UNIVERSITÉ DU QUÉBEC À MONTRÉAL

L'IMPACT DES PERTURBATIONS DU CYTOSQUELETTE SUR L'EXPRESSION ET LA FONCTION DE LA LOW-DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN 1 (LRP-1)

MÉMOIRE

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DE LA MAÎTRISE EN BIOLOGIE

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RÉSUMÉ

Les glioblastomes multiformes (GBM), considérés parmi les cancers les plus agressifs du cerveau, représentent entre 1-2% de l'ensemble des tumeurs adultes. Quoique plutôt rares, ceux-ci sont particulièrement difficiles à traiter, d'où le sombre pronostic relié à la maladie. De plus, les causes de la pathologie sont encore peu connues. Un obstacle considérable dans le traitement des GBM, et dans les maladies du système nerveux central (SNC) en général, est le passage de la barrière hémato-encéphalique (BHE) par les substances pharmacologiques. Angiopep-2, un court peptide inerte, a été développé comme système de vectorisation de médicaments au cerveau. Cette molécule est internalisée sélectivement à travers la BHE par un mécanisme dépendant de la protéine *Low-Density Lipoprotein Receptor-Related Protein-1* (LRP-1). La conjugaison de molécules thérapeutiques à Angiopep-2 a permis le développement de médicaments ciblant le cerveau. LRP-1 est un large récepteur membranaire présent à la surface des cellules endothéliales de la BHE, en plus d'être exprimé dans les cellules cancéreuses du cerveau. LRP-1 possède de nombreuses fonctions, dont l'internalisation de ligands extracellulaires. Il est également associé à la survie et à la migration cellulaire. De plus, LRP-1 est impliqué dans la détermination du grade et du phénotype du cancer du cerveau. Il a été démontré que l'expression et la fonction de LRP-1 étaient régulées par la protéine *Membrane Type-1 Matrix Metalloproteinase* (MT1-MMP). MT1-MMP est capable de cliver LRP-1, et joue conséquemment un rôle dans la régulation des fonctions qui dépendent de LRP-1. Dans notre recherche, nous avons décidé d'explorer la régulation de l'expression de LRP-1, par MT1-MMP, dans une lignée de cellules de gliome. Nous avons traité le modèle cellulaire U87-GM avec de la Concanavaline A (ConA), connue pour sa capacité à induire l'activation et la transcription de MT1-MMP. Nos résultats ont démontré une rapide internalisation ainsi qu'une dégradation de LRP-1. Cependant, par répression génique de MT1-MMP et de d'autres médiateurs impliqués dans la voie de signalisation de la ConA, ainsi que par l'utilisation d'inhibiteurs sélectifs, nous avons établi que cet effet n'était pas médié par MT1-MMP, ni par une interaction directe entre la ConA et la cellule. Sachant que les effets de la ConA impliquent des perturbations du cytosquelette d'actine, nous avons inhibé la polymérisation de l'actine et des microtubules et découvert que les effets sur LRP-1 étaient dus à la perturbation de l'actine. De plus, ce processus d'internalisation et de dégradation de LRP-1 a mené à une réduction significative de la capacité des cellules U87 à internaliser l'Angiopep-2 et l'alpha-2 macroglobuline, deux ligands de LRP-1. Nous concluons donc que l'intégrité du cytosquelette d'actine est requise pour les fonctions de LRP-1 à la surface de la cellule. Nos données démontrent également la nécessité de retrouver LRP-1 à la surface cellulaire dans la vectorisation de molécules thérapeutiques, couplées à l'Angiopep-2, dans les cellules cancéreuses du cerveau. Enfin, nos données peuvent être ajoutées à la littérature grandissante qui suggère l'exploration de la Concanavaline A comme une potentielle entité thérapeutique.

SUMMARY

Glioblastoma multiforme (GBM) is one of the most aggressive types of brain tumours, constituting 1 to 2% of all adult tumours. Though relatively rare, GBMs have a dire prognosis and are very difficult to treat. The causes of GBMs are relatively unknown making the study of the molecular pathologies that lead to this disease important, as well as enabling the development of more efficacious treatment strategies. A considerable obstacle in the treatment of GBMs, and in general, disorders of the central nervous system (CNS), is the ability to penetrate the blood-brain barrier (BBB) pharmacologically. Angiopep-2 is a short inert peptide that was designed as a drug delivery system: it is selectively internalized across the BBB in a low-density lipoprotein receptor-related protein 1 (LRP-1)-dependent manner. This has allowed for the successful development of drug molecules that are conjugated to Angiopep-2, allowing delivery to the brain. LRP-1 is a large receptor, present on the cell surface of BBB endothelial cells, as well as being expressed in brain and brain tumour cells. It has many functions, including the internalization of extracellular ligands. It is also associated with cell survival and cell migration. In addition to this, LRP-1 has been implicated in both cancer grade and phenotype. LRP-1 expression and function has already been shown to be regulated by Membrane Type 1 Matrix Metalloproteinase (MT1-MMP, also known as MMP-14). MT1-MMP has been shown to be able to cleave LRP-1, playing a role in the regulation of LRP-1-mediated functions. In our research, we decided to explore MT1-MMP regulation of LRP-1 in a glioma cell line, and see whether this would impact on the internalization of Angiopep-2, a therapeutic avenue which holds great promise in the treatment of GBM and other CNS disorders. We treated the GBM cell model, U87, with the plant lectin Concanavalin A (ConA), known to induce the activation and transcription of MT1-MMP, and found that there was rapid internalization and degradation of LRP-1. However, through gene silencing of MT1-MMP and other known mediators of ConA-signalling, as well as the use of selective inhibitors, we established that this effect was not mediated by MT1-MMP, nor via a direct cell-ConA interaction. Knowing that ConA's effects involve disruption to the actin cytoskeleton, we selectively disrupted the actin and microtubule cytoskeleton and found that the effects on LRP-1 were due to disruption of the actin, and not microtubule, cytoskeleton. Furthermore, this internalization and degradation of LRP-1 led to a significant reduction in the capacity of U87 cells to internalize α2Macroglobulin and Angiopep-2, a natural and synthetic ligand of LRP-1, respectively. We conclude that actin cytoskeleton integrity is required for proper LRP-1 cell surface functions. Furthermore, our data demonstrate the pivotal requirement of cell surface LRP-1 functions in the vectorized transport of therapeutic Angiopep bioconjugates into brain cancer cells. In addition, these data can be added to growing literature base supporting the claim that ConA merits further study as a potential therapeutic entity.

Key Words

Glioblastoma, Brain cancer, LRP-1, Concanavalin-A, Cytoskeleton

ABBREVIATIONS

BBB	Blood-Brain Barrier
ConA	Concanavalin A
CNS	Central Nervous System
ECM	Extracellular Matrix
GBM	Glioblastoma Multiforme
MMP	Matrix Metalloproteinase
LRP-1	Low Density Lipoprotein-Related Receptor 1
MMP-2	Matrix Metalloproteinase 2
MT1-MMP	Membrane Typre-1 Matrix Metalloproteinase
COX-2	Cyclooxygenase-2
CytoD	Cytochalasin-D
EGCG	Epigallocatechin 3-gallate
ER	Endoplasmic reticulum
TLR	Toll-like receptor
UPS	Ubiquitin-dependent proteasome system
TJ	Tight junctions
GPCR	G-protein-coupled receptor
VEGF	Vascular endothelial growth factor
ERK	Extracellular signal–regulated kinases

Chapter I
Literature Review

1. Introduction

Cancer is a pathology where populations of cells gain a phenotype where they no longer follow the intricate and extensive mechanisms that regulate homeostasis. This enables non-homeostatic growth of certain cells in an uninhibited manner. (Liotta & Kohn, 2001) This is a dynamic, multi-step process that in general takes many years to develop. A highly selective process is required to overcome the innate mechanisms that inhibit the occurrence of cancers. These cells are highly proliferative, and have often lost cell-cell contact induced growth inhibition - they gain these phenotypes through: genomic; proteomic; post-translational; epigenetic modifications that generate the highly complex alterations in cancerous tissues. (Hanahan & Weinberg, 2011) Hannah et al., in their seminal paper describe the 'hallmarks of cancer' (

Figure 1) – six characteristics required for progression to the neoplastic state. (Hanahan, Weinberg, & Francisco, 2000) However, as described by Lazebnik, these hallmarks are also characteristics of benign tumours, except for 'Tissue invasion and metastasis'. (Lazebnik, 2010) Thus, it is important to consider the use of the words 'tumour' and 'cancer', as they are not interchangeable. This process of invasion is multifaceted and involves many steps that are interlinked and interdependent (Figure 2), which ultimately result in metastatic invasion. Initially, as biomedical research began to understand the genetic component of the disease, interest was concentrated and hope was placed, in a simplistic view of single cell lineages being responsible for the cancer phenomena – however, it is now understood that there is a very complex interplay between heterogeneous pre-cancerous populations, and non-cancerous, but associated host cells that provide an essential component to the development of a malignant pathology. This includes the induction of angiogenesis, modification of the tumour environment, the recruitment of immune and stem cells, and the conditioning of potential metastatic sites. (Siemann, Dietmar, 2010)

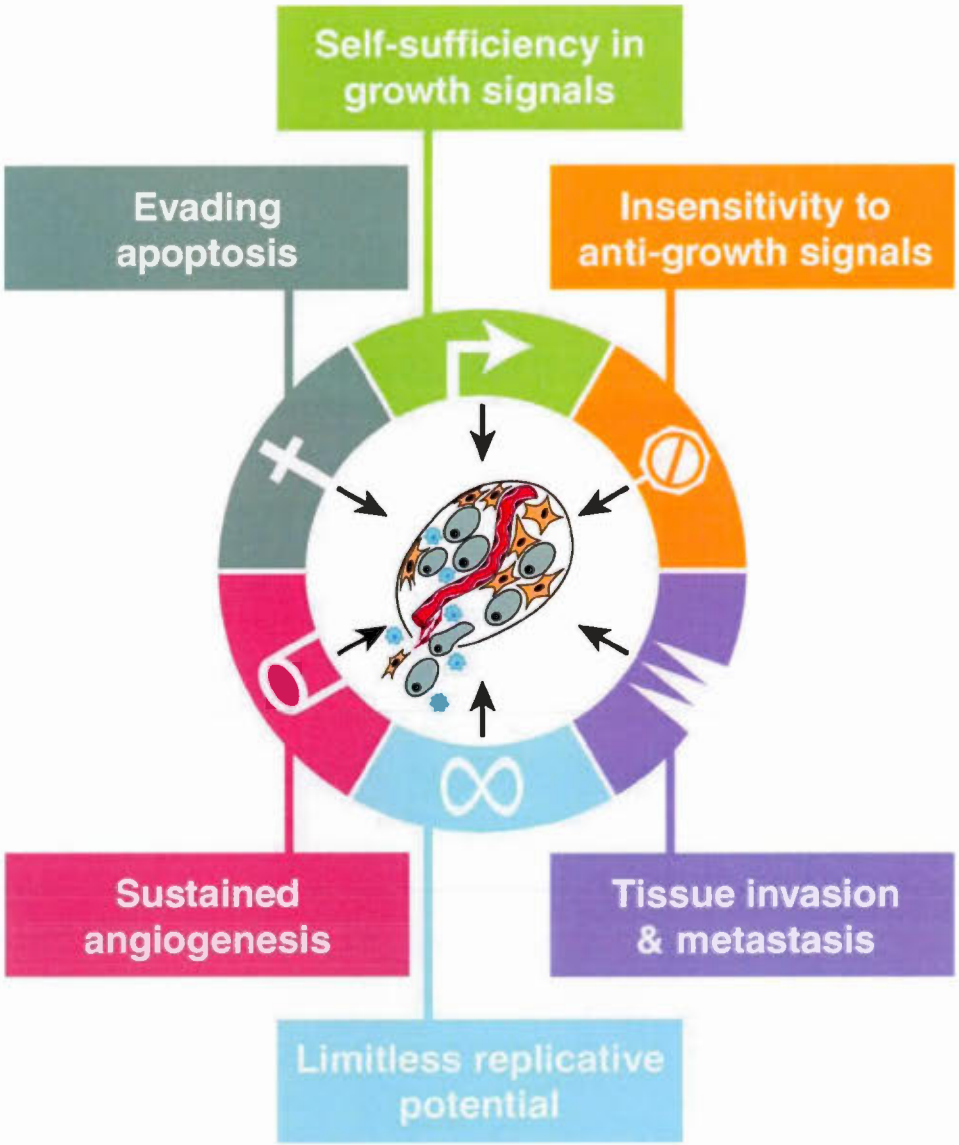

Figure 1 The acquired 'hallmarks' of cancer. Cancer cell genotypes involve the manifestation of six essential alterations in cell physiology that collectively dictate malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Each of these physiologic changes— novel capabilities acquired during tumour development— represents the successful breaching of an anticancer defence mechanism hardwired into cells and tissues. Adapted from (Hanahan et al., 2000)

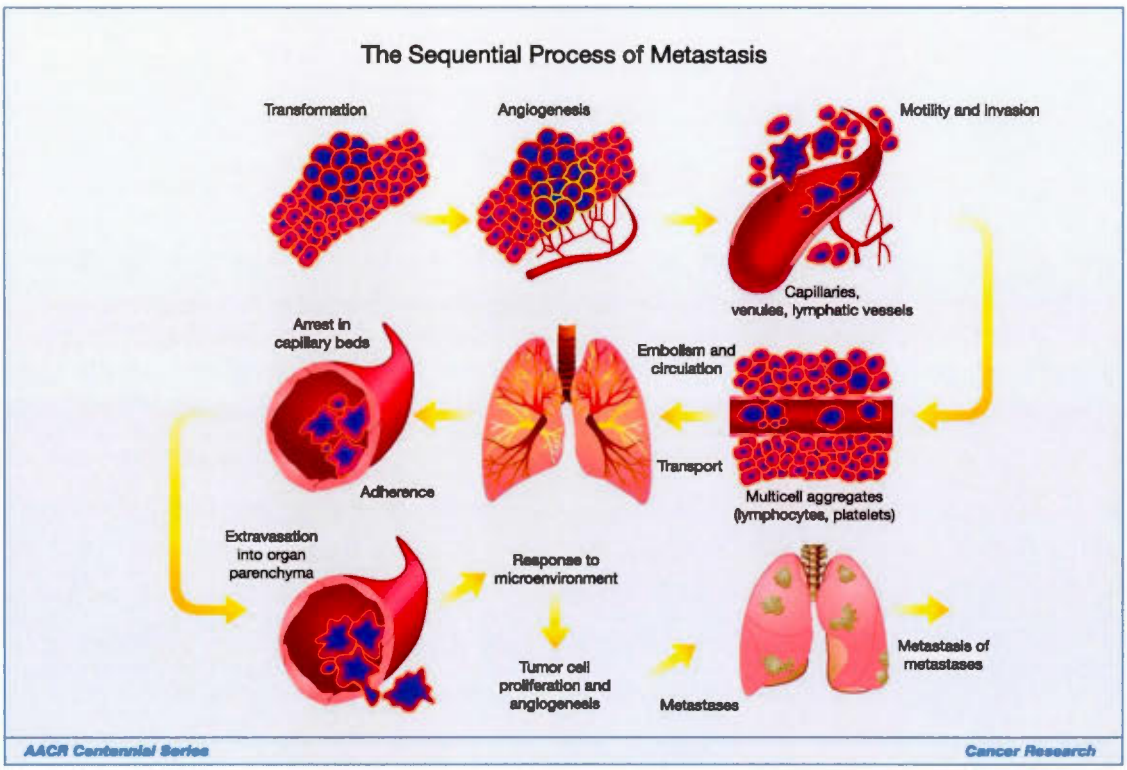

Figure 2 'Cancer metastasis consists of sequential, interlinked, and selective steps. The outcome of each step is influenced by the interaction of metastatic cellular subpopulations with homeostatic factors. Each step of the metastatic cascade is potentially rate limiting such that failure of a tumour cell to complete any step effectively impedes that portion of the process, thus, the interplay between the many factors is essential to understanding, and perhaps identifying the factors most influential in the process of benign tumours becoming cancerous'. (Talmadge & Fidler, 2010)

1.1 Brain Tumours

Brain tumours are a relatively rare disorder, with an incidence of 2-10 cases per 100,000 people, globally. (Bondy et al., 2008) The majority of *de novo* brain tumours are of neuroepithelial origin, and are called gliomas. These are graded according to their aggressiveness, from I to IV. Grade IV gliomas, or glioblastoma multiforme (GBM), are the most common glioma, and the most aggressive (Ohgaki & Kleihues, 2007) - GBM tumours represent only 1-2% of all adult tumours however, they are ultimately untreatable. (Erpolat et al., 2009)(Wen & Kesari, 2008) This has led to the dire prognosis

of this pathology – treatment strategies are ineffective (despite surgical intervention extending life expectancy). The mean time from diagnosis to death with treatment is 14 months, and the 5 year survival rate is 10%. (Bhujbal, de Vos, & Niclou, 2014) Their histopathological hallmarks include substantial vascularisation - GBMs being one of the most highly vascularised neoplasms (Visted & Lund-Johansen, 2003). In addition to primary brain tumours, secondary metastases are a major challenge and common in breast, colorectal, melanoma and lung cancer. (Bhujbal et al., 2014)

1.2 Glioblastomas

The aetiology of GBM is relatively unknown, with only the exception of therapeutic irradiation being associated with an increased risk of malignancy. (Visted & Lund-Johansen, 2003) Associations with head injuries, foods containing N-nitroso compounds, occupational risk factors and electromagnetic fields have been inconclusive. (Wen & Kesari, 2008) However, there is some suggested evidence of immunological factors contributing: atopic individuals having a reduced risk for glioma development; patients with GBMs who have elevated IgE levels having an improved prognosis. Interestingly, only 5% of GBM cases have familial association. (Wen & Kesari, 2008) Brain tumours, and many cancers in general, can be characterised by aberrant receptor tyrosine kinase (RTK) signalling. In GBMs, malignant transformation can be attributed to growth factor signalling. Roughly 90% of GBM tumours have aberrant RTK signalling, with 45% involving the epidermal growth factor receptor (EGFR) family of RTKs. (McLendon et al., 2008) RTKs have been targeted in several clinical trials, but have all ultimately failed to show any benefit as a treatment. (Johansson et al., 2013)

1.3 Treatment

Standard treatment of GBM involves, as much where possible, tumour resection followed by radiation therapy and temozolomide-based chemotherapy. (Kauer, Figueiredo, Hingtgen, & Shah, 2012) Radiation and chemotherapy does improve life expectancy - 18.9 vs 9.8 months without, (Erpolat et al., 2009) however this is still far behind advances made in other cancer treatments, and the benefits of chemotherapy in GBM treatment are debated. (Sarin, 2009) Despite resection of the tumour being effective, nearly 100% of GBM cases are fatal, (Lesniak & Brem, 2004) with 80-90% recurrence of the tumour occurring within 2 cm of the resection cavity. (Bhujbal et al., 2014) There are of course several factors that can be attributed to this phenomenon.

However, it is relatively well accepted that the biggest barriers to effective treatment of the residual, infiltrating cells, involves, among others: poor therapeutic access to CNS because of the blood brain barrier (BBB); (Muldoon et al., 2007) vascular dysfunction at the tumour; (Jain, Tong, & Munn, 2007) and, a short half-life of therapeutic molecules once *in vivo.* (Sarin, 2009) Pre-clinical studies have shown that the ineffectiveness of many treatment strategies can be attributed to poor BBB penetration. For example, the use of monoclonal antibodies against EGFRvIII in a murine model led to tumour shrinkage in subcutaneous melanomas but not in intracranial brain metastases due to poor BBB penetration. (J.H. Sampson, L.E. Crotty, S. Lee, G.E. Archer, D.M. Ashley, C.J. Wikstrand, 2000; Lesniak & Brem, 2004)

1.4 Therapeutic Barriers Of The CNS

One of the major obstacles in addressing pathologies of the CNS such as brain tumours; HIV encephalopathies; epilepsy; cerebrovascular disease; and neurodegenerative diseases), is achieving therapeutic concentrations of pharmacological agents within the CNS. (Misra, Ganesh, & Shahiwala, 2003) Several factors contribute to this phenomenon. Primarily, this is caused by the permeability of the BBB: due to its specialized function in protecting the CNS, which in the ensuing text will be discussed, it is very difficult to select agents which are BBB-permeable. (Lesniak & Brem, 2004) There are also other substantial factors, which are not limited to the CNS, that affect therapeutic concentrations at the desired site. Especially with chemotherapeutic agents, active transport out from tumour cells and BBB endothelial cells by efflux pumps such as P-glycoprotein results in diminished concentrations of the drug. (Schinkel et al., 1997) Furthermore, within the CNS there is a variance in drug volume distribution determined by cellular uptake, interactions with lipids and proteins, as well as accumulation in subcellular compartments. (Muldoon et al., 2007) With systemic delivery of agents, there is substantial plasma protein binding: for chemotherapeutic agents this can often be 90%, with only 10% free drug. However, this can be even higher: for example, the alkylating agent Chlormbucil is 99% plasma protein bound. (Muldoon et al., 2007) Another substantial barrier to effective targeting of cancer cells is the high tumoural interstitial pressure. This can often be above 50 mmHg; whereas the surrounding tissue will be at 2 mmHg. (Muldoon et al., 2007) This high interstitial pressure diminishes the transcapillary flow, significantly reducing the delivery of drugs to the tumour. This is likely caused by:

blood- vessel leakiness; interstitial fibrosis; contraction of the interstitial matrix mediated by stromal fibroblasts. (Heldin, Rubin, Pietras, & Ostman, 2004)

1.5 The Blood Brain Barrier (BBB)

The BBB acts to protect the CNS from fluctuations in the composition of the blood (hormones, amino acids, glucose, certain toxins etc.). It tightly regulates the passage of materials across the CNS-cardiovascular interface through anatomical, physicochemical, and biochemical mechanisms. The neurovascular unit is the anatomical feature forming the BBB, found at the endothelial cell – brain capillary site (Figure 3), deriving its properties from a specialized basal membrane, perycites and astrocytic endfeet. These features co-operate to produce the BBB, and to tightly control the passage of molecules across the interface. The continuous tight junctions that join the endothelial cells in the brain capillaries limit the diffusion of molecules across the BBB (the major proteins involved in this are summarised in Figure 4). The basal membrane provides structural support for the capillary and specific proteins present in the basement membrane play a part in the development and establishment of the BBB. (Obermeier, Daneman, & Ransohoff, 2013) Astrocytic foot processes release specific factors and are necessary for the development of the BBB. Transport carriers for glucose and essential amino acids facilitate the movement of these solutes into the brain – neuronal cells are unable to synthesize these essential amino acids, it is taken up from the blood. Secondary transport systems appear to cause efflux of small molecules and non-essential amino acids from the brain to the blood. Sodium ion transporters on the luminal membrane and Na/K-ATPases and on the anti-luminal membrane account for the movement of sodium from the blood to the brain - the large number of mitochondria present in brain endothelial cells provide energy for the functioning of these Na/K-ATPase. The major pathways for the movement of material into the CNS compartment (described in Figure 5) include: paracellular routes for small water-soluble molecules; transcellular for lipid soluble molecules, transport protein-mediated and receptor-mediated cellular internalization and transport.[22–25]

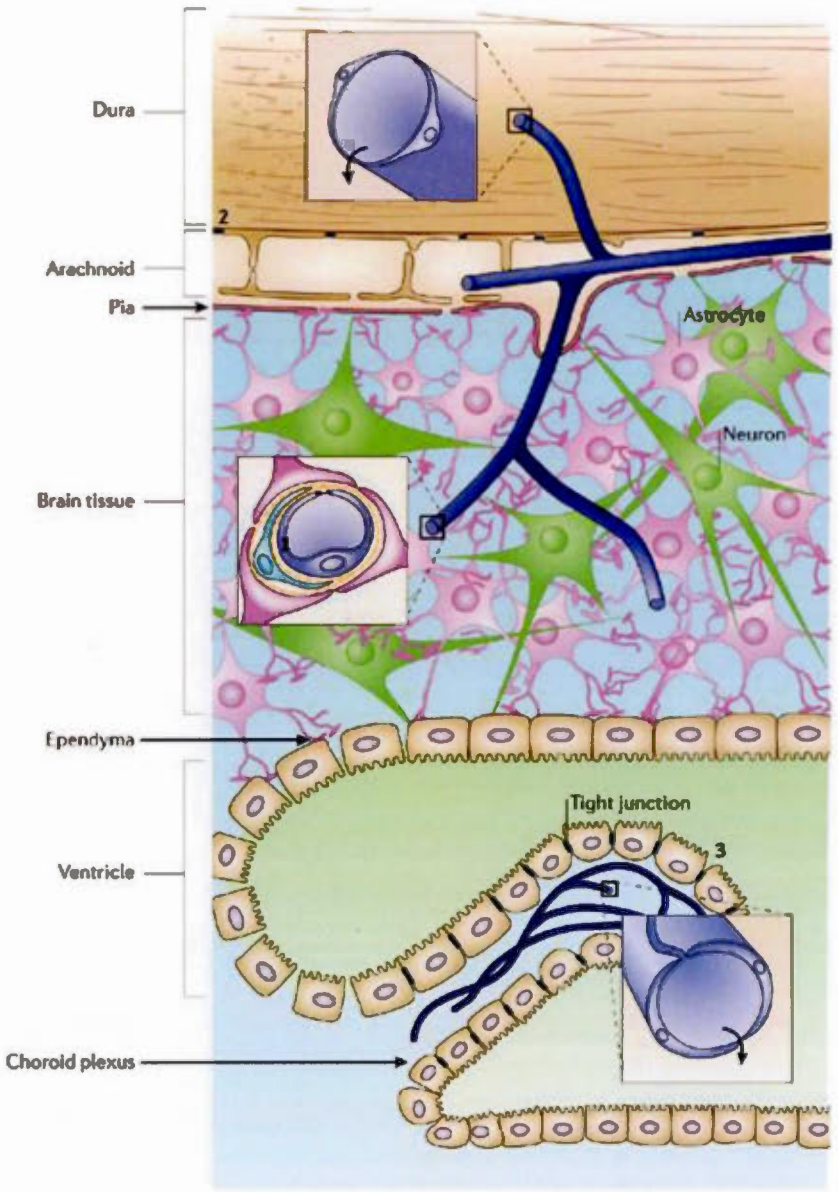

Figure 3 Location of barrier sites in the CNS. Barriers are present at three main sites: the brain endothelium forming the blood–brain barrier (BBB) (1), the arachnoid epithelium (2) forming the middle layer of the meninges, and the choroid plexus epithelium (3), which secretes cerebrospinal fluid (CSF). At each site, the physical barrier is caused by tight junctions that reduce the permeability of the paracellular (intercellular cleft) pathway. Modified from. (Abbott, Rönnbäck, & Hansson, 2006)

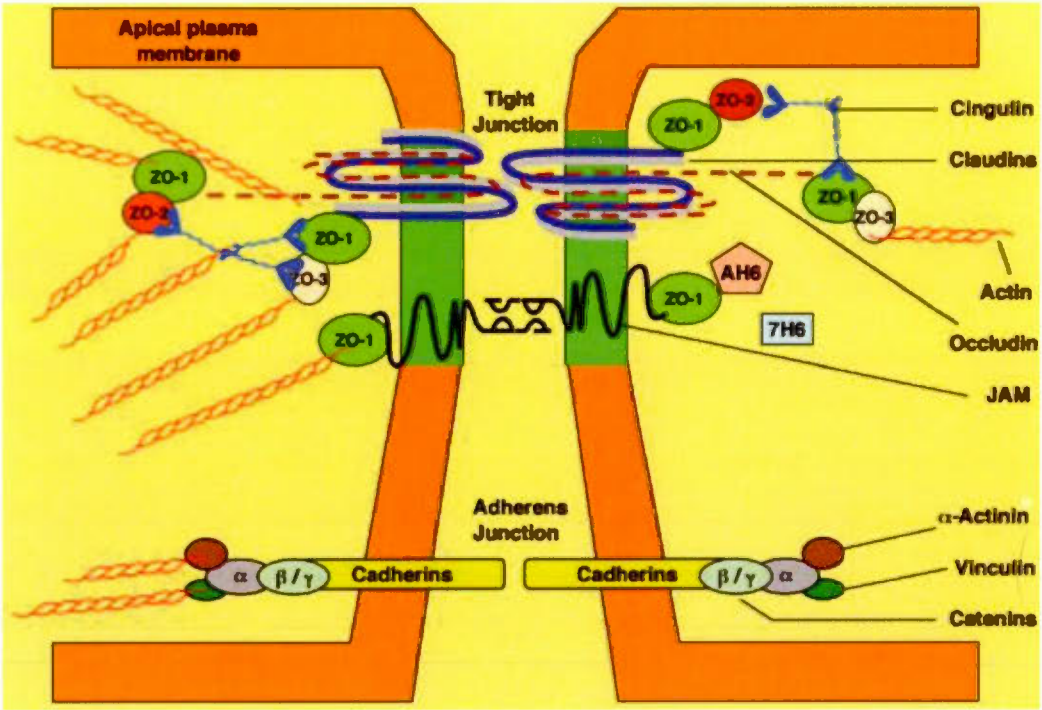

Figure 4 'The major proteins associated with tight junctions (TJs) at the BBB are shown. The tight junction is embedded in a cholesterol-enriched region of the plasma membrane (shaded). Three integral proteins—claudin 1 and 2, occludin and junctional adhesion molecule (JAM)—form the tight junction. Claudins make up the backbone of the TJ strands forming dimers and bind homotypically to claudins on adjacent cells to produce the primary seal of the TJ. Occludin functions as a dynamic regulatory protein. The tight junction also consists of several accessary proteins, which contribute to its structural support. The zonula occludens proteins (ZO-1 to 3) serve as recognition proteins for tight junctional placement and as a support structure for signal transduction proteins. AF6 is a Ras effector molecule associated with ZO-1. 7H6 antigen is a phosphoprotein found at tight junctions impermeable to ions and molecules. Cingulin is a double-stranded myosin-like protein that binds preferentially to ZO proteins at the globular head and to other cingulin molecules at the globular tail. The primary cytoskeletal protein, actin, has known binding sites on all of the ZO proteins'. (Nag, 2003)

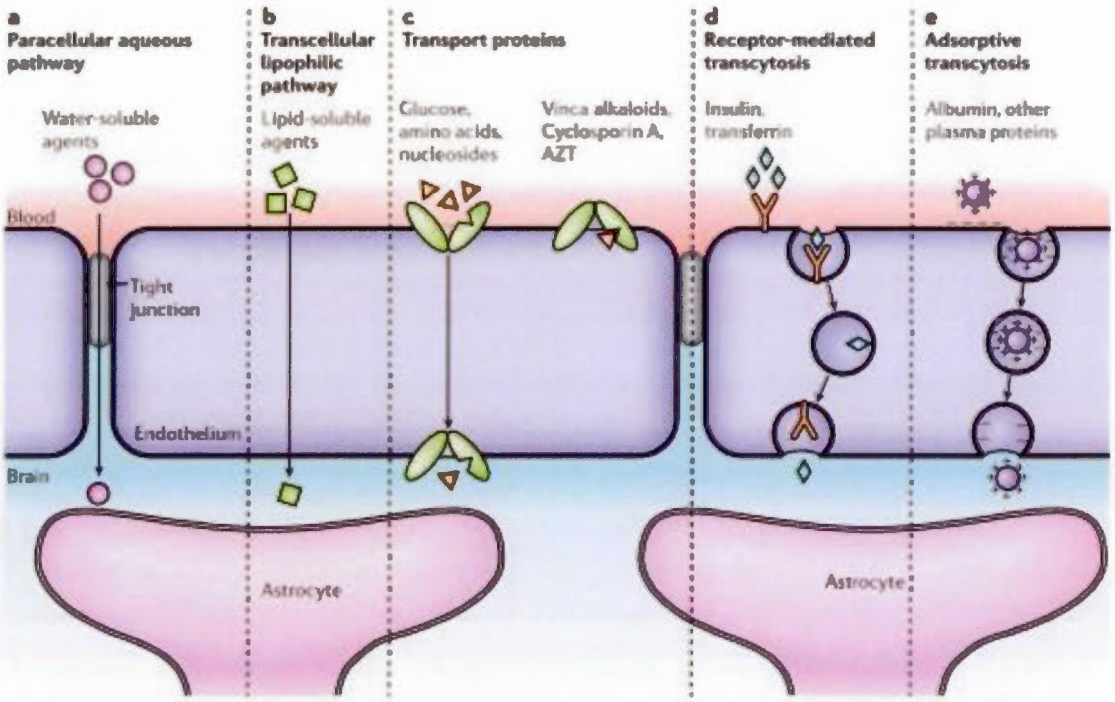

Figure 5 'Pathways across the blood–brain barrier. A schematic diagram of the endothelial cells that form the blood– brain barrier (BBB) and their associations with the perivascular endfeet of astrocytes. The main routes for molecular traffic across the BBB are shown. A) Normally, the tight junctions severely restrict penetration of water-soluble compounds, including polar drugs. B) However, the large surface area of the lipid membranes of the endothelium offers an effective diffusive route for lipid-soluble agents. C) The endothelium contains transport proteins (carriers) for glucose, amino acids, purine bases, nucleosides, choline and other substances. Some transporters are energy-dependent (for example, P-glycoprotein) and act as efflux transporters. AZT, azidothymidine. D) Certain proteins, such as insulin and transferrin, are taken up by specific receptor-mediated endocytosis and transcytosis. E) Native plasma proteins such as albumin are poorly transported, but cationization can increase their uptake by adsorptive-mediated endocytosis and transcytosis. Drug delivery across the brain endothelium depends on making use of pathways b–e; most CNS drugs enter via route b'. (Abbott et al., 2006)

1.6 The Cytoskeleton

The cytoskeleton's role is integral to almost every cellular function: it is the structure that gives cells their shape, mediates the controlled movement of subcellular structures such as organelles, as well as facilitating the organisation of cellular processes. (Albert, B., 2010) The cytoskeleton consists of three major classes of molecules that differ in size and in protein composition. Microtubules are the largest type of filaments and they are composed of a protein called tubulin. Actin filaments are the smallest type and they are made of a protein called actin. Intermediate filaments, as their name suggests, are mid-sized. (Albert, B., 2010)

The actin cytoskeleton plays an integral role in the spatial order of internal structures, providing mechanical forces and mediating adherence of the cell, it provides the contractile forces as well as linking mechanical stresses to biological responses. (Fischer & Fowler, 2015) The actin cytoskeleton is also essential for the transport of cargos through the cell cytosol, as well as the mediation of the internalization of extracellular cargos, and the creation of membrane vesicles. (Albert, B., 2010)

These structures also function, to a certain extent, as detectors for mechanical signals: crosstalk between the actin cytoskeleton and microtubule cytoskeleton plays a role in mediating the polarization of cells during division, morphological changes, as well as migration. This can be crucial for the development of tissues, as well as the homeostatic balance between non-metastatic, and metastatic cells. In migrating cells, growing microtubules that reach into the leading edge promote Rac activation and the formation of short, branched F-actin for lamellipodia formation. In essence, the cell can respond the extrinsic, physical cues that can be translated into biological responses, which lead to the formation of the required structures. (Akhshi, Wernike, & Piekny, 2014)

The cytoskeleton is also an integral component of vesicular trafficking: during endocytosis, transport within the cell and, exocytosis. (Albert, B., 2010) The cytoskeleton also plays an integral part in modulating membrane curvature and tension. Furthermore, it is involved in the regulation of clathrin coated pit internalisation: GPCRs interacting with the actin cytoskeleton regulating the rate of the internalisation of cargos, as well as perhaps, adding in the creation of forces during dynamin fission. In addition, it has been

suggested that the interplay between the cytoskeleton and the plasma membrane allows for mechanisms that facilitate internalisation of large volumes of membrane during, for example, pinocytosis or macropinocytosis.

Metastatic disease, or the movement of cancer cells from one site to another, is a complex process, as previously described, which can also involve dramatic remodelling of the cytoskeleton. The various components of the cytoskeleton are highly integrated and their functions are well orchestrated in normal, physiological cells. (Albert, B., 2010) In contrast, in metastatic pathologies, mutations and abnormal expression of cytoskeletal and cytoskeletal-associated proteins can play an important role in the ability of cancer cells to metastasize, as well as to resist treatment strategies. Studies on the role of actin and its interacting partners have highlighted key signalling pathways, such as the Rho GTPases (where they can mediate the formation of different types of F-actin that confer changes in cortical tension and contraction, and can be regulated by microtubules) and downstream effector proteins that, through the cytoskeleton, mediate tumour cell migration, invasion and metastasis. Improved understanding of how the cytoskeleton and its interacting partners influence tumour cell migration and metastasis has led to the development of novel therapeutics against aggressive and metastatic disease. (Fife, McCarroll, & Kavallaris, 2014)

1.7 Matrix Metalloproteinases (MMPs)

The plethora and complexity of the acquired phenotypes that enable cellular transformation to neoplastic tumours are exquisitely complex – in some cases dependent, and others independent of one another. The invasive and metastatic ability of tumour cells, especially in GBM cases, contributes significantly to their morbidity and mortality. This gained phenotype is often as a result of deregulation of the relationship between the cell and the extracellular matrix (ECM). This is a complex interaction involving proteases (mainly MMPs), their inhibitors and a plethora of signalling molecules that orchestrate this delicate interaction. (Siemann, Dietmar, 2010)

The MMPs are a family of zinc-dependent endopeptidases that are able to degrade nearly all the components of the ECM including, but not limited t,o fibrillar and nonfibrillar collagens, fibronectin, laminin and basement membrane proteoglycans.

(Ulasov, Yi, Guo, Sarvaiya, & Cobbs, 2014) as well as other non-matrix substrates implicated in tumour establishment. The family consists of more than 23 members, both secreted and membrane-anchored, that are synthesised as zymogens and many, furin-activatable. (Siemann, Dietmar, 2010) (Egeblad & Werb, 2002)

The MMPs play a significant role in the regulation of the tumour microenvironment. They are heavily involved in tissue remodelling, including specific physiological processes such as cell migration and proliferation. (Siemann, 2010) This involves the degradation of the ECM (the different MMPs being specific for varying substrates), and also the release of cytokines and growth factors from degraded basement membrane. Of the MMPs, MMP-2 and -9, as well as MT1-MMP, have been studied extensively due to their involvement in migration, invasion and metastasis (Siemann, Dietmar, 2010).

1.8 MT1-MMP

MT1-MMP is a transmembrane MMP, and the most extensively studied, that plays a major role in cell motility. Among its functions, it is often found at the leading edge of migrating and invading cells – along with its inhibitor TIMP2, MT1-MMP, in a multi-step process, is responsible for MMP-2 activation. (Sato et al., 1994, Ries et al., 2007) Together, these MMPs are able to target many substrates in the ECM, including cell-adhesion molecules such as αV integrin subunit precursor (MT1-MMP), lamininV (both) and CD44 (MT1-MMP) - both MMPs playing integral roles in angiogenesis and cell invasion. (Egeblad & Werb, 2002; Itoh, 2006) To summarize, as shown in figure 6, MT1-MMP's major role in ECM proteolysis is amplified by its ability to activate MMP-2 (as well as MMP-13). This is coupled with the processing and degradation of many cell-adhesion molecules, with the release of ECM fragments that promote both growth and migration. (Itoh, 2006)

MT1-MMP has been shown to play an integral role in angiogenesis, and is likely linked to its role in tumorigenesis where its upregulation is involved in the formation of MT1-MMP-VEGFR2-Src complexes that result in the activation of Akt and mTOR (Eisenach et al., 2010). However, adding to the complexity of its role is the result that in

MT1-MMP deficient mice, there is normal vascular formation. (Holmbeck et al., 1999) Whether the functions of MT1-MMP, during development, can be compensated by other systems, or that the function in vasulogensis of MT1-MMP become important following development in unknown.

There are several other biological functions of MT1-MMP that merit mention. These include MT1-MMP's modulation of the inflammatory response of macrophages. MT1-MMP can trigger the expression and activation of a phosphoinositide 3-kinase d (PI3Kd)/Akt/GSK3b signaling cascade which in turn, MT1- MMP-dependent PI3Kd activation regulates the immunoregulatory Mi-2/NuRD nucleosome remodeling complex that is responsible for controlling macrophage immune responses. (Shimizu-Hirota et al., 2012) MT1-MMP also regulates Notch signalling to maintain normal B-cell development in bone marrow, which occurs through the cleavagee of Notch ligand Delta-like 1, found on bone marrow stromal cells cell surface. (Jin et al., 2011)

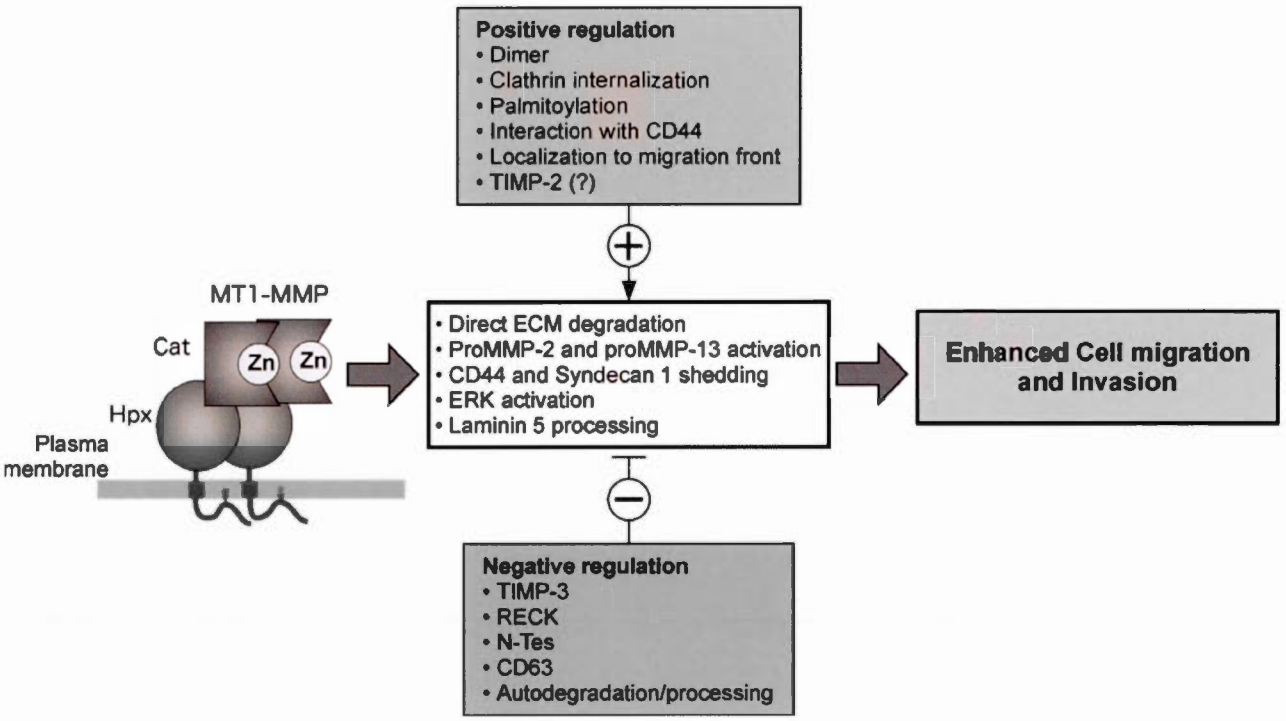

Figure 6 'Biological activities of MT1-MMP (MT1-MMP) and their regulation. MT1-MMP enhances cell migration and invasion by direct ECM degradation, activation of proMMP-2 and proMMP-13, CD44 and syndecan-1 shedding, ERK activation, and laminin 5 processing. These activities are positively (+) and negatively (-) regulated by a variety of processes. Disturbing one of the positive regulation processes may be enough to inhibit MT1-MMP-dependent cell migration'. (Itoh, 2006)

1.9 MT1-MMP Targets

As an MMP, MT1-MMP has a broad range of targets. In it's function of pericellular proteolysis of ECM macromolecules. MT1-MMP degrades Collagen I, II, II; Gelatin; Laminin I and IV; Fibronectin; Vitronectin; Aggrecan; Fibrin; Nidogen; Perlecan; Lumican. Furthermore, MT1-MMP is able to interact with cell surface proteins on nearby cells. Here, it is able interact with targets such as: CD44; Transglutaminase; LRP-1; Syndecan-1; Extracellular Signal Regulated Kinase (ERK) as well as VEGF. (Ulasov et al., 2014) Due to the plethora of possible interactions that MT1-MMP, and the

MMPs, in general, have, a developed and integrated knowledge of these functions will help better direct MMP inhibitor therapies that have, thus far, failed. (Egeblad & Werb, 2002)

1.10 LRP-1

LRP-1 is a member of the LDL receptor family of endocytic receptors (Figure 7) and is formed of two subunits, one extracellular of 515 kDa; one cytoplasmic of 85 kDa. The mature receptor is generated from its pro-form through cleavage of the 600 kDa peptide by Furin. (Willnow et al., 1996)

This family contains several functionally dynamic receptors involved in the cellular internalization of many circulating ligands. LRP-1 is found predominantly in the CNS and liver where it plays a significant role: specific for many ligands relevant to cholesterol homeostasis, it is also involved in the clearance of proteins, especially within the CNS. On binding to ligands, LRP-1 and its ligands undergo clathrin-mediated endocytosis, where the ligand is further processed or degraded in lysosomes – LRP-1 is then recycled back to the cell surface or to other intracellular compartments. (Nubile, 2007)

LRP-1 is one of three LDLRs that contain NPXY motifs – it has two within its intracellular 85 kDa signalling subunit. LRP-1 plays a role in intra-cellular signalling and has been found to be implicated in many essential functions (KO in mice being embryonically lethal). (A. P. Lillis, Van Duyn, Murphy-Ullrich, & Strickland, 2008) For example, LRP-1 plays an important role as a pro-survival signalling element; it has been implicated in varying forms of Alzheimer 's disease (having both positive and negative effects). (Nubile, 2007)

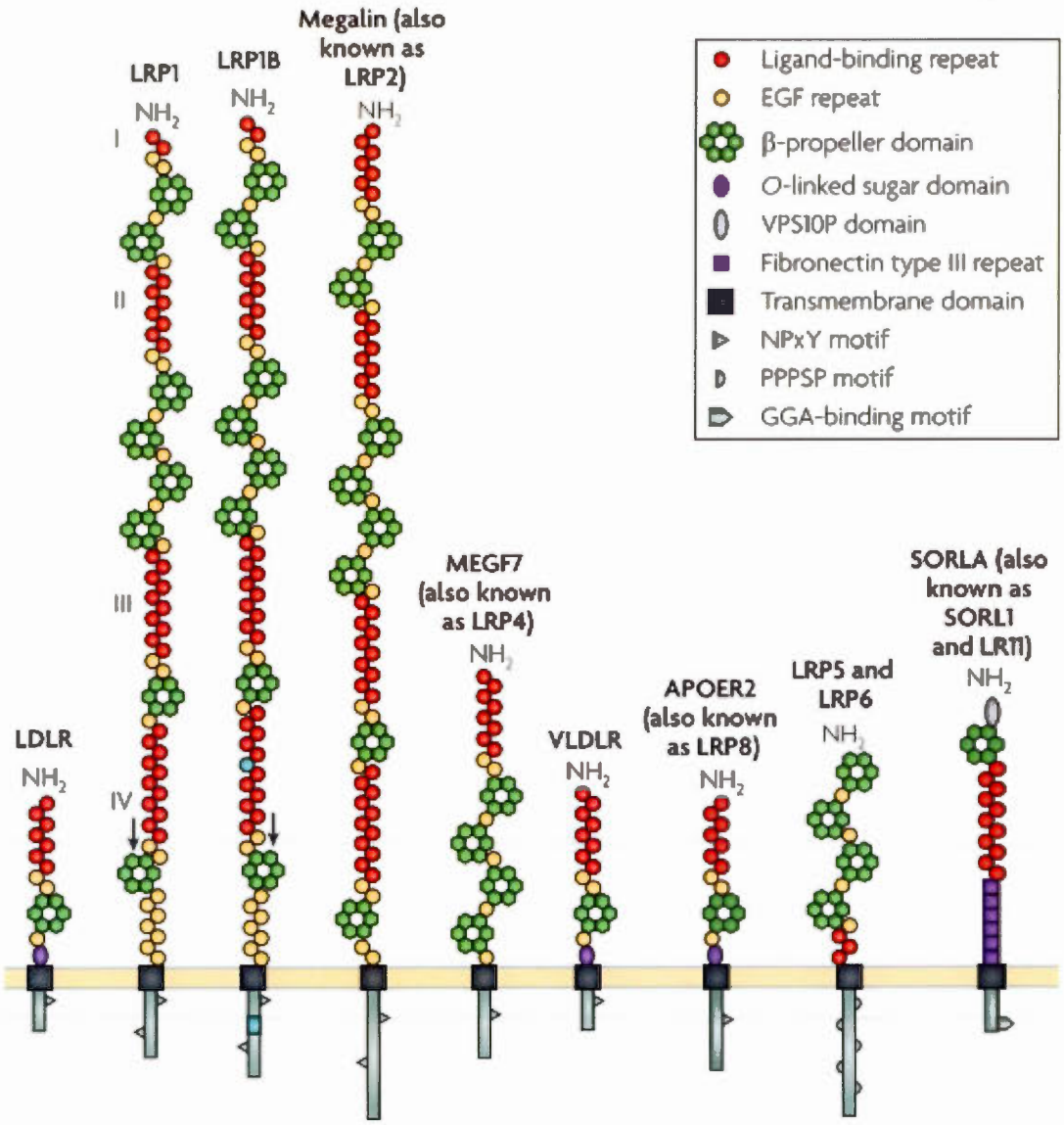

Figure 7 The LDLR family of receptors. 'The structural organization of the low-density lipoprotein receptor (LDLR) family members. All of the receptors are type I receptors that contain a single membrane-spanning domain and a relatively short cytoplasmic tail. The extracellular regions of these receptors contain three characteristic modules: ligand-binding repeats (also called complement-type repeats), epidermal growth factor (EGF) repeats and YWTD-containing β-propeller domains. The furin cleavage sites in LDLR-related protein 1 (LRP1) and LRP1B are indicated by arrows. The four clusters of ligand-binding repeats in LRP1 are labelled (I–IV). Highlighted in blue are the two extra sequences in LRP1B (compared with LRP1), which are encoded by two extra exons: a ligand-binding repeat in the fourth ligand-binding domain and a 33-amino acid insert in the cytoplasmic tail. LRP5, LRP6 and sortilin-related receptor with A-type repeats (SORLA; also known as SORL1 and LR11) are distant members of the family with atypical structural arrangements. Several other LDLR family members with poorly defined functions, including LRP3, LRP9 and LRP12'. (Bu, 2009)

In cancers, the literature suggests both positive and negative effects of LRP-1 status and function. Initially, it appeared that LRP-1 had protective properties in the malignant phenotype, namely due to it's ability to clear the extracellular compartment of proteases. LRP-1 expression has also been found to be downregulated in the most malignant gliomas. However, in the past decades, studies have emerged suggesting that LRP-1 does in fact play a significant role in the malignant phenotype of cancer cells.

It has been shown that LRP-1 is tethered to the actin network and focal adhesions sites; it was suggested that through this interaction, activating ERK signalling pathways and inhibiting JNK pathways, LRP-1 contributes to the cancer cell adhesive state, favouring invasion. Furthermore, it was demonstrated that LRP-1-dependent MAPK signalling contributes to cytoskeleton architecture organisation, and the mediation of adhesive complex turnover. (Langlois et al., 2010) An example of the LRP-1 paradox is, despite LRP-1's role in clearing extracellular proteases that it has been shown to be involved in the induction of both MMP-2 and 9, albeit in endometrial explants and not cancer cell models. (Selvais et al., 2009)

LRP-1 is involved in cell survival, proliferation and focal adhesion complex composition, and turnover (B Langlois, Emonard, Martiny, & Dedieu, 2009) – LRP-1 being found on the invasive front of invading cancer cells. Furthermore, novel interactions with the CD44 protein implicate LRP-1 in both its internalization and recycling, with LRP-1 / CD44 complexes being found at the migratory front of carcinoma cells. (Perrot et al., 2012) There are several studies demonstrating that LRP-1 blockade reduces the invasive phenotype of cancer cell models. In both carcrinoma and GBM cells LRP-1 silencing has shown to reduce cell invasion and migratory capacity, despite elevated levels of MMP-2 in the extracellular compartment. (Dedieu et al., 2008)

Another interesting function of LRP-1, which may have implications in the malignant phenotype, is its roles in cell survival: LRP-1, in primary neurones, was demonstrated to have an anti-apoptopic function where it is able to regulate the insulin receptor, as well as the Akt survival pathway (Fuentealba, Liu, Kanekiyo, Zhang, & Bu, 2009) (an affected target in ConA mediated cell death). LRP-1 also plays a significant role in the vascular system, where it is well studied. Recently a study has shown the potential of LRP-1 to contribute to the recruitment of monocytes to the tumour

compartment, that in turn are attributed with the ability to promote vascularisation of the tumour. (Staudt et al., 2013)

In all, LRP-1's roles are yet to be fully characterised and understood. However, it is evident that due to the plethora of roles it has, and can have, in a potentially environment / compartment specific manner, further understanding of its regulation have far-reaching implications. One can speculate that LRP-1 does play a role in the transition from benign to malignant tumour, and that it contributes to this phenotype in multiple ways.

1.11 Natural and Synthetic Ligands Of LRP-1; Vectorized Drug Delivery

As described previously, LRP-1 has many functions including lipoprotein metabolism, degradation of proteases, activation of lysosomal enzymes and cellular entry of bacterial toxins and viruses. This broad role is reflected in the number of ligands it binds, with LRP-1 being reported to bind up to 60 ligands, including: apolipoprotein E-enriched lipoproteins (chylomicron and VLDL remnants), α2Macroglobulin, uPA uPA/PAI-1, neuroserpin, neuroserpin/tPA complexes, MMP-9, MMP-13, MMP-2, HIV Tat protein, as well as several growth factors, to name a few. (Lillis, Mikhailenko, & Strickland, 2005)

Synthetic ligands of LRP-1 have been developed for the vectorized delivery of drugs. Angiopep-2 is a 19 amino acid peptide whose sequence is derived from the kunitz domain of aprotinin (and other peptides that are substrates for BBB transcytosis) that is selectively transported from the blood to the CNS compartment. It was developed with the aim of providing a delivery vehicle for pharmacological agents that are otherwise excluded from the CNS compartment by BBB selectivity. (Demeule et al., 2008) Angiopep-2 has been shown to be transcytosed and internalized in a LRP-1 dependent manner, a receptor that is significantly expressed in brain endothelial cells, as well as some brain tumours. Furthermore, it is not a substrate for the P-glycoprotein efflux pump, making it a highly attractive vehicle for molecules that are otherwise ejected from the CNS and tumour compartments. (Demeule et al., 2008) Chemotherapeutic conjugates of Angiopep-2 have been demonstrated, both *in vitro* and *in vivo,* to be as effective in killing

tumour cells - as well as showing improved BBB penetration and increased tumour penetration - as un-conjugated forms. This includes species conjugated to Doxorubicin, Nocodazole and Paclitaxel. (Bertrand et al., 2011; Ché et al., 2010) Angiopep conjugates are currently in clinical-phase trials, and appearing to be efficacious: the paclitaxel conjugate showing promising results for breast cancer patients with secondary brain metastases.

1.12 The Proprotein Convertase Furin

Furin is a ubiquitously expressed protein that mediates the proteolytic maturation, by cleavage, of proprotein substrates in the secretory pathway. Though not a substantial topic in this thesis, it is germane to discuss as both MT1-MMP and LRP-1, who both contain the consensus site that furin cleaves (positioned after the carboxy-terminal arginine residue in the sequence –Arg–X–Lys/Arg–Arg –), undergo Furin-mediated proteolytic maturation. On its discovery, Furin was thought to be a housekeeping protein, but has now been shown to have many roles and play an integral role in both normal physiology and pathology. Furin is a 794 amino acid peptide that resides predominantly in the trans-golgi network – its location being determined by signalling sequences in its cytoplasmic domain. The 83 amino acid pro-domain aids the peptide in its folding and activation. This process of maturation occurs in a similar way to that of Furin's actions on other proproteins – it is autoactivated in a compartment and pH specific manner, using its 'measure once, cut twice' rule. (Thomas, 2002) Furin is an essential protein in embryogenesis and homeostasis, as well as being implicated in some major pathologies. For example, Furin is the principal endoprotease for the 16 kDa β-nerve growth factor, a critical player in neuronal cell death / survival balance, as well as the transmembrane receptor Notch. Furin has also implications in neurodegenerative diseases such as Alzheimer's where it is involved in APP processing and the activation of both α- and β-secretase (members of the ADAM family of metaloproteiases). (Thomas, 2002) Furthermore, Furin has been implicated in several cancers: upregulated in GBMs as well as non-small cell lung carcinomas and squamous-cell carcinomas of the head and neck. (Mbikay et al., 1997) Furin is able to increase the malignancy of tumours through its ability to activate MT1-MMP, which, in turn, via MMP-2, increases degradation of the ECM (depicted in Figure 8). Furin, *in vitro,* has been targeted pharmacologically, where

its inhibition resulted in decreased cell motility and invasiveness in CHO and HT1080 cell lines. (Coppola et al., 2008)

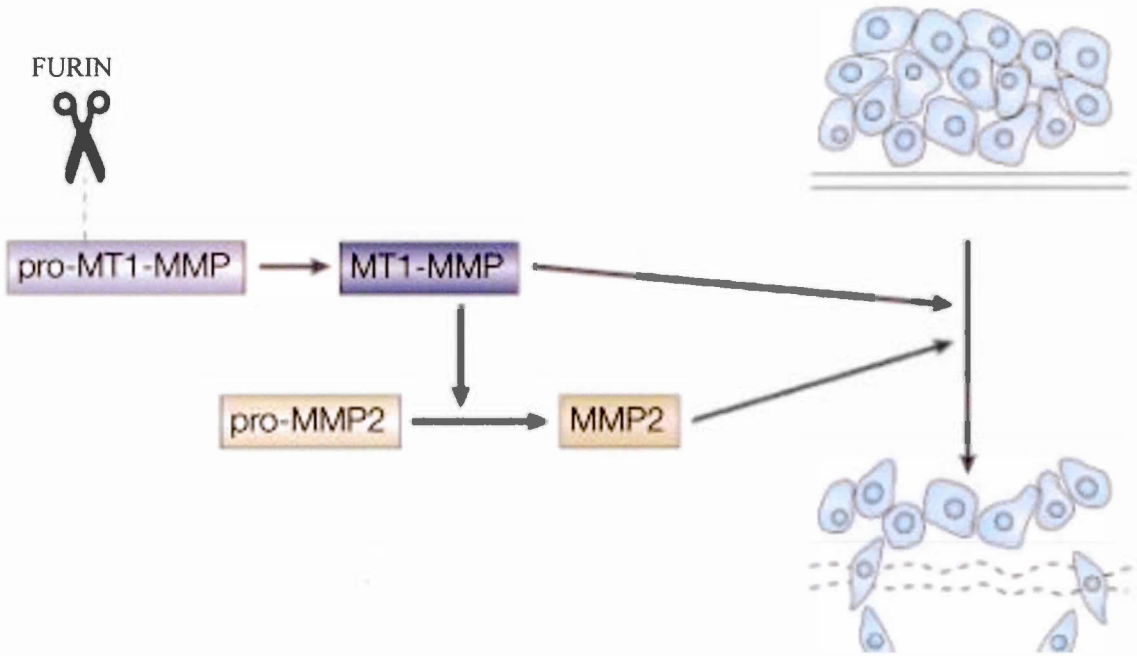

Figure 8 Furin (scissors in figure) is involved in the maturation of MT1-MMP, leading to a subsequent increase in MMP-2 activation, contributing to the metastatic phenotype of tumours. Adapted from. (Thomas, 2002)

1.13 Plant Lectins

Plant lectins are a family of Ca^{2+}/Mn^{2+} - dependent carbohydrate binding proteins that are able to bind selectively and reversibly to free sugars on glycoproteins and glycolipids. (Vijayan & Chandra, 1999) The specificity of the binding has lead to the classification of the lectins into 12 families. Over the past 20 years the lectins have been used to label and identify malignant vs benign tumours, evaluating the glycosylation state of malignancies (Mody, Joshi, & Chaney, 1995) and recently, their introduction into microarrays for high throughput analysis of protein glycosylation. (Z. Liu, Luo, Zhou, & Zhang, 2013) Furthermore, increased interest has been placed in the anti-tumour properties exhibited by these lectins. For example, they have apoptopic and autophagic inducing properties (the programmed cell death signalling network illustrated in figure 9)

, (Zhang, Chen, Ouyang, Cheng, & Liu, 2012) as well as anti-angiogenic. (Li, Yu, Xu, & Bao, 2011)(Z. Liu et al., 2013)

There is a substantial volume of literature reporting the multiple effects of the plant lectins on cancers throughout the tissues of the body. This includes several reports on the induction of apoptopic cell death induced by the lectins ConA, Polygonatum cyrtonema lectin and Mistletoe lectins.

1.14 Concanavalin A

ConA has been reported to induce both the extrinsic (Fas family of death receports dependent) and the intrinsic (mitochondrial dependent) pathways of apoptopic cell death. (Z. Liu et al., 2013) For example, apoptosis via the intrinsic pathway has been reported in both human melanoma A375 cells (B. Liu et al., 2009) and hepatocellular carcinoma HepG2 cells; (Zhongyu Liu, Li, Ding, & Yang, 2010). Furthermore, in U87 GBM cells, it has been shown to up-regulate COX-2 expression, as well as down-regulate Akt expression via IKK/NF_kB-dependent pathways. (Pratt et al., 2012) ConA induces apoptosis by inhibiting the Akt survival pathways as well as activating Fox01a-Bim signalling in both ovarian and Li-Fraumeni syndrome cells. (Z. Liu et al., 2013)

ConA has also been shown to induce autophagic cell death via a BCL-2/adenovirus EIB 19kDa-interacting protein 3 (BNIP-3) – mediated pathway. (Lei & Chang, 2009a) The mechanisms of ConA-induced cell death are varied (ConA's mutli-faceted effects resulting from various interactions, and modulations of a host of pathways). (Li et al., 2011) One mechanism includes the association of ConA with mannose moieties at the plasma membrane, clathrin-dependent internalization to mitochondria and the initiation of autophagic cell death. (Lei & Chang, 2009b)

Furthermore, autophagy induced by ConA can be abrogated through the silencing of MT1-MMP; though not by catalytic inhibition. (Pratt et al., 2012) This suggests that MT1-MMP-mediated ConA-induced autophagy is signalled, in the case of MT1-MMP, via the non-catalytic, cytoplasmic domain. (Pratt et al., 2012) Another interesting property of the plant lectins, especially of ConA, is the ability to perturb the cytoskeleton. (Vijayan & Chandra, 1999)

Interestingly, several of the cellular effects induced by Concanavalin A (ConA) are mediated, in part via MT1-MMP. This includes MT1-MMP-mediated-MMP-2 activation; COX-2 induction, independent of the MT1-MMP catalytic domain; MT1-MMP activation and transcription; the induction of autophagy biomarkers via MT1-MMP's cytoplasmic signalling domain. (Akla, Pratt, & Annabi, 2012; Annabi et al., 2009; Annabi et al., 2014; Pratt, Roy, & Annabi, 2012; Sina et al., 2010) Furthermore, of interest, is the ability of MT1-MMP to cleave LRP-1 in malignant cells – MT1-MMP acting as a 'sheddase', leading to the N-terminal being shed into the extracellular milieu. (Rozanov et al., 2004)

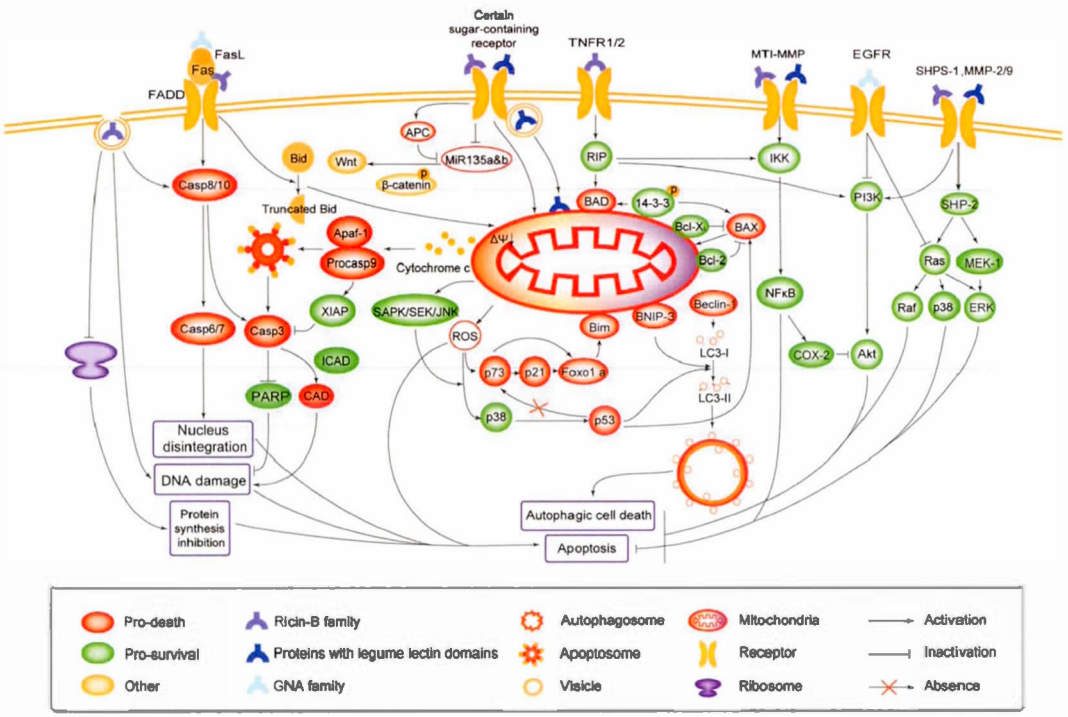

Figure 9 The programmed cell death (PCD) signalling network which can be regulated by plant lectins, especially ConA. In particular, the pathways regulated by the fas family of death receptors and MT1-MMP. Modified from (Fu et al., 2011)

2 Hypothesis

LRP-1 is a crucial player in GBM biology and a target for GBM treatments (as well as other CNS disorders). It also plays an integral role in both physiology, and the pathology of several diseases. As it has begun to be studied in more depth, reports are emerging showing a direct link to the cytoskeleton. Given this, further understanding of its regulation is certainly pertinent for our collective understanding. Within our group, MT1-MMP has been extensively studied, and has been shown in several other groups to be able to regulate LRP-1 expression. The plant lectin ConA presents as an entity with therapeutic potential, but its ability to induce the activation and transcription of MT1-MMP (alongside other well characterised effects, such as its ability to disrupts the ctyoskeleton), presents it as a useful tool to explore and investigate several potential cellular events that may impact on LRP-1. Thus, whether ConA is able to modulate LRP-1 in GBM cells, and affect the internalization of LRP-1 ligands, is of interest. Furthermore, given MT1-MMP's role in the GBM phenotype (as well as other malignancies), further probing its effects, linked to the malignant processes that can be modelled with ConA, will contribute to our understanding of the phenotypic transformation essential for transition to malignancy.

Hypothesis: Treating U87 cells with ConA at a concentration able to induce MT1-MMP activation will lead to MT1-MMP mediated proteolytic processing of LRP-1. The processing of LRP-1 will in turn lead to a reduced capacity of LRP-1-ligand internalization.

2.1 Aims and Objectives

- Further characterise ConA mediated effects in U87 cells – specifically MT1-MMP processing and MT1-MMP mediated events
- Explore the role of ConA and its effects on MT1-MMP in LRP-1-dependent vectorized drug delivery

Chapter II

3 Results

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Impact of Concanavalin-A-mediated cytoskeleton disruption on low-density lipoprotein receptor-related protein-1 internalization and cell surface expression in glioblastomas

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Running title : LRP-1 expression is decreased upon cytoskeleton disruption

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Keywords: Glioblastoma, Brain cancer, LRP-1, Concanavalin-A, Cytoskeleton

3.1 Summary

The low-density lipoprotein receptor-related protein 1 (LRP-1) is a multiligand endocytic receptor which plays a pivotal role in controlling cytoskeleton dynamics during cancer cell migration. Its rapid endocytosis further allows efficient clearance of extracellular ligands. Concanavalin-A (ConA) is a lectin used to trigger *in vitro* physiological cellular processes including cytokines secretion, nitric oxide production, and T lymphocytes activation. Given that ConA exerts part of its effects through cytoskeleton remodeling, we questioned whether it affected LRP-1 expression, intracellular trafficking, and cell surface function in grade IV U87 glioblastoma cells. Using flow cytometry and confocal microscopy, we found that loss of the cell surface

600-kDa mature form of LRP-1 occurs upon ConA treatment. Consequently, internalization of the physiological □2-Macroglobulin and of the synthetic Angiopep-2 ligands of LRP-1 was also decreased. Silencing of known mediators of ConA, such as the membrane type-1 matrix metalloproteinase, and the Toll-like receptors (TLR)-2 and TLR-6, was unable to rescue ConA-mediated LRP-1 expression decrease, implying that the loss of LRP-1 was independent of cell surface relayed signaling. The ConA-mediated reduction in LRP-1 expression was emulated by the actin-cytoskeleton disrupting agent Cytochalasin-D, but not by the microtubule inhibitor Nocodazole, and required both lysosomal- and ubiquitin-proteasome system-mediated degradation. Our study implies that actin cytoskeleton integrity is required for proper LRP-1 cell surface functions, and that impaired trafficking leads to specialized compartmentation and degradation. Our data also strengthen the biomarker role of cell surface LRP-1 functions in the vectorized transport of therapeutic Angiopep bioconjugates into brain cancer cells.

3.2 Introduction

Low density lipoprotein receptor-related protein 1 (LRP-1) is a member of the LDL receptor family of endocytic receptors formed of one extracellular 515 kDa subunit, and one cytoplasmic 85 kDa subunit;[1] the mature receptor having been generated by the cleavage of a 600 kDa propeptide by Furin.[2] The LRP family contains several functionally dynamic receptors involved in the cellular internalization of more than 40 circulating physiological ligands, including apolipoprotein E[3], α2-Macroglobulin[4], factor VIII[5], lipoproteins[6] and Amyloid-β.[7] Once ligand is bound, the LRP-1/ligand complex undergoes clathrin-mediated endocytosis in order that the ligand be further targeted within specialized intracellular compartments.[8] Disruption of the LRP-1 gene in mice was found to be embryonically lethal, presumably because LRP-1 transduces intracellular signalling and is involved in many essential functions.[9]

Over the past few decades, it has emerged that LRP-1 plays a significant role in the malignant phenotype of brain cancer cells, where it is tethered to the actin network and focal adhesion sites.[10] Through LRP1-dependent actin network remodeling, the activating ERK and inhibiting JNK signalling pathways contribute to the adhesive states of cancer cells which favor invasion.[11] Intriguingly, both positive and negative effects of the LRP-1 status and function have been reported for cancer cells. LRP-1 displayed protective

properties in the CNS malignant phenotype, due to its ability to clear the extracellular compartment of proteases.[12] The status of LRP-1 expression was also assessed in human glioma cell lines[13], in *in vivo* glioblastomas[14], and was found to be particularly elevated in U87 glioblastoma cells[15] as well as CD133+ pediatric brain tumor cells.[4] Several studies have also demonstrated that LRP-1 blockade reduced the invasive phenotype in numerous cancer cell models.[16] In glioblastoma cells, LRP-1 silencing reduced cell invasion and migration abilities, despite elevated levels of MMP-2 in the extracellular compartment.[16] Furthermore, its cell surface interactions with the CD44 protein implicated LRP-1 in both internalization and recycling, with LRP-1/CD44 complexes being found at the migratory front of carcinoma cells.[17] This association of LRP-1 compartmentation at the leading edge of migrating/invading cancer cells is relevant for its role in brain tumor development, and understanding of its cell surface expression will be crucial for the development of future therapeutic strategies. Interestingly, both LRP-1 and CD44 are cleaved by MT1-MMP,[18,19] a transmembrane matrix metalloproteinase that plays a fundamental role in cell motility.[20] Regulation of the invasive phenotype of glioma cells involving a MT1-MMP/CD44/Caveolin-1 interaction has been described[21,22] through, in part, its rapid trafficking/recycling to the plasma membrane from trans-Golgi network/endosome storage compartments.[23]

Recently, the ligand internalization functions and recycling of LRP-1 to the cell surface have been exploited for the vectorized transport of synthetic cargo peptides, termed Angiopep, through the blood-brain barrier (BBB) and to the brain.[24,25] This successful strategy led to the design of receptor-mediated internalization strategies through high brain permeable anticancer drugs such as paclitaxel-Angiopep bioconjugates to gliomas.[26-29] How cytoskeletal remodeling alters LRP-1 cell surface availability and functions in ligand internalization have not yet been explored. Here, we used Concanavalin-A (ConA), a lectin regulating MT1-MMP cell surface proteolytic functions[30,31] as well as MT1-MMP catalytic independent inflammation and autophagy cell signaling,[32,33] to trigger molecular alterations of the cytoskeleton[34,35] and assessed its impact on LRP-1 ligand internalization functions.

3.3 Experimental Procedures

Materials : Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). HyGLO chemiluminescent HRP antibody detection reagents

were from Denville Scientific Inc. (Rockford, IL). Micro bicinchoninic acid protein assay reagents were from Pierce (USA). The MMP inhibitor Ilomastat and the anti-LRP-1 light chain mAb (5A6) were purchased from EMD Millipore (Etobicoke, ON). Angiopep-2 and α2-Macroglobulin were gifts from Angiochem Inc (Montreal, QC). The antibody against murine LRP Heavy Chain (8G1) was from Calbiochem (San Diego, CA), the anti-COX-2 antibody (610203) was from BD Biosciences (San Jose, CA), and the anti-GAPDH (Ab8245) and anti-Ubiquitin (Ab7780) antibodies were from Abcam (Toronto, ON). The R-phycoerythrin (PE)-conjugated mouse antibodies against human CD91 and IgG1 κ Isotype were from BD Biosciences (Mississauga, ON). Horseradish peroxidase-conjugated donkey anti-rabbit and anti-mouse IgG secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). The anti-MT1-MMP hinge region antibody (M3927), Concanavalin-A, Cytochalasin-D, Nocodazole, Furin inhibitor II, Tofacitinib, SB203580, PP2, U0126, Acetyl-11-keto-beta-boswellic acid, sodium dodecylsulfate (SDS) and bovine serum albumin (BSA) were from Sigma-Aldrich (Oakville, ON).

Cell culture : The human U87 glioblastoma cell line (American Type Culture Collection, HTB-14) was maintained in Eagle's Minimum Essential Medium (EMEM, Wisent, 320-006CL) containing 10% (v/v) calf serum (HyClone Laboratories, SH30541.03), 1 mM sodium pyruvate (Sigma-Aldrich Canada, P2256), 100 units/ml penicillin and 100 mg/ml streptomycin (Wisent, 250-202-EL). Cells were incubated at 37°C with 95% air and 5% CO_2.

Total RNA isolation, cDNA synthesis and real-time quantitative RT-PCR : Total RNA was extracted from cell monolayers using TriZol reagent (Life Technologies, 15596-018). For cDNA synthesis, 2 μg of total RNA were reverse-transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, 4368814). cDNA was stored at -80°C prior to PCR. Gene expression was quantified by real-time quantitative PCR using iQ Sso Fast EvaGreen Supermix (Bio-Rad). DNA amplification was carried out using a CFX connect

Real-Time System (Bio-Rad) and product detection was performed by measuring binding of the fluorescent dye EvaGreen to double-stranded DNA. The QuantiTect primer sets were provided by QIAGEN: LRP1 (Hs_LRP1_1_SG QT00025536), MT1-MMP (Hs_Mmp14_1_SG QT00001533), TLR-2 (Hs_TLR2_1_SG, QT00236131), TLR-6 (Hs_TLR6_1_SG, QT00216272), GAPDH (Hs_GAPDH_2_SG QT01192646) and β-actin (Hs_Actb_2_SG QT01680476). The relative quantities of target gene mRNA compared against two internal controls, GAPDH and β-actin RNA, were measured by following a ΔC_T method employing an amplification plot (fluorescence signal vs. cycle number). The difference (ΔC_T) between the mean values in the triplicate samples of target gene and those of GAPDH and β-actin mRNAs were calculated by the CFX manager Software version 2.1 (Bio-Rad) and the relative quantified value (RQV) was expressed as $2^{-\Delta C}{}_T$.

Transfection method and RNA interference : Cells were transiently transfected with 10 nM siRNA against MT1-MMP (Hs_MMP14_6 HP validated siRNA; QIAGEN SI03648841), TLR-2 (HS_TLR2_1_SG QIAGEN QT00236131), TLR-6 (HSTLR6_1_SG QIAGEN QT00216272), or scrambled sequences (AllStars Negative Control siRNA; QIAGEN, 1027281) using Lipofectamine 2000 (Invitrogen, 11668). Every specific gene knockdown was evaluated by qRT-PCR as described above.

Gelatin zymography : Gelatin zymography was used to assess the extracellular levels of proMMP-2 and MMP-2 activities. Briefly, an aliquot (20 µl) of the culture medium was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a gel containing 0.1 mg/ml gelatin (Sigma-Aldrich Canada, G2625). The gels were then incubated in 2.5% Triton X-100 (Bioshop, TRX506.500) and rinsed in deionized distilled water. Gels were further incubated at 37°C for 20 hours in 20 mM NaCl, 5 mM CaCl2, 0.02% Brij-35, 50 mM Tris–HCl buffer, pH 7.6 and then stained with 0.1% Coomassie Brilliant blue R-250 (Bioshop,

CBB250) and destained in 10% acetic acid, 30% methanol in water. Gelatinolytic activity was detected as unstained bands on a blue background.

Immunoblotting procedures : Following treatments or transfection, U87 cells were washed with PBS and lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.1% Triton) in the presence of phosphatase and protease inhibitors on ice for 30 minutes. Cell debris was pelleted by centrifugation for 10 min at high speed. Protein concentration was quantified using a micro bicinchoninic acid protein assay kit (Thermo Fisher Scientific Inc). Proteins (30 □g) from control and treated cells were separated by SDS-PAGE. After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes which were then blocked for 1 hour at room temperature with 5% non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween-20 (TBST; Bioshop, TWN510-500). Membranes were further washed in TBST and incubated with the indicated primary antibodies (1/1,000 dilution) in TBST containing 3% bovine serum albumin and 0.1% sodium azide (Sigma-Aldrich Canada, S2002), followed by a 1 hour incubation with horseradish peroxidase-conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories, 711-035-152) or goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, 115-035-062) at 1/2,500 dilutions in TBST containing 5% non-fat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, RPN3004, Baie d'Urfé, QC).

Binding and uptake assays of Angiopep-2 and α2-Macroglobulin : Cells were incubated with 250 nM Alexa^{488}-α2-Macroglobulin / Ringer HEPES or Alexa^{488}-Angiopep-2 / Ringer-HEPES or Ringer-HEPES alone for 1 hour at 37°C in the dark and washed 3 times with PBS / BSA (5%) / EDTA (2 nM). Fluorescence was then measured in the FL1-A channel using a C6 Accuri flow cytometer (BD Biosciences, Mississauga, ON).

Confocal fluorescent microscopy assays of Angiopep-2 uptake and LRP-1 expression : Cells were incubated with 50 nM Alexa^{488}-Angiopep-2 in EMEM without phenol red for 18 hours at 37°C, 5% CO_2. Cells were fixed in 4% formaldehyde (Fisher Scientific, Ottawa, ON) for 20 minutes. Immunostaining was performed under non-permeabilizing conditions for 1 hour with the anti-LRP Heavy Chain antibody (2 µg/ml) in 1% BSA / PBS / NaN_3, followed by Rhodamine Red-X donkey anti-mouse IgG (Invitrogen, Burlington, ON). A solution of 10 µg/ml DAPI diluted in PBS was used to stain the nuclei. Fluorescence was then monitored by confocal microscopy using a Nikon Eclipse Ti confocal microscope and NIS Elements software.

LRP-1 cell surface immunophenotyping : Cells were collected and re-suspended in a solution of binding buffer. Then, cells were incubated with either a PE mouse α-human CD91 or a mouse IgG1 κ Isotype control antibody for 1 hour at room temperature in the dark. Cells were washed 3 times with PBS. Fluorescence was then examined by flow cytometry in the FL2-A channel with a C6 Accuri. The results obtained were quantified as the difference between the geometric means of LRP-1-PE and PE isotype control antibodies.

Fluorescent microscopy : U87 cells were seeded onto coverslips where they were treated with either Concanavalin-A, Cytochalasin-D or Nocodazole. Subsequently, cells were either incubated and/or immunolabelled followed by fixation. Images were acquired using a Nikon Eclipse Ti confocal microscope and NIS Elements software. Images were then deconvoluted and, where indicated, colocalisation analysis was carried out with AutoQuant X software.

Statistical data analysis : Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired t-test. Probability values of less than 0.05 were considered significant and an asterisk (*) identifies such significance in the figures.

3.4 Results

Cytoskeleton remodelling alters LRP-1 trafficking and cell surface expression. The lectin Concanavalin-A (ConA) was used to investigate whether cytoskeleton remodelling affected LRP-1 trafficking and recycling process to the cell surface in U87 glioblastoma cells. While untreated cells expressed basal levels of MT1-MMP and of the 85-kDa LRP-1 subunit, ConA-activated U87 cells resulted in a loss of the 85-kDa transmembrane LRP-1 subunit from whole cell lysates upon a 24 hours dose-response with ConA (Fig.1A). As expected, ConA treatment also led to the induction of cyclooxygenase (COX)-2 expression[31,32] and was correlated with both MT1-MMP proteolytic processing into its 43-kDa species, and with proMMP-2 activation (Fig.1A).[30,31,36] Densitometric analysis of immunoblots showed that LRP-1 decrease in expression correlated with the ConA-mediated effects on COX-2 induction and MT1-MMP activation (Fig.1B). Following this, a time-course assay was performed with various concentrations of ConA. A loss in LRP-1 expression occured within 30 minutes of treatment with ConA (Fig.1C, upper panel), and LRP-1 expression was completely abolished from lysates by 12 hours (Fig.1C, lower panel). Having established that the LRP-1 expression declined in cell lysates, we questioned whether a mature 600-kDa LRP-1 still trafficked and compartmentalized at the cell surface. We performed immunophenotyping by flow cytometry to assess the rate at which LRP-1 was being internalized from and recycled to the cell surface. U87 cells were incubated for the indicated times with ConA and non-permeabilized cells immunomarked as described in the Methods section. Following 15 minutes of ConA treatment, cell surface LRP-1 had decreased to 25% of that seen with the control treatment, and to 10% of control at 2 hours of treatment (Fig.1D, closed circles). LRP-1 gene expression, as assessed by qRT-PCR, was unaltered (Fig.1D, open circles). Altogether, the combined data leads us to hypothesize that mis-trafficking of LRP-1 to the cell surface possibly leads to its compartmentalized degradation.

LRP-1 decrease is independent of MT1-MMP catalytic activity but correlates with ConA-mediated MT1-MMP proteolytic processing. To explore the link between ConA and LRP-1, we next examined whether MT1-MMP was involved in mediating the degradation of LRP-1 in ConA-activated U87 cells. ConA is classically used to trigger MT1-MMP-mediated activation of proMMP-2, which is further reflected through MT1-MMP proteolytic activation.[31,32] The broad acting MMP catalytic inhibitor Ilomastat was

thus used and validated through the inhibition of ConA-mediated proMMP-2 activation. Neither proMMP-2 activation nor generation of the proteolytically processed MT1-MMP 43-kDa species were observed in Ilomastat-treated cells (Fig.2A). This effect was emulated by the efficient silencing of MT1-MMP using RNA interference (Fig.2B), followed by treatment with ConA (Fig.2C). Furthermore, we tested whether Furin-mediated LRP-1 maturation to the cell surface was involved. We found that inhibition of the proprotein convertase Furin, which is implicated in the maturation of LRP-1 into its 85-kDa and 515-kDa subunits, did not prevent the decrease in the 85-kDa LRP-1 subunit expression, though partially inhibiting MT1-MMP proteolytic processing into its 43-kDa species (Fig.2A). This observation potentially suggests that, upon cytoskeleton disruption, a pool of intracellular LRP-1 protein trafficking is rather directed towards degradation than recycled to the cell surface.

LRP-1 loss is mediated by both the ubiquitin-dependent proteasome system and by lysosomes in ConA-activated U87 cells. As LRP-1 has been reported to be degraded by the ubiquitin-dependent proteasome system (UPS) and within lysosomes,[37] we next examined whether either of these systems was active in ConA-activated U87 cells. As almost complete disappearance of LRP-1 from cell lysates was observed within 6 hours of treatment, we chose to treat U87 cells for this duration with various doses of UPS inhibitors, MG132 or Lactacystin, in the presence or absence of ConA. Inhibition of the UPS was confirmed by immunodetection of ubiquitin, and a significant rescue of LRP-1 was observed (Fig.3A). Treatment with epigallocatechin 3-gallate (EGCG), a green tea catechin known to inhibit the UPS,[38] was unable to rescue the ConA-mediated LRP-1 decrease (Fig.3B). To assess whether lysosomal degradation was involved, we treated coverslip-seeded U87 cells with ConA (30 μg/ml) for 0, 2 and 6 hours, after which we labelled both lysosomes and LRP-1 in order to evaluate whether these were colocalized. We found that, within 2 hours of treatment, significant colocalization between LRP-1 and lysosomes occurred, suggesting that the loss of LRP-1 is performed within this compartment (Fig.3C).

LRP-1 decreases in ConA-activated U87 cells is independent from ConA-mediated cell signalling. Having ruled out MT1-MMP as a mediator of the observed ConA effects (Fig.2), we next tested whether ConA receptors TLR-2 and TLR-6 were involved. Blocking TLR-2 *in vivo* was shown to attenuate experimental hepatitis induced by ConA in mice.[39] In parallel, TLR-6 gene silencing was found to abrogate ConA-induced CSF-2

and CSF-3 transcriptional regulation.[40] RNA interference allowed us to silence the expression of TLR-2 and TLR-6, which was followed by a 6 hour treatment with ConA, this did not abrogate the ConA-induced LRP-1 degradation (Fig.4A). We next inhibited several intracellular signalling pathways which could play a role in ConA signalling. However, despite some modulation observed in LRP-1 immunodetections, it appeared that this rapid regulation and almost complete loss in LRP-1 was independent from the aforementioned mechanisms (Fig.4B).

Altered LRP-1 trafficking is triggered upon ConA-mediated alterations in actin cytoskeleton integrity. Having been unable to identify receptor-mediated signalling pathway linking ConA to the internalization and degradation of LRP-1, we explored ConA's ability to rather directly disrupt the cytoskeleton as a cause of the observed effects. In order to compare ConA to other cytoskeleton-disrupting agents, U87 cells were treated with Cytochalasin-D (CytoD), an inhibitor of actin polymerization, and Nocodazole, an inhibitor of microtubule polymerization. We found that CytoD, in contrast to Nocodazole, emulated the effects caused by ConA in cell lysates (Fig.5A). The internalization of LRP-1 from the cell surface, as measured by flow cytometry, was again also emulated by CytoD but not by Nocodazole (Fig.5B). An immunofluorescent assay of CytoD- and Nocodazole-treated U87 cells failed to show colocalization between LRP-1 and lysosomes, although a decrease in LRP-1 fluorescence was observed by microscopy (Fig.5C) when cells were treated with CytoD, but not with Nocodazole (Fig.5D). This suggests that, although common actin cytoskeleton perturbations are involved between the CytoD and ConA actions, differential compartmentation processes regulate ConA-mediated LRP-1 decreased expression. Furthermore, one can also hypothesize that LRP-1 incapacity to recycle back to the cell surface triggers its intracellular proteolytic degradation.

Reduced uptake of LRP-1 ligands results from LRP-1 loss from the cell surface. Having established a rapid loss of LRP-1 from the cell surface, we next questioned whether this would lead to any ligand-mediated internalization functional impairment. Thus, we carried out cellular uptake assays with physiological and synthetic LRP-1 ligands in ConA-activated cells. Following 5 minutes of incubation with ConA, the cellular uptakes of both Alexa^{488}-Angiopep-2 and Alexa^{488}-α2-Macroglobulin decreased by ~60% (Fig.6A). To assess whether the observed degradation of cell surface LRP-1 correlated with the increasing concentrations of ConA, we carried out an uptake assay

with Alexa^{488}-Angiopep-2 following 2 hours of ConA treatment (Fig.6B). We observed a significant decrease in uptake by up to ~60% when treated with 30 μg/ml ConA. To further support our findings, we treated U87 cells with varying concentrations of ConA for 2 hours, after which we incubated the cells with Alexa^{488}-Angiopep-2 and labelled cell surface LRP-1 (Fig.6C). Quantification of these markers showed that the uptake of Alexa^{488}-Angiopep-2 correlated directly with LRP-1 cell surface expression, whereas high cell surface LRP-1 status resulted in high Angiopep-2 internalization (Fig.6D).

3.5 Discussion

In the current study, we questioned whether cytoskeleton remodeling, as it dynamically occurs in invading cells, altered endocytic processes such as those that regulate LRP-1 recycling and cell surface availability. We found that LRP-1 rapidly exited the cell surface of ConA-activated U87 glioblastoma cells, a phenomenon that was unrelated to MT1-MMP's catalytic activity. However, LRP-1 decreases correlated with ConA-induced MT1-MMP intracellular proteolytic activation and with induction of the inflammation biomarker COX-2 expression. Consequently, decrease in cell surface LRP-1 resulted in diminished binding to its physiological □2-Macroglobulin or synthetic Angiopep-2 ligands. Our study implies that cytoskeleton integrity is required for LRP-1 expression and recycling to the plasma membrane, and strengthens the pivotal requirement of cell surface LRP-1 functions in the vectorized transport of therapeutic Angiopep bioconjugates into brain cancer cells.

Although the intracellular compartments involved in endocytic recycling processes remain to be well-defined, LRP-1's tethering to the actin cytoskeleton and focal adhesion sites is an aspect of our study which confirms its absolute requirement for activated □2-Macroglobulin or Angiopep-2 internalization processes to efficiently occur. Further, we demonstrate that LRP-1's expression and function can be significantly regulated upon ConA-mediated cytoskeleton reorganization which mimics the migrating/invading cell phenotype.[11,41] As a consequence, the mis-trafficking of endosomal LRP-1 proteins may indeed affect cell migration, a process that is essential for development, tissue remodeling, and wound healing, as well as LRP-1 ligand recycling functions altered in many abnormal pathological states.[42,43] To migrate directionally, cells indeed require to coordinate temporal and spatial cytoskeleton rearrangements through actin

polymerization and focal adhesion turnover in order to generate the forces required for directional movement and cell surface protein availability.[44] Among the processes which could regulate LRP-1 cell surface availability, the recycling endosome is an organelle in the endocytic pathway where plasma membrane proteins are internalized by endocytosis and processed back to the cell surface for reuse. This allows the cell to maintain constituents of the plasma membrane on cell surfaces.[45] Evidence using Brefeldin-A, a vesicular trafficking inhibitor that trapped MT1-MMP within the cell, demonstrated similar induction of endoplasmic reticulum (ER) stress than ConA.[31] While Nocodazole was ineffective, CytoD, a potent inhibitor of actin polymerization, reduced LRP-1 expression to a level similar to that seen with ConA. Possible non-specific effects of CytoD, which may alter various other processes within the cell, must here be acknowledged. The use of Latrunculin B, a specific inhibitor of cytoskeleton polymerization,[46] may ultimately help in demonstrating LRP1 degradation is indeed mediated by cytoskeletal disruption. This is now mentioned in the Discussion section of the revised manuscript. Interestingly, silencing of MT1-MMP prevented ConA from inducing both ER stress and COX-2 expression, but was unable to prevent ConA-mediated LRP-1 attenuation (this study). Inhibition of furin-dependent MT1-MMP proteolytic processing by ConA was also ineffective at reversing the LRP-1 decrease in our hands, although a potential mature ~600 kDa LRP-1 unprocessed form may have been expected to be observed and required to be further investigated.

The use pharmacological endocytosis and lysosomes inhibitors, as well as proteasome inhibitors (as performed in Fig.3 with the use of MG132 and Lactacystin), may in the future studies help in our data interpretation regarding LRP-1 recycling processes. Of the recent molecular players demonstrated to link vesicular trafficking processes to cancer cell migration and invasiveness, the functions of the Rab family of small GTPases in regulating vesicular transport has raised intriguing mechanistic insights.[47] For instance, a Rab11-dependent recycling pathway was reported to regulate α2-Macroglobulin/LRP1-induced cellular migration of Müller glial cells by a mechanism that involved MT1-MMP intracellular trafficking to the plasma membrane.[48] Furthermore, a specific subset of RabGTPases was found to control cell surface exposure of MT1-MMP, ECM degradation and three-dimensional invasion of macrophages.[49] Whether any of these Rab proteins are also involved in LRP-1 internalization is unknown. Such evidence, however, points to the existence of possible crosstalk between these processes. LRP-1 internalization and

recycling back at the cell surface from early endosomes is a rapid (0.5 minutes) and high capacitive process,[50] and it becomes reasonable to hypothesize that disruption of these networks may rather lead to compartmentalized degradation of LRP-1 possibly by the UPS and via lysosome. Interestingly, EGCG, a green tea catechin known to inhibit several ConA- and MT1-MMP-mediated processes,[22,40,51] and which also inhibited some Ubiquitin-Proteasome properties,[52] had no effect on the ConA-mediated LRP-1 decrease. Given that neither TLR-2 silencing, TLR-6 silencing nor inhibition of multiple signalling pathways resulted in any reversal of LRP-1 decrease, we conclude that LRP-1 decreases are unrelated to any cell surface ConA-induced signaling pathway but rather caused through ConA's capacity to alter cytoskeleton integrity and, hence, trafficking/recycling processes.

Finally, ConA is a plant lectin that has also been used for its properties in inducing a plethora of cellular events including cell proliferation and cell death/survival, as well as molecular biomarkers expression such as cytokines secretion,[40,53] nitric oxide synthesis,[54] inflammatory COX-2 expression,[30-32,36] autophagy BNIP-3 expression,[33,55] and activation and transcription of MT1-MMP.[56,57] ConA has thus begun to be explored as a potential therapeutic entity due to its ability to induce both autophagy and apoptosis in human cancer cell models including hepatoma, glioblastoma, melanoma, and breast cancer cells.[54,58-60] It is thus considered as a potential antineoplastic in preclinical or clinical trials for cancer therapeutics. Interestingly, it has been inferred that LRP-1 could mediate toxin-induced autophagy and apoptosis in a human gastric epithelial cell model.[61] Due to ConA's potential as a therapeutic avenue, it will be useful to understand how it collectively affects MT1-MMP and LRP-1 regulation in tumour cells.

3.6 Acknowledgments

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The abbreviations used are : BBB, Blood-brain barrier; ConA, Concanavalin-A; COX-2, Cyclooxygenase-2; CytoD, Cytochalasin-D; ECM, Extracellular matrix; EGCG,

Epigallocatechin 3-gallate; ER, Endoplasmic reticulum; LRP-1, Low density lipoprotein receptor-related protein-1; MMP-2, matrix metalloproteinase-2; MT1-MMP, membrane type-1 matrix metalloproteinase; TLR, Toll-like receptor; UPS, Ubiquitin-dependent proteasome system

3.7 Figure Legends

Fig.1 : **ConA triggers a rapid decrease of LRP-1 expression in U87 cells.** (A) Serum-starved U87 cells were treated for 24 hours with various concentrations of Concanavalin-A (ConA). Cell lysates were isolated and processed for the immunodetection of COX-2, MT1-MMP, LRP-1 (85-kDa subunit), and GAPDH. Conditioned media were harvested in order to assess proMMP-2 activation using gelatin zymography (bottom panel). (B) Representative densitometry analysis of LRP-1 to COX-2 expression (left panel) and LRP-1 to MT1-MMP proteolytic processing (43-kDa form / 55-kDa form ratio, right panel). (C) Serum-starved U87 cells were treated with various concentrations of ConA. Cell lysates were isolated, and immunodetection of LRP-1 for the indicated times performed. (D) Representative cell surface immunophenotyping of LRP-1, as measured by flow cytometry (closed circles), and assessment of LRP-1 gene expression as measured by qRT-PCR (open circles) are presented relative to time of treatment with ConA 30 □g/ml.

Fig.2 : **ConA-induced LRP-1 degradation is independent of MT1-MMP catalytic or proteolytic maturation.** (A) Serum-starved U87 cells were treated (or not) for 24 hours with ConA (30 μg/ml) or with Ilomastat (25 μM) ± Furin inhibitor II (50 μM) (as a co-treatment, or as a 24 hours pre-treatment). Conditioned media were harvested to assess proMMP-2 activation by gelatin zymography (bottom images). (B) U87 cells were transiently-transfected with siScrambled (siScr) or siMT1-MMP and transfection efficienty confirmed by qRT-PCR, then (C) followed by treatment for 24 hours with or without ConA (30 μg/ml). Following treatments, cell lysates were isolated for immunodetection of MT1-MMP, LRP-1 (85-kDa subunit), or GAPDH. Conditioned media were harvested to assess proMMP-2 activation by gelatin zymography (bottom of panel).

Fig.3: **LRP-1 degradation in ConA-activated U87 cells is mediated both by lysosomes and by the UPS.** U87 cells were treated for 6 hours, in the presence or absence of ConA (30 μg/ml) with (A) the UPS inhibitors MG132 (Left panels) or Lactacystin (Right panels) or (B) EGCG (25 μM). In all conditions, cell lysates were isolated for immunodetection of MT1-MMP, LRP-1 (85-kDa subunit),

GAPDH and Ubiquitin (panel A only). (C) U87 cells were seeded onto coverslips where they were treated (or not) with ConA (30 μg/ml) for 2 and 6 hours. Cells were then incubated with Lysotracker (red), fixed, and stained for LRP-1 (green). Images were acquired using a Nikon Eclipse Ti confocal microscope and using NIS Elements software. Deconvolution and colocalization analysis was carried out with AutoQuant X software. Colocalization analysis revealed Pearson's Correlation Coefficients (t=0h, 0.59; t=2h, 0.99; t=6h, 0.99) and 2D histogram plots of colocalization inset (green, y-axis (LRP-1); red, x-axis (Lysotracker)).

Fig.4: **LRP-1 degradation in ConA-activated U87 cells does not require cell surface-mediated cell signalling.** (A) U87 cells were transiently-transfected with 10 nM of either siScr, siTLR-2 or siTLR-6, and transfection efficacy assessed by qRT-PCR. (B) Following ConA treatment, all cell lysates were used for immunodetection of LRP-1 (85-kDa subunit) and GAPDH. (C) U87 cells were treated for 6 hours in the presence or absence of ConA (30 μg/ml) in combination with the JAK/STAT inhibitor Tofacitanib (CB, 30 μM), the MAPK inhibitor SB203580 (SB, 10 μM), the Src inhibitor PP2 (PP2, 10 μM), the MEK inhibitor U0126 (U0, 20 μM), or the MAPK inhibitor Acetyl-11-keto-beta-boswellic acid (AKBA, 20 μM).

Fig.5: **Disruption of the actin cytoskeleton is responsible for LRP-1 internalization and degradation.** (A) U87 cells were treated for 6 hours with ConA (30 μg/ml), CytoD (1 and 0.1 μM) or Nocodazole (300 and 30 nM). Cell lysates were then isolated for immunodetection of LRP-1 (85-kDa subunit), MT1-MMP and GAPDH. (B) U87 cells were treated for 2 hours with ConA (30 μg/ml), CytoD (1 μM) or Nocodazole (300 nM). Cells were then harvested for immunophenotyping of cell surface mature 600-kDa LRP-1 by flow cytometry. (C) U87 cells were seeded onto coverslips and treated with CytoD (1 and 0.1 μM) or Nocodazole (300 and 30nM) for 6 hours. Cells were then incubated with Lysotracker (red), fixed, and stained for LRP-1 (green), or for the nucleus with DAPI (blue). Images were acquired with a Nikon Eclipse Ti confocal microscope and using NIS Elements software, deconvolution and colocalization analysis was carried out with AutoQuant X software. (D) Mean cell surface LRP-1-associated fluorescence was measured using ImajeJ software and divided by number of nuclei (arb. units).

Fig.6: **ConA-induced LRP-1 internalisation and degradation leads to a reduced uptake of LRP-1 ligands.** U87 cells were treated with ConA (30 μg/ml) for up to 120 minutes, followed by incubation with Alexa^{488}-labelled LRP-1 ligands (A) α2-Macroglobulin or (B) Angiopep-2 (left panel). Uptake of ligands was

measured by flow cytometry. (B) U87 cells were treated with various concentrations of ConA (0-30 μg/ml) for 2 hours, following which Angiopep-2 uptake was assessed. (C) U87 cells were plated on coverslips and treated with various concentrations of ConA for 2 hours, then subsequently incubated with Alexa^{488}-labelled Angiopep-2 (Top panels, green). Fixed cells were stained for LRP-1 (bottom panels, red) and images were acquired with a Nikon Eclipse Ti confocal microscope using NIS Elements software. Images shown are from a representative experiment. (D) Relative Angiopep-2 internalisation as a function of LRP-1 cell surface expression. Linear regression ($R^2 = 0.92$).

3.8 Figures

Fig.1

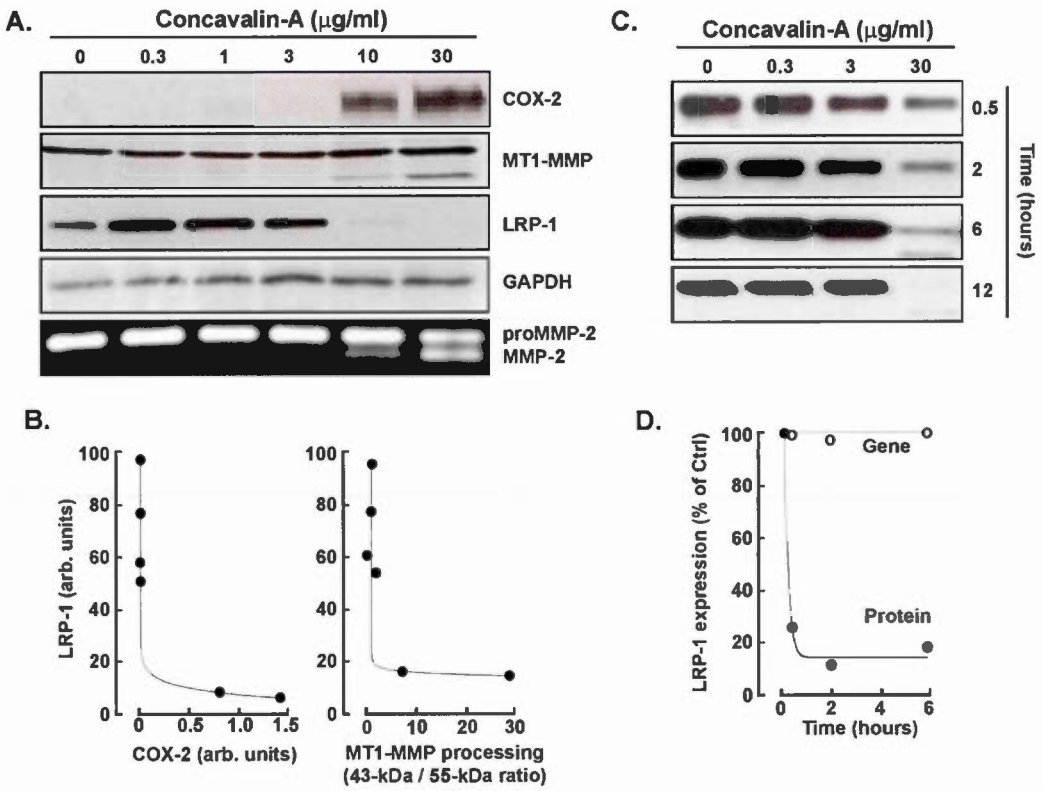


Fig.2

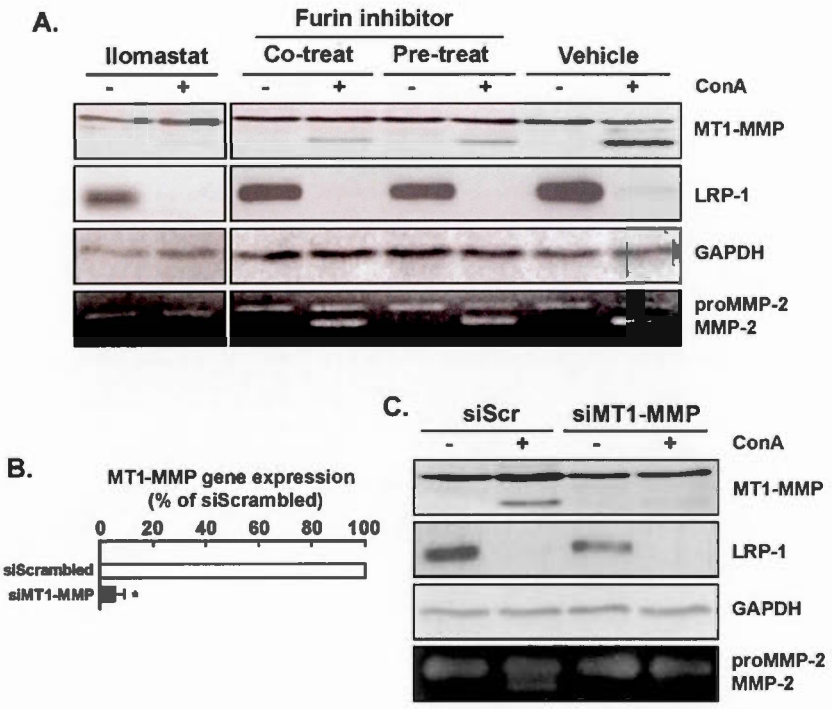

A.
Furin inhibitor
Ilomastat
Co-treat
Pre-treat
Vehicle
- + - + - + - +
ConA
MT1-MMP
LRP-1
GAPDH
proMMP-2
MMP-2
B.
MT1-MMP gene expression
(% of siScrambled)
0 20 40 60 80 100
siScrambled
siMT1-MMP
C.
siScr
siMT1-MMP
- + - +
ConA
MT1-MMP
LRP-1
GAPDH
proMMP-2
MMP-2

Fig.3

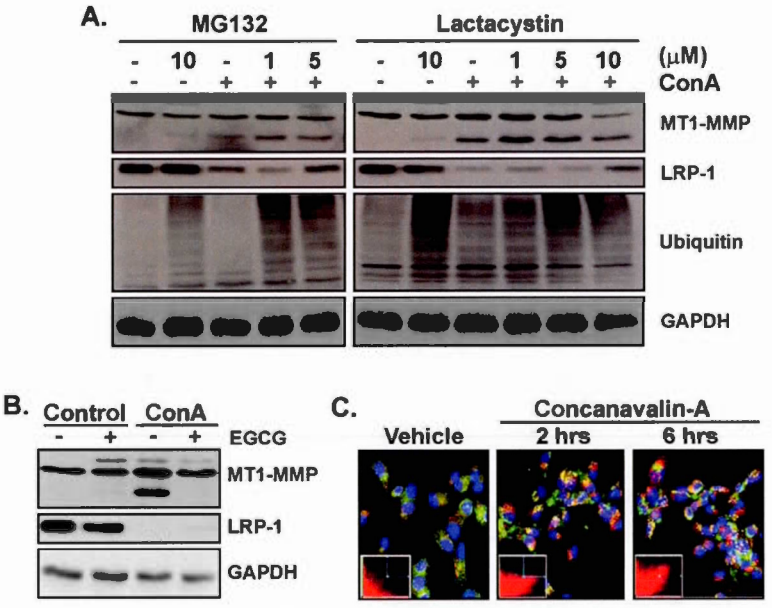

A.
MG132
Lactacystin
- 10 - 1 5 - 10 - 1 5 10 (μM)
- - + + + - - + + + + ConA
MT1-MMP
LRP-1
Ubiquitin
GAPDH
B.
Control ConA
- + - + EGCG
MT1-MMP
LRP-1
GAPDH
C.
Concanavalin-A
Vehicle
2 hrs
6 hrs

Fig.4

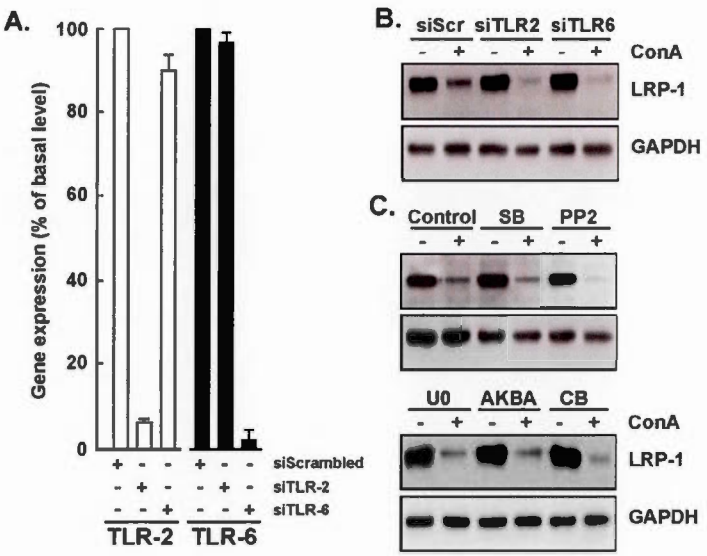

A.
Gene expression (% of basal level)
100
80
60
40
20
0
siScrambled
siTLR-2
siTLR-6
TLR-2
TLR-6
B.
siScr
siTLR2
siTLR6
ConA
LRP-1
GAPDH
C.
Control
SB
PP2
U0
AKBA
CB
ConA
LRP-1
GAPDH

Fig.5

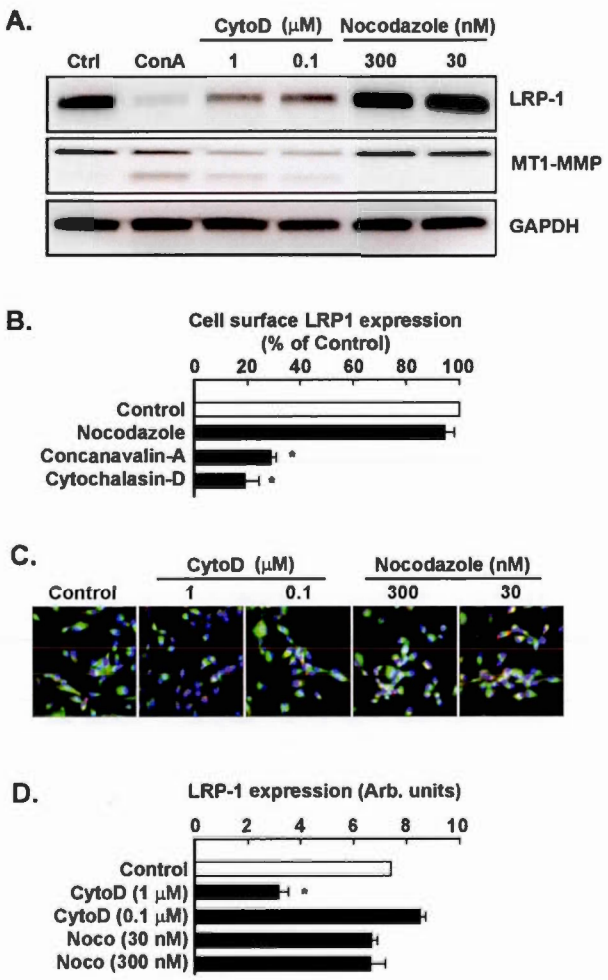
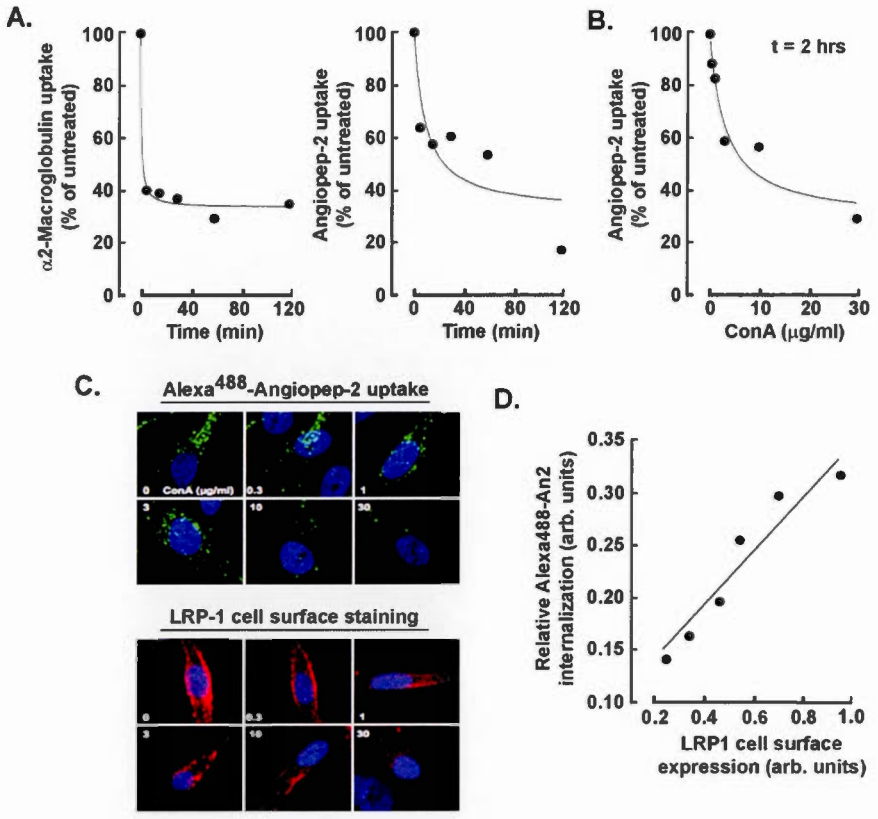

A.
CytoD (μM)
Nocodazole (nM)
Ctrl
ConA
1
0.1
300
30
LRP-1
MT1-MMP
GAPDH
B.
Cell surface LRP1 expression
(% of Control)
0
20
40
60
80
100
Control
Nocodazole
Concanavalin-A
Cytochalasin-D
C.
CytoD (μM)
Nocodazole (nM)
Control
1
0.1
300
30
D.
LRP-1 expression (Arb. units)
0
2
4
6
8
10
Control
CytoD (1 μM)
CytoD (0.1 μM)
Noco (30 nM)
Noco (300 nM)

Fig.6


A.
α2-Macroglobulin uptake (% of untreated)
Time (min)
Angiopep-2 uptake (% of untreated)
Time (min)
B.
t = 2 hrs
Angiopep-2 uptake (% of untreated)
ConA (μg/ml)
C.
Alexa488-Angiopep-2 uptake
0 ConA (μg/ml)
0.3
1
3
10
30
LRP-1 cell surface staining
D.
Relative Alexa488-An2 internalization (arb. units)
LRP1 cell surface expression (arb. units)

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Chapter III

4 Conclusions

Our finding that ConA-mediated cytoskeleton disruption results in the rapid internalization and complete degradation LRP-1 will contribute to both the study of LRP-1, and that of the malignant phenotype in GBMs. Furthermore, one can imagine that these new data may give context to future studies involving modulation of LRP-1 and/or the cytoskeleton.

Given that our laboratory has reported that MT1-MMP is able, when overexpressed in U87 cells, to reduce cell surface LRP-1 expression, it was somewhat unexpected to find that the phenomena of ConA-induced-LRP-1 processing was independent of MT1-MMP. It would have been expected to see some kind of reduction in the LRP-1 degradation found when inhibiting MT1-MMP catalytic function. However, as we did not, the processing of LRP-1 in MT1-MMP overexpressing cells may be much less significant than to that we see in our current study. Furthermore, the reduction in expression of LRP-1 in MT1-MMP over-expressing cells, is via a direct interaction of proteolytic cleavage. Due to the relative speed at which we observe LRP-1 being trafficked away from the cell surface (within minutes) and degraded within the cell (<30 minutes; Figure 1 – Chapter 2), any induction of MT1-MMP-mediated proteolysis of LRP-1, that could have been expected in our ConA-activated U87s, would take longer to show an effect than for LRP-1 to exit the cell surface (as observed in our study), and thus unavailable for MT1-MMP cleavage.

The finding that LRP-1 exited the cell surface as a result of actin-cytoskeleton disruption further contributes to body of evidence describing LRP-1's role in cytoskeleton processes. (Boucher et al., 2008; Lillis et al., 2005) This is especially relevant due to the nature of migration and invasion, both in physiological and pathological contexts, where the cytoskeleton is involved in the mechanical and physical changes that provide a movement component to migration and invasion (Albert, B., 2010). Furthermore, the cytoskeleton is involved in the mediation of attachment and anchoring of the cell to either the basement membrane and/or cells (Fischer & Fowler, 2015). Given the important role LRP-1 plays in clearance of extracellular proteases (Etique, Verzeaux, Dedieu, & Emonard, 2013), it would seem logical for this function of LRP-1 to be repressed in the

migratory front of cells. However, this would be a naïve and pre-emptive conclusion to draw from the data we present. This phenomena of such significant LRP-1 regulation on, what should be considered extreme, modulation of the actin cytoskeleton, merits further study. It would be extremely pertinent to determine whether the internalization and degradation of LRP-1 is as a result of disruption to the actin cytoskeleton, without doubt. Furthermore, to develop on the hypothesis that this ConA-mediated actin cytoskeleton disruption mimics the migratory / invasive phenotype, it would be revealing to quantify and localise LRP-1 during the migratory process. Additionally, by visualising the cytoskeleton during this process it may be possible to correlate both events to support the claim that LRP-1 is exiting the cell surface as a result in the dynamic changes that occur during cellular migration. Given that our study was carried out in an aggressive glioma cell line, whether this process - of LRP-1 internalization – is a characteristic of a highly malignant phenotype would also be interesting to probe (since interest in LRP-1 status in several different pathologies is being explored as a possible indicator, negative and positive).

Interestingly, it has recently been demonstrated that LRP-1 can contribute to the recruitment of monocyte to the tumour compartment, attributed to its ability to promote vascularisation of the tumour (Staudt et al., 2013). As we have demonstrated that ConA is able to rapidly remove LRP-1 from the cell surface, this provides more support for ConA to be further studied as a potential therapeutic entity given the growing implications of LRP-1 in pathological mechanisms. Despite being unable to give certainty to the link between ConA and CytoD to LRP-1 degradation, this is strong evidence for caution and diligence to be used when disrupting the actin-cytoskeleton alongside LRP-1 targeting drug delivery methods, such as the Angiopep peptides. Figure 6 showing significant functional impairment of LRP-1-mediated internalization of ligands at ConA (3 μg/ml) (Figure 6B), as well as functional impairment within 5 minutes of ConA exposure (Figure 6A). Functional impairment was maximally around 40-60% of control, most likely due to the nature of both Angiopep-2 and α2-Macroglobulin (neither being purely LRP-1 specific ligands). Figure 6C showed clearly a correlation between LRP-1 cell surface expression and the internalization of Angiopep-2.

Due to the dynamic role the actin cytoskeleton can play in cancers, interest has been placed in developing agents that are anti-actin. However, there are no current anti-

actin agents used in chemotherapy due to their indiscriminate inhibition of the filaments of the muscle sarcomere. Recently, Stehn et al, (Stehn et al., 2013) demonstrated that it was possible to "target specific actin filament populations fundamental to tumour cell viability based on their tropomyosin isoform composition. This improvement in specificity provides a pathway to the development of a novel class of anti-actin compounds for the potential treatment of a wide variety of cancers." Importantly, with increased specificity, cardiac muscle was unaffected by the inhibition. As the metastatic state is one of the fundamental transformations that differentiate benign from potentially fatal tumours, being able to inhibit this specific process may well be significant. Given the possible implications of actin cytoskeleton disruption on LRP-1, our study provides the initial evidence that warrants further experimental inquiry into the effects of actin cytoskeleton-targeting-therapy on LRP-1 status. Therefore, a pertinent next step in our research would be to explore these potential novel classes of chemotherapeutic agents on LRP-1 status within both cancerous and physiological cell models.

An emerging role for microtubules in tumour cell metastasis is being unravelled and there is increasing interest in the crosstalk between key microtubule interacting proteins and the actin cytoskeleton, which may provide novel treatment avenues for metastatic disease. (Fife et al., 2014) Perhaps another useful avenue to explore subsequently to our results - having confirmed that the LRP-1 depletion seen in our research is certainly due to actin cytoskeleton disruption - would be to identify the regulatory proteins involved in this regulation of LRP-1. As mentioned briefly in the results section (5.5 Discussion, Chapter 2), there is a RabGTPase family of proteins that play an integral role in regulating MT1-MMP trafficking in primary human macrophages. (Wiesner et al., 2013) These proteins, specifically Rab5a, Rab8a and Rab14, in mediating the tethering of vesicles to target organelles, give a means of control in the regulation of endocytotic and exocytotic processes of MT1-MMP. It is also suggested that Rab7a plays a role in mediating the delivery of vesicle to lysosomes for degradation. An attractive next step in developing our understanding of LRP-1 internalisation and degradation during ConA-meditated actin cytoskeleton disruption, would be to asses whether these sets of RabGTPases, or whether others - given that there are over 70 known mammalian Rab GTPases (Hutagalung & Novick, 2011) – play a similar role in U87 cells. An initial gene silencing screening of Rabs 5a, 8a, and 14, as well as 7a, would present as an attractive first step in exploring whether similar mechanisms occur in our model. The

identification of a specific mechanism linking the internalization and degradation of LRP-1 would be an interesting addition to the understanding of LRP-1 regulation and functions. Perhaps the regulation of LRP-1 is mediated by its own set of Rab GTPases, or the disruption of the actin cytoskeleton results in perturbations of LRP-1 endocytic and exocytic processes, via Rab proteins. Given the relationship LRP-1 and MT1-MMP share, it would be attractive to assume that the same family of proteins regulate their processing. However, this would be short sighted given the roles played by other small G protein families: both Arf or Rho have an integral role, alongside the Rab proteins, during endocytosis and exocytosis, and are also an avenue worth exploring to find link between LRP-1 regulation and cytoskeleton disruption. (Doherty & McMahon, 2009) Given that Rho small G proteins are involved in cytoskeleton regulation, it would be interesting to see whether this is as a result of, or a cause of the phenomena we see. (Doherty & McMahon, 2008)

As has been discussed, LRP-1 functions are still not fully characterized, and it can be presumed that much of the paradoxical information we have on LRP-1 function are due to the lack of a fully integrated understanding. A good example of this is, despite LRP-1's role in clearing extra-cellular proteases from the extra-cellular compartment, LRP-1 has been shown to be involved in the induction of both MMP-2 and -9, albeit in endometrial explants and not cancer cell models. (Selvais et al., 2009) Thus, phenomena such as this may well become more clear following a broader understanding of the different capacities LRP-1 has: it is able to induce and transmit several intracellular processes, that may or may not, be linked to its functions in the extracellular compartment. Given that LRP-1 status has been associated with both protective and deleterious effects in pathologies, it will be interesting to see how these superficially nonsensical phenomena are explained.

To conclude, due to LRP-1's broad implications, both positive and negative, in significant pathologies such as neurodegeneration, cardiovascular disease and many forms of cancer, our contribution to the understanding of its regulation will be welcomed. Furthermore, our work provides data to support the claim that ConA, as a potential therapeutic entity, merits continued exploration.

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