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AUTOTAXIN PROMOTES CANCER CELL INVASION VIA THE LYSOPHOSPHATIDIC ACID RECEPTOR 4

Par

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Département de pédiatrie, Service d'immunologie

Mémoire présenté à la Faculté de médecine et des sciences de la santé
en vue de l'obtention du grade de
maître ès sciences (M.Sc.) en immunologie

15 mai 2010

EVALUATEURS:
Pre. Claire Dubois (programme d'immunologie)
Pr. Gilles Dupuis (programme d'immunologie)
Pre. Nathalie Rivard (programme de biologie cellulaire)

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SUMMARY

Tumor metastasis is a fundamental property of malignant cancer cells and the major cause of death in cancer patients. Recent studies indicate that tumor cell invasion and metastasis may be initiated by the formation of the actin-rich cell protrusions with ECM degradation activity, invadopodia. However, despite extensive research on the biology of invadopodia, very little is known about their specific inducers during tumor progression. Autotaxin (ATX) is a secreted lysophospholipase whose expression levels within tumors correlates strongly with their aggressiveness and invasiveness. ATX produces lyosophosphatidic acid (LPA), a phospholipid with known tumor promoting functions that acts through the G-protein coupled receptors, LPA_{1-6}. Recently, overexpression of ATX and LPA receptors (LPA_{1-3}) has been linked to increased tumor invasion and metastasis *in*

vivo, however, the role of other LPA receptors (LPA_{4-6}) as well as the exact mechanisms by which ATX induces tumor metastasis remain poorly characterized.

In order to determine the involvement of ATX and LPA in invadopodia production, we used the fibrosarcoma HT-1080 cells stably transfected with ATX or shRNA targeting ATX in fluorescent matrix degradation assays. Our results demonstrate that ATX is implicated in the production of invadopodia resulting in an increase in both their formation and function. Using LPC or LPA, the substrate and product of ATX, we further show that invadopodia production is dependent on the production of LPA from LPC. Among the LPA receptors, LPA_4 has the highest expression in HT1080 cells. Using LPA_4 shRNA as well as agonists and inhibitors of the cAMP pathway, we provide evidence that LPA_4 signaling through the cAMP-EPAC-Rap1 axis, regulates invadopodia formation downstream of ATX. Furthermore, inhibition of Rac1, a known effector of Rap1 and invadopodia formation, abolished EPAC-induced invadopodia production, suggesting downstream participation of Rac1. Finally, results using LPA_4 shRNA support the requirement of this receptor for *in vitro* cell invasion and *in vivo* metastasis formation.

Our results suggest that ATX through LPA_4 is a strong inducer of invadopodia formation that correlates with the ability of the cells to invade and metastasize. This study also revealed an unexpected signaling pathway for cell invasion involving LPA_4-driven cAMP production and subsequent activation of the EPAC-Rap1-Rac1 axis.

Key words: invadopodia, autotaxin, lysophosphatidic acid (LPA), cAMP, metastasis

L' AUTOTAXIN INDUIT L'INVASION DES CELLULES CANCÉREUSES VIA LE RÉCEPTEUR DE TYPE 4 DE L'ACIDE LYSOPHOSPHATIDIQUE

RÉSUMÉ

La formation des métastases est une propriété fondamentale des cellules cancéreuses malignes ainsi que cause principale de décès chez les patients atteints de cancer. Des etudes recent indique que l'invasion tumorale et la formation des metastases peut etre initié par la formation des protrusions riches en actine et capables de dégrader la matrice extracellulaire, appeles des invadopodes. Cependant, malgré les recherches importantes sur la biologie des invadopodes, les informations concernant les initiateurs spécifiques de ces structures lors de la progression tumorale demeurent limitées. L'autotaxin (ATX) est une lysophospholipase sécrétée dont les niveaux d'expression corrèlent avec l'agressivité et le potentiel invasif des tumeurs. L'ATX produit l'acide lysophosphatidique (LPA), un phospholipide impliqué dans la progression tumorale qui agit par l'intermédiaire de récepteurs couplés aux protéines G, LPA_{1-6}. Il a été récemment démontré que la surexpression de l'ATX et des récepteurs LPA_{1-3} cause une augmentation de l'invasion tumorale et de la formation de métastases *in vivo,* cependant, le rôle d'autres récepteurs, soit les LPA_{4-6}, ainsi que les mécanismes exacts par lesquels l'ATX induit la formation de métastases demeurent peu connus.

Afin d'étudier l'influence de l'ATX sur la production d'invadopodes, nous avons transfecté des cellules de fibrosarcome, les HT-1080, avec des gène codant soit pour l'ATX ou des ARNm interférant. Ces cellules ont été testées dans des essais de

production d'invadopodes utilisant de la matrice fluorescente et des techniques d'immunofluorescence afin de visualiser de façon simultanée la dégradation de la matrice et les composantes caractéristiques de ces structures. Nos résultats indiquent que l'ATX est impliquée dans la formation et les fonctions des invadopodes . Par l'ajout du LPC ou du LPA, le substrat et le produit de l'ATX, nous avons montré que la production d'invadopodes est dépendante de la production de LPA du LPC. Parmi les récepteurs du LPA, le LPA4 possède l'expression la plus élevée chez les cellules HT-1080. Par le biais de shARNs spécifiques au LPA4 ainsi que d'agonistes et d'inhibiteurs de la voie de l'AMPc, nos résultats indiquent que la voie de signalisation AMPc-EPAC-Rap1, induite par l'activation du LPA4, régule la formation d'invadopodes en aval de l'ATX. De plus, l'inhibition de Rac1, un effecteur connu de Rap1 et de la formation d'invadopodes, abolit la production d'invadopodes induite par l'activation d' EPAC, suggérant la participation de Rac1 en aval de EPAC. Enfin, les résultats d'expériences utilisant des shARNs du LPA4 confirment l'implication de ce récepteur dans l'invasion des cellules *in vitro* et la formation de métastases.

En conclusion, nos résultats suggèrent que l'ATX via le LPA_4 est un initiateur puissant de la formation d'invadopodes par les cellules tumorales, ce qui corrèle avec leur habileté à former des métastases. Cette étude a également révélée l'existence d'une voie inattendue de signalisation cellulaire dans l'invasion, impliquant la production d'AMPc dépendante de LPA_4 et l'activation subséquente de l'axe EPAC-Rap1-Rac1.

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LIST OF ABBREVIATIONS USED

AC:	adenylyl cylase
ADAM:	a disintegrin and metalloproteinase
ADF:	actin depolymerizing factor
AGK:	acyl-glycerol kinase
AKAP:	a-kinase anchoring proteins
AMP:	adenosine monophosphate
Arap3:	Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 3
ARF:	ADP Ribosylation Factors
Arp2/3:	actin-related proteins
ATF1:	activating transcription factor 1
ATP:	adenosine Triphosphate
ATX:	autotaxin
BAD:	bcl-2-associated death promoter
bFGF:	basic fibroblast growth factor
Bmp-2:	bone morphogenic protein 2
CalDAG-GEF1:	calcium- and diacylglycerol-binding guanine nucleotide exchange factor 1
cAMP:	cyclic adenosine 3',5'-monophosphate
CBP:	CREB binding protein
CDC25-HD:	CDC25-homology domain

CNG:	cyclic-nucleotide gated ion channel
cPA:	cyclic phosphatidic acid
CREB:	cAMP response element-binding
CREM:	cAMP response element modulator
CRIB:	Cdc42/Rac1-interactive binding
CTX:	cholera toxin
DA:	dominant active
DAG:	diacylglycerol
DDW:	aspartic acid-aspartic acid-tryptophan motif
DEP:	dishevelled, Egl-10, pleckstrin
DOCK4:	dedicator of cytokinesis 4
DPP4:	dipeptidyl dipeptidase IV
EBV:	Epstein-Barr virus
ECM:	extracellular matrix
Edg:	endothelial differentiation gene
EGF:	epidermal growth factor
EPAC:	exchange protein activated by cAMP
E.R:	endoplasmic reticulum
ERK1/2:	extracellular signal-regulated kinases
F-actin :	filamentous actin
FAK:	focal adhesion kinase
FGF:	fibroblast growth factor

GAP:	GTPase activating protein
GDI:	guanosine nucleotide dissociation inhibitors
GDP:	guanosine diphosphate
GEF:	guanine exchange factor
GPCR:	G-protein coupled receptor
GPI:	glycosyl-phosphatidylinositol-anchored protien
GPR:	G-protein receptor
GSK3alpha:	glycogen synthase kinase 3
GTP:	guanosine triphosphate
GTPase:	guanosine triphosphate hydrolysing enzyme
HKD:	HxKxxxxD/E
HUVECs:	human umbilical vascular endothelial cells
IFN-γ:	interferon gamma
IL-1β:	interleukin 1-beta
IL-4:	interleukin 4
IP3:	inositol triphosphate
JNK:	c-Jun N-terminal kinases
KDELR:	endoplasmic reticulum protein retention receptor
LDL:	low density lipoprotein
LDV:	leucine-aspartic acid-valine
LIMK:	LIM motif-containing protein kinase
LPA:	lysophosphatidic acid

LPA_4:	lysophosphatidic receptor 4
LPAAT:	lysophosphatidic acid acyl transferases
LPC:	lysophosphatidyl choline
LPS:	lipopolysaccharide
lysoPLD:	lysophospholipase D
MAG:	monoacylglycerol
MAPK:	mitogen activated protein kinase
MEF:	mouse embryonic fibroblasts
MMP-2 (7, 9):	matrix metalloproteinase 2 (7, 9)
MORPHO:	modulator of oligodendrocyte remodeling and focal adhesion organization
mRNA:	messenger ribonucleic acid
MT1-MMP:	membrane-type 1 MMP
NFAT:	nuclear factor of activated T-cells
NFkB:	nuclear factor kappa β
N-myc:	neuroblastoma derived V-myc myelocytomatosis viral related oncogene
NPP2:	nucleotide pyrophosphatase/phosphodiesterase 2
NTA:	N-terminal acidic domain
N-WASP:	neuronal Wiskott-Aldrich syndrome protein
PA:	phosphatidic acid
PAI-1:	type 1 plasminogen activator inhibitor

PAK:	p21-activated protein kinase
PDE:	phosphodiesterase
PH:	pleckstrin homology
PI3K:	phosphoinositide 3-kinases
PKA:	protein kinase A
PKC:	protein kinase C
PLA1 or 2:	phospholipase A1 or A2
PLC:	phospholipase C
PLD:	phospholipase D
PRD:	proline rich domain
PTX:	Pertussis toxin
RA:	ras-association
Rap1 (2):	ras-proximate-1 (2)
RAPGEF3:	rap guanine nucleotide exchange factor 3
RAPL:	regulator of adhesion and polarization enriched in lymphocytes
RAS:	rat sarcoma
REM:	ras-exchange motif
RGD:	arginine-glycine-aspartic acid
Riam:	rap1–GTP-interacting adapter molecule
Rims:	rab3-interacting molecules
Rit:	ras-like protein in tissues
RNA:	ribonucleic acid

ROCK:	rho-associated coiled-coil-forming protein kinase
RT-PCR:	reverse transcriptase polymerase chain reaction
S1P:	sphingosine-1-phosphate
sAC:	soluble AC
SEM:	standard error
SFK:	src family kinase
SH3:	src-homology 3 domain
shRNA:	short hairpin RNA
SRE:	serum response element
Tbx2:	T-box transcription factor 2
TGF-β:	transforming growth factor beta
Tiam1:	T-cell lymphoma invasion and metastasis-inducing protein 1
tmAC:	transmembrane AC
tyr:	tyrosine
uPA:	urokinase-type plasminogen activator
uPAR:	urokinase receptor
VASP:	vasodilator-stimulated phosphoprotein
VCA:	verprolin-cofilin-acidic
VEGF:	vascular endothelial growth factor
vzg-1:	ventricular zone gene 1
WASP:	Wiskott-Aldrich syndrome protein
WAVE:	WASP family verprolin-homologous protein

1- INTRODUCTION

1.1 Autotaxin

1.1.1 Identification and structural characteristics

Autotaxin (ATX), also known as Nucleotide pyrophosphatase/phosphodiesterase 2 (NPP2), was originally isolated from the culture medium of human melanoma cells (A2058) in 1991 (STRACKE *et al.,* 1992). It was identified as a novel 125-kDa autocrine motility stimulating factor with a basic pI of 7.7 +/- 0.2 (STRACKE *et al.,* 1992). Following its initial discovery, ATX was identified as one of the seven members of the nucleotide pyrophosphatase/phosphodiesterase (NPP) enzyme family due to its homology with phosphodiesterases (MURATA *et al.,* 1994). All members of the NPP family have structurally related catalytic domains and nucleotide pyrophosphatase/phosphodiesterase activity whereby they hydrolyze pyrophosphate or phosphodiester bonds in nucleotides and other extracellular molecules. However, the NPP family members have very different substrate specificity (JANSEN *et al.,* 2005) and are therefore implicated in diverse pathological processes (STEFAN *et al.,* 2005).

ATX was originally thought to be an integral membrane protein like NPP1 because of their overall structural similarity (MOOLENAAR, 2002;STRACKE *et al.,* 1997). However, researchers have demonstrated that the N-terminal 27-residue hydrophobic domain of ATX is in fact a signal peptide that is removed by a signal peptidase during translation (JANSEN *et al.,* 2005). ATX has also been shown to have a more prominent

cytoplasmic distribution than NPP1, consistent with its N-terminal hydrophobic domain being a signal peptide and not a signal anchor as in NPP1 (JANSEN *et al.,* 2005;KOIKE *et al.,* 2006). Following the removal of this pre-peptide, the ATX pro-protein follows the classical secretory pathway, where proteins are transported outside the cell from the E.R via the Golgi apparatus. This was verified by the fact that ATX secretion was arrested by brefeldin A, an inhibitor of such transport (JANSEN *et al.,* 2005). These observations were consistent with the finding that the majority of ATX is present in the culture medium of several cell types such as Glioblastoma Multiforme cells (JANSEN *et al.,* 2005;KISHI *et al.,* 2006). ATX is therefore synthesized as a pre-pro-protein and functions as a secreted protein (JANSEN *et al.,* 2005;KOIKE *et al.,* 2006). C-terminal to the peptidase cleavage site is a consensus sequence for furin, or related pro-protein convertases such as PACE4. It is not known whether cleavage by furin occurs before or after secretion, however, this cleavage is not required for ATX secretion (JANSEN *et al.,* 2005;KOIKE *et al.,* 2006). The removal of an N-terminal octapeptide by furin, however, is associated with enhanced motility stimulating activity of ATX in certain reports (JANSEN *et al.,* 2005). ATX cleaved by furin was shown to have a 30% increase in activity over the non-furin cleaved form in HEK293 cells (KOIKE *et al.,* 2006). Three ATX isoforms have been found in the medium of cells and have been characterized by Giganti *et al* (2008) as ATX α, β, and γ. ATX α, originally discovered in A2058 cells, lacks exon 21; ATXβ, a splice variant reported in human teratocarcinoma (LEE *et al.,* 1996) lacks exons 12 and 21; and ATXγ (PD-1α), isolated from brain lacks exon 12. The mRNA transcripts of these three isoforms were also shown to have different tissue distributions. ATXβ mRNA has

high expression in peripheral tissues while ATXγ mRNA has highest expression in the brain. The ATXα mRNA isoform had the lowest expression levels in both the brain and peripheral tissues. The measured activity of ATXα protein was also very low probably due to its cleavage by an unknown factor that results in a protein of 55-66kDa with little or no enzymatic activity (GIGANTI *et al.,* 2008).

Besides its pre and pro protein domains, ATX contains a Modulator of Oligodendrocyte Remodeling and Focal adhesion Organization (MORPHO) domain implicated in oligodendroglial process network formation and focal adhesion organization (DENNIS *et al.,* 2008). It also contains an EF-hand-like motif that contributes to the function of the MORFO domain, an inactive nuclease-like domain that is essential for the catalytic activity of ATX, and two cysteine-rich somatomedin B domains (YUELLING and FUSS, 2008). The somatomedin B domain, which is derived from the amino terminus of vitronectin, forms a presumed binding site for type 1 plasminogen activator inhibitor (PAI-1), and urokinase plasminogen activator receptor (uPAR) (SEIFFERT and LOSKUTOFF, 1991;SEIFFERT *et al.,* 1994). This suggests a relationship of ATX with extracellular matrix proteins. In fact, ATX has recently been shown to contain an integrin binding domain (RGD) within its somatomedin B domains, that may be implicated in lymphocyte trafficking (KANDA *et al.,* 2008). Finally, ATX has a catalytic domain, structurally similar to that of the NPP family, that functions as a lysophospholipase D (UMEZU-GOTO *et al.,* 2002). This will be discussed in more detail in the following section. The structural domains of ATX are illustrated in Figure 1.

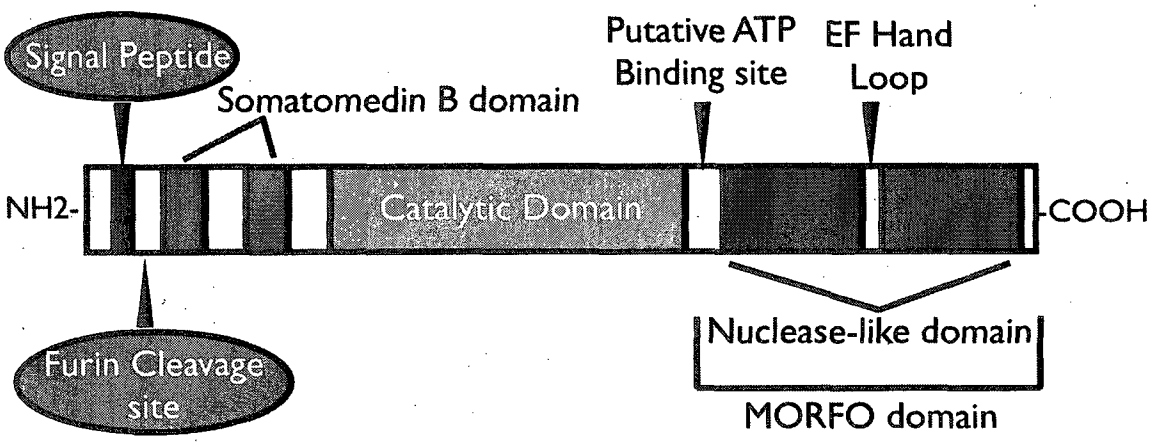

Figure 1: The major structural domains of ATX. ATX contains an N-terminal signal peptide that is removed during translation. Following this is a consensus sequence for furin cleavage that may result in enhance activity of the protein. ATX also has two somatomedin B domains, an inactive nuclease-like domain that contains an EF hand loop motif, and an ATP binding site. Finally there is a catalytic domain which functions as a lysophospholipase D and a MORPHO domain implicated in oligodendrocyte remodeling.

1.1.2 Enzymatic activity

ATX was originally thought to hydrolyze pyrophosphate or phosphodiester bonds in nucleotides due to its inclusion in the NPP family (BOLLEN *et al.,* 2000). However, autotaxin, has recently been shown to be molecularly identical to extracellular plasma lysophospholipase D (lysoPLD), whose activity was first discovered in human plasma in 1983 (YAMASHITA *et al.,* 1983). LysoPLD is responsible for catalyzing the production of Lysophosphatidic acid (LPA) from Lysophosphatidylcholine (LPC) by hydrolysis (UMEZU-GOTO *et al.,* 2002), see figure 2. cPA (cyclic phosphatidic acid), an analog of LPA and intermediate in LPA formation, can also be produced from LPC by ATX (TSUDA *et al.,* 2006). LPC is the main physiological substrate for ATX/lysoPLD. In fact, ATX has a 25-fold lower Km and thus higher affinity for LPC than for nucleoside substrates, which are the natural substrates for other members of the NPP family (XIE and Meier, 2004). ATX/LysoPLD also has a higher affinity for unsaturated acyl-LPCs as compared to saturated or ether-linked species (TOKUMURA *et al.,* 1999). ATX, interestingly, 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 at the same catalytic site used for the hydrolysis of nucleotides (GIJSBERS *et al.,* 2003). ATX requires a metal ion, such as Co^{2+}, for optimal lysoPLD activity. It can also be enhanced by Ca^{2+} and Mg^{2+}, which may act by stabilizing the structure of ATX, by protecting it from thermal denaturation and proteolysis, or by regulating the catalytic activity of ATX (TOKUMURA *et al.,* 1998). Plasma ATX is

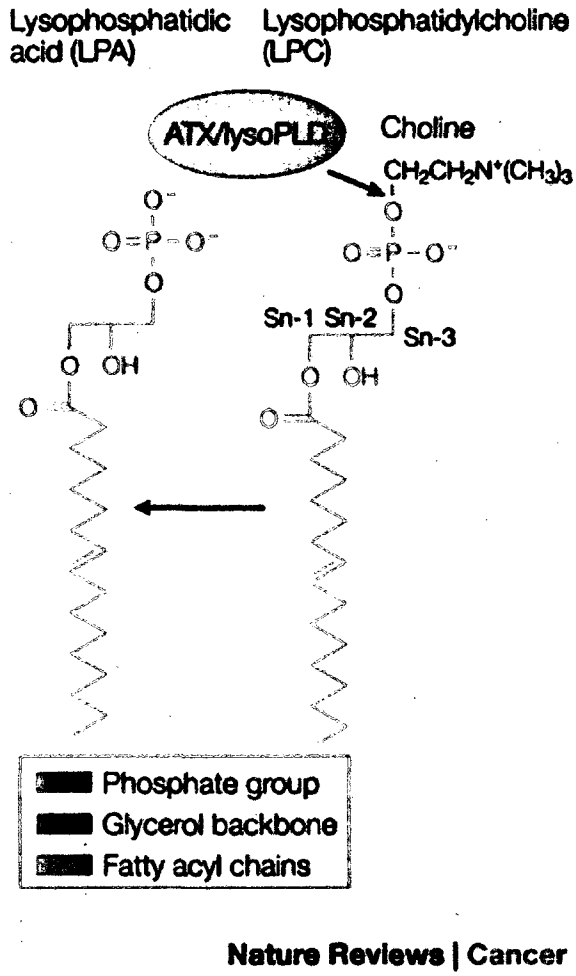

Figure 2: Representation of the production of LPA from LPC by ATX. The lysoPLD activity of ATX is responsible for hydrolyzing the bond between choline and the phosphate group of LPC. This results in release of choline and LPA. (Modified figure from: MILLS and MOOLENAAR, 2003).

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). LPA, generated from LPC, is considered to be responsible for the majority of ATX biological effects and will be discussed in the LPA section.

1.1.3 Expression and role in normal cells and tissues

ATX is ubiquitously expressed and is, therefore, synthesized by a variety of normal cells and tissues. Particularly high expression of ATX has been found in the brain (MILLS and MOOLENAAR, 2003), kidneys and lymph nodes (KANDA *et al.,* 2008). ATX is implicated in many processes during normal development such as adipogenesis (SIMON *et al.,* 2005), and central nervous system development that includes neurite morphology (DENNIS *et al.,* 2005). It is also implicated in intestinal cell motility through activation of PLC-gamma and phosphorylation/recruitment of villin (KHURANA *et al.,* 2008). ATX has been shown to regulate myelination by controlling cytoskeletal organization and FAK phosphorylation in oligodendrocytes (FOX *et al.,* 2004). More recently, ATX has been shown to be implicated in immune functions due to the findings that mast cells in submucosal connective tissue secrete ATX (MORI *et al.,* 2007) and that ATX was shown to promote the entry of lymphocytes into secondary lymphoid organs (KANDA *et al.,*

2008). Besides these advancements, the most well known role of ATX is in vascular development (KHURANA *et al.,* 2008;SATO *et al.,* 2005).

ATX has been shown to be essential for blood vessel formation during development. ATX deficiency using knockout technology in mice leads to embryonic lethality due to impaired vessel formation in the yolk sac and embryo (VAN MEETEREN *et al.,* 2006). The vascular defects in ATX deficient mice also resemble those in mice lacking genes involved in cell migration and adhesion such as the fibronectin and focal adhesion kinase genes. Results of this 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).

1.1.4 Expression and role in pathologies

ATX has been implicated in numerous pathologies including Alzheimer's disease, chronic hepatitis C, multiple sclerosis, neuropathic pain, obesity and rheumatoid arthritis, but its most investigated and presumably most important role is in tumorigenesis (FERRY *et al.,* 2003;HAMMACK *et al.,* 2004;INOUE *et al.,* 2008a;INOUE *et al.,* 2008b;UMEMURA *et al.,* 2006;WATANABE *et al.,* 2007;ZHAO *et al.,* 2008). ATX has been shown to be up-regulated in malignancies including breast, lung, colon, ovarian, stomach, thyroid and brain cancer, correlating with the invasiveness of these cancer cells (KEHLEN *et al.,* 2004;KISHI *et al.,* 2006;YANG *et al.,* 2002;YANG *et al.,* 1999). ATX has been shown to augment cellular characteristics associated with tumor aggressiveness, including cell

proliferation, cell survival, cell motility, invasion and angiogenesis. ATX acts extracellularly and stimulates the metastatic cascade at multiple levels by acting as a tumor cell motility factor as well as a strong inductor of the angiogenic response (NAM *et al.,* 2000;NAM *et al.,* 2001). Specifically, ATX-transfected Ras-transformed NIH3T3 cells were shown to be more invasive, tumorigenic, angiogenic and metastatic than mock-transfected controls (NAM *et al.,* 2000;NAM *et al.,* 2001). ATX, especially in the presence of LPC, has been shown to increase chemotaxis and proliferation in multiple cell lines. It has also been demonstrated that several cancer cell lines release significant amounts of LPC into the culture medium (UMEZU-GOTO *et al.,* 2002). ATX promotes proliferation of A2058, MDA-MB231, CHO-K1 and Edg2-RH7777 cancer cells, but not RH7777 cells that lack LPA receptors. Research, therefore, suggests that autocrine or paracrine production of LPA via ATX contributes to tumor cell motility, survival and proliferation (BRINDLEY, 2004;UMEZU-GOTO *et al.,* 2002). Recently over-expression of ATX (or LPA_{1-3}) has been shown to increase tumor invasion and metastasis of breast cancer cells (LIU *et al.,* 2009) while 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.

1.1.5 Regulation of expression

Although ATX has important roles in tumor progression, mostly due to its aberrant expression in various malignant cells (described above), little is known about the factors

that regulate its expression in cells. This section will detail the few stimulators and inhibitors currently known.

ATX expression can be induced by retinoic acid in a neuroblastoma cell line with N-myc amplification that is responsive to the differentiation inducing effects of retinoic acid (DUFNER-BEATTIE *et al.,* 2001). ATX is also one of many genes up-regulated during Bmp-2 mediated mesenchymal development (BACHNER *et al.,* 1998) indicating that cell differentiation might trigger ATX expression in certain cell lines. Lipopolysaccharide (LPS) induces ATX expression in the monocytic THP-1 cells via JNK and p38MAPK, resulting in enhanced immune cell migration (LI and ZHANG, 2009). Fibroblast-like synoviocytes from patients with rheumatoid arthritis have increased expression of ATX that can be down regulated by anti-inflammatory cytokines including IL-1β, IL-4 and IFN-γ (SANTOS *et al.,* 1996). In thyroid carcinoma cell lines, the growth factors EGF and bFGF have been shown to stimulate ATX activity while the anti-inflammatory cytokines IL-4, IL-1β and TGF-β reduced its expression (KEHLEN *et al.,* 2004). Therefore, pro-inflammatory stimuli seem to increase ATX expression while anti-inflammatory cytokines have the opposite effect. Expression of α6β4 integrin, which correlates with an invasive and migratory phenotype in advanced breast carcinomas, leads to increased expression of ATX mediated by up-regulation and activation of NFAT1 that binds to the ATX promotor (CHEN and O'CONNOR, 2005). ATX expression is increased by more than 100-fold in cells transformed by the viral oncoprotein v-Jun (BLACK *et al.,* 2004) and Epstein-Barr virus, an oncogenic herpesvirus. Infection of Hodgkin lymphoma

cells with EBV also results in induction of ATX (BAUMFORTH *et al.,* 2005). 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). Therefore it seems that molecular cues associated with cancer progression can induce ATX expression while tumor suppressors seem to reduce its expression. Each of the above mentioned stimuli have been investigated in very few cell types. Therefore, the precise signaling pathways and transcription factors responsible for ATX regulation remain mostly unknown so many more studies are needed to define exactly how ATX expression is regulated.

1.2 Lysophosphatidic acid

1.2.1 Identification

Lysophospholipids have been known for decades as components in the biosynthesis of cells membranes with short half lives that range from seconds to minutes (CHOI *et al.,* 2010;SHIMIZU, 2009). They were originally thought to act as intracellular messengers (GERRARD and ROBINSON, 1984) or to mediate effects due to intrinsic chemical properties, such as calcium binding activity (SIMON *et al.,* 1984). However, they were later found to have cell signaling roles (VOGT, 1963) and it is now known that most bioactive lipids act on cell surface GPCRs to mediate intracellular signaling (SHIMIZU, 2009). Many lysophospholipids, including LPA, have similar effects on cellular functions as polypeptide growth factors (HILL and TREISMAN, 1995). LPA (1 or 2-acyl-sn-glycerol-3-phosphate), a glycerolysophospholipid, was the first LP to be recognized as a major lipid mediator in serum exerting growth factor like activities at submicromolar

concentrations (TOKUMURA *et al.*, 1978), including mitogenic and morphological effects on many cell types (ISHII *et al.*, 2009).

LPA has a glycerol backbone, single carbon chain, and a polar headgroup (see figure 2) (MEYER ZU HERINGDORF and JAKOBS, 2007). There are multiple molecular species of LPA, consisting of acyl- or ether-linked chains with various numbers of carbons and degrees of unsaturations. The acyl chain can be 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). The biological activities of LPA depend on the carbon chain length and degree of unsaturation as well as the position and linkage type of the carbon chain attached to the glycerol backbone (MEYER ZU HERINGDORF and JAKOBS, 2007).

1.2.2 Production and degradation of LPA

LPA is detected in serum, plasma and many other biological fluids and tissues such as saliva (SUGIURA *et al.*, 2002), follicular fluid (TOKUMURA *et al.*, 1999), seminal fluid (HAMA *et al.*, 2002), and malignant effusions (WESTERMANN *et al.*, 1998). 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 able 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). LPA is found at a concentration of approximately 154pmol in cells, 0.1-6.3 μM in blood and 80-100nM in plasma

(HOSOGAYA *et al.*, 2008;KISHIMOTO *et al.*, 2003). As mentioned previously, the lysophospholipase D activity of autotaxin is responsible for the majority of LPA produced in vivo from the substrate LPC (UMEZU-GOTO *et al.*, 2002). However, other enzymes and pathways remain responsible for some LPA production. These other routes of LPA synthesis include de novo LPA biosynthesis in cells through intermediate lipid metabolism, resulting in intracellular LPA, or liberation and subsequent enzymatic conversions of precursor glycerophospholipids, resulting in extracellular LPA (GOETZL and An, 1998). Extracellular LPA can be produced by the action of many different enzymes including phospholipase A1 or A2 (PLA1 or 2), monoacylglycerol kinase or glycerol-3-phosphate acyltransferase (PEBAY *et al.*, 2007). PLA1/2 for example produce LPA by deacylating phosphatidic acid (PA) that is first generated intracellularly from phospholipids or diacylglycerol (AOKI *et al.*, 2008). Figure 3 summarizes the pathways of LPA production and degradation.

LPA has short half-life attributed to its rapid degradation by lipid phosphate phosphatases, integral membrane proteins that dephosphorylate LPA to monoacylglycerol (MAG) (BRINDLEY *et al.*, 2002), or by acylation of LPA to PA by the action of acyl transferases (LPAAT) (YAMASHITA *et al.*, 2001). To counteract these 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;PAGES *et al.*, 2001).

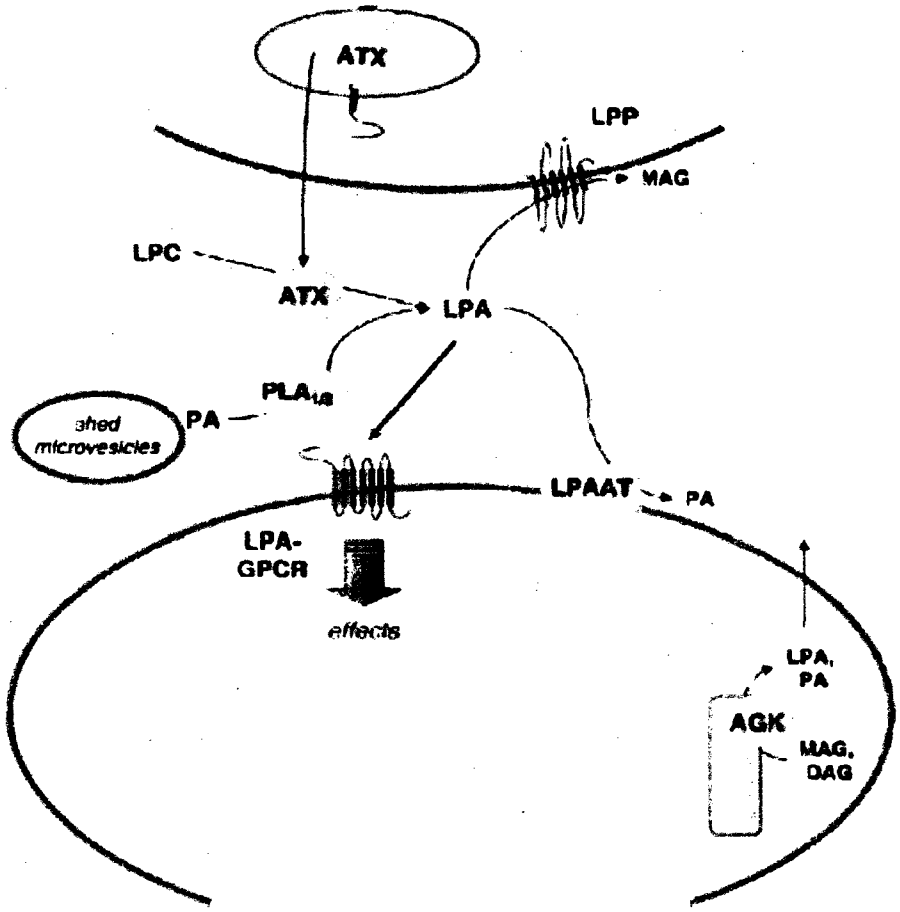

Figure 3: Production and degradation pathways for LPA. The major producer of LPA in vivo is ATX which converts LPC to LPA. Other enzymes that can also produce LPA include acylglycerol kinase (AGK) which produces LPA from MAG and PLA1/2 which can produce LPA from PA. The two major degradation pathways for LPA are acylation by LPAAT resulting in PA and phosphorylation by LPPs resulting in MAG. (Modified figure from: MEYER ZU HERINGDORF and JAKOBS, 2007).

1.2.3 LPA receptors

The first evidence of the possible existence of G-protein-coupled receptors (GPCRs) specific for LPA was the observation, by van Corven et al. in 1989, that the proliferative effects of LPA on fibroblasts were pertussis toxin sensitive and therefore mediated by G-proteins (VAN CORVEN *et al.*, 1989). GPCRs are 7 transmembrane receptors that couple to various trimers of G-proteins to mediate intracellular signaling. Further studies also supported the existence of LPA-specific receptors (THOMSON *et al.*, 1994;VAN DER BEND *et al.*, 1992). Thomson et al. (1994) found LPA-specific high affinity binding sites on cell membranes and suggested that GPCRs could be responsible for mediating the LPA binding to membranes and induce a transient increase in intracellular Ca^{2+} levels. Van der Bend et al. (1992) found that a ^{32}P-labeled LPA analog binds to a membrane protein of 38-40 kDa in various cells types that was proposed to be a specific cell-surface LPA receptor (VAN DER BEND *et al.*, 1992). However, it was not until 1996 (HECHT *et al.*, 1996) that the first LPA receptor gene was identified in mice during the course of studies designed to identify novel GPCR genes associated with the production of neurons in mice (CHUN *et al.*, 1999;CHUN, 1999;MASANA *et al.*, 1995).

LPA_1, originally named ventricular zone gene 1(vzg-1) due to its increased localization in this area of the brain, was also known as endothelial differentiation gene 2 (Edg-2) due to its sequence similarity to an orphan receptor called Edg-1 (endothelial differentiation gene 1) cloned from endothelial cells (HLA and Maciag, 1990). Edg-2/LPA_1 was subsequently cloned and identified as an LPA receptor in humans in 1997 (AN *et al.*, 1997). LPA_1 is a 41 kDA (364 a.a) protein with seven transmembrane domains, in

concordance with the structure for GPCRs (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). Interestingly, LPA_1 is also expressed in several cancers (FURUI *et al.*, 1999) such as HeLa carcinoma, SW480 colorectal adenocarcinoma, A549 lung carcinoma, and G361 melanoma, but undetectable in many leukemia lines and Burkitt's lymphoma (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). In humans, LPA_2 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). Therefore, LPA_2 is less widely distributed than LPA_1. LPA_2 has also been found to be expressed in various cancer cell types (AN *et al.*, 1998a). A third related gene, Edg-7 now known as LPA_3 has 60% amino acid similarity to mouse LPA_1 and 2. LPA_3 has a more restricted expression pattern than LPA_1 and 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). The Edg family has 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). However, signaling induced by these receptors was unable to account for all the cellular effects of LPA. The existence of additional receptors was later implied by several reports

(CONTOS *et al.*, 2002;HOOKS *et al.*, 2001) due to the fact that cell lines not expressing the Edg receptors were shown to have mitogenic responses to LPA.

Recently, a distinct group of LPA receptors unrelated to the Edg family receptors have been identified. In 2003 a fourth LPA receptor (LPA_4/p2y9/GPR23) was identified that was structurally distant from the Edg receptors (NOGUCHI *et al.*, 2003) (LPA_4 will be discussed in detail in the LPA_4 section). 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).

LPA_5 (GPR92-93) (KOTARSKY *et al.*, 2006;LEE *et al.*, 2006) was an orphan GPCR that was identified as an LPA receptor due to its close relation to LPA_4 (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).

LPA_6 (p2y5), was originally reported in 1996 as an orphan GPCR encoded in an intron of the retinoblastoma gene (HERZOG *et al.*, 1996). LPA_6 has ubiquitous expression including in hair follicle cells, epidermis (PASTERNACK *et al.*, 2008), intestine (LEE *et*

al., 2009) and a leukemia cell line (YOON *et al.*, 2006) with high expression in human umbilical vascular endothelial cells (HUVECs) (YANAGIDA *et al.*, 2009).

1.2.4 Signaling pathways

The major cellular effects mediated by LPA are either growth related or cytoskeletal-dependent effects (GOETZL and An, 1998). Upon receptor activation, G-alpha subunits are separated from G-beta-gamma subunits, which remain together, now in their active state bound to GTP (WATTS and NEVE, 2005). The Edg family LPA GPCRs mediates effects by coupling to G_i, G_q or $G_{12/13}$ depending on cell type, receptor expression levels, or 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 all three of these G-proteins while LPA_3 only couples to G_q and G_i (FUKUSHIMA *et al.*, 1998;ISHII *et al.*, 2000)(see figure 4 for an overview of the signaling pathways). Signaling by LPA_4 will be discussed in further detail later and signaling of the newest LPA receptors, LPA_5 and LPA_6, is less well characterized and will not be discussed here.

G_i, which is pertussis toxin sensitive, mediates inhibition of cAMP production as well as stimulation of protein kinases that recruit the ras-raf cascade and activate MAP kinases, resulting in proliferation and differentiation. G_i induces FAK (focal adhesion kinase) activation leading to focal adhesion formation. PI3K is also activated downstream of G_i leading to Akt activation and cell survival or Rac activation and cell migration. Finally, tyrosine kinase-dependent induction of tyrosine phosphatases can also be promoted by G_i signaling (CHUPRUN *et al.*, 1997;GAITS *et al.*, 1996).

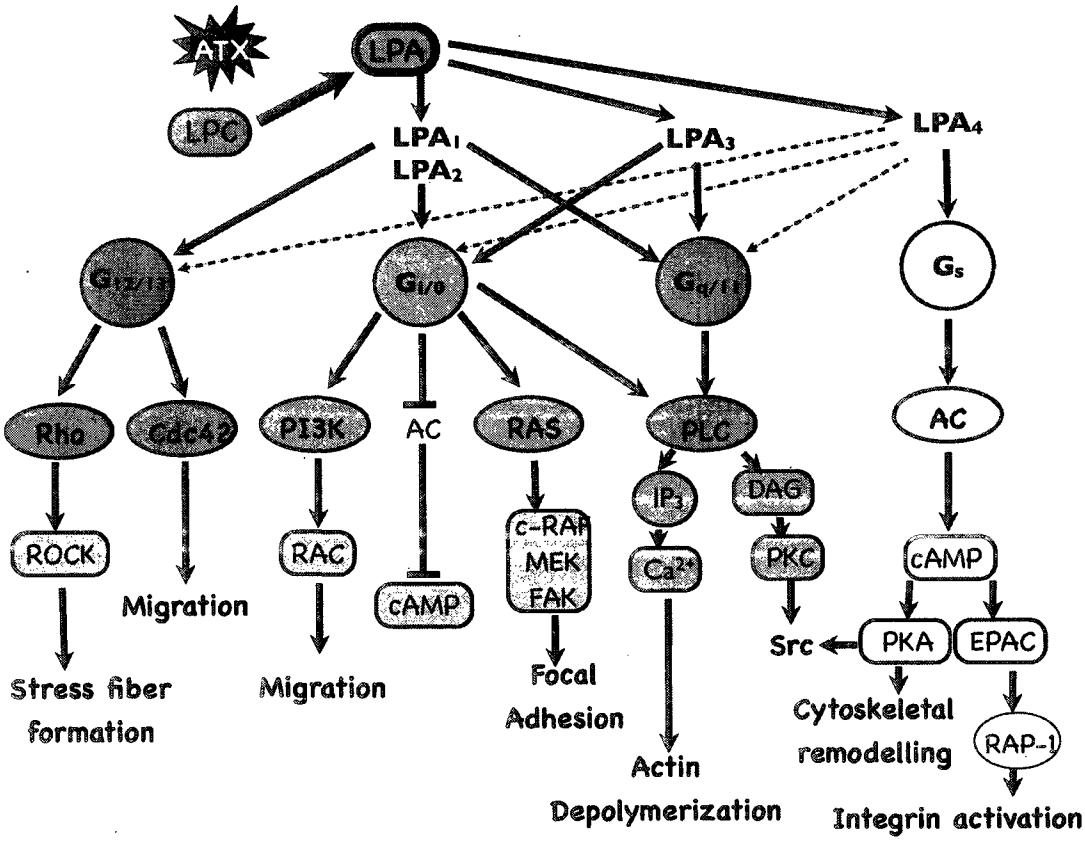

Figure 4: Signaling pathways of the LPA receptors. The major LPA signaling pathways are illustrated here and include signaling through four different G-protein families, $G_{12/13}$, G_i, G_q, an G_s. Signaling through these G-proteins can activate diverse proteins including RhoA, PI3K, Ras, PLC and AC. The major downstream effects of LPA signaling include cytoskeletal remodeling, cell migration, stress fiber formation, focal adhesion formation and integrin activation.

G_q mediates phospholipase C (PLC) activation, liberating inositol triphosphate (IP3) that causes mobilization of intracellular Ca^{2+} that can result in actin depolymerization. PLC activation downstream of G_q can also stimulate MAP kinases directly or through induction of diacylglycerol (DAG), which activates some PKC isozymes, which in turn can activate MAP kinases by activating Raf-1, (GHOSH *et al.*, 1997). Furthermore, PKC-δ can activate src kinase activity indirectly through protein tyrosine phosphatase alpha (BRANDT *et al.*, 2003). Finally, $G_{12/13}$ stimulates RhoGTPase pathways that contribute to SRE-mediated transcription as well as mediating cytoskeletal dependent functions such as stress fiber formation and actin polymerization. $G_{12/13}$ also promotes activation of PLD, PI3K, RhoA and Cdc42 (FROMM *et al.*, 1997). $G_{\beta\gamma}$ 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).

1.2.5 Implication in physiological and pathological processes

LPA signaling 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). For instance, LPA has been identified as a main platelet-activating lipid of mildly oxidized LDL and human atherosclerotic lesions (SIESS *et al.*, 1999). In platelets, LPA induces human platelet shape change and platelet aggregation. LPA signaling is also implicated in neuropathic

pain responses and induces neuropathic pain and demyelination of the dorsal root similar to what is observed after nerve injury (FUJITA *et al.*, 2007).

LPA is highly implicated in embryonic development of the nervous system . It is an important mediator of physiological and pathological processes in the central nervous system and influences all neuronal cell types. Both apoptotic and survival effects of LPA have been reported and these opposing effects may be due to concentration differences or differential expression of LPA receptors as well as cell maturation and cell density (TAKUWA *et al.*, 2002). For example, LPA has been demonstrated to mediate proliferative effects and morphological changes in Ventricular Zone neuroblasts (CONTOS *et al.*, 2000a;FUKUSHIMA *et al.*, 2000), and to influence neuronal differentiation (DOTTORI *et al.*, 2008;SPOHR *et al.*, 2008), including neurite formation by differentiating neurons (FUKUSHIMA *et al.*, 2000). LPA was also shown to protect Schwann cells from apoptosis (WEINER and CHUN, 1999) and to promote cell survival of early postmitotic cortical neurons (KINGSBURY *et al.*, 2003). However, LPA also induces growth cone collapse and neurite retraction as well as apoptosis in hippocampal neurons (FUKUSHIMA, 2004). Apoptotic effects of LPA are observed in neurons cultured for over one week while survival effects are seen in neurons cultured for 1-2 days (FUJIWARA *et al.*, 2003).

Another important role of LPA is in reproduction. LPA is found in normal follicular fluid suggesting its involvement in normal physiology of ovaries (TOKUMURA *et al.*, 1999). LPA may also be involved in male and female reproductive physiology and pathology

(TOKUMURA *et al.*, 1999). LPA receptor mediated signaling has been implicated in many processes involved in reproduction such as ovarian functions (CHEN *et al.*, 2008;TOKUMURA *et al.*, 1999), with LPA_4 being highly expressed in human and mouse ovary (NOGUCHI *et al.*, 2003), spermatogenesis, with degradation of LPA being associated with reduced spermatogenesis and LPA_{1-4} expression in human testis (CHOI *et al.*, 2010;NOGUCHI *et al.*, 2003;YE, 2008), fertilization (GARBI *et al.*, 2000), early embryo spacing (HAMA *et al.*, 2007;YE *et al.*, 2005), decidualization (SHIOKAWA *et al.*, 2000) and pregnancy maintenance (ZIECIK *et al.*, 2008). LPA signaling has also been implicated in pathologies of the reproductive system including ovarian, prostate and endometrial cancers (LPA_2 and MMP-7 implicated) (GUO *et al.*, 2006;HOPE *et al.*, 2009;SUTPHEN *et al.*, 2004) and endometriosis (WEI *et al.*, 2009;WOCLAWEK-POTOCKA *et al.*, 2009).

These physiological effects of LPA are mediated by signaling through various LPA receptors whose effects can be elucidated by 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. 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). LPA_1, therefore, seems to play an important role in the central nervous system. 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.

Finally, the most important role of LPA for this study is in tumor progression. LPA is known to be a potent tumor promoting molecule and influences many cellular processes implicated in tumorigenesis. LPA has an effect on the cellular motility of cancer cells by mediating cytoskeletal rearrangements via the Rho GTPases Rho and Rac (IMAMURA *et al.*, 1993;STAM *et al.*, 1998). This leads to stimulation or inhibition of cell migration or invasion depending on the cell type. LPA can induce proliferation and mitogenic signaling of prostate cancer cells (BUDNIK and MUKHOPADHYAY, 2002). LPA also stimulates migration and proliferation of human 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). LPA is also present in the ascites from ovarian cancer patients (WESTERMANN *et al.*, 1998;XU *et al.*, 1998), and a significant increase in blood LPA has been found in patients with ovarian carcinoma at the first stage of this disease, suggesting an important contribution of LPA to this pathology (FANG *et al.*, 2000). Malignant progression has also been shown to correlate with differential expression of various LPA receptor subtypes (CONTOS *et al.*, 2000b). LPA_1 over-expression in breast carcinoma cells leads to metastatic spread to bone (BOUCHARABA *et al.*, 2004), while LPA_1 signaling has been shown to mediate stimulation of motility of human pancreatic cancer cells (YAMADA *et al.*, 2004) and induction of metastasis by human colon carcinoma cells (SHIDA *et al.*, 2003). LPA_2 is over-expressed in invasive ductal

carcinoma (KITAYAMA *et al.*, 2004) as well as ovarian cancer (ERICKSON *et al.*, 2001) and promotes mitogenic signaling in human colon cancer cells (YUN *et al.*, 2005). Finally, LPA_3 expression increases the aggressiveness of ovarian carcinoma (YU *et al.*, 2008). Also, as mentioned in the previous ATX section, over-expression of LPA_{1-3} receptors or their pharmacological inhibition results in promotion or inhibition of cancer cell invasion, tumor progression, and metastasis (LIU *et al.*, 2009;ZHANG *et al.*, 2009).

1.3 LPA receptor 4

1.3.1 Expression and role in physiological processes

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). Increased mRNA expression has also been documented at implantation sites in the uterus (ISHII *et al.*, 2009). The roles of LPA_4 in physiology and disease have only started to be uncovered. To date, LPA_4 has been shown to induce Rho-mediated neurite retraction and stress fiber formation as well as cell aggregation and rounding (LEE *et al.*, 2007;YANAGIDA *et al.*, 2007), AC stimulation leading to increased cAMP levels (LEE *et al.*, 2007;NOGUCHI *et al.*, 2003), and G_q and G_i-mediated calcium mobilization (LEE *et al.*, 2007;NOGUCHI *et al.*, 2003;YANAGIDA *et al.*, 2007). LPA_4 may play a role in neuronal development including neurogenesis and neuronal migration as it increases cAMP and the transcription factor CREB, which is essential to neuronal differentiation (RHEE *et al.*, 2006;YANAGIDA *et al.*, 2007). In a recent study, LPA_4, coupled to cAMP,

has been found to inhibit osteogenic differentiation reducing bone volume and trabecular thickness (LIU *et al.*,) signifying a role in bone homeostasis.

1.3.2 Implications in cancer

There is little information to date on the implications of LPA_4 in cancer. One study has found that LPA_4 expression inhibits motility and invasion of B103 neuroblastoma cells, which do not endogenously express LPA_4. In this study, knockdown of LPA_4 in MEFs increased cell migration, and LPA_4 expression decreased PI3K reducing Akt and Rac activation levels while increasing Rho activation. It is important to note that in the cell type studied, LPA_4 couples only to G_q and $G_{12/13}$ (LEE *et al.*, 2008). However, another recent study has found that expression of the LPA_4 receptor can induce transformation and anchorage independent growth in Myc/tbx2 -transformed cells. Myc and tbx2 are cooperating partners in cell transformation. The authors found that expression of LPA_4 (or LPA_1 or LPA_2) in Myc/tbx2-MEFs induced a transformed phenotype observed by the increased ability of the cells to grow in soft agar (anchorage-independent growth) as well as inducing tumor formation in vivo when these cells were subcutaneously injected in mice. These effects were found to necessitate G_i signaling and activation of PI3K and ERK1/2 pathways, particularly with prolonged activation of ERK (TAGHAVI *et al.*, 2008). Therefore the role of LPA_4 in tumorigenesis is currently unclear and probably depends on cell type and G-protein coupling.

1.3.3 Major signaling pathway

LPA4 has been shown to be capable of signaling through G_i, activating PI3K and ERK1/2 (TAGHAVI *et al.*, 2008), $G_{12/13}$ resulting in Rho activation (LEE *et al.*, 2007) and G_q inducing increases in calcium (TAGHAVI *et al.*, 2008). However, the major signaling pathway of LPA4, different from those previously discussed for the other receptors, is signaling through G_s resulting in the production of cAMP (NOGUCHI *et al.*, 2003).

1.3.3.1 Production of cAMP

cAMP (cyclic adenosine 3'.5'-monophosphate) was first identified as a second messenger nucleotide found to have a fundamental role in the cellular response to extracellular stimuli and, therefore, control a diverse range of cellular processes (ROBISON *et al.*, 1968). GPCRs appear to be the main receptors responsible for causing an accumulation of intracellular cAMP in response to ligand binding. The heterotrimeric G proteins coupled to GPCRs regulate ACs (adenylyl cyclases) in response to various cellular stimuli. The G_s G-protein activates adenylyl cylclases (as does G_q in some instances) while the G_i family of G-proteins inactivates ACs (WATTS and NEVE, 2005). These G-proteins, upon receptor activation, separate from $G\beta\gamma$ and are converted to their GTP-bound state in which they can exert their distinctive regulatory functions on ACs (WATTS and NEVE, 2005). AC are 12 transmembrane domain proteins which are generally bound to the inside of cell membranes. Class III ACs are responsible for cAMP production (WATTS and NEVE, 2005). In humans there are 9 transmembrane AC enzymes (tmAC) and one soluble AC (sAC). However, the soluble form occurs primarily in mature spermatozoa and will not be further discussed here (JAISWAL and CONTI, 2003;WATTS

and NEVE, 2005). $G_s\alpha$ activates ACs by inducing a conformational change in the catalytic site upon interaction (SKIBA and HAMM, 1998). Activated AC converts ATP (adenosine triphosphate) to cAMP by creation of a cyclic phosphodiester bond with the alpha-phosphate group of ATP resulting in increased intracellular cAMP concentrations. cAMP can then be converted to AMP by cAMP-specific phosphodiesterases (PDE), see figure 5. Growth factors and PI3K can also down-regulate cAMP signaling by activating Akt and subsequently PDEs, facilitating the conversion of cAMP to AMP (ROBISON *et al.*, 1968; DEGERMAN *et al.*, 1997).

1.3.3.2 Spatial regulation of effectors

GPCRs are confined to specific domains of the cell membrane in association with intracellular organelles or the cytoskeleton. The ACs that they activate are found anchored nearby (JARNAESS and TASKEN, 2007) resulting in targeted cAMP production depending on the extracellular ligand and receptor activated. cAMP is further regulated through its degradation by cAMP-specific phosphodiesterases (PDEs), the only known mechanism of cAMP inactivation (JARNAESS and TASKEN, 2007). The cellular localization of PDEs is controlled by anchoring to specific subcellular compartments and recruitment into multi-protein signaling complexes, therefore, targeting them to specific subcellular locations. This allows increased cAMP concentrations in certain areas of the cell and not in others, where it will be degraded instead (JARNAESS and TASKEN, 2007). The level of intracellular cAMP is, therefore, spatially and temporally regulated by the balance between the activities of ACs and cyclic nucleotide PDEs (JARNAESS and TASKEN, 2007).

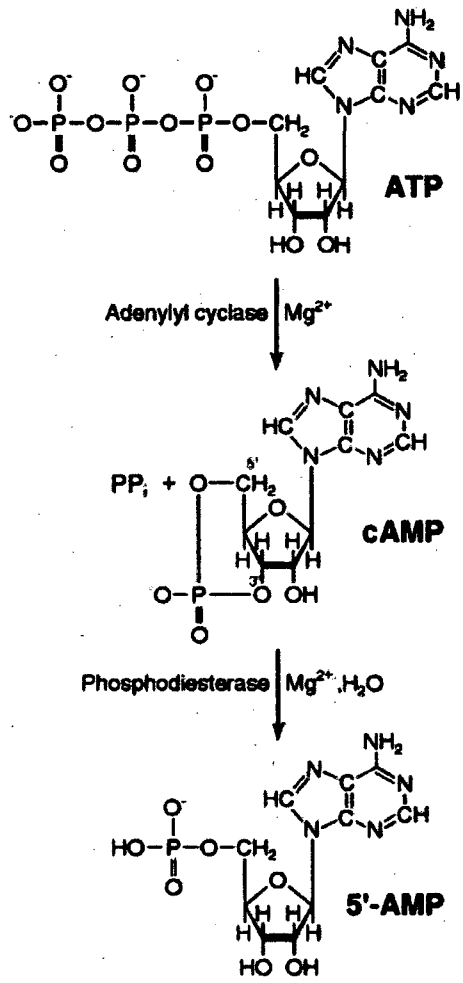

Figure 5: Production and degradation of cAMP. Activated AC converts ATP (adenosine triphosphate) to cAMP by creation of a cyclic phosphodiester bond with the alpha-phosphate group of ATP. cAMP can then be degraded to AMP by the action of cAMP-specific PDEs. From: DUMAN and NESTLER, 1999)

cAMP effectors are also spatially regulated. PKA (Protein kinase A/cAMP-dependent protein kinase), a major effector of cAMP (to be elaborated in the cAMP effector section) is tethered to specific intracellular locations by AKAPs (A-kinase anchoring proteins), which anchor the regulatory subunits (see figure 6A). AKAPs are scaffolding proteins that form multi-protein complexes to integrate cAMP signaling (SCOTT and MCCARTNEY, 1994). There are over 50 members of the AKAP family (WONG and SCOTT, 2004) which all have similar functions while being structurally diverse (JARNAESS and TASKEN, 2007). These proteins all contain a PKA binding domain and a unique targeting domain, with several containing additional interaction sites for formation of multivalent signaling complexes (JARNAESS and TASKEN, 2007). Some AKAPs are known to interact with both PDEs and PKA for example. Targeting of PKA isozymes is important for many physiological processes such as cAMP regulation of ion channels in the nervous system, regulation of the cell cycle involving microtubule dynamics, steroidogenesis, reproductive function, immune response and numerous intracellular transport mechanisms (TASKEN and AANDAHL, 2004).

EPAC (exchange protein activated by cAMP/cAMP-regulated guanine exchange factor) proteins, other effectors of cAMP (see cAMP effector section), are also spatially regulated. First these proteins have been shown to also interact with AKAP signaling complexes which may be responsible in part for their localization, that requires specific anchoring, to various cell compartments such as the cytosol, nucleus, nuclear envelope, and plasma membrane, resulting in different cellular functions (PONSIOEN *et al.*, 2009). EPAC localization has also recently been found to be directly regulated by cAMP. In a

2009 study by Ponsioen *et al.* the authors found that cAMP binding to EPAC induced translocation of EPAC to the plasma membrane due to a conformational change that also reveals its catalytic site for Rap activation. The translocation of EPAC was found to be due to passive diffusion and depended on its DEP domain and is a dynamic and reversible event (PONSIOEN *et al.*, 2009).

Therefore, cAMP is produced at a specific location, due to receptor and AC localization, and its diffusion in the cell is controlled by PDE. This results in targeting of increased cAMP to specific effectors, for example, PKA or EPAC, and their associated substrates. PKA and EPAC are also anchored close to specific effectors resulting in a controlled and specific response to cAMP increases, depending on the receptor activated, that will mediate a distinct biological effect.

1.3.3.3 cAMP effectors and roles in cellular functions

cAMP has been found to be implicated in virtually all cellular responses such as proliferation, differentiation, apoptosis, gene transcription, metabolism, secretion, cell division and neurotransmission (CHENG *et al.*, 2008). Therefore, it is also implicated in many pathologies including diabetes, heart failure and cancer to name a few (CHENG *et al.*, 2008). The response to cAMP is cell type and cell context specific, and in different situations can mediate opposing effects. For example, cAMP has been shown to either inhibit or stimulate cell proliferation depending on the cell type studied or the stimuli used (BEAVO and BRUNTON, 2002;STORK and SCHMITT, 2002). The cAMP effector EPAC was shown to induce Akt phosphorylation in WRT cells and macrophages increasing gene expression and proliferation (CASS *et al.*, 1999), while in adipocytes

EPAC was shown to inhibit Akt phosphorylation thereby reducing proliferation (ZMUDA-TRZEBIATOWSKA *et al.*, 2007). cAMP has also been shown to inhibit keratinocyte migration (MCCAWLEY *et al.*, 2000) or to enhance it (IWASAKI *et al.*, 1994), depending on the concentration of cAMP used. The role of cAMP in cancer is vague, while it has been shown to inhibit tumorigenesis (O'CONNOR *et al.*, 1998), these effects are likely to be cell type and context dependent and it is likely that cAMP can mediate opposing effects on tumorigenesis as it does on other cellular processes. For example one study has shown that cAMP suppresses MMP-2 activation (LEE *et al.*, 2006), while another study showed that elevation of cAMP increased the expression and activity of MMP-2 (TSURUDA *et al.*, 2004), a molecule known to be implicated in tumor cell invasion. The responses to cAMP are mediated by its three main effectors, CNGs (cyclic-nucleotide gated ion channel) whose activation by cAMP allows calcium influx, as well as PKA and EPAC two intracellular cAMP receptors whose diverse cellular functions will be discussed in the following paragraphs.

PKA is the best known cAMP effector and before the discovery of EPAC it was thought to mediate nearly all of the effects of cAMP. PKA is a heterotetramer composed of two regulatory subunits that when bound to cAMP dissociate from two catalytic subunits releasing the inhibition of PKA activity (KIM *et al.*, 2007). PKA is a broad specificity serine/threonine kinase that phosphorylates many different substrates including cytoplasmic or nuclear substrates, enzymes and transcription factors (KIM *et al.*, 2007). Some of the processes that PKA regulates include metabolism, learning and memory (ABEL and Nguyen, 2008), exocytosis (SZASZAK *et al.*, 2008), transcription, cell cycle

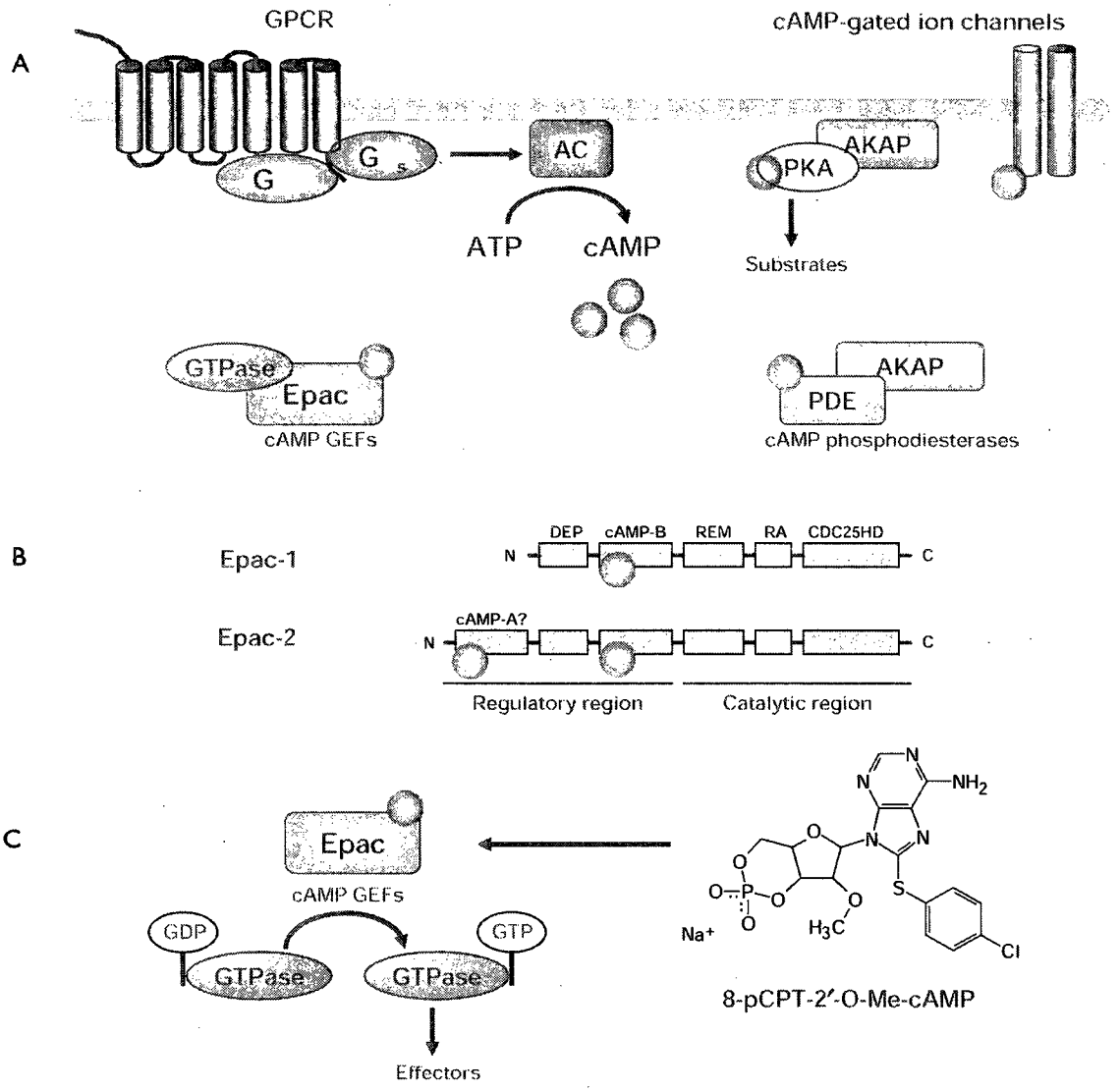

Figure 6: Overview of cAMP production and major effectors. A) cAMP is produced from ATP by AC that is activated by the G_s G-protein of a GPCR. Degradation of cAMP is performed by PDE anchored to specific subcellular compartments by AKAPs. The three major effectors of cAMP, CNG-ion channels, PKA and EPAC are illustrated here. PKA is anchored close to the AC producing cAMP due to the action of AKAPs. B) The structural domains of EPAC1 and EPAC2 are shown. C) The cAMP analog 8-pCPT can activate EPACs GEF activity. (Figure from: ROSCIONI *et al.*, 2008)

progression and apoptosis (LORENOWICZ *et al.*, 2008). For example, PKA inhibits the interaction of 14-3-3 proteins with BAD and NFAT to promote cell survival (SASTRY *et al.*, 2007). It activates KDELR (endoplasmic reticulum protein retention receptor), which promotes retrieval of proteins from Golgi to ER therefore maintaining the steady state of the cell (CABRERA *et al.*, 2003). Increased cAMP levels promote survival of neuronal cells by inactivating GSK3alpha and beta via PKA-dependent mechanisms and thus prevents oncogenesis and neurodegeneration (TANJI *et al.*, 2002). PKA also mediates ERK activation controlling cell proliferation, and enhances release of stored energy, (lipolysis) (CALIPEL *et al.*, 2006). Finally, PKA phosphorylates many transcription factors such as CREB, CREM and ATF1, allowing them to interact with transcriptional co-activators CBP and p300 to activate transcription (DANIEL *et al.*, 1998).

EPAC is the newest member of cAMP regulated proteins. EPAC proteins were originally identified in 1998 (DE ROOIJ *et al.*, 1998) as having cAMP binding and GEF (guanine exchange factor) domains. To date there exist 2 isoforms of the protein, EPAC-1 and EPAC-2 also known as RAPGEF3 and RAPGEF4. EPAC1 protein is widely expressed in tissues such as blood vessels, kidney, adipose tissue, central nervous system, ovary and uterus but not peripheral leukocytes, while EPAC2 has limited expression mainly in the central nervous system (DE ROOIJ *et al.*, 1998;KAWASAKI *et al.*, 1998;KILPINEN *et al.*, 2008). Like PKA, EPAC has cAMP regulatory binding sites that, when bound to cAMP, allow the protein to be active and therefore mediate its GEF activity. EPAC1 contains a DEP (dishevelled, Egl-10, pleckstrin) domain responsible for membrane anchoring, a cAMP binding domain, and a Ras-association domain (RA). For catalytic

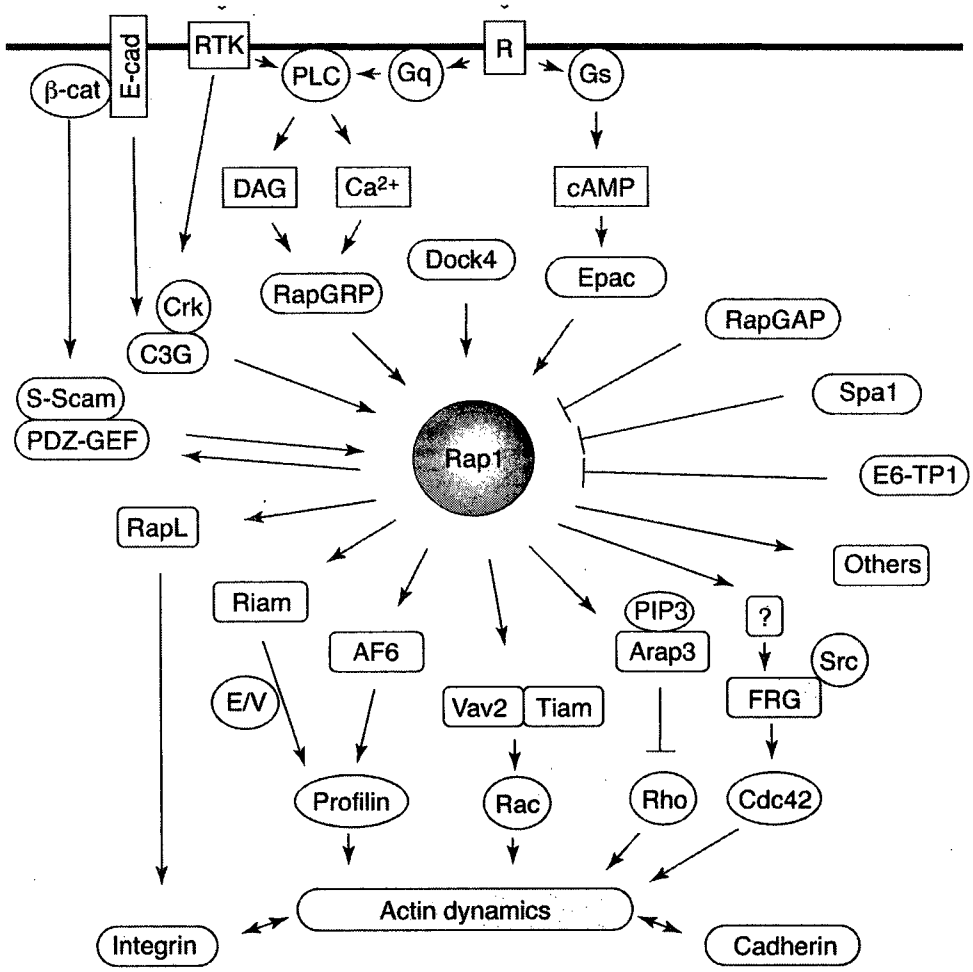

Figure 7: Activators and effectors of Rap1. Rap1 can be activated by E-cadherin, receptor tyrosine kinase and GPCR signaling that induce various RapGEFs such as RapGRP (CalDAG-GEF) and EPAC. Various RapGAPs, including Spa, are able to inhibit Rap1 activation. The many effectors of Rap1 are implicated mainly in actin dynamics or integrin activation. (Figure from: BOS, 2005)

activity, EPAC1 contains a CDC25-homology domain (CDC25-HD) for GEF activity with a high specificity for Rap1 and 2 (Ras-proximate-1 and 2), and a Ras-exchange motif (REM) required for catalytic function. EPAC2 differs by containing an additional cAMP-binding domain (DE ROOIJ *et al.*, 2000) see figure 6B. Since its discovery over 10 years ago, EPAC has been found to mediate many of the effects previously attributed to PKA. EPAC has been shown to play an important role in cAMP-mediated insulin secretion, cell adhesion, exocytosis/secretion, cell differentiation, proliferation, gene expression, apoptosis, neurotransmitter release, heart functions and circadian rhythm (CHENG *et al.*, 2008;PONSIOEN *et al.*, 2009). Briefly, some of the other EPAC effectors besides Rap (which will be discussed in following section), include R-Ras which is implicated in cell proliferation, survival and gene expression (JESUS *et al.*, 2006), Rims, which is implicated in exocytosis (WANG *et al.*, 1997) and Rit, which is implicated in neuronal signaling (ROSCIONI *et al.*, 2008;SHI *et al.*, 2006).

1.3.3.4 EPAC and Rap1

The main effectors of EPAC are Rap1 and Rap2, members of the Ras-like small GTPases, which are activated by EPACs guanine nucleotide exchange factor (GEF) activity and have themselves GEF activity for many other proteins. Rap2 has been less well studied than Rap1 and will not be further discussed here. Rap1 can be activated by a variety of stimuli that include activated receptor tyrosine kinases, GPCRs and cytokine receptors. They can also be activated by other specific GEFs, besides EPAC, that include calcium- and diacylglycerol-binding guanine nucleotide exchange factor 1 (CalDAG-GEF) (BOS *et al.*, 2001). Rap1 is best known for its inside out activation of integrins resulting in

integrin-mediated cell adhesion. Over-expression of Rap1 results in activation of integrins and increased adhesion to fibronectin while expression of Spa1, a specific GTPase activating protein (GAP) for Rap1 that results in inactivation of Rap1, causes inhibition of cell adhesion (BOS *et al.*, 2003;TSUKAMOTO *et al.*, 1999). Rap1 appears to regulate integrins by increasing both their affinity (conformational activation) and avidity (redistribution of integrins into clusters) (BOS *et al.*, 2003) and appears to be capable of regulating all integrins associated with the actin cytoskeleton (ENSERINK *et al.*, 2004). The main Rap1 effector directly linked to integrin activation is RAPL (regulator of adhesion and polarization enriched in lymphocytes), which is found mainly in lymphocytes and regulates homing and adherence of these cells (BOS, 2005).

The other major effect of Rap1 is the regulation of actin dynamics. Some of the Rap1 effectors implicated in actin dynamics include afadin, a multidomain adaptor protein (BOETTNER *et al.*, 2000); Riam (Rap1–GTP-interacting adapter molecule), which is implicated in cell spreading and integrin-mediated adhesion as well as binding to profilin; VASP (Vasodilator-stimulated phosphoprotein) which is involved in actin regulation; Arap3, which has ArfGAP and RhoGAP domains resulting in inactivation of Arf6 and RhoA; and finally the Rac specific GEFs Vav2 and Tiam1 (BOS, 2005). The interaction of Rap1 with Vav2 and Tiam1 resulting in Rac1 activation is the most direct link between Rap1 and actin dynamics. Rac1, to be discussed in the following section on invadopodia, is a small GTPase of the RhoGTPase family responsible for actin polymerization through activation of WAVEs and the Arp2/3 complex (EDEN *et al.*, 2002). Rap1 binds to the PH domain of Vav2 and Tiam1 resulting in translocation of these GEFs to the plasma

membrane at sites of cell protrusion in contact with the extracellular matrix (ARTHUR *et al.*, 2004). Arthur et al. (2004) have found that Rac1 is both required and sufficient to mediate Rap1 induced cell spreading and is therefore an important mediator of Rap1. See figure 7 for an overview of Rap activators and effectors.

Contrary to the other cAMP mediators, Rap1 is known to be involved in malignant progression. However, like cAMP, this role is not straightforward and Rap1 has also been shown in certain instances to inhibit cancer progression. When Rap1 was originally discovered in 1989, it was identified as a gene product that restored a malignant phenotype of K-Ras-transformed fibroblasts (KITAYAMA *et al.*, 1989). Since then, other studies have supported the role of Rap1 in promoting malignancy. For example mice deficient for SPA-1, which would lead to uncontrolled Rap1 activation, develop myeloid disorders that resemble human chronic myelogenous leukemia ((KOMETANI *et al.*, 2004). Activation of Rap1 has also been shown to promote prostate cancer metastasis (BAILEY *et al.*, 2009) while Rap1GAP acts as a tumor suppressor gene for pancreatic cancer (ZHANG *et al.*, 2006a). Rap1 also regulates migration of melanoma cells (GAO *et al.*, 2006). However, loss of function mutations of DOCK4, a specific Rap1 activator, were found in human and mouse tumor cells and transfection of Wild-type DOCK4 into these tumor cells reduced their growth and invasion in vivo (YAJNIK *et al.*, 2003) suggesting a role of Rap1 in inhibition of tumorigenesis. Therefore, it seems that either defective or excess Rap1 can lead to malignant phenotypes via different biological effects on different cell lines.

1.4 Invadopodia

1.4.1 Structure

In order for cancer cells to metastasize they first need to degrade surrounding ECM and cross the basement membrane to enter blood vessels and travel to distance sites in the body (WOODHOUSE *et al.,* 1997). Cancer cells have been shown to generate protrusions called invadopodia to facilitate their migration and invasion through tumor stroma and the basement membrane of blood vessels during the process of metastasis (CHEN, 1989; KELLY *et al.,* 1998; WOLF and Friedl, 2009). Invadopodia can be defined as actin rich, ventral cell membrane protrusions with proteolytic activity (MUELLER and Chen, 1991). Invadopodia contain cortactin and phosphotyrosines, are not confined to cell periphery but rather are in proximity to the Golgi, and are directly associated with sites of substrate degradation. Invadopodia structures are stable with a long half-life of two hours or more. Some of the important molecules found at invadopodial protrusions, which will be discussed in more detail later, are integrins, tyrosine kinase signaling machinery, proteases, actin and actin-associated proteins (BOWDEN *et al.,* 1999;MUELLER *et al.,* 1992;MUELLER *et al.,* 1999;NAKAHARA, 1998). However, there is no known single specific marker of invadopodia to distinctively distinguish them from other structures such as podosomes.

Invadopodia structures have been shown, by electron microscopy, to originate from profound invaginations of approximately 8 μm in width and 2 μm in depth (BALDASSARRE *et al.,* 2003;CHEN, 1989). From these invaginations extend many surface protrusions that penetrate into the matrix (invadopodia), with diameters ranging

from 100's of nanometers to a few micrometers and a length of approximately 500 nm (BALDASSARRE *et al.,* 2003). The connections between these protrusions and the cell body have been shown to be very narrow (BALDASSARRE *et al.,* 2006). Another important feature in cells producing invadopodia is a polarization and proximity of the Golgi toward invadopodial protrusions, suggesting a possible relationship between membrane/protein transport and proteolytic activity (BALDASSARRE *et al.,* 2003).

While the molecular and physiochemical cues that trigger invadopodia biogenesis are not well known, one possible mechanism of invadopodia initiation is the activation of integrins by their extracellular substrates ((NAKAHARA *et al.,* 1996;NAKAHARA, 1998). For example, alpha6beta1 activation promotes Src-dependent tyr phosphorylation of p190RhoGAP affecting the actin cytoskeleton. This activates membrane-protrusive and proteolytic activity leading to invadopodia formation and cell invasion (MUELLER *et al.,* 1999). The following sections will detail the various components necessary for full functioning of an invadopodium.

1.4.2 Actin remodeling

Invadopodia are formed as small clusters consisting of a few large actin-rich dots that extend into the matrix substratum. Actin remodeling is therefore essential for the formation and stability of invadopodial protrusions and involves coordinated action of many proteins such as Arp2/3, N-WASP, cortactin, and cofilin to spatially and temporally regulate actin polymerization (POLLARD and BORISY, 2003). These proteins which 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.,* 2005) will be discussed in greater detail in the following paragraphs.

The Arp2/3 complex consists of 7 proteins, two related proteins Arp2 and Arp3 along with five unique polypeptides ARPC1-5 (GOLEY and WELCH, 2006). When activated, this protein complex initiates the nucleation of a new actin filament from an existing filament at a 70 degree angle, known as branched actin polymerization (MULLINS *et al.,* 1998;WELCH *et al.,* 1998). Arp2/3 is, therefore, responsible for actin rearrangement implicated in the formation of lamellipodia, filopodia, invadopodia and cell motility in general (GOLEY *et al.,* 2004). N-WASP is part of a family including WASP and WAVEs that have a common C-terminal catalytic verprolin-cofilin-acidic (VCA) domain essential for functional activation of the Arp2/3 complex (MULLINS *et al.,* 1998;WELCH *et al.,* 1998). WASP family proteins integrate multiple upstream signals to induce actin polymerization through the Arp2/3 complex (MILLARD *et al.,* 2004). Their major upstream regulators are Cdc42 and Rac1 (EDEN *et al.,* 2002) however N-WASP is also regulated by phosphorylation by Src family kinases. N-WASP is ubiquitously expressed and abundant in the brain (MIKI *et al.,* 1998). 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). It is important to note as well that dysfunctions of Arp2/3 are associated with cancer metastasis and 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).

For example, WAVE2, regulated by Rac1, is a primary regulator of melanoma cell invasion and metastasis (KURISU *et al.,* 2005).

Cortactin is another essential element of invadopodia and is often used as a marker because cortactin clusters located at the basement membrane near the center of the cell and not in the cell periphery are used to identify invadopodia structures (GIMONA and BUCCIONE, 2006). Cortactin is an actin-binding and scaffolding protein that coordinates cell migration, cytoskeletal remodeling, and intracellular protein transport (AMMER and WEED, 2008). The N-terminal acidic domain (NTA) of cortactin contains a DDW motif (aspartic acid-aspartic acid-tryptophan motif) that binds and weakly activates the Arp2/3 complex directly through the N-terminal region of Arp2/3, or indirectly by binding N-WASP via the SH3 domain of WASP and activating it (LUA and LOW, 2005;WEAVER *et al.,* 2001). Cortactin also acts by stabilizing branched actin filaments and, therefore, regulates actin assembly mediated by Arp2/3 (URUNO *et al.,* 2001). Cortactin can also bind to dynamin2, via an SH3 domain, Src, via a proline rich region and filamentous actin (F-actin) with the fourth of its cortactin repeats (DALY, 2004). Cortactin was originally identified as a major substrate of Src (WU *et al.,* 1991) and is tyrosine phosphorylated in response to stimuli that induce actin cytoskeleton remodeling, such as FGF, EGF, or integrins. Phosphorylation of some of its tyrosines, Y421/466/482 in the proline rich domain (PRD) has been shown to be required for motility and metastatic dissemination of breast cancer cells (LI *et al.,* 2001). Phosphorylation of cortactin by Src might influence the interactions of actin nucleators and actin turnover. As well Src phosphorylation of cortactin is important for invadopodia

function (ARTYM *et al.,* 2006). Along with its cytoskeletal remodeling functions cortactin might also regulate MMP secretion at sites of degradation, another critical component of invadopodia to be discussed in the next section (CLARK *et al.,* 2007). Cortactin is ubiquitously expressed, however, as with the other proteins discussed thus far, cortactin is frequently up-regulated in many cancers including breast, head and neck carcinoma, and bladder cancer (SCHUURING, 1995).

Cofilin is a member of the actin depolymerizing factor (ADF)/cofilin family and is a small ubiquitous protein (19kDa). Cofilin is able to bind 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 resulting 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. Cofilin, therefore, seems to have a role in stabilization and/or the maturation process of invadopodia (YAMAGUCHI *et al.,* 2005). Cofilin is also implicated tumor cell invasion and metastasis (WANG *et al.,* 2007).

Finally invadopodia are also enriched with integrins, which form bridges between the cytoskeleton and ECM potentially linking the two aspects necessary for fully functional invadopodia, formation and matrix degrading activity (CHEN, 1990).

1.4.3 ECM degradation

Degradation of the extracellular matrix (ECM) constitutes the function of invadopodia. This process requires many proteases to physically degrade ECM proteins. It also needs membrane trafficking to sustain prolonged degradation by transporting protease-delivering carriers from the Golgi to the ECM as well as an appropriate substrate (ECM) that can affect the activity of invadopodia depending on its rigidity, matrix density and cross-linking (ALEXANDER *et al.,* 2008;CALDIERI *et al.,* 2009a).

The majority of proteolysis at invadopodial structures is due to the metalloproteinase family, which includes matrix metalloproteinases (MMPs) and ADAMs (a disintegrin and metalloproteinase). There are over 25 members of the MMP family that are broadly divided into membrane and soluble types that together can degrade virtually all ECM components (EGEBLAD and WERB, 2002). MMPs are synthesized as inactive proenzymes that become activated through proteolytic removal of their pro-domain (VAN HINSBERGH *et al.,* 2006). Membrane-type1 MMP (MT1-MMP), which degrades collagen, fibronectin and laminins is one of the most important enzymes for invadopodia functions (NAKAHARA *et al.,* 1997). It has been shown to be a master regulator of protease-mediated cell invasion through activation of a cascade of proteases including the gelatinase MMP-2 (HOLMBECK *et al.,* 2004). MT1-MMP over-expression or knockdown results in an increase or decrease of invadopodia formation and function (ARTYM *et al.,* 2006;NAKAHARA *et al.,* 1997). Therefore, recruitment of MT1-MMP to invadopodia might establish the focused zone of MMP activation around this structure. 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-MUNOZ *et al.,* 2006). Interestingly MMPs might also play a role in invadopodia formation as broad-spectrum MMP inhibitors abolish formation and not just functioning of invadopodia (AYALA *et al.,* 2008;BALDASSARRE *et al.,* 2006). The other members of the metalloprotease family are ADAMs. They function as sheddases (enzymes that cleave proteins at the cell surface leading to the release of ectodomains) and cleave growth factors and cytokine precursors into their active forms while their disintegrin domain interacts with integrins especially the beta1 component (HUOVILA *et al.,* 2005).

Serine proteinases are also implicated in invadopodia-mediated degradation. Seprase and DPP4 (dipeptidyl dipeptidase IV) are transmembrane serine peptidases implicated in ECM degradation and shown to colocalize with invadopodia (GHERSI *et al.,* 2006). These two serine proteases are activated by oligomerization and are also able to bind beta1 integrins in a collagen dependent manner and degrade collagen, fibronectin, and laminins (MONSKY *et al.,* 1994). Seprase and DPP4 are also up-regulated in melanoma and breast carcinoma cells (CHEN, 2003). The urokinase-type plasminogen activator (uPA) proteolytic system has also been implicated in invadopodia (ARTYM *et al.,* 2002;KINDZELSKII *et al.,* 2004). uPAR (urokinase-type plasminogen activator receptor) is found in a complex with seprase at sites of invadopodia formation and activates plasminogen which can subsequently activate various MMPs (LIJNEN, 2001). Matrix degrading proteases also activate and release growth factors such as VEGF (Vascular

endothelial growth factor) locally from the matrix, which might enable continued induction of invadopodia through receptor signaling (EBRAHEM *et al.,* 2010).

1.4.4 Signaling

Signaling plays a major role in coordinating the formation and functioning of invadopodia. Activation of integrins and receptor tyrosine kinases induces intracellular cascades involving PKC, Src, Rho GTPases and tyrosine kinases for invadopodia generation. Some of the most important signaling pathways involve tyrosine kinases, such as Src and Src-family kinases whose role in invadopodia has been well established. In fact, tyrosine phosphorylation is a marker of invadopodia and SFK activity is absolutely required for invadopodia formation and degradation (MUELLER *et al.,* 1992). Serine/threonine kinases such as ERK1/2 and PAK have also been implicated in invadopodia biogenesis possibly playing a role in cortactin phosphorylation (AYALA *et al.,* 2008;TAGUE *et al.,* 2004). The ARF-family of GTPases are also involved as they control actin rearrangement (AL-AWAR *et al.,* 2000) and have been localized to invadopodia (TAGUE *et al.,* 2004).

Finally, the most important signaling molecules for this study are the Rho family GTPases. These are small G-proteins that when activated exchange GDP for GTP resulting in a conformational change that allows them to interact with downstream effectors. This active state is later terminated by hydrolysis of bound GTP to GDP (BISHOP and HALL, 2000). Among their various effector targets are protein kinases and actin nucleators allowing them to influence cell shape, morphology, polarization, motility

and metastasis formation (HALL, 2005). Most Rho GTPases act on various cellular membranes and affect the morphology of these membranes through alterations of the associated cytoskeleton. Many also affect specific steps of vesicle trafficking between different intracellular compartments (MATOZAKI *et al.,* 2000). They are also usually activated on membranes by GEFs (ROSSMAN *et al.,* 2005). Post-translational modifications are also critical for RhoGTPase interaction with membranes (ex prenylation, palmitoylation) and can enhance their interaction with various cellular membranes and define their localization to specific membrane compartments (CUSHMAN and CASEY, 2009). Phosphorylation also regulates activity and localization of some Rho GTPases such as RhoA (ROSSMAN *et al.,* 2005).

Rac1 and Cdc42 can both activate Arp2/3 through their effectors Sra-1 and N-WASP respectively. Cdc42 binds WASP specifically through WASPs CRIB domain and Cdc42 binding results in unfolding of the protein making the VCA domain accessible to Arp2/3 (CALDIERI *et al.,* 2009a). Cdc42 in particular has been shown to act upstream of invadopodia formation with DA mutants inducing dot-like degradation (NAKAHARA *et al.,* 2003). RhoA has also been suggested to play a role due to the fact that p190Rho-GAP activates membrane protrusive activity (NAKAHARA *et al.,* 1998). All of these GTPases require GEFs (stimulate release of GDP allowing GTP to bind), GAPs (catalyze GTP hydrolysis converting to GDP) and GDIs to function efficiently and many GEF have been identified as oncogenes (ROSSMAN *et al.,* 2005). While Rac1 has not been directly related to invadopodia formation, it can induce actin polymerization through activation of Arp2/3 and could be implicated in recruitment of cortactin, both essential to invadopodia

formation (BAUMER *et al.,* 2008). 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.,* 2009a).

Finally, there are also numerous adaptor molecules and effectors downstream of these signaling cascades that are localized to and regulate invadopodia such as cortactin, (GIMONA and BUCCIONE, 2006); dynamin2, a large GTPase which regulates actin organization via cortactin (KRUCHTEN and MCNIVEN, 2006), and also has a specialized function in tubulation and constriction of cell membranes and, therefore, affects membrane trafficking (BALDASSARRE *et al.,* 2003); Tks5, a scaffolding protein and Src substrate that binds and modulates N-WASP (OIKAWA *et al.,* 2008); IQGAP1, which regulates Cdc42 and Rac1 modulating cytoskeletal architecture (NABESHIMA *et al.,* 2002); and Paxillin, a scaffolding protein that coordinates the actions of Rho GTPases and forms a complex with cortactin at invadopodia (BOWDEN *et al.,* 1999), to name a few. Figure 8 provides an overview of the structure of an invadopodium and many of the proteins implicated.

1.4.5 Implication of invadopodia in metastatic process

Invadopodia are considered to be an in vitro model for cell invasion due to the fact that focal delivery of proteases is crucial for physiological ECM remodeling events and tumor cell invasion (BASBAUM and WERB, 1996). Focal ECM degradation requires tight coordination between polarized trafficking, signaling events and cytoskeletal/membrane

remodeling and invadopodia are likely the place where this integration occurs (GIMONA and BUCCIONE, 2006). Furthermore, invadopodia biogenesis correlates highly 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). Invadopodia-like structures formed by migrating cancer cells undergoing intravasion have been imaged by multiphoton microscopy while whole animal imaging has allowed real-time visualization of invadopodia-like protrusions in tumor cells invading through tissues (CONDEELIS and SEGALL, 2003;YAMAGUCHI *et al.,* 2005). 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.,* 2006b). In summary, invadopodia are organelles where many functions associated with cancer aggressiveness converge such as tyrosine kinase signaling, protease secretion and targeting, and cytoskeletal rearrangement and may, therefore, reveal to be good therapeutic targets. 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).

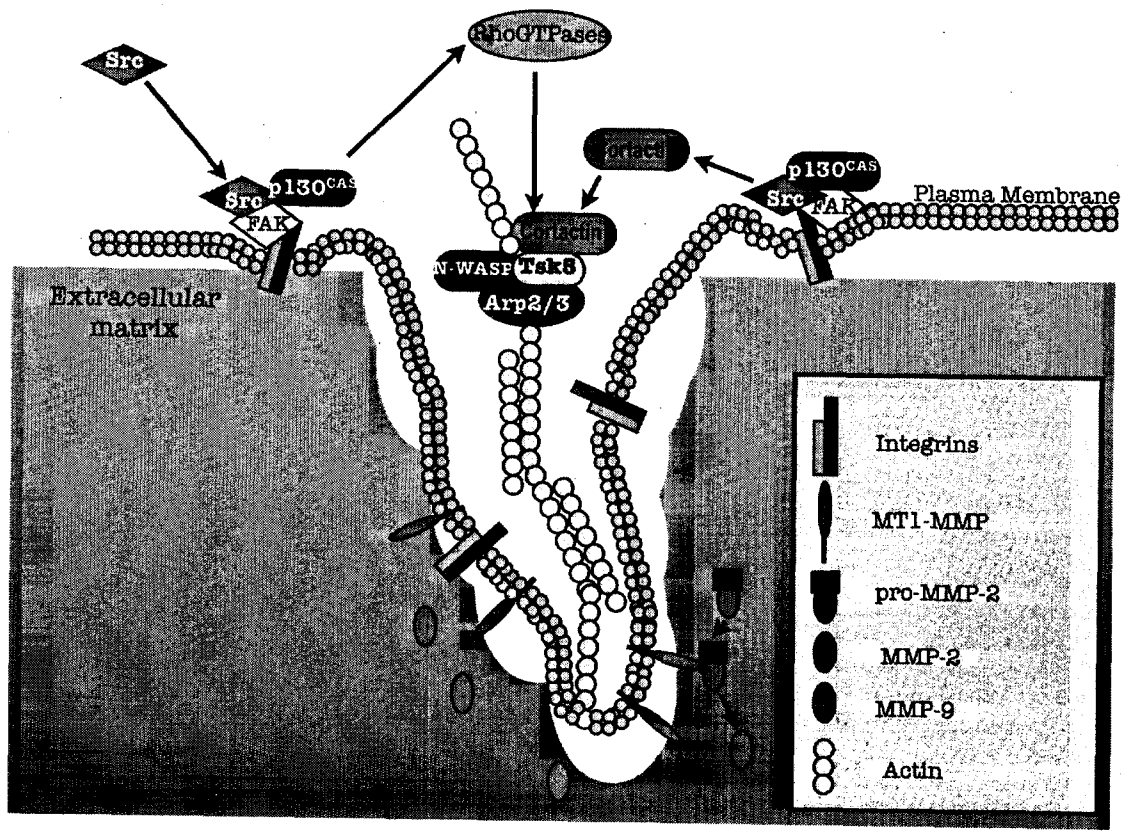

Figure 8: Invadopodium. A plasma membrane protrusion is formed due to excessive actin polymerization induced by RhoGTPases signaling. Various molecules are implicated in this actin polymerization including N-WASP, Arp2/3 and cortactin. Cortactin can be further activated by Src which may be found in focal adhesion complexes containing FAK and integrins, in close proximity to invadopodia. Metalloproteinases including MMP-2 and MMP-9 are secreted at invadopodia and can be activated by MT1-MMP which is enriched at invadopodia membrane protrusions. (ARSENAULT, D. Unpublished)

1.5 Objectives and pertinence

Autotaxin has been implicated in the invasiveness and metastatic potential of numerous cancer cell lines (KEHLEN *et al.*, 2004;KISHI *et al.*, 2006;YANG *et al.*, 2002;YANG *et al.*, 1999). It is responsible for producing the majority of LPA *in vivo*, which in turn signals by activating GPCRs (BRINDLEY, 2004;UMEZU-GOTO *et al.*, 2002). Over-expression of ATX or LPA receptors has recently been linked to increased tumor invasion and metastasis of breast cancer cells (LIU *et al.*, 2009). Similarly, knockdown of ATX and LPA receptors has been shown to inhibit tumor cell migration and cause tumor regression in mice (ZHANG *et al.*, 2009). These results suggest an important role for ATX and LPA receptors in cancer progression and tumor metastasis, however the precise signaling pathways and cellular effects involved remain largely unknown. In order for cancer cells to metastasize, they first need to degrade surrounding tissues. Invadopodia are invasive subcellular structures formed by cancer cells that are increasingly being recognized as important mediators of matrix degradation necessary for cell invasion and metastasis (CONDEELIS and SEGALL, 2003;YAMAGUCHI *et al.*, 2005). The formation of these structures requires specific signaling events and recruitment of effectors, which are slowly being unraveled (YAMAGUCHI *et al.*, 2006). The hypothesis of this research was that ATX, through LPA production and LPAR signaling, induces the formation of the invasive structures, invadopodia, leading to metastasis of cancer cells. The objectives of this research were to evaluate the implication of ATX and LPA on invadopodia production and to determine the receptor and signaling pathways used to promote invadopodia and metastasis formation.

The first objective of the research presented in this memoire was to determine the involvement of ATX and LPA in invadopodia production. For this, we transfected cells with ATX or shRNA against ATX. We then investigated the response of these cells in invadopodia assays. We also studied the effects of LPC and LPA on various cancer cell types in invadopodia assays. The second objective of this memoire was to establish whether LPA receptors were implicated in invadopodia production and to define the receptor(s) implicated. For this, we used a broad-spectrum inhibitor of LPA receptors and observed the effects on invadopodia production. We also measured the mRNA expression levels of the various LPA receptors in the HT1080 cell line. In addition, we used LPA_4 shRNA to study the requirement of this receptor for invadopodia production. The effect of LPA_4 knockdown on *in vitro* cell invasion and *in vivo* metastasis formation was also studied, using a 3D invasion assay and a lung metastasis assay, respectively. The third objective of this research was to ascertain the downstream signaling pathways involved in ATX-induced invadopodia. As LPA_4 primarily couples to G_s, resulting in increased intracellular cAMP concentrations, we investigated signaling by the cAMP pathway. For this, we evaluated the effect of various activators and inhibitors of cAMP and its downstream effectors in the invadopodia assay.

2 - ARTICLE

2.1 Preface

The manuscript presented in this memoire entitled "Autotaxin Promotes Cancer Invasion via the Lysophosphatidic Acid Receptor 4: Participation of the cAMP/EPAC/Rac1 Signaling Pathway in Invadopodia Formation" presents the results of research performed in the context of my master's program. This article has been accepted for publication in the peer reviewed journal, Cancer Research and will appear in the June issue. The authors of this article are Kelly Harper, Dominique Arsenault, Stephanie Boulay-Jean, Annie Lauzier, Fabrice Lucien and Claire M. Dubois. The form for authorization of integration of an article into a memoire with signatures from all authors is attached. 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. Dominique Arsenault, a Ph.D. student, performed the experiments represented in Figure 6A and 6B as well as helping capture images for figure 1. Stephanie Boulay-Jean, a stagiaire, performed the experiment presented in figure 2D. Annie Lauzier, a post-doctoral student, performed the western blot presented in figure 4D. Fabrice Lucien, a master's student, participated in the WB presented in figure 2C. Dr. Claire Dubois is my research director. It was with her guidance that I planned the experiments and developed this project.

2.2 RESUME

L'habileté des cellules cancéreuses à envahir les tissus et former des métastases est la cause principale de décès chez les patients atteints de cancer. L'autotaxin (ATX) est une lysophospholipase sécrétée dont les niveaux d'expression corrèlent avec l'agressivité et le potentiel invasif des tumeurs. L'ATX est l'enzyme majeure impliquée dans la production de l'acide lysophospatidique (LPA), un phospholipide qui est connu pour agir principalement par l'intermédiare de récepteurs LPA_{1-3}. L'invasion des cellules tumorales et la formation des métastases sont dépendantes de la capacité des cellules cancéreuses à dégrader la membrane basale. Ce processus peut être initié par la formation de protrusions riches en actine, appelées invadopodes. Dans cette étude, nous démontrons que l'ATX est impliquée dans la formation des invadopodes chez divers types cellulaires cancéreux. Cet effet est dépendant de la production de LPA. De plus, nous montrons que la signalisation via le LPA_4 chez les cellules de fibrosarcome régule la formation des invadopodes en aval de l'ATX, c'est à dire à travers l'activation de EPAC par l'AMPc et l'activation ultérieure de Rac-1. Les résultats utilisant l'ARN interférent du LPA_4 renforce l'idée que le récepteur LPA_4 est nécessaire à l'invasion cellulaire et la formation de métastases in vivo. Ce travail suggère que le blocage du récepteur au LPA, LPA_4, dans les cellules de fibrosarcome pourrait être une avenue intéressante afin d'améliorer l'efficacité du traitement des métastases chez les patients atteints de cancer. Du fait que les récepteurs au LPA et l'ATX sont actuellement des cibles thérapeutiques pour des essais précliniques, ces découvertes devraient stimuler des études ultérieures visant à évaluer le profil

d'expression et l'issue clinique du LPA_4, en conjoncture avec les autres récepteurs au LPA, dans plusieurs types de cancers.

2.3 Manuscript

Autotaxin Promotes Cancer Invasion via the Lysophosphatidic Acid Receptor 4: Participation of the cAMP/EPAC/Rac1 Signaling Pathway in Invadopodia Formation

Kelly Harper[1], Dominique Arsenault[1], Stephanie Boulay-Jean[1], Annie Lauzier[1], Fabrice Lucien[1] and Claire M. Dubois[1,2]

[1]From the Immunology Division, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, Quebec, Canada, J1H 5N4.

Running Title: **Autotaxin Promotes Cancer Invasion via LPA_4**

Key Words: Autotaxin, Lysophosphatidic acid (LPA), invadopodia, EPAC, Rac1

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Abstract

The ability of cancer cells to invade and metastasize is the major cause of death in cancer patients. Autotaxin (ATX) is a secreted lysophospholipase whose level of expression within tumors correlates strongly with their aggressiveness and invasiveness. ATX is the major enzyme involved in the production of LPA, a phospholipid that is known to act mostly through its three first characterized receptors, LPA_{1-3}. Tumor cell invasion across tissue boundaries and metastasis are dependent on the capacity of invasive cancer cells to breach the basement membrane. This process can be initiated by the formation of the actin-rich cell protrusions, invadopodia. In this study, we demonstrate that ATX is implicated in the formation of invadopodia in various cancer cells types and this effect is dependent on the production of LPA. We further provide evidence that LPA_4 signaling in fibrosarcoma cells regulates invadopodia formation downstream of ATX, a process mediated through the activation of EPAC by cAMP and subsequent Rac-1 activation. Results using LPA_4 shRNA support the requirement of the LPA_4 receptor for cell invasion and in vivo metastasis formation. This work presents evidence that blocking the LPA receptor, LPA_4, in fibrosarcoma cells could provide an additional tool to improve the efficacy of treatment of metastasis in patients. Because LPA receptors and ATX are currently being targeted in preclinical trials, the current findings should stimulate future studies to evaluate the expression pattern and clinical outcome of LPA_4, together with other LPA receptors, in various cancer patients.

Introduction

Cancer remains a leading cause of death worldwide despite relentless efforts in basic research and clinical management of the disease. Autotaxin (ATX), a secreted motility stimulating factor that was originally isolated from the culture supernatant of human melanoma cells (A2058) (1), has been linked with progression of many types of cancer. Up-regulation of ATX in malignancies including breast, lung, colon, ovarian, stomach and brain cancer was correlated with invasiveness and metastatic potential (2-4). ATX has been found in many biological fluids including blisters, cerebrospinal, peritoneal and, synovial, as well as plasma (5-8). Through its extracellular actions, ATX augments cellular functions closely associated with tumor aggressiveness and metastasis that include proliferation, survival, motility, invasion and angiogenesis (9, 10). ATX has recently been shown to be molecularly identical to lysophospholipase D, which catalyzes the production of the majority of lysophosphatidic acid (LPA) from lysophosphatidylcholine (LPC) in vivo (11). Consequently, LPA is thought to be responsible for the majority of ATXs effects (12). LPA is a bioactive lipid and a major constituent of serum whose effects are similar to those of ATX (11, 13). LPA is also implicated in many pathophysiological conditions such as atherosclerosis, hypertension, ischemia reperfusion injury, and, pertinent to this study, cancer (14). LPA mediates cell type-specific responses by engaging high affinity G-protein-coupled receptors (GPCRs) (15).

There are currently six known LPA receptors. LPA_1 - LPA_3 are members of the endothelial differentiation gene (edg) receptor family whereas the more recently de-orphaned LPA_4 -LPA_6 belong to the purigenic cluster of GPCRs (16-18). Most research

has focused on the first identified LPA receptors while the signaling and physiological roles of LPA_4-LPA_6 have received less attention. LPA signals via at least four distinct G-protein families, G_i, G_q, $G_{12/13}$, and G_s and their downstream effectors. The major signaling pathways that become activated include the PI3K and Ras pathways which depend on G_i, PLC which depends on G_q, Rho GTPases which are associated with $G_{12/13}$ signaling and cAMP production downstream of G_s (13, 19). Many of these signaling routes have been shown to be involved in tumor survival and invasion. (19). Over-expression of ATX and LPA receptors (LPA_{1-3}) has been recently linked to increased tumor invasion and metastasis of breast cancer cells (20). Moreover, pharmacological inhibition of both ATX and LPA receptors (mostly LPA_{1-3}) has been shown to decrease cell migration in vitro and cause tumor regression in mice (21). These studies uncovered an important role for ATX and LPA_{1-3} receptors in tumor metastasis, a fundamental property of malignant cancer cells and the major cause of death in patients.

Metastatic tumor cells must first degrade the surrounding tissues and reach the blood stream to travel to distant sites and form new tumors. Cancer cells have been shown to generate protrusions called invadopodia to facilitate their migration and invasion through tumor stroma and the lining of the blood vessels during the process of metastasis (22, 23). Invadopodia are formed by highly invasive cancer cells. They are actin-rich ventral membrane protrusions which possess ECM degrading activity (24). The formation of these structures necessitates the convergence of many different signaling pathways and molecules, which are slowly being unraveled. Besides actin, invadopodia contain actin regulatory proteins, adhesion molecules, membrane remodeling and signaling proteins and extracellular matrix-degrading enzymes (25). The Rho GTPases,

including Rac1 and Cdc42, have repeatedly been shown to promote invadopodia production through their actions on the cytoskeleton (26). Formation of invadopodia is enhanced by extracellular matrix rigidity (27), some growth factors such as EGF (22) or expression of wild-type or activated forms of the src tyrosine kinase (28). Therefore, despite much research on invadopodia biology, very little is known about specific inducers and receptors during tumor progression.

Because of the implications of ATX and LPA signaling in metastasis and their activation of pathways, which could be implicated in invadopodia formation, we have investigated the involvement of ATX and LPA receptors in invadopodia production. Using the invasive fibrosarcoma cell line, HT1080, we observed that ATX through the LPA_4 receptor is a strong inducer of invadopodia formation that correlates with the ability of the cells to invade and metastasize. This study also revealed an unexpected signaling pathway for cell invasion involving LPA_4-driven cAMP production and subsequent activation of the EPAC-Rap1-Rac1 axis.

Materials & Methods

Reagents. 1-oleoyl-*sn*-glycerol-3-phosphate sodium salt 18:1 (LPA), L-α-lysophosphatidyl choline from egg yolk (LPC-egg), 1-oleoyl-*sn*-glycero-3-phosphocholine 18:1 (LPC-18:1), 2'-O-dibutyryladenosine 3', 5'-cyclic monophosphate sodium (dibutyryl cAMP), 8-(4-chlorophenylthio)-2'-O-methyladenosine 3'-5'-cyclic monophosphate monosodium hydrate (8-pCPT), 8-bromoadenosine 3'-5'-cyclic monophosphate sodium (8-bromo cAMP), H-89 dihydrochloride hydrate, LPA receptor antagonist (Ki16425), forskolin, cholera toxin (CTX), and pertussis toxin (PTX) were purchased from Sigma-Aldrich (St. Louis, MO). The Rac1 inhibitor NSC23766 and Rap1 inhibitor GGTI-298 were from Calbiochem (EMD Chemical Inc, La Jolla, CA) and myristoylated PKI (14-22) amide (PKI) was from Biomol International LP (Plymouth Meeting, PA). ATX cDNA construct and ATX antibody (84A) were a kind gift from Dr. Tim Clair (Center for Cancer Research, NCI, NIH). shRNA against ATX, LPA_4, or EPAC was from SABiosciences (Frederick, MD). The anti-tubulin antibody was from Sigma-Aldrich, the anti-cortactin antibody was from Millipore (Temecula, CA), the anti-EPAC antibody was from Cell signaling (Boston, MA) and Texas Red phalloidin and all secondary antibodies were from Invitrogen (Molecular Probes, Eugene, OR).

Cell culture and transfections. HT1080 human fibrosarcoma, MDA-MB231 and MCF-7 human breast cancer, A549 human lung cancer and U87 human glioblastoma were obtained from the American Type Culture Collection (ATCC, Rockville, MD). All cell lines were cultured in minimal essential medium (MEM) (Wisent, St-Bruno, QC, Canada) supplemented with 10% FBS (Gibco BRL, Burlington, ON, Canada) and 40μg/ml of

gentamycine (Shering Canada Inc., Pointe-Claire, QC, Canada) in a humidified 95% air/ 5% CO_2 incubator at 37°C. Stable transfections with ATX cDNA or shRNA against ATX, LPA_4 or EPAC shRNA, or scramble shRNA (negative control) were performed with the Fugene reagent from Roche Diagnostics (Mannheim, Germany), according to the manufacturer's protocol.

Real time RT-PCR. Total RNA was isolated using the TRI-Reagent (Invitrogen, Carlsbad, CA) protocol as previously described (29) and quantitative Real-Time PCR was performed on a Rotor-Gene 3000 (Corbett Research, Kirkland, QC, Canada). The following primer pairs were selected for LPA_4: (forward) 5'-AAAGATCATGTACCCAA TCACCTT-3', (reverse) 5`-CTTAACAGGGACTCCATTCTGAT-3' and for human acidic ribosomal phosphoprotein PO (RPLPO): (forward) 5'-GATTACACCTTCCCACTTGC-3', (reverse) 5'-CCAAATCCCATATCCTCGTCCG-3'. The cycling program was: initial denaturation at 95°C for 15 min, 40 amplification cycles with denaturing T° of 95°C for 30 s, annealing T° of 55°C for 30 s and final extension at 72°C for 30 s.

Western blotting. Cells were lysed on ice in RIPA buffer. Supernatant samples were recovered by centrifugation (13000rpm for 30min at 4°C) and protein concentration was determined using the BCA reagent (Biolynx Inc, Brockville, ON). Immunoblotting was performed as previously described (30).

Invadopodia assays. Coverslips were prepared as previously described (31), using Oregon-green gelatin (Invitrogen, ON, Canada). Thirty thousand cells were seeded on

each coverslip, allowed to adhere and incubated in MEM with 0.5% FBS. Following various incubation times as described within the figure legends, cells were fixed with 1% paraformaldehyde for 30 min at 4°C and stained with DAPI (Invitrogen) for 5 min at room temperature. Cells were visualized by fluorescence microscopy and cells forming invadopodia were counted. Invadopodia were identified by areas of matrix degradation characterized by loss of green fluorescence. Three X 100 cells were counted per coverslip.

Fluorescence Microscopy. Cells were grown on gelatin coated coverslips and fixed with 1% paraformaldehyde in PBS for 30 min at 4°C, permeabilized with 0.05% saponine (Sigma-Aldrich) in PBS for 20 min and blocked with 2% BSA in PBS for 30 min. For calculation of the number of invadopodia per cell, cells were then incubated with anti-cortactin antibody for 2 h, secondary antibody 488-conjugated anti-mouse for 1 h at 4°C followed by Texas Red phalloidin for 45 min and DAPI for 5 min. 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). Images were taken with a FV1000 scanning confocal microscope (Olympus, Tokyo, Japan) coupled to an inverted microscope using a 63x oil immersion objective. 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.

3D invasion assays. The 3D invasion assay was modified from a previously described technique (32). Collagen type I 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 ($2x10^4/100\mu l$ of serum-free MEM) were deposited on top of the collagen gel and incubated for 36 h. The cells were then labeled with CellTrace™ calcein green AM (Invitrogen) 1h prior 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 5 μm layers within the gel.

In vivo metastasis assay. Mice were housed and manipulated under pathogen free conditions, in accordance with the guidelines of the local institutional animal care facility. Experimental metastatic potential of HT1080 transfected cells was measured by the lung colonization assay. Briefly, 2 x 10^5 cells in 0.1 ml of PBS were injected into the tail vein of 5- to 7-week-old female CD1 nude mice. Twenty-nine days later the mice were sacrificed and the lungs were fixed with Bouin's solution (Sigma-Aldrich). Metastatic colonies on the lung surface were counted macroscopically.

Results

Autotaxin induces the formation of matrix degrading invadopodia through the production of LPA from LPC.

Since ATX is upregulated in many cancer cell lines and its expression correlates with cell invasion (2-4), we investigated whether ATX is implicated in invadopodia production. As shown in Fig. 1A, ATX over-expression in HT1080 human fibrosarcoma cells induced a significant 5-6 fold increase in the percentage of invadopodia producing cells as compared to parental HT1080 cells. Furthermore, the over-expression of ATX resulted in a significant increase of the gelatin degradation area (Fig. 1B), as well as the number of invadopodia formed per cell identified by colocalization of actin and cortactin, two known markers of invadopodia (28) (Fig. 1C). Confocal microscopy analysis of ATX degradation areas showed that they were associated with cell membrane protrusions, which were characterized by punctate actin cores that extended from the basal membrane of the cell into the underlying matrix (Fig. 1D). These observations suggested that ATX is involved in both invadopodia formation and function.

To determine whether the impact of ATX was due to the ability of the enzyme to produce LPA from LPC, HT1080 cells were incubated in the presence or absence of LPC or LPA during the invadopodia assay. Results showed concentration-dependent increases in the percentage of invadopodia-producing cells after 10h of incubation (Figs. 2A and B), showing that both the substrate and the product of ATX enzymatic activity have the ability to induce the production of invadopodia. Next, depletion of ATX by shRNA abolished LPC- but not LPA-induced invadopodia formation(Fig. 2C), confirming that LPC-derived LPA was a key metabolite in invadopodia production. We further

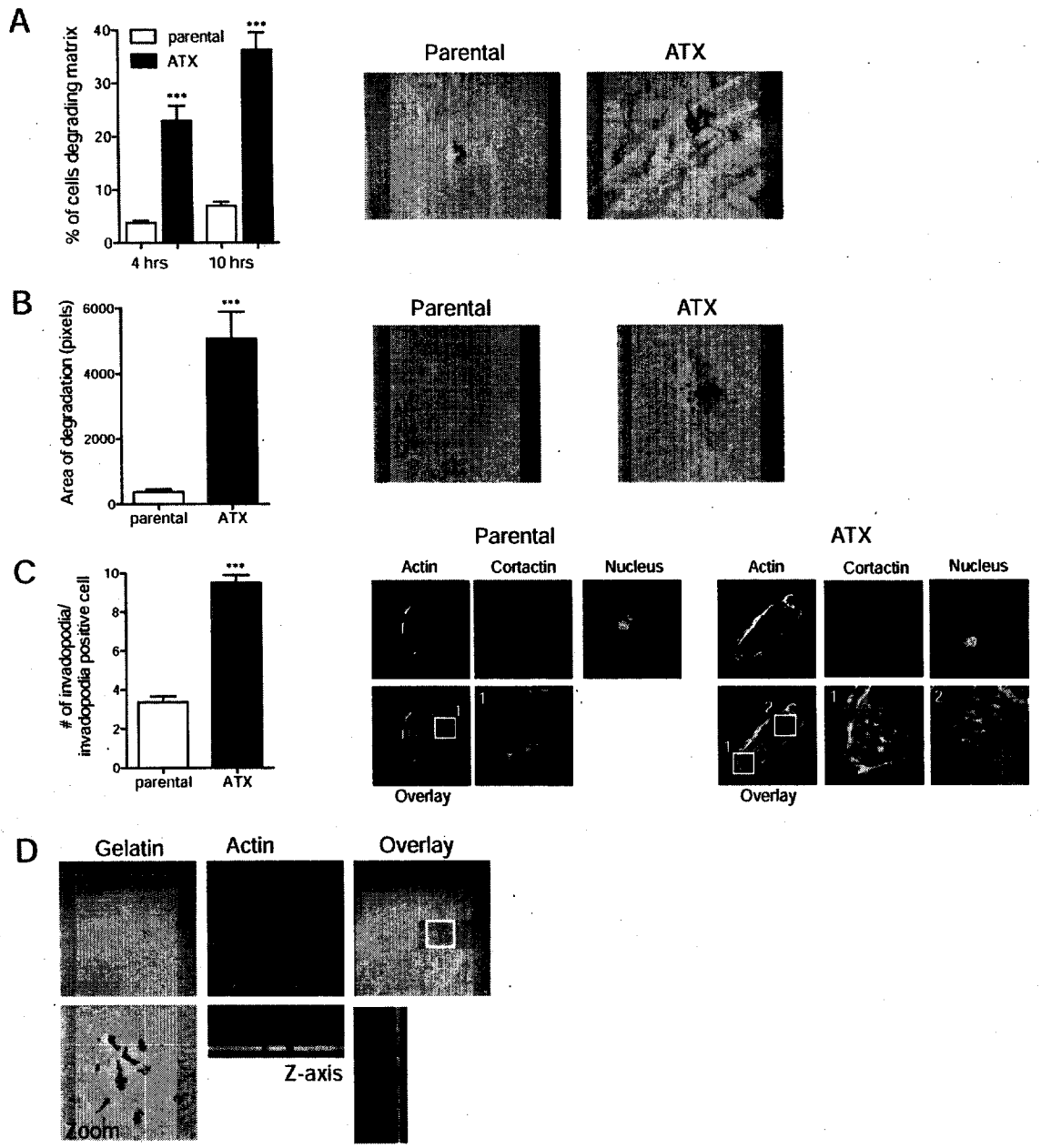

A
% of cells degrading matrix
parental
ATX
4 hrs
10 hrs
Parental
ATX
B
Area of degradation (pixels)
parental
ATX
Parental
ATX
C
# of invadopodia/ invadopodia positive cell
parental
ATX
Parental
ATX
Actin
Cortactin
Nucleus
Overlay
D
Gelatin
Actin
Overlay
Z-axis
Zoom

Figure 1. Autotaxin induces invadopodia formation in HT1080 cells

Parental and ATX-overexpressing HT1080 cells were cultured on fluorescent gelatin for 10 h (**A** and **B**), or on non-fluorescent gelatin for 4 h (**C**). **A**, The percentage of invadopodia-producing cells are shown; n=3-9. Representative images of parental and ATX-transfected cells with associated matrix degradation are shown (10X magnification). **B**, Quantification of ECM degradation area/cell; n=3, with representative images of degradation by parental and ATX transfected cells (40X magnification). **C**, Number of F-actin (green) and cortactin (red) positive invadopodia per cell; n=2, with representative confocal images showing colocalization of actin and cortactin spots (magnification 60X). **D,** A representative confocal image of ATX-transfected cells showing actin-rich invadopodia that extend into the matrix substratum (F-actin, red; gelatin, green). Columns, mean; bars, SEM; ***, $p < 0.0001$.

investigated whether the findings observed in the HT1080 cell line applied to other malignant cell lines. LPC induces invadopodia production in MDA- MB231 (breast cancer), A549 (lung cancer), CaCo2 (colon cancer) and U87 (glioblastoma) cell lines. In contrast, MCF-7(breast cancer), that has an intrinsic defect in cell invasion, failed to respond to LPC (Fig. 2D). These results indicate a role for ATX in the formation of invadopodia by a wide range of neoplastic cells.

LPA receptor 4 is implicated in the formation of invadopodia through the activation of the G_s-cAMP-EPAC pathway.

Since the cellular effects of LPA are due to its binding and activation of various LPA-specific GPCRs (15) and some of these receptors have been linked to tumor invasion (20, 21), we further investigated the role of these receptors and their downstream signaling pathways in mediating the effects of ATX and LPA on invadopodia production. Treatment of ATX over-expressing cells with a broad spectrum LPA receptor antagonist (Ki16425) resulted in a significant decrease in invadopodia production (Fig. 3A), suggesting an important role for these receptors in transducing the ATX/LPA-dependent invasive function. We next assessed the relative expression levels of the most characterized LPA receptors (LPA_{1-4}) in HT1080 cells to identify which of the receptors were more likely to mediate the effects of ATX/LPA on invadopodia production. In agreement with previously published results (2), HT1080 cells expressed high levels of LPA_4 with only a minimal expression of LPA_{1-3} (Fig. 3B). Next, to further assess the role of LPA_4 in invadopodia production, HT1080 cells were transfected with LPA_4 shRNA. Results showed that LPA_4 inhibition abolished invadopodia production induced by LPA or

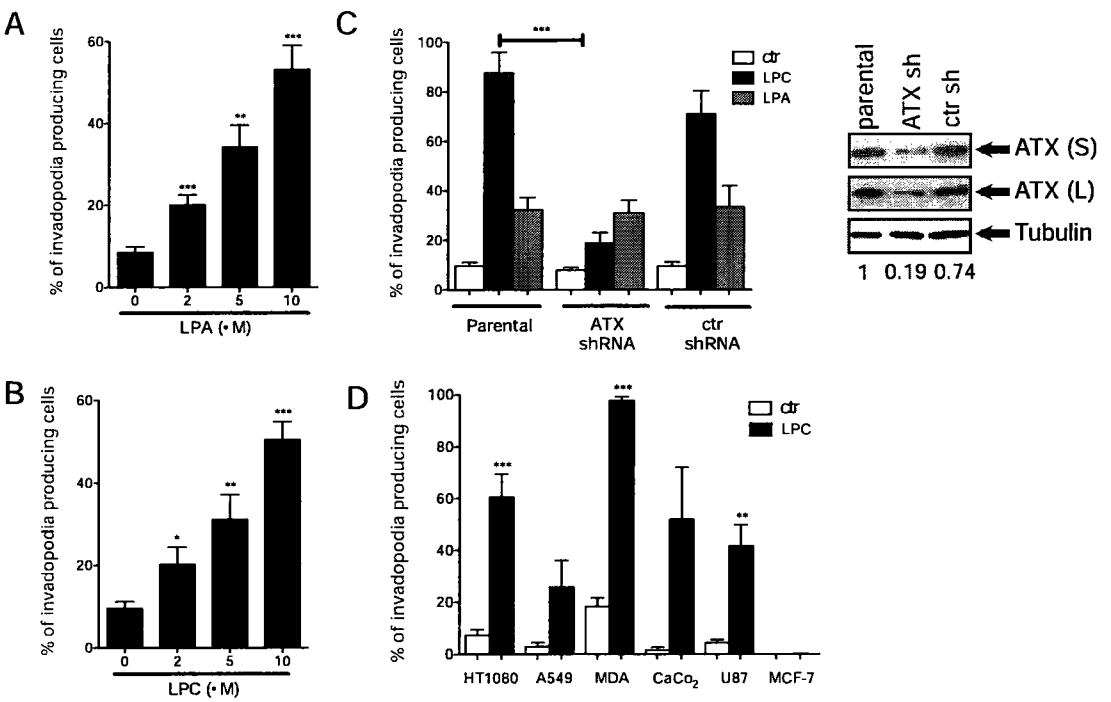


Figure 2. ATX-induced invadopodia production is dependent on LPA production from LPC

A and **B**, HT1080 cells were cultured for 10h with various concentrations of LPC-18:1 (**A**) , or LPA (**B**) and the percentage of invadopodia-producing cells was calculated; n=3-4. **C,** parental HT1080 or cells transfected with ATX shRNA or ctr shRNA were cultured on fluorescent gelatin in the presence or absence (ctr) of LPA(10μM) or LPC - egg (10μM) and the percent of invadopodia-producing cells was determined; n=3. Western blot analysis of supernatants (S) and cell lysates (L) with antibodies directed against ATX was performed. Ratios of ATX to tubulin are shown. One representative blot of 2 is shown. **D,** Cell lines were cultured in invadopodia assays in the presence or absence (ctr) of LPC-egg (10μM) for 10 h (HT1080, A549, MDA-MB231) or 24 h (U-87, MCF-7). The percent of invadopodia-producing cells is shown; n=2-4. Columns, mean; bars, SEM; *, p<0.01 ; **, p<0.001 ; ***, p<0.0001.

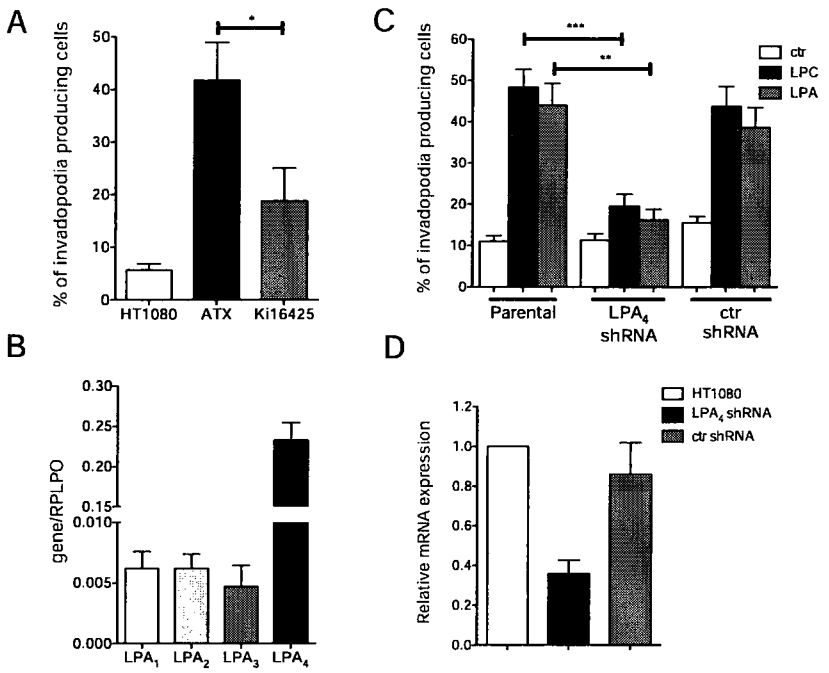


Figure 3. LPA4 is implicated in invadopodia production by HT1080 cells.

A, ATX-overexpressing cells were treated with Ki16425 (25μM) for 10h in an invadopodia assay; n=3. **B,** LPA receptors were measured by RT-PCR; n=3. **C,** Effect of LPA_4 shRNA on invadopodia production by unstimulated (ctr) or LPA (10μM) or LPC-18:1 (10μM) stimulated cells; n=3-4. **D,** LPA_4 mRNA was measured by RT-PCR; n=2. Columns, mean; bars, SEM; *, $p<0.01$; **, $p<0.001$; ***, $p<0.0001$.

LPC (Fig. 3C), indicating the important contribution of this receptor in ATX-induced invadopodia production.

LPA_4 has been shown to couple to the G_s family of G-proteins that triggers the activation of adenylyl cyclase resulting in an increase in cAMP accumulation (33). To verify the involvement of this pathway in invadopodia formation, ATX over-expressing cells were incubated overnight with cholera toxin (CTX) that interferes with G_s function. While short-term incubation with CTX is known to activate G_s, prolonged exposure ultimately results in its depletion (34). Depletion of G_s resulted in a drastic reduction of the percent of cells producing invadopodia (Fig. S1). Consistent with this result, we next investigated whether increasing intracellular levels of cAMP would result in increases in invadopodia formation. Exposing parental HT1080 cells to forskolin (an activator of adenylyl cyclase) or to dibutyryl cAMP (a stable analog of cAMP) resulted in 1.5-to 3-fold increases in cells producing invadopodia (Fig. 4A). cAMP exerts its effects by activating the cAMP-dependent protein kinase A (PKA) and the recently discovered exchange protein directly activated by cAMP (EPAC) (35). To determine which of these cAMP effectors was responsible for invadopodia production we took advantage of cAMP analogs known to specifically activate PKA (8-bromo-cAMP) or EPAC (8-pCPT). Results indicated that EPAC activation induced a 2.5-fold increase in invadopodia-positive cells whereas PKA activation had no significant effect (Fig. 4B and C). In addition the PKA inhibitors H-89 and PKI did not affect ATX-induced invadopodia formation (Fig. S2), confirming the lack of involvement of PKA. To further assess the role of EPAC in invadopodia formation we transfected HT1080 cells with shRNA against

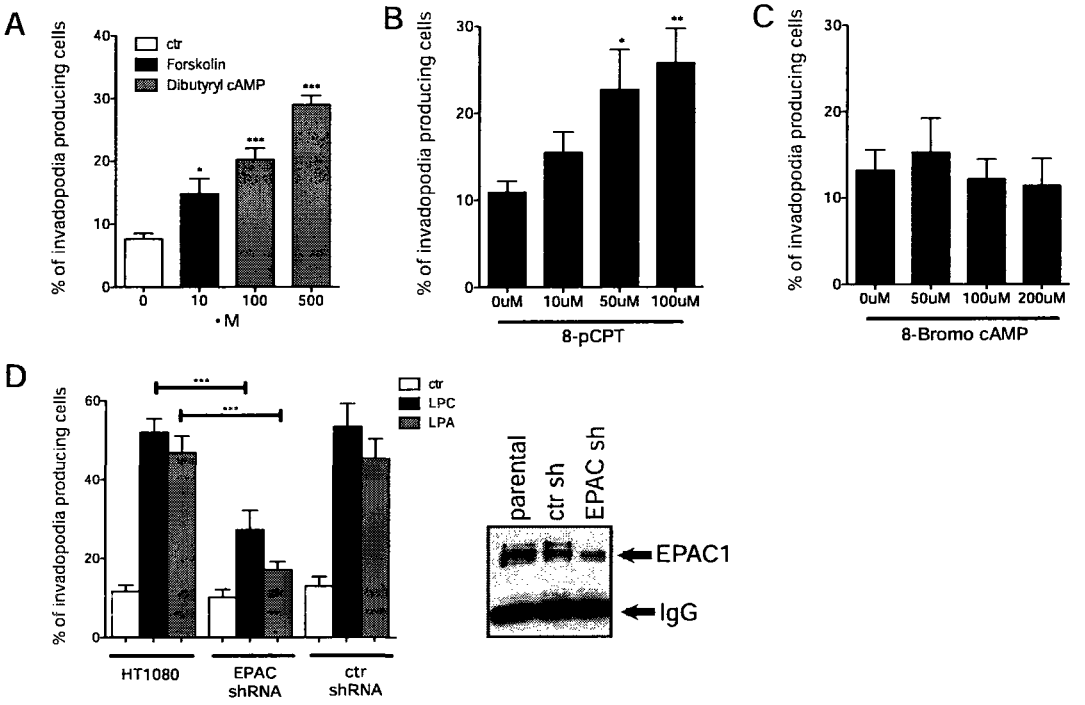


Figure 4. LPA_4 signals through G_s-cAMP-EPAC for invadopodia production

HT1080 cells were incubated with or without (ctr), forskolin or dibutyryl cAMP (**A**), 8-pCPT (**B**), or 8-bromo cAMP (**C**) for 10 h in invadopodia assays; n=3-5. **D**, parental HT1080 or cells transfected with EPAC shRNA or ctr shRNA were cultured in the presence or absence (ctr) of LPA (10μM) or LPC-18:1 (10μM) and the percent of invadopodia-producing cells was determined; n=3. Immunoprecipitation of EPAC from equal amounts of protein per sample is also shown. Columns, mean; bars, SEM; *, $p<0.01$; **, $p<0.001$; ***, $p<0.0001$.

EPAC and observed that EPAC inhibition reduced LPA- and LPC- induced matrix degradation (Fig. 4D). Taken together, the results suggested that invadopodia production by ATX is promoted by a G_s-cAMP-EPAC signaling pathway.

Rac1 activation downstream of EPAC is required for invadopodia production.

EPAC is a nucleotide exchange factor that activates the small G-protein Rap1. Selective inhibition of Rap1 using GGTI-298 abolished EPAC-induced invadopodia production (Fig. S3), suggesting its involvement downstream of EPAC. Rap1 has many effectors, including the small G-protein Rac1 (36), which can induce actin polymerization by the Arp2/3 complex, an event essential to invadopodia formation (37). We therefore used the Rac1 inhibitor, NSC23766, to investigate whether Rac1 could be a downstream target of EPAC for invadopodia production. Rac1 inhibition abolished 8-pCPT induced invadopodia production, whereas the effects induced by LPC and LPA were decreased by approximately 50% (Fig. 5A-C). These results suggested that although Rac1 appears to be the essential downstream mediator of EPAC and Rap1 in invadopodia production, LPC and LPA might activate additional pathways that are independent of the EPAC-Rac1 interaction.

LPA_4 is implicated in 3D invasion and metastasis.

After determining the implication of LPA_4 in invadopodia formation we further investigated the contribution of this receptor in cell invasion through a 3D matrix and metastasis formation in vivo. For the invasion assay, parental HT1080 cells or cells transfected with LPA_4 shRNA were seeded on top of a type I collagen matrix and

incubated with or without LPC and LPA. The results demonstrated that cells transfected with LPA_4 shRNA lost the ability to invade deeply into a 3D matrix in response to LPC or LPA (Fig. 6A-B). Also, mice injected with LPA_4 shRNA transfected cells developed significantly less lung metastasis compared to those injected with control shRNA (Fig. 6C). These results indicated an essential role for LPA_4 in HT1080 fibrosarcoma invasion both in vitro and in vivo and further strengthen the relevance of invadopodia formation for cancer cell invasion and metastasis.

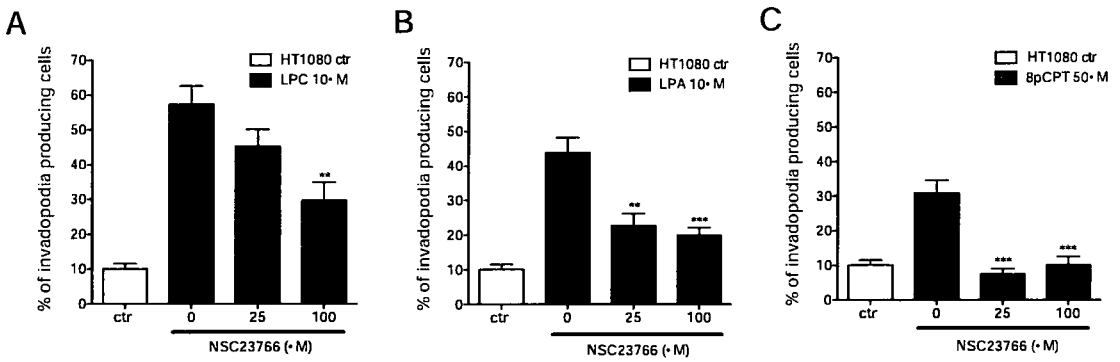


Figure 5. Rac1 activation downstream of EPAC is required for invadopodia production.

A, B, and C, HT1080 cells were incubated with the Rac-1 inhibitor (NSC23766) for 30 min prior to stimulation with LPC-18:1 (10μM) **(A),** LPA (10μM) **(B)**, or 8-pCPT (50μM) **(C),** in invadopodia assays. The control (ctr) represents unstimulated HT1080 cells; n=3-4. Columns, mean; bars, SEM; *, p<0.01 ; **, p<0.001 ; ***, p<0.0001.

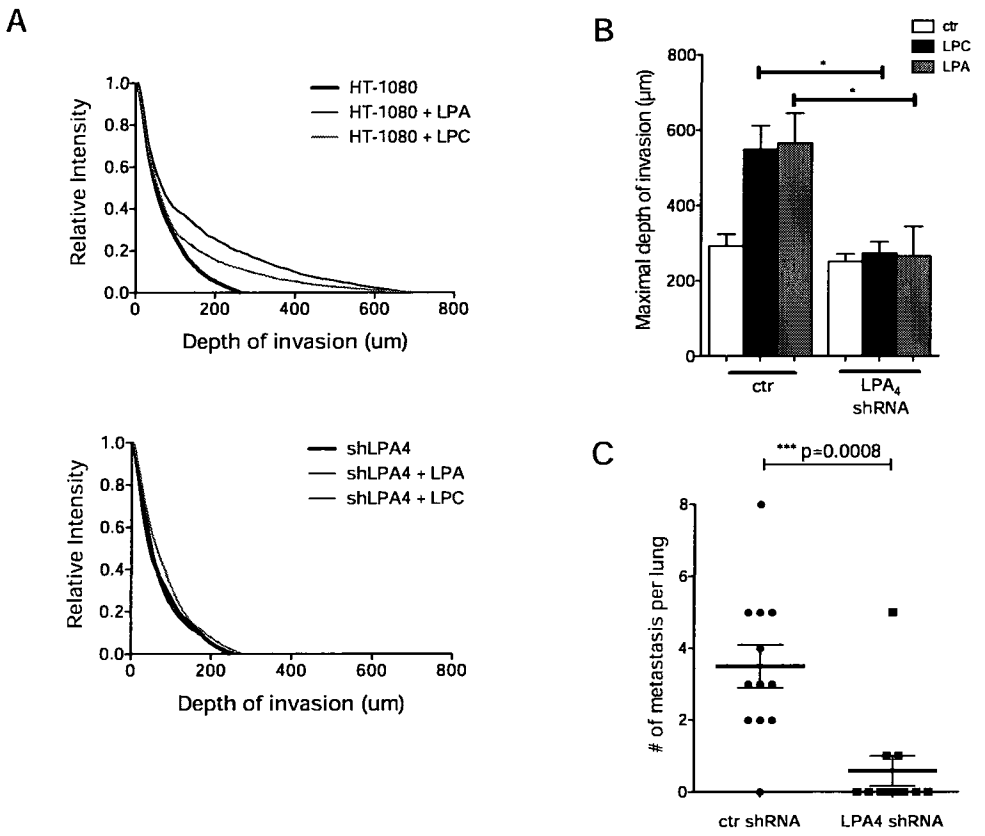
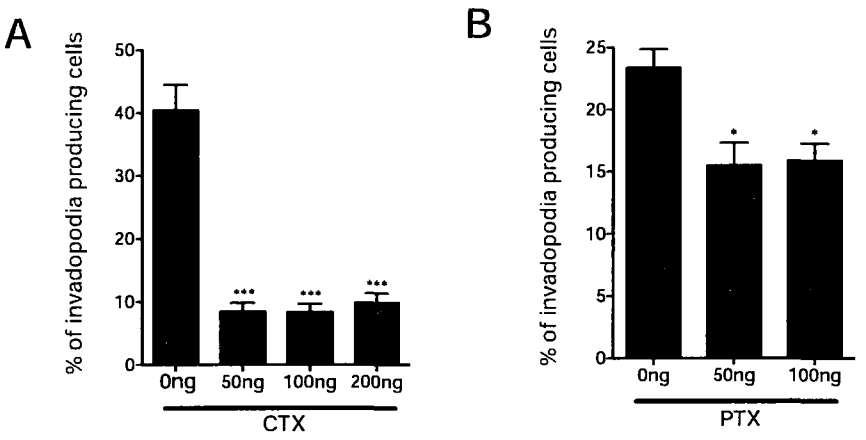


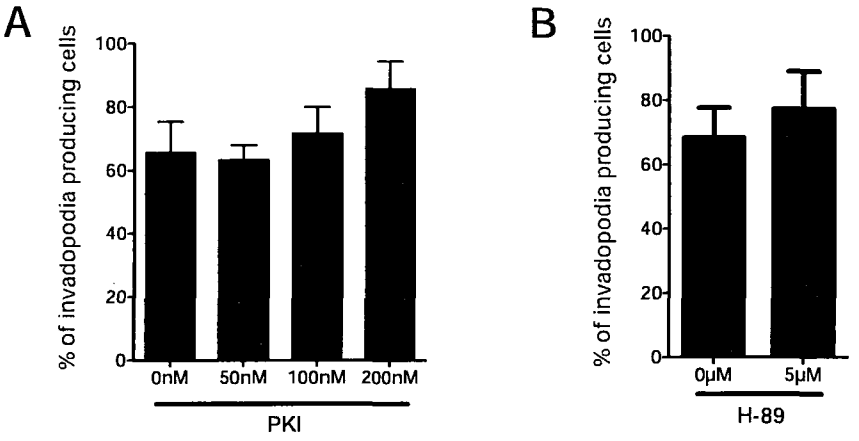
Figure 6. LPA_4 is implicated in cell invasion in vitro and in vivo.

A and B, Parental HT1080 or LPA_4 shRNA-transfected cells were incubated on type I collagen in 3D invasion assays in the presence or absence (ctr) of LPA (10μM) or LPC-18:1 (10μM). Relative intensity of cell staining according to depth of invasion is shown (**A**), graph represents maximal depth of invasion (**B**); n=3. Columns, mean; bars, SEM; *, p<0.01; **, p<0.001. **C,** CD1 nude mice were injected with HT1080 cells transfected with control shRNA (n=12) or LPA_4 shRNA (n=12). The number of metastases counted on the lung surface for each mouse is shown. Results are from two separate experiments. Means +/- SEM are shown; *, p=0.0008.

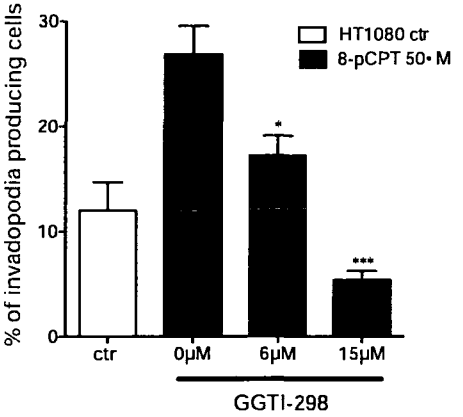
Supplementary figure S1



Supplementary figure S2



Supplementary figure S3



Supplementary Figure S1. Involvement of G_s and G_i in ATX-induced invadopodia production.

A, For G_s depletion HT1080 cells over-expressing ATX were incubated overnight with CTX at the indicated concentrations. Cells were then plated on fluorescent gelatin in the presence or absence of CTX for 10h and the percentage of invadopodia-producing cells was calculated. **B,** ATX over-expressing HT1080 cells were incubated overnight with pertussis toxin (PTX) to inhibit G_i. Cells were plated on fluorescent gelatin in the presence or absence of PTX for 10h and the percentage of invadopodia producing cells was calculated. Columns, mean; bars, SEM; *, $p<0.01$; ***, $p<0.0001$.

Supplementary Figure S2. PKA inhibitors have no effect on ATX-induced invadopodia production.

A and B, ATX over-expressing HT1080 cells were incubated in the presence or absence of PKA inhibitors, PKI **(A)** or H-89 **(B)** in invadopodia assays. The percent of invadopodia producing cells was calculated. Columns, mean; bars, SEM.

Supplementary Figure S3. Rap1 inhibition suppresses EPAC-induced invadopodia production.

HT1080 cells were incubated with the Rap-1 inhibitor (GGTI-298) in the presence of 8-pCPT (50μM) in invadopodia assays. The control (ctr) represents unstimulated HT1080 cells. The percentage of invadopodia producing cells was calculated; n=3-4. Columns, mean; bars, SEM; *, $p<0.01$; ***, $p<0.0001$.

Discussion

In this study, we demonstrated that ATX is implicated in the production of invadopodia and that this effect is dependent on the production of LPA from LPC, both of which can mediate invadopodia production in various cancer cell types expressing ATX and LPA receptors. We further provide the first evidence that LPA_4 signaling regulates invadopodia formation downstream of ATX and LPA in HT1080 cells, a process mediated through the activation of EPAC by cAMP and subsequent Rac-1 activation. RNA interference experiments further support the requirement of LPA_4 for in vitro cell invasion and in vivo metastasis.

ATX and LPA have been shown to be involved in tumor invasion and metastasis (20, 21) and expression of ATX was correlated with increased invasion of many malignant cell lines (2-4). ATX is also known to augment functions associated with tumor progression and metastasis such as proliferation, survival, motility, invasion and angiogenesis. (9, 10, 12). A very recent study indicated that LPA can induce the formation of actin dots that resembles invadopodia (38). Here, we further showed that ATX, through the production of LPA, regulated the formation and function of the invasive structures, invadopodia, in tumor cells. Invadopodia are implicated in the first steps of mesenchymal invasion due to their ability to make cytoskeletal protrusions enriched in matrix degrading proteases (22, 23). The role of ATX in invadopodia production may be part of the mechanism to explain how ATX affects tumor cell invasion and metastasis

LPA receptors have also been implicated in tumorigenesis. LPA has been shown in various studies to stimulate cell motility and to modulate tumor cell invasion, mediated mainly by LPA_1 and $G_{i/o}$ coupling protein (19, 39). LPA_4, that we investigated in this

study, has so far been poorly linked to tumorigenesis. LPA_4 is widely expressed in embryonic tissues and its mRNA expression has been shown to be increased at implantation sites in the uterus (33, 40), suggesting a role in matrix degradation and invasion into the uterus wall. Among the few studies of LPA_4 functions in tumor cells, the report of Lee et al. (41) suggests that LPA_4 signaling inhibits cell motility and invasion by over-activation of RhoA and inhibition of PI3K resulting in decreased Rac1 activation. Conversely, LPA_4 has been shown to induce cell transformation and anchorage independent growth in Myc-transformed cells (42). Our finding that LPA_4 is implicated in the formation of invadopodia through G_s signaling provides a novel role for this LPA receptor and adds an alternative pathway by which ATX and LPA can favor tumor invasion. The discrepancy between our results and those of Lee et al. (41), showing a negative role for LPA_4 in the regulation of motility and invasion, may be due to the fact that these authors used a LPA_4 over-expressing cell type in which the LPA_4 response is coupled to G_q and $G_{12/13}$ but does not mediate effects through G_s as suggested herein with endogenously LPA_4 expressing HT1080 cells.

In addition to the role of LPA_4 in tumor invasion, one important observation of our study is the finding that cAMP and EPAC are involved in invadopodia production. Although the role of cAMP in various cell functions has been studied for decades, information regarding its contribution to cell invasion is scarce. Increases in cAMP have previously been correlated with anti-invasiveness of intestinal cancer cells (43). However, many of the effects of cAMP seem to be contradictory and depend on the cell types studied. For example, depending on cell context, cAMP either stimulates or inhibits cell division. It also causes reversion to a normal phenotype in some transformed cells,

while being important for differentiation of many cell types (44). These observations may be related to the independent or opposing functions of the two main effectors of cAMP, PKA and EPAC (35). Here, we clearly showed that cAMP accumulation led to the production of invadopodia structures and that downstream activation of EPAC and Rap1, but not PKA, were involved in this function. EPAC is an established GEF for Rap-1, a small GTPase, which has been implicated in malignancy (36) mainly through the inside-out activation of integrins that are associated with cell invasion (45). Invadopodia structures are enriched in integrins that interact with the metalloproteinase MT1-MMP to trigger cancer cell invasion (46). It is therefore possible that one of the mechanisms by which EPAC affects invadopodia formation is through the inside-out activation of integrins by Rap-1.

We also observed that invadopodia formation induced by EPAC was dependent on Rac1 activation. Rap1 is know to activate Rac1 indirectly, through integrin signaling, or more directly, due to the activation of the Rap1 effectors Vav2 and Tiam (45). Recently EPAC and Rap1 have been implicated in translocation of Rac1 to the cell membrane, resulting in the recruitment of the Rac effector cortactin (47). Because Rap1 as well as EPAC activities are spatially regulated in cells (48), it is possible that specific activation of EPAC-Rap1 at sites of invadopodia formation could result in translocation of Rac1 to these sites, leading to the recruitment of cortactin, an essential component of forming invadopodia. Our findings that stimulation of the cAMP-EPAC pathways affects invadopodia production through Rac-1 activation identifies an alternative pathway for the activation of Rac-1 at the sites of invadopodia formation.

Whereas Rac1 inhibition abolished EPAC-induced invadopodia production, the effects of LPC and LPA were only reduced by approximately 50%. These observations suggested that LPC and LPA triggered the activation of pathways that were independent of the EPAC-Rac1 axis. In support of this, our results further showed a minor role for the G_i G-protein in invadopodia formation (S1). It is possible that in addition to G_s, G_i could also couple to LPA_4 due to G_s/G_i switching, a phenomenon whereby phosphorylation of the receptor causes it to switch coupling specificity from G_s to G_i (49). In addition, preliminary results from our laboratory (Harper et al. unpublished) indicate that inhibition of Rock, a downstream effector of RhoA, but not PI3K, causes a partial decrease in invadopodia formation induced by ATX. This would implicate a potential involvement of the Rock pathway as well as other LPA receptors such as LPA_1 or LPA_2 in invadopodia formation, which remains to be further elucidated.

To date studies have indicated that LPA is involved in growth and metastasis of various cancers including ovarian and breast mainly through its action on LPA_{1-3} receptors (20, 21, 39). The expression of LPA receptors is cell-specific, and each can elicit different and to some extent overlapping responses upon LPA binding (13, 19). Our work presents evidence that inhibition of the LPA receptor LPA_4 could provide an additional tool to improve the efficacy of treatment of metastasis. Because LPA receptors and ATX are currently being targeted in preclinical trials, (50), the current findings should stimulate studies to evaluate the expression pattern and clinical outcome of LPA_4, together with other LPA receptors and ATX, in various cancer patients.

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3 - DISCUSSION

Metastasis is the leading cause of cancer patient mortality and is a complex process that involves many steps in order for the cells to reach other parts of the body. One important step in the process of metastasis is the degradation of surrounding ECM and basement membranes. This allows the tumor cells to leave the original tumor site and enter blood or lymphatic vessels in order to travel to distant sites in the body where they will again need to degrade surrounding tissues to invade and form a new tumor (WOODHOUSE *et al.,* 1997). Invadopodia are invasive structures that are thought to be responsible for this degradative capacity of cancer cells and are therefore thought to be implicated in an important step of metastasis (BASBAUM and WERB, 1996;CONDEELIS and SEGALL, 2003).

In the manuscript presented herein, we have shown that LPA_4 is implicated in invadopodia production. LPA_4 is also important for invasion into a 3D matrix as well as metastasis formation in a mouse model, further correlating the relationship between invadopodia production and metastasis formation. Furthermore, ATX through the production of LPA and downstream signaling through LPA_4, was found to be implicated in invadopodia biogenesis by cancer cells. Invadopodia are complex structures that require the coordinated action of many proteins and signaling molecules in a small subcellular space (GIMONA and BUCCIONE, 2006). Therefore the mechanisms that promote the specific localization of the proteins and signaling molecules discussed in this

paper, including ATX, LPA_4 and cAMP-EPAC-Rac1, could potentially explain in part why ATX is able to promote invadopodia production.

The effect of ATX on invadopodia biogenesis may be due in part to increased localized secretion of ATX towards sites of forming invadopodia. In various studies, the Golgi apparatus has been shown to reorient toward and come in close proximity to invadopodia (BALDASSARRE *et al.,* 2003). Because ATX follows the classical secretory pathway for its secretion, this might allow the concentrated delivery of ATX-containing vesicles toward the extracellular space surrounding invadopodia. Another possibility is that secreted ATX could remain near invadopodial structures, by binding to integrins, which were found enriched at invadopodia (CHEN, 1990). ATX has potential RGD and LDV integrin-binding sites (KANDA *et al.,* 2008), and its binding to activated integrins was recently shown to be a mechanism that targets secreted ATX to lymphocytes undergoing recruitment and promotes their entry into secondary lymphoid organs (KANDA *et al.,* 2008). Either of these possibilities or both could lead to an increased local production of LPA by ATX in the vicinity of forming invadopodia that could then signal via GPCRs to promote further invadopodia formation. To determine the implication of integrins on ATX localization and its effect on invadopodia, one possibility is to use integrin-directed antibodies or integrin blocking peptides in the invadopodia assay, similar to those used in the study by Kanda et al. However this approach is non-specific and would likely result in reduced cell attachment, among other effects. To increase the specificity, we could perform mutagenesis experiments to mutate the RGD and/or LDV binding sites in ATX and compare the impact of overexpressed wild type or mutated ATX on invadopodia

production. In order to determine if there is directed secretion or binding of ATX to invadopodia sites we could also perform fluorescence or confocal microscopy to visualize the localization of ATX in cells forming invadopodia in conjunction with secretion/ recycling vesicles or plasma membrane markers. We can further use these microscopy techniques to determine the effect of the above-mentioned integrin binding site mutations on ATX localization.

LPA might also promote invadopodia formation due to a specific relocalization or targeting of LPA receptors to sites of forming invadopodia, resulting in enhanced LPA signaling at invadopodia. GPCRs and G-proteins are known to be localized to specific lipid domains and lipid rafts are thought to regulate their signaling cascades (ALLEN *et al.,* 2007;PATEL and INSEL, 2009). For example, the delta-opioid receptor, when activated, is found concentrated in sphingomyelin enriched bilayers (i.e. lipid rafts) (ALVES *et al.,* 2005). G_s trafficking has also been shown to be regulated by caveolin-1 and lipid microdomains (ALLEN *et al.,* 2009). Lipid rafts are specialized lipid microdomains enriched in cholesterol, saturated lipids (sphingolipids) and raft-associated proteins that include caveolin-1 (BROWN and LONDON, 1998;SIMONS and IKONEN, 1997). They also contain glycosyl-phosphatidylinositol anchored proteins, are associated with the cytoskeleton, and are resistant to detergent extraction (BROWN *et al.,* 2000). Lipid rafts have been implicated in signal integration, membrane trafficking, and protein sorting (JACOBSON *et al.,* 2007;MAYOR and RAO, 2004;SIMONS and TOOMRE, 2000). The lipid raft/caveolae domains are thought to compartmentalize proteins involved in specific signaling tasks. Cholesterol increases rigidity of the membrane

therefore regulating lateral mobility and compartmentalization of proteins. This can affect the probability of receptor interaction with downstream signaling partners and optimize their spatiotemporal interactions to increase signaling efficiency (SIMONS and TOOMRE, 2000). Furthermore, cholesterol may play a role in stabilizing the conformation of some GPCRs (ALBERT and BOESZE-BATTAGLIA, 2005;SCHERTLER and HARGRAVE, 2000).

Invadopodial structures have recently been associated with increases in cholesterol and caveolin. Therefore, invadopodia seem to have specific lipid characteristics similar to lipid rafts. Caveolin-1 has been determined to be a regulator of invadopodia biogenesis by modulating lipid domains through the transport of cholesterol (CALDIERI *et al.,* 2009b), while lipid rafts and caveolin-1 were shown to be required for invadopodia formation and subsequent ECM degradation by human breast cancer cells (YAMAGUCHI *et al.,* 2009). In the study of Yamaguchi et al., lipid rafts were found to be enriched, internalized, and dynamically trafficked at invadopodia sites. Furthermore, various invadopodia-associated components such as cortactin and MT1-MMP were found localized in lipid raft enriched membrane fractions (CALDIERI *et al.,* 2009b;YAMAGUCHI *et al.,* 2009). Therefore, specific GPCRs, for example LPA_4 in the case of this memoire, could potentially be localized to, or trafficked in, lipid rafts at invadopodia leading to enhanced signaling. This could account for localized cytoskeletal rearrangements induced by LPA signaling that are required for invadopodia formation. To verify if LPA_4 is localized to invadopodia and whether this is dependent on lipid rafts we could perform fluorescence microscopy to visualize the localization of LPA_4 along with

invadopodia markers. We could then use techniques to disrupt lipid rafts, such as cholesterol sequestration, and determine the effect on LPA_4 localization.

Spatial regulation of numerous signaling and effector molecules is another important aspect of invadopodia formation as it requires the coordinated action of many different signaling pathways in a specific cellular space (GIMONA and BUCCIONE, 2006). This ties in well with the cAMP-EPAC-Rap1 pathway found to be implicated in our work. It is well known that cAMP and its effectors, EPAC (PONSIOEN *et al.,* 2009) and PKA, undergo tight spatial and temporal regulation (JARNAESS and TASKEN, 2007) to promote different cellular functions. As mentioned in the introduction, the EPAC effector Rap1 influences Rac1 activation by the relocalization of the Rac1 effectors Vav2 and Tiam1 to sites of membrane protrusion actively engaging the ECM, in Hela cells (ARTHUR *et al.,* 2004). Invadopodia can be considered to be membrane protrusions engaging the ECM which leads us to speculate that there could be specific activation of Rac1 by its effectors Vav2 and Tiam1, induced by EPAC-Rap1 signaling, at sites of invadopodia production. Furthermore, EPAC activation has been shown to induce Rac1 translocation to the cell membrane in microvascular endothelium resulting in recruitment of cortactin to the cell membrane, an important component of invadopodia (BAUMER *et al.,* 2008). Therefore, the postulated specific localization of either ATX or LPA_4 would lead to activation of the cAMP pathway in the vicinity of invadopodia. Due to the spatial regulation of cAMP and its effectors the signaling induced by the cAMP pathway would then be efficiently confined to the area of invadopodia production, specifically the cell membrane.

In addition to promoting actin rearrangement essential for invadopodia formation, ATX may also stimulate invadopodia functions. ATX has been shown to increase the expression of uPA through a G_i-PI3K-Akt-NFkB signaling pathway in human melanoma cells (LEE *et al.,* 2006). LPA is also capable of increasing uPA activity in ovarian cancer cells, through Ras-Raf signaling, as well as in endometrial carcinoma cells (LI *et al.,* 2005;WANG *et al.,* 2010). As discussed in the introduction section, uPA is a serine protease that interacts with uPAR to convert plasminogen to plasmin which degrades collagen type IV, fibronectin and laminin as well as activating MMP-2, -3, -9 and uPA (DANO *et al.,* 1985;LIJNEN, 2001;SCHMITT *et al.,* 1992). Complexes containing uPAR and seprase (another serine proteinase) have been localized to invadopodia with this association being dependent on the microtubule cytoskeleton and integrins (ARTYM *et al.,* 2002). Interestingly, uPAR is a GPI-linked transmembrane protein and has therefore been associated with caveolae and lipid rafts, which are enriched at invadopodia (STAHL and MUELLER, 1995). This involvement of uPA in invadopodia function is an interesting avenue to further explore. Whether this would imply LPA_4 or other LPA receptors remains to be elucidated.

Supplementary results of the manuscript presented herein suggest an implication of other receptors in ATX-induced invadopodia production. For example, inhibition of G_i with PTX was found to partially inhibit ATX-induced invadopodia production. Inhibition of Rock, a downstream effector of $G_{12/13}$-RhoA, also partially decreased ATX effects. Because G_i and $G_{12/13}$ seem to be partially implicated in invadopodia production, we

believe that in addition to LPA_4, LPA_1 and LPA_2, which can signal through both of these G-proteins, could also be involved. In the article by Taghavi et al., in which LPA_4 expression was found to induce transformation and anchorage independent growth, LPA receptors 1 and 2, but not 3, were shown to mediate a similar effect (TAGHAVI *et al.,* 2008). LPA_1 and LPA_2 have also been highly implicated in tumorigenesis, migration and invasion of many cancer cell types. For example, LPA_1 is involved in promoting motility of human pancreatic cancer cells (YAMADA *et al.,* 2004), neoplastic cells (HAMA *et al.,* 2004) and breast cancer cells (HORAK *et al.,* 2007), as well as metastasis of human colon cancer cells (SHIDA *et al.,* 2003) and breast cancer cells (BOUCHARABA *et al.,* 2006). LPA_2 has been implicated in mitogenic signaling of human colon cancer cells (YUN *et al.,* 2005) as well as mediating cell motility of ovarian cancer cells (JEONG *et al.,* 2008). Therefore signaling pathways of multiple LPA receptors might also play a role in the metastatic cascade by promoting migration following invadopodia-mediated ECM degradation. It is important to note that in our study we used HT1080 fibrosarcoma cells that have high expression of LPA_4 and low expression of LPA_{1-3}. However, any of the LPA_{1-3} receptors could potentially be upregulated by invadopodia signaling events or extracellular cues from the degraded matrix during invadopodia-mediated degradation. Therefore, LPA_4 through G_s-cAMP signaling and Rap1-Rac1 activation might lead to increased invadopodia formation and subsequent activation of other LPA receptors might then promote migration of the cell through the matrix that has been degraded.

In this memoire, we have added both LPC, the substrate, and LPA, the product of ATX activity, to invadopodia assays to determine their effects. In our first experiments, we

used a natural source of LPC derived from egg yolk, that was prepared by the action of phospholipase A on egg L-α-phosphatidylcholine (LPC-egg), that unexpectedly induced dramatically more invadopodia production than LPA in the same experiments (Fig. 2C). In order to understand these results and to be more rigorous, we changed our source of LPC for a synthetic reagent with the same hydrophobic tail length and degree of unsaturation as the LPA. Using synthetic LPC (18:1) and LPA (18:1) , we observed that both LPC and LPA, used at the same concentration, induced similar percentages of cells to produce invadopodia. However, in the literature, LPA has been shown to be much more potent than LPC in inducing cell migration often measured by a wound healing assay. In these publications, LPA was shown to be maximally effective at 0.5 μM, while LPC required 5-10 μM concentrations for a similar effect (GAETANO *et al.,* 2009). In order to determine why our results differed from those of other authors, we tested the effects of LPC and LPA in a wound healing assay. Using this assay, we observed that for the same concentration of reagents used, LPA had a stronger impact on cell migration than LPC, as measured by the number of cells that had moved into the scratch area (Harper et al., unpublished results). Therefore, it seems that the observed discrepancy can neither be attributed to the cell type, nor to the source of LPC and LPA, but possibly to the biological assay used. One possibility for the strong response to LPC in the invadopodia assay could be due to the postulated relocalization of ATX and LPA receptors at sites of forming invadopodia (as discussed above). In this scenario, ATX might produce LPA from the added LPC in closer proximity to the responding receptor, thereby requiring less LPC.

In this memoire, we have studied the roles of ATX, LPA and the LPA GPCR LPA_4 in tumor cell invasion and invadopodia production in HT1080 cells. We have discovered a novel role for LPA_4 and cAMP-EPAC-Rac1 signaling in ATX-induced invadopodia production. Each of these proteins and signaling molecules can be spatially regulated by various mechanisms. It will be interesting to determine in future studies whether ATX and molecules involved in the LPA_4 signaling pathway are spatially regulated during invadopodia formation and to define the mechanisms involved.

From a more clinical or physiological point of view, finding a way to target and inhibit the production of invadopodia by cancer cells could be a good way to prevent the metastasis of tumor cells. Without invadopodia the tumor cells should no longer be able to cross high-density matrix such as basement membranes and this should therefore prevent their migration and invasion to distant sites. Furthermore invadopodia are non-essential structures that are not formed by normal cells and therefore targeting them would likely result in fewer side effects than conventional chemotherapies and should not affect non-cancerous cells (WEAVER, 2006).

In this study we have found that blocking LPA_4 or ATX could provide new targets to potentially inhibit the production of invadopodia by cancer cells. LPA_4 is one of the more recently identified LPA receptors and therefore there is little information on the implications of this receptor in physiology and disease. LPA_4 is highly expressed in embryonic tissues while in adults it has low expression in many tissues (LEE *et al.*, 2007;NOGUCHI *et al.*, 2003). Studies so far have found that it has a role in neuronal

development and bone homeostasis and possibly during pregnancy (ISHII *et al.*, 2009;LIU *et al.*, 2010;YANAGIDA *et al.*, 2007). Due to the findings of our current work it will be interesting to further investigate the roles of LPA_4 and determine if this receptor is essential for other biological functions in the adult. It will also be interesting to determine why LPA_4 is over-expressed in many cancer cell lines and if factors in the tumor microenvironment can cause an increase in LPA_4 expression levels. As LPA_4 is highly expressed in a wide range of highly invasive cancers such as ovarian, glioblastoma, colon, fibrosarcoma, lung, prostate and melanoma (KISHI *et al.*, 2006), the results presented herein can be postulated to apply to a variety of cancers. Further studies are needed to determine which cells types are affected by LPA_4 knockdown and if the cAMP-EPAC-Rac1 pathway is used by different cell lines to promote invadopodia production.

LPA receptors (1-3) and ATX are currently being targeting in preclinical trials to treat cancer (MURPH and MILLS, 2007) so the design and utilization of inhibitors that also target LPA_4 could be beneficial as it seems to mediate signaling through a different pathway than the other LPA receptors. ATX and LPA_4 are both highly expressed during embryogenesis but in the adult have a lower and more restricted expression. Their over-expression may therefore be a good determinant for identifying potentially aggressive cancers. Early detection of cancer cells that possess metastatic potential could improve the treatment and prognosis of this disease. Metastasis is a complicated process yet to be fully understood. The results of our study will hopefully help shed some new light on this process.

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