of MMPs to promote matrix degradation.

1.2.1 The stages of invadopodia formation

As research has focused on delineating the underlying mechanisms involved in invadopodia formation and function, distinct stages in their production have come to light (Artym et al., 2006; Beaty and Condeelis, 2014; Murphy and Courtneidge, 2011). There are three recognized steps necessary for fully functioning invadopodia: precursor formation, stabilization, and maturation. Invadopodia are initiated mainly by growth factors or ECM-rigidity signals, which will be further discussed in section 1.2.2. Similar to other cellular protrusions such as lamellipodia and filipodia, invadopodia require spatially and temporally regulated remodeling of actin, which constitutes the core of their structure (Alblazi and Siar, 2015). See Figure 1 for a schematic representation of an invadopodium and the important proteins involved in its formation and function.

Invadopodia precursor structures are formed within seconds and are inherently unstable (Artym et al., 2006; Sharma et al., 2013). As actin remodeling is essential for the formation of invadopodial protrusions, it is unsurprising that many essential actin regulatory proteins are recruited to the cell surface around an actin-cortactin complex during the formation of invadopodia precursor structures. These include Arp2/3 (Actin related proteins 2 and 3), N-WASP (neural Wiskott-Aldrich syndrome protein) and cofilin. These proteins have all been either localized to invadopodia or shown to be essential for invadopodia formation (Artym et al., 2006; Baldassarre et al., 2006; Yamaguchi et al., 2005a).

As mentioned, the core of the precursor structure is composed of actin and cortactin, an actin-binding scaffolding protein (Ammer and Weed, 2008). Cortactin is an essential element of invadopodia and is often used as a prominent marker of invadopodia structures. Cortactin clusters, located at the basement membrane near the center of the cell, and not in the cell periphery, can identify invadopodia structures (Gimona and Buccione, 2006). Cortactin binds to or interacts with filamentous actin (F-actin), Arp2/3, N-WASP, dynamin2 and Src to coordinate cell migration, cytoskeletal remodeling, and intracellular

protein transport (Ammer and Weed, 2008; Daly, 2004; Lua and Low, 2005; Weaver et al., 2001). It can act by stabilizing branched actin filaments and, therefore, regulates actin assembly mediated by Arp2/3 (Uruno et al., 2001). Cortactin is a substrate of Src and tyrosine phosphorylation of cortactin, driven by growth factor stimulation or integrin activation, has been implicated in motility and metastatic dissemination of breast cancer cells (Li et al., 2001) as well as being important for invadopodia function (Artym et al., 2006). Along with its cytoskeletal remodeling functions, cortactin might also regulate MMP secretion at focal sites of degradation during invadopodia maturation discussed below (Clark et al., 2007). Additionally, cortactin is frequently upregulated in many cancers including breast, head and neck, and bladder cancers suggesting that cortactin must play an important role in tumor progression, possibly through its role in invadopodia production (Schuuring, 1995).

Important actin regulatory proteins, Arp2/3 and N-WASP, are recruited to the actin-cortacin core of invadopodia. Proteins of the WASP family, including N-WASP, are responsible for functional activation of the Arp2/3 complex, which explains why these two proteins are recruited to invadopodia together (Mullins et al., 1998; Welch et al., 1998). The Arp2/3 complex serves as a nucleation site on an existing filament to initiate the growth of a new actin filament at a distinctive 70-degree angle, creating branched actin polymerization (Mullins et al., 1998; Welch et al., 1998). Therefore, Arp2/3 is responsible for actin rearrangement implicated in the formation of lamellipodia, filopodia, invadopodia and cell motility in general (Goley et al., 2004). Proteins of the WASP family integrate multiple upstream signals, from Rho GTPases such as Cdc42 and Rac1, to induce actin polymerization through the Arp2/3 complex (Eden et al., 2002; Millard et al., 2004). Besides affecting actin polymerization through Arp2/3, N-WASP is also implicated in endocytic and phagocytic processes and may promote internalization of degraded matrix components or recycling of invadopodia components (Innocenti et al., 2005; Lorenzi et al., 2000). Similar to cortactin, Arp2/3 and WASP family proteins are found to be up-regulated in some tumors and invasive cells (Otsubo et al., 2004; Semba et al., 2006; Yamaguchi and Condeelis, 2007).

Finally, another essential component for the assembly of nascent invadopodia is cofilin. Cofilin is a member of the actin depolymerizing factor (ADF)/cofilin family that binds monomeric and filamentous actin (Paavilainen et al., 2004). It is an essential regulator of actin dynamics at the plasma membrane through its ability to sever actin filaments. This results in disassembly of F-actin from the rear of migrating cells and recycling of actin monomers to the leading edge for further polymerization (Paavilainen et al., 2004). Depletion of cofilin results in small, short-lived and, therefore, poorly degrading invadopodia (Yamaguchi et al., 2005a). Cofilin is also implicated in tumor cell invasion and metastasis (Wang et al., 2007).

The next step in invadopodia formation is the stabilization of the precursor structure. This is achieved by recruitment of the scaffold protein Tks5/FISH (tyrosine kinase substrate 5/five sh3 domains), which tethers the proteins involved in the formation of nascent invadopodia structures to the lipid membrane via PI(3,4)P2 (Sharma et al., 2013). The adaptor protein Tks5 contains five SH3 domains and one Phox homology (PX) domain, bringing membrane and cellular components together (Saini and Courtneidge, 2018). Binding of Tks5 to N-WASP occurs via its SH3 domains (Oikawa et al., 2008), while its PX domain interacts with PI(3,4)P2 anchoring these proteins to the cell membrane. These events result in its specific localization to the invadopodium core structure. Furthermore, Tks5 has been shown to be essential for invadopodia formation in many different cell types (Abram et al., 2003; Mader et al., 2011; Oikawa et al., 2008; Seals et al., 2005; Stylli et al., 2009), and its colocalization with cortactin can identify invadopodia structures (Eddy et al., 2017). Additionally, Tks5 is involved in the generation of reactive oxygen species (ROS) that are implicated in signaling promoting invadopodia production (Saini and Courtneidge, 2018). The PI(3,4)P2, that Tks5 binds to, has been shown to accumulate at invadopodia sites a few minutes after the core initiation, this correlates with the arrival of SHIP2 at the same site. The increase in PI(3,4)P2 at invadopodia sites therefore seems to be regulated by SHIP2, a 5'-inositol phosphatase that is known to regulate PI(3,4)P2 levels (Sharma et al., 2013). Inhibition of SHIP2 or Tks5 has no effect on invadopodia precursor formation while significantly reducing the formation of mature invadopodia and blocking subsequent degradation, confirming their role in the maturation of the precursor structure (Sharma et

al., 2013).

The third and final stage of invadopodia production is maturation, which consists of further actin polymerization to elongate the protrusion as well as recruitment of MMPs and subsequent degradation of the ECM. Recently, β1 integrins have been shown to be required for the formation of mature degradation-competent invadopodia. Integrins of the β1 family are localized to invadopodia structures and promote metastasis of numerous cancer types including breast, ovarian, pancreatic and skin cancers (Grzesiak et al., 2011; Huck et al., 2010; Lahlou and Muller, 2011; Mitra et al., 2011; Trikha et al., 1994). Integrins of the β1 family promote the maturation of invadopodia through activation of Arg (Abelson-related gene). Cortactin is phosphorylated by Arg, which subsequently promotes the actin-severing activity of cofilin, resulting in actin polymerization at invadopodia sites. Cortactin phosphorylation mediated by Arg, triggered by β1 integrins, is therefore a key switch for invadopodia maturation (Beaty et al., 2013).

An equally important part of the maturation process is the recruitment and activation of proteases that degrade ECM proteins. Invadopodia have been shown to degrade multiple extracellular substrates such as collagen type I and IV, fibronectin and laminin (Kelly et al., 1994). The majority of proteolysis at invadopodial structures is due to the metalloproteinase family members, which includes matrix metalloproteinases (MMPs) and ADAMs (a disintegrin and metalloproteinase). A key metalloproteinase that is enriched at the invadopodia-associated plasma membrane is MT1-MMP/MMP14 (membrane type-1 matrix metalloproteinase), which plays an important role in activating many of the subsequently recruited and secreted MMPs. The metalloproteinase, MT1-MMP degrades collagen, fibronectin and laminins and is a master regulator of protease-mediated cell invasion through activation of a cascade of proteases including the gelatinase MMP-2 (Holmbeck et al., 2004; Nakahara et al., 1997). Therefore, recruitment of MT1-MMP to invadopodia might establish a focal zone of MMP activation around this structure. In fact, MT1-MMP over-expression or knockdown has been shown to increase or decrease invadopodia degrading ability (Artym et al., 2006; Nakahara et al., 1997). Two gelatinases MMP-2 and MMP-9 have also been localized to invadopodia and are known to degrade

type IV collagen, a major component of basement membrane (Redondo-Muñoz et al., 2006). Serine proteinases and the urokinase-type plasminogen activator (uPA) proteolytic system are also implicated in invadopodia-mediated matrix degradation. Seprase and DPP4 (dipeptidyl dipeptidase IV) are transmembrane serine proteinases implicated in ECM degradation, shown to localize at invadopodia through binding with β1 integrins (Artym et al., 2002; Ghersi et al., 2006; Kindzelskii et al., 2004; Monsky et al., 1994; Mueller et al., 1999). Urokinase-type plasminogen activator receptor (uPAR) is found in a complex with seprase at sites of invadopodia formation and activates plasminogen which can subsequently activate various MMPs (Lijnen, 2001).

Recruitment and docking of proteases to invadopodia structures can be mediated by β1 integrins, adding another aspect to their role in invadopodia maturation (Jacob and Prekeris, 2015). Integrins of the β1 family can also recruit NHE-1 to invadopodia sites, via talin and moesin, which contributes to invadopodia maturation in two ways. By acidifying the extracellular space, NHE-1 activates proteases around the invadopodia structure. The subsequent increase in intracellular pH, due to the extrusion of protons from the cytosol, results in cofilin activation that promotes actin polymerization (Beaty et al., 2014; Brisson et al., 2012). Cortactin has also be implicated in the localized delivery of proteases to invadopodia, as the plasma membrane delivery of MT1-MMP was found to correlate with cortactin expression levels (Clark et al., 2007).

1.2.2 Inducers of invadopodia

Many stimuli have now been identified that initiate invadopodia formation, most of them being microenvironmental signals. Several oncogenes have also been implicated in invadopodia initiation such as constitutively active mutants of Src or Ras. In pancreatic cancer invadopodia production was dependent on K-Ras (Neel et al., 2012), while Src has been repeatedly demonstrated to be essential for invadopodia production by phosphorylating cortactin and Tks5 (Bailey et al., 2016; Burger et al., 2014; Pignatelli et al., 2012; Williams and Coppolino, 2014). In certain contexts, such as hypoxia, Notch mediates cell contact-dependent signaling resulting in paracrine activation of EGFR to induce invadopodia (Díaz et al., 2013; Pignatelli et al., 2016). However, the most common stimuli are growth factors that activate receptor tyrosine kinases (RTK) or serine/threonine

receptors, such as TGF-βR. Numerous growth factors, including EGF (Mader et al., 2011), PDGF (Eckert et al., 2011), VEGF (Hoshino et al., 2013a), HGF (Rajadurai et al., 2012), HB-EGF (Díaz et al., 2013), TGF-β (Pignatelli et al., 2012), and SDF-1 (Hoshino et al., 2013a) can induce invadopodia production. For example, EGF activation of the EGFR was shown to induce invadopodia formation in breast cancer cells via Src- and Arg-induced phosphorylation of cortactin (Mader et al., 2011). Finally, ECM proteins can also initiate invadopodia maturation through the activity of adhesion receptors such as integrins, CD44 and discoidin domain receptors (DDRs) (Di Martino et al., 2016; Eddy et al., 2017). For example, Di Martino *et al*. found type I collagen to be a potent inducer of invadopodia via its activation of the discoidin domain receptor DDR1 (Di Martino et al., 2015). In another study, dense fibrillar collagen was found to induce invadopodia in human breast cancer cells through activation of α2β1 integrin, while alternative integrin α5β1 were necessary for invadopodia production in a fibronectin rich environment (Artym et al., 2015). Additionally, α6β1 activation promotes Src-dependent Tyr phosphorylation of p190RhoGAP, a key regulator of Rho GTPase signaling, affecting the actin cytoskeleton. This event activates membrane-protrusive and proteolytic activity leading to invadopodia formation and cell invasion (Nakahara et al., 1998). Matrix rigidity also promotes invadopodia production (Alexander et al., 2008; Parekh and Weaver, 2016; Parekh et al., 2011).

Activation of these integrins and RTKs induce many intracellular cascades involving PKC, Src, Rho GTPases and tyrosine kinases for invadopodia generation. In fact, increased total tyrosine phosphorylation is a marker of invadopodia (Mueller et al., 1992). Serine/threonine kinases, such as ERK1/2 and PAK, have also been implicated in invadopodia biogenesis, possibly through their involvement in cortactin phosphorylation (Ayala et al., 2008; Tague et al., 2004). Cell shape, morphology, polarization, motility and metastasis formation is influenced by RhoGTPases through their activation of protein kinases and actin nucleators (Hall, 2005). For example, Rac1 and Cdc42 can both activate Arp2/3 through their effectors Sra-1 and N-WASP, respectively (Caldieri et al., 2009). In particular, Cdc42 has been shown to act upstream of invadopodia formation, with constitutively active mutants of Cdc42 inducing dot-like degradation (Nakahara et al.,

2003). Also, RhoA has been suggested to play a role due to the fact that p190Rho-GAP activates membrane protrusive activity (Nakahara et al., 1998). Additionally, Rac1 can also promote invadopodia formation, possibly through activation of Arp2/3, inducing actin polymerization, or recruitment of cortactin, both essential to invadopodia formation (Fung et al., 2008; Revach et al., 2016). The Rho GTPase effector, ROCK, as well as Rac1 and Cdc42 also activate PAKs, which phosphorylate and activate LIMKs, which then phosphorylate cofilin to permit its role in actin dynamics at invadopodia (Ayala et al., 2008; Caldieri et al., 2009). In summary, microenvironment signals from growth factors, the ECM and also tumor hypoxia, discussed in the previous section (Arsenault et al., 2013; Díaz et al., 2013; Lucien et al., 2011; Md Hashim et al., 2013), promote the initiation of invadopodia in cancer cells under different cellular contexts.

1.2.3 Implication of invadopodia in the metastatic process

Recent progress suggests an essential role for invadopodia in tumor invasion and metastasis (Paz et al., 2014). Invadopodia biogenesis has previously been shown to correlate closely with well-established assays for invasive capability such as invasion through matrigel-coated transwell chambers and xenograft metastasis models (Bowden et al., 1999; Coopman et al., 1998; Thompson et al., 1992). Enrichment of invadopodia markers such as cortactin and Tks5 have also been found at the invading front of human tumors in tissue samples, consistent with invadopodia-mediated invasion (Seals et al., 2005; Zhang et al., 2006). Invadopodia-like structures have been imaged in migrating cancer cells undergoing intravasion, while intravital imaging has allowed real-time visualization of invadopodia-like protrusions in tumor cells invading through connective tissues (Condeelis and Segall, 2003; Yamaguchi et al., 2005b). The visualization and characterization of invadopodia in breast cancer models suggest that invadopodia are key mediators of intravasation (Eckert et al., 2011; Gligorijevic et al., 2012; Kedrin et al., 2008; Roh-Johnson et al., 2014). For example, 3D time-lapse imaging of breast cancer cells showed formation of protrusions that were positive for cortactin and proteolytic activity (Gligorijevic et al., 2012). Furthermore, invadopodia were recently found to be required for cancer cell extravasation (Leong et al., 2014). In this study by Leong *et al*, cancer cells were shown to extend invadopodia into the extravascular space prior to extravasation of the cells. This was dependent on essential invadopodia components such as Tks5 and cortactin, as their genetic or pharmacological

inhibition blocked cancer cell extravasation and metastatic colony formation *in vivo* (Leong et al., 2014). Therefore the ability to form invadopodia correlates with the invasive and metastatic potential of tumor cells making them ideal targets for anti-metastasis therapy (Yamaguchi, 2012). Also, these structures are not important for cell viability and their disruption may, therefore, have less side effects than some of the current treatments (Weaver, 2006).

1.3 The LPA signaling axis

Lysophosphatidic acid (LPA; 1 or 2-acyl-sn-glycerol-3-phosphate) is a bioactive lipid implicated in a plethora of biological activities and processes including cancer (van Meeteren and Moolenaar, 2007). It was the first lysophospholipid found to exert growth factor-like activities, at submicromolar concentrations, and is an important lipid mediator (Tokumura et al., 1978). In this section we will examine the major aspects of the LPA signaling axis including the enzymes controlling the production and degradation of LPA. LPA acts on cell surface GPCRs to mediate intracellular signaling (Shimizu, 2009) therefore this aspect will be addressed to provide an understanding of the biological effects of LPA. Finally, LPA has been found to promote many of the hallmarks of cancer through activation of its various LPA GPCRs (Houben and Moolenaar, 2011), therefore the implication of members of the LPA signaling axis during tumorigenesis will also be examined in this section.

1.3.1 LPA structure and sources

A glycerol backbone, a single carbon chain, and a polar headgroup compose the structure of LPA (Figure 2) (Meyer zu Heringdorf and Jakobs, 2007). Multiple different species of LPA with varying carbon chain lengths and degrees of unsaturation have been identified. Additionally, their carbon chains may be either acyl- or ether-linked with the acyl chain esterified at either the sn-1 or sn-2 position of the glycerol backbone (1-acyl-LPA or 2-acyl-LPA) while ether-linked LPAs carry an alkyl or alkenyl linkage at the sn-1 position (1-alkyl-LPA or 1-alkenyl-LPA). All of these factors contribute to the differing biological activities of individual LPA species (Meyer zu Heringdorf and Jakobs, 2007). The concentration of LPA is approximately 154 pmol in cells, 0.1-6.3 μM in blood and 80-100nM in plasma (Hosogaya et al., 2008; Kishimoto et al., 2003). This lipid has been

detected in many biological fluids including serum, plasma, saliva, follicular fluid, seminal fluid, and malignant effusions (Hama et al., 2002; Sugiura et al., 2002; Tokumura et al., 1999; Westermann et al., 1998). The major cellular sources of LPA include platelets and adipocytes (Eichholtz et al., 1993; Valet et al., 1998), while postmitotic neurons, lymphoid cells, endometrial cells, erythrocytes and cancer cells are also reported to produce LPA (Aoki et al., 2008; Smyth et al., 2008; Ye, 2008). Therefore, LPA may act as a circulating as well as a locally produced paracrine mediator (Takuwa et al., 2002).

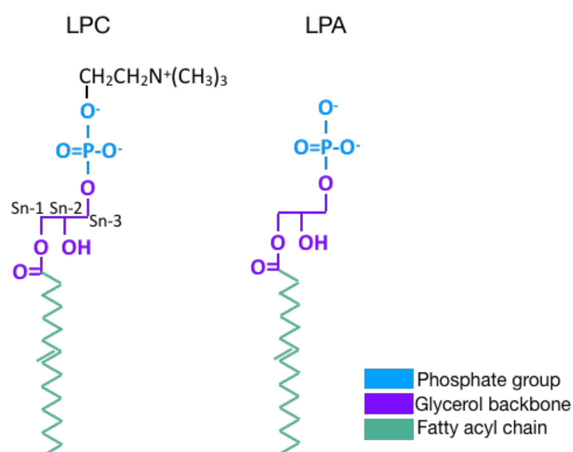

Figure 2 Structure of LPC and LPA

Representation of the structures of LPC and LPA, each composed of a glycerol backbone, phosphate group, and fatty acyl chains. LPC has an additional choline headgroup. The lysophospholipase D activity of ATX is responsible for hydrolyzing the bond between choline and the phosphate group of LPC to produce LPA. *Adapted from: (Mills* and *Moolenaar, 2003)* with permission from Springer Nature, license # 4382541352781.

1.3.2 Production and degradation of LPA

Various enzymes are involved in the production and degradation of LPA as detailed in Figure 3. Intracellular LPA can be produced by de novo LPA biosynthesis through intermediate lipid metabolism (Goetzl and An, 1998). Extracellular LPA can be produced from precursor glycerophospholipids by the action of many different enzymes including phospholipase A1 or A2 (PLA1 or 2), monoacylglycerol kinase or glycerol-3-phosphate acyltransferase (Pébay et al., 2007). For example, PLA1/2 produces LPA by deacylating phosphatidic acid (PA) that is first generated intracellularly from phospholipids or diacylglycerol (Aoki et al., 2008). However, the majority of LPA produced *in vivo* depends

on the lysophospholipase D activity of autotaxin, which will be discussed in the next subsection, 1.3.2.1 (Umezu-Goto et al., 2002). Additionally, two families of proteins, lipid phosphate phosphatases (LPP) and acyl transferases, are responsible for the rapid degradation of LPA resulting in a short half-life for this lipid mediator. The LPA Acyl transferase (LPAAT) family mediates the acylation of LPA to PA (Yamashita et al., 2001). The LPPs, integral membrane proteins, that dephosphorylate LPA to monoacylglycerol (MAG), are the major LPA degrading enzymes that terminate LPA signaling (see section 1.3.2.2) (Brindley et al., 2002). To counteract these degradative effects, extracellular LPA is normally bound to proteins such as albumin, fatty acid binding protein, or gelsolin which act to increase the stability and facilitate transport of LPA (Aoki, 2004; Gaits et al., 1997; Mills and Moolenaar, 2003; Pagès et al., 2001). Another mechanism regulating LPA levels in the blood is the rapid trans-cellular uptake of LPA into the liver, which therefore acts as an important buffering system controlling LPA bioavailability (Salous et al., 2013).

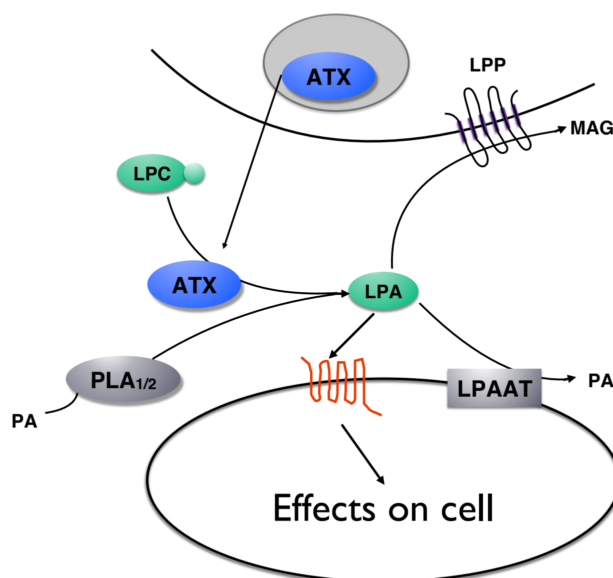

Figure 3 Mechanisms of LPA production and degradation

The major producer of LPA *in vivo* is secreted ATX which converts LPC to LPA. Other enzymes can also produce LPA such as PLA1/2, which can produce LPA from PA. LPA may be degraded to PA by the action of LPAAT, or to MAG by the action of LPPs. *Adapted from:(Meyer zu Heringdorf and Jakobs, 2007)* with permission from Elsevier, license # 4382550417670.

1.3.2.1 Autotaxin as the main producer of LPA

Autotaxin (ATX), also known as ENPP2 (ectonucleotide pyrophosphatase/ phosphodiesterase 2), was originally identified as a novel 125-kDa autocrine motility stimulating factor after its isolation from the culture medium of human melanoma cells (A2058) in 1991 (Stracke et al., 1992). It was subsequently found to be present in the culture medium of several other cancer cell types including glioblastoma and breast cancer (Gaetano et al., 2009; Jansen et al., 2005; Kishi et al., 2006). The ATX protein is a secreted protein that is synthesized as a pre-pro-protein and processed by a signal peptidase and pro-protein convertase (such as furin) to remove the pre, N-terminal 27-residue hydrophobic domain, and pro domain of ATX, respectively (Jansen et al., 2005; Koike et al., 2006). The ATX pro-protein follows the classical secretory pathway, where proteins are transported outside the cell from the E.R via the Golgi apparatus (Jansen et al., 2005). Four ATX isoforms have been identified, ATX α, β, γ, and δ with differing tissue distributions (Giganti et al., 2008; Hashimoto et al., 2012). High expression of ATXβ mRNA is found in peripheral tissues, ATXγ mRNA is expressed mostly in brain, ATXα mRNA has lower expression levels in all tissues, and ATXδ is highly expressed in the small intestine and spleen (Giganti et al., 2008; Hashimoto et al., 2012). Originally discovered in A2058 cells, ATXα lacks exon 21, while ATXβ, a splice variant reported in human teratocarcinoma (Lee et al., 1996) lacks exons 12 and 21 and is the major isoform of ATX. ATXγ (PD-1α) was isolated from the brain and lacks exon 12 and ATXδ is identical to ATXβ except for a deletion of four amino acids in the linker region and is the second most common isoform (Giganti et al., 2008; Hashimoto et al., 2012). All isoforms, except ATXα, were found to be fully active with no differences in catalytic efficiency or substrate specificity. The ATXα isoform is cleaved in exon 12, which is unique to this isoform, resulting in a less active cleaved 55-66kDa form (Giganti et al., 2008; Hashimoto et al., 2012).

1.3.2.1.1 Structure and activity of ATX

The ATX protein contains several structural domains illustrated in Figure 4. These include a Modulator of Oligodendrocyte Remodeling and Focal adhesion Organization (MORFO) domain, implicated in oligodendroglial process network formation and focal adhesion organization (Dennis et al., 2008), an EF-hand-like motif that contributes to the function of the MORFO domain, an inactive nuclease-like domain, and two cysteine-rich somatomedin

B domains (Yuelling and Fuss, 2008). The somatomedin B domain (SMB), which is derived from the amino terminus of vitronectin, forms a presumed binding site for type 1 plasminogen activator inhibitor (PAI-1), and uPAR (Seiffert and Loskutoff, 1991; Seiffert et al., 1994). The N-terminal SMB-2 domain has also been shown to bind to β1 and β3 integrins thereby localizing ATX to platelets and cells including lymphocytes (Fulkerson et al., 2011; Hausmann et al., 2011). Additionally, the ATXα isoform can bind to heparin sulfate proteoglycans, due to the arginine/lysine rich clusters in its 53 amino acid polybasic insertion. This isoform was therefore found to bind abundantly to cultured mammalian cells (Houben et al., 2013; Perrakis and Moolenaar, 2014). Finally, ATX has a catalytic domain which has lysophospholipase D (lysoPLD) activity, producing LPA from LPC (Umezu-Goto et al., 2002). The crystal structure of ATX has been solved and reveals a central catalytic domain interacting with the SMB domains on one side and the nuclease domain on the other side (Nishimasu et al., 2011).

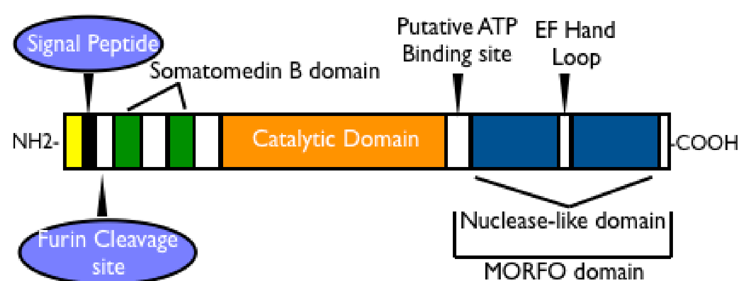

Figure 4 Structural domains of ATX

ATX is processed by a signal peptidase and pro-protein convertase to remove pre and pro N-terminal domains. ATX contains a MORFO domain implicated in oligodendrocyte remodeling, an EF hand-like motif, an inactive nuclease-like domain and a putative ATP binding site. ATX also has two somatomedin B domains implicated in binding to integrins. Finally there is a catalytic domain which functions as a lysophospholipase D to produce LPA.

The main physiological substrate for ATX/lysoPLD is LPC, which subsequently produces LPA from LPC by hydrolysis removing the choline head group (Figure 2) (Umezu-Goto et al., 2002). Cyclic phosphatidic acid (cPA), an analog of LPA and intermediate in LPA formation, may also be produced by ATX from LPC (TSUDA *et al.,* 2006). Furthermore, ATX/LysoPLD has a higher affinity for unsaturated acyl-LPCs as compared to saturated or

ether-linked species (Tokumura et al., 1999). Interestingly, ATX does not contain the HKD motifs critical for the catalytic activity of the phospholipase D (PLD) superfamily (Xie and Meier, 2004). The hydrolysis of lysophospholipids by ATX is instead a metal-assisted reaction that occurs via a nucleotidylated threonine requiring a metal ion such as Co^{2+} (Gijsbers et al., 2003). Calcium and Magnesium could also enhance ATX activity by stabilizing the structure of ATX, by protecting it from thermal denaturation and proteolysis, or by directly regulating the catalytic activity of ATX (Tokumura et al., 1998). Plasma ATX is constitutively active (Yuelling and Fuss, 2008), however, its catalytic activity depends on an essential disulfide bridge between the catalytic and nuclease-like domains (Jansen et al., 2009) as well as glycosylation of Asn-524 (Jansen et al., 2007). Human ATX activity can be inhibited by EDTA, phenanthroline and ATP as well as by its products LPA, cPA and S1P (Baker et al., 2006; van Meeteren et al., 2005).

1.3.2.1.2 Regulation of ATX expression

Although ATX is ubiquitously expressed and is, therefore, synthesized by a variety of normal cells and tissues, there are several stimulators and inhibitors of its expression. Expression of ATX can be induced by cell differentiation mediators such as retinoic acid, in a neuroblastoma cell line with N-myc amplification (Dufner-Beattie et al., 2001), and Bmp-2, during mesenchymal development (Bächner et al., 1998). Ligands of TLRs, including Lipopolysaccharide (LPS) induce ATX expression via JNK and p38MAPK or IFN-β or α resulting in enhanced immune cell migration (Li and Zhang, 2009; Song et al., 2015). Growth factors such as EGF and bFGF have been shown to stimulate ATX in thyroid carcinomas (Kehlen et al., 2004), while anti-inflammatory cytokines such as IL-4, IL-1β, IFN-γ and TGF-β reduce ATX expression in auto-immune and cancer cells (Kehlen et al., 2004; Santos et al., 1996). Therefore, pro-inflammatory stimuli seem to increase ATX expression while anti-inflammatory cytokines have the opposite effect. Furthermore it seems that molecular cues associated with cancer progression can induce ATX expression while tumor suppressors seem to reduce its expression. During cancer progression ATX can be upregulated by α6β4 integrin, via NFAT, which correlates with an invasive and migratory phenotype in advanced breast carcinomas, (Chen and O'Connor, 2005), and by the viral oncoprotein v-Jun (Black et al., 2004) or the oncogenic Epstein-Barr virus (EBV)

in different cancer cell lines (Baumforth et al., 2005). In neuroblastoma cells, ATX is upregulated by Ap1 and Sp transcription factors (Farina et al., 2012). In contrast, a candidate tumor suppressor gene for breast cancer, CST6, when expressed in breast cancer cells, down-regulated the expression of ATX (Song et al., 2006). Interestingly, radiotherapy has been shown to increase ATX production in the adipose tissue of breast cancer patients (Meng et al., 2017). Finally, ATX expression and secretion can be regulated by its own product, LPA. However this feedback regulation can be overcome by inflammatory cytokines such as TNF-α or IL-1β (Benesch et al., 2015). Each of the above mentioned stimuli have been investigated in very few cell types. Therefore, more studies are needed to define exactly how ATX expression is regulated in most cell types.

1.3.2.1.3 ATX role in physiology and pathology

Many normal development processes such as adipogenesis (Simon et al., 2005) and central nervous system development show involvement of ATX (Dennis et al., 2005). A major role in vascular development is played by ATX (Khurana et al., 2008; Sato et al., 2005). In fact, ATX knockout mice are embryonic lethal due to impaired vessel formation in the yolk sac and embryo (van Meeteren et al., 2006). The vascular defects in atx-deficient mice resemble those in mice lacking genes involved in cell migration and adhesion such as the fibronectin and focal adhesion kinase genes. Results of such study indicated that the loss of LPA production and downstream GPCR signaling is responsible for the phenotype observed in atx knockout mice (van Meeteren et al., 2006). Furthermore, ATX stimulates cytoskeletal reorganization in different cell types including intestinal cells, oligodendrocytes and lymphocytes, to regulate cell motility, myelination, and immune functions (Fox et al., 2004; Kanda et al., 2008; Khurana et al., 2008; Mori et al., 2007). Interestingly, ATX plays an important role in inflammation increasing cytokine production and lymphocyte infiltration, thereby aggravating many inflammatory conditions including asthma, pulmonary fibrosis and rheumatoid arthritis (Knowlden and Georas, 2014; Valdés-Rives and González-Arenas, 2017). Furthermore, ATX is involved in the wound healing response where it induces platelet aggregation and keratinocyte proliferation, migration and differentiation (Benesch et al., 2016). Additionally, ATX has been implicated in numerous pathologies including Alzheimer's disease, chronic hepatitis C, multiple sclerosis,

neuropathic pain, and obesity, but its most investigated and presumably most important role is in tumorigenesis, which will be discussed in section 1.3.4 (Ferry et al., 2003; Hammack et al., 2004; Inoue et al., 2008a, 2008b; Umemura et al., 2006; Watanabe et al., 2007; Zhao et al., 2008).

1.3.2.2 Lipid phosphate phosphatases

Lipid phosphate phosphatases were first characterized in 1991, by Jamal *et al.* (Jamal et al., 1991). They were found to be magnesium independent, N-ethylmaleidimide-insensitive members of the phosphatidic acid phosphatase (PAP) family and were named type 2 PAPs (PAP2s) but were later renamed lipid phosphate phosphatases (LPPs) due to the fact that they dephosphorylate a wide variety of lipid phosphates. These characteristics separate them from the magnesium dependent class I PAPs, which are responsible for dephosphorylating phosphatidic acid specifically (Donkor et al., 2007). In contrast, LPPs can dephosphorylate LPA, S1P, C1P and PA to produce monoacyl glycerol, sphingosine, ceramide or diacyglycerol, respectively (Dillon et al., 1997; Waggoner et al., 1996). There are three members of the mammalian LPP family each coded by a separate gene *PPAP2A*, *PPAP2B* and *PPAP2C,* which correspond to LPP1 (and splice variant LPP1a), LPP3 and LPP2 respectively. The first in this family to be cloned was LPP1 in 1996 (Kai et al., 1996), followed shortly thereafter by LPP3 (Kai et al., 1997). The 3 LPP proteins have differing catalytic efficiencies with LPP1 being most efficient at degrading LPA species with less activity at degrading S1P and C1P (LPA > PA > S1P > C1P). LPP2 prefers PA as a substrate (PA > C1P > LPA > S1P) and LPP3 mostly degrades LPA and PA (LPA=PA > C1P > S1P) (Brindley and Waggoner, 1998).

1.3.2.2.1 Structure and function of LPPs

The LPPs are transmembrane proteins with 6 transmembrane alpha helices and 3 extracellular catalytic domains, whose C and N terminal domains are cytoplasmic (Figure 5) (Zhang et al., 2000b). The catalytic domains, C1, C2 and C3 work together to dephosphorylate lipid phosphates. C1 is responsible for substrate recognition while both C2 and C3 are involved in the phosphotransferase reaction (Sigal et al., 2005). Various residues are essential for the functioning of these catalytic domains. A conserved histidine in C3 acts as a nucleophile important for the formation of a phospho-histidine intermediate.

Another conserved histidine in C2 is responsible for breaking the phosphate bond to release the dephosphorylated lipid product. Specific arginine and lysine residues in C1 are involved in stabilizing the transition state (Sigal et al., 2005). The main function of LPPs is the dephosphorylation of LPA and S1P resulting in attenuation of their downstream signaling, although the sphingosine produced from this reaction can reenter the cell and be converted to S1P again. The degradation of C1P by LPPs produces ceramide that may also be recycled by the cell. The degradation of extracellular PA may increase the uptake of diacylglycerol by the cells (Roberts and Morris, 2000).

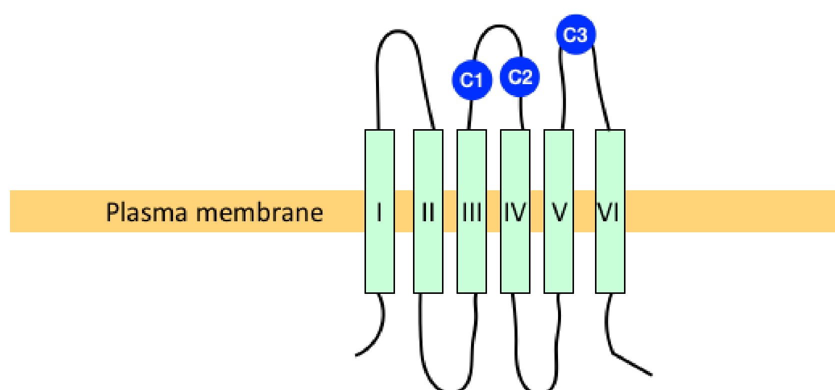

Figure 5 Lipid phosphatase phosphatase structure and catalytic domains

Illustration of the structure and orientation of the LPPs in the plasma membrane. LPPs have six transmembrane domains (I-VI) and their C- and N-terminal domains are located intracellularly. Three conserved catalytic domains (blue circles) are located in the extracellular loops and work together to dephosphorylate lipid phosphates. *Adapted from: (Tang et al., 2015)*

The LPPs are mainly responsible for degrading lipid phosphates found in the extracellular space due to the location of their catalytic domains but may also have some intracellular actions. Besides being found at the plasma membrane LPPs are localized to the endoplasmic reticulum (Barilà et al., 1996) and Golgi (Kai et al., 1997) where their catalytic domains presumably face into the lumen. The LPPs seem to regulate some signaling pathways, including ERK and mobilization of calcium. This later pathway was found to be independent of LPP catalytic activity. The mechanisms used by LPPs to regulate these intracellular signaling pathways have not yet been elucidated, but may involve dephosphorylation of intracellular substrates (Pyne et al., 2004; Samadi et al.,

2011). Intracellular PA may be an intracellular target of LPPs and when hydrolyzed produces DAG which can activate PKCs. In support of this theory, depletion of LPP3 was found to decrease levels of de novo DAG resulting in impaired protein trafficking (Gutiérrez-Martínez et al., 2013). Intracellular LPPs may also degrade the C1P and S1P found within the cell that are both involved in inflammation and apoptotic signaling (Maceyka et al., 2002; Pettus et al., 2005). Additionally, intracellular S1P can affect gene transcription through interactions with histone deacetylases (Hait et al., 2009), and TNF-α signaling (Alvarez et al., 2010), processes that may therefore be regulated by LPP activity. The LPPs have also been shown to have some non-catalytic functions. Interestingly, LPP3 can interact with integrins at the plasma membrane via its RGD domain. Specifically, LPP3 has been shown to interact with αVβ3 and α5β1 to promote endothelial cell-cell adhesion (Humtsoe et al., 2003).

1.3.2.2.2 Regulation of LPPs

Importantly, LPP1 and LPP3 are downregulated in many tumor cells, however, little is known about the regulation of expression of the different LPPs (Samadi et al., 2011). One study found that gonadotropin-releasing hormone could increase LPP3 expression in ovarian cancer cells (Imai et al., 2000). Subsequently, tetracyclines were also found to increase the protein levels and plasma membrane expression of all three LPP members (Tang et al., 2016). Another form of regulation of LPPs appears to be in their subcellular localization. The LPPs may form homo- and heterodimers that can be found in different subcellular locations in the cell, therefore differences in dimerization might regulate the subcellular distribution of the LPPs and therefore result in spatial regulation of LPA and S1P signaling (Long et al., 2008). Furthermore, LPP1 and LPP3 were found to localize to distinct lipid raft domains, which may provide unique environments for each LPP (Kai et al., 2006).

1.3.2.2.3 Role of LPPs in physiology and pathology

Some insight into the roles of LPPs in physiology and pathology has been attained through knockout mice or mice overexpressing the various LPPs, which suggest non-redundant roles for each LPP. Mice overexpressing LPP1 were found to have a 50% decrease in birth weight as well and some abnormalities related to fur growth, such as decreased numbers of

hair follicles and disrupted hair structure. Furthermore, LPP1 seems to be implicated in fertility as mice overexpressing LPP1 had reduced fertility associated with impaired spermatogenesis for males and decreased litter sizes for females. Interestingly, these mice had no significant changes in their plasma LPA concentration (Yue et al., 2004). In contrast, mice with LPP1 knockdown had increased levels of LPA but no obvious phenotypic effects (Tomsig et al., 2009). Tumor progression can be regulated by LPP1 due to its effects on LPA-induced signaling, which will be further discussed in section 1.3.4. The LPP2 knockout mice are fertile and viable (Zhang et al., 2000a) however increased expression of LPP2 caused premature entry into the S-phase of the cell cycle. This is opposite to the effects of LPP1 and LPP3 whose overexpression inhibits cell growth and migration (Morris et al., 2006). Consistent with its cell cycle-promoting activity, LPP2 expression is increased in carcinomas and sarcomas (Flanagan et al., 2009). Finally LPP3 knockout mice have the most drastic defects with embryonic death at gestational day 9.5. This is due mainly to its important role in vascular development as LPP3 knockout mice do not form chorioallantoic placenta or yolk sac vessels. Axis patterning is also affected by LPP3, as a shortening of the anterior-posterior axis was observed in some of the knockout mice (Escalante-Alcalde et al., 2003). Selective LPP3 deficiency in endothelial cells increases vascular permeability and sensitivity to inflammation-induced vascular leak, effects that can be blocked by inhibiting LPA production or LPA receptor signaling (Panchatcharam et al., 2014). Furthermore, LPP3 is required for maintaining blood vessel function and plays a causal role in atherosclerosis as well as being associated with coronary artery disease (Schunkert et al., 2011). Similar to LPP1, LPP3 also plays an important role in tumorigenesis (see section 1.3.4).

1.3.3 LPA receptors and signaling

Extracellular LPA is known to mediate its effects on the cell through activation of specific G-protein coupled receptors (GPCRs). The GPCRs are 7 transmembrane domain receptors that couple to various trimers of G-proteins to mediate intracellular signaling. Although Van Corven *et al*. postulated the presence of LPA-specific GPCRs in 1989, it wasn't until 1996 that the first LPA receptor gene was identified (van der Bend et al., 1992; Chun, 1999; Chun et al., 1999; van Corven et al., 1989; Hecht et al., 1996; Masana et al., 1995; Thomson et al., 1994). To date 6 LPA receptors have been identified that have both

redundant and unique effects and belong to two distinct families, the endothelial differentiation gene (Edg) and purinergic receptor families.

1.3.3.1 LPA receptor identification

The Edg family receptors have some common structural features such as lack of a cysteine residue in the first extracellular loop found in most GPCRs and they share 50-57% amino acid identity in humans (An et al., 1998a; Ishii et al., 2009). The Edg-2/ LPA_1 is a 41 kDa (364 a.a) protein cloned and identified as an LPA receptor in humans in 1997 (An et al., 1997; Chun et al., 1999; Contos and Chun, 1998). In human tissues, LPA_1 is widely expressed in almost all tissues with high expression in brain, heart, placenta and digestive tract and lowest expression in liver and peripheral blood leucocytes (An et al., 1998a). A second LPA receptor, LPA_2, originally known as Edg-4, was subsequently identified due to sequence similarity with Edg-2/ LPA_1 (Chun, 1999; Contos and Chun, 1998). The LPA_2 is less widely expressed in humans, it is detected in testis, pancreas, prostate, thymus, spleen, and peripheral blood leukocytes and is almost undetectable in brain, heart, placenta, and digestive tract contrary to LPA_1 (An et al., 1998a). Similar to LPA_1, LPA_2 is also expressed in various cancer cell types (An et al., 1998a). The third related gene, Edg-7 or LPA_3 has a more restricted expression pattern than LPA_1 and LPA_2, having abundant expression only in human testis, prostate, heart and frontal regions of the cerebral cortex (Im et al., 2000) as well as pancreas, lung and ovary (Bandoh et al., 1999; Im et al., 2000). Because signaling induced by these receptors was unable to account for all the cellular effects of LPA the existence of additional receptors was investigated. (Contos et al., 2002; Hooks et al., 2001).

In 2003, a fourth LPA receptor (LPA_4/p2y9/GPR23) was identified that was structurally distant from the Edg receptors (Noguchi et al., 2003). This stimulated the identification of two additional LPA receptors, LPA_5 and LPA_6. These three receptors are more closely related to the purinergic receptors (purino-receptor cluster), indicating that they arose from different ancestor genes than the Edg family receptors (Ishii et al., 2009; Yanagida et al., 2013). The LPA_4/p2y9/GPR23 is widely expressed in embryonic tissues including brain and stem cells (Lee et al., 2007). In adults, it is abundant in ovary and is weakly expressed in many tissues including pancreas, prostate, spleen, small intestine, colon, skeletal muscle, brain, placenta, lung, liver, skin, heart, thymus and bone marrow (Noguchi et al., 2003).

The LPA_5 (GPR92-93) was an orphan GPCR that was identified as an LPA receptor due to its close genetic relation to LPA_4 (Kotarsky et al., 2006; Lee et al., 2006). Low levels of LPA_5 mRNA are expressed in embryonic brain, heart, placenta (Kotarsky et al., 2006) and platelets (Amisten et al., 2008), while high levels were found in small intestine (specifically in the lymphocyte compartment) and moderate levels in skin, spleen, stomach, thymus, lung, liver, dorsal root ganglion cells (Oh et al., 2008) and embryonic stem cells (Lee et al., 2006). The LPA_6 (p2y5), was originally reported in 1996 as an orphan GPCR encoded in an intron of the retinoblastoma gene (Herzog et al., 1996). The LPA_6 is ubiquitously expressed (Pasternack et al., 2008) with high expression in human umbilical vascular endothelial cells (HUVECs) (Yanagida et al., 2009). Expression of LPA_6 was also found in a leukemia cell line (Yoon et al., 2006).

1.3.3.2 Signaling pathways of LPARs

LPA GPCRs mediate effects by coupling to heterotrimeric G-proteins of the G_i, G_q, $G_{12/13}$, or G_s families. The specific G-protein that a receptor will couple to depends not only on the receptor sequence but also on cell type, receptor expression levels, and amounts of available G-proteins (An et al., 1998b; Bandoh et al., 1999; Im et al., 2000). LPA_1 and LPA_2 can signal through G_i, G_q and $G_{12/13}$, while LPA_3 only couples to G_q and G_i (Fukushima et al., 1998; Ishii et al., 2000). The LPA_4 has the broadest coupling specificity with a potential to signal through any of the four G-protein families (Lee et al., 2007). The LPA_5 only couples to G_q and $G_{12/13}$, while LPA_6 signals through G_i and $G_{12/13}$ (Lee et al., 2006; Yanagida et al., 2009). See Figure 6 for an overview of the signaling pathways.

Upon receptor activation, Gα subunits are activated through exchange of GDP for GTP. This destabilizes the Gα-Gβγ complex so that the Gα or Gβγ subunit can now interact with downstream effectors (Oldham and Hamm, 2008). $G\alpha_i$-mediated signaling results in inhibition of cAMP production, as well as activation of MAP kinases leading to cell proliferation and differentiation. Focal adhesion kinase (FAK) and PI3K are also activated by $G\alpha_i$, promoting focal adhesion formation or cell survival and migration, respectively. Tyrosine kinase-dependent induction of tyrosine phosphatases can also be promoted by $G\alpha_i$ signaling (Chuprun et al., 1997; Gaits et al., 1996). Phospholipase C (PLC) activation is

mainly mediated by Gα_q, producing two important second messengers, inositol triphosphate (IP3) and diacylglycerol (DAG). Mobilization of intracellular Ca^{2+},by IP3, can result in actin depolymerization. Some isozymes of PKC are activated by DAG resulting in activation of MAP kinases via Raf-1 (Ghosh et al., 1997), or activation of Src, indirectly through protein tyrosine phosphatase alpha, specifically by PKC-δ (Brandt et al., 2003). G$\alpha_{12/13}$ stimulates RhoGTPase pathways that contribute to SRE-mediated transcription as well as mediating cytoskeletal dependent functions such as stress fiber formation and actin polymerization. G$\alpha_{12/13}$ also promotes activation of PLD, PI3K, RhoA and Cdc42 (Fromm et al., 1997). Finally, signaling through Gα_s results in the production of the second messenger cAMP (cyclic adenosine 3'.5'-monophosphate) through activation of adenylyl cylclase (AC) (Noguchi et al., 2003; Watts and Neve, 2005). Virtually all cellular responses such as proliferation, differentiation, apoptosis, gene transcription, metabolism, secretion, cell division and neurotransmission have been found to be affected by cAMP (Cheng et al., 2008; Robison et al., 1968). $G_{\beta\gamma}$ dimers may also participate in signaling by recruitment of PLC (Barr et al., 2000; Sankaran et al., 1998) and association with PI3K (Kurosu et al., 1997; Maier et al., 1999; Stoyanov et al., 1995).

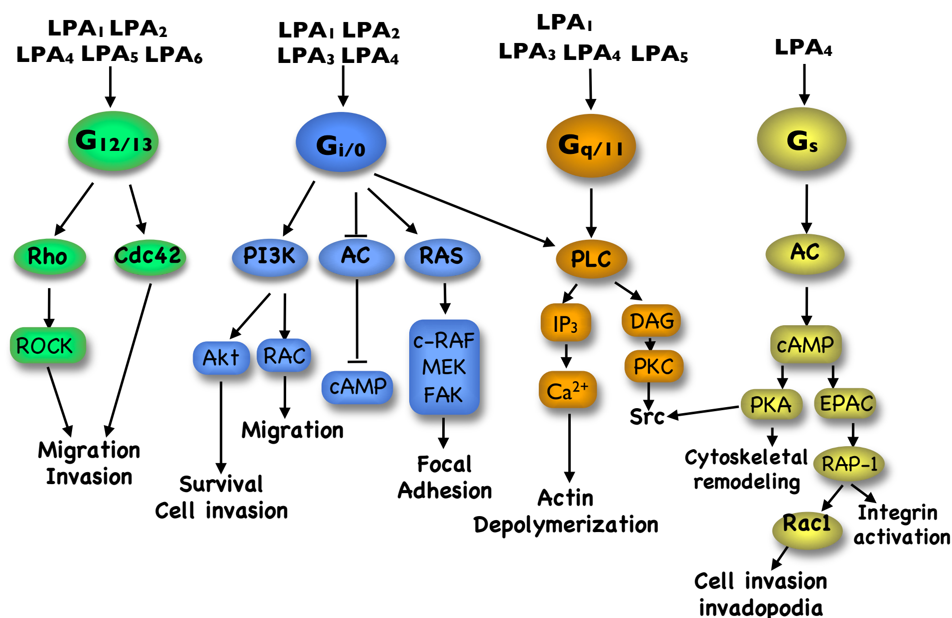

Figure 6 LPAR signaling

The major LPA signaling pathways, downstream of LPARs 1-6, are illustrated here and include signaling through four different G-protein families, G$\alpha_{12/13}$, Gα_i, Gα_q, and Gα_s.

Signaling through these G-proteins can activate diverse proteins including RhoA, PI3K, Ras, PLC and AC. The major downstream effects of LPA signaling include cytoskeletal remodeling, cell migration, stress fiber formation, focal adhesion formation and integrin activation.

1.3.3.3 Crosstalk of LPARs with RTKs

An additional aspect of LPA receptor signaling is their potential to transactivate RTKs to mediate growth factor-like effects. In this way, numerous GPCRs of the lipid, peptide, chemokine, nucleotide, steroid and even orphan receptor families are known to cross-communicate with RTKs, including VEGFR (Thuringer et al., 2002), PDGFR (Tsai et al., 2014), EGFR (Gschwind et al., 2002) and IGFR (Köse, 2017; Oligny-Longpré et al., 2012; Wang, 2016). The GPCR-mediated EGFR transactivation is the most prominent and well-studied crosstalk mechanism in various cancer cell lines (Köse, 2017) and has been shown to be mediated by a variety of GPCR agonists including LPA, thrombin, S1P, endothelin-1, prostaglandin E2 and parathyroid hormone (Cattaneo et al., 2014). In the case of LPA, various studies have identified both ligand-dependent and ligand-independent mechanisms of LPA-induced transactivation of various RTKs, such as c-MET (Fischer et al., 2004), tropomyosin receptor kinase A (TrkA) (Nan et al., 2016) and EGFR depending on cell type and physiological context (Bhola and Grandis, 2008; Cattaneo et al., 2014). Among these, EGFR represents the prototypical RTK in the context of LPAR transactivation. For example, LPA has been shown to mediate EGFR transactivation via increased ligand shedding of the EGFR ligands HB-EGF, amphiregulin (AR) or TGF-alpha. Shedding of these ligands was found to be dependent on MMPs or ADAM10, 15 or 17 depending on the cell types investigated. Other cell type-dependent factors involved in ligand-dependent transactivation were activation of Src kinases and MAPK signaling (Brusevold et al., 2014; Gschwind et al., 2002, 2003; Schäfer et al., 2004; Umata et al., 2001; Xu et al., 2007; Yoo et al., 2013; Zhao et al., 2006). Originally, LPA was found to transactivate EGFR through a ligand-independent mechanism, as the phosphorylation occured within 5 minutes, however the mechanism was not elucidated (Daub et al., 1996). One mechanism of ligand-independent transactivation of RTKs involves the generation of ROS, which can directly activate kinases via protein-protein interactions or indirectly through inactivation of protein

tyrosine phosphatases (PTPs) (Cattaneo et al., 2014; Finkel, 2000). In this respect, LPA has been shown to induce EGFR phosphorylation, in as little as 4 minutes, by increasing ROS production (Cunnick et al., 1998; Fischer et al., 2004). Another ligand-independent mechanism involves intracellular tyrosine kinases such as Src, Pyk and Fyn that can directly phosphorylate the cytoplasmic domains of RTKs (Cattaneo et al., 2014). The prototypical example is Src known to play a prominent role by directly phosphorylating EGFR and VEGFR (Cattaneo et al., 2014). For example, G_i-coupled LPA receptors were found to mediate Src-dependent EGFR phosphorylation in COS-7 cells (Luttrell et al., 1997). Conversely, RTKs have also been shown to transactivate GPCRs providing an alternative mechanism for GPCR activation. Similarly to GPCR transactivation of RTKs, RTK-mediated GPCR transactivation can be through production of GPCR ligands or by ligand-independent mechanisms involving formation of GPCR-RTK complexes (Delcourt et al., 2007). Some examples of RTK ligands shown to transactivate GPCRs are IGF-1, Insulin, PDGF, EGF, and NGF (Delcourt et al., 2007). For example, PDGF was found to activate S1P1 through ligand synthesis or physical interaction (Hobson et al., 2001; Waters et al., 2003), IGF-1 transactivates CXCR4 by formation of a CXCR4-IGF-1R complex (Akekawatchai et al., 2005), NGF induces production of S1P to transactivate S1P1 (García-Sáinz et al., 2010), and insulin receptor, EGFR or PDGFR activity phosphorylates B2-adrenoceptors increasing their functional activity (Cattaneo et al., 2014; Valiquette et al., 1995). In the case of LPA receptors, EGF signaling through EGFR has been found to increase the production of LPA in human ovarian cancer cells (Snider et al., 2010), while NGF, via TrkA, activates LPARs through physical interactions (Moughal et al., 2004, 2006). Therefore, various crosstalk mechanisms between RTKs and GPCRs, such as LPARs, add to the complexity of receptor signaling and thereby influence biological outcomes.

1.3.3.4 Implication of LPA signaling in physiological and pathological processes

Signaling by LPA is implicated in diverse biological processes that include tissue remodeling, wound healing (Watterson et al., 2007), angiogenesis, platelet aggregation, cardiovascular function (Smyth et al., 2008), neurogenesis, myelination, olfaction, neuropathic pain, reproduction, adipogenesis (Ye, 2008), and immunomodulation (Moolenaar et al., 2004; Noguchi et al., 2009). The effects of each receptor on these

processes have been elucidated by the use of receptor knockout mice. Knockout studies have shown that LPA_1 is implicated in the initiation of neuropathic pain and is important for proliferation of astrocytes. The LPA_1-null mice display 50% lethality, and survivors have abnormal phenotypes such as reduced body size, craniofacial dysmorphism and reduced brain mass as well as a suckling defect (Contos et al., 2000a). Therefore, LPA_1 seems to play an important role in the central nervous system. The LPA_2-null mice show no obvious phenotypic abnormalities and, therefore, might have redundant functions with LPA_1 as they both couple to the same G-proteins (Choi et al., 2008). Female LPA_3-null mice display delayed embryo implantation, altered embryo spacing, and reduced litter size (Ye et al., 2005), suggesting that this receptor is implicated in reproductive functioning. The LPA_4 knockout mice have no apparent abnormalities, however there is 30% lethality due to some blood vessel defects during embryogenesis (Lee et al., 2008; Sumida et al., 2010). The LPA_5 knockout mice also have no apparent phenotypic defects however they do experience decreased pain sensitivity and recover faster from inflammation. These mice were also found to have less social exploration, nocturnal hyperactivity and anxiety, suggesting a role in the central nervous system (Callaerts-Vegh et al., 2012). Finally, there have been no studies so far on LPA_6 knockout mice however this receptor seems to be related to hair growth as a mutation in LPA_6 was detected in patients with hypotrichosis simplex (Pasternack et al., 2008).

1.3.4 Implications of the ATX/LPA axis in cancer

Finally, the most relevant role of the LPA/ATX axis for this study is in tumor progression. As a potent tumor-promoting molecule, LPA influences many cellular processes implicated in tumorigenesis. Supporting this notion is the fact that the enzyme responsible for producing the majority of LPA *in vivo*, ATX, has been shown to be up-regulated in various malignancies including breast, lung, colon, ovarian, stomach, thyroid and brain cancer, correlating with the invasive potential of these cancer cells (Kehlen et al., 2004; Kishi et al., 2006; Yang et al., 2002, 1999). In fact, ATX was found to be one of the top 40 most upregulated genes in patients with highly metastatic cancer (Euer et al., 2002) In contrast, the LPA degrading enzymes LPP1 and LPP3 have been found to be downregulated in some cancer types such as breast, lung and ovarian cancer (Tang et al., 2015). Together, these studies suggest a global increase in LPA levels surrounding tumor cells. In fact, this has

been documented in ovarian and pancreatic cancer where high levels of LPA are present in the ascites from these patients (Westermann et al., 1998; Xu et al., 1998; Yamada et al., 2004). Furthermore, malignant progression has also been shown to correlate with differential expression of the various LPA receptor subtypes (Contos et al., 2000b). Increased expression of LPA_1 has been observed in bladder cancer and in breast carcinoma cells, where it leads to metastatic spread to bone (Boucharaba et al., 2004; Kataoka et al., 2015), LPA_2 is over-expressed in invasive ductal carcinoma (Kitayama et al., 2004), ovarian cancer (Erickson et al., 2001) and colorectal cancer (Shida et al., 2004). The expression of LPA_3 increases the aggressiveness of ovarian carcinoma and is also highly expressed in triple-negative breast cancer (breast cancer cells negative for estrogen receptors, progesterone receptors and human epidermal growth factor receptor 2) (Sun et al., 2015; Yu et al., 2008). Finally, elevated LPA_2 and LPA_6 expression in hepatocellular carcinoma correlates with invasion and recurrence (Enooku et al., 2016). Therefore, the increase in LPA production, decrease in LPA degradation, and increased LPA receptor levels in various cancer cell types all emphasize the importance of LPA as an extracellular signaling molecule in cancer.

Cellular characteristics associated with tumor aggressiveness, including cell proliferation, cell survival, cell motility, invasion, angiogenesis, resistance to treatment and metastasis have long been known to be augmented by ATX. (Benesch et al., 2018; Leblanc and Peyruchaud, 2015; Nam et al., 2000, 2001). For example, ATX-transfected Ras-transformed NIH3T3 cells were shown to be more invasive, tumorigenic, angiogenic and metastatic than mock-transfected controls (Nam et al., 2000, 2001). Research suggests that it is the autocrine or paracrine production of LPA via ATX that contributes to these effects on cancer cells. It has been demonstrated that several cancer cell lines release significant amounts of LPC into the culture medium and the presence of LPC promotes ATX-induced increases in chemotaxis and proliferation in multiple cell lines (Umezu-Goto et al., 2002). Furthermore, ATX promotes proliferation of A2058, MDA-MB231, CHO-K1 and Edg2-RH7777 cancer cells, but not RH7777 cells that lack LPA receptors (Brindley, 2004; Umezu-Goto et al., 2002). In contrast, the overexpression of the LPA degrading enzymes LPP1 and LPP3 have been shown to decrease proliferation and colony forming ability of

ovarian cancer cells while increasing their levels of apoptosis. Furthermore, ovarian cancer cells overexpressing LPP3 had decreased tumor growth in nude mice (Tanyi et al., 2003a). In breast and thyroid cancer cells the overexpression of LPP1 decreased cell growth in 3D culture and LPA stimulated migration as well as decreasing tumor growth and metastasis by up to 80%, compared to overexpression of an inactive LPP1 (Tang et al., 2014). The LPP1 knockout mice were also found to have increased cancer cell seeding implicating LPA signaling in the establishment of the pre-metastatic niche (Nakayama et al., 2015). Therefore increased production of LPA, by ATX, promotes tumorigenesis while reduced levels of LPA, due to the action of LPPs, has a negative effect on tumor progression.

Unsurprisingly, LPA has been shown to modulate many of the same characteristics as ATX, affecting cell motility, proliferation and metastasis as well as protecting cancer cells from radiation therapy (Benesch et al., 2018). For example, LPA mediates cytoskeletal rearrangements via the Rho GTPases Rho and Rac affecting cancer cell motility (Imamura et al., 1993; Stam et al., 1998). This leads to stimulation or inhibition of cell migration or invasion depending on the cell type. Proliferation and mitogenic signaling of prostate cancer cells can be induced by LPA (Budnik and Mukhopadhyay, 2002). Also, LPA stimulates migration and proliferation of human colon carcinoma cells (DLD1) as well as their adhesion to collagen type I and secretion of endothelial growth factor and IL-8, all of which can lead to an increased metastasizing potential of DLD1 carcinoma cells (Shida et al., 2003). These effects are mediated by LPA receptors depending on cell type. For example, LPA_1 signaling has been shown to mediate stimulation of motility of human pancreatic cancer cells (Yamada et al., 2004), induction of metastasis by human colon carcinoma cells (Shida et al., 2003), spontaneous metastasis of breast cancer cells (David et al., 2012) and hepatocellular carcinoma invasion (Park et al., 2011). In addition, LPA_2 promotes mitogenic signaling in human colon cancer cells (Yun et al., 2005), endometrial cancer invasion (Hope et al., 2009), and invasion and migration of ovarian cancer cells (So et al., 2005). Finally, LPA_3 has been found to increase motility and invasion of pancreatic cancer cells (Kato et al., 2012) and LPA_5 can increase proliferation and motility of rat lung and liver tumor cells (Okabe et al., 2011).

Interestingly, overexpression of ATX or LPA_{1-3} alone in mice was found to induce a chronic inflammation contributing to the development of mammary carcinoma as well as increased tumor invasion and metastasis of breast cancer cells in these transgenic mice (Liu et al., 2009). In contrast, pharmacological inhibition of ATX and LPA receptors was shown to decrease cell migration *in vitro* and cause tumor regression in mice (Zhang et al., 2009), further supporting the role of ATX in cancer progression. Several publications indicate that ATX and LPA receptors are also implicated in the production of the degradative structures, invadopodia (described above section 1.2). Specifically, ATX overexpression was found to increase invadopodia production, which was blocked by LPA_4 knockdown in HT1080 fibrosarcoma cells. This correlated with a decrease in 3D cell invasion and metastasis *in vivo* upon LPA_4 knockdown (Harper et al., 2010). In prostate cancer cells, LPA was shown to promote functional invadopodia formation through NF-kB and RhoA signaling (Hwang et al., 2016). In melanoma cells, LPA triggered invadopodia through Gi coupled receptors and Cdc42 signaling (Kedziora et al., 2016). Therefore, the effects of the LPA signaling axis on cancer cell invasion may be mediated by their control of invadopodia biogenesis.

1.4 Hypothesis

Metastasis is the leading cause of cancer patient mortality yet therapeutic targets for the treatment of metastatic cancer remain elusive. Recent evidence suggests that invadopodia are important mediators of cancer cell metastasis, being essential for blood vessel intravasation and extravasation, suggesting that the components and regulators of these invasive structures could be used as therapeutic targets for blocking metastasis. The hypoxic tumor microenvironment is an important driver of tumor cell invasion and metastasis, in part through regulation of invadopodia. The LPA signaling axis, which is activated in tumors, has also been implicated in invadopodia production suggesting interplay between these two aspects of the tumor microenvironment. However, our knowledge of the influence of the hypoxic microenvironment on the LPA signaling axis in the context of cancer cell invasion and metastasis is lacking.

Based on the above statements, we formulate the following hypothesis:

The hypoxic tumor microenvironment promotes cancer cell invasion and metastasis through positive regulation of the LPA signaling axis.

1.4.1 Objectives

Objective #1

Determine if hypoxia-mediated effects on invasion and metastasis are dependent on LPA receptors

Objective #2

Define signaling pathways involved in invadopodia production downstream of LPA receptors in hypoxic cells

Objective #3

Elucidate the implication of the processes involved in production and degradation of LPA in hypoxia-induced invadopodia formation and metastasis

2. ARTICLE 1

The Hypoxic Microenvironment of Tumors Promotes Invadopodia Formation and Metastasis through Cooperation between LPA1 and EGF Receptors

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Statut de l'article: accepté dans Molecular Cancer Research

Avant-propos: I personally performed the bibliographic research, experimental planning and the majority of the experiments. I also wrote and participated in the correction of the manuscript with guidance from my supervisor Dr. Claire Dubois.

Résumé :
L'hypoxie, une caractéristique commune des tumeurs solides, a été impliquée de manière critique dans l'invasion cellulaire et le développement des métastases, mais les mécanismes sous-jacents demeurent méconnus. Nos études antérieures avaient démontré que l'axe de signalisation du récepteur de l'acide lysophosphatidique, LPA4, médie la production de structures subcellulaires invasives, les invadopodes, qui sont connues pour être nécessaires à la formation de métastases. Nous démontrons maintenant que LPA1 est le récepteur commun et majeur utilisé pour la production d'invadopodes induite par l'hypoxie dans diverses lignées cellulaires cancéreuses. L'utilisation généralisée du LPA1 n'est pas due à une augmentation de l'expression de LPA1 mais repose plutôt sur une transactivation de l'EGFR induite par Src. La phosphorylation du Y845-EGFR, médiée par LPA1 en hypoxie, conduit à l'activation de PI3K/Akt, un événement qui augmente la capacité des cellules à produire des invadopodes. En outre, la phosphorylation de Y845-EGFR est régulée à la hausse dans les zones hypoxiques des tumeurs et une combinaison d'inhibiteurs du EGFR et du LPA1 a agit de façon synergique afin de supprimer la production de métastases *in vivo*. Ces résultats mettent en lumière l'axe LPA1-EGFR en tant que cible potentielle pour limiter la progression des métastases chez les patients atteints de cancer.

The Hypoxic Tumor Microenvironment Promotes Invadopodia Formation and Metastasis through LPA1 Receptor and EGFR Cooperation

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ABSTRACT

Hypoxia, a common feature of solid tumors, has been critically involved in cell invasion and metastasis but the underlying mechanisms remain poorly understood. Previously, it has been observed that the lysophosphatidic acid receptor 4 (LPA_4) signaling axis mediates production of the degradative subcellular structures invadopodia, which are known to be required for metastasis. Here, it is demonstrated that LPA_1 (LPAR1) is a common and major receptor used for hypoxia-induced invadopodia production in various cancer cell lines. The widespread use of LPA_1 was not due to increased LPA_1 expression but rather relied on Src-mediated crosstalk with EGFR. Phosphorylation of Y845-EGFR mediated by LPA_1 under hypoxia led to PI3K/Akt activation, an event that increases the ability of cells to produce invadopodia. Moreover, phospho-Y845-EGFR was upregulated in hypoxic zones of tumors and a combination of EGFR and LPA_1 inhibition synergistically suppressed metastasis in an *in vivo* model in chick embryos.

IMPLICATIONS

This study uncovers a LPA_1-EGFR signaling axis that is used for cell invasion in hypoxia and suggest a potential target to impede cancer metastasis.

INTRODUCTION

Metastasis is the leading cause of mortality in cancer patients. However, effective therapies targeting the disseminated disease remain a major challenge in clinical management of the disease. Tumor microenvironment is increasingly recognized to play a significant role in many of the hallmarks of cancer, notably as an important modulator of tumor cell phenotypes driving cell invasion and metastasis (1). A common feature of the tumor microenvironment is hypoxia, or low concentrations of oxygen, within solid tumors. Tumor hypoxia arises because of inadequate delivery of oxygen as a result of insufficient and defective vasculature and high oxygen consumption rate of cancer cells (2). Hypoxic tumors are aggressive, resistant to chemotherapy, and prone to recurrence (3). It has been recently reported that tumor microenvironment stimuli such as hypoxia and the associated acidic pH induce cell invasion and metastasis through activation of sodium-hydrogen exchangers, growth factors, metalloproteases or RhoGEFs (4). In these reports, the increase in cellular invasion was associated with production of invadopodia, which are specialized cell structures required for cancer cell dissemination.

Invadopodia are actin-rich and proteolytically active subcellular structures generated by cancer cells that promote their migration and invasion through tumor stroma and the basement membrane of blood vessels during the process of metastasis (5). These structures are relevant to the cell invasion process as they have been observed in migrating cancer cells undergoing intravasion as well as in tumor cells invading through tissues (6,7). More recently, invadopodia have been shown to be essential for cancer cell extravasion and metastasis *in vivo* through genetic analysis and pharmacological inhibition of molecules specifically involved in their initiation, maturation or function (8). These studies have provided direct *in vivo* evidence of the functional role of invadopodia in metastasis, making

them relevant therapeutic targets to counter pathological cell invasion. Generation of invadopodia requires tight coordination between polarized cellular trafficking, signaling events and cytoskeletal remodeling. Whereas these events have been extensively studied, many of the upstream inducers that drive invadopodia formation and activity remain unknown (9).

An emerging participant in cancer progression is the ATX-LPA receptor signaling axis. Lysophosphatidic acid (LPA) is a bioactive lipid produced mainly by Autotaxin (ATX) that signals through 6 known LPA G-protein coupled receptors (GPCRs) (LPA_{1-6}). These receptors activate various signaling components that include cAMP, PLCβ, PLCε, small rhoGTPases, Ras, ERK and PI3K that regulate many pathophysiological processes including cancer (10). We have previously reported that LPA_4 , through the cAMP-Rap1-Rac1 axis, was involved in invadopodia production by cancer cells and that this event correlated with their metastatic efficiency (11). The ability of LPA to stimulate invadopodia production has since been confirmed in prostate and melanoma cells (12,13). Furthermore, over-expression of ATX or LPA_{1-3} has been shown to induce tumorigenesis and metastasis in a mouse model (14), whereas their pharmacological inhibition decreased cell migration *in vitro* and caused tumor regression in mice (15). Although these findings suggested the critical implication of LPA receptors in tumor progression, the interplay between LPARs and hypoxia within the context of cell invasion has not yet been addressed. Here, we report that hypoxia promotes cell invasion through a mechanism that involves Src-mediated cross-communication between the LPA_1 receptor and EGFR. Our findings suggest a mechanistic rationale for targeting both LPA_1 and EGFR to counteract metastasis.

MATERIALS & METHODS

Reagents. 1-oleoyl-sn-glycerol-3-phosphate 18:1 (LPA) sodium salt and LPA receptor antagonist (Ki16425) were purchased from Sigma-Aldrich (St. Louis, MO). Inhibitors of EGFR (Tyrphostin AG 1478), PLC (U-73122), and PI3K (LY294002) were purchased from Biomol International (Plymouth Meeting, PA). Inhibitors of MEK (PD 98059), ROCK (Y-27632), Src (PP2) and RAP1 (GGTI-298) were from Calbiochem (EMD Chemical Inc, La Jolla, CA). LPA_1 receptor antagonist AM095 was from APExBIO (Houston, TX). Corning Matrigel Basement Membrane Matrix was from Corning (Bedford, MA). The antibody directed against LPA_1 was from Abnova (Walnut, CA) and, against LPA_4, from Novus Biologicals (Littletone, CO). Antibodies directed against EGFR (D38B1), pTyr, phospho-EGFR (Y845), phospho-AKT1 (Ser473), pan AKT, Src 32G6 and phospho-Src (416) were purchased from Cell Signaling (Danvers, MA). The anti-GRK2 antibody was from Santa Cruz (Dallas, Texas). The anti-tubulin antibody was from Sigma-Aldrich and, Texas Red-conjugated phalloidin, DAPI (4',6-Diamidino-2-Phenylindole), and all secondary antibodies were from Invitrogen (Molecular Probes, Eugene, OR). Hypoxyprobe kit containing pimonidazole HCl and anti-pimonidazole mouse antibody was from Hypoxyprobe (Burlington, MA).

Cell culture and transfections. HT1080 human fibrosarcoma, MDA-MB231 human breast cancer and U87 human glioblastoma cells were obtained from the American Type Culture Collection (Rockville, MD). All cell lines were routinely tested for mycoplasma using the MycoSEQ Mycoplasma Detection Kit (all negative) (Thermo Fisher Scientific). Cells were grown for no more than 25 passages in total for any experiment. Cells were cultured in minimal essential medium (MEM) (Wisent, St-Bruno, QC) supplemented with 10% FBS

(Gibco BRL, Burlington, ON) and 40 µg/ml of gentamicin (Wisent) in a humidified 95% air/5% CO_2 incubator at 37ºC. For hypoxic stimulations, cells were cultured in an INVIVO2 400 hypoxic chamber (Ruskinn, Sanford, ME) under an atmosphere of 1% O_2 and 5% CO_2. In the case of stable transfections with shRNA against LPA_1 or LPA_4 (SABiosciences, Frederick, MD), cells were seeded at a density of 1 x 10^5 cells per well in a 6-well culture plate the day before transfection. Transfections were performed using the FuGENE reagent (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's protocol. Stable transfectants were obtained by selection with Puromycin 2 µg/ml (Invivogen, San Diego, CA). In the case of lentiviral transductions, cells were seeded at a density of 3 x 10^5 cells per 10 cm^2 Petri dish and infected with 1 ml of viral stock in 2 ml of optiMEM supplemented with 2 µl Polybrene (10 mg/ml) (EMD Millipore, Etobicoke, ON), cell populations were used. Mission lentiviral shRNA targeting EGFR (TRCN0000010329), Src (TRCN0000195339), ADAM12 (TRCN0000047033), ADAM17 (TRCN0000052172), or a scramble sequence were used (Sigma-Aldrich). Viral particles were generated by transient transfection of 293T cells using a ViraPower lentiviral expression system (Invitrogen Thermo Fisher Scientific, Burlington, ON).

Real-time RT-PCR. Total RNA was isolated using the TRIzol (Invitrogen, Carlsbad, CA) protocol, as described (11), and 1 µg of RNA was reverse transcribed to complementary DNA (cDNA) using a QuantiTect reverse transcription kit (Qiagen, Mississauga, ON). Transcribed cDNA was then analyzed by real-time PCR using a hot start SYBR Green qPCR master mix (BiMake, Houston, TX). Primer pairs are described in the supplementary Material and Methods section. Quantitative real-time PCR was performed on a Rotor-Gene 3000 (Corbett Research, Kirkland, QC). The cycling program was as follows: the initial

denaturation was performed at 95ºC for 15 min, followed by 40 amplification cycles with annealing temperature of 55ºC for 30 s and, final extension at 72ºC for 30 s. For calculation of copy numbers, cloned plasmid DNA was used to generate a standard curve for each target and samples were normalized with a reference gene (RPLP0) as previously described (16).

Western blotting. Cells were lysed in a RIPA buffer (50mM Tris HCL, 150 mM NaCl, 0.1% Na-deoxycholate, 4mM EDTA, 1% NP40) with protease inhibitors 1/10 (cOmplete Mini EDTA-free from Roche Diagnostics (Indianapolis, IN)) and phosphatase inhibitors 1/10 (10mM NaF, 1mM Na_3VO_4, 20mM B-glycerophosphate). Supernatant samples were recovered by centrifugation (13000 rpm for 30 min at 4ºC) and protein concentrations determined using the BCA reagent (Biolynx Inc, Brockville, ON). Immunoblotting was performed as previously described (11). In the case of immunoprecipitation experiments, 1 mg of total protein was immunoprecipitated using an anti-EGFR antibody (dilution, 1:100). The membranes were probed overnight with primary antibodies. The secondary antibody was a peroxidase-conjugated anti-rabbit or anti-mouse antibody, depending on the source of primary antibody used (Amersham, Baie d'Urfé, QC). Immunoblots were revealed using the Luminata™ Western HRP Chemiluminescence substrate (Millipore, Etobicoke, ON). Band intensities were analyzed using the Quantity One software (Bio-Rad Laboratories, Mississauga, ON, Canada).

Invadopodia assay. Coverslips were prepared as described (11), using Oregon-Green^{488}-conjuagted gelatin (Invitrogen, ON). Thirty thousand cells were seeded on each coverslip and allowed to adhere. Following a 10 h or 16 h incubation period under the conditions

described in Figure legends, cells were fixed with 2% paraformaldehyde for 10 min at room temperature. Nuclei were stained with DAPI and F-actin was stained using Texas-Red-conjugated phalloidin. Cells were visualized and images were taken using a Zeiss Axioskop fluorescence microscope. Cells forming ECM-degrading invadopodia were identified based on cells with at least one F-actin-enriched area of matrix degradation (characterized by loss of green fluorescence). Three fields of 100 cells (magnification 40X) were counted per coverslip to quantify the percentage of cells forming ECM-degrading invadopodia.

RTK phosphorylation array. HT1080 cells were incubated under normoxia or hypoxia for 30 or 60 min in serum-free medium. Proteins were extracted and analyzed using the human PathScan RTK Signaling Antibody Array kit (Cell Signaling Technology), according to the manufacturer's instructions. Fluorescent images were captured using an Odyssey Infrared Imaging System and fluorescence intensities semi-quantitated using the ImageJ software.

ELISA assays. HT1080 cells were plated at a density of 1 x 10^6 cells per 10 cm^2 Petri dish. On the next day, the medium was removed and replaced with serum-free medium for overnight starvation. Cells were then incubated for 8 h in an atmosphere of 21% O_2 or 1% O_2 and supernatants were collected and centrifuged to remove cellular debris and concentrated. Ligands for EGFR were detected using Quantikine ELISA kits for human EGF, TGF-alpha (R&D Systems) or HB-EGF (Abcam), according to the manufacturer's instructions.

Chorioallantoic membrane assays. Fertilized eggs from white leghorn chicken were obtained from the Public Health Agency of Canada (Nepean, ON). The project was

approved by the Ethics Committee on Animal Research of the University of Sherbrooke (Protocol # 054-13) and all experimental procedures involving embryos were conducted in accordance with regulations of the Canadian Council on Animal Care. Chorioallantoic membrane (CAM) assays were performed as we described (17), with the following modifications. Cell suspensions were mixed with the appropriate inhibitors prior to grafting on the CAM and the eggs were then returned to the incubator for another 6 days. The cells were treated with DMSO (vehicle), AM095, AG1478 or a combination of the two inhibitors. At the end of the experiments, livers from the chick embryos were removed and immediately snap-frozen in liquid nitrogen and stored at -80°C until DNA extraction. Genomic DNA extraction was performed using DNAzol reagent (Invitrogen), according to the manufacturer's instructions. Amplification of Alu repeats by qPCR was performed as described (18), using the hot start Taq PCR kit (Qiagen) and relative changes in metastasis were then calculated as $2^{\Delta\Delta CT}$.

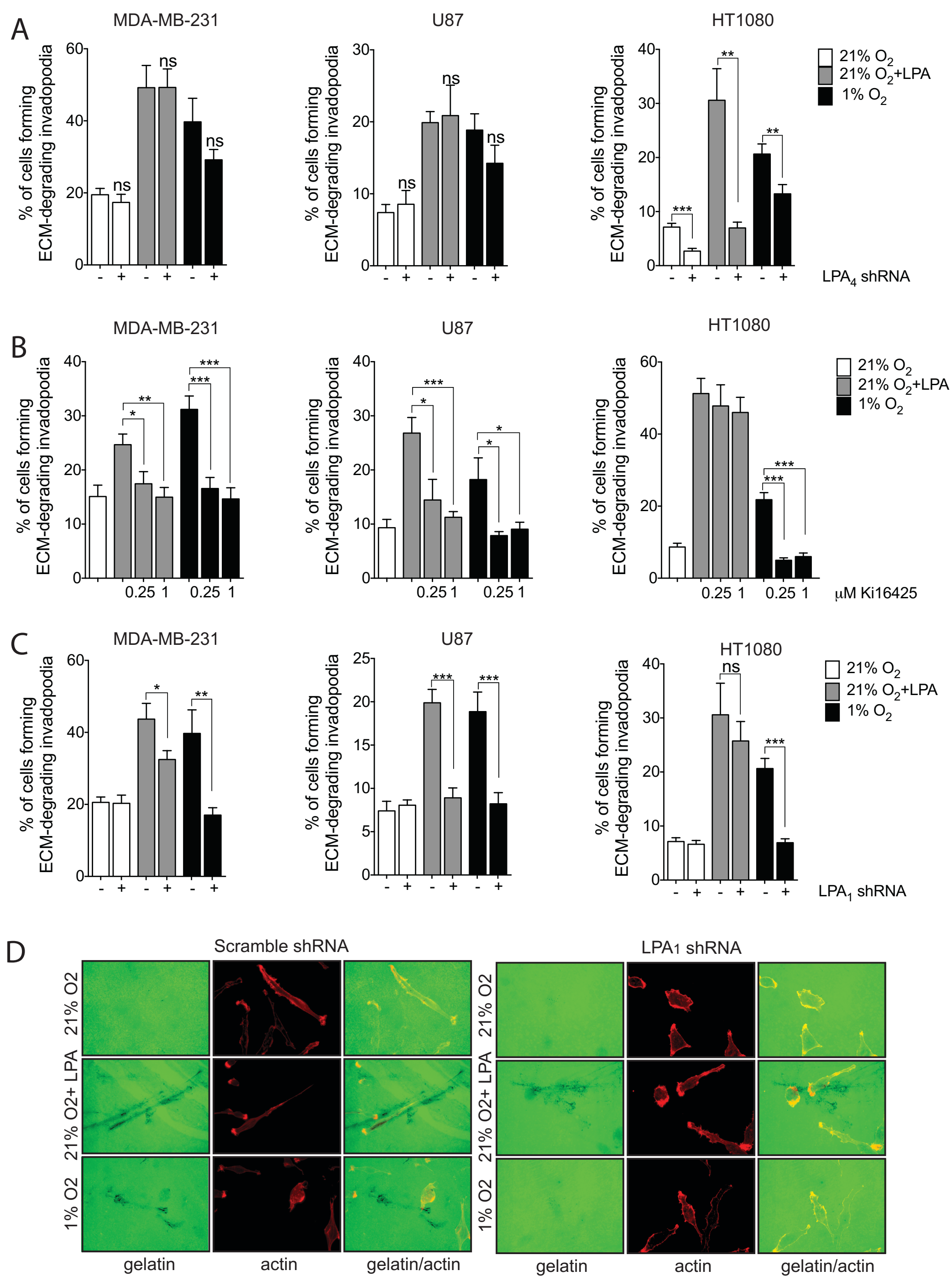
Immunohistochemistry. Immunohistochemistry of tumor hypoxia in tumors grown on CAM was performed as we described (17). Tumor sections were double stained for pimonidazole, in combination with phospho-Y845-EGFR antibody (1:50). For quantification of phospho-Y845-EGFR staining intensity at least 6 representative areas from at least 3 separate tumors for each condition were captured using an Axioskop 2 phase-contrast/epifluorescence microscope (Carl Zeiss Inc., Thornwood, NY). Fluorescence intensities were analyzed using the Image Pro software (Media Cybernetics, Bethesda, MD), and results are expressed as the sum of labeling intensity (density) relative to total area.

Statistical analysis. The GraphPad software was used for statistical analysis. Paired or unpaired Student's t-test were used to assess statistical significance, which was set at a P value <0.05.

RESULTS

LPA_1 is the major LPA receptor used in hypoxia for invadopodia production

We have previously reported that LPA_4 signaling was involved in invadopodia production in HT1080 cancer cells cultured under normoxic conditions (11). Since LPARs can have cell type and context dependent effects on cell motility and invasion (19), and since several GPCRs are known to play a role in hypoxia-mediated signaling (20), we sought to investigate which LPAR and downstream signaling were involved in invadopodia formation under hypoxic conditions. To address this issue, we first examined the impact of LPA_4 knockdown on the percentage of cells forming ECM-degrading invadopodia under normoxic or hypoxic conditions using various cancer cell lines, namely MDA-MB-231 breast cancer, U87 glioblastoma and HT1080 fibrosarcoma cells. Unexpectedly, shRNA-mediated knockdown of LPA_4 in MDA-MB-231 or U87 cells had no effect on the percentage of cells with invadopodia in hypoxia and had only a partial effect in HT1080 cells. As previously reported, LPA_4 knockdown resulted in complete inhibition of LPA-induced invadopodia-forming cells in normoxic HT1080 cells (11), whereas it had no effect under these conditions in MDA-MB-231 and U87 cells (Fig 1A and Supp Fig1A). Since one interpretation of these results is the utilization of LPA receptors other than LPA4, for invadopodia production in hypoxia, cells were incubated with the LPAR inhibitor Ki16425, which inhibits preferentially LPA1 > LPA3 >> LPA2 (21). Interestingly, low concentrations of Ki16425, which are selective for LPA1, were effective at reducing the increase in the percentage of cells producing invadopodia under hypoxia in all three cells lines. In MDA-MB-231 and U87 cells, Ki16425 treatment also diminished LPA-induced invadopodia under normoxic conditions (Fig 1B). These results suggest a common usage of LPA1 under hypoxic conditions and a selective usage under normoxia by MDA-MB-231



A
MDA-MB-231
U87
HT1080
% of cells forming ECM-degrading invadopodia
21% O2
21% O2+LPA
1% O2
LPA4 shRNA
B
µM Ki16425
C
LPA1 shRNA
D
Scramble shRNA
LPA1 shRNA
21% O2
21% O2+ LPA
1% O2
gelatin
actin
gelatin/actin

Figure 1 LPA_1 is essential for hypoxia-induced cancer cell invasion.

(A-C) MDA-MB-231, U87 or HT1080 cells were cultured on fluorescently-labeled gelatin for 10 h or 16 h, under normoxia (21% O_2) or hypoxia (1% O_2) in the presence/absence of LPA (10 μM). The percentages of cells forming ECM-degrading invadopodia in the case of, (**A**) cells stably transfected with shRNA against LPA_4, (**B**) cells treated with the LPA receptor inhibitor Ki16425, or (**C**) cells stably transfected with shRNA against LPA_1 are shown ($n \geq 3$ independent experiments). (**D)** Representative immunofluorescence images of matrix degradation by scrambled or LPA_1 shRNA-transfected HT1080 cells cultured for 10 h on fluorescently-labeled gelatin in an atmosphere of 21% O_2, 21% O_2 in the presence of LPA, or 1% O_2 are shown (magnification 40X). F-actin staining [red] and Oregon Green488-conjugated gelatin [green]. Bars represent the mean ± SEM. The asterisks (*) correspond to $P < 0.05$ (*), $P < 0.01$ (**) and, $P < 0.001$ (***).

and U87 cells. To confirm the role of LPA1, we silenced its expression using LPA1-targeted shRNA (Supp Fig1B). Knockdown of LPA_1 decreased the percentage of invadopodia-forming cells induced by hypoxia in all three cell lines, further underscoring the prevailing LPA_1 usage under this condition. In addition, LPA_1 knockdown also decreased the percentage of LPA-induced invadopodia-forming cells in MDA-MB-231 and U87 cells (Fig 1C). Immunofluorescence pictures revealed a marked difference in matrix degradation between scrambled and LPA_1 shRNA transfected HT-1080 cells cultured under hypoxic conditions compared to the similar levels of degradation observed in LPA-stimulated cells under normoxic conditions (Fig 1D). Overall, these results suggested that LPA_1 is a common and major LPA receptor used for the production of hypoxia-induced ECM-degrading invadopodia in various cancer cell lines.

LPA_1 usage in hypoxic HT1080 cells is not related to changes in LPA_1 or GRK2 levels of expression

The differential use of LPA receptors under normoxic and hypoxic conditions led us to investigate the gene expression profile of LPA receptors in HT1080, MDA-MB-231 and

U87 cell lines. Results from qPCR experiments showed that both MDA-MB-231 and U87 cells expressed predominantly mRNA for the LPA1 receptor, which is consistent with the use of this receptor for invadopodia production under both normoxic and hypoxic conditions (Fig 2A,B). In contrast, and as previously described (22), HT1080 cells expressed mainly LPA4, which correlates with its role in invadopodia formation in normoxia. Of interest, these cells displayed low levels of LPA1, despite the major use of this receptor in hypoxia (Fig 2C). To further investigate this issue, we examined whether modulation of LPA1 expression or activity could explain the main usage of this receptor in hypoxic HT1080 cells. Analysis of both mRNA and protein levels showed no significant increases in LPA1 expression under hypoxic conditions, despite a significant increase in mRNA expression of CAIX, a well-recognized hypoxia-regulated gene (Fig 2D,F,G). Since the G-protein regulatory kinase, GRK2, has been shown to be decreased in hypoxic brain cells resulting in LPA1 overactivation (23), we next examined whether GRK2 levels were modulated in hypoxic HT1080 cells. No significant changes in either mRNA or protein expression of GRK2 were observed in HT-1080 cells subjected to hypoxic conditions for up to 16 h (Fig 2E,G). Taken together, these results indicated that modulation of LPA1 or GRK2 expression did not account for LPA1 usage in hypoxic HT-1080 cells.

LPA_1 usage in hypoxic cells involves crosstalk with EGFR

The GPCRs and LPARs are known to cross-communicate with RTKs to mediate additional signaling events and growth factor-like effects (24). Furthermore, hypoxia has been shown to increase the expression or activation of various RTKs (25), suggesting the possibility of RTK participation in LPA1-induced invadopodia formation in hypoxia. To address this possibility, we initially used a phospho-specific RTK antibody array that measures the

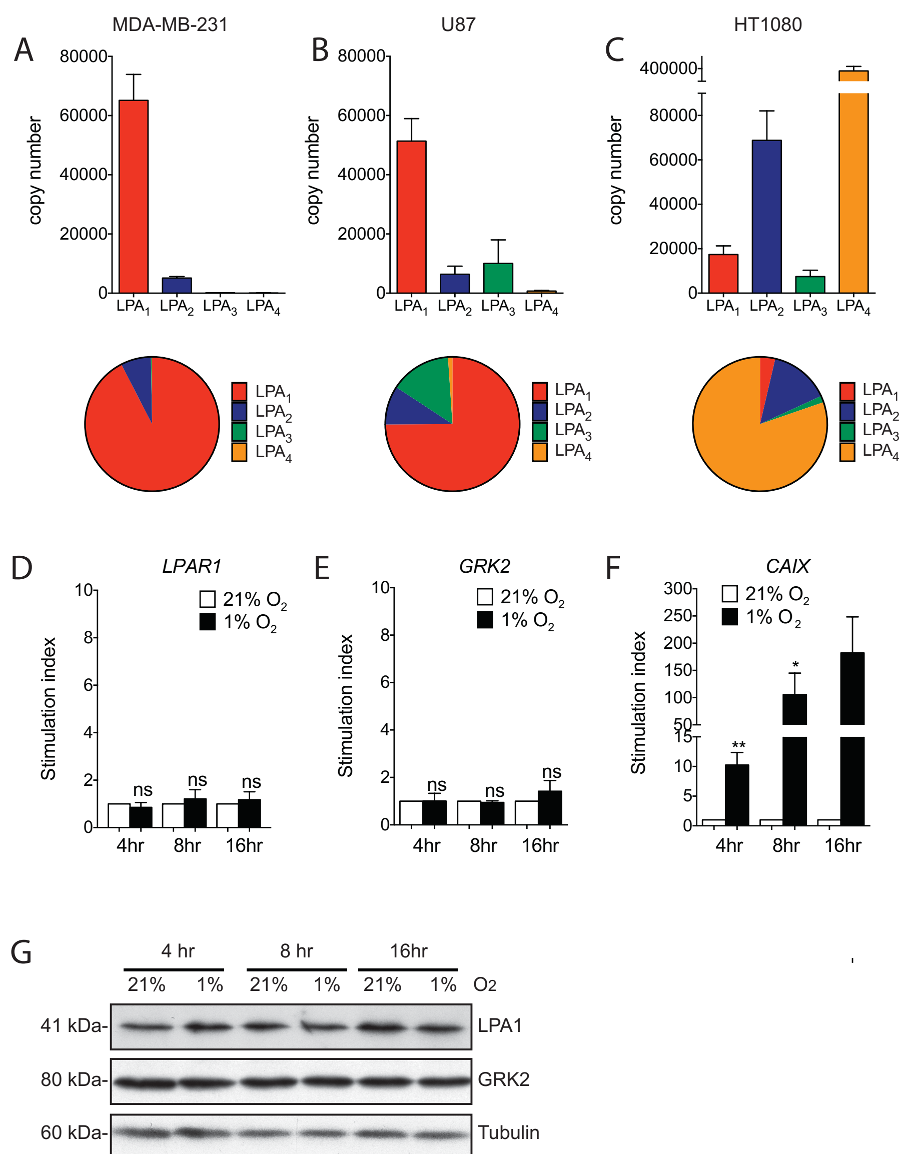


Figure 2 LPA_1 and GRK2 expression is not modulated under hypoxic conditions.

(A-C) qPCR analysis of mRNA isolated from **(A)** MDA-MB-231, (**B**) U87, or (**C**) HT1080 cells. Graphs show relative expression of *LPAR1*, *LPAR2*, *LPAR3* and *LPAR4* mRNA in copy number. *RPLP0* was used to normalize the data (n = 3 independent experiments). (**D-F)** mRNA expression of **(D)** *LPAR1,* **(E)** *GRK2,* or (**F**) *CAIX* in HT1080 cells cultured in the presence of 21% O_2 or 1% O_2 for 4 h, 8 h, or 16 h. *RPLP0* was used to normalize the data (n = 4 independent experiments). (**G**) Protein levels of LPA_1 and GRK2 analyzed by Western blotting following incubation of HT1080 cells for 4 h, 8 h, or 16 h under hypoxia (1% O_2). Tubulin was used as a loading control. One representative blot of 2 independent experiments is shown. Bars represent the mean ± SEM. The asterisks (*) correspond to $P < 0.05$ (*) and, $P < 0.01$ (**). ns = not significant.

levels of activation of common RTKs and signaling nodes that include MetR, PDGFR, VEGFR, IGFR, EGFR and Akt. Results showed that EGFR was a main RTK activated under hypoxic conditions in HT1080, MDA-MB-231 and U87 cell lines (Fig 3A,B and Supplementary Fig 2A). Immunoprecipitation of EGFR followed by Western blotting using anti-phosphotyrosine antibodies revealed that hypoxia or LPA stimulation resulted in a rapid phosphorylation of EGFR with maximal effects observed after 1 h and 30 min of stimulation, respectively (Fig 3C). In addition, Ki16425 used at a concentration that preferentially inhibits LPA1, completely blocked hypoxia-induced EGFR phosphorylation (Fig 3D,E), while it had no effect on LPA-induced phosphorylation in normoxia (Supplementary Fig2 B), suggesting the participation of LPA1 solely under hypoxic conditions in HT1080 cells. Similar inhibition of hypoxia-induced EGFR phosphorylation by Ki16425 was observed in MDA-MB-231 and U87 cell lines (Supplementary Fig2 C,D), indicating that LPA1-EGFR crosstalk in hypoxic cells is not associated with a particular cell type.

The involvement of EGFR in hypoxia-induced invadopodia production downstream of LPA1 was next investigated. EGFR knockdown in HT1080 cells was found to significantly block the increase in the percentage of cells forming invadopodia under hypoxic conditions whereas this procedure did not affect invadopodia induced by LPA under normoxic conditions (Fig 4A, Supplementary Fig3). Similar findings were observed in MDA-MB-231 and U87 cells (Fig 4A). In contrast, in cells where LPA1 had been knocked down by shRNA treatment, inhibition of EGFR had no effect on invadopodia production whether cells were grown under normoxic or hypoxic conditions or were stimulated with LPA (Fig 4B). Overall, these results suggested that EGFR was preferentially involved in the ability of

cells to produce invadopodia under hypoxic conditions and that this event occurred downstream of LPA1.

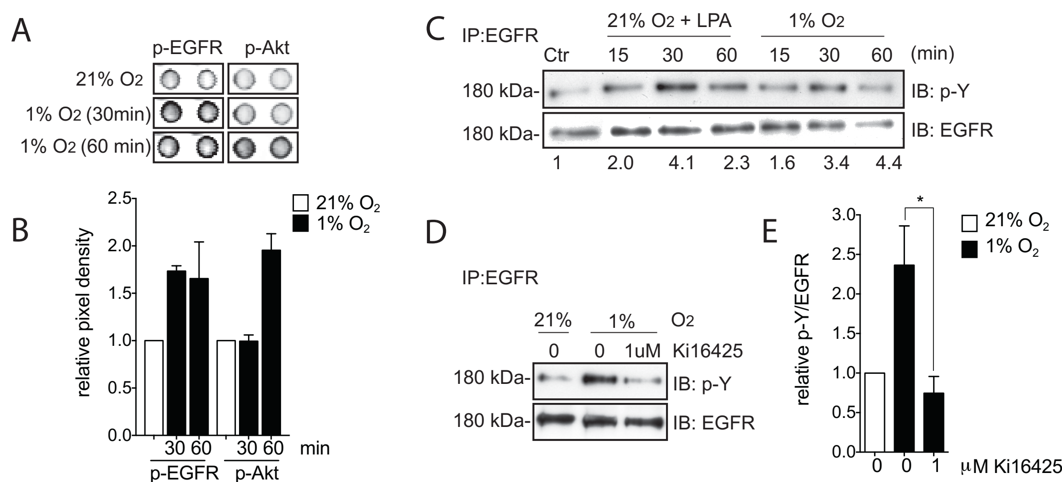


Figure 3 EGFR is transactivated by LPA_1 under hypoxic conditions.

(A-B) HT1080 cells were incubated under 21% O_2 or 1% O_2 for 30 or 60 min and cell lysates were subjected to phospho-RTK array analysis. (**A**) One representative array, showing phospho-EGFR and phospho-Akt, of two independent experiments is shown. (**B**) The associated graph shows the mean phosphoprotein fluorescence intensities for each cell lysate from two independent experiments. (**C-E)** HT1080 cell lysates were immunoprecipitated using an anti-EGFR antibody and immunoblotted with anti-phospho-tyrosine (pY) and total EGFR antibodies. (**C)** One representative blot which shows a time course of LPA (10 μM) or hypoxic (1% O_2) stimulations. (**D)** One representative blot showing hypoxic stimulation (30 min) in the presence/absence of Ki16425 (1 μM). (**E**) Corresponding graph showing densitometric analysis of p-Y/EGFR ratios of 3 independent experiments. Bars represent the mean ± SEM (* $P< 0.05$). The asterisk (*) corresponds to $P < 0.05$ (*).

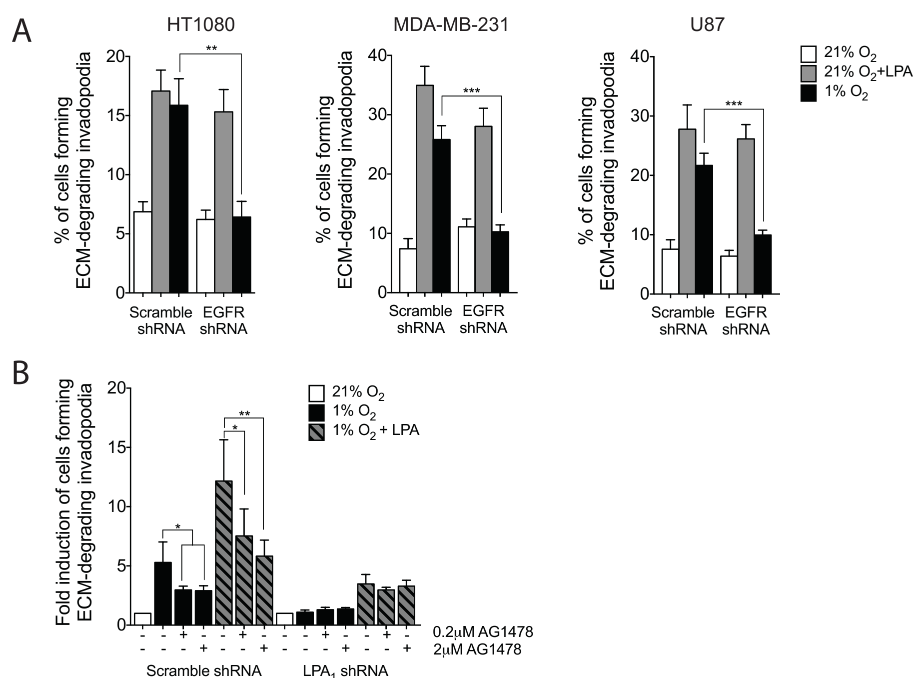


Figure 4 EGFR is necessary for hypoxia-induced invadopodia formation downstream of LPA₁.

(A,B) Cells were cultured on fluorescently-labeled gelatin for 10 h and the percentages of cells forming ECM-degrading invadopodia are shown. (**A**) HT-1080, MDA-MB-231 or U87 cells transduced with scramble or EGFR-targeting shRNA were incubated in 21% O_2 in the presence/absence of LPA (10 μM) or in 1% O_2. (**B**) Cells transfected with scramble or LPA_1-targeting shRNA were incubated in 21% O_2, 1% O_2 or 1% O_2 in the presence of LPA (10 μM) with or without the addition of the EGFR inhibitor AG1478. Bars represent the mean ± SEM. The asterisks (*) correspond to $P < 0.05$ (*), $P < 0.01$ (**) and, $P < 0.001$ (***).

Src is a mediator of LPA_1-EGFR crosstalk

Transactivation of RTKs mediated by GPCRs can involve different mechanisms that include increased ligand availability, through protease-mediated shedding of the ligands, or direct phosphorylation of the RTK by a downstream kinase (26). To address the first possibility, we investigated whether hypoxic HT1080 cells showed increased release of the major EGFR ligands involved in GPCR-RTK crosstalk, EGF, HB-EGF and TGF-alpha. Results from ELISA assays showed the absence of increases in the release of EGF, HB-

EGF or TGF-alpha under hypoxic conditions (Fig 5A). Furthermore, shRNA-mediated knockdown of ADAM17 or ADAM12, two major proteases involved in the shedding of EGFR ligands, did not affect hypoxia-induced EGFR phosphorylation (Fig 5B-D). These results indicate that increased ligand availability is unlikely to be the mechanism responsible for the rapid increase in EGFR phosphorylation under hypoxia.

Since one of the main kinases known to be involved in phosphorylation-induced transactivation of EGFR is Src (26), we next examined the contribution of this kinase to the hypoxia-induced LPA1-phosphoEGFR axis. Western blot results showed a clear increase in Src Y416 phosphorylation after 45 min of exposure to hypoxia and that upregulated Src phosphorylation was reduced to basal levels following inhibition of LPA1 (Fig 5F,G). Similarly, shRNA-mediated knockdown of Src, or inhibition of its activity by PP2 strongly repressed EGFR phosphorylation induced by hypoxia (Fig 5B,E; Supp Fig 4). These findings clearly indicate that Src-mediated phosphorylation of EGFR is the likely mechanism of EGFR transactivation by LPA1 in hypoxic cells.

To gain insight into the significance of the Src-mediated EGFR transactivation mechanism in the ability of cells to form ECM-degrading invadopodia under hypoxic conditions, Src shRNA-transduced cells were tested in invadopodia assays. Cells with Src knockdown failed to affect the increase in the percentage of invadopodia forming cells induced by LPA under normoxic conditions, which is consistent with our previous results that had identified an alternative mechanism downstream of LPA_4 (11). In contrast, Src knockdown resulted in inhibition in the percentage of hypoxia-induced invadopodia producing cells, in agreement with its involvement in EGFR transactivation. As a control, knockdown of ADAM17- or

ADAM12 did not affect invadopodia production under hypoxia (Fig 5H), consistent with their observed lack of involvement in EGFR phosphorylation (Fig 5B).

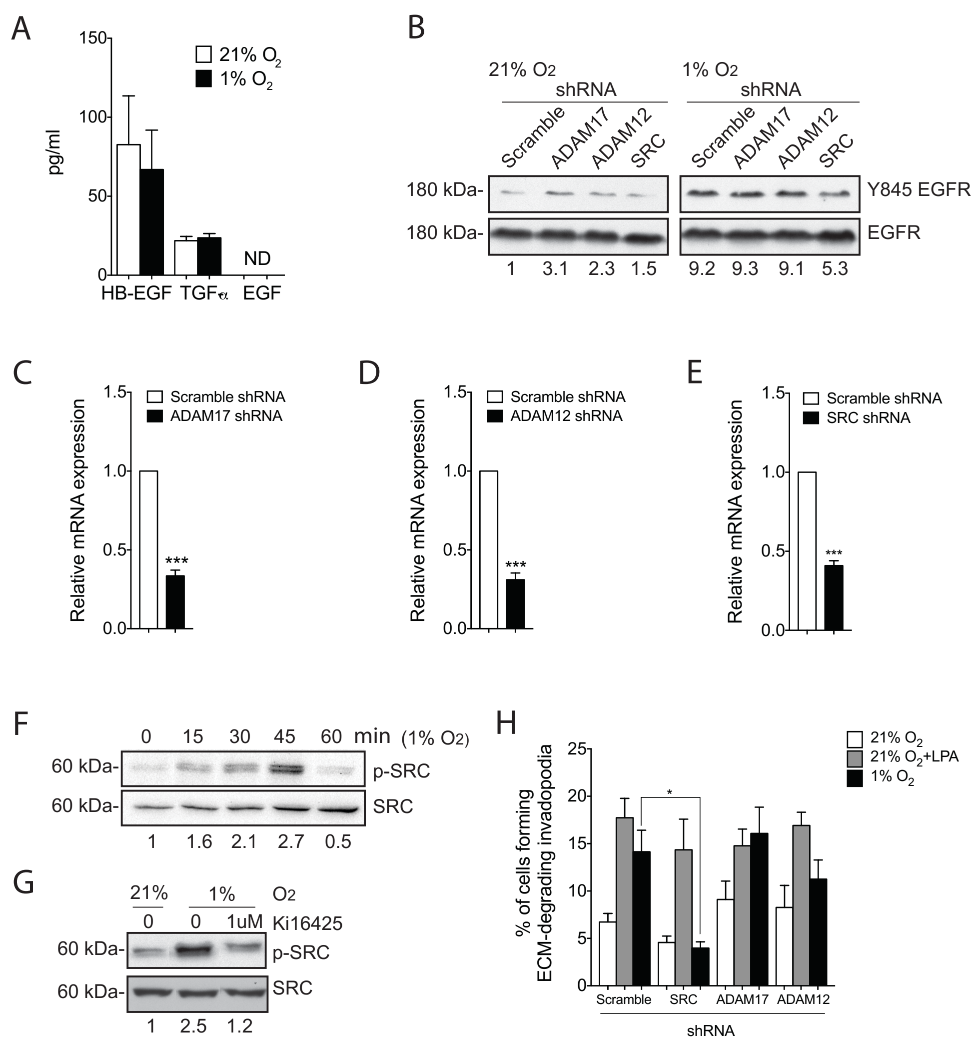


Figure 5 Mechanism of transactivation of EGFR by LPA_1 under hypoxia.

(A) Detection of HB-EGF, TGFα, and EGF by ELISA was performed on concentrated cell supernatants from HT1080 cells incubated for 8 h in the presence of 21% O_2 or 1% O_2. Results are presented in pg/ml (n = 3). ND = non-detectable. (**B**) Western blot of phospho-Y845 EGFR and total EGFR in HT1080 cells transduced with scramble, ADAM17, ADAM12, or Src-targeted shRNA and incubated under 21% O_2 or 1% O_2 for 30 min. Densitometric analysis of phospho-Y845/EGFR ratio is shown below the blots. One

representative blot of 3 independent experiments is shown. (**C-E**) Quantification of (**C**) *ADAM17*. (**D**) *ADAM12* and (**E**) *SRC* mRNA levels by qPCR using *RPLP0* as a reference gene (n = 3). (**F**) HT1080 cell lysates were analyzed by Western blotting using anti-phospho-Src and Src antibodies. Densitometric analysis of phospho-Src/Src ratio is shown below the blots. One representative blot of a time-course of hypoxic (1% O_2) stimulations is shown. (**G**) HT1080 cells were incubated for 45 min in 1% O_2 with or without Ki16425 (1 μM). One representative blot is shown. **(H)** HT1080 cells transfected with scramble, ADAM17, ADAM12, or SRC shRNA were cultured on fluorescently-labeled gelatin for 10 h in 21% O_2 or 1% O_2 in the presence or absence of LPA (10 μM). The percentage of cells forming ECM-degrading invadopodia is shown (n = 3). Bars represent the mean ± SEM. The asterisks (*) correspond to P < 0.05 (*) and, P < 0.01 (**) and, P < 0.001 (***).

LPA_1-EGFR axis signals through PI3K/Akt to promote hypoxia-induced invadopodia production

We next investigated the involvement of the main LPA_1 and EGFR downstream signaling pathways in hypoxia-induced invadopodia production using pharmacological inhibitors of ROCK (Y-27632), PLC (U-73122), MEK (PD 98059) or PI3K (LY294002). Inhibition of Rap1 (GGTI-298), which has previously been reported to be downstream of LPA_4 for invadopodia production (11), was used as a control. Among the pathways tested, only inhibition of MEK or Rap1 signaling significantly decreased the percentage of cells forming invadopodia induced by LPA in normoxia or induced by hypoxia. In contrast, PI3K inhibition exclusively decreased invadopodia production induced by hypoxia (Fig 6A). Increasing the concentration of the PI3K inhibitor did not affect LPA-stimulated invadopodia-forming cells in normoxia, but efficiently blocked the stimulatory effect of hypoxia alone or in the presence of LPA (Fig 6B). As expected, Rap1 inhibition reduced the percentage of cells forming invadopodia under both normoxic and hypoxic conditions (Fig 6C). Given that cells mostly used LPA_1 in hypoxia (Fig 1C), these results suggest that HT-1080 cells use PI3K signaling downstream of the LPA_1-EGFR axis for invadopodia production only under hypoxic conditions. To support this interpretation, we compared the

effect of PI3K inhibition in LPA_1- or LPA_4-knockdown HT1080 cells. Results showed that inhibition of the PI3K pathway in hypoxic LPA_1-knockdown cells did not affect invadopodia production whereas Rap1 inhibition resulted in a strong inhibitory effect (Fig 6D). Conversely, in LPA_4-knockdown cells, PI3K inhibition decreased invadopodia production whereas Rap1 had no effect (Fig 6E), which is in line with our previously published results (11). These findings clearly indicate that invadopodia production in hypoxic cells requires PI3K signaling downstream LPA1.

A major effector downstream of PI3K, Akt, has been reported to be implicated in invadopodia generation as well as production of several matrix metalloproteases important for their ability to degrade the extracellular matrix (27,28). To confirm activation of the PI3K/Akt pathway under hypoxic conditions, Akt phosphorylation was assessed by Western blotting. Results showed that Akt was phosphorylated in response to LPA after 30 min, whereas a sustained phosphorylation from 1 h up to 6 h was observed following hypoxic stimulation (Fig 6F,G). Furthermore, addition of the LPA_1 inhibitor Ki16425 or the EGFR inhibitor AG1478 resulted in a reduction of Akt phosphorylation induced by 1 h or 6 h of hypoxic stimulation (Fig 6 H,I). These results suggest that PI3K/Akt activation under hypoxia is mediated by LPA_1-induced transactivation of EGFR, an event that promotes invadopodia production.

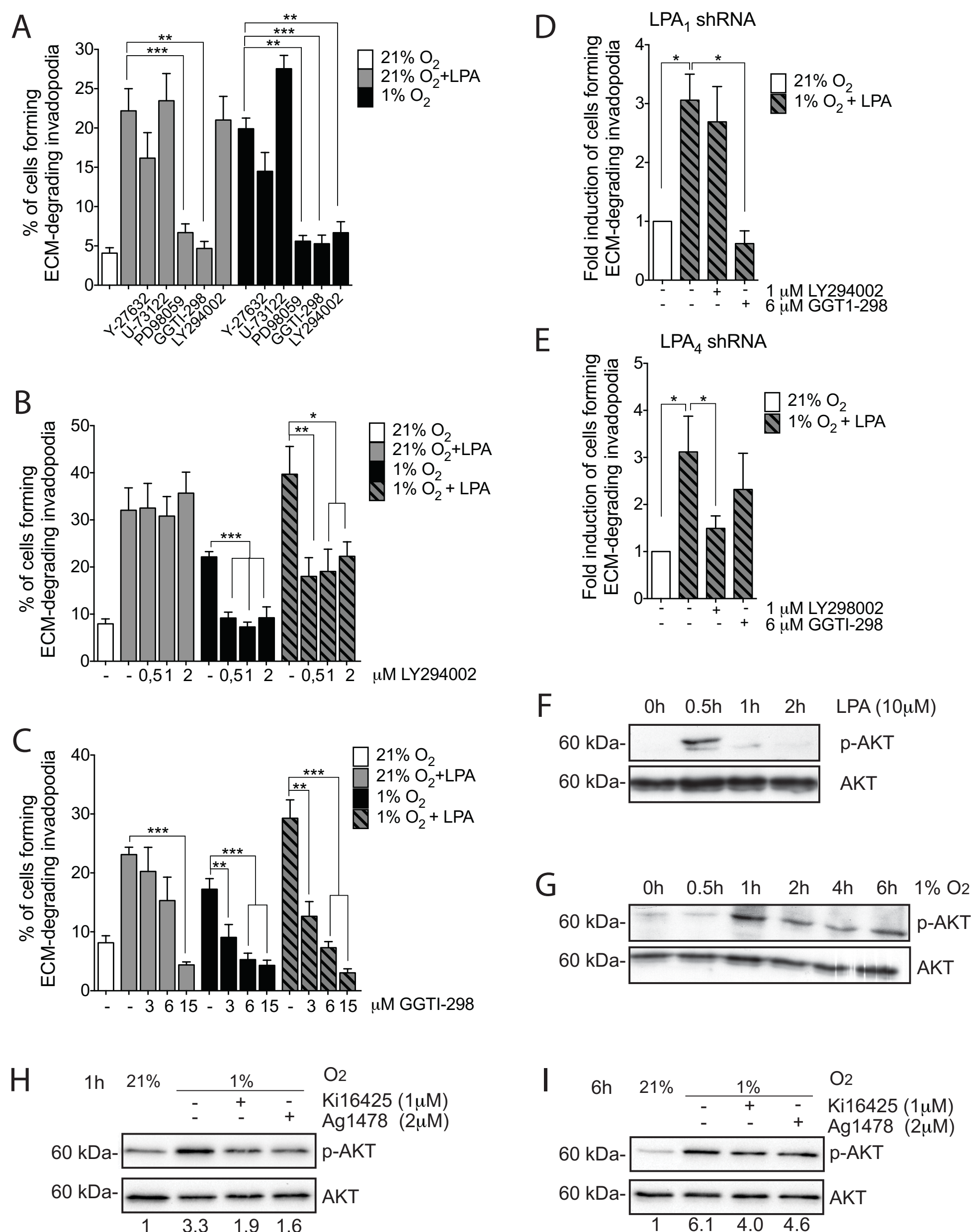


Figure 6 LPA_1 signals through PI3K/AKT to promote hypoxia induced invasion, an effect mediated by transactivation of EGFR.

(A-C) HT1080 cells were cultured on fluorescently-labeled gelatin for 10 h under 21% O_2 or 1% O_2 with or without addition of LPA (10 μM) and the following inhibitors of, (**A**) ROCK (Y-27632 2 μM), PLC (U-73122 0.5 μM), MEK (PD 98059 2 μM), Rap1 (GGTI-298 6 μM) or PI3K (LY294002 0.5 μM) or (**B**) a dose-response of the cells treated with LY294002 or (**C**) GGTI-298. The percentage of cells forming ECM-degrading invadopodia is shown (n ≥ 3 independent experiments). (**D-E)** GGTI-298 (6 μM) or LY294002 (1 μM) were added to cells expressing **(D)** LPA_1-directed shRNA or **(E)** LPA_4-directed shRNA and incubated in 1% O_2 in the presence of LPA (10 μM), or in 21% O_2. The percentage of cells forming ECM-degrading invadopodia is shown (n = 4). (**F-G)** Western blots of cell lysates

from HT1080 cells incubated with (**F**) LPA (10 μM) or in (**G**) 1% O_2 for 0 to 6 h. Representative blots of phospho-Akt (p-Akt) and total Akt are shown. (**H-I)** Western blots of p-Akt and total Akt in HT1080 cells incubated under 21% O_2 or 1% O_2 with addition of Ki16425 (1 μM) or AG1478 (2 μM) for (**H**) 1 h or (**I**) 6 h. Blots are representative of 3 independent experiments. Results of densitometric analysis of p-Akt/Akt ratios are shown below the blots. Bars represent the mean ± SEM. The asterisks (*) correspond to $P < 0.05$ (*), $P < 0.01$ (**) and, $P < 0.001$ (***).

A combined inhibition of LPA_1 and EGFR reduces metastasis in an *in vivo* avian model

To assess whether our observations that hypoxia promoted cell invasion through crosstalk between LPA_1 and EGFR were relevant to cancer metastasis therapy *in vivo*, we used an *ex ovo* chorioallantoic membrane (CAM) xenograft model in chicken embryos (29). Since HT1080 tumors grown in the CAM assay had been previously shown to develop hypoxia (17), the CAM model allows us to evaluate the effects of LPAR and EGFR receptor inhibition on spontaneous metastasis arising from tumors containing hypoxic areas. First, CAM bearing HT1080 tumors (grown for 6 days) were injected with pimonidazole. After 30 min, the tumors were excised and we determined the presence of phospho-Y845-EGFR within hypoxic zones of the tumors. Results showed a significant increase in phospho-Y845-EGFR staining within pimonidazole-positive regions of the tumors (Fig7A). This finding was consistent with increased EGFR phosphorylation that was observed in hypoxic cells (Fig 3A-C). Next, we analyzed the effect of targeting LPA_1 alone or in combination with EGFR inhibition using the EGFR inhibitor (AG1478) and/or AM095, a potent and selective LPA1 inhibitor used for *in vivo* studies (30). HT1080 cells, in the presence of increasing concentrations of AM095 or AG1478, were inoculated in 9-day-old CAMs. A time period of 6 days was allowed for tumors to develop, livers were then removed and spontaneous metastasis (primate Alu repeats by qPCR) was measured (18). Results showed

a dose-dependent decrease in the numbers of metastatic cells found in chick embryo livers with maximal effects observed at a concentration of 10 μM of AM095 and 10 μM of AG1478 (Fig 7B,C). To define whether these inhibitors can provide therapeutic gain when combined, tumors were treated with suboptimal concentrations of AM095 (0.2 μM) and AG1478 (0.2 μM). A synergistic effect on metastasis was observed with a combined inhibition of EGFR and LPA_1 (Fig 7D). We next analyzed the levels of phospho-EGFR in hypoxic zones of tumors treated with the above inhibitors. Maximum EGFR inhibition drastically decreased EGFR phosphorylation, as expected. Similarly the maximum dose of AM095 decreased p-EGFR, confirming its role in suppressing hypoxia-induced EGFR phosphorylation as observed in *in vitro* assays (Fig7E). Finally, suboptimal concentrations of both inhibitors induced a strong reduction of EGFR phosphorylate in hypoxic zones of tumors, similar to the diminution observed with the highest concentration of EGFR inhibitor (Fig 7 F-G)

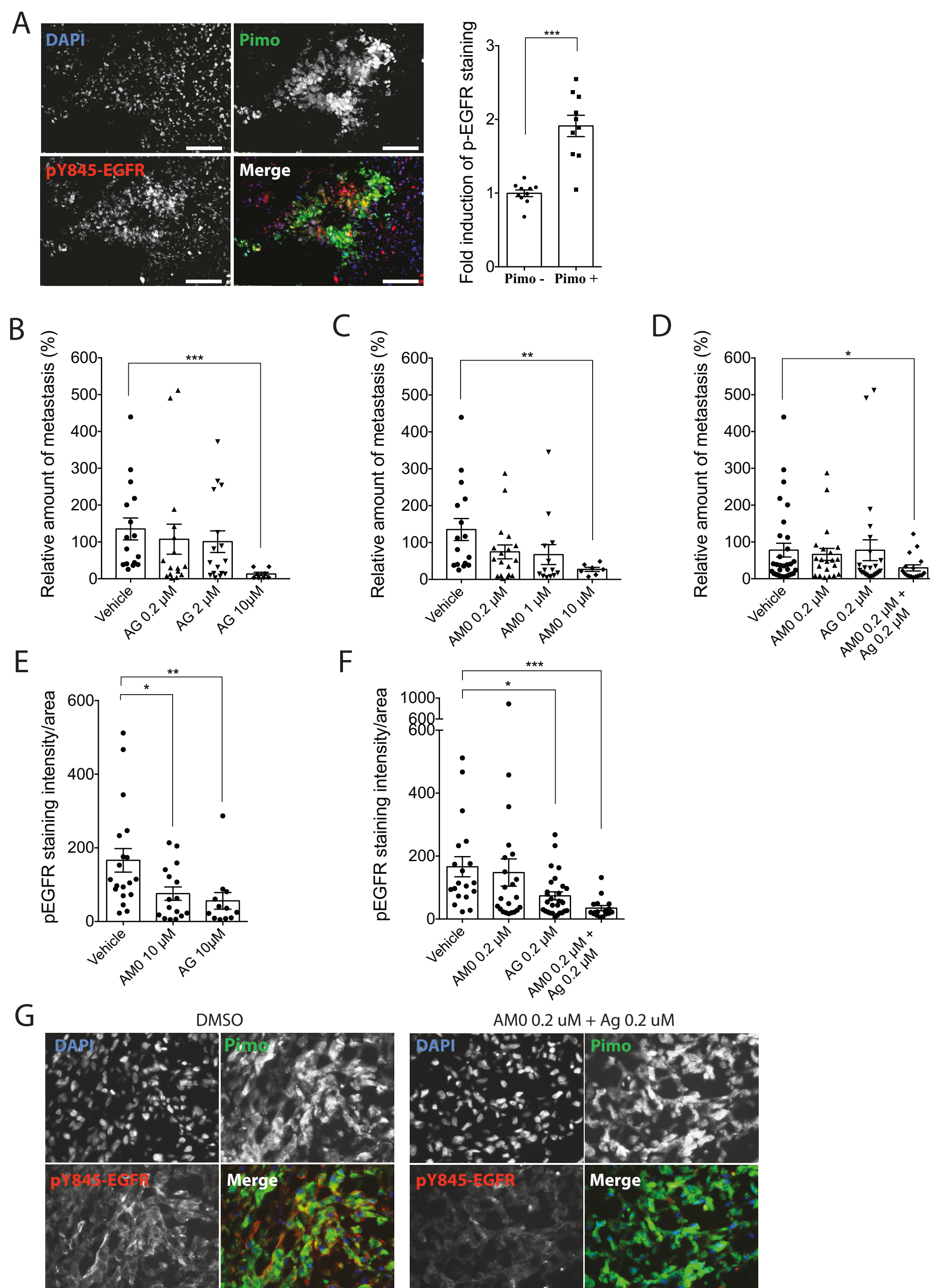


Figure 7 LPA$_1$ and EGFR are implicated in spontaneous metastasis in a chorioallantoic membrane.

(A) Representative immunohistochemistry images of HT1080 xenograft tumors showing hypoxic regions (Pimonidazole (Pimo); green) and phospho-EGFR (red) staining. Nuclei were stained with DAPI (blue). Scale bars correspond to 100 µm. The associated graph shows phospho-EGFR relative labeling intensities in Pimo+ and Pimo-zones of tumors. **(B-G)** HT1080 cells (3 x 10^5) were inoculated in the presence/absence of the LPA$_1$ inhibitor

AM095 or the EGFR inhibitor AG1478, to the CAM of chick embryos. Embryonic livers were harvested after 6 d of tumor growth for genomic DNA extraction and tumors were processed for immunohistochemistry. The ability of HT1080 cells to disseminate in embryonic livers was quantitated as the relative amount of metastasis normalized to the amount of host DNA. Dose response to, (**B**) AG1478, (**C**) AM095 and, (**D**) a combined treatment with AM095 (0.2 μM) and Ag1478 (0.2 μM). (**E,F**) Relative labeling intensities of phospho-EGFR in hypoxic (pimo+) zones of tumors treated with (**E**) AM095 (10 μM) or AG1478 (10 μM) or, (**F**) a combined treatment with AM095 (0.2 μM) and Ag1478 (0.2 μM). (**G**) Representative immunohistochemistry images of HT1080 xenograft tumors showing phospho-EGFR staining in hypoxic (Pimo+) zone of tumors treated with AM095 (0.2 μM) and Ag1478 (0.2 μM). Hypoxic regions (Pimonidazole (Pimo); green) and phospho-EGFR (red) staining. Nuclei were stained with DAPI (blue). Scale bars correspond to 50 μm. Bars represent the mean ± SEM. The asterisks (*) correspond to $P < 0.05$ (*), $P < 0.01$ (**) and, $P < 0.001$ (***).

DISCUSSION

In this study, we uncovered an essential role for LPA_1 in hypoxia-induced invadopodia production and that this event was dependent on Src-mediated cross-communication with EGFR. Furthermore, we found that hypoxic cells use PI3K/AKT signaling downstream of the LPA_1-EGFR axis as a dominant invadopodia-inducing signaling node. Tumor xenograft experiments further support the requirement of LPA_1 and EGFR for *in vivo* metastasis, and suggest that targeting both receptors could be an effective strategy to improve metastasis.

Hypoxia, a prevalent feature of the tumor microenvironment, is a potent inducer of cancer cell aggressiveness, invasion and metastasis. However, the nature of the mechanisms involved in these processes remains to be fully understood (3). Although there have been a few clues that hypoxia and LPA signaling are intertwined (23,31,32), the observations reported here are, to the best of our knowledge, the first study that identifies LPA_1 as the main receptor involved in invadopodia production triggered by the hypoxic microenvironment. The ability to establish the role of a particular LPAR is of importance since these receptors can have redundant or opposing effects on cell motility and cell

invasion. These cellular properties are cell-type and context-dependent, suggesting that the therapeutic use of pan-LPAR antagonists ought to be considered with caution. For example, both LPA_1 and LPA_3 can be positive or negative regulators of cell motility and invasion, depending on the cell type involved (19). The LPA receptor LPA_4 has also been shown to inhibit cell motility and invasion in mouse embryonic fibroblasts while promoting invasion and metastasis in human fibrosarcoma cells (11,33). Our findings of the common use of LPA_1 in a predominant tumor microenvironment highlight the potential benefit of LPA_1-directed therapy to fight cancer metastasis and progression.

A discrepancy between LPA receptor gene expression levels and usage led us to investigate the influence of hypoxia on LPA_1 expression and activity. Long-term exposure to hypoxia has been shown to increase LPA_1 expression in retinal ganglion cells (23), and to induce overactivation of LPA_1 in fetal brain through downregulation of the G-protein regulatory kinase GRK2 (34). To our surprise, exposure of the cells to hypoxia for an extended period of time (16 h) did not modulate mRNA or protein expression of LPA_1 or GRK2. These findings prompted us to explore alternatives for the common use of LPA_1 under hypoxia. We found that the RTK, EGFR, was transactivated by LPA_1 leading to invadopodia production solely under hypoxia (as compared to normoxia) suggesting that receptor crosstalk is a mechanism by which LPA_1 promotes invadopodia in this condition.

LPA_1-mediated transactivation of EGFR is in concordance with previous studies that identified both ligand-dependent and ligand-independent mechanisms of LPA-induced transactivation, depending on cell type and physiological context (35). Under hypoxic conditions, we found a ligand-independent mechanism of EGFR transactivation that involved Src kinase-mediated phosphorylation of tyrosine-845 (Y845) on EGFR. Phosphorylation of this tyrosine residue has been associated with survival, proliferation and

malignancy of various cancer cells, including glioma and breast cancer cells, as well as being a highly predictive value for prognosis correlating with worse prognosis and poor response to chemotherapy (36). In addition, colocalization of p-EGFR and hypoxic markers in human tumor biopsies was associated with a critical subpopulation prone to local recurrence, metastasis formation or treatment resistance (37). The finding that Y845-EGFR is phosphorylated in response to LPA_1 activation under hypoxia *in vitro* and localized in hypoxic zones of tumors suggests the possibility that this could be used, in conjuncture with hypoxic markers, as predictive tools of the clinical outcome of LPA_1- and EGFR-directed therapy.

We also uncovered PI3K/Akt signaling as an effector of LPA_1 and EGFR crosstalk that lead to invadopodia production in hypoxia. Acting through Akt, PI3K has previously been implicated in the regulation of invadopodia formation, as well as their ability to degrade the extracellular matrix (27). Although our findings showed that PI3K/Akt signaling was only involved in hypoxia-induced invadopodia production, we also observed phosphorylation of Akt in response to LPA under normoxic conditions. This apparent discrepancy may be partly explained by the fact that Akt phosphorylation in normoxic cells was transient with a peak effect reached after 30 min of LPA stimulation. In contrast, we observed a sustained activation (up to 6 h) in response to hypoxia. Although few publications have directly compared the effects of acute versus sustained Akt phosphorylation, Goel *et al.* found differential effects on G1 progression, suggesting distinct consequences on biological responses (38). Furthermore, sustained Akt phosphorylation has been associated with enhanced tumor survival as well as increased cytoskeletal rearrangement and migration (39,40), suggesting that under hypoxic

conditions, this event also plays a role in invadopodia formation, as these structures require actin remodeling (5).

The effect of hypoxia on Src-mediated EGFR-LPA_1 crosstalk and sustained Akt phosphorylation may be due to relocalization of both receptors and Src in specific subcellular domains such as lipid rafts, thus facilitating their interactions. Hypoxia has been reported to rapidly increase cellular cholesterol levels, thereby influencing the composition, number and size of lipid rafts (41). Lipid rafts function as organizing centers for the assembly of signaling molecules, their increased rigidity optimizes spatiotemporal interactions and compartmentalizes proteins involved in specific signaling tasks (42,43). Interestingly, both RTKs and GPCRs can localize to lipid rafts, which provide a platform that facilitates interactions between EGFR and GPCRs, therefore enhancing the efficiency of transactivation (44). Lipid rafts have also been identified as key plasma membrane microdomains for Src-EGFR functional interactions as well as PI3K/Akt activation (42,45). It is therefore conceivable that, under hypoxic conditions, the recruitment of LPA_1, Src, and EGFR in lipid rafts facilitates their interactions and that results in prolonged Akt activation and invadopodia formation. This possibility is in keeping with the finding that lipid rafts and caveolin-1 are required for invadopodia production in breast cancer cells (46).

A combination of suboptimal doses of inhibitors revealed a synergistic effect between LPA_1 and EGFR inhibitors in blocking metastasis. Combination therapy using lower doses of inhibitors are of clear clinical interest in order to overcome toxicity and resistance, two major issues associated with the use of RTK-targeted therapies (47). It has been suggested that resistance to RTK inhibitors is linked to activation or crosstalk with receptors that could include IGF-1R, c-MET, and LPARs (EDGRs) (48). More specifically, blocking $LPA_{1/3}$ receptors with Ki16425 has been shown to prevent and delay resistance to

the RTK inhibitor Sunitinib® *in vivo* (49), whereas inhibiting Src or Y845EGFR phosphorylation reverses resistance to anti-EGFR monoclonal antibodies (50). These results suggest that blocking LPA_1-induced transactivation of EGFR could also overcome resistance to EGFR inhibitors. In addition, EGF has been shown to increase LPA production, suggesting there can be bidirectional crosstalk between LPA and EGF receptors (51). Pre-clinical studies that combine the therapeutic potential of LPA_1 and EGFR in various types of solid tumors should therefore be a valuable research direction.

In conclusion, our study provides insight into a mechanism of hypoxia-induced cell invasion, which implies a major role for LPA_1 in activating two major players in tumorigenesis: Src and EGFR. Targeting LPA_1, alone or in combination with EGFR inhibitors, could benefit cancer patients by blocking upstream inducers of invadopodia, a key component of the metastatic process. Since metastasis remains the most deadly aspect of cancer, efficient interference with this process is of the utmost importance to increase cancer patient well-being and survival.

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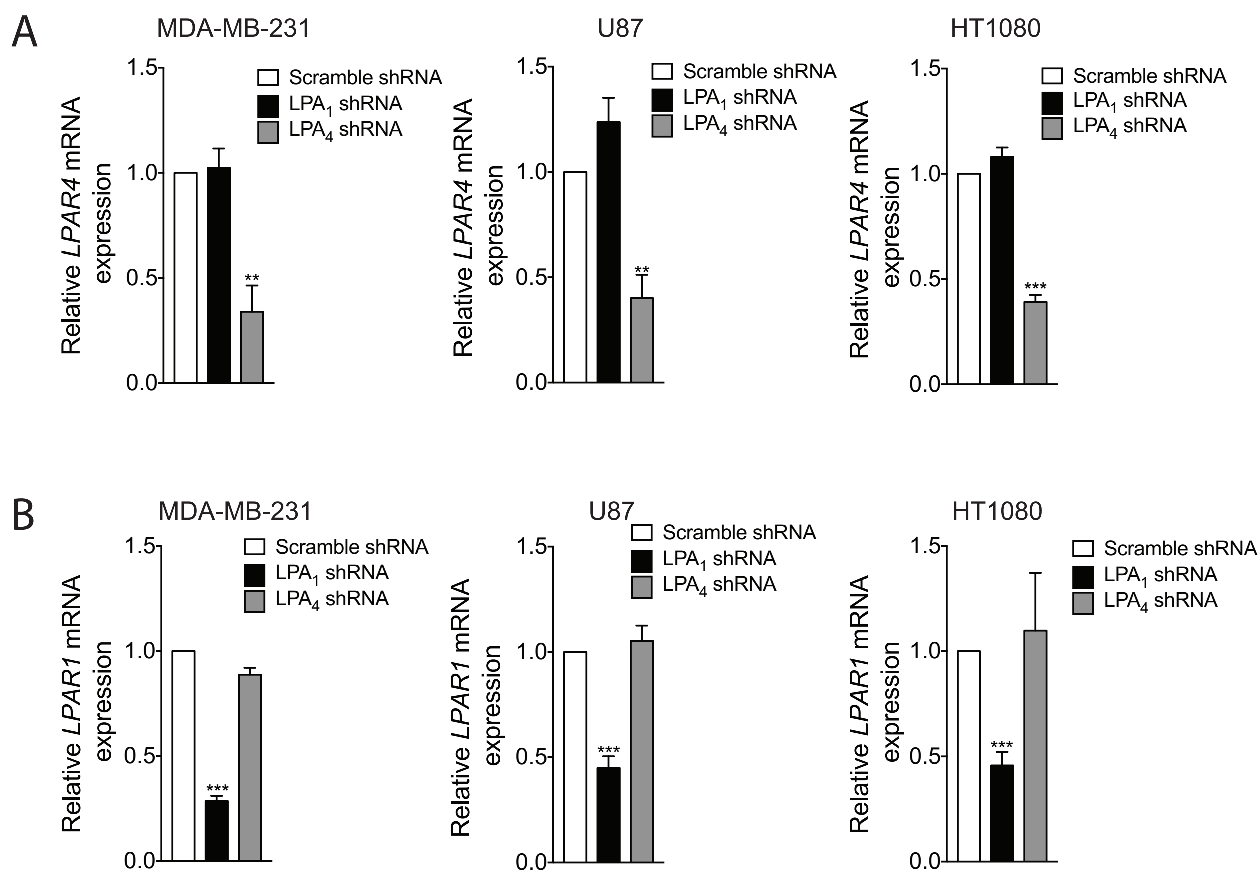
Author contributions

KH performed the majority of the experiments and interpretation of the data. KH and CMD planned the experiments and wrote the manuscript. RRL performed some of the experiments involving Western blotting of phospho-proteins. MC performed acquisition and quantification of CAM tumor images. KBG participated in acquisition of data from the CAM assays and revision of the manuscript. CMD supervised the study. All authors approved the final version of the manuscript.

Supplementary Materials & Methods

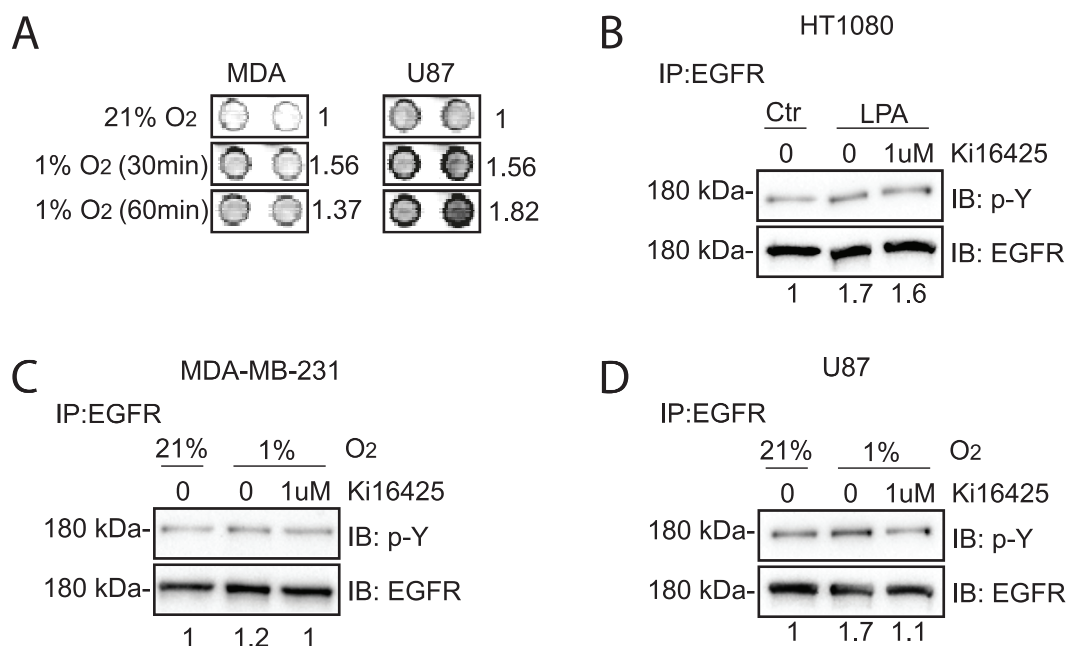
Real-time RT-PCR. The following primer pairs were selected for LPA_1: (forward) 5'-AATCGAGAGGCACATTACCGG-3', (reverse) 5'-TGTGGACAGCACACGTCTAG-3'; LPA_2: (forward) 5'-CATCATGCTTCCCGAGAACG-3', (reverse) 5'-GGGCTTACCAAGGATACGCAG-3', LPA_3: (forward) 5'-TCGCGGCAGTGATCAAAAACAGA-3', (reverse) 5'-ATGGCCCAGACAAGCAAAATGAGC-3'; LPA_4: (forward) 5'-AAAGATCATGTACCCAATCACCTT-3', (reverse) 5'-CTTAACAGGGACTCCATTCTGAT-3'; EGFR: (forward) 5'-CAAGGAAGCCAAGCCAAATG-3' , (reverse) 5'-CCGTGGTCATGCTCCAATAA-3' ; Src: (forward) 5'-GCTTGTGGGTGATGTTTGAC-3', (reverse) 5'-CTGGACTCTTGGCTCTTCTATG-3' ; ADAM17: (forward) 5'-CGTGGTGGTGGATGGTAAA-3' , (reverse) 5'-ATGTGGGCTAGAACCCTAGA-3' ; ADAM12: (forward) 5'-TCTGGACTGGAGGAAGATGAA-3' , (reverse) 5'-GATGGTGGTCCCTTGGAAATAA-3' ; CAIX: (forward) 5'-CCTCAAGAACCCCAGAATAATGC-3', (reverse) 5'-CCTCCATAGCGCCAATGACT-3'; GRK2: (forward) 5'-CTTCCAGCCATACATCGAAGAG-3', (reverse) 5'-CCGTGTGAACTTATCGCTCTC-3'; and housekeeping gene RPLPO: (forward) 5'-GATTACACCTTCCCACTTGC-3', (reverse) 5'-CCAAATCCCATATCCTCGTCCG-3'.

Supplementary Figures

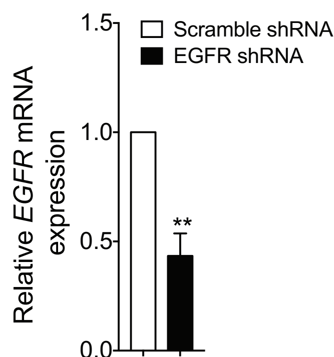


Supplementary Figure 1. **qPCR assessment of LPA receptor shRNA-mediated knockdown**

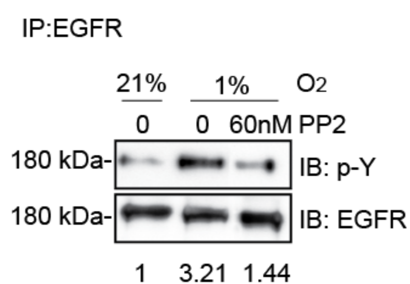
MDA-MB-231, U87, or HT1080 cells were transfected with scramble, LPA_1 or LPA_4 targeted shRNAs. mRNA levels of **(A)** *LPAR4* or **(B)** *LPAR1* were quantified by qPCR. *RPLP0* was used as a reference gene (n = 3). Bars represent the mean ± SEM. The asterisks (*) correspond to $P < 0.01$ (**) and, $P < 0.001$ (***).



Supplementary Figure 2. EGFR is transactivated by LPA_1 under hypoxic conditions. **(A)** MDA-MB-231 or U87 cells were incubated under 21% O_2 or 1% O_2 for 30 or 60 min and cell lysates were subjected to phospho-RTK array analysis. (**A**) One representative array showing phospho-EGFR is shown with results from densitometric analysis (in fold) shown beside the array. (**B-D**) HT1080, MDA-MB-231 or U87 cell lysates were immunoprecipitated using an anti-EGFR antibody and immunoblotted with anti-phospho-tyrosine (pY) and total EGFR antibodies. Densitometric analysis of p-EGFR/EGFR ratio are shown below the blots. (**B**) One representative blot showing LPA (10 µM) stimulation in 21% O_2 in the presence/absence of Ki16425 (1 µM) in HT1080 cells. (**D-E**) One representative blot showing hypoxic stimulation (30 min) in the presence/absence of Ki16425 (1 µM) in MDA-MB-231 (**C**) and U87 (**D**) cells.



Supplementary Figure 3. **qPCR assessment of shRNA-mediated knockdown of EGFR.** HT1080 cells were transduced with scramble or EGFR-targeted shRNA mRNA levels of *EGFR* were quantified by qPCR using *RPLP0* as a reference gene (n = 3). Bars represent the mean ± SEM. The asterisk (**) corresponds to P < 0.01.



Supplementary Figure 4. Src inhibition blocks EGFR phosphorylation in hypoxia. Representative immunoblot of HT1080 incubated in 21% O_2 or 1% O_2 in the presence or absence of the Src inhibitor, PP2. Cell lysates were immunoprecipitated using an anti-EGFR antibody and immunoblotted with anti-phosphotyrosine (pY) and EGFR antibodies. Densitometric analysis of p-EGFR/EGFR ratio are shown below the blots.

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3. CHAPTER 2: HYPOXIA PROMOTES INVASION THROUGH REGULATION OF LPA SYNTHESIS AND DEGRADATION ENZYMES

Article 1 presented in this thesis demonstrated that under hypoxia cancer cells rely on LPA_1 signaling for the production of invadopodia and metastasis. In the Article we uncovered a signaling pathway that included transactivation of EGFR by LPA_1 exclusively under hypoxia, however exactly why this occurs solely in hypoxic cells remains to be determined. Signaling by LPA is known to be affected by the amount of LPA available to interact with receptors. Therefore in chapter 2 of this thesis, our goal was to gain a more complete understanding of the regulation of LPA signaling by hypoxia in cancer cells by studying the effects of hypoxia on the enzymes involved in controlling LPA levels that could modulate LPAR signaling. LPA production is mainly regulated by ATX, which promotes tumor progression and whose expression is upregulated in various cancers. In contrast, important LPA degrading enzymes, the LPPs, are downregulated in cancer cells and play a negative role in tumor progression. Yet, the factors involved in shaping the differential expression of these LPA regulatory components remain unknown. In the following chapter we first investigated the influence of hypoxia on ATX and LPP mRNA and protein expression in various cancer cell lines. We further determined their involvement in hypoxia-induced invadopodia production using invadopodia assays and shRNA targeting ATX or LPP1/2/3. Finally we studied the localization of these LPA-producing and -degrading enzymes in hypoxic cells by confocal microscopy.

3.1 MATERIALS and METHODS

3.1.1 Reagents

1-oleoyl-sn-glycerol-3-phosphate 18:1 (LPA) sodium salt and 1-oleoyl-*sn*-glycero-3-phosphocholine 18:1 (LPC) were from Sigma-Aldrich (St. Louis, MO). Plasmid ATX cDNA construct was a kind gift from Dr. Tim Clair (Center for Cancer Research, NCI, NIH). shRNA against ATX or scramble (ctr) shRNA was from SABiosciences (Frederick, MD). Mission lentiviral shRNA targeting LPP1 (TRCN0000010720 and TRCN0000002579), LPP2 (TRCN0000002583 and TRCN0000002584), LPP3 (TRCN0000358710 and TRCN0000358709), or a scramble sequence, were from Sigma-Aldrich. ATX, LPP1 and LPP3 antibodies were from Abcam (Cambridge, MA). The anti-tubulin antibody was from Sigma-Aldrich. Texas Red phalloidin, DAPI (4',6-diamidino-2-phenylindole), the lipophilic tracer DiD, and all secondary antibodies were from Invitrogen (Molecular Probes, Eugene, OR). Fibrillar collagen type I was from R&D Systems (Minneapolis, MN).

3.1.2 Cell culture and transfection

HT1080 human fibrosarcoma, U87 human glioblastoma, and MDA-MB231 human breast cancer cell lines were cultured as described in article 1. In the case of stable transfections, with ATX, pcDNA3.1 or shRNA against ATX or scramble sequence, cells were seeded at a density of 1 x 10^5 cells per well in a 6-well culture plate the day before the transfection. Transfections were performed with the Fugene reagent from Roche Diagnostics (Mannheim, Germany), according to the manufacturer's protocol. Stable transfectants were obtained by antibiotic selection, G418 600µg/ml (Gibco) for ATX and pcDNA3.1 transfections and Puromycin 2µg/ml (Invivogen, San Diego, CA) for all shRNA transfections. For lentiviral transductions, with LPP1, LPP2, LPP3 or scramble shRNA cells were seeded at a density of 3 x 10^5 cells per 10 cm^2 Petri dish and infected with 1 ml of viral stock in 2 ml of optiMEM supplemented with 2 µl Polybrene (10 mg/ml) (EMD Millipore, Etobicoke, ON). Viral particles were generated by transient transfection of 293T cells using a ViraPower lentiviral expression system (Invitrogen Thermo Fisher Scientific, Burlington, ON).

3.1.3 Real time RT-PCR

Total RNA was isolated using the TRIzol (Invitrogen, Carlsbad, CA) protocol as previously described (Harper et al., 2010) and 1ug of RNA was reverse transcribed to complementary DNA (cDNA) using a QuantiTect reverse transcription kit (Qiagen, Mississauga, ON). cDNA was then analyzed by real time PCR using a hot start SYBR Green qPCR master mix (BiMake, Houston, TX). The following primer pairs were selected for:
CAIX: (forward) 5'-CCTCAAGAACCCCAGAATAATGC-3',
(reverse) 5'-CCTCCATAGCGCCAATGACT-3'; ATX: (forward)
5'-TGAAACAGCACCTTCCCAAA-3', (reverse) 5'-CCAAAGGTTTCCTTGCAACA-3';
LPP1: (forward) 5'-GTCGAGGGAATGCAGAAAGA-3', (reverse)
5'- CCTTCATCCTGGCTTGAAGATA-3';
LPP2: (foward) 5'- CCTACCGTCCAGATACCATCA-3',
(reverse) 5'- GTTGAAGTCCGAGCGAGAATAG-3';
LPP3: (forward) 5'- CAAATCAGAAGGAGCCAGAGAA-3',
(reverse) 5'- CAGCAAGAGCAACTCCTACAA-3'; and housekeeping gene RPLP0: (forward) 5'-GATTACACCTTCCCACTTGC-3', (reverse)
5'-CCAAATCCCATATCCTCGTCCG-3'. Quantitative Real-Time PCR was performed on a Rotor-Gene 3000 (Corbett Research, Kirkland, QC, Canada). The cycling program was as follows: initial denaturation at 95ºC for 15 min, 35 amplification cycles with annealing Tº of 59ºC for 30 s and final extension at 72ºC for 30 s. Results were calculated as $2^{\Delta\Delta CT}$.

3.1.4 Western blotting

Western blotting was performed as described in article 1.

3.1.5 Invadopodia assay

Coverslips were prepared as previously described (Harper et al., 2010), using Oregon-Green^{488}-conjugated gelatin (Invitrogen, ON). Thirty thousand cells were seeded on each coverslip and allowed to adhere. Following various incubation times as described within the figure legends, cells were fixed with 2% paraformaldehyde for 10 min at room temperature. Nuclei were stained with DAPI and F-actin was stained using Texas-Red-conjugated phalloidin. Cells were visualized by fluorescence microscopy using a Zeiss Axioskop fluorescence microscope. Cells forming ECM-degrading invadopodia were

identified based on cells with at least one F-actin-enriched area of matrix degradation (characterized by loss of green fluorescence). Three fields of 100 cells (magnification 40X) were counted per coverslip to quantify the percentage of cells forming ECM-degrading invadopodia. To quantify the areas of degradation, pictures of fluorescent gelatin were acquired and captured into ImagePro imaging software (MediaCybernetics) and degradation areas were calculated in pixels for a total of at least 20 cells per coverslip.

3.1.6 Immunofluorescence

Cells were grown on non-fluorescent gelatin coated coverslips and fixed with 2% paraformaldehyde in PBS for 10 min at room temperature. Where indicated cells were permeabilized with 0.05% saponine (Sigma-Aldrich) in PBS for 20 min and blocked with 2% BSA in PBS for 30 min. Then, cells were incubated with the appropriate primary antibodies, for 2h, and secondary antibodies, for 1h or fluorescent phalloidin, for 45 min, as indicated within the Figure Legends. Images were taken with a FV1000 scanning confocal microscope (Olympus, Tokyo, Japan) coupled to an inverted microscope using a 63x oil immersion objective. For calculation of the number of invadopodia per cell, cells were incubated with anti-cortactin antibody and Texas Red phalloidin. Colocalization of actin and cortactin was visualized using an Axioskop 2 phase-contrast/epifluorescence microscope and 20 cells were counted per condition (Carl Zeiss, Inc., Thornwood, NY).

3.1.7 3D invasion assay

Collagen type I 3D matrix was prepared as follows: Aliquots (50 µL) of Agarose containing 10% FBS were deposited in a 96 well culture plate. Aliquots (50 µL) of fibrillar collagen type I (R&D Systems, Minneapolis, MN) were then prepared following manufacturer's instructions and layered on top of the Agarose. Cells ($2x10^4$/100µl in serum-free MEM) were deposited on top of the collagen gel and incubated for 24 h. The cells were then labeled with CellTraceTM calcein green AM (Invitrogen) 1h prior to the end of incubation. Cells were then washed with PBS and fixed with 3% glutaraldehyde for 30 minutes followed by confocal microscopy analysis using a FV1000 Olympus confocal microscope. Collagen matrix pellets were scanned along the Z-axis. Cells that had invaded the collagen were imaged and quantitated at each 5µm layer within the gel.

3.2 RESULTS

3.2.1 Hypoxia induces ATX and represses LPP expression

Previously, we established that hypoxic cells use LPA_1 signaling to promote invadopodia production and metastasis. HT1080 cells, in particular, were found to use the LPA_1-Src-EGFR signaling path for invasion only under hypoxic conditions (see Article1). To gain a more complete understanding of the LPA signaling axis under hypoxia, we investigated whether hypoxia also has an effect on enzymes controlling LPA levels that could modulate LPAR signaling. As previous work from our laboratory has shown that both hypoxia and ATX can increase invadopodia production (Harper et al., 2010; Lucien et al., 2011), and ATX was found overexpressed in many cancer cell lines (Kehlen et al., 2004; Kishi et al., 2006; Yang et al., 2002, 1999), we investigated whether hypoxia could modulate ATX expression levels to drive invadopodia production. To address this question, we first investigated the hypoxic regulation of the LPA producing enzyme, ATX in three diverse cancer cell lines, HT1080 fibrosarcoma, U87 glioblastoma, and MDA-MB231 breast cancer. A significant increase in ATX (*ENPP2*) mRNA expression was observed following 8 or 16 hours of hypoxic stimulation in HT1080 cells. This was correlated with an increase in ATX protein levels at 16 hours (Fig 1 A-B). In the other cell lines tested, preliminary results indicated no significant modulation in ATX expression (Fig 1 C-D).

LPA degrading enzymes also play an important role in controlling LPA levels. Lipid phosphate phosphatases (LPPs), which are major LPA-degrading enzymes, have been found downregulated in various cancers (Tang et al., 2015). Therefore, we investigated whether hypoxia is a contributing factor to the decreased expression of LPPs observed in tumor cells. A small but significant inhibition of LPP1 (*PLPP1*) expression upon hypoxic stimulation was observed in the U87 and MDA-MB231 cell lines at 4h or 16h with no significant modulation in HT1080 at all time points tested (Fig 2 A-C). In contrast, LPP2 mRNA expression was not significantly inhibited in any of the cell lines tested, but rather had a tendency to be increased in hypoxic conditions, particularly in HT1080 and MDA-MB231 cells (Fig 2 D-F). Interestingly, hypoxia had a more pronounced effect on LPP3, significantly decreasing the mRNA expression levels of LPP3 by up to 40%, in all cell lines

tested (Fig 2 G-I). Together these results demonstrate that hypoxia increases the expression of the LPA-producing enzyme ATX and decreases the expression of LPA degrading enzymes LPP1 and LPP3, two events that could potentially lead to higher overall levels of LPA.

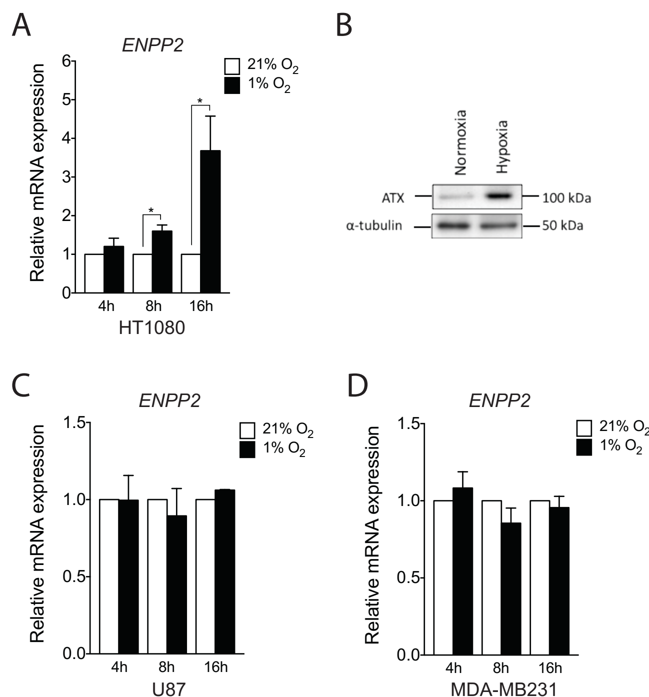

Figure 1 ATX expression is modulated in hypoxia

(A, B) HT0180 cells were incubated under normoxic (21%O_2) or hypoxic (1%O_2) conditions. **(A)** mRNA expression of *ENPP2* (Autotaxin) was evaluated by qPCR following 4, 8, or 16 hours of stimulation. RPLPO was used to normalize the data. N=5 **(B)** ATX protein levels were analyzed by western blotting after 16 hours of stimulation, with alpha-tubulin as a loading control. One representative blot is shown. **(C-D)** Cells were incubated under normoxic (21%O_2) or hypoxic (1%O_2) conditions for 4, 8 or 16 hours. mRNA expression of *ENPP2* (Autotaxin) was evaluated by qPCR in **(C)** U87 cells, or **(D)** MDA-MB231 cells. RPLPO was used to normalize the data. N=2. *Bars represent the mean ± SEM (* P< 0.05).*

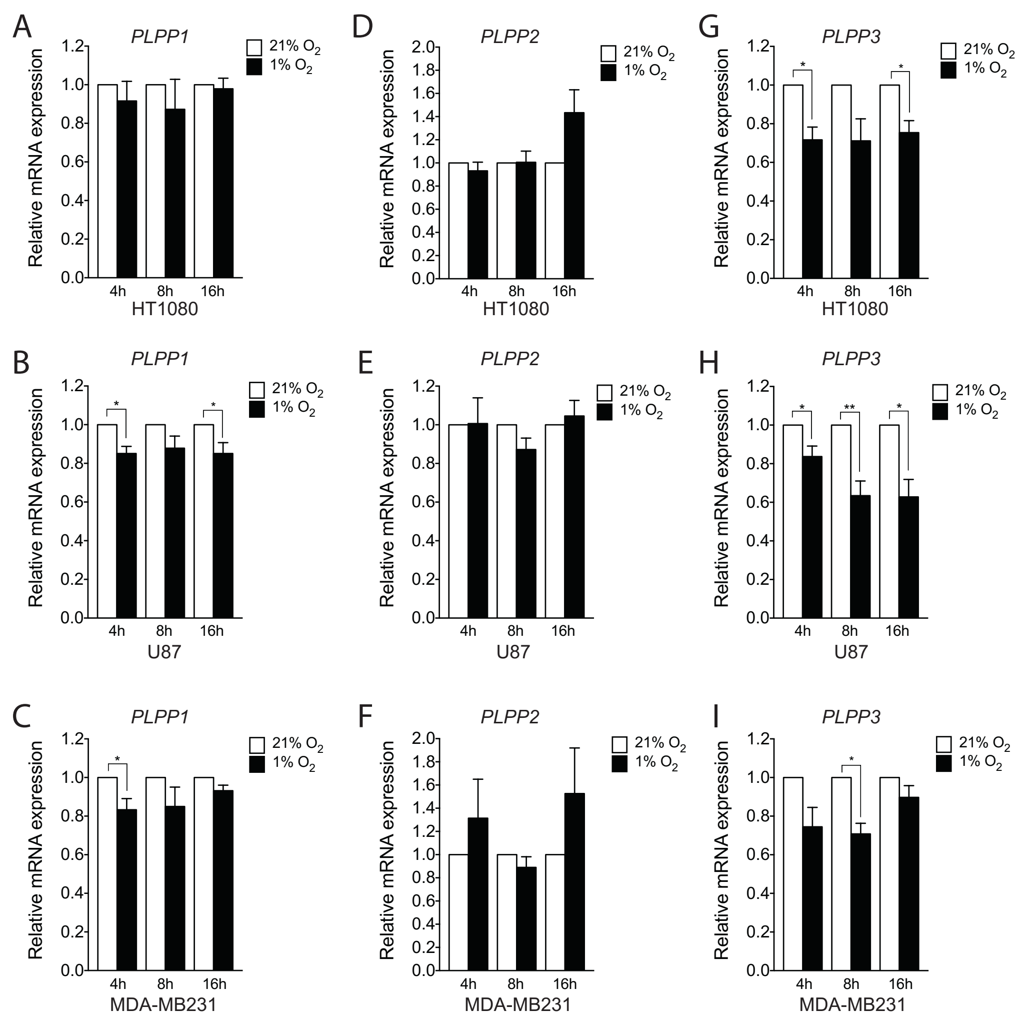

Figure 2 Lipid phosphate phosphatase expression is modulated in hypoxia

(A-I) Cells were incubated under normoxic (21%O_2) or Hypoxic (1%O_2) conditions for 4, 8 or 16 hours. mRNA expression of **(A-C)** *PLPP1* (LPP1), **(D-F)** *PLPP2* (LPP2) or **(G-I)** *PLPP3* (LPP3) was evaluated by qPCR in **(A,D,G)** HT1080, **(B,E,H)** U87, or **(C,F,I)** MDA-MB231 cells. RPLPO was used to normalize the data. N=3 or more. *Bars represent the mean ± SEM (* $P< 0.05$, ** $P< 0.01$).*

3.2.2 Activity of the LPA producing enzyme ATX is required for hypoxia-induced invadopodia production

Since ATX expression was increased in hypoxia, we next investigated its involvement in hypoxia-induced invadopodia production. To this aim, we first compared the effects of ATX overexpression to hypoxic stimulation on invadopodia production. Both ATX

overexpression and hypoxic stimulation induced an increase in the percentage of cells forming ECM-degrading invadopodia (Fig 3A). The effect of ATX overexpression was superior to hypoxia on the percent of cells forming invadopodia at both time points. Both stimuli also increased the number of invadopodia formed per cell and the area of degradation per cell (Fig 3B,C). While ATX overexpression induced slightly more invadopodia per cell, hypoxia on the other hand had a more important effect on the area of degradation per cell. These results indicate that ATX may have a role in invadopodia formation, while hypoxia may play a more important role in ECM degradation. Next, using ATX-targeted shRNA, we observed that ATX inhibition abolished invadopodia production induced by hypoxia suggesting an important role for ATX in hypoxia-induced invadopodia production (Fig 3D). Furthermore, under hypoxic conditions the addition of the product (LPA), but not the substrate (LPC) of ATX restored invadopodia production in ATX knockdown cells to the same level as in control cells (Fig 3E). These results demonstrate that exogenous LPA can override ATX inhibition, suggesting that the effects of ATX on hypoxia-induced invadopodia production are due to its ability to produce LPA.

3.2.3 Lipid phosphate phosphatases modulate invadopodia production

Since LPP1 and LPP3 expression levels were reduced in hypoxic cells, we sought to determine whether inhibiting their expression in normoxic cells could recapitulate some effects of hypoxia on invadopodia production. Results showed that LPP1 and LPP3 knockdown, by targeted shRNA, induced significant increases in the percentage of cells forming ECM-degrading invadopodia (Fig 4A). In contrast, LPP2-targeted shRNA had no effect on invadopodia production (Fig 4A), which is consistent with the lack of inhibition of LPP2 gene expression in hypoxia (Fig 2). These results suggest that LPP1 and LPP3 have an inhibitory role in invadopodia production, most likely through their ability to degrade LPA.

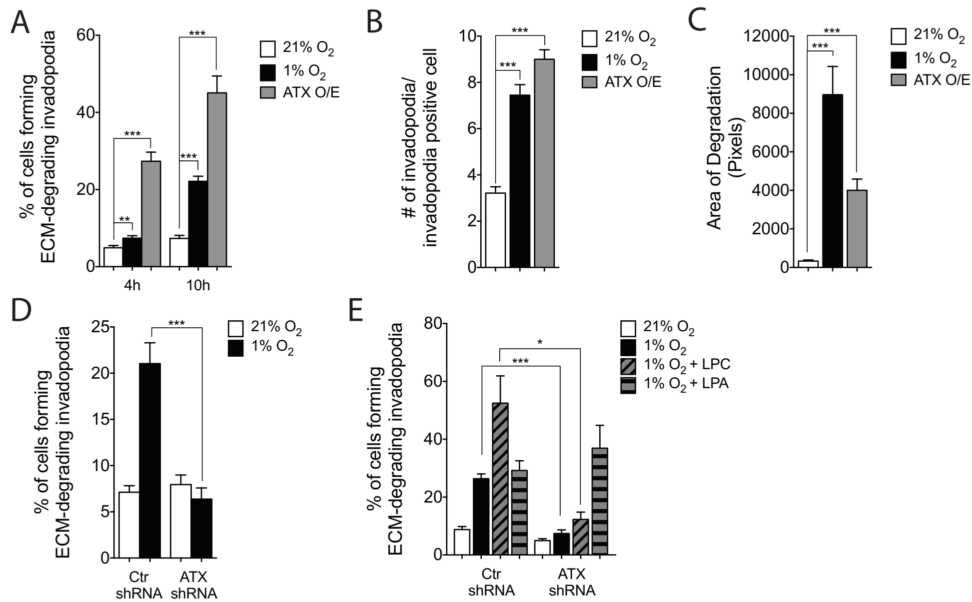

Figure 3 ATX is essential for hypoxia-mediated invadopodia production

(A) HT1080 cells or cells overexpressing ATX (ATX O/E) were cultured for 4h or 10h on fluorescently labeled gelatin in normoxia (21%O_2) or hypoxia (1%O_2). The percentage of cells forming ECM-degrading invadopodia is shown. N=5. **(B)** HT1080 cells or cells overexpressing ATX (ATX O/E) were cultured on non-fluorescent gelatin for 4h in normoxia (21%O_2) or hypoxia (1%O_2). Actin and cortactin were labeled by immunofluorescence and the number of invadopodia per cell was counted based on actin/cortactin colocalization. N=3. **(C)** HT1080 cells or cells overexpressing ATX (ATX O/E) were cultured for 10h on fluorescently labeled gelatin in normoxia (21%O_2) or hypoxia (1%O_2). Quantification of ECM degradation area per cell is shown. N=3. **(D-E)** Cells transfected with scramble (Ctr) or ATX targeted shRNA were cultured for 10h on fluorescently labeled gelatin. The percentage of cells forming ECM-degrading invadopodia is shown for cells cultured in **(D)** normoxia (21%O_2) or hypoxia (1%O_2), or **(E)** normoxia (21%O_2), hypoxia (1%O_2), hypoxia with LPC (1%O_2 + LPC), or hypoxia with LPA (1%O_2 + LPA). N=3. *Bars represent the mean ± SEM (* $P< 0.05$, ** $P< 0.01$, *** $P< 0.001$).*

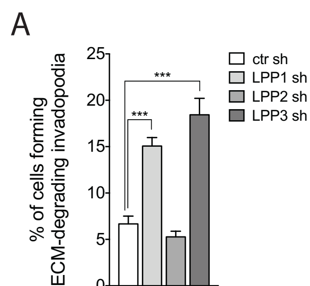

Figure 4 LPP1 and LPP3 contribute to invadopodia production

(A) HT1080 cells transduced with scramble (ctr), LPP1, LPP2, or LPP3 shRNA were incubated for 10h on fluorescently labeled gelatin in normoxia (21%O_2). The percentage of cells forming ECM-degrading invadopodia is shown. Results from two sets of shRNA for each of LPP1, LPP2, and LPP3 were combined. N=3. *Bars represent the mean ± SEM (*** P< 0.001).*

3.2.4 Hypoxia affects the spatial localization of ATX and LPPs

Results demonstrated that ATX knockdown exerted strong inhibitory effects on invadopodia production induced by hypoxia (Fig 3D), however, ATX expression was not affected in most cells lines tested with a somewhat late, 8-16h, induction in HT0180 cells (Fig 1 A-D). As discussed in the introduction, secreted ATX is known to bind to integrins or heparin sulfates on the cell surface, which may result in localized production of LPA close to LPA receptors (Fulkerson et al., 2011; Houben et al., 2013). Therefore, we sought to determine whether hypoxia modulates the cellular localization of ATX. First, HT1080 cells were permeabilized and stained for ATX and actin. Strong ATX staining, at what appears to be the leading edge of cells, was observed in hypoxia, compared to a more diffuse staining under normoxic conditions (Fig 5A). Furthermore, ATX was localized at or near the cell surface in these hypoxic cells, as seen in the z-axis view (Fig 5A). Because secreted ATX is recruited to the cell-surface, we also performed ATX staining in non-permeabilized cells. Co-staining of the cells with the lipophilic marker DiD and ATX showed localized ATX staining at the cell surface. In hypoxic cells, a prominent cell-surface staining was orientated towards the leading edge, while in normoxic cells less pronounced cell-surface staining of ATX was observed (Fig 5B). These results indicate that there is either an increased secretion of ATX and/or increased recruitment of ATX by cell-surface proteins in hypoxia.

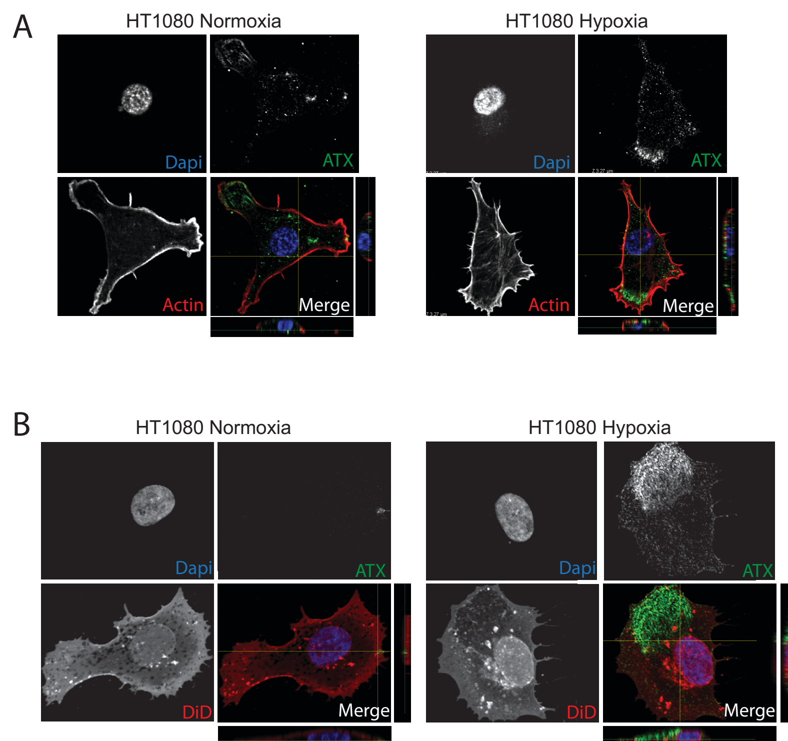

Figure 5 ATX localization is altered in hypoxic cells

(A-B) Representative immunofluorescence images of HT1080 cells cultured on non-fluorescent gelatin for 4h in normoxia (21%O_2) or hypoxia (1%O_2) are shown. **(A)** Representative images of cells permeabilized and stained for ATX (green) or F-actin (red). Nuclei were stained with DAPI (blue). Magnification 60X. **(B)** Representative images showing ATX (green) and DiD (red) staining in non-permeabilized cells. Nuclei were stained with DAPI (blue). Magnification 60X.

Given the changes in ATX localization observed in Figure 5, it was of interest to investigate if hypoxia also modulates the localization of the LPA degrading enzymes, LPP1 and LPP3. Furthermore, since there was no significant decrease in LPP1 expression in hypoxic HT1080 cells, but there was a significant increase in invadopodia production in LPP1 knockdown HT1080 cells, an alternative mechanism of LPP1 regulation would seem likely. Indeed, double immunofluorescence staining of LPP1 and ATX, or LPP3 and ATX, in non-permeabilized cells shows overlapping staining in normoxic cells, while hypoxic cells display staining for each protein in distinct cellular localizations. In hypoxia, ATX staining is concentrated towards the leading edge, as in Figure 5, while LPP1, or LPP3, staining is located at the trailing edge with no apparent overlap with ATX (Fig 6A-B). Furthermore, results from LPP3 and ATX co-staining show clearly less LPP3 staining in

hypoxic cells than normoxic cells (Fig 6B). These results suggest an altered localization of LPP1 and LPP3 in hypoxia as well as a decrease in LPP3 expression. This decrease in LPP3 staining observed in hypoxic cells could be due to the observed reduction in its expression levels in hypoxia (Fig 2).

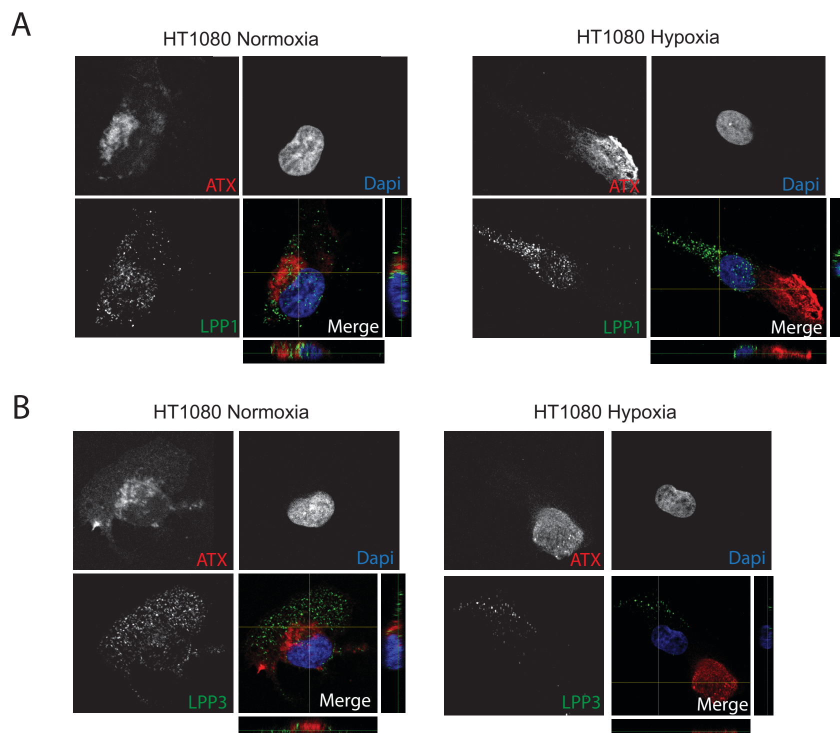

Figure 6 LPP1 and LPP3 localization is altered in hypoxic cells

(A-B) Representative immunofluorescence images of HT1080 cells cultured on non-fluorescent gelatin for 4h in normoxia (21%O_2) or hypoxia (1%O_2) are shown. **(A)** Cells were stained for LPP1 (green) and ATX (red). Nuclei were stained with DAPI (blue). Magnification 60X. **(B)** Representative images of cells stained for LPP3 (green) and ATX (red). Nuclei were stained with DAPI (blue). Magnification 60X.

3.2.5 ATX promotes cancer cell migration and invasion

Immunofluorescence results showing ATX localization at the leading edge of cells, distinct from the location of LPP1 and LPP3 expression in hypoxic cells, suggests a function in cell migration. To investigate the possible role of ATX in hypoxia-induced cell migration we used a more physiological 3-dimentional (3D) invasion assay in type I collagen matrices. These assays require formation of ECM-remodeling cell protrusions, cell elongation, and

stable cell orientation for directionally persistent cell migration in order for cells to invade deeply into the collagen matrix (Thievessen et al., 2015). Control shRNA cells seeded on top of 3D collagen gels were found to migrate deeper into the matrix when incubated in hypoxic conditions (Fig 7A-B). In contrast, cells transfected with shRNA targeting ATX did not exhibit this increased invasive migration in response to hypoxia (Fig 7A-B). These results indicate that ATX is necessary for hypoxia-mediated increases in directionally persistent cell migration.

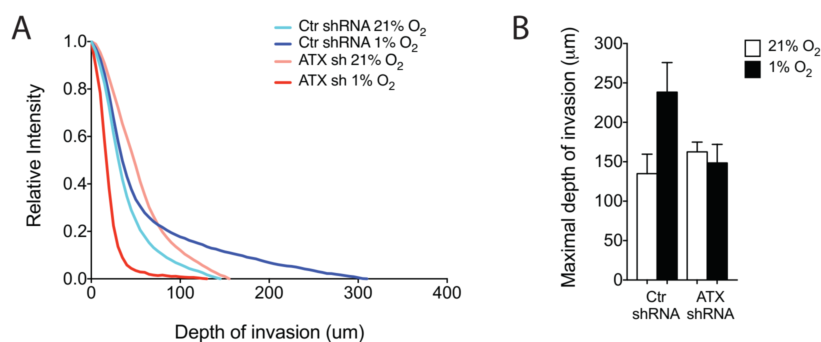

Figure 7 ATX is necessary for hypoxia-induced 3D invasion and migration

(A-B) Cells transfected with scramble (Ctr) or ATX targeted shRNA were incubated on type I collagen in 3D invasion assays in normoxia (21% O_2) or hypoxia (1% O_2) for 24h. **(A)** The relative intensity of cell staining according to depth of invasion is shown. **(B)** The maximal depth of invasion is shown for each condition. N=2-3. *Bars represent the mean ± SEM.*

4. DISCUSSION

Tumor metastasis is the leading cause of cancer patient mortality, however due to the complexity of the processes involved there are currently no specific metastasis inhibitors in clinical use. The hypoxic tumor microenvironment is an important driver of tumor progression and the metastatic phenotype, therefore a better understanding of the mechanisms involved in hypoxia-induced invasion is warranted. In this thesis, we have identified hypoxia as a master regulator of the LPA signaling axis. In the first part (chapter 1) of this thesis, we discovered a mechanism of LPA_1-Src-EGFR crosstalk under hypoxic conditions that mediates hypoxia-induced invadopodia production and metastasis through PI3K/Akt signaling. Our results also revealed the therapeutic potential of a combination of LPA_1 and EGFR inhibition on tumor metastasis. Furthermore, in chapter 2 of this thesis, hypoxia was found to modulate the expression and localization of the LPA producing (ATX) and degrading (LPP1, LPP3) enzymes, thereby identifying another mechanism by which hypoxia could modulate LPA signaling. The upregulation of ATX or downregulation of LPP1 or LPP3 were all found to promote invadopodia production, supporting the essential role of LPA signaling in this process.

4.1 Discussion of Article

Mounting evidence suggests a crucial role of the LPA/LPAR axis in cancer cell invasion and promising studies are underway to investigate the therapeutic potential of LPAR antagonists in pathologies such as idiopathic pulmonary fibrosis (Kihara et al., 2015). Pan-LPAR antagonists are likely to have cross-reactivity with other GPCRs as well as systemic side effects. It is therefore imperative to identify the specific LPA receptors involved under aggressive microenvironment conditions, such as hypoxia, in order to develop proper targeted therapies for cancer cell invasion and metastasis. The findings, presented in the manuscript section of this thesis, raise the interesting possibility that LPA_1 usage can be a hallmark of hypoxia-induced invadopodia production and point to a potential benefit of LPA_1-directed therapy to counteract cancer cell invasion. The LPA signaling axis, including LPA receptors, are promising clinical targets to treat chronic inflammatory conditions, fibrosis, and cancer (Benesch et al., 2018). Various phase I and phase II clinical

trials are currently underway to investigate the efficacy of various inhibitors in blocking pulmonary fibrosis, systemic sclerosis, and lesions caused by traumatic brain injury (Benesch et al., 2018; Desroy et al., 2017). Due to the many similarities between inflammation and fibrosis, and cancer associated pathways, these LPA signaling inhibitors have the potential to be useful for cancer therapy as well. However currently there are no approved cancer therapies that target the LPA signaling axis. Our identification of the essential role of LPA_1 in hypoxia-induced invadopodia production and metastasis suggest that some LPA_1 antagonists, such as BMS-986020 (NCT01766817) that is being tested in clinical phase II by Bristol-Myers-Squibb, could be useful for cancer therapy. In addition to cancer cell invasion and metastasis, LPA signaling also plays an important role in tumor-driving inflammation (Benesch et al., 2018). Therefore, an LPA_1 antagonist may have even more beneficial effects in the clinic by blocking both inflammation and cell invasion.

The most likely use of such inhibitors will be in combination with other approved therapies in order to increase efficacy and potentially overcome toxicity, by reducing the amounts of other drugs used. Due to the complex signaling networks involved in tumor progression, which interact through crosstalk and feedback loops, single therapeutic agents often have limited efficacy (Yap et al., 2013). Recently, combination therapy of two targeted drugs has been shown to be more beneficial than mono-therapies (Bayat Mokhtari et al., 2017; Yap et al., 2013). For example, a combination of VEGF and EGFR inhibitors showed improved efficacy over individual inhibitors for the treatment of renal cell carcinoma (Hainsworth et al., 2005), combined BRAF and MEK inhibition had a synergistic effect in melanoma (Flaherty et al., 2012), and combined suppression of Rictor and EGFR resulted in complete tumor regression in an orthotopic glioblastoma mouse model (Verreault et al., 2013). In agreement with the benefits of combination therapy, our results suggest that LPA_1 inhibition acts synergistically with EGFR inhibition to block metastasis. Inhibitors of EGFR are often not well tolerated, mostly due to gastrointestinal and cutaneous toxicity, and occasionally more severe side effects such as pulmonary or hepatic toxicity (Harari, 2004; Hidalgo et al., 2001). Furthermore, despite positive early responses most patients eventually develop acquired resistance to EGFR inhibitors, which been linked to activation or crosstalk from another receptor (Rosenzweig, 2012; Steeg and Theodorescu, 2008).

Therefore LPA1-EGFR combination therapy could be a useful strategy to reduce EGFR inhibitor side effects and overcome resistance, by reducing the required dose of EGFR inhibitor and disrupting a crosstalk mechanism. Due to the heterogeneous nature of human tumors it will be important to determine if a patients tumor will respond to EGFR and LPA1 inhibition in order for such a combination therapy to be useful. It would therefore be of interest to test for expression of these receptors in tumor biopsies, or even use the CAM model and directly test the inhibitors on the patient tumor samples implanted on the CAM.

The identification of LPA_1-EGFR crosstalk under hypoxic conditions, that promotes invadopodia biogenesis and metastasis, identifies this crosstalk as a potential target to block the effects of the hypoxic microenvironment on invasion and metastasis. Therefore, a better understanding of how LPA_1-EGFR crosstalk is promoted under hypoxia could help to better target this phenomenon. While we uncovered a ligand-independent, Src-mediated mechanism of transactivation, exactly why this occurs solely in hypoxic cells remains to be determined. Various possibilities will be examined below.

One possible explanation for the LPA_1-EGFR crosstalk observed in hypoxic conditions could be the generation of ROS. As mentioned in the introduction, ROS have been implicated in GPCR-RTK crosstalk with one mechanism involving inactivation of PTPs resulting in increased tyrosine phosphorylation levels (Cattaneo et al., 2014; Finkel, 2000). For example, EGFR transactivation by a GPCR, FPRL1, was mediated by increased ROS generation that modulates Src kinase activity (Cattaneo et al., 2011). Phosphorylation of EGFR induced by LPA was also found to require ROS (Cunnick et al., 1998). Increased production of ROS in hypoxia has been extensively documented (Tafani et al., 2016). For example, hypoxia was shown to increase ROS via activation of NADPH oxidase in pulmonary smooth muscle cells (Rathore et al., 2008). Hypoxia was also shown to generate hydrogen peroxide in pulmonary artery endothelial cells (Porter et al., 2014). In addition, hypoxia was shown to increase ROS generation, involved in activation of HIF pathways, in A549 lung cancer cells (Goyal et al., 2004). In concordance with these studies, we have found an increase in ROS production induced by hypoxia in the HT1080 cells line, an effect that was dependent on LPA_1 activation (Discussion Figure 1, Annex 1).

Interestingly, Diaz *et al.* detected ROS at sites of invadopodia formation and found that ROS generation was necessary for invadopodia formation (Diaz et al., 2009). It is therefore possible that, under hypoxia, local production of ROS would enhance invadopodia production by increasing tyrosine phosphorylation of TKs, such as Src or EGFR, which are involved LPA1-EGFR crosstalk. To verify the involvement of ROS in this process we could determine the effect of ROS inhibition on the LPA$_1$-EGFR crosstalk mechanism and invadopodia production, using antioxidants such as NAC (N-acetyl-L-cysteine) or specific shRNA targeting essential components of the NADPH complex such as p22PHOX.

Another potential explanation for the LPA$_1$-EGFR crosstalk in hypoxia is a change in receptor localization under this condition. Relocalization of the receptors and signaling components to specific microdomains, such as lipid rafts, where they would be in close proximity could presumably facilitate their crosstalk. Lipid raft formation can be controlled by hypoxia and GPCRs, EGFR, Src and Akt can all be relocalized to lipid rafts (Danza et al., 2013; Irwin et al., 2011; Jensen et al., 2013; Liu et al., 2014; Simons and Toomre, 2000). Therefore, increased lipid raft formation in hypoxia might promote the interaction of LPA$_1$, EGFR and Src leading to their cross communication. To determine if lipid rafts are involved in LPA$_1$-mediated transactivation of EGFR, we can use a variety of techniques. First, we can assess whether LPA$_1$ and EGFR are localized in lipid rafts by biochemical raft isolation using ultracentrifugation. Lipid raft fractions can then be identified by the absence of transferrin receptors and the presence of flotillin and GM1, a lipid raft-specific ganglioside (Macdonald and Pike, 2005; Wolf et al., 1998). Localization of LPA$_1$ and EGFR in the lipid raft fractions can then be evaluated by Western blotting. Confocal microscopy analysis using super-resolution stimulated emission depletion (STED) microscopy could also be used to visualize the colocalization of LPA$_1$ and EGFR in lipid rafts following lipid raft staining with labeled cholera toxin subunit B, which binds to GM1 (Wolf et al., 1998), and staining of LPA$_1$ and EGFR. We can also determine if LPA$_1$ and EGFR colocalize with caveolin, which identifies a subset of lipid rafts (Zheng et al., 2011). We can further use techniques to disrupt lipid rafts, such as methyl-beta cyclodextrin (M□CD) induced depletion of cholesterol (Mahammad and Parmryd, 2015) and determine

the effects on EGFR transactivation, interaction of LPA_1 and EGFR by coimmunoprecipitation, and invadopodia formation.

Altered receptor trafficking in hypoxia might also mediate crosstalk. Abnormal trafficking is involved in malignant transformation and endocytosis-associated proteins are often deregulated in cancer (Mosesson et al., 2008). Some of the mechanisms involved in defective trafficking and unbalanced recycling are influenced by hypoxia in the tumor microenvironment (Wang and Ohh, 2010). For example, hypoxia has been found to globally inhibit endocytosis, via a caveolin-1 dependent mechanism. However certain proteins were found to have enhanced internalization under hypoxic conditions, including several RTKs such as EGFR, DDR1, IGFR1 and ROR2, and several integrins such as ITGA1, 2, or 3 and ITGB1 and 5 (Bourseau-Guilmain et al., 2016). This suggests that hypoxia has protein-specific effects on trafficking. For example, hypoxia was found to mediate delivery of integrin containing vesicles to the leading edge of invasive cancer cells (Yoon et al., 2005). In another study, hypoxia was found to delay endocytosis resulting in reduced EGFR degradation and prolonged EGFR signaling using a mechanism involving downregulation of the protein rabaptin-5 (Wang et al., 2009). In the manuscript presented herein, we discovered a ligand-independent mechanism of LPA_1 transactivation of EGFR via Src kinase-mediated phosphorylation of EGFR on Y845. Phosphorylation of this residue was previously found to result in endosomal distribution of EGFR. In the study by Medts *et al*. activated Src phosphorylated EGFR on Y845 and triggered EGFR endocytosis via clathrin-coated vesicles. Importantly, the Src-EGFR interaction was sufficient to trigger EGFR activation without the requirement of an extracellular ligand (Medts et al., 2010). Internalization of EGFR in response to Src activation also resulted in sequestration of EGFR in perinuclear/recycling endosomes that avoid lysosomal degradation (Medts et al., 2010). This is reminiscent of the effect of hypoxia on EGFR internalization, which also delayed EGFR degradation. In addition, Src seems to play an important role in trafficking of other receptors, as some proteins involved in GPCR endocytosis and trafficking are Src substrates, for example, dynamin, a large GTPase involved in fusion of clathrin-coated vesicles. Phosphorylation of dynamin mediated by Src is required for internalization of GPCRs such as B2-adrenergic and M1 muscarinic receptors (Luttrell and Luttrell, 2004).

Therefore, Src activity could control LPA_1 trafficking as well. Altogether, this suggests that altered trafficking in hypoxia could bring LPA_1 and EGFR together in endosomes where EGFR is known to signal from (Di Guglielmo et al., 1994; Murphy et al., 2009). Furthermore, the crosstalk mechanism involving Src kinase may play a role in the altered trafficking.

Preliminary results from investigation into LPA_1 and EGFR localization show that they are found colocalized at specific microdomains in the cell including what appear to be membrane ruffles and large vesicles inside the cells (Discussion Figure 2, Annex 1). This could be due to internalization via macropinocytosis, an alternative form of endocytosis. Macropinocytosis is an actin-driven process whereby a membrane protrusion forms a large vesicle that is subsequently internalized into the cell (Michalopoulou et al., 2016). Macropinocytosis has also been shown to be dependent on lipid rafts (Conner and Schmid, 2003; Liu et al., 2002; Nichols and Lippincott-Schwartz, 2001), and therefore could be responsible for internalizing the components found in lipid rafts that were implicated in our study, such as LPA1, EGFR, Src and Akt. Another observation suggesting that the vesicles detected in our preliminary study are macropinosomes is the fact that they are found close to membrane ruffles, which is consistent with studies indicating that macropinosomes originate from ruffles (Michalopoulou et al., 2016). Interestingly, Rac1 and Cdc42 are major regulators of macropinocytosis along with Arp2/3 and PI3K (Dumontier et al., 2000; Hoeller et al., 2013). All of these are also implicated in invadopodia formation, and PI3K was the major signaling pathway leading to LPA1-regulated invadopodia in hypoxia (Eddy et al., 2017). Macropinocytosis also shares another link with invadopodia as both depend on CARMIL2, an actin capping protein regulator, for their formation and the loss of this protein inhibits macropinocytosis and impairs cell migration (Lanier et al., 2015, 2016). Therefore, macropinocytosis may contribute to the formation of invadopodia and these two events could work together to increase cellular invasion.

Macropinocytosis is a nutrient-scavenging pathway that allows the cell to recuperate nutrients via uptake of extracellular proteins (Recouvreux and Commisso, 2017). Hypoxic tumors are deprived of nutrients and hypoxic cells have been shown to support growth by

scavenging lipids (Kamphorst et al., 2013; Recouvreux and Commisso, 2017). Therefore, we might suspect that hypoxia could induce macropinocytosis to internalize lipids to support their metabolic needs. However, regulation of macropinocytosis by hypoxia is not clear at this time as there was only one study that found that low pH could modulate macropinocytosis in a cell-dependent manner (Gündel et al., 2017). However, there is a very interesting link between Src, EGFR signaling and macropinocytosis. Firstly, Src can induce macropinocytosis (Mettlen et al., 2006; Veithen et al., 1996) and EGFR is known to be internalized via macropinocytosis (Orth et al., 2006). In a study by Donepudi *et al*., Src and activated EGFR were found to be colocalized and traffic together in large endocytic vesicles identified as macropinosomes (Donepudi and Resh, 2008). Under this condition, EGFR activation was found to be prolonged in a Src-kinase dependent manner, presumably because EGFR in macropinosomes could evade degradation. In fact, macropinosomes were found to remain distinct from lysosomes (Donepudi and Resh, 2008), and as discussed above, EGFR is able to signal from intracellular vesicles (Murphy et al., 2009; Wang et al., 2002). Macropinocytosis has been shown to be regulated by GPCRs in podocytes (Chung et al., 2015). Therefore, the mechanism of crosstalk that we identified, involving Src kinase activation of EGFR, could be driving the production of invadopodia observed in hypoxia due to increased EGFR internalization and activation in macropinocytic vesicles. To investigate the involvement of macropinocytosis we can use inhibitors to block the formation of macropinosomes and 70kDa fluorescent dextran as a probe to detect and quantify them.

Scaffolding proteins play an important role in assembling multi-protein signaling complexes to facilitate their concerted interactions and functions. It is therefore plausible that a scaffolding protein could also be involved in LPA_1-EGFR crosstalk. An interesting candidate for this is NHERF1/EBP50 (Na+/H+ Exchanger Regulatory Factor/ERM Binding Protein 50) which is a PDZ-domain containing scaffolding protein that recruits a variety of transmembrane proteins, including RTKs and GPCRs, into functional complexes (Saponaro et al., 2014; Weinman et al., 2006). Interestingly, NHERF1 was also found to interact with an LPA receptor, LPA_2 (Oh et al., 2004) and has important effects on EGFR trafficking and function (Bellizzi et al., 2015; Lazar et al., 2004). By bringing together multiple proteins

NHERF1 plays a role in cell signaling, cytoskeletal remodeling and receptor trafficking (Oh et al., 2017). Many cancers overexpress NHERF1 in association with poor prognosis (Saponaro et al., 2014). In a study by Cardone *et al*. the hypoxic tumor microenvironment was found to regulate the expression and localization of NHERF1. Briefly, they observed an upregulation of NHERF1 expression and a redistribution of NHERF1 to leading edge pseudopodia in hypoxic cells (Cardone et al., 2007). Recently, LPA was also found to stimulate NHERF1 translocation to the plasma membrane, specifically to protrusive structures, in ovarian cancer cells. In this study, NHERF1 was also found to be essential for LPA-induced cell migration. Unfortunately, this study did not examine the involvement of LPA receptors (Oh et al., 2017). Therefore, NHERF1 is an important scaffolding protein that can be relocalized to protrusive structures under hypoxic conditions, and that can potentially interact with both EGFR and LPA_1 in order to bring them together at specific microdomains in hypoxia. NHERF1 may therefore facilitate LPA1-EGFR crosstalk in hypoxic cells. It would be interesting to knockout NHERF1 expression and measure the effects on LPA_1-EGFR crosstalk as well as visualizing their subcellular localization by confocal microscopy and ultimately the outcome of invadopodia production.

Finally, the PI3K/Akt pathway was found to mediate invadopodia production downstream of LPA_1-EGFR crosstalk. The involvement of Akt is in concurrence with other studies, which have shown that Akt may promote invadopodia through several potential mechanisms. For example, Akt can activate Rac1, which could lead to the recruitment of cortactin, which is essential for the formation of invadopodia precursor structures (Han et al., 1998; Head et al., 2003; Henderson et al., 2015). The degradative capacity of invadopodia can also be augmented by Akt through increased secretion of MMP9, an important protease involved in ECM degradation (Cho et al., 2008). Additionally, the PI3K/Akt pathway may be an interesting target for cancer therapy, especially in light of the recent research into PI3K isoform specific inhibitors. Clinical trials with pan-PI3K inhibitors were found to have disappointing results possibly due to their lack of specificity (Rodon et al., 2013). This is not surprising, as different PI3K isoforms are known to have divergent biological effects, as illustrated by the different phenotype of knockout mice (Thorpe et al., 2015). Subsequently, PI3K inhibitors selective for p110α, p110β, p110δ, or

p110γ were developed and are currently being tested in phase I and phase II clinical trials (Chaussade et al., 2007; Thorpe et al., 2015). Therefore, PI3K isoforms could potentially provide additional selective targets to inhibit metastasis. It would be of interest to investigate which specific isoform is implicated downstream of LPA_1-EGFR crosstalk in hypoxia to mediate invadopodia production and metastasis. To this aim, we could test the different isoform selective inhibitors in invadopodia assays and *in vivo* metastasis studies. We can also measure the relative levels of each isoforms, and determine if they are modulated by hypoxia. Results from these experiments should give us an indication of which one is dominant in mediating signaling. After determining the isoform/s involved we may want to test them in conjunction with the inhibitors of EGFR and/or LPA_1 to determine if we can achieve a synergistic effect in blocking invadopodia and metastasis.

4.2 Discussion of Chapter 2

Signaling by LPA is known to be affected by the amount of LPA available to interact with receptors. Increased levels of LPA have been found in the ascites from ovarian and pancreatic cancer patients compared to control groups (Westermann et al., 1998; Xiao et al., 2001; Xu et al., 1998; Yamada et al., 2004). Interestingly, ovarian ascites fluid is hypoxic suggesting that hypoxia could be responsible for the observed increase in LPA levels (Kim et al., 2006). The potential role of hypoxia is supported by the demonstration of increased LPA levels in a chronic-hypoxia pulmonary hypertension rat model (Shlyonsky et al., 2014). Shlyonsky *et al.* observed an increase in LPA serum content as well as increased LPA staining in the lungs of rats exposed to hypoxia. This was accompanied by an increase in ATX mRNA levels in the lung tissues of hypoxic rats (Shlyonsky et al., 2014). Elevated levels of LPA have also been measured in patients with ischemic cerebrovascular disease (Li et al., 2008). Therefore, conditions of hypoxia, or low oxygen concentration, seem to increase LPA levels in non-malignant pathologies, potentially through upregulation of ATX.

In the second chapter of this thesis, our goal was to gain a more complete understanding of the regulation of LPA signaling by hypoxia in cancer cells by studying the effects of hypoxia on the enzymes involved in controlling LPA levels. LPA production is mainly

regulated by ATX, which promotes tumor progression and whose expression is upregulated in various cancers (Benesch et al., 2014a; Kehlen et al., 2004; Kishi et al., 2006; Yang et al., 2002, 1999). In contrast, important LPA degrading enzymes, the LPPs, are downregulated in cancer cells and play a negative role in tumor progression (Tang et al., 2015; Tanyi et al., 2003a, 2003b). Yet, the factors involved in shaping the differential expression of these LPA regulatory components remain unknown. Since hypoxia is associated with both increased LPA levels and tumor progression (Hockel et al., 1996; Shlyonsky et al., 2014), we investigated the impact of the hypoxic microenvironment on ATX and LPP expression. We observed an increase in ATX expression in hypoxic HT1080 cells, which correlates with the increased LPAR signaling in these cells discovered in Article 1. This finding identifies hypoxia as a novel inducer of ATX in cancer cells that could be responsible for ATX upregulation in fibrosarcomas. In contrast, ATX expression was not modulated in U87 or MDA-MB-231 cells exposed to the same hypoxic condition. The lack of significant increase of ATX expression in the U87 cell line may be explained by the fact that ATX is known to be already overexpressed in glioblastomas (Kishi et al., 2006). Breast cancer stromal cells as well as the mammary fat pad have been identified as major sources of ATX for breast tumor cells (Benesch et al., 2014b; Popnikolov et al., 2012). This may provide an explanation for the lack of increased ATX expression observed in hypoxia-stimulated MDA-MB-231 breast cancer cells. Certainly, protein levels of ATX both in cell lysates and supernatants, since ATX is a secreted protein, will need to be measured in the U87 and MDA-MB-231. There is a discrepancy between mRNA and protein abundance for many genes, as differential expression of mRNA has been found to explain at most 40% of the variation in protein expression (Tian et al., 2004; Wang, 2008). Therefore, we could observe an increase in ATX protein expression despite little change in ATX mRNA expression in these cell lines.

Results demonstrated that LPP1 and LPP3 expression is also modulated by hypoxia. Hypoxia significantly decreased LPP3 mRNA levels in all cell lines tested. Expression of LPP1 was similarly decreased, albeit to a lesser extent, with significant down modulation observed only in the MDA-MB-231 and U87 cell lines. These results suggest that although U87 cells express high levels of ATX, the reduced expression of LPA degrading enzymes

in hypoxia could be necessary to sustain high levels of LPA, since LPA is normally rapidly degraded by these enzymes (Brindley et al., 2002). Breast cancer MDA-MB-231 cells do not appear to increase ATX levels; therefore, the reduced expression of LPA degrading enzymes in hypoxia could be the main mechanism involved in the regulation of LPA levels under hypoxic conditions in these cells (Li et al., 2008; Shlyonsky et al., 2014). Result also highlight the fact that it will be important to look at both ATX and LPP expression levels when investigating autocrine production of LPA levels in cell lines or pathological contexts. In contrast to LPP1 and LPP3, LPP2 expression was not decreased. This is consistent with previous studies indicating that LPP1 and LPP3 are downregulated in some cancers (Tang et al., 2015) and their overexpression inhibits cell proliferation and migration as well as tumor growth and metastasis (Tang et al., 2014; Tanyi et al., 2003a). On the other hand, LPP2 has increased expression in cancer and opposing effects to LPP1 and LPP3, as it induces cell cycle progression (Morris et al., 2006). Interestingly, when we look at the specificity of these different LPPs, LPP1 and LPP3 degrade mostly LPA while LPP2 prefers PA as a substrate, suggesting an important role for LPP1 and LPP3, but not LPP2, in controlling LPA-mediated signaling (Brindley and Waggoner, 1998). We have therefore identified hypoxia as a novel modulator of LPP1 and LPP3 expression, uncovering an important microenvironment factor that may contribute to their low expression in tumors. Again, these results will need to be validated by western blotting of protein levels to confirm that the decreased mRNA levels correlate with lower protein expression. Overall, the increase in ATX expression and/or decrease in LPP1 and LPP3 expression in hypoxic cells may contribute to increased LPA levels in the tumor microenvironment (Westermann et al., 1998; Xiao et al., 2001; Xu et al., 1998; Yamada et al., 2004).

Hypoxia is known to modulate gene expression, through various transcriptions factors, primary among them is HIF1. Therefore, it will be of interest to determine if the modulation of ATX, LPP1 and LPP3 expression under hypoxia is dependent on HIF1 or other transcription factors known to be modulated by hypoxia. The expression of ATX can be modulated by a variety of transcription factors including AP1, SP and NFkB (Farina et al., 2012; Wu et al., 2010) that can also be activated under hypoxic conditions (Achison and Hupp, 2003; Beitner-Johnson and Millhorn, 1998; Koong et al., 1994; Millhorn et al.,

1997). In contrast, very little is known concerning LPP1 and LPP3 gene regulation; so far, the only transcription factor associated with increased LPP1 expression is p73, a p53-related transcription factor (Ishida et al., 2007). In this study by Ishida *et al*., several p53 responsive element-like sequences were identified in the promotor of LPP1 indicating that p53 may also play a role in regulation of LPP1 expression. However, no modulators of low LPP expression have been discovered so far. It would therefore be of interest to determine the involvement of HIF1 and other transcription factors in hypoxia-induced down modulation of LPP1 or LPP3. This could be assessed by various means including the use of pharmacological inhibitors of the transcription factors or knockout of their expression with shRNA.

Following the identification of increased ATX and decreased LPP1 and LPP3 levels in hypoxic cells it will be important to determine if these modulations have an effect on the global LPA production by these cells. For this, we could measure LPA levels by mass spectrometry, as described by Wijesinghe et al. (Wijesinghe et al., 2011). This method can also identify specific LPA species to determine which ones might be preferentially produced in hypoxia. Different LPA species may have differing biological activities and can preferentially activate specific LPA receptors. For example, LPA_3 prefers unsaturated LPA species and LPA_{1-3} prefer acyl to alkyl LPA (Bandoh et al., 1999; Okusa et al., 2003; Tigyi, 2010; Yanagida and Ishii, 2011). It would also be of interest to verify if changes in total LPA levels or production of specific species of LPA depends on the activities of ATX or LPP1 or LPP3. For this we can knockdown their expression with shRNA or overexpress these enzymes and analyze LPA levels. The identification of specific species of LPA produced in hypoxia could provide additional clues as to how hypoxia mediates its effects, for example does hypoxia increase the production of an LPA species that preferentially activates LPA_1, potentially explaining the importance of this receptor in hypoxia?

Since ATX expression was increased in hypoxic HT1080 cells and ATX is known to promote cell invasion (Liu et al., 2009), the involvement of ATX in hypoxia-induced invadopodia production was investigated. We first determined that both ATX overexpression and hypoxic stimulation were strong inducers of invadopodia, increasing

the percentage of cells forming invadopodia, the number of invadopodia formed per cell, and the area of degradation. These results correlate with previous studies that showed increased invadopodia production in hypoxic cells or cells overexpressing ATX (Harper et al., 2010; Lucien et al., 2011). Interestingly, overexpression of ATX was found to induce a much higher percentage of cells forming invadopodia than hypoxic stimulation, suggesting ATX may play an important role in the initiation of invadopodia formation. Hypoxic cells on the other hand were more aggressive at degrading the matrix compared to ATX overexpressing cells. This is most likely due to the effect of hypoxia on factors distinct from ATX, which increase the degradative capacity of the cells. For example, hypoxia can increase the expression of many matrix-degrading proteases such as MT1-MMP, MMP-2 and MMP-9 (Hanna et al., 2013; Semenza, 2012). Furthermore, the acidic extracellular pH associated with hypoxic conditions can also drive matrix degradation by augmenting the secretion and activation of these proteases (Rozhin et al., 1994; Stock and Schwab, 2009). Therefore, it would be of interest to determine what specific aspects of invadopodia formation and function are affected by ATX overexpression versus hypoxic stimulation to fully delineate their roles in invadopodia biogenesis.

Our results also identify ATX as a novel and essential mediator of hypoxia-induced invadopodia, as ATX knockdown completely blocked this event. Furthermore, LPA, but not LPC, was able to efficiently rescue ATX shRNA-mediated knockdown of invadopodia in hypoxic conditions, indicating that the effects of ATX are most likely due to its production of LPA and downstream signaling through LPARs. These results are consistent with our previously published results in normoxic conditions, and studies with ATX knockout mice, indicating that LPA production and GPCR signaling were responsible for ATX effects (Harper et al., 2010; van Meeteren et al., 2006). Furthermore, this finding provides additional evidence that LPA signaling plays a role in hypoxia-induced invadopodia, complementing the findings from Article 1. Perhaps part of the explanation for increased LPA_1 signaling in hypoxia was due to increased activity of ATX in hypoxia. The ATX/LPA axis may therefore be a major factor in hypoxia-induced invasion and metastasis.

As LPP1 and LPP3 have negative effects on cancer progression and metastasis (Tang et al., 2014, 2015) and were downregulated in hypoxic conditions, we investigated the result of inhibiting their expression on invadopodia production. Knockdown of the LPA-degrading enzymes, LPP1 and LPP3, resulted in an increase in invadopodia production, an effect similar to overexpression of the LPA-producing enzyme, ATX. These results are in concordance with the decreased expression of LPP3 observed in hypoxic HT1080 cells in this study, as well as decreased LPP1 and LPP3 found in breast, lung, and ovarian cancers (Tang et al., 2015). This is the first time that LPPs have been shown to negatively affect invadopodia production therefore providing a potential mechanism by which they mediate their negative effects on cancer metastasis (Tang et al., 2014). To further our study on the implication of LPPs in hypoxia-induced invadopodia production overexpression of LPP1 or LPP3 in hypoxic conditions will be an asset. It will also be important to verify that the effects of LPPs are due to their ability to degrade of LPA specifically and not to non-catalytic effects (Tang et al., 2015). One way to verify the involvement of LPP1 or LPP3 LPA-degrading activity is to use a non-hydrolysable analogue of LPA that can therefore not be degraded by these LPPs (Brindley et al., 2002). Addition of a non-hydrolysable LPA analogue should reverse the effects of LPP overexpression on invadopodia production if their effects are due to their catalytic activity. Another approach would be to express a catalytically inactive form of LPP1 or LPP3 (Tang et al., 2014) and measure the effects on invadopodia and LPA production.

In addition to changes in gene expression, hypoxia may also mediate effects by altering the trafficking and subcellular localization of proteins (Bourseau-Guilmain et al., 2016). Therefore we studied the subcellular localization of ATX, LPP1 and LPP3 under hypoxic conditions. Firstly, we observed a striking concentration of ATX staining at the leading edge of hypoxic cells, compared to less, and more diffuse ATX staining in normoxia. ATX staining was localized to the exterior cell surface as non-permeabilized cells also displayed strong ATX staining. This is interesting, since ATX has been shown to bind to β1 and β3 integrins, localizing ATX to the surface of platelets or mammalian cells such as lymphocytes (Fulkerson et al., 2011; Hausmann et al., 2011; Kanda et al., 2008). Furthermore, β1 integrins are dynamically relocalized to the leading edge of polarized

migrating cells, suggesting they could be involved in recruiting ATX to this area (Shafaq-Zadah et al., 2016). In addition, hypoxia enhances integrin expression, including β1, to promote metastasis (Ju et al., 2017), and β1 integrins are required for the formation of mature degradation-competent invadopodia, as discussed in the introduction (Huck et al., 2010; Lahlou and Muller, 2011). Therefore, increased β1 expression in hypoxia may recruit ATX to the cell surface where the enzyme can augment local LPA concentrations in the vicinity of LPA receptors. Furthermore, binding of ATX to β1 integrins could potentially localize ATX to sites of invadopodia formation where ATX could produce LPA and activate LPAR signaling directly at invadopodia sites. We intend to further investigate the localization of ATX at invadopodia by colocalization with invadopodia markers such as cortactin or Tks5 and areas of matrix degradation. To further investigate the role of integrin binding in ATX localization under hypoxia we could construct ATX mutants lacking integrin-binding sites. These mutants would also be useful to determine the effect of altered ATX binding on invadopodia production or other biological effects.

Interestingly, if ATX does interact with integrins this could potentially link it to the possible NHERF-LPA1-EGFR complex described in section 4.1 of this discussion (Bellizzi et al., 2015; Saponaro et al., 2014). In a study by Antelmi *et al*. phosphorylated ezrin, a cytoskeletal linker protein, was found to bind to both NHERF1 and β1 integrins at sites of functionally active invadopodia (Antelmi et al., 2013; Vaheri et al., 1997). Interestingly this NHERF-1-ezrin-β1 complex also contained EGFR (Antelmi et al., 2013). Therefore if NHERF-1 forms a complex with LPA_1 and EGFR and β1 integrins recruit ATX, all of these proteins could be brought together in a functional complex by NHERF-1/β1 joint interaction with ezrin. This is an intriguing possibility to further pursue, as localized production of LPA, by ATX bound to integrins, in close proximity to LPA_1 would favor LPA1 signaling. Furthermore if LPA_1 is already in a complex with EGFR, mediated by NHERF1, crosstalk would be promoted as well. Therefore it could be of interest to investigate LPA_1 and EGFR localization in relation to ATX in hypoxic cells and determine if the NHERF1 scaffolding protein or integrins play a role in bringing these proteins together.

There are other mechanisms that could also be implicated in ATX recruitment to the cell surface. The ATXα isoform was shown to bind heparan sulfate proteoglycans at the cell surface, which could also localize ATX to this cell compartment (Houben et al., 2013; Perrakis and Moolenaar, 2014). To investigate the involvement of this mechanism in ATX cell-surface localization we can use heparinase to cleave proteoglycans, thereby preventing ATX binding to them. Another alternative possibility is the delivery of ATX via exosomes. Recently exosomes were found to bind ATX and act as a delivery mechanism. Briefly, secreted ATX can bind the surface of cell-secreted exosomes, where it remains active and carries LPA. Exosome-bound ATX can then bind to cells through integrins and deliver the LPA for signaling (Jethwa et al., 2016). Exosomes can promote tumor progression and metastasis (Whiteside, 2016), and have key roles in various steps of invadopodia biogenesis (Hoshino et al., 2013b). In fact, invadopodia have been identified as important docking and secretion sites for exosomes (Hoshino et al., 2013b). Furthermore hypoxia can induce exosome secretion to promote invasive behavior of cancer cells (Ramteke et al., 2015). Therefore an intriguing possibility for future study is whether hypoxia releases exosomes containing ATX that promote invadopodia production.

Co-staining of ATX and LPP1 or LPP3 revealed a subcellular spatial segregation of these enzymes under hypoxic conditions. Staining of ATX was localized to the leading edge, while LPP1 or LPP3 staining was localized towards the trailing edge, of hypoxic cells, and distinct from ATX staining. Furthermore, we observed less staining of LPP3 in hypoxia, which could potentially be due to the downregulation of LPP3 expression in hypoxia. Quantification of LPPs and ATX staining intensities in different regions of the cells (leading edge versus trailing edge) should add further support to these observations. Our finding of ATX localization at the leading edge of cells distinct from the location of LPP1 and LPP3 expression in hypoxic cells suggests a function in cell migration. This compartmentalization of ATX and LPP1 or LPP3 under hypoxia might lead to uncontrolled LPA production towards the leading edge of the cell promoting migration by activating LPA receptors (Zhang et al., 2009).

To support this possibility, we investigated the role of ATX in hypoxia-induced cell migration using a more physiological 3-dimentional (3D) collagen invasion assay, which requires formation of ECM-remodeling cell protrusions, cell elongation, and stable cell orientation for directionally persistent cell migration in order to invade deeply into this substrate (Thievessen et al., 2015). Indeed, we observed that cell migration induced by hypoxia was efficiently blocked by ATX shRNA, suggesting an important role for ATX in this process. This is in concordance with other publications identifying important roles for LPA and ATX in cell migration, but is the first time ATX was shown to mediate hypoxia-induced cell migration (van Meeteren et al., 2006; Zhang et al., 2009). It would be of interest to investigate the involvement of spatial segregation between ATX and LPP1/LPP3 in directionally persistent cell migration in 3D cell migration assays. One possibility is to utilize GFP- and RFP-tagged ATX and LPPs in order to visualize their localization in cells migrating through a 3D collagen matrix, ideally we would use CRISPR/Cas9 technology to tag endogenous proteins, avoiding the need for overexpression (Leonetti et al., 2016). To complement this study, we may also knockdown ATX expression and label either cell membranes and/or the actin cytoskeleton, before incubation in the 3D assay, to analyze changes in cell shape or actin-based protrusions in ATX deficient cells.

There is another intriguing possibility as to how hypoxia may affect ATX, LPP1 and LPP3 activity that involves changes in pH. As discussed in the introduction, one of the major effects of hypoxia in the tumor microenvironment is a reverse acid-outside pH gradient (Cassavaugh and Lounsbury, 2011; Chiche et al., 2010). Hypoxia increases the activity of the sodium hydrogen exchanger, NHE-1, a principal regulator of cellular pH, to increase the extrusion of protons (Casey et al., 2010; Lucien et al., 2011). The exchanger, NHE-1, is preferentially localized to the leading edge of migrating cells where it results in localized extracellular acidification (Ludwig et al., 2013; Stock et al., 2007). Localized acidification produced by NHE-1 at invadopodia microdomains is also essential for hypoxia-induced invadopodia (Busco et al., 2010; Lucien et al., 2011). Therefore, distinct pH nanodomains at the leading edge or invadopodia may result in differential activation of pH-dependent proteins at these sites.

As ATX is a secreted protein and LPP1 and LPP3 are membrane proteins with their catalytic sites facing the extracellular space, in order to degrade extracellular LPA, they would all be exposed to changes in the extracellular pH under hypoxia. One of the ways that pH affects proteins is by altering the protonation state of histidine residues, that act as pH sensors due to the fact that their pKa is close to physiological pH (Webb et al., 2011). Various histidine-containing proteins have been found to experience altered protein-protein interactions or activity following changes in pH (Dillon et al., 2012; Kelly et al., 2002; Kim et al., 2010; Mueller et al., 2008; Webb et al., 2011; Zhang et al., 2013). At low pH histidine is protonated and positively charged, while at high pH it is unprotonated and negatively charged. Most interestingly ATX, LPP1 and LPP3 all contain histidine residues that are important for their catalytic activity. Several important histidine residues are found within ATX, H316, H360 and H475, which are metal-binding residues (Gijsbers et al., 2003; Koh et al., 2003), and H226 and H434 involved in substrate interactions, all of which were found to be essential for ATX catalytic activity and biological effects. Mutation of any of these histidine residues to glutamine, which mimics the non-protonable form of histidine, attenuated the activity of ATX towards LPC and reduced cell migration in response to ATX (Koh et al., 2009). The extracellular catalytic sites of LPP1 and LPP3 also contain conserved histidine residues. As mentioned in the introduction the conserved histidine in C3 acts as a nucleophile, and therefore must be in its unprotonated state to form the phospho-histidine intermediate (Bischoff and Schlüter, 2012; Sigal et al., 2005). This information suggests that the acidic extracellular pH found in hypoxic conditions may very well increase the activity of ATX specifically at the leading edge or invadopodia. Whether these changes would simultaneously inhibit LPP1 and LPP3 activity would likely depend on their localization at acidic subcellular sites of the cells or other cell compartments.

To investigate whether this is the case we can envision a variety of experiments. First we can alter the extracellular pH and measure the amount of LPA produced, in this way we can determine if hypoxia-mediated changes in LPA levels are dependent on pH. For this we can use pH neutralizers such as NH_4Cl or chloroquine, or add medium adjusted to a specific pH to the cells. We can also measure the effects of pH modulation specifically on ATX activity using ATX activity assays that are sold commercially or on LPP activity with

non-commercial assays for LPP activity (Han and Carman, 2004). To investigate more specifically the role of each histidine residue we can perform mutagenesis experiments for ATX, LPP1, and LPP3. The histidine residues can be substitued by arginine, to mimic the protonated state and therefore acidic conditions, representing hypoxic conditions, or by glutamine, to mimic the non-protonated state and therefore more alkaline conditions. With these mutants we can again assess LPA levels as well as biological effects such as invadopodia production or cell migration to determine if these histidine residues are playing an essential role in the functioning of the enzymes. To determine if NHE-1-mediated changes in pH and associated formation of distinct pH nanodomains can affect LPP or ATX activity in hypoxia we can use shRNA targeting NHE-1 and measure the outcomes previously described. Interestingly, NHE-1 may itself be affected by LPA signaling creating a potential positive feedback loop. Signaling by LPA has been shown to contribute to the phosphorylation of NHE-1 in its cytoplasmic tail via Rock and p90Rsk, resulting in increased cell migration (Wallert et al., 2015). Studies of the reciprocal effects of LPA signaling and NHE-1 activity in hypoxia will be interesting to pursue as we previously found hypoxia to increase NHE-1 activity through p90Rsk-mediated phosphorylation of its cytoplasmic tail (Lucien et al., 2011).

Finally, the identification of ATX and LPP involvement in invadopodia production and their regulation by hypoxia makes them relevant targets to block the LPA signaling axis and therefore reduce cancer cell invasion. Interestingly, there has been much research into the development of ATX inhibitors, particularly following the identification of its crystal structure, which aided the design of rationale inhibitors (Benesch et al., 2014a). Similar to LPA receptors, ATX inhibitors have been developed and are currently in clinical trials for pulmonary fibrosis (Benesch et al., 2018). Although in light of our observations of localized plasma-membrane expression of ATX in hypoxic cells, blocking ATX-cell interactions might be a more selective approach to inhibit the specific effects of hypoxia on ATX-induced invasion. This approach has the potential to generate fewer side effects than global ATX inhibition. As for the LPPs, increasing their reduced expression levels in hypoxic cancer cells could be a novel target for cancer therapy (Benesch et al., 2016). Increasing low LPP1/3 expression through gene overexpression has been shown to limit

tumor progression (Benesch et al., 2018) and interestingly, tetracyclines can increase the expression of LPPs through stabilization of the protein (Tang et al., 2016). Therefore, tetracyclines or other potential inducers of LPP gene, or protein, expression or, stabilization could be used to inhibit LPA signaling. Clearly, more information on the mechanism of LPPs inhibition by hypoxia could provide novel targets for the control of LPP expression levels. The results of our study indicate that ATX or LPPs could be targeted as anti-metastasis therapies, due to their implication in invadopodia production, which is essential for cancer cell invasion and metastasis.

4.3 Conclusions and Perspectives

Metastasis is the main cause of cancer patient mortality making it vital to elucidate novel ways to block the metastatic cascade. Invadopodia make ideal targets to block the metastatic process due to their essential role in cancer cell intravasation and extravasation. Our work suggests that hypoxia is a master regulator of the LPA signaling axis for invadopodia production, highlighting the importance of taking into account the influence of the tumor microenvironment when investigating cell invasion processes. The LPA signaling axis is revealed in this thesis to be a relevant target to overcome hypoxia-induced increases in cell invasion and metastasis, therefore therapies targeting this axis could be beneficial to counteract the detrimental effects of tumor hypoxia on cancer patient survival. The use of LPA_1 inhibitors, already in clinical trials for fibrosis, in conjunction with EGFR inhibitors, currently used in the clinic for cancer patients, is a promising avenue of therapeutic targeting. Eventual development of ways to interfere with ATX recruitment to the cell surface in hypoxia and also ways to increase LPP expression may provide new therapeutic targets and warrant investigation. Currently, there are no approved cancer therapies that target LPA signaling therefore this provides an exciting opportunity for developing novel strategies that could improve upon current cancer therapies.

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ANNEXES

Annex 1: Discussion Figures

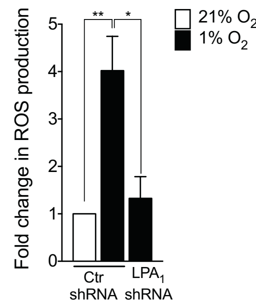

Figure 1 Increased ROS production in hypoxia is dependent on LPA_1

HT1080 cells transfected with scramble (Ctr) or LPA_1 shRNA were incubated in normoxia (21% O2) or hypoxia (1% O2) for 60 minutes. The fold change in ROS production is shown, measured using CellROX^{tm} Green Reagent. *Bars represent the mean ± SEM (* P< 0.05, ** P< 0.01).*

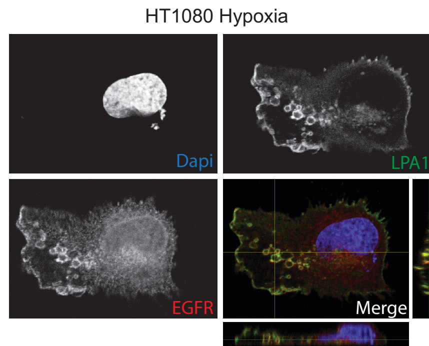

Figure 2 LPA1 and EGFR are colocalized in hypoxic HT1080 cells

Representative image of LPA1-GFP (green) transfected HT1080 cells cultured on non-fluorescent gelatin for 4h in hypoxia (1% O_2). Cells were permeabilized and stained for EGFR (red), and nuclei were stained with DAPI (blue). Magnification 60X.