The role of the relaxin receptor RXFP1 in brain cancer

By

Usakorn Kunanuvat

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba in partial fulfilment for the requirements

of the degree of

MASTER OF SCIENCE

Department of Human Anatomy and Cell Science

University of Manitoba

Winnipeg

ABSTRACT

Relaxin (RLN2) promotes cell migration/invasion, cell growth, and neoangiogenesis through binding to the relaxin receptor RXFP1 in many types of cancers. However, there have been no studies to determine the role of this system in brain tumors, especially in Glioblastoma Multiforme (GB), the most lethal primary brain tumor in adults. GB is a systemic brain disease and aggressively invades brain tissue. In this study, we have identified RXFP1 receptor, but not RLN2, in GB cell lines and primary GB cells from patients. RLN2 treatment resulted in a significant increase in migration of GB cell line and primary GB cells. To determine molecular mechanisms that facilitate RXFP1-mediated migration in GB cells, we employed a pseudopodia assay and 2D LC-MS/MS to investigate the protein composition at cell protrusions (pseudopodia) during GB cell migration. We also observed the expression of known mediators promoting tissue invasion upon RLN2 treatment. We identified PGRMC1, a candidate protein from 2D LC-MS/MS as a novel relaxin target protein in RXFP1-expressing brain tumor cells. RLN2 treatment also caused an increase in cathepsin (cath)-B and -L and enhanced production of as the small Rho-GTPases Rac1 and Cdc42 in GB cells. Collectively, these findings indicate that RXFP1-induced cell migration is mediated by the upregulation and intracellular actions of Rac1, Cdc42 and by cath-B and cath–L who serve as matrix modulating factors to facilitate brain tumor cells migration. PGRMC1 also contributes to RXFP1-mediated cell migration through an as yet unknown mechanism. RLN2 is not present in the brain. We determined the role of a peptide ligand of RXFP1, the newly discovered C1q/TNF related peptide (CTRP)8-derived P74 peptide, in promoting migration in GB cells. Similar to relaxin, P74 was found to have pro-migratory effects on GB cells. The biological activity of this peptide was also similar to relaxin and caused the upregulation of cath-B, cath-D and cath-L in the primary GB cells, thus, indicating that P74 might serve as a novel RXFP1 activating peptide ligand. We conclude that RXFP1 receptor signaling plays a key role in brain tumors cell migration and invasion.

ACKNOWLEDGEMENTS

In the first place I am heartily thankful to my supervisor, Dr. Thomas Klonisch for bringing me to Winnipeg and giving me a great opportunity to work on my Master's Degree. You are just a great supervisor. Your supervision, advice, and guidance from the very early stage of this research gave me extraordinary experiences throughout the work. I have encountered many obstacles performing experiments as is true for every student. Fortunately, you provided me with unflinching encouragement and support in various ways. Sometimes, things did not turn out exactly the way I intended to and I felt depressed and downhearted. You always encouraged me with our lab's motto "Never ever give up". That made me converts the negative energies of my struggles into building a solid foundation and continue research. Thank you for continuously supporting me and pushing me to my limits to complete my Master's studies, and for all the patience and great support you gave me since the beginning.

I would like to express my gratitude to Dr. Sabine Hombach-Klonisch. I am much indebted to you for your valuable advice in science discussions, supervision in the lab, using your precious time to help me to overcome problems and challenges I faced many times during my lab experiments. Without your help I would not be this far. Thank you for believing in me, helping me to believe in myself and encouraging me to pursue excellence.

I would like to acknowledge the advice and guidance of my committee members, Dr. John A. Wilkins and Dr. Rajinder Bhullar. Thank you for using your precious time reading this thesis and for your valuable and critical comments that were essential in further improving my work. I thank you both for providing the necessary infrastructure, resources and expertise to accomplish parts of my research work. You have been always very generous to me.

I express my sincere thanks to my lab members; Aleksandra, Adrian, Suchitra and Manoj. I had the fortune of working at this exciting lab. While we are multicultural, we get along together very well and are friends. You guys were fabulous. I had a great time and learned a great deal by working together with you. Thank you for being good friends who are always so supportive and encouraging. I do appreciate your help in everything.

Special thanks go to Dana Henderson and Neha Khanna for taking time to check my English in my thesis and polish my thesis until it was in the stage that it was to my satisfaction.

I extend my warm thanks to Peyman Ezzati who always kindly granted me his time, helped me and answered the many questions on Proteomics and the analysis of my data.

I would also like to extend a huge and warm thank you to all Thai students in Winnipeg. All of you make me feel that I am not alone while I was far away from home. You guys have made a mini Thai community where we helped and supported each other in every way despite that we knew each other only when we came here. Special thanks to Ruedee and family, Narison and Thatchawan who always helped and supported me and stayed at my side no matter what. I felt so lucky to know each of you. Thank you for being "big sisters" to me.

Likewise, my appreciation goes to my friends in Thailand who always encouraged me when I felt bad and laughed with me on the days of my successes. Most importantly, you had the faith in me that I will overcome all the challenges.

I will forever be thankful to my former supervisors, Dr.Tewin Tencomnao and Dr. Wanida Laiwatttanapaisal. You have been helpful in providing advice many times during my undergraduate school and also when I was here. Thank you both for giving me the opportunity to get to know Dr. Klonisch and his team which then paves the way of my life to the success.

Last but not least, my deepest gratitude goes to my family for their unconditional love and support throughout my life; this thesis would have been impossible without the love and patience of my family. This thesis is dedicated to all members of my family who have been a constant source of love, support and strength for all these years.

To Mom, I have no suitable word that can fully describe your everlasting love to me. Thank you for a long distance calls every day and every time I was facing problems. You are always by my side even though we are a thousand miles apart. To Dad, thank you for your unequivocal support and everlasting love for me. To both of you, thank you both for your warm and constant support for giving me strength and confidence during my work.

To my sister, Saranrat and my brother, Dhanachart , thank you for taking good care of Mom and Dad while I was here and thank you for your love and support in everything that I do.

To Grandpa, this work is dedicated to you. I promised you that I will fight to get my degree from North America. Notwithstanding, you are not with me anymore but I do believe that you know I did it. To Grandma who lovingly supports me, regardless of where in the world I am.

To my aunt, thank you for so many encouraging emails that always make me feel positive every time I read them. Your optimistic thinking always makes me feel that tomorrow everything will be better when things go wrong no matter what I do.

Finally, I would like to thank everybody who has helped me directly or indirectly and so was important to the successful realization of this thesis, as well as expressing my apologies that I could not mention personally one by one.

Usakorn Kunanuvat

CONTENTS

LIST OF ABBREVIATIONS

2D LC-MS/MS	two dimensional high performance liquid chromatography – mass spectrometry analysis
AC	adenylyl cyclase
AC2	adenylyl cyclase 2
ADAMs	a disintegrin and metalloproteinase
AKAP79	A-kinase-anchoring protein 79
Akt (PKB)	protein kinase B
ARP2/3 complex	actin-related protein complex
bFGF	basic fibroblast growth factor
bp	base pairs
cAMP	cyclic 3′–5′ adenosine monophosphate
cath	cathepsin
cGMP	cyclic guanosine monophosphate
CHO-K1	chinese hamster ovary cells
CRE	cAMP-response element
CTRPs	complement C1q tumor necrosis factor (TNF) related proteins
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
ECM	extracellular matrix
EPIL	early placenta insulin-like
ER	endoplasmic reticulum

ERK1/2	extracellular signal-regulated kinase ½
FBS	fetal bovine serum
Fn14	fibroblast growth factor-inducible14
FSHR	follicle-stimulating hormone receptor
FTC-133	human follicular thyroid carcinoma-primary metastasis
FTC-236	human follicular thyroid carcinoma- neck lymph node metastasis
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GB	glioblastoma
GBM	glioblastoma multiforme
GPCR	G-protein coupled receptor
GPM	global proteome machine
GR	glucocorticoid receptor
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IAA	iodoacetamide
IGF	insulin-like growth factors
INSL3	insulin/relaxin-like peptides
LDL-A	low-density lipoprotein class A
LGR	leucine-rich repeat containing G protein-coupled receptor
LGR7	leucine-rich G protein coupled receptor 7
LGR8	leucine-rich G protein coupled receptor 8
LHR	luteinizing hormone receptor
LRR	leucine-rich repeat

MAPKs	mitogen activated protein kinases
MMPs	matrix metalloproteinases
MS	mass spectrum
mTOR	mammalian target of rapamycin
NF- κB	nuclear factor of kappaB
NO	nitric oxide
NOS II	nitric oxide synthase 2
NOS III	endothelial nitric oxide synthase 3
N-WASP	neural wiskott-aldrich syndrome protein
One way-ANOVA	one way analysis of variance
PANTHER	protein analysis through evolutionary relationships
PBS	phosphate buffer saline
PBS	primary brain tumor study
PCR	polymerase chain reaction
PGRMC1	membrane-associated progesterone receptor component 1
PI3K	phosphatidyl inositol 3 kinase
PKA	protein kinase A
PKCζ	protein kinase C zeta
PP	protein pilot
PPARgamma	peroxisome proliferator-activated receptor gamma
rhRLN2	recombinant human relaxin
RLF	relaxin-like factor
RT	room temperature

RT-PCR	reverse transcriptase-polymerase chain reaction
RXFP1	relaxin family peptide receptor1
RXFP2	relaxin family peptide receptor2
RXFP3	relaxin family peptide receptor3
RXFP4	relaxin family peptide receptor4
RXFPs	relaxin family peptide receptors
SEM	scanning electron microscopy
siRNA	small interfering RNA
SP	signal peptide
TAE	tris-acetate-EDTA
TBS	tris-buffered saline
TfR1	transferrin receptor protein1
TGF β1	transforming growth factor beta1
TIMPs	tissue inhibitors of metalloproteinases
TMZ	temozolomide
TNF	tumor necrosis factor
TSHR	thyroid-stimulating hormone receptor
uPA	urokinase-type plasminogen activator
VEGF	vascular endothelial growth factor
WHO	world health organization

LIST OF TABLES

LIST OF FIGURES

LIST OF COPYRIGHTED MATERIAL FOR WHICH PERMISSION WAS OBTAINED

This figure was published in van der Westhuizen, E. T., M. L. Halls, et al. (2008). "*Relaxin family peptide receptors--from orphans to therapeutic targets.*" Drug Discov Today 13(15-16): 641, figure2. Copyright © Elsevier, 2008. Permission obtained: September 20th, 2012.

This figure was published in Bathgate, R. A., R. Ivell, et al. (2006). "*International Union of Pharmacology LVII: recommendations for the nomenclature of receptors for relaxin family peptides.*" Pharmacol Rev 58(1): 25, figure8. Copyright © 2006 The American Society for Pharmacology and Experimental Therapeutics. Permission obtained: August 23rd, 2012.

This figure was published in Halls, M. L. and D. M. Cooper (2010). "*Sub-picomolar relaxin signalling by a pre-assembled RXFP1, AKAP79, AC2, beta-arrestin 2, PDE4D3 complex."* EMBO J 29(16): 2784, figure10. Copyright © 2010, Rights Managed by Nature Publishing Group. Permission obtained: August 22nd, 2012.

CHAPTER 1: INTRODUCTION

1.1 The relaxin family of peptides

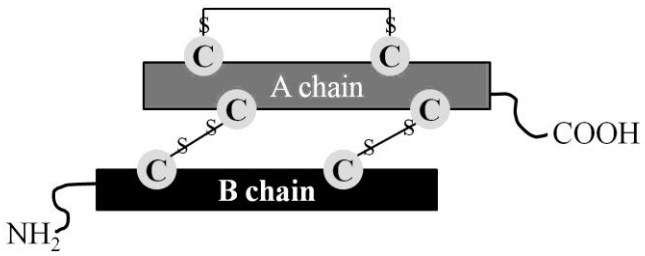
Overview

The insulin/relaxin superfamily of peptide hormones is made up of ten peptide hormones including; insulin, insulin-like growth factors (IGF) I and II, and seven members of the relaxin-like peptide family, which are a sub group of this superfamily. In humans, the relaxin family of peptide hormones consists of seven members on the basis of structural similarity. They are relaxin-1 (Hudson, Haley et al. 1983) , relaxin-2 (Hudson, John et al. 1984) and relaxin-3, also known as insulin-like peptide 7 (Bathgate, Samuel et al. 2002) plus, the insulin/relaxin-like peptides INSL3 also called Leydig insulin-like peptide and relaxin-like factor (RLF) (Adham, Burkhardt et al. 1993) , INSL4 also known as placentin and early placenta insulin-like (EPIL)(Chassin, Laurent et al. 1995), INSL5 (Conklin, Lofton-Day et al. 1999) and INSL6 (Lok, Johnston et al. 2000). These seven genes are located on three different chromosomes (Wilkinson, Speed et al. 2005). *RLN1* and *RLN2* genes are present in a tight cluster with *INSL4* and *INSL6* genes on chromosome 9, at 9p24. *RLN3* and *INSL3* are mapped on chromosome 19 at 19p13.3 and 19p13.2, respectively, whereas *INSL5* is the only member that is located at position 1p31.1 on chromosome 1.

Structure of the relaxin family peptides

All peptides within the relaxin family share high structural similarity due to the presence of six cysteine residues that form three disulfide cross-links. One intrachain disulfide bond within the A-chain, and two interchain disulfide bonds between the A- and B-chains (Fig.1.1).

Figure1.1 Structure of the processed mature active form of relaxin family peptide hormones



1.1.1 The discovery of relaxin

Relaxin, a 6 kDa peptide hormone was discovered by F. L. Hisaw in 1926. In 1983, the first human relaxin gene was identified and named H1-relaxin (Hudson, Haley et al. 1983) followed by the discovery of a second H2-relaxin gene in 1984 (Crawford, Hudson et al. 1984). Unlike most mammals which possess only the *RLN1*-encoded relaxin-1 peptide, humans and higher primates possess two separate genes, the *RLN1*-encoded relaxin-1 peptide, and the *RLN2*-encoded relaxin-2 peptide. These separate genes were thought to be a consequence of a gene duplication event (Gunnersen, Fu et al. 1996). H2-relaxin is the functional equivalent of H1-relaxin in other species. A third H3-relaxin gene was later found in humans and other species (Bathgate, Samuel et al. 2002).

Production and structure of relaxin

Relaxin is first synthesized as a 21 kDa preprohormone with a signal peptide and three distinct regions or chains designated A, B and C. In endoplasmic reticulum (ER), the signal peptide is cleaved and results in prorelaxin, which is stored in secretory granules prior to secretion. The prorelaxin becomes a mature 6 kDa relaxin as a result of the cleavage of the C-chain which is transported to the cell membrane where it is released into the bloodstream (Adham, Burkhardt et al. 1993).

Like insulin and other members within the relaxin family peptides, the structure of the processed form of relaxin is comprised of two polypeptide chains, the A- and B- chain. There are 22 amino acids in the A-chain (Schwabe, McDonald et al. 1976) and 32 amino acids in the B-chain (Schwabe and McDonald 1977). Both A-chain and B-chain make up for the molecular weight of around 6 kDa. X-ray crystallography revealed the tertiary structure of relaxin and demonstrated that the A-chain is comprised of two alpha helices connected by a short loop, while the B-chain is composed of one alpha helix and one strand. The two chains are linked via two disulphide bridges. The amino acid motif Arg^{13}-x-x-x-Arg^{17} –x-x-Ile^{20} located in the middle of the B-chain forms a binding surface required for the interaction of relaxin with the large extracellular domain of its G protein coupled receptor (GPCR), the relaxin family peptide receptor 1 (RXFP1) (Bullesbach and Schwabe 2000). Recently, the interaction of relaxin with RXFP1 has been shown to involve residues Tyr^{16}, Lys^{17}, and Phe^{23} at C-terminal part of the A-chain (Park, Semyonov et al. 2008). The activity of relaxin signaling through RXFP1 depends on interactions of the RXFP1 with both the B- and A-chains.

Expression of relaxins

H2-relaxin is expressed in a wide range of reproductive organs. It is mainly produced in the corpus luteum but also expressed in endometrium, decidual placenta, trophoblast, mammary gland, prostate and seminal vesicles (Hudson, John et al. 1984; Winslow, Shih et al. 1992; Gunnersen, Fu et al. 1996; Bryant-Greenwood, Yamamoto et al. 2009). In non-reproductive organs, H2-relaxin has been found in human atrial and ventricular myocardium and blood vessels (Dschietzig, Richter et al. 2001). H2-relaxin is the major stored and

circulating form of the relaxin peptides (Winslow, Shih et al. 1992) and a cognate ligand for RXFP1 receptor. Throughout this thesis, the human *RLN2* gene and peptide product H2-relaxin will be referred to as relaxin. For the other isoform of relaxin, *RLN1* gene is expressed in the decidua, placental trophoblast, and prostate gland (Hansell, Bryant-Greenwood et al. 1991; Gunnersen, Fu et al. 1996) as well as breast (Tashima, Mazoujian et al. 1994), human atrial and ventricular myocardium, arteries and veins (Dschietzig, Richter et al. 2001). *RLN3* appears to be predominantly expressed in the brain, especially in regions known to be involved in the regulation of hypothalamo-pituitary secretory function and stress response. (Bathgate, Samuel et al. 2002).

1.2 Relaxin family peptide receptors (RXFPs)

Overview

The most significant advancement in the area of relaxin has been the elucidation of the receptors for relaxin in 2002. The relaxin family peptide receptors (RXFPs) are a group of four GPCRs which have been identified to date as receptors for the relaxin family peptides. There are four members in RXFPs group including the leucine-rich repeat (LRR)-containing RXFP1 and RXFP2, and the small peptide-like RXFP3 and RXFP4. These four receptors were originally named as leucine-rich G protein coupled receptor 7 (LGR7), leucine-rich G protein coupled receptor 8 (LGR8), GPCR135 and GPCR142 respectively. (Bathgate, Ivell et al. 2006).

There is binding of the ligands to the various RXFP receptors due to the similarity of the structure of the peptides within relaxin family (Table 1.2). The process by which the relaxins activate their receptors is complicated and depends on compatible structural features

of both the ligands and the receptors. RXFP1 is able to bind to H2-relaxin, H1- relaxin, H3-relaxin and INSL3, but its main activating and high affinity binding ligand is H2-relaxin (Hsu, Nakabayashi et al. 2002; Sudo, Kumagai et al. 2003). RXFP2 is the endogenous receptor for INSL3 (Kumagai, Hsu et al. 2002) and RXFP3 (GPCR135; SALPR) is activated by relaxin-3 and INSL5. Unlike relaxin-3, INSL5 binds to RXFP3 with poor affinity and acts as a weak antagonist (Liu, Kuei et al. 2005). RXFP4 (GPCR142; GPR100) interacts with INSL5 (main ligand) and weakly with H3 relaxin.

Table 1.2 Ligand Potencies of receptors for relaxin and relaxin-related peptides

Receptor	Ligand Potencies	References
RXFP1(LGR7)	H2-relaxin >H1-relaxin> H3-relaxin >> INSL3	(Hsu, Nakabayashi et al. 2002; Sudo, Kumagai et al. 2003)
RXFP2(LGR8)	INSL3 > H2-relaxin >> H3-relaxin	(Kumagai, Hsu et al. 2002; Sudo, Kumagai et al. 2003)
RXFP3(GPCR135; SALPR)	H3-relaxin > H3-relaxin (B chain)>>INSL5	(Liu, Eriste et al. 2003)
RXFP4(GPCR142; GPR100)	INSL5 = H3-relaxin > H3- relaxin (B chain)	(Liu, Eriste et al. 2003; Liu, Kuei et al. 2005)

1.2.1 **Relaxin family peptide receptor 1 (RXFP1)**

RXFP1 structure

The RXFP1 receptor was identified based on its homology to the other members of the glycoprotein hormone receptor subfamily and shares the highest homology of about 60% amino acid sequence identity with the RXFP2 receptor (Hsu, Nakabayashi et al. 2002). RXFP1 belongs to a subfamily of the rhodopsin-like GPCR family called leucine-rich repeat containing G protein-coupled receptor (LGR). Three important domains of RXFP1 receptor were identified, including the ectodomains which comprise over half the size of each receptor, the typical seven transmembrane domain-spanning regions followed by the cytoplasmic domain of the C-terminal tail. The ectodomain can be further divided into two parts. The first part is the major component of the ectodomain and contains ten leucine-rich repeats (LRRs) and their capping structures. Located at the extreme amino terminus of the RXFP1 is the unique cysteine-rich low-density lipoprotein class A or LDL-A module (Fig.1.2.1-1), a distinctive structural feature of RXFP1 not observed in other human GPCR (Hsu 2003).

There are two ligand binding sites in RXFP1 which are necessary for inducing receptor activation. The primary high affinity ligand-binding site of RXFP1 located at specific residues in the LRRs of the ectodomain interacts with B-chain residues of the relaxin and results in a conformational change of the receptor. The secondary low affinity ligand binding site is in the second extracellular loop of the transmembrane region which interacts with the A-chain of relaxin (Sudo, Kumagai et al. 2003; Halls, Bond et al. 2005).

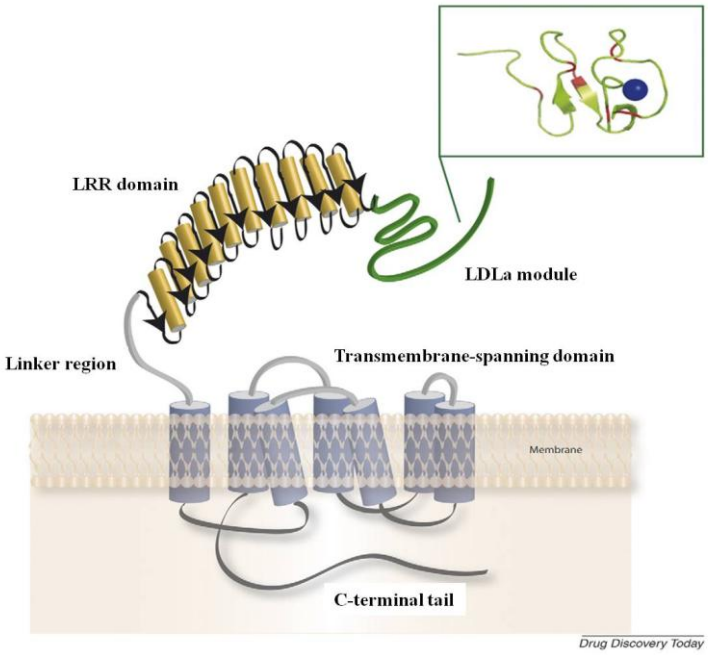
Apart from the two ligand binding sites in RXFP1, the presence of the unique LDL-A domain at N-terminus of receptor is essential for inducing receptor activation. A mutant

RXFP1 receptor with the LDL-a domain replaced by the LDL-a domain of the LDL receptor LB2 binds relaxin normally but does not permit signaling (Hopkins, Layfield et al. 2007). Beside its role in ligand-induced receptor signaling, the LDL-a domain also plays a role in receptor maturation and trafficking to the cell membrane (Kern, Agoulnik et al. 2007).

Alternative splicing of RXFP1, which is a common occurrence with GPCRs, is an important feature and may modulate the function of the receptor. RXFP1 contains 17 introns and 18 exons; as a result of differential splicing, numerous splice variants have been described for RXFP1 (Muda, He et al. 2005; Scott, Layfield et al. 2006; Kern and Bryant-Greenwood 2009) and other members of LGR family, such as follicle-stimulating hormone receptor (FSHR) (Song, Park et al. 2002; Gerasimova, Thanasoula et al. 2010), thyroid-stimulating hormone receptor (TSHR) (Ando, Sarlis et al. 2001), and luteinizing hormone receptor (LHR) (Madhra, Gay et al. 2004; Nakamura, Yamashita et al. 2004). Some variants contained transmembrane domains whereas other splice forms were composed exclusively of ectodomain fragments. Further analysis of the localization of these variants demonstrated that some were expressed on the cell surface, while others were retained within the ER and, yet, other variants were partially secreted into the media (Muda, He et al. 2005; Scott, Layfield et al. 2006; Kern, Hubbard et al. 2008). So far, most of these identified splice variants do not bind to ligand (Muda, He et al. 2005; Kern, Hubbard et al. 2008). Those variants that lack LDL-a module and are expressed at the cell surface have been shown to bind to the ligand but lost the ability to generate a signal (Scott, Layfield et al. 2006). Additionally, some of these variants function as dominant-negative counterparts of the wild type RXFP1 by preventing its homodimerization, maturation, and subsequent trafficking to

the cell surface, resulting in loss of function of receptor (Scott, Tregear et al. 2005; Scott, Layfield et al. 2006; Kern, Hubbard et al. 2008).

Figure 1.2.1-1 Structure of RXFP1 receptor

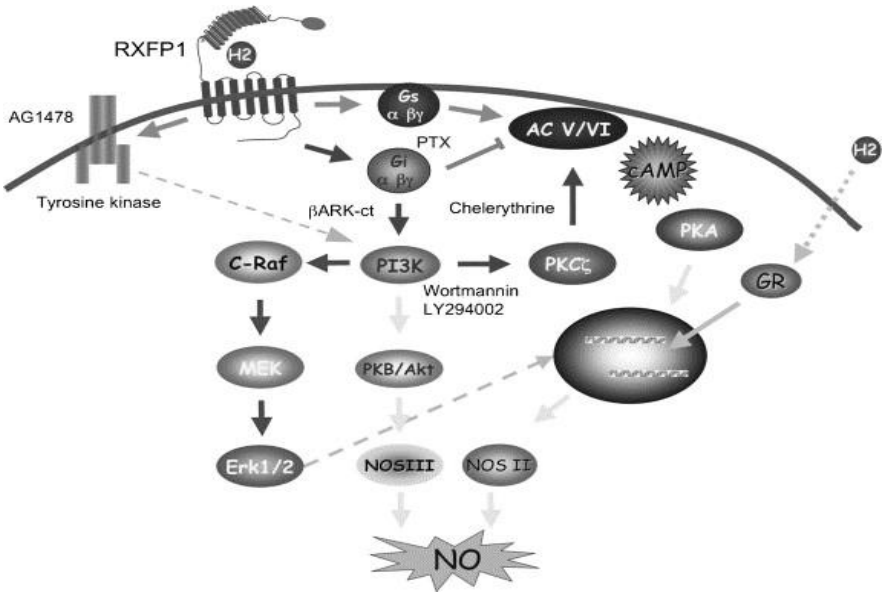


Adapted from (van der Westhuizen, Halls et al. 2008) with permission

Signaling pathways of RXFP1

Binding of relaxin to its native GPCR RXFP1 induces the cell type specific activation of multiple pathways (Kong, Shilling et al. 2010). These pathways include cyclic 3′–5′ adenosine monophosphate (cAMP) signaling, mitogen activated protein kinases (MAPKs) signaling, nitric oxide (NO) signaling, peroxisome proliferator-activated receptor gamma (PPARgamma) signaling, and interaction with glucocorticoid receptor (GR) (Fig.1.2.1-2).

Figure 1.2.1-2 Potential signaling pathways of relaxin-RXFP1



Adapted from (Bathgate, Ivell et al. 2006) with permission

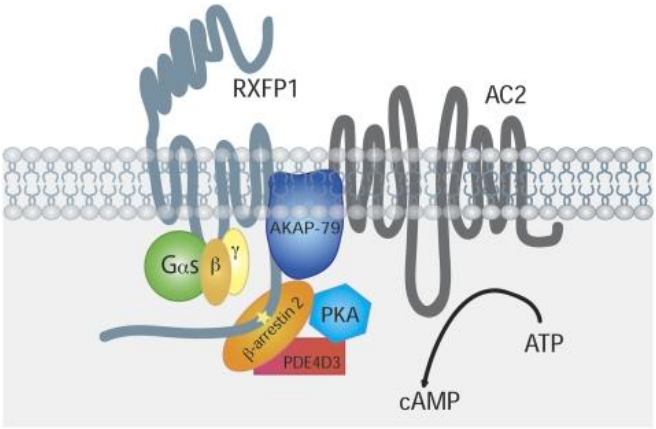
The elevation of both intracellular and extracellular cAMP is the most extensively studied to date as are the major signaling pathways activated in cells expressing RXFP1 (Fei, Gross et al. 1990; Bartsch, Bartlick et al. 2001). The increase in cAMP has been found to be biphasic and involves the interaction of the receptor with up to three distinct type of G-protein coupling, $G_{\alpha s}$, $G_{\alpha oB}$ and G_{i3} (Halls, Bathgate et al. 2006). The coupling of RXFP1 to the combination of these G-proteins varies with cell type and creates diverse cellular responses. For example, RXFP1 expressed in HEK293 cells, primary cultures of rat cardiac fibroblast and THP-1 cell lines can couple to all three G-protein pathways. In contrast, endogenous expression of RXFP1in primary cultures of rat renal myofibroblast or colon carcinoma cell lines can only couple to $G_{\alpha s}$ and $G_{\alpha oB}$ pathways (Halls, Hewitson et al. 2009). The biphasic nature of cAMP accumulation suggests that it may be involved in more than

one pathway. In the initial phase of RXFP1 activation which occurs within 1-2 minutes upon ligand binding the cAMP increase is likely caused by an activation of $G_{\alpha s}$ and inhibition of $G_{\alpha oB}$. A delayed cAMP increase occurs after 10-20 minutes and is mediated by the $\beta\gamma$ subunits of G_{i3} which results in the activation of the phosphatidyl inositol 3 kinase (PI3K) and protein kinase C zeta (PKCζ). Activated PKCζ then translocates to the cell membrane to activate adenylyl cyclase (AC) which produces the second phase of cAMP accumulation. cAMP can go on to activate protein kinase A (PKA), which has the ability to phosphorylate many signaling proteins. The activation of the G_{i3}-$G_{\beta\gamma}$ -PI3K- PKCζ cAMP pathway by RXFP1 requires the last 10 amino acids of the receptor C terminus especially Arg(752) and the coupling of the receptor to this pathway depends on the localization in membrane raft microdomains (Halls, van der Westhuizen et al. 2009). For the cAMP downstream targets $G_{\alpha s}$ and $G_{\alpha oB}$, the pathway affects cAMP-response element (CRE) important for control of gene transcription. Meanwhile, the G_{i3} pathway is the only pathway that affects the nuclear factor kappaB (NF- κB) mediated gene transcription (Halls, Bathgate et al. 2007).

The concentration of relaxin that interacts with RXFP1 affects the cAMP signaling response. The classical pathway of cAMP production generates physiological actions by coupling RXFP1 to the $G_{\alpha s}$, $G_{\alpha oB}$ and G_{i3} and occurs in response to nanomolar concentrations of relaxin. However, further complexity of relaxin stimulated cAMP signaling pathway may also occur in signalosomes that specifically respond to sub-picomolar concentrations of relaxin (Halls and Cooper 2010). These pre-assembled RXFP1 containing protein complexes are essential for generating the signaling since they facilitate the activation of downstream targets. RXFP1 is pre-coupled to adenylyl cyclase 2 (AC2) via the A-kinase-anchoring protein 79 (AKAP79). When an ultra-low concentration of relaxin binds to RXFP1, there is

an activation of AC2 that results in the production of cAMP. The negative regulation of these signalosomes occurs when β-arrestin-2 binds to RXFP1 and recruits two other proteins, the phosphodiesterase PDE4D3 and PKA to the protein complex (Fig.1.2.1-3). Importantly, the signalosomes will dissociate with the activation of RXFP1 at nanomolar concentrations of relaxin. Thus, relaxin stimulates a non-classical mechanism of cAMP production through signalosomes at picomolar concentrations. At nanomolar and higher concentrations, relaxin activates the classical signaling pathway which induces cAMP production through Gαs, $G_{\alpha oB}$ and G_{i3}.

Figure 1.2.1-3 The components of the constitutively assembled RXFP1 signalosome



Adapted from (Halls and Cooper 2010) used under a Creative Commons Attribution-Non-commercial licence: http://creativecommons.org/licenses/by-nc/2.5/

Relaxin has also been shown to stimulate the activation of MAPK and extracellular signal-regulated kinase (ERK1/2) in some cell types which endogenously express RXFP1. These cell types include human endothelial stromal cells, THP-1 mononuclear leukemia cells, primary human smooth muscle cells from coronary artery and pulmonary artery, and Hela cervical cancer cells. In THP-1 cells, relaxin induces

MAP kinase pathway activation and this is essential for increasing the expression of the gene for vascular endothelial growth factor (VEGF) (Zhang, Liu et al. 2002; Dschietzig, Bartsch et al. 2003). Additionally, relaxin binds to RXFP1 and activates PI3K/ Akt/ ERK1/2 and PKCζ pathway to induce MMP-9 expression in fibrochondrocytes (Ahmad, Wang et al. 2012).

Binding of relaxin to RXFP1 also activates NO production and induces vasodialation via two different pathways. The first pathway occurs when relaxin binds to RXFP1 and activates PI3K. Activated PI3K stimulates Akt which results in the activation of endothelial nitric oxide synthase III (NOS III) via phosphorylation of Ser1179. The following is another pathway which is involved in the increase of cAMP levels in cells, which then activates PKA. Activated PKA can phosphorylate and inactivate the inhibitor subunit of transcription factor NF- κB, IkB-α, which leads to the translocation of NF- κB into the nucleus, and increases nitric oxide synthase II (NOSII) gene expression (Nistri and Bani 2003).

More recently, relaxin has been shown to activate the PPARgamma pathway via signals through RXFP1 in HEK-293T cells over-expressing RXFP1. These results were confirmed in endogenously expressing RXFP1+ THP1 cells (Singh and Bennett 2009; Singh and Bennett 2010). PPARgamma is a nuclear receptor that induces transcription of target genes. Further it was reported that relaxin infusion in non-pregnant rats also increased wall thickness and inner diameter of brain parenchymal arterioles, effects that are known to be mediated by PPARgamma signaling (Chan and Cipolla 2011).

Apart from acting via RXFP1, relaxin can also bind to and activate GR, a steroid receptor to inhibit endotoxin-stimulated tumor necrosis factor and interleukin-6 secretion by

human macrophages. This effect is independent of RXFP1 since inactivated relaxin which cannot bind to RXFP1 still causes this effect in cells. The results of this study suggest that there may be cross-talk between RXFP1 and GR pathways in cells expressing both receptor systems (Dschietzig, Bartsch et al. 2009).

Distribution of RXFP1 receptor

The RXFP1 receptor distribution has been observed both at a transcriptional level and protein level using reverse transcriptase polymerase chain reaction (RT-PCR), Northern blot or in situ hybridization for mRNA detection and immunohistochemistry or receptor autoradiography for protein detection. Human RXFP1 transcripts have been identified in reproductive organs, including the placenta, uterus, ovary, testis and prostate as well as in many non-reproductive organ sites such as the brain, kidney, adrenal, skin, lung, liver, thyroid, salivary glands, heart, muscle, peripheral bloods cells and bone marrow (Hsu, Nakabayashi et al. 2002; Luna, Riesewijk et al. 2004; Mazella, Tang et al. 2004; Bathgate, Ivell et al. 2006). Human RXFP1 protein has been detected in uterus, cervix, vagina, nipple, and breast (Ivell, Balvers et al. 2003; Bond, Parry et al. 2004; Luna, Riesewijk et al. 2004; Krusche, Kroll et al. 2007) . The expression of RXFP1 is consistent with known relaxin binding sites.

1.3 Function of Relaxin-RXFP1 system

1.3.1 Physiological functions

Relaxin is commonly known as a pregnancy hormone and has had a long history as a reproductive hormone since its discovery in 1926 (Fevold 1930; Hisaw 1926). RXFP1 expression has been detected in many sites in reproductive organs corresponding to relaxin

binding sites and action. In the human, relaxin is produced mainly by the corpus luteum, in both pregnant and non-pregnant females, and was detected during both the follicular and luteal phases of the menstrual cycle (Wreje, Kristiansson et al. 1995). During menstruation the body produces relaxin that rises to a peak around 30-150 pg/ml within approximately 14 days of ovulation and then declines (Bryant, Panter et al. 1975). During pregnancy, the primary sources for relaxin are the corpus luteum and placenta. Relaxin can be detected in serum in conception cycles during the time of missed period. Relaxin secretions rapidly rise and peak at a concentration of 1-2 ng/mL by the middle of the first trimester of pregnancy, then it gradually declines by approximately 20% and remains stable until delivery (Weiss 1991).

In female reproductive tissues, relaxin has a diverse range of effects in maternal adjustments of pregnancy (Conrad 2011). These include ovarian follicle growth and ovulation (Shirota, Tateishi et al. 2005), preparation of the endometrium for implantation, facilitating embryo implantation by thickening of the uterus and extensive vascularisation (Einspanier, Muller et al. 2001), development of mammary nipples and mammary glands (Sherwood 2004), and preparation of the uterus and cervix for pregnancy and delivery by cervical softening and widening of the pubic symphysis together with stimulating collagen remodeling within the birth canal for parturition (Sherwood 2004; Parry and Vodstrcil 2007). The action of relaxin to inhibit spontaneous myometrial contraction was observed in some species but not in humans, sheep and cows (Sherwood 2004; Bathgate, Ivell et al. 2006). Additionally, relaxin acts through RXFP1 to promote epithelial and stromal cell proliferation and inhibit apoptosis in rat (Burger and Sherwood 1998) and mouse cervix and vagina in order to facilitate parturition (Yao, Agoulnik et al. 2008). Relaxin's action on glucose

metabolism may involve enhancement of insulin action by the ability of relaxin to increase the affinity of insulin binding to human and rat adipocytes. Thus, relaxin might have a protective effect against gestational diabetes in pregnancy (Weiss 1991).

In males, relaxin is produced by the prostate gland and secreted into the seminal fluid. Studies conducted in the past have suggested a role for relaxin in improving sperm motility (Lessing, Brenner et al. 1986; Sasaki, Kohsaka et al. 2001; Sherwood 2004).

Mice deficient in relaxin or RXFP1 were generated to study the function of this ligand-receptor system. Female RXFP1 knockout mice showed normal fertility and litter size. However, severe impairment of nipple development during pregnancy as well as defective parturition reflecting impaired cervical ripening was observed in those mice (Krajnc-Franken, van Disseldorp et al. 2004). These data are in line with the phenotype of relaxin knockout female mice (Zhao, Roche et al. 1999) which support the role of relaxin in nipple development and facilitating birth by softening the cervix (Sherwood 2004). Male mice deficient in relaxin showed a decrease in fertility with disrupted spermatogenesis associated with an increased apoptosis of spermatocytes (Samuel, Tian et al. 2003). These phenotype observations can also be seen in RXFP1 knockout male mice (Krajnc-Franken, van Disseldorp et al. 2004).

1.3.2 Disease related functions

There is evidence to suggest that relaxin and RXFP1 can have multiple and diverse effects on several tissues and cells outside reproductive organs with significant therapeutic and clinical implications (Conrad and Novak 2004; Adams, Schott et al. 2012; Lee, Choi et al. 2012; Nair, Samuel et al. 2012).

1.3.2-1 Anti-fibrotic agent

One of the relaxin's actions that has been of increasing interest for researchers is its ability to stimulate the breakdown of collagen, a major component of all organs within the body. Relaxin not only stimulates collagen remodeling within the birth canal in preparation for parturition but also acts on cells and tissues to inhibit fibrosis. This is a process involving tissue scarring as a result of excessive collagen deposition that occurs in various internal organs, including lungs, liver, kidney and skin and causes loss of organ function. Relaxin may serve as a potential antifibrotic therapeutic agent. Recent *in vitro* and *in vivo* studies have confirmed that relaxin acts at multiple levels to inhibit fibrogenesis and collagen overexpression associated with fibrosis in several organs such as lung (Tang, Samuel et al. 2009) , kidney (Hewitson, Ho et al. 2010) , liver (Bennett, Heimann et al. 2009) and heart (Du, Xu et al. 2009). The general mechanism of relaxin for inhibiting fibrogenesis in these organs is inhibition of the activity of transforming growth factor β1 (TGF-β1), a potent profibrotic factor, and the down regulation of Smad signaling (Heeg, Koziolek et al. 2005; Mookerjee, Hewitson et al. 2009). This results in the decrease of collagen types I and III expression and promotes fibronectin degradation. Furthermore, relaxin alters the production and/or activity of matrix metalloproteinases (MMPs), their endogenous inhibitors called the tissue inhibitors of metalloproteinases (TIMPs) and other proteolytic enzymes to promote the ECM degradation (Unemori, Pickford et al. 1996; McDonald, Sarkar et al. 2003; Mookerjee, Unemori et al. 2005).

1.3.2-2 Cardiovascular protection

Heart and blood vessels are specific relaxin targets. Relaxin is a potent vasodilator of the systemic and coronary circulation (Teichman, Unemori et al. 2009). Several mechanisms for vasodilaton via relaxin have been reported. These include upregulation of the expression of the inducible NO synthase isoform in vascular smooth muscle cells which results in the stimulation of the production of endogenous NO (Failli, Nistri et al. 2002; Masini, Nistri et al. 2004), increasing the level at which MMPs modify the extracellar matrix of the vessel wall (Conrad and Novak 2004) and the activation of the PI3K-Akt-endothelial NOs (eNOS) vasodilatory pathway (McGuane, Debrah et al. 2011). In addition, relaxin has been shown to inhibit apoptosis in cardiomyocytes through the Akt/ protein kinase B (PKB) pathway (Moore, Tan et al. 2007). These effects suggest that relaxin may serve a potential theraputic role against cardiovascular disease (Bani and Bigazzi 2011). Currently, relaxin is in phase III clinical trials for the treatment of acute heart failure due to its ability to induce vasodilation and influence renal function (Teerlink, Metra et al. 2009).

1.3.2-3 Wound healing

The relaxin/RXFP1 system has been shown to be involved in the regulation of wound healing by increasing vasodilation together with organization of collagen around an injury site, reducing tissue granulation, inflammation, and necrosis at a wound site, and, finally, promoting angiogenesis (Stewart 2009; Brecht, Bartsch et al. 2011). The action of vascularization is through the local induction of VEGF and/or basic fibroblast growth factor (bFGF) as demonstrated in THP-1 cells *in vitro* and ischemic wound sites *in vivo* (Baylis and

Corman 1998). Moreover, the injection of relaxin improves muscle regeneration after traumatic injury by stimulating blood vessel and tissue development (Li, Negishi et al. 2005).

1.3.2-4 Neoplastic tissues

Relaxin and RXFP1 have been detected in various tumor types, including breast cancer, prostate cancer, thyroid cancer, colorectal cancer, endometrial cancer and lung cancer, with increased ligand-receptor gene expression in neoplastic tissues compared to normal healthy tissue in the same organ (Tashima, Mazoujian et al. 1994; Hombach-Klonisch, Buchmann et al. 2000; Alfonso, Nunez et al. 2005; Hombach-Klonisch, Bialek et al. 2006; Kamat, Feng et al. 2006; Silvertown, Ng et al. 2006). Relaxin and RXFP1 were also detected in neighboring normal stromal cells suggesting autocrine and paracrine functions for this ligand-receptor system in tumor tissues (Klonisch, Bialek et al. 2007). Relaxin/RXFP1 signaling has been linked to a number of critical tissue and cellular functions important for tumor growth, tumor angiogenesis and tumor cell migration/invasion. These events can be seen in various types of cancers presented in the following section.

Prostate cancer

The injection of relaxin producing PC-3 human prostate cancer cell line into mice increased growth of PC3 prostate tumor xenografts. Relaxin was also shown to generate an advanced angiogenic tumor phenotype that coincides with increased VEGF gene expression (Silvertown, Ng et al. 2006). RT-PCR analysis revealed significantly elevated relaxin gene activity in recurrent human prostate cancer tissues as compared to normal tissues. In the same study, stimulation with relaxin increased cell proliferation, invasiveness, and adhesion in both androgen-responsive LNCaP and androgen receptor–negative PC-3 human prostate

adenocarcinoma cells (Feng, Agoulnik et al. 2007). Opposite effects were observed after suppression of endogenous relaxin/RXFP1 expression (Feng, Agoulnik et al. 2007). Suppression of relaxin signaling in PC-3 human prostate cancer cells by RXFP1-specific small interfering RNA (siRNA) inhibited tumor growth *in vivo* through increased apoptosis and decreased proliferation (Feng, Agoulnik et al. 2010).

Breast cancer

In MCF-7 breast cancer cells, relaxin at low nanomolar concentrations, caused an increase in cell proliferation whereas stimulating the cells at high micromolar concentrations resulted in the inhibition of cell proliferation and changes in cell differentiation (Bani, Riva et al. 1994). Treatment with relaxin in two human breast cancers cell lines MCF-7 and SK-BR3 promoted *in vitro* invasiveness by increasing MMP levels in these cells (Binder, Hagemann et al. 2002). Furthermore, overexpression of relaxin in CF.33MT, canine mammary cell line, increased a significant migratory action but did not affect mitogenesis (Silvertown, Geddes et al. 2003). The concentration of relaxin in serum of patients with active metastasis breast cancer was found to be significantly higher than in a control population of healthy people and patients with other diseases, indicating the involvement of relaxin in promoting an invasion phenotype in human breast cancer patients (Binder, Simon et al. 2004).

Thyroid cancer

In two human follicular thyroid carcinoma cell lines, FTC-133 and FTC-238, recombinant relaxin and secreted prorelaxin caused an RXFP1-dependent increase in migration without affecting tumor cell proliferation (Hombach-Klonisch, Bialek et al. 2006). Injection of relaxin transfectants of human thyroid carcinoma cells (FTC133-RLN2) in the

nude mice caused large and fast-growing tumors with significantly increased number of proliferating cells. These grafted tumors contained multiple blood vessels, suggesting increased vascularisation. This was confirmed by the detection of endothelail cell marker CD31 in tumor sections suggesting that relaxin can enhance *in vivo* tumor growth and angiogenesis (Radestock, Willing et al. 2010). Recently, relaxin has been shown to be involved in the upregulation of MMPs and induce the formation of MMP-14-enriched invadopodia that lead to increased collagenolytic thyroid cancer cell invasion (Bialek, Kunanuvat et al. 2011).

Endometrium cancer

Similar to other types of cancer, high levels of relaxin have been detected by immunohistochemical tests in high-grade tumors with increased depth of myometrial invasion of emdometrium cancer patients. Quantitative RT-PCR showed significantly higher RXFP1 receptor expression in cancer samples. The same study showed that patients with high relaxin expressing tumors had a significantly shorter survival time (Kamat, Feng et al. 2006).

Intracellular pathways and mediators associated with the activation of the relaxin-RXFP1 in cancer

Major efforts have been undertaken to understand the role and molecular mechanism of the relaxin/RXFP1 system in tumor growth, angiogenesis and tumor cell migration/invasion.

Tumor cell growth and angiogenesis

Relaxin may utilize NO as a possible mechanism to promote tumor cell growth. In a concentration-dependent way, NO can either suppress or promote tumor cell growth. NO promotes tumor cell growth at high concentrations, whereas low levels of NO results in inhibiting tumor cell growth (Chinje and Stratford 1997). The other mechanism that is involved in relaxin's action in tumor growth is the PKA pathway. Relaxin has been shown to activate PKA in many cell types including the human MCF-7 and THP-1 cells (Parsell, Mak et al. 1996). Activation of PKA phosphorylates NF- κB and increases the transcriptional activity of NF- κB which promotes tumor growth (Zhong, SuYang et al. 1997). Impairing of PKA and NF- κB signaling was shown to inhibit relaxin-mediated castrate-resistant growth of prostate cancer cell lines and induce apoptosis (Vinall, Mahaffey et al. 2011).

Tumor growth depends on blood supply and blood vessel formation. Angiogenesis, thus, constitutes an important component in the control of cancer progression. (Kerbel 2008). The process of angiogenesis is regulated by several different pro- and anti-angiogenic factors. An imbalance between these factors could cause a tumor to switch to an angiogenic phenotype (Huang and Bao 2004). The action of relaxin in angiogenesis is thought to involve the upregulation of VEGF, one of the main pro-angiogenic factors. VEGF is also an enzyme required for NO synthesis, called NO synthase (NOS), to promote neovascularization and enhanced blood flow carrying oxygen and cellular nutrients to the tumor (Parsell, Mak et al. 1996; Silvertown, Ng et al. 2006).

Tumor cell invasion

The cellular pathways that involve the MMPs, a family of 23 zinc containing enzymes and their inhibitors called TIMPs, are important actors in the degradation of ECM

components. The roles of MMPs in cancer are numerous and include the following functions: (i) provide a suitable microenvironment for tumor cells to establish by remodeling of the ECM and cleavage of membrane bound growth factors which affect tumor progression (Kessenbrock, Plaks et al. 2010) (ii) activate pro-angiogenic factors such as bFGF and VEGF to promote angiogenesis (Egeblad and Werb 2002; Han, Fahd et al. 2011) , (iii) degrade basement membrane components associated with capillary and lymphatic vessels to help tumor cells invade the surrounding cells. MMPs also promote the migration of tumor cells by removing sites of cell adhesion and cleaving the cell-cell and cell-matrix receptor interactions (McCawley and Matrisian 2001). Relaxin upregulates the expression and/or secretion of collagenase 1 (MMP-1) and 3 (MMP-13), gelatinase A (MMP-2) and B (MMP-9), stromelysin1 (MMP-3) and transmembrane metalloproteases such as MMP-14 (MT1-MMP) in different cancer types. A broad spectrum of MMP-inhibitors abolished the effect of relaxin on the upregulation of MMPs expression indicating that there is an association between the upregulation of MMPs expression and relaxin-induced invasiveness in cancer cells (Binder, Hagemann et al. 2002; Kamat, Feng et al. 2006; Bialek, Kunanuvat et al. 2011).

The signaling cascades contributing to relaxin's modulation of MMPs expression and activity in cancer remain largely unknown. However, two recent studies have shown that relaxin signals through RXFP1via ERK1/2 signaling pathways to upregulate MMPs in fibrocartilaginous cells (Ahmad, Wang et al. 2012), human dermal fibroblasts and primary rat renal myofibroblasts (Chow, Chew et al. 2012). These finding suggested that ERK1/2 could be one of the signaling pathways that mediates the effect of relaxin on the upregulation of MMPs expression in cancer cells.

Lysosomal acid hydrolases of the cathepsin (cath) family is another relaxin target enzyme, which were identified in thyroid cancer that facilitates the action of the relaxin/RXFP1 system in tumor cell migration and invasion. Cathepsins are a class of proteases which are localized in lysosomes and classified into cysteine, serine, and aspartate hydrolases based on structure and crucial amino acid in the active site of the protease (Rawlings and Barrett 1999). A main physiological role for cathepsins is their involvement in the turnover of proteins delivered to the lysosomes. However, several members of the cathepsins have been implicated in cancer progression, in particular, the cysteine cathepsins-B and -L and the aspartate cathepsin-D. Relaxin significantly increased production and secretion of cathepsin-L and cathepsin-D. In thyroid cancer cells, both proteases contribute to cell migration/invasion through elastin matrices. Furthermore, relaxin has an impact on the intracellular distribution of procathepsin-L, as a different distribution of procathepsin-L in relaxin over-producing thyroid cancer cells was observed as compared to control cells (Hombach-Klonisch, Bialek et al. 2006).

S100A4 is also another major target molecule of relaxin in tumor cell migration/ invasion that has been identified recently. This protein is a member of the S100 family of calcium-binding proteins and directly involved in tumor metastasis in various human cancer types (Chen, Zheng et al. 2012; Dahlmann, Sack et al. 2012; Yang, Zhao et al. 2012). Human thyroid carcinoma cells when expressing relaxin or treated with recombinant human relaxin showed a strong increase in S100A4 production at both the transcriptional and protein level and promoted cell migration. The upregulation of S100A4 by relaxin also promoted xenograft angiogenesis. Thus, S100A4 is a major mediator of the actions of relaxin on

thyroid carcinoma cell motility and in vivo thyroid tumor angiogenesis (Radestock, Willing et al. 2010).

Our group have found that the small Rho-like GTPase proteins Rac1 and Cdc42 which are important for formation of laemellopodia and filopodial pseudopodia, respectively, were upregulated in relaxin-producing thyroid cancer cells and contributed to the high motility of these cells. In addition, Rac1 and Cdc42 activity is markedly increased in these relaxin producing clones (data unpublished). Both Rac1 and CdC42 act upstream of a signaling cascade which causes dynamic changes in the actin cytoskeletal organization and enhances cell motility, suggesting that relaxin/RXFP1 signals through Rac1 and Cdc42 to increase tumor cell motility by the formation of lamellopodia and filopodia at the leading edge of migration cells.

1.4 P74 peptide and Complement C1q tumor necrosis factor (TNF) related protein 8 (CTRPs) (C1q/TNF8 or CTRP8)

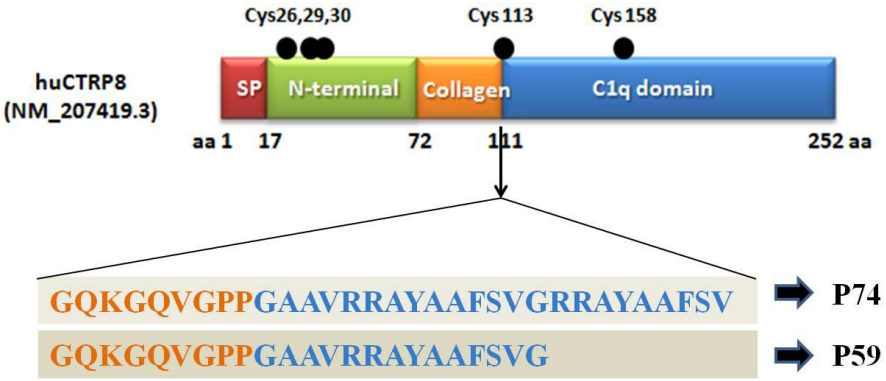
1.4.1 P74 is a short, linear peptide derived from the collagen domain of the CTRP8

As a result of a search for peptide agonists for the GPCR family, Compugen Ltd. (Tel Aviv, Israel) were able to identify multiple natural peptide ligands including two novel peptides derived from the same precursor, P74 (CGEN-25009) and P59 (CGEN-25010) that can activate RXFP1 and RXFP2 receptors (Shemesh, Shemesh et al. 2008). Further examination of P74 and P59 was done by testing for cAMP accumulation (Halls, Bathgate et al. 2006) as well as a GPCR activation assay. The two assays demonstrated that P74 and P59 treatment showed an increase in cAMP production with both RXFP1- and RXFP2-

transfected Chinese hamster ovary cells (CHO-K1) as compared to untransfected CHO-K1 cells, suggesting that these two peptides stimulate a cellular pathway through activation of RXFP1 and RXFP2 receptors (Shemesh, Hermesh et al. 2009).

Both P74 and P59 are short linear peptides derived from a collagen-containing domain of complement C1q tumor necrosis factor-related protein 8 Swiss-Prot ID; C1QT8_HUMAN (CTRP8). The sequences of these two peptides are similar to the CTRP8 with a slight modification to enhance stability and functionality (Shemesh, Shemesh et al. 2008). These two peptides share the same sequence, with the exception that P74 contains a duplication of a specific motif. While P74 is 33 amino acids in length, P59 is shorter by 10 amino acids from the C-terminus (Fig.1.4.1).

Figure 1.4.1 Sequences of P74 and P59 which are short linear peptides derived from a collagen-containing domain of the CTRP8 protein



Relaxin signaling through RXFP1 has been shown previously to induce a matrix degradation in human lung fibroblasts *in vitro* and inhibit pulmonary fibrosis *in vivo* (Unemori, Pickford et al. 1996). P74, a simple peptide agonist for RXFP1was used to investigate the anti-inflammatory and anti fibrotic activity of relaxin both in *in vitro* assays

and in an *in vivo* bleomycin-induced pulmonary fibrosis model. Similar to relaxin, P74 was found to inhibit the effect on TGF β1-induced collagen deposition in human dermal fibroblasts and also to enhance MMP-2 expression. The biological activity of P74 in the stimulation of cAMP, cyclic guanosine monophosphate (cGMP), and NO in the THP-1 human cell line was also similar to relaxin. In bleomycin-induced pulmonary fibrosis mouse model, administration of P74 in a preventive or therapeutic mode resulted in a significant reduction in lung inflammation and injury (Pini, Shemesh et al. 2010).These finding indicated that P74 might serve as a novel RXFP1 activating peptide ligand.

P74 is derived from C1q tumor necrosis factor (TNF) and related protein 8 (CTRP8). CTRP8 might also serve as a novel ligand for RXFP1 receptor for several reasons: (i) P74 and CTRP8 share a collagen and globular C1q domain, suggesting that either of these structures may be involved in RXFP1 binding; (ii) while P74 is being chemically synthesized as a peptide, CTRP8 is a secreted glycoprotein and could interact with RXFP1 in various tissue types. Therefore, CTRP8 is worthy of further attention and additional investigation as a possible novel ligand for RXFP1 receptor.

1.4.2 CTRPs family and CTRP8

The sequence homology between C1q tumor necrosis factor (TNF) and related protein (CTRP) family members and adiponectin, so called adiponectin paralogs, suggested an involvement of CTRPs in metabolism and immunity (Wong, Wang et al. 2004). Both CTRPs and adiponectin belong to the C1q/TNF protein superfamily (Kishore, Gaboriaud et al. 2004). Currently, fifteen members of the CTRPs (CTRPs 1 through 15) have been identified (Schaffler and Buechler 2012). They are secreted proteins circulating in plasma

and form homotrimers as their basic structural units (Pajvani, Du et al. 2003; Kishore, Gaboriaud et al. 2004). Unlike adiponectin which acts as an adipokine produced by adipocytes to monitor and control metabolism in the whole body, (Fasshauer, Paschke et al. 2004; Lihn, Pedersen et al. 2005) CTRPs are widely expressed by many different tissues and play important and diverse roles in various biological properties, including the immune, endocrine, skeletal, neuronal, vascular, and sensory systems (Kishore, Gaboriaud et al. 2004).

CTRP8 gene expression and structure

Peterson and his group identified and characterized a novel adiponectin paralog, known as CTRP8 (Peterson, Wei et al. 2009). The CTRP8 gene is expressed in humans and some vertebrates such as cat, squirrel, and bats. The CTRP8 gene is absent in the mouse genome and, thus, prevents gene deletion studies. The function of CTRP8 is still unresolved. In human tissue, CTRP8 transcripts are expressed in low levels; testis and lung are two major sites of CTRP8 expression in humans (Peterson, Wei et al. 2009). Like other members in the CTRPs family, the CTRP8 protein of 252-amino acids consists of four domains including a signal peptide (SP), an N-terminus with two conserved Cysteine residues, a collagen domain with fourteen Gly-X-Y repeats, and a C-terminal C1q globular domain (Fig. 1.4.1). The CTRP8 amino acid sequence is highly conserved among humans and other vertebrates sharing 57% to 98% identity. Among CTRP family members, CTRP1 and CTRP6 show the closest sequence homology to CTRP8. When CTRP8 is secreted from mammalian cells, it forms homotrimers (Schaffler and Buechler 2012).

1.5 Brain tumors

Overview

There are two main types of brain tumors: primary brain tumors originate in the brain itself, whereas secondary brain tumors are often referred to as brain metastases. Secondary brain tumors are made of cancerous cells from tumors located at distant sites in the body that metastasized to the brain (Schouten, Rutten et al. 2002). Our study focuses on primary brain tumors. The properties and characteristics of gliomas, in particular the glioblastoma multiforme (GBM) World Health Organization (WHO) classification grade IV are reviewed.

Primary Brain Tumors

The incidence of primary brain tumors worldwide is approximately 7 per 100,000 individuals per year (Furnari, Fenton et al. 2007). Brain tumors present as different histopathological types and can occur at any age. Brain tumors are the second most common type of childhood cancer after leukaemia and about 300 children are diagnosed with a brain tumor each year world-wide. GBM are more common in older people.

Types and grading of primary brain tumor

There are over 120 different types of primary brain tumors classified using an international standard introduced by the WHO on the basis of the cell type that tumors are derived from and the given grade according to histological appearances (Louis, Ohgaki et al. 2007). Primary brain tumors are generally named according to the cell of origin of the tumor. About half of all primary brain tumors develop from the cells that support the nerve cells of the brain, called glial cells. In contrast, some primary brain tumors grow outside the brain itself usually from the lining tissues (meninges), and are most frequently called meningiomas. The grading systems also consider a malignancy scale which is an indication of

aggressiveness to benefit treatment planning and predict outcomes for brain tumors. The grading of tumors varies from one (I) to four (IV), with one (I) being the most benign and four (IV) the most malignant.

Gliomas make up the majority of primary tumors which account for 30 – 40 % of all brain tumors. Gliomas originate from glial cells and tend to grow in the cerebral hemispheres but may also occur in the brain stem, optic nerves, spinal cord, and cerebellum. Various types of glial cells including astrocytes, oligodendrocytes and ependymal cells can develop three subgroups of glioma referred to as astrocytomas, oligodendrogliomas, and ependymomas respectively (Riemenschneider, Jeuken et al. 2010). Astrocytomas are the most common type of glioma in both adults and children. This type of glioma is divided into two main groups according to the WHO guidelines. The first group is focal astrocytomas including pilocytic astrocytoma (WHO grade I), which is more often diagnosed in children. The other astrocytomas are called diffuse astrocytomas since these do not have a clear boundary between the tumor and normal brain tissue. Depending on the degree of malignancy, diffuse astrocytomas can be subdivided as diffuse astrocytomas and variants (WHO grade II), anaplastic astrocytomas (WHO grade III), and glioblastoma and variants (WHO grade IV) (Huttner 2012). Oligodendrogliomas represent 5% to 6% of all gliomas. Tumors derived from oligodendrocytes include grade II (oliogodendrogliomas) and grade III neoplasms (oligoastrocytoma). This type of glioma is more slowly progressive and sensitive to chemotheraputics (Ohgaki and Kleihues 2005). Ependymomas originate from the ependymal cells that line the ventricles of the brain and the center of the spinal cord. Ependymomas are relatively rare tumors in adults, accounting for only 2-3% of primary brain tumors.

Glioblastoma multiforme (GBM)

WHO grade IV GBM is the most common and lethal type of malignant brain cancer in adults and accounts for more than 50% of primary malignant brain tumors (Schwartzbaum, Fisher et al. 2006). The current therapy for GBM involves total surgical resection followed by combination of radiation therapy and temozolomide (TMZ), chemotherapeutic drug that has been shown to prolong patient survival (Villano, Seery et al. 2009; Fialho, Salunkhe et al. 2012). Even with substantial progress of early diagnosis and a combined therapeutics approach, the mean survival time of adult patients with GBM is only about 15 months after diagnosis (Smith and Jenkins 2000; Stupp, Mason et al. 2005). GBMs are located preferentially in the cerebral hemispheres and spread along white matters tract. These tumors are more frequently found de novo (primary GBMs), without any evidence of a less malignant precursor lesion. Less that 10% of these tumors develop from lower-grade astrocytomas (WHO grade II) or anaplastic astrocytomas (WHO grade III), so called secondary GBMs (Winger, Macdonald et al. 1989). GBMs are characterized by extensive heterogeneity at the cellular and molecular levels. Microscopically, GBMs are composed of mixed pleomorphic tumor cells, ranging from small-cell tumors with scant cytoplasm to multinucleate giant cells. High mitotic activity, including atypical forms, are frequent along with neovascularisation, and an area of central necrosis surrounded by a highly proliferating peripheral region (Brat, Prayson et al. 2008). On the molecular level, GBMs are characterized by intense resistance to apoptosis and genomic instability (Furnari, Fenton et al. 2007). Recently the WHO subdivided GBMs into three variants including gliosarcomas, giant cell and small cell glioblastomas (Louis, Ohgaki et al. 2007).

Gliobalstoma and cell migration and invasion

One of the clinically most challenging hallmarks of malignant GBMs is their invasive behavior, which is characterized by rapid and infiltrative growth into the surrounding brain tissue and therefore enhances the destructiveness of the GBMs (Demuth and Berens 2004). This invasiveness is a major cause of death in GBM patients since no effective therapy is available. GBM tumor cells infiltrate the normal brain cells usually along blood vessels and white matter tracts, extending along either neuronal fiber perivascular spaces or subependymal spaces. This means they are often found at a long distance from the original tumor sites (Rutka, Apodaca et al. 1988). Another prominent characteristic of GBM invasiveness which makes this deadly tumor uncontrollable is that the speed of cell migration is higher than other types of cancer, up to 100 μm/h (Gerlee and Nelander 2012).

Invasion of GBM tumors is facilitated by migration and degradation of ECM barriers (Teodorczyk and Martin-Villalba 2010). Like other cancers, GBM tumor cell migration involves a complex series of events in a continuous cycle. The migrating cell becomes polarized upon the exposure to a chemoattractant molecule which leads to the formation of membrane protrusions at the leading edge. The leading front then binds to ECM proteins, inducing forward movement of the cell body and release/retraction of the rear part of the cells (Friedl and Brocker 2000; Yamaguchi, Wyckoff et al. 2005). The complicated process of GBM cell migration and invasion is stimulated by both autocrine and paracrine factors that act on a large array of cell surface-bound receptors to activate various signaling pathways to facilitate each mechanism (Hoelzinger, Demuth et al. 2007). Additionally, growth factors, adhesion molecule and ECM components are all implicated in GBM cell migration (Demuth and Berens 2004). In the initiation phase of cell migration, cytoskletal rearrangement is required to form membrane

protrusions including the extension at the leading edge of pseudopodia, lamellipodia, filopodia, and invadopodia. These membrane protrusions are the prerequisite for the onset and maintenance of cell motility in glioblastoma (GB) cells. Several recent reviews have highlighted the important role of each of these structures, particularly invadopodia with focal degradation of the ECM, in facilitating invasion of the brain (Stylli, Kaye et al. 2008; Sibony-Benyamini and Gil-Henn 2012; Yamaguchi 2012). There are many different actin regulatory proteins which include cortactin, Neural Wiskott-Aldrich syndrome protein (N-WASP), Actin-related protein complex (ARP2/3 complex) , cofilin, fascin and formin and are involved in the formation of cell protrusions (Lorenz, Yamaguchi et al. 2004; Yamaguchi, Lorenz et al. 2005; Lizarraga, Poincloux et al. 2009; Li, Dawson et al. 2010). Each actin regulator is controlled by Rho family GTPases, including Rac1, RhoA and Cdc42 which are key signaling elements that mediate receptor-initiated signaling in the regulation of glioblastoma invasion (Khalil and El-Sibai 2012). These GTPases regulate cell morphology and actin dynamics, and stimulate cell squeezing through narrow extracellular spaces that are typical of the brain parenchyma. Rho stimulates the formation of stress fibers and focal adhesion, while Rac is responsible for the laemellopodia formation. Cdc42 acts on the formation of filopodia and regulates cell polarity (Nobes and Hall 1999; Ridley, Schwartz et al. 2003). Attachment of cells to the ECM is also necessary for glioblastoma cell invasion, and mostly meditated by integrins. This adhesion molecule has been reported to initiate diverse intracellular signalling pathways including PI3K, Akt, mammalian target of rapamycin (mTOR) and MAP kinases to promote glioblastoma cell migration and invasion (D'Abaco and Kaye 2007; Weber, Parat et al. 2011). In order to degrade ECM barriers, GB cells secrete several classes of proteolytic enzymes including MMPs e.g. MMP-2, MMP-9 and MMP-14), a disintegrin and metalloproteinase (ADAMs), cathepsins and

urokinase-type plasminogen activator (uPA) (Levicar, Nuttall et al. 2003; Chetty, Vanamala et al. 2012). Each of these proteases may act alone, or together with the others to support the invasive behavior of brain tumor cells.

1.6 Rational for this study

The role of the relaxin/RXFP1 system on tumor cell migration and invasion has been shown for various types of cancer. Brain cancer is considered to be one of the most aggressive human cancers with a highly invasive nature that shortens the survival time of patients. So far neither relaxin nor RXFP1 expression has been studied in brain cancer, and the role of the relaxin/RXFP1 system in brain cancer is currently unknown. This is the first time that we demonstrate a possible link between brain cancer and the role of RXFP1 which is expressed in brain cancer patients. Our goal is to identify molecular pathways that facilitate the RXFP1-mediated increase in migration of brain cancer cells and relate these data to primary cells from brain cancer patients. This study contributes to a better understanding of the molecular mechanisms involved in brain tumor cell invasion and can lead to the identification of molecular targets for therapeutic intervention in the case of this devastating cancer.

1.7 Hypothesis and objectives

1.7.1 Hypothesis

RXFP1 enhances glioblastoma cell migration and brain tissue invasion.

1.7.2 Objectives

1. To study the bioactivity of RXFP1 in brain cancer cell migration/invasion
2. To determine RLN2/RXFP1-induced pseudopodial target proteins at the leading edge of migration
3. To determine the role of P74, a new peptide ligand for RXFP1in promoting migration in GB cells

CHAPTER 2: MATERIALS AND METHODS

Cell culture

Cell lines

Human glioblastoma astrocytoma cell lines U87MG, T98G, LN-18, A-172, LNT-229, U-251, and U-373 were propagated in Dulbecco's Modified Eagle's medium and F-12 1:1 (DME/F12, Hyclone, Thermo Scientific, Waltham, MA, USA) and 10% fetal bovine serum (FBS, Invitrogen, ON, Canada) and maintained at 37°C in a humidified atmosphere with 95% air and 5% CO_2. The cells were routinely passaged every 2 or 3 days.

Primary human brain tumor cells

Primary human brain tumor cells were isolated from surgically resected tumor specimens from brain tumor patients at the local Health Science Centre. The samples were obtained following informed consent, with approval from University and Pathology ethics boards. Tumor tissues derived from different histological types of brain cancer glioblastoma (GB), astrocytoma, meningioma, oligodendroglioma) were washed twice with sterile phosphate buffer saline (PBS) without calcium and magnesium, minced and tissue pieces were digested with 0.5 mg/mL collagenase and 10 µg/mL DNAse (Sigma, St Louis, MO, USA) for 20-40 min at 37°C in DME/F12 medium with frequent shaking. Enzymatic activities were inhibited by adding culture medium (DME/F12 containing 10% FBS) and digested tissue pieces were filtered through a 40 µm pore nylon mesh (BD Bioscience, San Diego, CA, USA) into a sterile conical tube. The mesh was washed once with culture medium and cells were centrifuged at 800 rpm for 5 min. The supernatant was removed and the cell pellet was

resuspended in hypotonic buffer (NH_4Cl, $KHCO_3$ and EDTA-Na_2) to lyse erythrocytes for 5 min at room temperature (RT) before adding sterile PBS and pelleting cells at 800 rpm for 5 min. The pellet was resuspended, seeded into 6-well plates, and grown in DME/F12 medium supplemented with 10% FBS, 1X penicillin/ streptomycin (Gibco/BRL, Grand Island, NY, USA) and 1X amphotericin B (Sigma, St Louis, MO, USA) and maintained at 37°C in a humidified atmosphere with 95% air and 5% CO_2.

Recombinant human relaxin and P74 peptide

Recombinant human relaxin (rhRLN2) was a generous gift from Corthera Inc. The P74 peptide used in experiments is derived from a modified protein sequence derived from Complement C1q tumor necrosis factor-related protein 8 (C1QT8_HUMAN) (Shemesh, Toporik et al. 2008). The peptides (P74 and scramble control) were chemically synthesized by EZBiolab (Carmel, IN, USA) and purity was verified by HPLC. Peptide sequences are:

P74 : GQKGQVGPPGAAVRRAYAAFSVGRRAYAAFSV

Scramble control: GSKMEGGSPGAPVQKRFFAFSVGRK

Total RNA Extraction and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from the human glioblastoma and astrocytoma cell lines and primary human brain tumor cells for RT-PCR analysis using the Trizol reagent (Invitrogen, ON, Canada) according to the manufacturers' protocols. RNA concentration was determined spectrophotometrically at 260 nm and 280 nm using a Nanovue spectrophotometer (GE Healthcare, ON, Canada). To assess the quality of total RNA samples, total RNA was separated by electrophoresis through a 1% agarose gel made in 1X Tris-acetate-EDTA (TAE) buffer containing 0.5 μg/mL ethidium bromide. Only samples with distinct 18S and 28S rRNA bands with a ratio of

approximately 2:1 showing no signs of degradation were considered for cDNAs synthesis. Total RNA (1μg) obtained from the human glioblastoma and astrocytoma cell lines and primary human brain tumor cells were used for cDNA synthesis. cDNAs were synthesized by using SuperScript II reverse transcriptase kit (Invitrogen, ON , Canada) and random hexamers (Promega, Madison, WI, USA). Briefly, 1 μg of total RNA was diluted to 0.1 μg/μL in a 10 μL volume with pure water followed by addition of 50 ng/μL random hexamers and 100 μmol/L deoxynucleotide triphosphate (dNTP) (Invitrogen, ON , Canada) and further incubation at 65°C for 10 min and subsequently chilling on iced water. Synthesis of cDNA was performed by adding RT master mix containing 5X First Strand buffer , 0.1 M DTT , and 200 unit/μL SuperScriptII reverse transcriptase. The reverse transcription reaction was incubated in a programmed thermal cycler under the following conditions: 25 °C 10 min, 42 °C 50 min, 72 °C 15 min, and cDNAs were stored at -20°C until used. All PCR reactions were carried out in 25μL (Table 2.1)

Table 2.1 PCR master-mix

Reagent	**One reaction volume (μL)**
Double distilled water (ddH_2O)	18.8
10X PCR Buffer, Minus Mg	2.5
50 mM Magnesium Chloride	1.0
100μmol/L dNTPs	0.5
20 nM Forward Primer	0.5
20 nM Reverse Primer	0.5
(5 U/μL) Taq DNA polymerase	0.2
cDNA	1
Total	**25**

For amplification of all transcripts of interest from cell lines and primary cells, the PCR cycles consisted of an initial denaturation step of 3 min at 95°C followed by 40 cycles of 1 min denaturation step at 95°C, annealing for 1 min at specific temperature (Table 2.2), and a 2 min elongation step at 72°C employing the PCR primer pairs (Table 2.2). Included in each PCR reaction was positive control cDNA from cell line or plasmid to increase confidence in negative PCR results. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also used in order to control for experimental variations in the amount of RNA used in RT-PCR. PCR products were separated on a 1% agarose gel in TAE buffer and visualized by ethidium bromide staining. The pictures of the gel were documented by a FluorChem-8900 chemiluminescence and gel imager (Alpha Innotech Corp, San Leandro, CA).

Table 2.2 Primer sequences used for RT-PCR

Target gene	Primer	Primer sequence from 5' to 3'	Annealing temperature (°C)	Size of product (bp)
RLN1	Forward	TCTGTTTACTACTGAACCAATTT	55	485
	Reverse	TTAGGCAACATTTCTCAAACAG		
RLN2	Forward	TCTGTTTACTACTGAACCAATTT	55	485
	Reverse	CATGGCAACATTTATTAGCCAA		
RLN3	Forward	ACGGGGTCAGGCTTTGCGGC	55	266
	Reverse	TCGGCTGCCCCGAAGAACCCC		
RXFP1	Forward	ACTTCCCAATATCCTTTTGAGGCA	60	492
	Reverse	CATGTGTTGACAGAGAGGTTTATC		
RXFP2	Forward	ATTCAAAGACTTACATCAGCTAAC	60	363
	Reverse	AAAAGGTGAGGTGATAGTTCCG		
CTRP8	Forward	ACGGCCCACTATAGACATCGAA	58	350
	Reverse	TGTAGTTCCAGGTGTGCACGTT		
GAPDH	Forward	CATCACCATCTTCCAGGAGCG	60	443
	Reverse	TGACCTTGCCCACAGCCTTG		

Abbreviations: bp, base pairs

BrdU assay

Cells were seeded at a density of $4x10^3$ cell per well in DME/F12 medium with 10% FBS in 96-well tissue culture plates and left overnight at 37°C. The medium was changed to serum- free medium and cells were incubated for additional 24 h at 37°C prior to rhRLN2 treatment. Next day the medium was removed and replaced with serum-free medium supplemented with rhRLN2 (100

pg/mL = 17 pM, and 100 ng/mL = 17 nM). Each concentration was tested in triplicate . Twenty-four hours after the addition of rhRLN2, a colorimetric BrdU cell proliferation ELISA (Roche Diagnostics, Canada) was used according to the manufacturer's instructions. Ten µL of BrdU labeling solution was added to each well of the 96-well plate. Cells were incubated for 3.5 h at 37°C in a water-saturated CO_2 incubator. Thereafter, plates were drained, fixed for 30 min and 100 µL of BrdU antiserum was added to each well. Plates were incubated for 60 min at RT, drained, washed, and incubated with 100 µL/well of substrate solution for 10 min at RT. Finally, 25 µL of 1M H_2SO_4 was added to each well, incubated for 1 min on a shaker at 300 rpm, and absorbance was measured within 5 min at 450 nm in an ELISA reader (Perkin Elmer, Boston, USA).

Cell viability assay (WST assay)

The WST assay measures dehydrogenase activity in mitochondria and is generally used as a measure of proliferation (Holthaus, Treccani et al. 2012). Both, the proliferation of the T98G cells upon relaxin treatment and the cytotoxicity of P74 peptide were assessed using the reagent WST (Roche Diagnostics, Canada). Cells were seeded and serum starved according to the same procedure as described for the BrdU assay. After treatment for 24 h with different concentration of rhRLN2 (100 pg/mL= 17 pM, and 100 ng/mL=17 nM) and P74 peptide (0.5 nM, 1nM, 5 nM, 10 nM, 100 nM and 3 µM), WST was applied and cells were incubated according to the instructions by the manufacturer (Roche Diagnostics, Canada). Conversion of WST to formazan was measured at 450 nm by microplate spectrophotometry (Perkin Elmer, Boston, USA). Untreated cells were defined as 100% viable cells.

Motility Assay

Before the motility experiments, cells were seeded at 30% confluence with DME/F12 medium with 10% FBS and left overnight at 37°C. The next day, cells were washed twice with 1X PBS and grown in DME/F12 medium with 1% FBS 24 h prior to the motility assay. Cellular motility and migration were evaluated in 24-well Transwell chambers (Costar, Corning, NY).The upper and lower culture compartments were separated by polycarbonate filters with 8-µm pore size. A total of $1x10^4$ cells (at 70% confluency) were put in DME/F12 medium plus 1% FBS and placed on the upper chamber of the filters. The following substances were added to the wells (lower chamber): either human recombinant RLN2 at 100 pg/mL (17pM), and 100 ng/mL(17nM) and P74 peptide (3µM) dissolved in DME/F12 medium with 1% FBS. DME/F12 medium containing 1% FBS served as a negative control and was placed in the bottom of the chamber. Migration was allowed to proceed for 24 h at 37°C in a humidified incubator at 5% CO_2, 20% O_2 atmosphere. Thereafter, cells on top of the filter were wiped off with cotton swabs and migrated cells that had traversed the membrane pores to the lower surface of the membrane were washed with chilled PBS, incubated for 5 min in 1:1 PBS/methanol (EMD, Quebec, Canada) and 10 min in methanol before staining with 0.1% toluidine blue in 2.5% sodium carbonate (Sigma, St Louis, MO, USA). Migrated cells were counted under the light microscope (Olympus, Markham, ON, Canada) at 10-fold magnification in five separate high-power fields per filter. Experiments were performed in triplicates.

Scanning Electron Microscopy (SEM)

The day before the experiment, cells were washed twice with 1X PBS and grown in DME/F12 medium with 1% FBS overnight. To allow the migrating cells to develop pseudopodia, experiment was performed in 6-well plates with insert filters containing 3.0 µm porous track-etched

polyethylene terephthalate membranes cell culture inserts (BD Bioscience, San Diego, CA, USA). Cells ($7.5X10^5$) were placed into the upper filter compartment and incubated with the companion plate in a 5%, 20% O_2 CO_2 atmosphere at 37 °C for 1 h to allow the tips of pseudopodia to penetrate the pores and reach the underside of the filters. Cells on both sides of the filter were rinsed with PBS, fixed with Karnovskys Fixative (4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2; all Sigma) for 1h at RT. Filters were further fixed for 1h at RT each in 1% OsO_4 and 2% uranyl acetate. Upon fixation, the filters were then prepared for SEM examination by dehydration in a series of graded ethyl alcohol solutions (50%, 70%, 95%, and 100%). The final dehydration step was accomplished in 100% methanol (gradient grade, EMD chemicals, ON, Canada). The filters were cut into small pieces, sputter-coated with gold-palladium (Edwards S150B, West Sussex, UK) and subsequently examined in a Cambridge Stereoscan 120 SEM (Cambridge, UK) with EDAX Genesis imaging software (Mahwah, New Jersey, USA). Images of cells on both sides of the filters were taken at 20 kV using various magnifications.

Pseudopodia assay

Pseudopodia assays were performed with slight modifications as described previously (Cho and Klemke 2002). Before the assay, U87MG GB cell lines were washed twice with 1X PBS and grown in DME/F12 medium with 1% FBS overnight. The next day, U87MG cells were pre-treated for 24 h with human recombinant RLN2 100 ng/mL or DME/F12 medium with 1% FBS (control). Purification of pseudopodia was performed in 6-well plates with insert filters containing 3.0 μm porous track-etched polyethylene terephthalate membranes (BD Bioscience, San Diego, CA, USA). Each filter was seeded with $7.5X10^5$ cells on the upper chamber and incubated in a 5% CO_2, 20% O_2 humidified incubator at 37°C for 1 h to allow the tips of pseudopodia to reach the underside of the

filter. This was done in the absence and presence of rhRLN2 at 100 ng/mL dissolved in DME/F12 medium with 1% FBS and added to the lower chamber only. After 1h, pseudopodia were isolated by dipping the underside of each filter in 100 µL of lysis buffer (50mM Tris-HCl, pH7.4, 3mM NaCl , 2mM $MgCl_2$, 0.1%SDS , 0.1 % NP-40 (Igepal), protease inhibitor cocktail; Sigma, St Louis, MO, USA) for 10 seconds. The remaining cell body was collected by adding 100 µL of lysis buffer onto the top of each filter. Pseudopodia and cell body lysates from ten filters were pooled separately and subjected to three freeze/ thaw cycles (dry ice for 3 min and thawed at 37°C for 1 min). Lysates were centrifuged at 13,000 x g for 20 min at 4°C and supernatants of pseudopodia lysates were dried in a speed vacuum (Thermo Fisher Scientific, Mississauga ON, Canada), resuspended in double distilled water to make a 10x concentrated pseudopodia supernatant and protein concentration was measured using the Micro BCA Protein Assay Kit (Pierce, ON, Canada).

Proteomic analysis

Pseudopodia for proteomic analysis were obtained from untreated and RLN2-treated U87MG cells as described in the pseudopodia assay section. Proteomic analysis was carried out at the Manitoba Centre for Proteomics and Systems Biology using two dimensional high performance liquid chromatography - mass spectrometry analysis (2D LC-MS/MS). The workflow and experimental design for Proteomics analysis performed in this study are shown in (Fig.2.1).

Sample preparation: To determine the quality of samples preparation, 15 µg of pseudopodia lysates from untreated and RLN2-treated U87MG cells were loaded and separated on a precast 4–12% bis-Tris-acrylamide gradient gel (Invitrogen, ON, Canada). The gels were stained using Gel-Code Blue Coomassie Pierce, ON, Canada). Each 100 µg protein sample was adjusted to 100 µL with 100 mM ammonium bicarbonate buffer. Samples were reduced with 10 mM dithiothreitol (DTT) for 40 min

at 56°C followed by alkylation using 50 mM iodoacetamide (IAA) for 30 min at RT. Excess IAA was neutralized by the addition of 17 mM DTT. Proteins were digested with sequencing-grade trypsin (Promega, Madison, WI, USA) at a 1:50 enzyme: substrate ratio overnight at 37°C. Samples were frozen at −80 °C and dried using a SpeedVac. Trypsin-digested peptides were purified using a reversed-phase Scalar C-18 (1 x 100 mm, 5 μm, 100 Å) column (Agilent Technologies, Inc., Santa Clara, CA, USA).

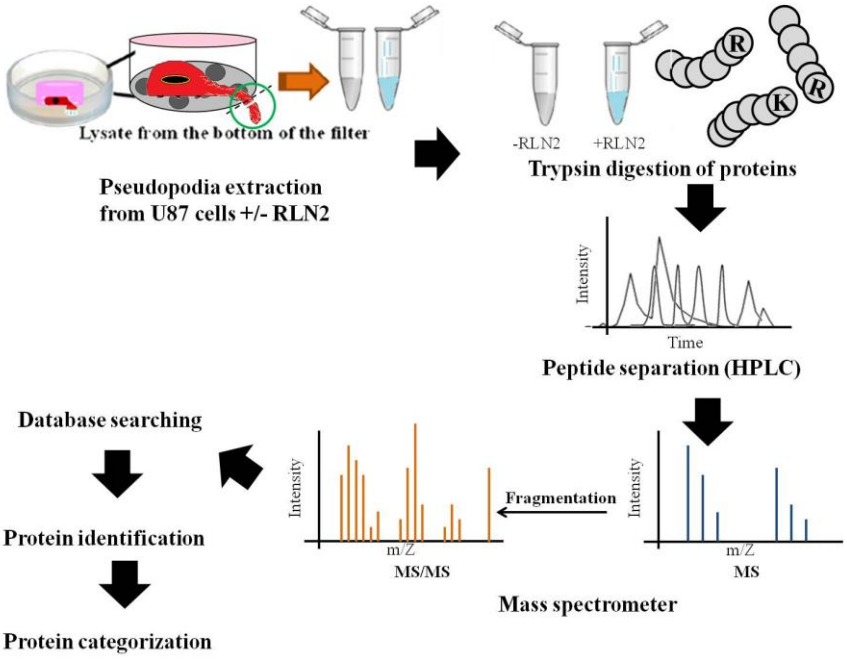
2D LC-MS/MS: Peptide digests (100 μg) were purified using a two dimensional LC method (Dwivedi, Dhindsa et al. 2009). Briefly, dried peptide samples were fractionated in the first dimension employing a high-pH (pH10) reversed-phase separation on C18 X-Terra column (1 x 100 mm, 5 μm, 100 Å; Waters Corporation, Milford, MA,USA) with an Agilent 1100 series high performance liquid chromatography (HPLC) system. Both eluents A (water) and B (90% acetonitrile) contained 20 mM ammonium formate buffer (pH 10.0). Using a gradient of 1% to 40% of solvent B (1% acetonitrile/min, 150 μL/min flow rate), twenty fractions were collected from the C18 X-Terra column separation, vacuum-dried, re-dissolved and applied to second dimension separation using a splitless nano-flow Tempo LC system (Eksigent, Dublin, CA) with sample injection via a PepMap100 trap column (0.3 x 5 mm, 5 μm, 100 Å, Dionex, Sunnyvale, CA). A 100 μm x 150 mm analytical column packed with 5 μm Luna C18(2) was used prior to MS/MS analysis. Both eluant A (2% acetonitrile in water) and eluant B (98% acetonitrile) contained 0.1% formic acid as ion-pairing modifier. A 0.35% acetonitrile/min linear gradient (0–35% B in 100 min, 500 nL/min) was used for peptide elution, followed by a 5-min wash with 80% eluent B. A QSTAR Elite QqTOF mass spectrometer (Applied Biosystems, Concord, ON, Canada) was used in standard MS/MS data-dependent acquisition mode with a nano-electrospray ionization source. Data acquisition in QSTAR Elite was set to positive ion mode using Analyst® QS 2.0 software (Applied Biosystems). Survey

MS spectra were collected (m/z 400 to 1500) for 1 sec followed by three MS/MS measurements on the most intense parent ions (80 counts/s threshold, +2 to +4 charge state, and m/z 100 to 1500 mass range for MS/MS), using the manufacturer's 'smart exit' setting. Parent ions previously targeted were excluded from repetitive MS/MS acquisition for 60 sec (mass tolerance of 50 mDa).

Database searching and protein identification: The MS/MS data were searched by two database search engines: ProteinPilot (PP) software version 2.0.1 (Applied Biosystems/ MDS Sciex, Concord, ON, Canada) which employs the Paragon™ search algorithm and The Global Proteome Machine (GPM) Version 2.0.0.4 (Beavis Informatics Ltd., Winnipeg, MB, Canada)(Craig and Beavis 2004) for peptide matching and protein identification. Search parameters were as follows: (i) Trypsin was selected as the enzyme (ii) one missed cleavage allowed; (iii) fixed modification, carbamidomethylation of cysteines; (iv) variable modification, oxidation of methionine; (v) peptide tolerance, 3.0 Da; and (vi) MS/MS tolerance, 0.4 Da. Those protein candidates with greater than or equal to 95% identification confidence were used for further analysis.

Protein Categorization: The identified proteins were classified based on the Protein Analysis Through Evolutionary Relationships (PANTHER) system (http://www.pantherdb.org), a unique resource that classifies genes and proteins by their functions. The PANTHER ontology, a highly controlled vocabulary (ontology terms) by biological process, molecular function and molecular pathway was used to categorize proteins into families and subfamilies with shared functions (Thomas, Campbell et al. 2003).

Figure 2.1 Outline of the experimental workflow for identifying candidate proteins at the leading edge involved in relaxin/RXFP1-induced migration in U87 GB cells. Pseudopodial proteins were isolated from rhRLN2-treated and untreated U87 cells. Protein samples were digested into peptides using trypsin. The peptides were separated by HPLC, eluted and transferred into a mass spectrometer. The mass spectrum (MS) revealed the masses and intensities of peptides eluting from the column at any given time. Fragmentation of individual peptides revealed the fragment MS/MS spectra, which contains information about the peptide sequence. The data from MS/MS spectra were used to identify peptides and identify the corresponding proteins using bioinformatic tools. Finally the identified proteins were categorized according to their molecular function.



Western blot analysis

For detection of target proteins, cells were seeded at 30% confluence ($1X10^5$ cells/ mL) with DME/F12 medium with 10% FBS and grown overnight at 37°C. Cells were washed twice with 1X PBS, grown in DME/F12 medium with 1% FBS for 24 h and then treated with either human recombinant RLN2 (100 pg/mL and 100 ng/mL) or P74 peptide (3µM) dissolved in DME/F12 medium with 1% FBS for 24 h prior to isolation with 250 µl of lysis buffer (50mM Tris-HCl, pH7.4, 3mM NaCl , 2mM $MgCl_2$, 0.1%SDS , 0.1 % NP-40 (Igepal), protease inhibitor cocktail; Sigma, St Louis, MO, USA). Protein concentration was determined by BCA Protein Assay Kit (Pierce, ON, Canada) prior to storage at -80^{o}C until Western blot analysis. Samples were incubated at 90°C for 5 min. Depending on the size of the protein of interest, 30 µg of whole cell lysate of each sample was loaded on a 10-15% SDS-PAGE gel. As a standard, 5 µl of Precision Plus Protein all blue standards (Thermo Fisher Scientific, ON, Canada) was loaded. After SDS-PAGE, gels were transferred to nitrocellulose membrane in transfer buffer (500mM glycine, 50mM Tris-HCl, and 20% methanol) at 100 volt for 1 h. Membranes were blocked with Tris-buffered saline (TBS/ 0.01% Tween 20 (TBST) plus 5% skimmed milk) for 1 hr at RT. All primary antibodies (Table 2.3) were dissolved in 5% milk in TBST and incubated with the membranes overnight at 4°C on a rotator. The next day, membranes were washed with TBST three times for 5 min prior to incubation with the appropriate secondary antibodies (Table 2.4) coupled to horseradish peroxidase (HRP) for 1 h at RT on a rotator. After washing the membranes 3X 5 min, signals were visualized using ECL reagent (Pierce) according to the manufacturer's instructions. Blots were exposed to an x-ray film and developed. If necessary, blot was stripped with stripping buffer (200 mM glycine adjusted p H to 2.5, and 0.005% Tween20) for 15 min and probed again.

Table 2.3 Primary antibody used for Western blot analysis

Primary Antibody	Dilution	Source	Expected size
PGRMC1	1:2000	#ab80941 Abcam, Canada	25kDa
TfR1 clone H68.4	1:500	# 13-6800 Invitrogen, Canada	85kDa
MMP-14	1:2000	#ab51074 Abcam, Canada	66 kDa
Cdc42	1:1000	#2462 Cell signaling technology	21kDa
Rac1	1:1000	#2467 Cell signaling technology	21kDa
Cathepsin-B	1:500	Dr. E. Weber-Martin Luther University, Halle ,Germany	* proform (40 kDa) ** heavy chain (HC) (30 kDa)
Procathepsin-L clone 2D4	1:500	Dr. E. Weber-Martin Luther University, Halle ,Germany	* proform (43 kDa) **single-chain(31kDa) *** heavy chain (HC) (24 kDa)
Cathepsin-D	1:500	Dr. E. Weber-Martin Luther University, Halle ,Germany	* proform (52 kDa) **heavy chain (HC) (25 kDa)
β-actin	1 :10000	#A5441 Sigma	43 kDa

Table 2.4 Secondary antibodies used for Western blot analysis

Secondary Antibody	Dilution	Source
Goat anti rabbit-HRP conjugated	1:10000	# 111-005-045 Jackson Immuno Research Laboratories
	1:2000	# 7074 Cell Signaling technology
Goat anti mouse –HRP conjugated	1:10000	#A5278 Sigma

Densitometry analysis of immunoblots

Densitometric analysis of bands was carried out with the ImageJ software (Abramoff et al., 2004). The gel analysis tool was used to obtain the absolute intensities for each experimental protein band and corresponding Beta actin band served as a loading control. The relative intensities were calculated from the ratio of protein bands to Beta actin absolute intensities.

Statistical analysis

The statistical analysis was carried out with the SPSS program, version 14.0 for Windows and Excel software. To test differences among treatments, one way analysis of variance (One way-ANOVA) was applied. When this test determined statistically significant differences, a posthoc comparison was made using the Tukey's test. Analyses with a confidence level of 95% ($p<0.05$) were considered significant.

CHAPTER 3: RESULTS AND FIGURES

3.1 *RXFP1* is expressed in human primary brain tumor cells and GB cell lines

We have established a cell bank of primary cells derived from patients with brain tumors in collaboration with the Department of Neurosurgery. In the present study, 32 cases of human primary brain tumor cells with known clinical pathology (Table 3.1) were selected. Total RNA was extracted to determine the expression of *RLN2* and *RXFP1* using RT-PCR analysis. We also checked for the expression of *RLN2* and *RXFP1* in several established GB cell lines (Fig. 3.1) and human astrocytes. We found that *RXFP1* was expressed in 15 of 32 (46.88%) primary cells and 4 of 7 (57.14%) GB cell lines, while human astrocytes showed very weak *RXFP1* expression. In both human primary cells and GB cell lines, *RXFP1* showed different expression levels (Fig. 3.1). The clinical pathology of the 15 cases of RXFP1 positive primary GB cells showed that 10 of 15 (66.67%) were glioblastoma WHO grade IV, 3 of 15 (20%) were recurrent cases of glioblastoma WHO grade IV, and 2 of 15 (13.33%) were meningioma. For two RXFP1 positive meningioma cases, one case was a meningioma WHO grade I, while the other case was an invasive meningioma WHO grade II. Rapid progression in growth and invasiveness in GB tumor growth may be linked to RXFP1 induction as exemplified in the one case of PBS-12 primary GB cells. Here, post-operatively the tumor progressed rapidly within 10 days (PBS-12.1; second operation) (Fig. 3.1A). This coincided with an increased expression of *RXFP1*. In contrast to *RXFP1* expression, none of the human primary brain tumor cells and GB cell lines expressed *RLN2* transcripts (Fig. 3.1). Although RXFP1 is the main and most specific receptor to H2-relaxin, both H1- and H3-relaxin are also known to show weaker interaction with RXFP1 (Hsu, Nakabayashi et al. 2002; Sudo, Kumagai et al. 2003; van der Westhuizen, Halls et al. 2008). Evidence shows that RLN2 induced both RXFP1 and RXFP2

receptors to stimulate cAMP production in cultured cells (Hsu, Nakabayashi et al. 2002; Hsu, Nakabayashi et al. 2003) indicating that circulating RLN2 can signal through both of these receptors *in vivo* to generate physiological actions. This information led us to investigate the expression of *RLN2* and *RXFP1* and the expression of the other relaxin isoforms *RLN1* and *RLN3* as well as RXFP2 in the human primary brain tumor cells, GB cell lines, and human astrocyte to determine a complete expression profile of this ligand-receptor system. None of the cases of primary cells, GB cell lines and human astrocytes expressed *RLN1, RLN3* and *RXFP2* transcripts (data not shown). In this study, we selected two RXFP1 positive human GB cell lines (U87MG and T98G) and some of RXFP1 positive human primary GB cells (PBS-1, PBS-10 and PBS-12.1) to study the function of RXFP1.

Table 3.1 Histopathological diagnosis of all 32 studied brain tumor cases correlates with *RXFP1* expression detected using RT-PCR

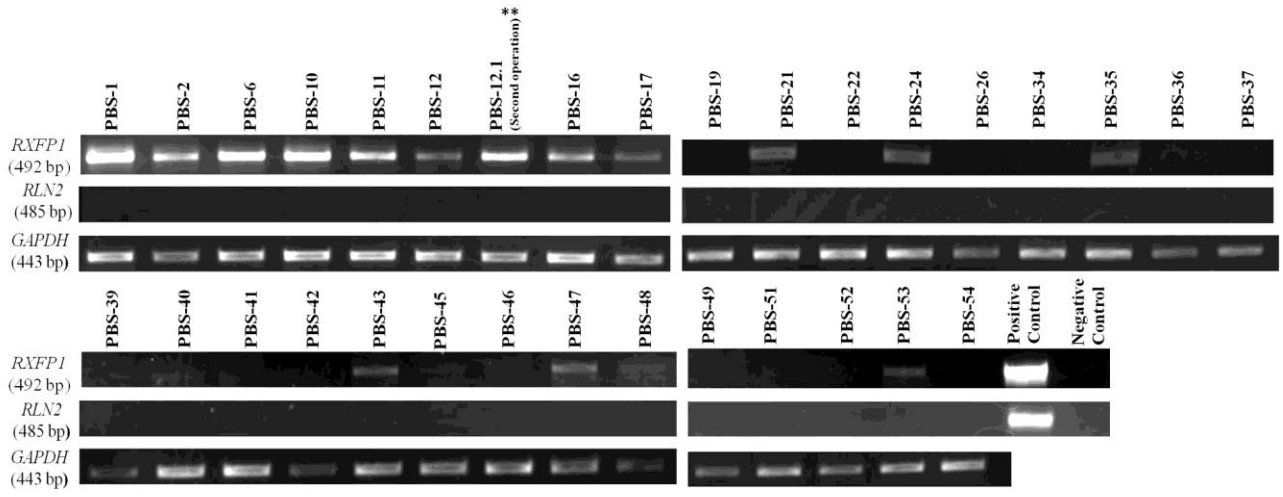
Patient ID	Histopathological diagnosis	RXFP1 expression
PBS-H2009:328-1	GB with oligodendroglial component	+
PBS-H2009:328-2	GB	+
PBS-H2009:328-6	GB (small cell variant)	+
PBS-H2009:328-10	GB	+
PBS-H2009:328-11	GB recurrent with oliogo component	+
PBS-H2009:328-12	GB (cyst)	+
PBS-H2009:328-12.1	GB (second operation)	+
PBS-H2009:328-16	GB	+
PBS-H2009:328-17	GB	+
PBS-H2009:328-19	GB	0

PBS-H2009:328-21	GB (recurrent)	+
PBS-H2009:328-22	Oligodendroglioma WHO gradeII, increased proliferative index and reactive changes	0
PBS-H2009:328-24	GB (recurrent)	+
PBS-H2009:328-26	GB	0
PBS-H2009:328-34	GB	0
PBS-H2009:328-35	GB	+
PBS-H2009:328-36	Meningioma WHO grade I	0
PBS-H2009:328-37	Oligodendroglioma WHO gradeII increased proliferative index	0
PBS-H2009:328-39	Atypical Meningioma; WHO grade II	0
PBS-H2009:328-40	GB	0
PBS-H2009:328-41	GB	0
PBS-H2009:328-42	Anaplastic Astrocytoma; WHO grade III	0
PBS-H2009:328-43	Meningioma with brain invasion; WHO grade II	+
PBS-H2009:328-45	Meningioma WHO grade I	0
PBS-H2009:328-46	GB	0
PBS-H2009:328-47	Meningioma WHO gradeI with increased proliferative index	+
PBS-H2009:328-48	GB with oligodendroglial component	0
PBS-H2009:328-49	GB	0
PBS-H2009:328-51	GB	0
PBS-H2009:328-52	Meningioma; WHO grade I, with increased proliferative index	0
PBS-H2009:328-53	GB	+
PBS-H2009:328-54	GB	0

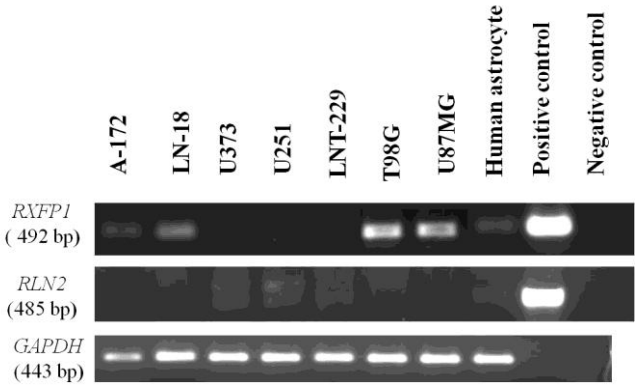
Abbreviations: PBS stands for Primary Brain tumor Study; GB: Glioblastoma multiforme; WHO: The World Health Organization; (0) indicates no expression of RXFP1 transcripts; (+) indicates expression of RXFP1 transcripts

Figure 3.1 Expression of *RXFP1*and *RLN2* in (A) human primary brain tumor cells and (B) in GB cell lines by RT-PCR. (A) None of the human primary brain tumor cells expressed *RLN2*. However, human primary brain tumor cells expressed *RXFP1* receptor at different levels. In one case (PBS-12), the onset of *RXFP1* expression correlated with fast progression of the GB. Primary PBS-12 GB cells showed a higher expression of *RXFP1* after 10 days of rapid progression (PBS-12.1) indicated by asterisk (**). (B) None of the GB cell lines expressed *RLN2* where as four GB cell lines, A-172, LN-18, T98G, and U87MG expressed *RXFP1*. While T98G and U87MG showed a strong expression of *RXFP1*, A-172, and LN-18 showed weak expression of the receptor. Human astrocytes showed weak expression of *RXFP1*.

A



B



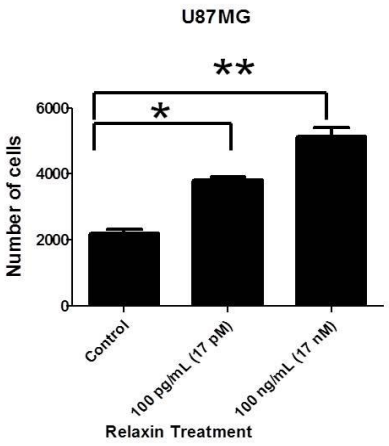
3.2 Recombinant human RLN2 (rhRLN2) increases cell motility of GB cell lines and primary GB cells

Transwell filter assays were employed to investigate the role of RXFP1 in rhRLN2-induced U87MG, T98G and PBS-10 cells motility. The upper and lower culture compartments were separated by filters with an 8 μm pore size. The cells were seeded into the upper chamber and incubated for 24 h in the absence (control) and presence of two different rhRLN2 concentrations. rhRLN2 at 100 pg/ mL (17 pM) and 100 ng/ mL (17 nM) were added as a chemoattractant in the lower chamber. Treatment of cells with rhRLN2 resulted in a significant increase (2–4-fold) in cell motility in U87MG and T98G and PBS-10 primary GB cells, although the magnitude of change was greater in U87MG cells. In U87MG cells, both 100 pg/ mL and 100 ng/ mL of rhRLN2 significantly increased cell motility in a concentration-dependent manner with P=0.002 and P=0.001, respectively (Fig. 3.2-1A). rhRLN2 at 100 ng/ mL significantly increased cell motility in T98G (Fig. 3.2-1B) and PBS-10 (Fig. 3.2-1C) with P=0.02 and P=0.005, respectively. Similar to U87MG cells, we observed the concentration-dependent increase in cell motility in PBS-10, while we did not observe this effect in T98G. Thus, we determined that rhRLN2 was highly effective in promoting migration of U87MG, T98G and also PBS-10 cells suggesting a functionally intact RXFP1 was expressed in these GB cells. These results also indicated that RXFP1 expression plays a role in GB cell motility and led us

to explore the potential mechanisms for this effect. To exclude a rhRLN2-induced increase in cell proliferation and cell number to be responsible for the increased number of migrated cells, two different proliferation based assays were employed. We performed BrdU assays on U87MG cells and WST assays on T98G cells. After 24 h exposure to rhRLN2, we found no significant increase in cell proliferation in U87MG (Fig. 3.2-2A) or T98G (Fig. 3.2-2B). Our results suggest that rhRLN2 does not act as a growth factor in U87MG and T98G cell lines and confirms that the higher number of migrated cells counted following rhRLN2 treatment was not due to an increase in cell proliferation.

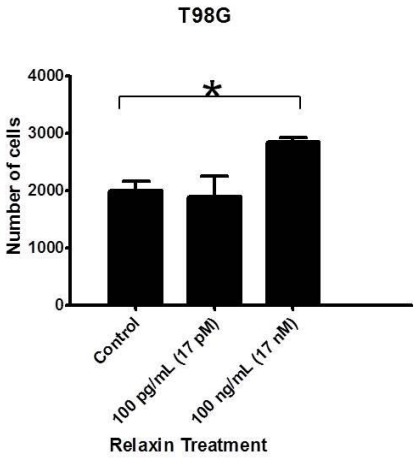
Figure 3.2-1 Effect of rhRLN2 on cell motility. Cell motility in U87MG, T98G and PBS-10 cells was assessed using transwell chambers. All U87MG, T98G and PBS-10 cells displayed an increase (2–4-fold) in cell motility. (A) 100 pg/ mL and 100 ng/ mL of rhRLN2 significantly increased motility in comparison to untreated U87MG cells. (B) only 100 ng/ mL of rhRLN2 significantly increased cell motility in T98G and (C) PBS-10. The results shown are representative of three separate experiments with three filter sets per experiment and are expressed as mean ± S.E.

A



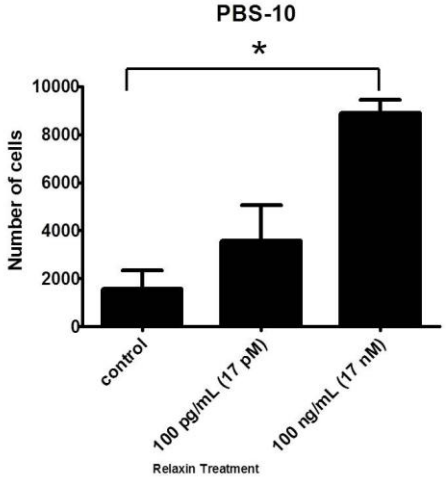
*P<0.01, ** P<0.01

B



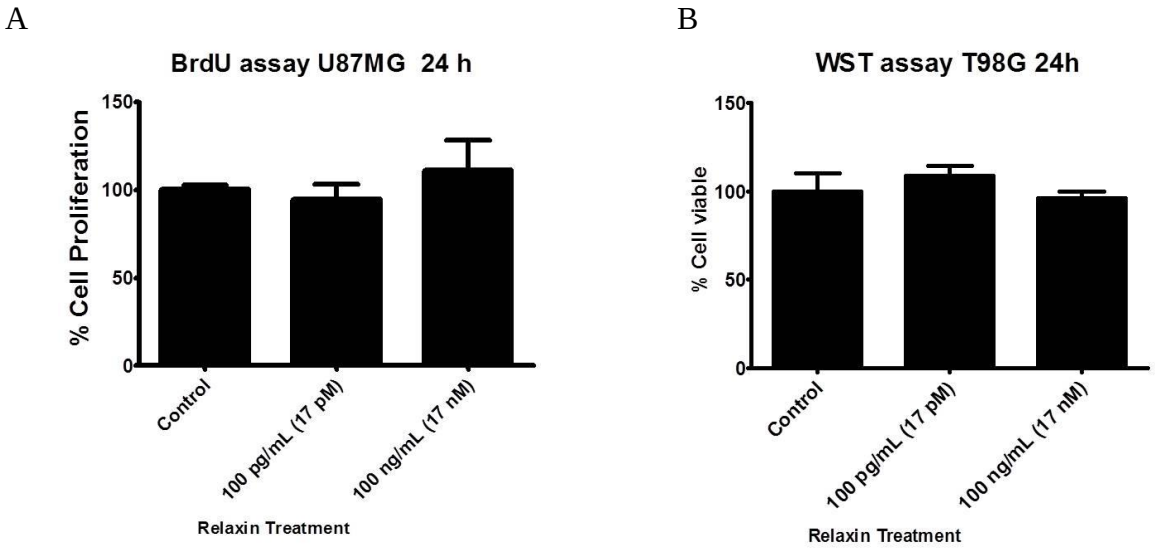
*P<0.05

C



*P<0.01

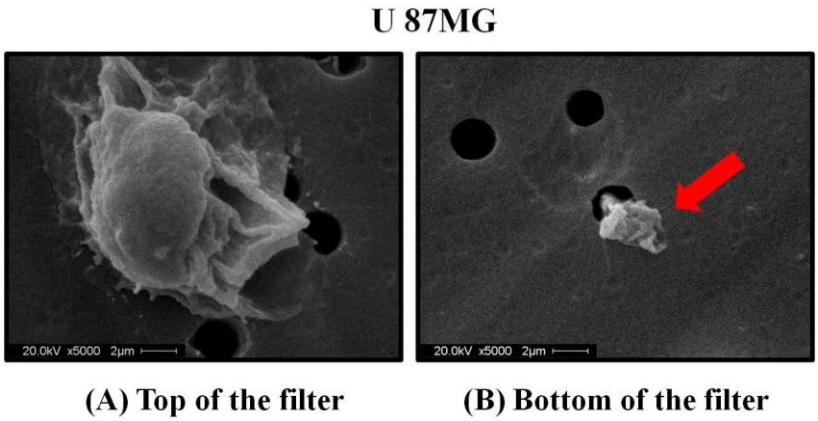
Figure 3.2-2 Effect of rhRLN2 on cell proliferation. BrdU proliferation assays were performed to study the effect of rhRLN2 in U87MG cells growth, while WST assays were employed to measure the cell viability of T98G upon rhRLN2 treatment. (A) rhRLN2 did not affect U87MG cell proliferation as determined by the BrdU incorporation assay. (B) WST assays showed no effect of rhRLN2 on T98G cell proliferation. The results shown are representative of three separate experiments with triplicate wells per experiment and are expressed as mean ± S.E.



3.3 Scanning electron microscopy (SEM) analysis of migrating U87MG cell line

An important step in GB cell migration and tissue invasion is the development of cell protrusions at the leading edge of the migration front. We employed SEM to visualize the migrating front of U87MG cells. Cells were plated on filters with no stimulus and allowed to migrate through a 3-µm for 1 h. Thereafter, U87MG cells partially traversed the filters by extending their pseudopodial protrusions to the underside of the filters (Fig. 3.3).

Figure 3.3 Scanning electron microscope images of U87MG cells after 1 h migration time. (A) on the top of the filters, cell showed pseudopodia at the leading edge trying to migrate through the pore of the filter (B) at the bottom of the filters, leading edge pseudopodial extensions started to appear at the pores.



3.4 Identification of candidate proteins at the migration front of U87MG cell line

To further investigate the potential mechanisms for relaxin/ RXFP1-induced cell migration**,** we used pseudopodia assays followed by 2D LC-MS/MS to identify and select candidate proteins involved in relaxin/ RXFP1-induced migration in the RXFP1 positive human GB cell line U87MG. Pseudopodial proteins were isolated from rhRLN2-treated and untreated U87MG cells and analyzed by LC-MS/ MS. The workflow and experimental design performed in this study are shown and described in the material and methods section. MS/ MS spectra were searched for protein identification using two database search engines: The Global Proteome Machine **(**GPM) software and ProteinPilot (PP) software. GPM uses a probability-based scoring algorithm to derive from a protein database the most likely peptide sequence matching the experiment spectrum while PP asscess each MS/MS spectrum from a data set to determine which spectra are worth scoring. Thus, PP acts as a filter to remove spectra that are less likely to yield reliable peptide identification. The

confidence level of the protein identification of GPM is represented by log (e) values where log (e)-1.3 equals a 95% confidence value. In contrast, PP uses different algorithrims to calculate protein confidence as represented by unused score, where unused score =1.3 equals a 95% confidence value. We used two search engines which have different parameters for higher reliability and increased sensitivity and accuracy of protein identification. PP software identified a total of 620 proteins in rhRLN2-treated U87MG cells, whereas 443 proteins were identified in untreated U87MG cells. The GPM software identified a total of 744 proteins and 564 proteins in rhRLN2-treated U87MG cells and untreated U87MG cells, respectively. The lists of identified proteins from both database search engines were combined in each group (rhRLN2-treated and untreated cells) in order to construct a more reliable protein list. Only those proteins identified in both search engines were considered for future analysis. This resulted in a total of 535 and 401 proteins identified both database search engines in rhRLN2-treated and untreated U87MG cells, respectively. Of those, 301 proteins were found in both rhRLN2-treated and untreated cells. By contrast, 234 out of 535 proteins and 100 out of 401 proteins were exclusively observed in rhRLN2-treated or untreated cells, respectively (Fig. 3.4-1). We further separately classified these proteins from each group including the uniquely proteins observed in rhRLN2-treated or untreated cells and the common proteins that are observed in both rhRLN2-treated or untreated cells according to their reported molecular functions using the PANTHER Classification System (www.pantherdb.org/) as shown in (Fig. 3.4-2). The majority of the identified proteins from each group were classified in similar functions including enzyme, cytoskeletal proteins, transporter/transfer/carrier proteins, nucleic acid binding proteins, signaling molecule, enzyme modulator, membrane traffic proteins, transcription factor, receptor, extracellular matrix proteins and chaperon. We calculated the percentages of identified proteins in each category by the total number of categories observed within each group and also observed similar percentage

of identified proteins in most of categories when compared between the three groups. However, the differences of the percentages of identified proteins in some categories which include cytoskeleton proteins, receptor, membrane trafficking proteins, extracellular matrix proteins, and chaperon were observed within each group (Table 3.4-1).

To identify candidate proteins at the leading edge involved in relaxin/ RXFP1-induced migration in U87MG GB cells, we focused mainly on the 234 proteins which were uniquely observed in the rhRLN2-treated cells. These 234 unique proteins detected in the rhRLN2-treated cells were classified into 11 groups which provided a broad functional overview of the pseudopodial proteome from rhRLN2-treated cells in the RXFP1 positive human GB cell line U87MG. Among these 11 groups, we initially focused on the receptors group since they may contain members that can serve as suitable tissue markers. Furthermore, binding of ligand to a cell surface receptor can stimulate signaling events that may be inhibited to suppress migration. Nine proteins were classified in this receptor group. We used several criteria to select candidate proteins involved in relaxin/ RXFP1-induced migration of U87MG cells. First, we scanned for proteins known to be involved in carcinogenesis and tumor cell migration/ invasion. Secondly, based on the log (e)-value from LC-MS/ MS analysis which is a statistical score indicating the significance of protein identification we considered only those proteins with a score of below log (e)-10. Another criteria was based on the number of unique peptide fragments reported from LC-MS/ MS analysis upon tryptic digest of a protein. Every MS/ MS spectrum is assigned to one or more proteins according to the spectrum of non-unique and unique peptides identified within this MS spectrum. Non-unique peptides are the peptide fragments that can be shared among multiple proteins. In contrast, unique peptides are the peptides that only occur in one protein and therefore increase identification confidence of that particular protein. In this study, we selected only the proteins which were identified at least by two

unique peptides. Of the nine cell-surface receptor proteins of the 234 unique proteins identified in the rhRLN2-treated cells, six candidate proteins were involved in carcinogenesis and tumor cell migration/ invasion (Table 3.4-2). Based on the criteria above, two proteins, transferrin receptor protein1 (TfR1) and membrane-associated progesterone receptor component 1 (PGRMC1) were selected for further studies. TfR1 is known to be overexpressed in gliomas and correlates with tumor grading (Recht, Torres et al. 1990). This receptor is implicated in the increase of tumor cell proliferation since growing tumor cells have an increased iron supply required for functioning of numerous iron-containing proteins (Richardson and Ponka 1997). Accordingly, it was suggested that TfR1 represents an important target for ligand-directed brain tumor immunotherapy for anti-proliferative effects (Wang, Tian et al. 2010). PGRMC1, a hormone receptor component and binding partner for P450 proteins, is over-expressed in breast tumors and in cancer cell lines from the colon, thyroid, ovary, lung, and cervix (Crudden, Loesel et al. 2005). *PGRMC1* mRNA was observed more frequently in high-grade than low-grade tumors (Carroll, Zhang et al. 1995; Gonzalez-Aguero, Ondarza et al. 2001). Moreover, this interesting protein promotes multiple phenotypes in cancer cells, including apoptotic resistance, anchorage-independent growth, invasion, tumor growth, and metastasis (Peluso, Romak et al. 2008; Peluso, Gawkowska et al. 2009; Ahmed, Rohe et al. 2010).

Figure 3.4-1 A total of 535 proteins were identified in rhRLN2-treated U87MG and 401 proteins were identified in untreated U87MG cells. Among those, 301 proteins were found in both rhRLN2-treated and untreated cells, while 234 out of 535 proteins and 100 out of 401 proteins were uniquely observed in the rhRLN2-treated cells or untreated cells, respectively

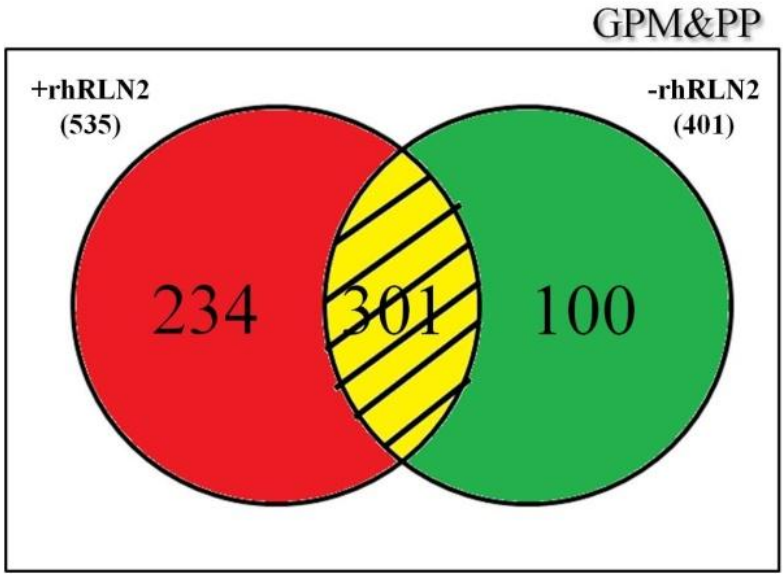
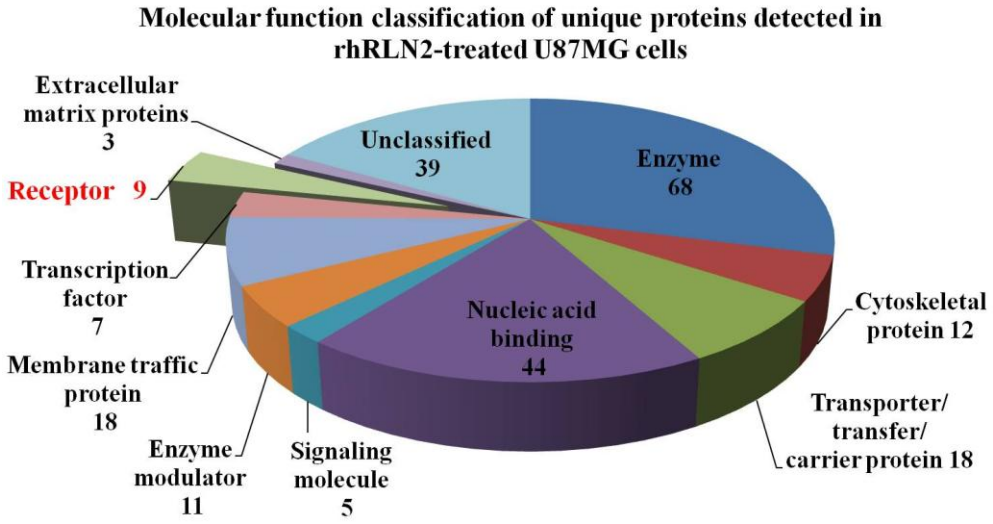
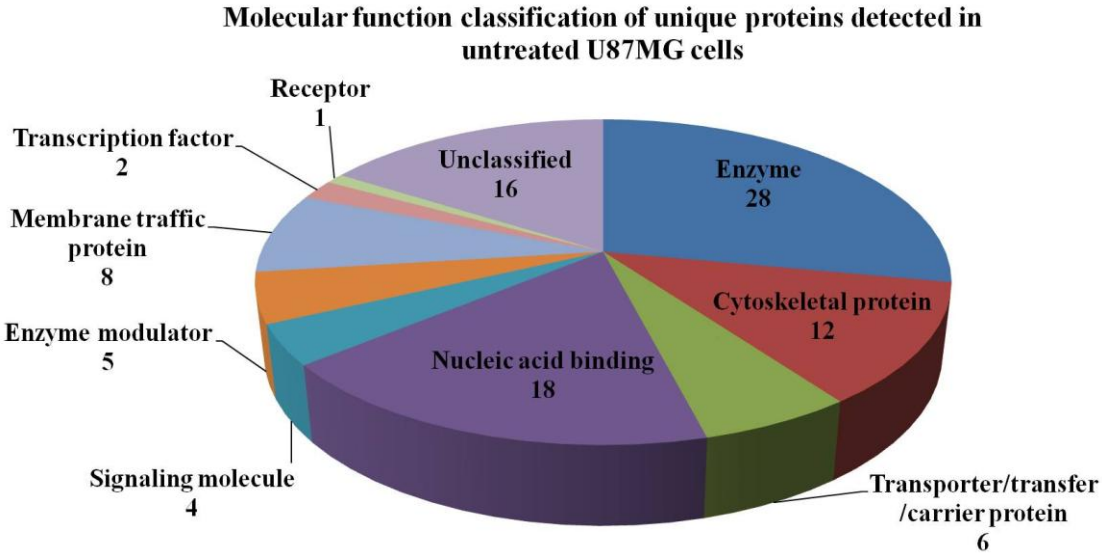


Figure 3.4-2 Classification by molecular function of the 234 unique proteins detected in rhRLN2-treated cells, the 100 unique proteins detected in untreated cells and the 301 common proteins that are observed in both rhRLN2-treated and untreated cells were done using the PANTHER database (http://www.pantherdb.org/). For the 234 unique proteins detected in rhRLN2-treated cells, which we focused on were classified to 11 categories. Of these categories, we selected the candidate proteins form the surface receptor group.





Molecular function classification of common proteins detected in untreated and rhRLN2-treated U87MG cells

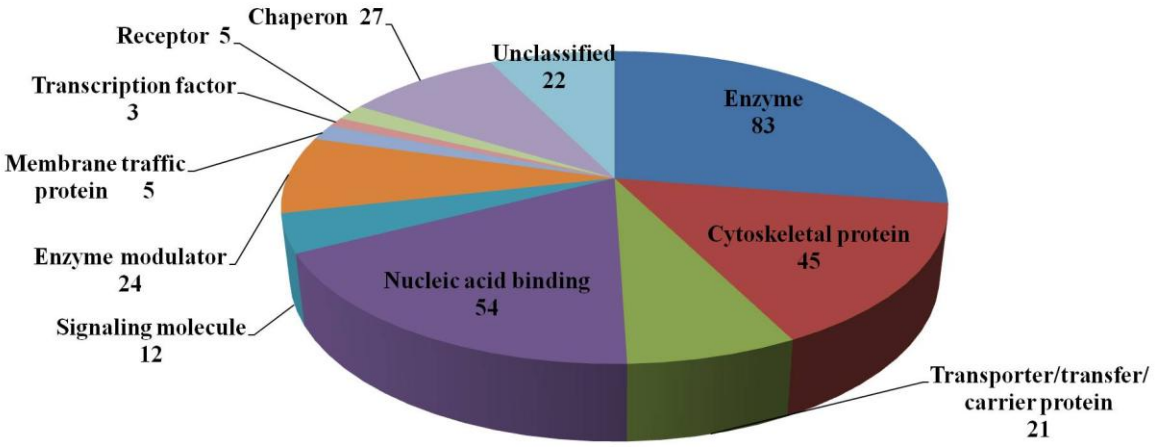


Table 3.4-1 Molecular function of proteins identified from rhRLN2-treated U87MG cells and untreated U87MG cells

Molecular function	Untreated	% of total	rhRLN2 treated	% of total	Common proteins	% of total
Enzyme	28	28.00	68	29.06	83	27.57
Cytoskeletal protein	12	12.00	12	5.00	45	14.95
Transporter/transfer/carrier proteins	6	6.00	18	8.00	21	6.98
Nucleic acid binding	18	18.00	44	18.80	54	17.94
Signaling molecule	4	4.00	5	2.00	12	3.99
Enzyme modulator	5	5.00	11	5.00	24	7.97
Membrane traffic proteins	8	8.00	18	8.00	5	1.66
Transcription factor	2	2.00	7	3.00	3	1.00
Receptor	1	1.00	9	4.00	5	1.66
Extracellular matrix proteins	0	0	3	1.00	0	0
Chaperon	0	0	0	0	27	8.97
Unclassified	16	16.00	39	16.00	22	7.31
Total	**100**	**100**	**234**	**100**	**301**	**100**

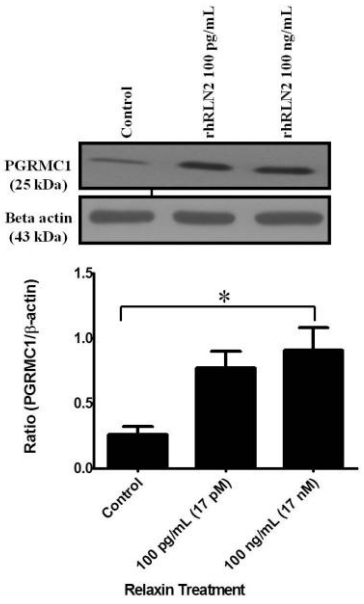
Table 3.4-2 The LC-MS/ MS data of the receptor category. Log (e)-value is the base -10 log of the expectation that any particular protein assignment was made at random, therefore, reflects the statistical score indicating the significance of protein identification. The lower the log (e)-value of the selected candidate proteins, the more significant the score which increases the chance that a peptide match is correct for this protein identification.

log(e)	Unique peptides	Name	Relationship between receptors and cancer	References
-34.6	4	Transferrin receptor protein	tumor growth, migration/ invasion	(Recht, Torres et al. 1990; O'Donnell, Yu et al. 2006; Chirasani, Markovic et al. 2009)
-17.9	3	Ribosome-binding protein 1	no relation to migration/ invasion	-
-17.3	3	Membrane-associated progesterone receptor	tumor growth, migration/ invasion	(Crudden, Loesel et al. 2005; O'Donnell, Yu et al. 2006; Ahmed, Rohe et al. 2010)
-12.3	2	Lysosome membrane protein 2	tumor metastasis	(Saitoh, Wang et al. 1992)
-11.0	2	Galectin-3-binding protein	cell migration/ metastasis	(Kim, Jung et al. 2011; Noma, Simizu et al. 2012)
-7.3	2	Tyrosine-protein phosphatase non-receptor type 1	tumor metastasis	(Wang, Liu et al. 2012; Zheng, Zhou et al. 2012)
-6.2	1	Signal recognition particle 9 kDa protein	no relation to migration/ invasion	-
-3.0	1	Niemann-Pick C1 protein	no relation to migration/ invasion	-
2.4	1	Zinc-alpha-2-glycoprotein	tumor suppressor, metastasis	Henshall, S.M., et al., (2006)

3.5 Verification of candidate proteins from 2D LC-MS/ MS analysis U87MG cell line

We employed Western blot analysis for the detection of TfR1 and PGRMC1 pseudopodial candidate proteins identified by 2D LC-MS/ MS analysis in of rhRLN2-treated U87MG cells. Protein extracts of purified pseudopodia (extracted from the lower part of the membrane) and cell bodies (extracted from the upper part of the membrane) from migrating rhRLN2-treated and untreated U87MG cells were used. However, we failed to detect both candidate proteins in the pseudopodial protein fraction. Even though we increased the number of the filters used for purifying pseudopodia from these cells, the concentration of the pseudopodial proteins was below the limit of detection. By contrast, we were able to detect the two candidate proteins, TfR1 and PGRMC1, in the protein extracts from the cell bodies in rhRLN2-treated and untreated U87MG cells. We also observed an upregulation of PGRMC1 in rhRLN2-treated cells when compared with the untreated U87MG cells. There was no change of TfR1 expression between rhRLN2-treated cells and the untreated cells (data not shown). Protein extracts of cellular bodies of the upper part of the membrane reflect an almost complete protein spectrum of the cells, except for parts of the invading pseudopodia. Since we successfully detected the two candidate proteins in U87MG cell bodies, we therefore decided to use whole cell lysates to try and confirm the expression of our candidate proteins in U87MG cells exposed to 100 pg/ mL and 100 ng/ mL rhRLN2 for 24 h; the latter relaxin concentration was used in the mass spectrometry experiments. Western blot analysis revealed that 100 ng/mL of rhRLN2 treatment significantly upregulated PGRMC1 protein levels ($P=0.033$) (Fig 3.5), but failed to do so with TfR1 (data not shown). We also observed the expression of both candidate proteins in T98G cell lines and some of primary RXFP1+ GB cells. However, there were no differences in TfRC1 and PGRMC1 expression between rhRLN2 -treated cells and untreated cells.

Figure 3.5 Western blot analysis and quantitative densitometry of the protein expressions of PGRMC1 in U87MG cell line (control, 100 pg/ mL and 100 ng/mL of rhRLN2). Treatment with 100 pg/ mL rhRLN2 caused a higher and borderline significant increase in PGRMC1 protein expression. This trend became significant when U87MG were treated with 100 ng/ mL of rhRLN2, resulting in a marked increase in the expression of PGRMC1 when compared with the control (P=0.033). Asterisk indicates P < 0.05 when compared to control (no treatment). For all panels, data represent three observations for each experiment and are expressed as mean±S.E.



*** P < 0.05**

3.6 Known mediators of relaxin-RXFP1 action in cell invasion are upregulated upon rhRLN2 treatment of GB cell lines and primary GB cells

The objective of this study was to investigate the role of the relaxin receptor RXFP1 in brain cancer and to determine RXFP1-mediated mechanisms in tumor cell migration and tissue invasion. We discovered PGRMC1 as a novel candidate that is upregulated in rhRLN2-treated U87MG cells. However, tumor cell migration and tissue invasion are complicated processes involving numerous

protein networks. Recent studies suggest that relaxin is produced by many types of cancer cells and acts on RXFP1 to induce metastasis via some mediators such as MMPs and their tissue inhibitors TIMPs (Binder, Hagemann et al. 2002; Bialek, Kunanuvat et al. 2011), lysosomal acid hydrolases (Hombach-Klonisch, Bialek et al. 2006), and small GTPases (unpublished data). This led us to hypothesize that some of these known mediators of relaxin's action might also be involved in relaxin/ RXFP1-induced migration in GB cells. To test our hypothesis, we used a similar approach as for detecting candidate proteins by mass spectrometry. We selected RXFP1 positive U87MG and T98G cell lines and incubated these cells for 24 h with 100 pg/ mL and 100 ng/ mL of rhRLN2. We also selected RXFP1 positive primary cells (PBS-1, PBS-10 and PBS-12.1) and incubated the cells for 24 h with rhRLN2 at 100 ng/ mL. Thereafter, we collected whole cell lysates and performed Western blot analysis. Along with candidate proteins identified by mass spectrometry analysis, we detected the expression of MMP-14 , the lysosomal acid hydrolases cath-B , cath-D, cath-L, and the small GTPases (Rac1 and Cdc42) (Table 3.6).

Table 3.6 Protein expression data for six relaxin mediators investigated in untreated (-rhRLN2) and rhRLN2-treated cells (+rhRLN2) U87MG cell lines and primary cells using Western blot analysis.

Relaxin targets	GB cell lines				Primary cells					
	U87MG		T98G		PBS-1		PBS-10		PBS-12.1	
	-rhRLN2	+rhRLN2	-rhRLN2	+rhRLN2	-rhRLN2	+rhRLN2	-rhRLN2	+rhRLN2	-rhRLN2	+rhRLN2
1. Matrix metello proteinases										
MMP-14	+	NC	+	NC	+	NC	+	NC	+	NC
2. Lysosomal acid anhydrases										
Cath-B	+	↑	0	0	+	↑	+	↑	+	↑
Cath-L	+	↑	0	0	+	↑	0	0	+	NC
Cath-D	+	↑	+	NC	+	↑	0	0	0	0
3. Small GTPases										
Rac1	+	↑	+	NC	+	↑	+	↑	+	↑
Cdc42	+	↑	+	NC	+	↑	+	↑	+	↑

(0) indicates no expression; (+) indicates protein expression; (↑) upregulation; (NC) no change

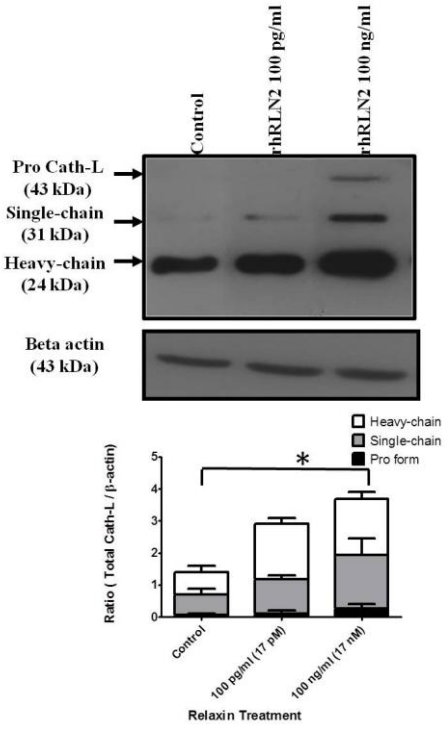
We observed that MMP-14 was present in all selected GB cell lines and primary GB cells but we observed no change in MMP-14 protein levels upon treatment with rhRLN2 (Table 3.6). The production of two cysteine cathepsins, cath-B and cath-L, and the aspartic protease cath-D in whole cell protein extracts of untreated and rhRLN2-treated GB cells was determined by Western blot. In GB cell lines, T98G was devoid of cath-B and cath-L expression as determined by Western blot analysis. By contrast, we found that T98G expressed cath-D but showed no significant change in cath-D expression (Table 3.6). U87MG were the GB cell line to express all three cathepsins. The three known immunoreactive forms of cath-L resembling the proform (43 kDa), single-chain (31

kDa), and heavy chain (24 kDa) as well as procath-D 52 kDa and heavy chain cath-D 25 kDa were detected in cellular protein extracted of U87MG cell lines. The amount of the three processing products of cath-L and two forms of cath-D were increased upon rhRLN2 treatment (both at 100pg/ mL and 100 ng/ mL). rhRLN2 treatment at 100 ng/mL showed a significant upregulation of the processed cath-L (heavy chain, 24 kDa) (P=0.026) (Fig 3.6-1A), whereas in U87MG treated with 100 ng/mL rhRLN2 the increase in cath-D heavy chain was insignificance (P=0.06) as shown by quantitative western blot analysis (Fig. 3.6-1B). These data suggested complete processing into the active form of cath-L and cath-D in rhRLN2-treated U87MG cells. The cath-B antibody only detected heavy chain cath-B in the U87MG cells where as no bands for the pro cath-B. We found that both 100 ng/ mL and 100 ng/ mL of rhRLN2 treatment resulted in a significantly increased production of heavy chain cath-B (P=0.034 and P=0.031, respectively) (Fig.3.6-1C) indicating complete processing and activation of this powerful protease in U87MG.

In the primary cells, Western blot analysis showed that cath-B exists as single-chain and heavy chain form in all selected cases of primary cells. Treatment with 100 ng/ mL of rhRLN2 increased the cath-B expression in all primary GB cells tested (PBS-1, PBS-10 and PBS-12.1) (Fig. 3.6-2A**).** The expression of cath-L varied among primary cells. PBS-10 cells showed no expression of cath-L, while cath-L was expressed in PBS-1 and PBS12.1 cells. No change in cath-L expression was observed upon rhRLN2 treatment in PBS-12 where as in PBS-1, rhRLN2 treatment (100 ng/ mL) caused an increase in all three cath-L forms [proform (43 kDa), single-chain (31 kDa), heavy chain (24 kDa)] (Fig. 3.6-2B). Western blot analysis revealed no expression of cath-D in PBS-10 and PBS-12.1. In contrast, procath-D (52 kDa) and heavy chain cath-D (25 kDa) were detected in PBS-1 and rhRLN2 at 100 ng/ mL slightly increased the expression of heavy chain cath-D as compared to untreated cells (Fig. 3.6-2B).

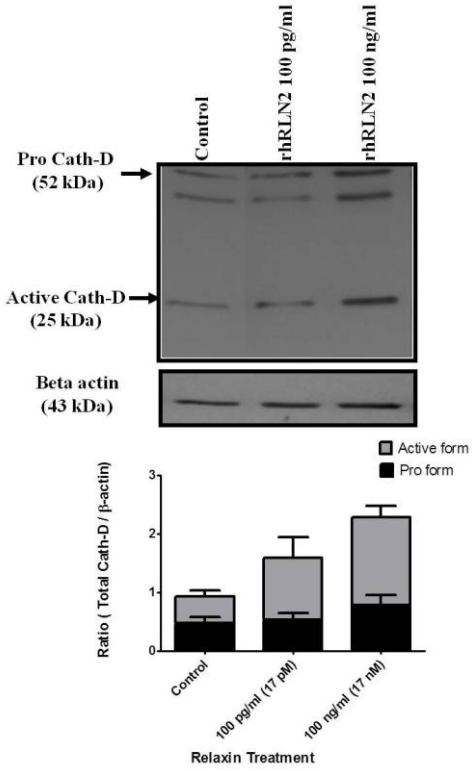
Figure 3.6-1 Protein levels of cath-B, -D and -L in untreated and rhRLN2-treated U87MG. (A) Immmunoblots of procath-L (43 kDa), single-chain cath-L (31 kDa) and heavy-chain cath-L active form (24 kDa) in U87MG. Densitometry analysis of all bands recognized by anti-cathL were plotted in different colors but within the same bar representing a total cath-L with black box denoted the proform , light grey box denoted the single chains and white box represented heavy chain form (active form) of cath-L. (B) Immunoblots for cath-D and densitometric analysis of procath-D (52 kDa) and processed cath-D (25 kDa) in untreated and rhRLN2-treated U87MG. Densitometry analysis of all bands recognized by anti-cath-D were plotted in different colors but within the same bar representing a total cath-D with black box denoted the proform, and light grey box denoted the active form. (C) Western blot detection of cath-B expression in untreated and rhRLN2-treated U87MG cells. Only the active form of cath-B was recognized by anti-cathB. Quantitative analysis of cath-B expression in U87MG treated with 100 pg/ mL and 100 ng/ mL rhRLN2 by densitometry showed a significantly increased in cath-B protein. Results from three independent experiments are shown as mean±S.E. ($P<0.05$). Beta-actin served as a loading control.

A.



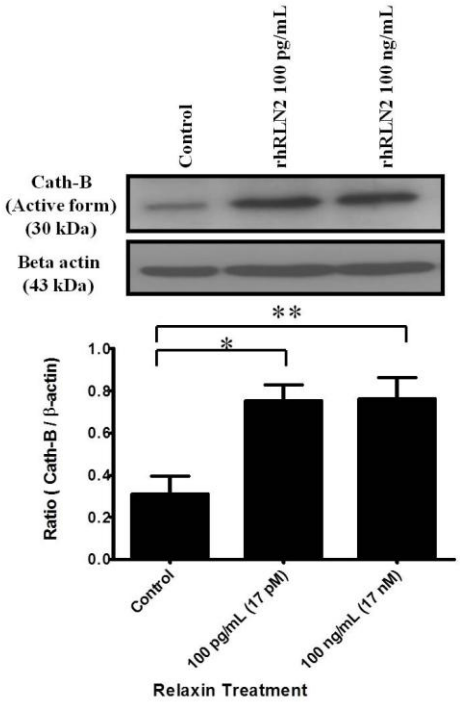
*P < 0.05

B.



P=0.06

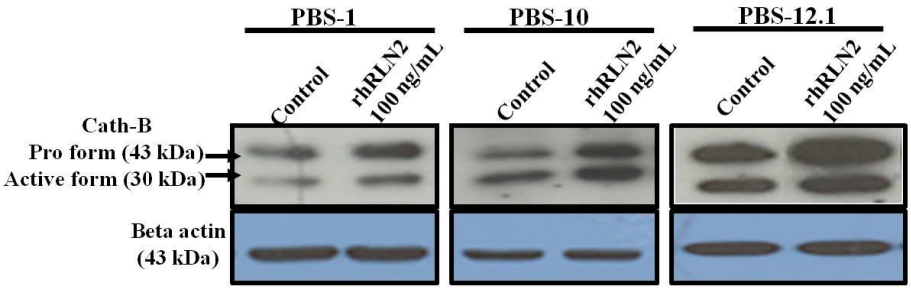
C.



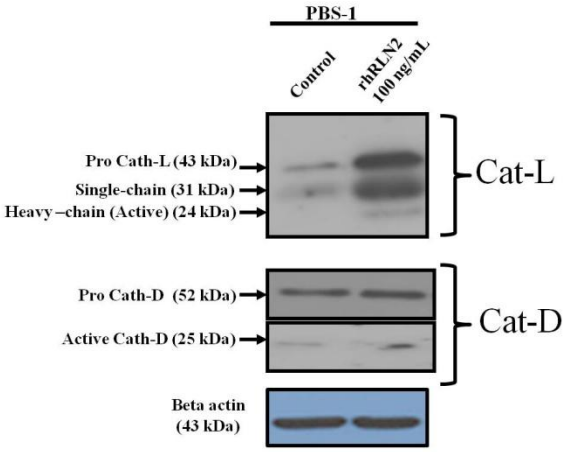
*P <0.05, **P<0.05

Figure 3.6-2 Protein levels of cath-B, -L and -D in primary cells upon rhRLN2 (100 ng/ml) treatment. (A) Representative Western blots showed a marked increase in cathB in PBS-1, PBS-10 and PBS-12.1. (B) Representative Western blots demonstrated an increase of cath-L and -D in PBS-1.

A.



B.

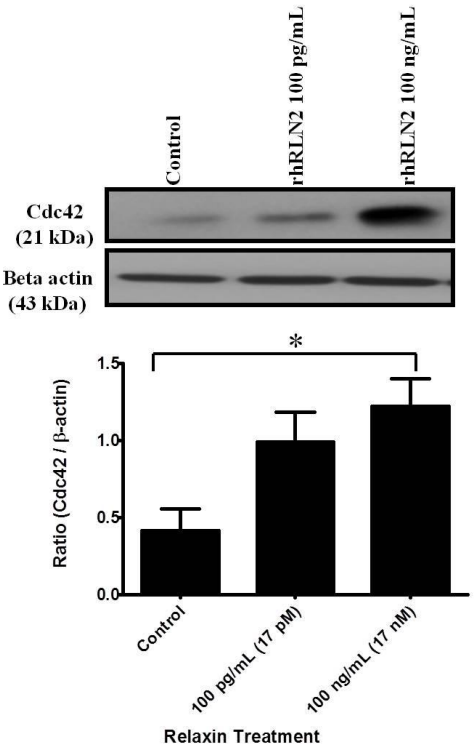


Cdc42 and Rac1 are members of the Rho family of small GTPases which play critical roles during invasion of brain cancer cells. Both Cdc42 and Rac1 were ubiquitously expressed in all selected GB cell lines and primary GB cells. We detected a significant increase in both Cdc42 (P=0.04) and Rac1 (P=0.04) expression in U87MG upon treatment with 100 ng/mL rhRLN2 (Fig 3.6-3A, 3B). Western

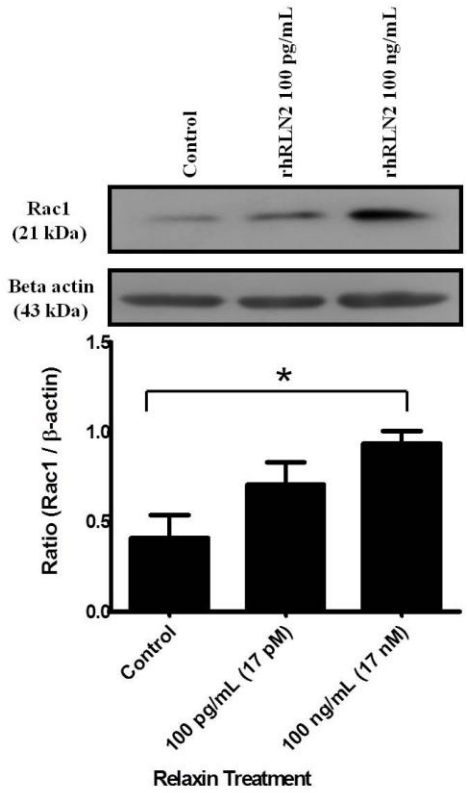
blot analysis of three cases of primary GB cells (PBS-1, PBS-10, PBS-12.1) showed a consistent increase in Rac1 and Cdc42 protein levels in rhRLN2-treated cells when compared with untreated cells (Fig 3.6-4A , 4B).

Figure 3.6-3 Protein levels of Cdc42 and Rac1 in untreated and rhRLN2-treated (100 ng/mL) U87MG. Immmunoblots of (A) Cdc42 and (B) Rac1 in U87MG. Densitometric analysis showed a significant upregulation of both Cdc42 and Rac1in rhRLN2-treated (100 ng/mL) U87MG cells.

A.



B.

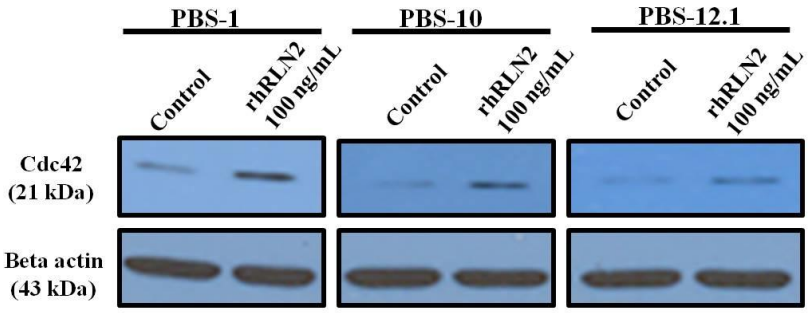


* P<0.05

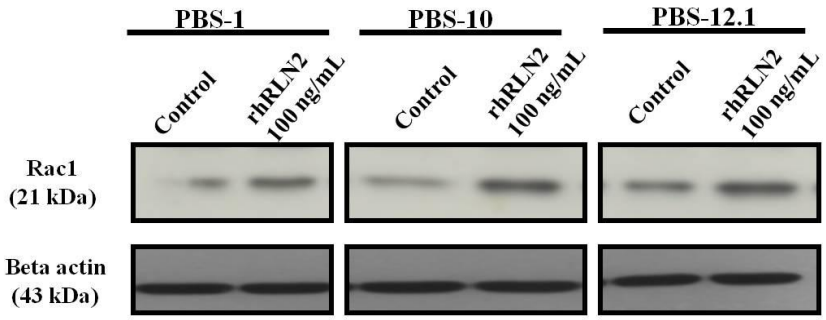
* P<0.05

Figure 3.6-4 Protein levels of Cdc42 and Rac1 in three cases of primary cells. Representative Western blots showed a marked increase in (A) Cdc42, (B) Rac1 in PBS-1, PBS-10 and PBS- 12.1 GB cells.

A.



B.



3.7 C1q tumor necrosis factor-related protein 8 (CTRP) 8 is a novel RXFP1 ligand

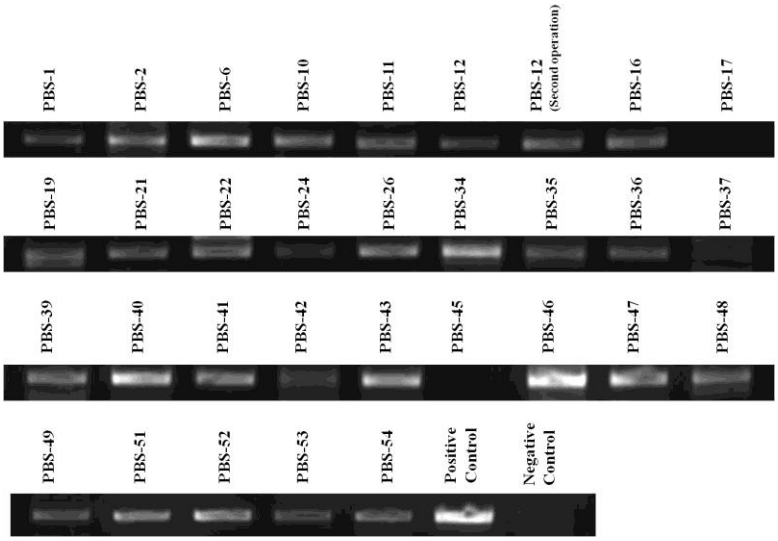
CTRP8 expression in human GB cell lines and primary GB cells

The fact that we did not detect the expression of either *RLN1* or *RLN2* in GB cells led us to hypothesize that there is another ligand which can bind to RXFP1 and activate this receptor in GB cells. It has recently been shown that a small peptide derived from the secreted multimeric C1q tumor necrosis factor-related protein 8 (C1QT8; also named CTRP8) was able to serve as a novel

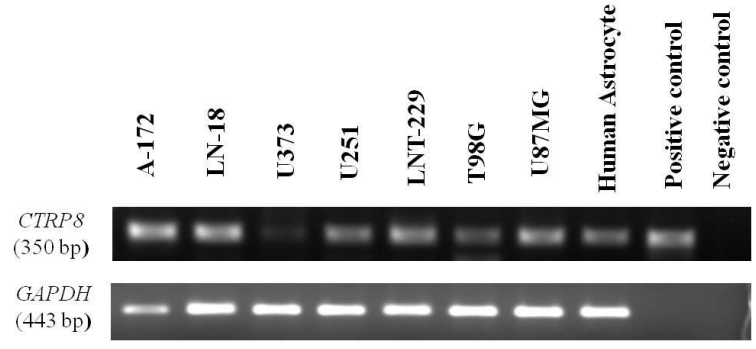
ligand for RXFP1 (Shemesh, Hermesh et al. 2009; Pini, Shemesh et al. 2010). Thus, we investigated by RT-PCR analysis the expression of *CTRP8* in GB cell lines, 32 cases of primary GB cells, and human astrocytes (Fig 3.7-1A, 1B). *CTRP8* transcripts at various levels of expression were detected in all GB cell lines. We also detected *CTRP8* transcripts in human astrocytes (Fig 3.7-1B). *CTRP8* transcripts were also detected in 29 of 32 (90.63%) cases of primary GB cells (Fig 3.7-1A). The expression of *CTRP8* in established and primary GB cells suggested that this secreted protein may have the potential to serve as a novel ligand for RXFP1 in the normal and diseased brain.

Figure 3.7-1 Expression of *CTRP8* in human primary GB cells (A) and in GB cell lines (B). PBS stands for Primary Brain tumor Study. (A) Human primary GB cells expressed *CTRP8* at different levels. (B) Human astrocytes and all GB cell lines expressed *CTRP8*.

A.



B.

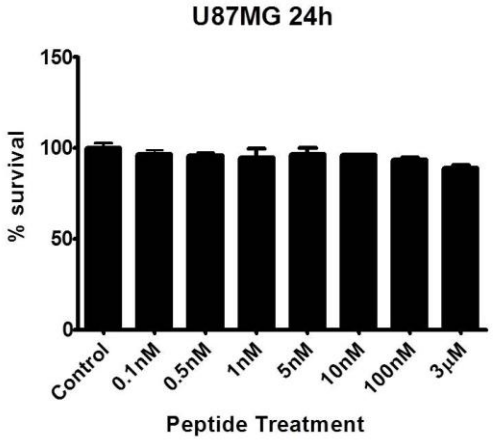


CTRP8-derived peptide P74 showed no toxicity in U87MG and T98G GB cell lines

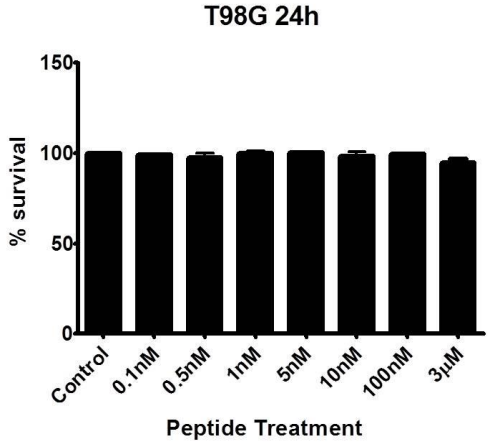
RT-PCR analysis revealed the *CTRP8* expression in GB cell lines and primary GB cells. We hypothesized that CTRP8 may act as an agonist in RXFP1 signaling. We used the previously described P74 peptide (Shemesh, Hermesh et al. 2009) encoding for the collagen domain and C1q globular domain of human CTRP8 and previously shown to interact with RXFP1 to investigate the role of this potential new peptide ligand for RXFP1-promoted migration in human GB cells. First, WST assays were employed to assess the cytotoxicity of the peptide in U87MG and T98G cell lines. Exposure of GB cells to concentrations of P74 peptide as high as 3 µM failed to cause cytotoxicity as determined by WST assays (Fig. 3.7-2).

Figure 3.7-2 Effect of P74 peptide on the viability of (A) U87MG and (B) T98G cells detected by WST assay. Cells were treated with 0.1nM, 0.5 nM, 1 nM, 5 nM, 10 nM, 100 nM and 3µM of the peptide for 24 h. Results on the cell viability measured by WST assay are given in percent related to untreated controls. Bars represent mean ± S.E. (n=3). P74 at all peptide concentrations tested was non-toxic to (A) U87MG and (B) T98G cells.

A.



B.

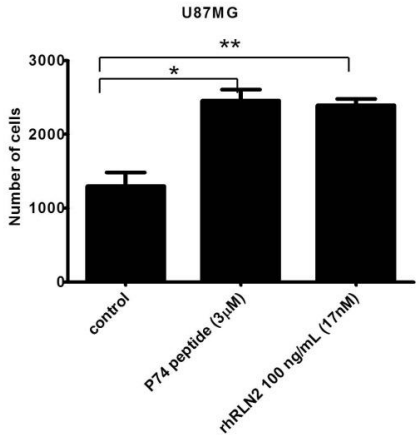


P74 increased cell motility of U87MG and T98G and RXFP1+ primary GB cells

U87MG and T98G GB cell lines as well as two cases of primary GB cells (PBS-1 and PBS-10) were used to evaluate the effect of P74 on RXFP1-mediated cell motility by employing transwell filter assays. The upper and lower culture compartments were separated by filters with an 8 μm pore size. The cells were seeded into the upper chamber and incubated for 24 h in the absence (control) or presence of P74 peptide at 3 μM added to the lower chamber. rhRLN2 (100 ng/ mL equaling 17 nM) was added to the lower chamber as a positive control. Similar to rhRLN2, treatment with P74 caused a significant increase in cell motility in U87MG (P=0.01) and T98G (P=0.03) cell lines and in the two cases of primary GB cells tested (PBS-1 (P=0.04) and PBS-10 (P=0.025) (Fig. 3.7-3). Scrambled control peptide was also used at 3 μM and showed no increase in cell motility.

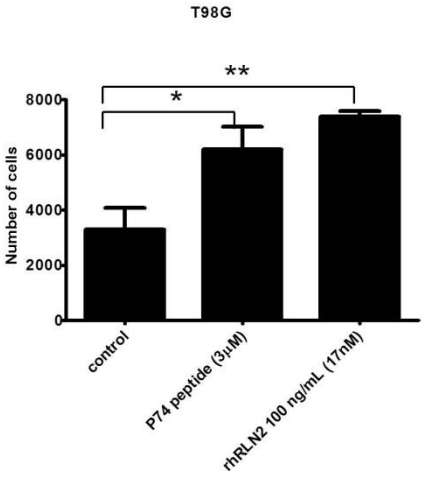
Figure 3.7-3 P74 induced effects on cell motility of U87MG, T98G, PBS-1 and PBS-10 GB cells in transwell motility assays. The cells were allowed to migrate for 24 h in the absence or presence of 3 µM P74 peptide and rhRLN2 (100 ng/ mL equaling 17 nM). The latter served as positive control. Quantitation of the number of migrated cells using bright field microscopy revealed a (A, B) 1.8-fold,, and (C,D) 2.3 fold significant increase in cell motility in the presence of P74 and rhRLN2, respectively, compared to scramble control and untreated cells. The results shown are representative of three separate experiments with three filter sets per experiment. Data are expressed as mean±S.E.

A.



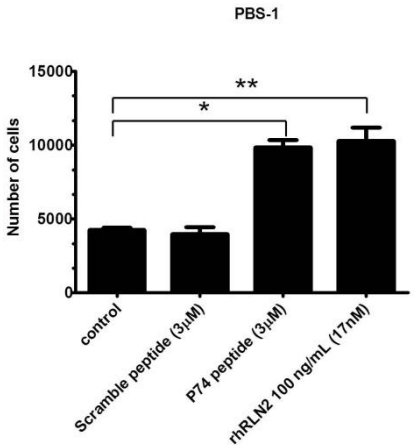
*P<0.05 , **P<0.05

B.



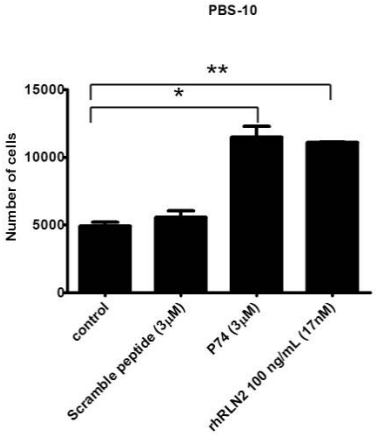
*P<0.05 , **P<0.05

C.



*P<0.05, **P<0.05

D.

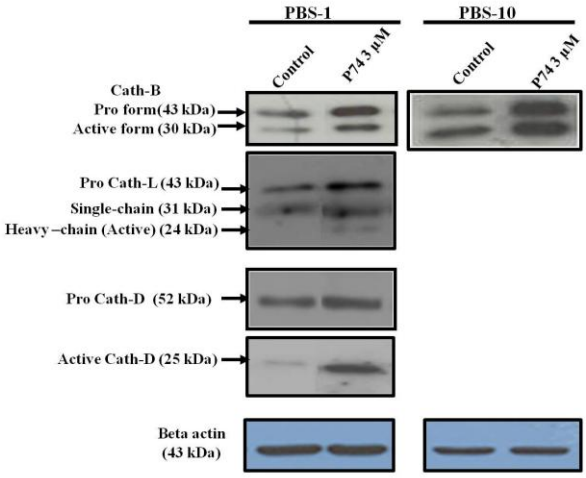


*P<0.05, **P<0.05

P74 peptide treatment upregulates cathepsins in primary GB cells

We had observed changes in cath-B, -D and cath-L production upon rhRLN2 treatment of U87MG, PBS-1 and PBS-10 cells. The same cells were selected for a 24h treatment with P74 peptide. Proteins were extracted and Western blot analysis revealed the presence of cath-B, -D, and -L (Fig.3.7-4). In contrast to rhRLN2 treatment, P74 treatment failed to induce changes in the expression of all three cathepsins as shown for relaxin with U87MG. However, P74 peptide treatment caused the upregulation of cath-B when compared with untreated cells in both PBS-1 and PBS-10. P74 peptide also caused an increase in cath-L and cath-D protein in PBS-1 (Fig.3.7-4). We were unable to detect cath-L and cath-D in PBS-10 (Table 3.6). These results indicate that similar to rhRLN2, the CTRP8-derived P74 peptide was able to cause the upregulation of cathepsins in GB cells suggesting that the interaction of this peptide with RXFP1 can regulate the quantity and activity level of different cathepsins and facilitate GB cell migration and tissue invasion.

Figure 3.7-4 Protein level of cath-B, -L and -D in primary GB cells upon P74 peptide treatment. (A) Representative Western blots showed a marked increase in cathB in PBS-1, and PBS-10 when treated with P74 peptide as compared to untreated cells. Marked increases in cath-L and cath-D were observed in PBS-1 when treated with the P74 peptide compared to untreated cells.



CHAPTER 4: DISCUSSION

We hypothesized that RXFP1 enhances glioblastoma cell migration and invasion. The main objective of our investigation was to determine the mechanisms by which RXFP1 can facilitate the invasion of glioblastoma brain tumor cells. In this study, we investigated the gene expression of RLN2 and RXFP1 in different types of brain tumors and also in GB cell lines by RT-PCR. In addition, we determined the expression of other relaxin isoforms, RLN1and RLN3, and RXFP2 receptor to provide a complete expression profile of this ligand -receptor system in human GB. Of all transcripts tested, RXFP1 was the only one expressed in brain tumor cell lines and primary cells. Previously, RXFP1 transcripts have been demonstrated in normal human brain using RT-PCR (Hsu, Nakabayashi et al. 2002). In rats, RXFP1 mRNA was located in the circumventricular organs and the neurosecretory magnocellular hypothalamus nuclei which are responsible for controlling plasma osmolality (Sunn, Egli et al. 2002; Burazin, Johnson et al. 2005). No information is currently available on the gene expression of the relaxin-RXFP1 ligand-receptor system in brain tumors. Earlier studies reported RXFP1 transcripts in many types of cancer such as breast, prostate, endometrium and thyroid cancer and plays an important role in cancer progression. (Radestock, Hoang-Vu et al. 2005; Hombach-Klonisch, Bialek et al. 2006; Kamat, Feng et al. 2006; Feng, Agoulnik et al. 2007). We identified here, for the first time, GB cell lines and human primary brain tumor cells as a novel source of RXFP1 receptor, whereas weak detection of RXFP1 mRNA was observed in human astrocytes. This suggested a potential role for this receptor in brain tumors and astrocytes. We showed that GB cell lines and 15 of 32 cases primary cells of human primary brain tumor cells with different clinical pathology expressed RXFP1 transcripts. RXFP1 expression was not restricted to one type of brain cancer but was found in GB WHO grade IV as well as menigioma and astrocytoma. The expression of RXFP1 in most cases of GB and the GB recurrent cases may

have important and specific roles in the high grade tumors and also in the recurrent brain tumors. The function of RXFP1 in cancer is related to tumor cell growth, cell invasion and angiogenesis (Silvertown, Summerlee et al. 2003). The highly invasive characteristic of GB prompted us to hypothesize that expression of RXFP1 may mediate cancer cell migration and invasion in GB.

To study the function of RXFP1 receptor in brain tumor cell migration, we used rhRLN2 due to the absence of RLN2 in the system. Numerous studies showed that activating RXFP1 signaling cascades via relaxin regulates the migratory capacity of both normal and tumor cells. Additionally, relaxin acted mainly as a chemoattractant rather than a chemokinetic agent in inducing migration of the cells. Relaxin has been shown to stimulate the increase of migration of bronchial epithelial cells in a wound healing assay (Wyatt, Sisson et al. 2002). Another study reported that relaxin was not a monocyte chemoattractant by itself but it did enhance the capacity of MCP-1 induced chemotaxis and increased the migratory activity of human leukocytes (Figueiredo, Mui et al. 2006). Recently, it has been demonstrated that relaxin acts as a chemoattractant to increase the direction of migration of bone marrow–derived endothelial cells in an RXFP1- and NO-dependent manner (Segal, Sautina et al. 2012). Relaxin promotes migration and invasion in an RXFP1-dependent manner in various types of cancers, including breast (Binder, Hagemann et al. 2002; Silvertown, Geddes et al. 2003) , thyroid (Hombach-Klonisch, Bialek et al. 2006; Radestock, Willing et al. 2010; Bialek, Kunanuvat et al. 2011) , and prostate (Silvertown, Ng et al. 2006). Similarly, we demonstrated that RLN2 increased the cellular motility of two human GB cell lines (U87MG and T98G) as well as primary human GB cells (PBS-10). The concentrations of RLN2 used in the motility assay were 100 pg/mL and 100 ng/mL, corresponding to RLN2 concentrations of 1-100 ng/mL that is typically used in most *in vitro* studies. One study went even higher and used 1 μg/mL of RLN2 (Filonzi, Cardoso et al. 2007). In our study, the 100 ng/mL of RLN2 significantly increased cellular motility in all cells tested, while

the 100 pg/mL of RLN2 caused a significant increased in motility only in U87MG cells. Previous studies have suggested that the concentration of relaxin that interacts with RXFP1 plays an essential role in activating the signaling pathway to generate the cellular responses. The RXFP1 was recently shown to be unique among GPCRs in that this receptor exists as a preassembled ready-to-fire membrane receptor complex able to respond to sub-picomolar concentrations of relaxin (Halls and Cooper 2010; Halls 2012) . It is possible that RXFP1 expressed in U87MG cells exists as a preassembled highly sensitive complex that then can be activated by a low concentration of RLN2 at 100 pg/mL (17 picomolar). By contrast, in T98G and PBS-10 cells these complexes may be absent or arranged in ways that would not allow them to be fully activated at picomolar RLN2 concentrations. We observed that the migration of these GB cells only occurred when RLN2 was added solely to the lower chamber of the well but not when it was added to both the upper and lower chambers (data not shown).Our finding supports the notion that relaxin acted mainly as a chemoattractant to induce cell migration.

Since RLN2 treatment caused increased migration in RXFP1 positive GB cells, we further determined the mechanism for RXFP1-mediated GB cell migration and tissue invasion. We first focused on the membrane protrusion of migrating GB cells. The basic mechanism of cell migration is due to the formation of pseudopodial membrane protusion which results from dynamic actin cytoskeleton remodeling (Chodniewicz and Klemke 2004). Pseudopodial protrusions and the formation of related invadopodia has long been associated with tumor cell migration and invasion (Guirguis, Margulies et al. 1987; Bialek, Kunanuvat et al. 2011; Zuzga, Pelta-Heller et al. 2012). A number of proteins in pseudopodia have been identified to facilitate cancer cell migration and invasion (Liu, Sun et al. 2003; Philippar, Roussos et al. 2008; Pichot, Arvanitis et al. 2010). The use of SEM allowed us to visualize and isolate pseudopodial extensions in migrating U87 MG cells. 2D

LC-MS/MS analysis identified the protein content in these pseudopodia which was subject to dynamic alterations, likely for the purpose of assisting the process of RXFP1-mediated GB cell migration and tissue invasion. Liquid chromatography coupled with tandem mass spectrometry has been widely used for proteome identification in complex cells and whole tissues (Washburn, Wolters et al. 2001) and the protein content present in the pseudopodia has been linked to the invasion of cancer cells (Wang, Hanley et al. 2006; Shankar, Messenberg et al. 2010). Our proteomics analysis revealed a total of 535 proteins and 401 proteins in RLN2-treated and untreated U87MG cells respectively. About 301 of those proteins detected were present in both RLN2-treated and untreated cells. A total of 234 of 535 proteins and 100 of 401 proteins were identified as unique proteins in RLN2-treated and untreated cells, respectively. This data can be considered in several ways: (i) the 234 proteins which were uniquely observed in RLN2-treated cells resulted from the effect of rhRLN2 treatment; (ii) the 100 proteins which were only observed in untreated cells were also noticeably downregulated upon RLN2 treatment; (iii) the 301 proteins which were found in both RLN2-treated and untreated cells may or may not have been affected by RLN2 since in this present study, we did not perform quantitative proteomics. Therefore, it is difficult to determine the effect of RLN2 on these proteins. One objective of this study was to identify proteins involved in RLN2-RXFP1 induced migration in U87MG cells. We focused on proteins uniquely observed in RLN2-treated cells and chose the receptor category as our first group. This was done because previous studies showed that brain tumor cells invasion includes the interaction of cell surface bound receptors with various autocrine and paracrine factors to activate multiple signaling pathways facilitating cell migration (Hoelzinger, Demuth et al. 2007). Several cell surface receptors have been shown to be upregulated in migrating brain tumor cells. The fibroblast growth factor-inducible14 (Fn14), which is a member of the tumor necrosis factor (TNF) superfamily receptors, was found to

be upregulated in migration-stimulated glioma cells *in vitro*. In radius motility assay, Fn14 is highly expressed in cells migrating at the outer tumor edge compared to the non-motile cells at the core, suggesting the association between the upregulation of this receptor and the effect on migration (Tran, McDonough et al. 2003). TROY is other member of tumor necrosis factor receptor and is over-expressed in glioma cells and enriched in lamellipodia. The increased expression of TROY enhances glioma cell migration and invasion by activating Rac1 signaling in a Pyk2-dependent manner (Paulino, Yang et al. 2010).

In selecting candidate proteins from the receptor category, we chose to use those involved in RXFP1-mediated GB cell migration since it may help to identify the receptor that plays a role in inducing cell migration. Blockage of the signaling through these newly identified receptors might attenuate brain tumor cell migration and invasion. TfR1 and PGRMC1 were two selected candidate proteins in our study because the expression of TfR1 has been known to be upregulated in brain tumors including astrocytomas and GB where the highest expression was observed (Recht, Torres et al. 1990) . Even though the major role of TfR1 in gliomas is to induce increased intracellular iron accumulation resulting in tumor cell proliferation (Hall, Godal et al. 1992) , activating TfR1 by transferrin has been demonstrated to promote endothelial cell migration and invasion in the hypertrophic cartilage during endochondral bone formation (Carlevaro, Albini et al. 1997). Moreover, transferrin secretion has been identified in oligodendrocytes and astrocytes in adult brain (Connor, Menzies et al. 1990). The presence of transferrin and TfR1 in the brain system suggested the possibility that TfR1 may play a role in brain tumor cell migration. PGRMC1 is induced in various types of cancers to promote the survival of tumor cells and induce resistance to damage upon exposure to chemotherapeutics (Crudden, Chitti et al. 2006; Peluso, Liu et al. 2008). Recently, PGRMC1 receptor has also been shown to enhance cell motility and invasion in A549 lung cancer

cells, while using siRNA to knockdown PGRMC1 caused a significant decrease in cell migration indicating the effect of PGRMC1 on A549 cell migration. (Ahmed, Rohe et al. 2010). Using Western blot analysis, we failed to confirm the presence of these two candidate proteins in isolated pseudopodial proteins from RLN2-treated and untreated U87MG cells. The concentration of these proteins in isolated pseudopodial proteins was below the detection level despite the fact that we had increased the number of the filters to obtain appropriate lysates. Our pseudopodia purification method was similar to a previous study with slight modifications (Cho and Klemke 2002). According to this reference method, the yields obtained for pseudopodia protein purification from COS-7 and NIH-3T3 cells are typically 25–30 µg of protein from each 24-mm well stimulated with chemokine for 30–60 min (Cho and Klemke 2002). Using different cell type and a different chemoattractant in our study might have resulted in less concentrated purified pseudopodia protein extract. Another way of purifying pseudopodia, which gave good yield of pseudopodia protein, has been described and this method was used to isolate pseudopodia protein from U87MG cells (Beckner, Chen et al. 2005). Optimization of the pseudopodia isolation method in our study should be considered for future studies. In contrast to the pseudopodia protein extracts, we were able to detect TfR1 and PGRMC1in the whole cell lysates of RLN2-treated and untreated cells. However, only PGRMC1 was found upregulated upon RLN2 treatment which is in agreement with our proteomic results. Although we were unable to use the pseuodopodia lysates to confirm the expression of our candidate proteins, detecting the upregulation of PGRMC1 upon RLN2 treatment in whole cell lysates provides new clues pointing to this novel candidate protein being involved in relaxin-RXFP1 induced migration in U87MG cells. We cannot exclude that TfR1 is not involved in relaxin-RXFP1 induced migration since our proteomic experiment has only been done once. The same procedure needs to be repeated to confirm the presence of these two candidate proteins in

pseudopodia extracted from RLN2-treated cells to prove that these proteins play a role in relaxin-RXFP1 induced migration. In addition, the presence of PGRMC1 in whole cell lysates of untreated cells and its upregulation in RLN2- treated cells suggest that this protein may re-distribute from the cell body to concentrate in the pseudopodia upon RLN2 treatment. Our proteomic data showed an absence of these proteins in pseudopodia of untreated cells. We also observed the expression of TfR1 and PGRMC1 in T98G and primary GB cells (PBS-1, PBS-10 and PBS-12.1) but found no difference in TfR1 and PGRMC1 expression in response to RLN2 treatment in these GB cells, indicating that the effect of RLN2 on upregulation of PGRMC1 may be U87MG cell-type specific.

Proteomics analysis is one way to identify proteins and these provide a spectrum of pseudopodial proteins that may be involved in relaxin-RXFP1 induced migration. However, to understand the mechanism of relaxin-RXFP1 induced migration, we also observed the expression of some proteins and known mediators of relaxin action in cell invasion in the whole cell lysates of GB cell lines (U87MG, T98G) and primary GB cells (PBS-1,PBS-10 and PBS-12.1). We utilized the same concentrations of RLN2 treatment that were shown to increase GB cell migration. Mediators of relaxin-RXFP1 action consist of MMPs and their tissue inhibitors (TIMP1-4), TIMPs (Binder, Hagemann et al. 2002; Bialek, Kunanuvat et al. 2011), lysosomal acid hydrolases of the cathepsin family (Hombach-Klonisch, Bialek et al. 2006), and small GTPases (unpublished data). The action of relaxin-RXFP1 on cancer cell migration and invasion is facilitated by MMPs including collagenase 1 (MMP-1) and 3 (MMP-13), gelatinase A (MMP-2) and B (MMP-9), stromelysin1 (MMP-3), and MMP-14 (MT1-MMP) (Binder, Hagemann et al. 2002; Kamat, Feng et al. 2006; Bialek, Kunanuvat et al. 2011). We found that MMP-14, which is one of the membrane anchored-MMPs (MT-MMPs) members, was expressed in all GB cell lines and primary cells but there were no detectable changes in MMP-14 expression upon RLN2 treatment. This contrasts with previous

studies in different cell models which demonstrated that RLN2 is involved in the upregulation of MMP-14 along with others MMPs. These studies suggested that the effect of relaxin on the upregulation of MMP species involved in cell migration and invasion may depend on certain cancer cell types. For example, in human follicular thyroid carcinoma cells relaxin specifically induced the expression of MMP-2 and MMP-14 (Bialek, Kunanuvat et al. 2011), whereas MMP-2,-7,-9,-13 and-14 were induced by relaxin in breast cancer cells (Binder, Hagemann et al. 2002). It is well known that MMP-14, MMP-2, and TIMP-2 are regulated in a coordinated fashion to promote cancer cell invasion. Activation of pro MMP- 2 requires the binding of the proenzyme of MMP-2 to the MMP-14/TIMP-2 complex located at the cell surface and is followed by the initiation of the activation cascade leading to the release of active processed MMP-2 enzyme into the extracellular matrix (Strongin, Collier et al. 1995). The net activity of MMP-14 and MMP-2 is regulated in a complex manner depending on TIMP-2 concentration (Itoh, Ito et al. 1998). Increasing expression of TIMP-2 and MMP-2 in GB tissue has been previously documented (Lampert, Machein et al. 1998). In highly invasive glioblastoma cell models, upregulation of TIMP-2 promotes MMP-2 activation and cell invasion is not associated with an increase of MMP-14 expression (Lu, Jong et al. 2004). MMP-9 has been reported in numerous studies to play a role in brain tumor cell migration and invasion (Lakka, Rajan et al. 2002; Gondi, Lakka et al. 2004; Liu, Wu et al. 2010).These findings suggested that relaxin might enhance brain tumor cell migration by affecting other member of MMPs and TIMPs, particularly MMP-2, TIMP-2 and MMP-9.

Lysosomal acid hydrolases of the cathepsin family are another group of proteinases responsive to relaxin (Hombach-Klonisch, Bialek et al. 2006). In normal cells, cathepsins are synthesized as prepro-enzymes that first undergo proteolytic posttranslational modification; the signal “pre” sequence is cleaved off within the ER followed by conversion to an intermediate form

in endosomes, and subsequent completion of the processing process in the acidic lysosomal compartments to the fully active mature protease. Proteolytic processing and activation of cathepsins may occur intracellularly (in the lysosome) and/ or extracellularly (von Figura and Hasilik 1986). In cancer the intracellular trafficking of cathepsins is frequently altered, resulting in the production and secretion of proforms (Recklies, Poole et al. 1982) and the active form of the enzymes with the redistribution from perinuclear lysosomes to the pericellular space (Roshy, Sloane et al. 2003). This altered intracellular trafficking and localization contributes to non-lysosomal functions that have been linked to tumor growth, migration, invasion and angiogenesis (Mohamed and Sloane 2006). In this study, we observed the expression of cathepsins under the effect of RLN2. We have identified cysteine cathepsins, cath-B and cath-L, but not aspartic cath-D, as a novel target of relaxin-RXFP1 in brain tumor cells. Both cath-B and -L have similar proteolytic properties and action in tumor progression (Arora and Chauhan 2002).They also play a role in promoting brain cancer cell invasion by degrading the basement membrane and inhibit apoptosis (Levicar, Dewey et al. 2003; Levicar, Nuttall et al. 2003; Zajc, Hreljac et al. 2006). Reduction of the activity of intracellular cath-B by a specific inhibitor resulted in decrease in invasion of human melanoma, prostate carcinoma cells (Szpaderska and Frankfater 2001) and human breast epithelial cells (Premzl, Zavasnik-Bergant et al. 2003).We observed a significant increase in the active form of both cath-B and -L upon RLN2 treatment in U87MG and primary GB cells and this correlated with a significant increase in GB cell migration which suggests a mechanism of relaxin-RXFP1 induced GB cell migration. Moreover, the expression of cath-B in U87MG and primary cells confirmed the previous reports of cath-B as being a biomarker of invasiveness in brain tumor (Colin, Voutsinos-Porche et al. 2009). Absence of the expression of cath-B in T98G coincided with low invasive phenotype as reported by Formolo et al. (Formolo, Williams et al. 2011).

Cath-D may cooperate with cath-B in the process of proteolysis and cancer progression as procath-B can be activated by cath-D to initiate the proteolytic cascade (Rao 2003). Cath-D serum levels have been found to be significantly higher in GB patients than in low-grade astrocytoma patients (Fukuda, Iwadate et al. 2005). RLN2 treatment resulted in increased levels of procath-D and active cath-D in U87MG and PBS-1 cells that were borderline to becoming statistically relevant. Thus, cath-D may not be the direct target for RXFP1 mediated cell migration in brain cancer.

Proteases such as MMPs, their inhibitors and cathepsins play key roles in promoting brain tumor cell migration and invasion. However, tumor cell migration and invasion can be also supported by other mechanisms that do not involve proteases but, instead, affect the cytoskeleton. Activation of the small GTPases are central regulators of cell protrusion and responsible for cell movement and cancer cell migration (Parri and Chiarugi 2010). We observed an increase in the expression of Rac1 and Cdc42 in U87MG and primary cells upon RLN2 treatment. This coincided with the increased motility in these cells. Our data strongly suggest a significant increase in levels of Cdc42 and Rac1 in U87MG and all primary cells. This is in accordance with many studies that have identified Rac1 and Cdc42 as two key signaling proteins that play critical roles in the invasive behavior of glioblastoma (Chan, Coniglio et al. 2005; Yiin, Hu et al. 2009; Fortin, Ennis et al. 2012). Currently, little information is available about the effects of relaxin-RXFP1 system on small GTPases that relate to tumor cell migration and invasion. Our group recently studied the effects of relaxin on Rho-like small GTPases in facilitating invasion of thyroid cancer cells. The relaxin transfected follicular thyroid carcinoma cell line formed large-sized invadopodia laden with MMP-14. There was also increased stress fiber formation in the invadopdia indicating that relaxin affected changes in the actin cytoskeleton. (Bialek, Kunanuvat et al. 2011). This was known to be the responsibility of Rho GTPases, which contribute to the increased invasion of ECM. One possible

mechanism of relaxin in facilitating brain tumors cell migration may be that relaxin increased the level of Rac1 and Cdc42. This induced the cytoskeleton reorganization and the formation the protrusion such as pseudopodia or lamellipodia.

The combined results of an increased cath-B and-L, Rac1 and Cdc42 and PGRMC1 expression upon RLN2 treatment might all contribute to the overall increase of brain tumor cell migration. The effect of inhibiting these proteins on cell migration is required for future studies to confirm the action of these proteins in facilitating GB cell migration.

Human brain cancer cells were devoid of RLN1/2 suggesting that other ligands may activate RXFP1 and facilitate GB cell migartion. The identification of short linear peptides, P74 and P59, derived from the transition region between collagen domain and globular C1q domain of CTRP8 protein as activators of RXFP1 (Shemesh, Toporik et al. 2008) suggested that instead of RLN2, the human CTRP8 may be a novel RXFP1 ligand. CTRP8 is known to be predominantly expressed in human lung and testis but not in normal brain (Peterson, Wei et al. 2009). However no data is currently available for the expression of this protein in brain tumor cells. We observed CTRP8 expression in 32 cases of primary cells as well as GB cell lines. RT-PCR revealed that 90% of the cases of the primary cells and 100% of the GB cell lines and human astrocyte expressed CTRP8. CTRP8 is one of at least 15 C1q/TNF protein family members which have been shown to play major role in the metabolic and immune system (Kishore, Gaboriaud et al. 2004). The function of CTRP8 is still unclear but some other members of this family such as CTRP3 (Akiyama, Furukawa et al. 2009), CTRP4 (Li, Wang et al. 2011), and CTRP6 (Takeuchi, Adachi et al. 2011) have been reported to play a role in cancer. We successfully validated previous findings of the CTRP8-derived peptide, P74, being an RXFP1 activator similar to relaxin both *in vitro* and *in vivo* (Pini, Shemesh et al. 2010). Our present study revealed that P74, similar to relaxin, significantly increased cell migration

in cell lines (U87MG and T98G) and primary GB cells. Moreover, P74 increased cath-B,-L and –D levels in PBS-1 and PBS-10 but failed to induce the increase of cath-B,-L and –D levels in U87MG and T98G. Thus, CTRP8-derived P74 peptide was functional and, similar to relaxin, exerted a pro-migratory effect on human GB cells. The effect of P74 on cathepsin expression was GB cell specific and it is conceivable that P74 activates different signaling pathway in GB cell lines and primary GB cells. P74 might bind and activate RXFP1 differently from relaxin. Relaxin is a structurally complex heterodimeric peptide, whereas P74 is a linear peptide which may trigger different signaling pathways but, in part at least, lead to the same cellular function.

In conclusion, we reported for the first time the involvement of RXFP1 in the increased cellular motility and migration of GB cell lines and primary human GB cells. These findings also suggested that RXFP1 activation increase in GB cell migration involved the small GTPases Rac1 and Cdc42 and extrinsic matrix modulating enzymes. Furthermore, this work has identified PGRMC1 as a novel relaxin target protein in RXFP1-expressing brain tumor cells. Our findings implicate a clinically relevant role for RXFP1 in GB invasion and future studies on the effect of RXFP1 suppression (e.g., siRNA) on brain tumor cell migration are required to confirm the role of this GPCR in facilitating GB migration. P74 promises to be a novel candidate ligand for RXFP1 in brain tumors and additional experiments will elucidate the biological mechanism of this peptide and/or CTRP8 in brain cancer cell invasion. Overall, we conclude that RXFP1 and its dependent signaling pathways may be novel targets for modulating the invasive behavior of brain tumor cells.

REFERENCES

Adams, J., S. Schott, et al. (2012). "A novel role for relaxin-2 in the pathogenesis of primary varicosis." PLoS One **7**(6): e39021.

Adham, I. M., E. Burkhardt, et al. (1993). "Cloning of a cDNA for a novel insulin-like peptide of the testicular Leydig cells." J Biol Chem **268**(35): 26668-72.

Ahmad, N., W. Wang, et al. (2012). "Relaxin induces matrix-metalloproteinases-9 and -13 via RXFP1: Induction of MMP-9 involves the PI3K, ERK, Akt and PKC-zeta pathways." Mol Cell Endocrinol **363**(1-2): 46-61.

Ahmed, I. S., H. J. Rohe, et al. (2010). "Progesterone receptor membrane component 1 (Pgrmc1): a heme-1 domain protein that promotes tumorigenesis and is inhibited by a small molecule." J Pharmacol Exp Ther **333**(2): 564-73.

Akiyama, H., S. Furukawa, et al. (2009). "Elevated expression of CTRP3/cartducin contributes to promotion of osteosarcoma cell proliferation." Oncol Rep **21**(6): 1477-81.

Alfonso, P., A. Nunez, et al. (2005). "Proteomic expression analysis of colorectal cancer by two-dimensional differential gel electrophoresis." Proteomics **5**(10): 2602-11.

Ando, S., N. J. Sarlis, et al. (2001). "Aberrant alternative splicing of thyroid hormone receptor in a TSH-secreting pituitary tumor is a mechanism for hormone resistance." Mol Endocrinol **15**(9): 1529-38.

Arora, S. and S. S. Chauhan (2002). "Identification and characterization of a novel human cathepsin L splice variant." Gene **293**(1-2): 123-31.

Bani, D. and M. Bigazzi (2011). "Relaxin as a cardiovascular drug: a promise kept." Curr Drug Saf **6**(5): 324-8.

Bani, D., A. Riva, et al. (1994). "Differentiation of breast cancer cells in vitro is promoted by the concurrent influence of myoepithelial cells and relaxin." Br J Cancer **70**(5): 900-4.

Bartsch, O., B. Bartlick, et al. (2001). "Relaxin signalling links tyrosine phosphorylation to phosphodiesterase and adenylyl cyclase activity." Mol Hum Reprod **7**(9): 799-809.

Bathgate, R. A., R. Ivell, et al. (2006). "International Union of Pharmacology LVII: recommendations for the nomenclature of receptors for relaxin family peptides." Pharmacol Rev **58**(1): 7-31.

Bathgate, R. A., C. S. Samuel, et al. (2002). "Human relaxin gene 3 (H3) and the equivalent mouse relaxin (M3) gene. Novel members of the relaxin peptide family." J Biol Chem **277**(2): 1148-57.

Baylis, C. and B. Corman (1998). "The aging kidney: insights from experimental studies." J Am Soc Nephrol **9**(4): 699-709.

Beckner, M. E., X. Chen, et al. (2005). "Proteomic characterization of harvested pseudopodia with differential gel electrophoresis and specific antibodies." Lab Invest **85**(3): 316-27.

Bennett, R. G., D. G. Heimann, et al. (2009). "Relaxin reduces fibrosis in models of progressive and established hepatic fibrosis." Ann N Y Acad Sci **1160**: 348-9.

Bialek, J., U. Kunanuvat, et al. (2011). "Relaxin enhances the collagenolytic activity and in vitro invasiveness by upregulating matrix metalloproteinases in human thyroid carcinoma cells." Mol Cancer Res **9**(6): 673-87.

Binder, C., T. Hagemann, et al. (2002). "Relaxin enhances in-vitro invasiveness of breast cancer cell lines by up-regulation of matrix metalloproteases." Mol Hum Reprod **8**(9): 789-96.

Binder, C., A. Simon, et al. (2004). "Elevated concentrations of serum relaxin are associated with metastatic disease in breast cancer patients." Breast Cancer Res Treat **87**(2): 157-66.

Bond, C. P., L. J. Parry, et al. (2004). "Increased expression of the relaxin receptor (LGR7) in human endometrium during the secretory phase of the menstrual cycle." J Clin Endocrinol Metab **89**(7): 3477-85.

Brat, D. J., R. A. Prayson, et al. (2008). "Diagnosis of malignant glioma: role of neuropathology." J Neurooncol **89**(3): 287-311.

Brecht, A., C. Bartsch, et al. (2011). "Relaxin inhibits early steps in vascular inflammation." Regul Pept **166**(1-3): 76-82.

Bryant-Greenwood, G. D., S. Y. Yamamoto, et al. (2009). "Relaxin stimulates interleukin-6 and interleukin-8 secretion from the extraplacental chorionic cytotrophoblast." Placenta **30**(7): 599-606.

Bryant, G. D., M. E. Panter, et al. (1975). "Immunoreactive relaxin in human serum during the menstrual cycle." J Clin Endocrinol Metab **41**(06): 1065-9.

Bullesbach, E. E. and C. Schwabe (2000). "The relaxin receptor-binding site geometry suggests a novel gripping mode of interaction." J Biol Chem **275**(45): 35276-80.

Burazin, T. C., K. J. Johnson, et al. (2005). "Localization of LGR7 (relaxin receptor) mRNA and protein in rat forebrain: correlation with relaxin binding site distribution." Ann N Y Acad Sci **1041**: 205-10.

Burger, L. L. and O. D. Sherwood (1998). "Relaxin increases the accumulation of new epithelial and stromal cells in the rat cervix during the second half of pregnancy." Endocrinology **139**(9): 3984-95.

Carlevaro, M. F., A. Albini, et al. (1997). "Transferrin promotes endothelial cell migration and invasion: implication in cartilage neovascularization." J Cell Biol **136**(6): 1375-84.

Carroll, R. S., J. Zhang, et al. (1995). "Steroid hormone receptors in astrocytic neoplasms." Neurosurgery **37**(3): 496-503; discussion 503-4.

Chan, A. Y., S. J. Coniglio, et al. (2005). "Roles of the Rac1 and Rac3 GTPases in human tumor cell invasion." Oncogene **24**(53): 7821-9.

Chan, S. L. and M. J. Cipolla (2011). "Relaxin causes selective outward remodeling of brain parenchymal arterioles via activation of peroxisome proliferator-activated receptor-gamma." FASEB J **25**(9): 3229-39.

Chassin, D., A. Laurent, et al. (1995). "Cloning of a new member of the insulin gene superfamily (INSL4) expressed in human placenta." Genomics **29**(2): 465-70.

Chen, D., X. F. Zheng, et al. (2012). "S100A4 silencing blocks invasive ability of esophageal squamous cell carcinoma cells." World J Gastroenterol **18**(9): 915-22.

Chetty, C., S. K. Vanamala, et al. (2012). "MMP-9 induces CD44 cleavage and CD44 mediated cell migration in glioblastoma xenograft cells." Cell Signal **24**(2): 549-59.

Chinje, E. C. and I. J. Stratford (1997). "Role of nitric oxide in growth of solid tumours: a balancing act." Essays Biochem **32**: 61-72.

Chirasani, S. R., D. S. Markovic, et al. (2009). "Transferrin-receptor-mediated iron accumulation controls proliferation and glutamate release in glioma cells." J Mol Med (Berl) **87**(2): 153-67.

Cho, S. Y. and R. L. Klemke (2002). "Purification of pseudopodia from polarized cells reveals redistribution and activation of Rac through assembly of a CAS/Crk scaffold." J Cell Biol **156**(4): 725-36.

Chodniewicz, D. and R. L. Klemke (2004). "Guiding cell migration through directed extension and stabilization of pseudopodia." Exp Cell Res **301**(1): 31-7.

Chow, B. S., E. G. Chew, et al. (2012). "Relaxin Signals through a RXFP1-pERK-nNOS-NO-cGMP-Dependent Pathway to Up-Regulate Matrix Metalloproteinases: The Additional Involvement of iNOS." PLoS One **7**(8): e42714.

Colin, C., B. Voutsinos-Porche, et al. (2009). "High expression of cathepsin B and plasminogen activator inhibitor type-1 are strong predictors of survival in glioblastomas." Acta Neuropathol **118**(6): 745-54.

Conklin, D., C. E. Lofton-Day, et al. (1999). "Identification of INSL5, a new member of the insulin superfamily." Genomics **60**(1): 50-6.

Connor, J. R., S. L. Menzies, et al. (1990). "Cellular distribution of transferrin, ferritin, and iron in normal and aged human brains." J Neurosci Res **27**(4): 595-611.

Conrad, K. P. (2011). "Emerging role of relaxin in the maternal adaptations to normal pregnancy: implications for preeclampsia." Semin Nephrol **31**(1): 15-32.

Conrad, K. P. and J. Novak (2004). "Emerging role of relaxin in renal and cardiovascular function." Am J Physiol Regul Integr Comp Physiol **287**(2): R250-61.

Craig, R. and R. C. Beavis (2004). "TANDEM: matching proteins with tandem mass spectra." Bioinformatics **20**(9): 1466-7.

Crawford, R. J., P. Hudson, et al. (1984). "Two human relaxin genes are on chromosome 9." EMBO J **3**(10): 2341-5.

Crudden, G., R. E. Chitti, et al. (2006). "Hpr6 (heme-1 domain protein) regulates the susceptibility of cancer cells to chemotherapeutic drugs." J Pharmacol Exp Ther **316**(1): 448-55.

Crudden, G., R. Loesel, et al. (2005). "Overexpression of the cytochrome p450 activator hpr6 (heme-1 domain protein/human progesterone receptor) in tumors." Tumour Biol **26**(3): 142-6.

D'Abaco, G. M. and A. H. Kaye (2007). "Integrins: molecular determinants of glioma invasion." J Clin Neurosci **14**(11): 1041-8.

Dahlmann, M., U. Sack, et al. (2012). "Systemic shRNA mediated knock down of S100A4 in colorectal cancer xenografted mice reduces metastasis formation." Oncotarget **3**(8): 783-97.

Demuth, T. and M. E. Berens (2004). "Molecular mechanisms of glioma cell migration and invasion." J Neurooncol **70**(2): 217-28.

Dschietzig, T., C. Bartsch, et al. (2009). "RXFP1-inactive relaxin activates human glucocorticoid receptor: further investigations into the relaxin-GR pathway." Regul Pept **154**(1-3): 77-84.

Dschietzig, T., C. Bartsch, et al. (2003). "Relaxin, a pregnancy hormone, is a functional endothelin-1 antagonist: attenuation of endothelin-1-mediated vasoconstriction by stimulation of endothelin type-B receptor expression via ERK-1/2 and nuclear factor-kappaB." Circ Res **92**(1): 32-40.

Dschietzig, T., C. Richter, et al. (2001). "The pregnancy hormone relaxin is a player in human heart failure." FASEB J **15**(12): 2187-95.

Du, X. J., Q. Xu, et al. (2009). "Reversal of cardiac fibrosis and related dysfunction by relaxin." Ann N Y Acad Sci **1160**: 278-84.

Dwivedi, R. C., N. Dhindsa, et al. (2009). "The effects of infliximab therapy on the serum proteome of rheumatoid arthritis patients." Arthritis Res Ther **11**(2): R32.

Egeblad, M. and Z. Werb (2002). "New functions for the matrix metalloproteinases in cancer progression." Nat Rev Cancer **2**(3): 161-74.

Einspanier, A., D. Muller, et al. (2001). "Characterization of relaxin binding in the uterus of the marmoset monkey." Mol Hum Reprod **7**(10): 963-70.

Failli, P., S. Nistri, et al. (2002). "Relaxin up-regulates inducible nitric oxide synthase expression and nitric oxide generation in rat coronary endothelial cells." FASEB J **16**(2): 252-4.

Fasshauer, M., R. Paschke, et al. (2004). "Adiponectin, obesity, and cardiovascular disease." Biochimie **86**(11): 779-84.

Fei, D. T., M. C. Gross, et al. (1990). "Cyclic AMP response to recombinant human relaxin by cultured human endometrial cells--a specific and high throughput in vitro bioassay." Biochem Biophys Res Commun **170**(1): 214-22.

Feng, S., I. U. Agoulnik, et al. (2007). "Relaxin promotes prostate cancer progression." Clin Cancer Res **13**(6): 1695-702.

Feng, S., I. U. Agoulnik, et al. (2010). "Suppression of relaxin receptor RXFP1 decreases prostate cancer growth and metastasis." Endocr Relat Cancer **17**(4): 1021-33.

Fevold, H. H., FL. Meyer, RK. (1930). "The relaxative hormone of the corpus luteum: its purification and concentration." J Am Chem Soc **52**: 3340-3348.

Fialho, A. M., P. Salunkhe, et al. (2012). "Glioblastoma multiforme: novel therapeutic approaches." ISRN Neurol **2012**: 642345.

Figueiredo, K. A., A. L. Mui, et al. (2006). "Relaxin stimulates leukocyte adhesion and migration through a relaxin receptor LGR7-dependent mechanism." J Biol Chem **281**(6): 3030-9.

Filonzi, M., L. C. Cardoso, et al. (2007). "Relaxin family peptide receptors Rxfp1 and Rxfp2: mapping of the mRNA and protein distribution in the reproductive tract of the male rat." Reprod Biol Endocrinol **5**: 29.

Formolo, C. A., R. Williams, et al. (2011). "Secretome signature of invasive glioblastoma multiforme." J Proteome Res **10**(7): 3149-59.

Fortin, S. P., M. J. Ennis, et al. (2012). "Cdc42 and the Guanine nucleotide exchange factors ect2 and trio mediate fn14-induced migration and invasion of glioblastoma cells." Mol Cancer Res **10**(7): 958-68.

Friedl, P. and E. B. Brocker (2000). "The biology of cell locomotion within three-dimensional extracellular matrix." Cell Mol Life Sci **57**(1): 41-64.

Fukuda, M. E., Y. Iwadate, et al. (2005). "Cathepsin D is a potential serum marker for poor prognosis in glioma patients." Cancer Res **65**(12): 5190-4.

Furnari, F. B., T. Fenton, et al. (2007). "Malignant astrocytic glioma: genetics, biology, and paths to treatment." Genes Dev **21**(21): 2683-710.

Gerasimova, T., M. N. Thanasoula, et al. (2010). "Identification and in vitro characterization of follicle stimulating hormone (FSH) receptor variants associated with abnormal ovarian response to FSH." J Clin Endocrinol Metab **95**(2): 529-36.

Gerlee, P. and S. Nelander (2012). "The impact of phenotypic switching on glioblastoma growth and invasion." PLoS Comput Biol **8**(6): e1002556.

Gondi, C. S., S. S. Lakka, et al. (2004). "Downregulation of uPA, uPAR and MMP-9 using small, interfering, hairpin RNA (siRNA) inhibits glioma cell invasion, angiogenesis and tumor growth." Neuron Glia Biol **1**(2): 165-76.

Gonzalez-Aguero, G., R. Ondarza, et al. (2001). "Progesterone receptor isoforms expression pattern in human astrocytomas." Brain Res Bull **56**(1): 43-8.

Guirguis, R., I. Margulies, et al. (1987). "Cytokine-induced pseudopodial protrusion is coupled to tumour cell migration." Nature **329**(6136): 261-3.

Gunnersen, J. M., P. Fu, et al. (1996). "Expression of human relaxin genes: characterization of a novel alternatively-spliced human relaxin mRNA species." Mol Cell Endocrinol **118**(1-2): 85-94.

Hall, W. A., A. Godal, et al. (1992). "In vitro efficacy of transferrin-toxin conjugates against glioblastoma multiforme." J Neurosurg **76**(5): 838-44.

Halls, M. L. (2012). "Constitutive formation of an RXFP1-signalosome: a novel paradigm in GPCR function and regulation." Br J Pharmacol **165**(6): 1644-58.

Halls, M. L., R. A. Bathgate, et al. (2006). "Relaxin family peptide receptors RXFP1 and RXFP2 modulate cAMP signaling by distinct mechanisms." Mol Pharmacol **70**(1): 214-26.

Halls, M. L., R. A. Bathgate, et al. (2007). "Comparison of signaling pathways activated by the relaxin family peptide receptors, RXFP1 and RXFP2, using reporter genes." J Pharmacol Exp Ther **320**(1): 281-90.

Halls, M. L., C. P. Bond, et al. (2005). "Multiple binding sites revealed by interaction of relaxin family peptides with native and chimeric relaxin family peptide receptors 1 and 2 (LGR7 and LGR8)." J Pharmacol Exp Ther **313**(2): 677-87.

Halls, M. L. and D. M. Cooper (2010). "Sub-picomolar relaxin signalling by a pre-assembled RXFP1, AKAP79, AC2, beta-arrestin 2, PDE4D3 complex." EMBO J **29**(16): 2772-87.

Halls, M. L., T. D. Hewitson, et al. (2009). "Relaxin activates multiple cAMP signaling pathway profiles in different target cells." Ann N Y Acad Sci **1160**: 108-11.

Halls, M. L., E. T. van der Westhuizen, et al. (2009). "Relaxin family peptide receptor (RXFP1) coupling to G(alpha)i3 involves the C-terminal Arg752 and localization within membrane Raft Microdomains." Mol Pharmacol **75**(2): 415-28.

Han, K. Y., D. C. Fahd, et al. (2011). "MT1-MMP Modulates bFGF-Induced VEGF-A Expression in Corneal Fibroblasts." Protein Pept Lett.

Hansell, D. J., G. D. Bryant-Greenwood, et al. (1991). "Expression of the human relaxin H1 gene in the decidua, trophoblast, and prostate." J Clin Endocrinol Metab **72**(4): 899-904.

Heeg, M. H., M. J. Koziolek, et al. (2005). "The antifibrotic effects of relaxin in human renal fibroblasts are mediated in part by inhibition of the Smad2 pathway." Kidney Int **68**(1): 96-109.

Hewitson, T. D., W. Y. Ho, et al. (2010). "Antifibrotic properties of relaxin: in vivo mechanism of action in experimental renal tubulointerstitial fibrosis." Endocrinology **151**(10): 4938-48.

Hisaw, F. (1926). "Experimental relaxation of the pubic ligament of the guinea pig." Proc Soc Exp Biol Med **23**: 661–663.

Hoelzinger, D. B., T. Demuth, et al. (2007). "Autocrine factors that sustain glioma invasion and paracrine biology in the brain microenvironment." J Natl Cancer Inst **99**(21): 1583-93.

Holthaus, M. G., L. Treccani, et al. (2012). "Osteoblast viability on hydroxyapatite with well-adjusted submicron and micron surface roughness as monitored by the proliferation reagent WST-1." J Biomater Appl.

Hombach-Klonisch, S., J. Bialek, et al. (2006). "Relaxin enhances the oncogenic potential of human thyroid carcinoma cells." Am J Pathol **169**(2): 617-32.

Hombach-Klonisch, S., J. Buchmann, et al. (2000). "Relaxin-like factor (RLF) is differentially expressed in the normal and neoplastic human mammary gland." Cancer **89**(11): 2161-8.

Hopkins, E. J., S. Layfield, et al. (2007). "The NMR solution structure of the relaxin (RXFP1) receptor lipoprotein receptor class A module and identification of key residues in the N-

terminal region of the module that mediate receptor activation." J Biol Chem **282**(6): 4172-84.

Hsu, S. Y. (2003). "New insights into the evolution of the relaxin-LGR signaling system." Trends Endocrinol Metab **14**(7): 303-9.

Hsu, S. Y., K. Nakabayashi, et al. (2003). "Relaxin signaling in reproductive tissues." Mol Cell Endocrinol **202**(1-2): 165-70.

Hsu, S. Y., K. Nakabayashi, et al. (2002). "Activation of orphan receptors by the hormone relaxin." Science **295**(5555): 671-4.

Huang, Z. and S. D. Bao (2004). "Roles of main pro- and anti-angiogenic factors in tumor angiogenesis." World J Gastroenterol **10**(4): 463-70.

Hudson, P., J. Haley, et al. (1983). "Structure of a genomic clone encoding biologically active human relaxin." Nature **301**(5901): 628-31.

Hudson, P., M. John, et al. (1984). "Relaxin gene expression in human ovaries and the predicted structure of a human preprorelaxin by analysis of cDNA clones." EMBO J **3**(10): 2333-9.

Huttner, A. (2012). "Overview of primary brain tumors: pathologic classification, epidemiology, molecular biology, and prognostic markers." Hematol Oncol Clin North Am **26**(4): 715-32.

Itoh, Y., A. Ito, et al. (1998). "Plasma membrane-bound tissue inhibitor of metalloproteinases (TIMP)-2 specifically inhibits matrix metalloproteinase 2 (gelatinase A) activated on the cell surface." J Biol Chem **273**(38): 24360-7.

Ivell, R., M. Balvers, et al. (2003). "Immunoexpression of the relaxin receptor LGR7 in breast and uterine tissues of humans and primates." Reprod Biol Endocrinol **1**: 114.

Kamat, A. A., S. Feng, et al. (2006). "The role of relaxin in endometrial cancer." Cancer Biol Ther **5**(1): 71-7.

Kerbel, R. S. (2008). "Tumor angiogenesis." N Engl J Med **358**(19): 2039-49.

Kern, A., A. I. Agoulnik, et al. (2007). "The low-density lipoprotein class A module of the relaxin receptor (leucine-rich repeat containing G-protein coupled receptor 7): its role in signaling and trafficking to the cell membrane." Endocrinology **148**(3): 1181-94.

Kern, A. and G. D. Bryant-Greenwood (2009). "Mechanisms of relaxin receptor (LGR7/RXFP1) expression and function." Ann N Y Acad Sci **1160**: 60-6.

Kern, A., D. Hubbard, et al. (2008). "Cloning, expression, and functional characterization of relaxin receptor (leucine-rich repeat-containing g protein-coupled receptor 7) splice variants from human fetal membranes." Endocrinology **149**(3): 1277-94.

Kessenbrock, K., V. Plaks, et al. (2010). "Matrix metalloproteinases: regulators of the tumor microenvironment." Cell **141**(1): 52-67.

Khalil, B. D. and M. El-Sibai (2012). "Rho GTPases in primary brain tumor malignancy and invasion." J Neurooncol **108**(3): 333-9.

Kim, Y. S., J. A. Jung, et al. (2011). "Galectin-3 binding protein promotes cell motility in colon cancer by stimulating the shedding of protein tyrosine phosphatase kappa by proprotein convertase 5." Biochem Biophys Res Commun **404**(1): 96-102.

Kishore, U., C. Gaboriaud, et al. (2004). "C1q and tumor necrosis factor superfamily: modularity and versatility." Trends Immunol **25**(10): 551-61.

Klonisch, T., J. Bialek, et al. (2007). "Relaxin-like ligand-receptor systems are autocrine/paracrine effectors in tumor cells and modulate cancer progression and tissue invasiveness." Adv Exp Med Biol **612**: 104-18.

Kong, R. C., P. J. Shilling, et al. (2010). "Membrane receptors: structure and function of the relaxin family peptide receptors." Mol Cell Endocrinol **320**(1-2): 1-15.

Krajnc-Franken, M. A., A. J. van Disseldorp, et al. (2004). "Impaired nipple development and parturition in LGR7 knockout mice." Mol Cell Biol **24**(2): 687-96.

Krusche, C. A., T. Kroll, et al. (2007). "Expression of leucine-rich repeat-containing G-protein-coupled receptors in the human cyclic endometrium." Fertil Steril **87**(6): 1428-37.

Kumagai, J., S. Y. Hsu, et al. (2002). "INSL3/Leydig insulin-like peptide activates the LGR8 receptor important in testis descent." J Biol Chem **277**(35): 31283-6.

Lakka, S. S., M. Rajan, et al. (2002). "Adenovirus-mediated expression of antisense MMP-9 in glioma cells inhibits tumor growth and invasion." Oncogene **21**(52): 8011-9.

Lampert, K., U. Machein, et al. (1998). "Expression of matrix metalloproteinases and their tissue inhibitors in human brain tumors." Am J Pathol **153**(2): 429-37.

Lee, W. J., I. K. Choi, et al. (2012). "Relaxin-expressing adenovirus decreases collagen synthesis and up-regulates matrix metalloproteinase expression in keloid fibroblasts: in vitro experiments." Plast Reconstr Surg **130**(3): 407e-17e.

Lessing, J. B., S. H. Brenner, et al. (1986). "Effect of relaxin on human spermatozoa." J Reprod Med **31**(5): 304-9.

Levicar, N., R. A. Dewey, et al. (2003). "Selective suppression of cathepsin L by antisense cDNA impairs human brain tumor cell invasion in vitro and promotes apoptosis." Cancer Gene Ther **10**(2): 141-51.

Levicar, N., R. K. Nuttall, et al. (2003). "Proteases in brain tumour progression." Acta Neurochir (Wien) **145**(9): 825-38.

Li, A., J. C. Dawson, et al. (2010). "The actin-bundling protein fascin stabilizes actin in invadopodia and potentiates protrusive invasion." Curr Biol **20**(4): 339-45.

Li, Q., L. Wang, et al. (2011). "Identification of C1qTNF-related protein 4 as a potential cytokine that stimulates the STAT3 and NF-kappaB pathways and promotes cell survival in human cancer cells." Cancer Lett **308**(2): 203-14.

Li, Y., S. Negishi, et al. (2005). "The use of relaxin improves healing in injured muscle." Ann N Y Acad Sci **1041**: 395-7.

Lihn, A. S., S. B. Pedersen, et al. (2005). "Adiponectin: action, regulation and association to insulin sensitivity." Obes Rev **6**(1): 13-21.

Liu, C., E. Eriste, et al. (2003). "Identification of relaxin-3/INSL7 as an endogenous ligand for the orphan G-protein-coupled receptor GPCR135." J Biol Chem **278**(50): 50754-64.

Liu, C., C. Kuei, et al. (2005). "INSL5 is a high affinity specific agonist for GPCR142 (GPR100)." J Biol Chem **280**(1): 292-300.

Liu, C., C. Sun, et al. (2003). "Overexpression of legumain in tumors is significant for invasion/metastasis and a candidate enzymatic target for prodrug therapy." Cancer Res **63**(11): 2957-64.

Liu, L., J. Wu, et al. (2010). "Astrocyte elevated gene-1 upregulates matrix metalloproteinase-9 and induces human glioma invasion." Cancer Res **70**(9): 3750-9.

Lizarraga, F., R. Poincloux, et al. (2009). "Diaphanous-related formins are required for invadopodia formation and invasion of breast tumor cells." Cancer Res **69**(7): 2792-800.

Lok, S., D. S. Johnston, et al. (2000). "Identification of INSL6, a new member of the insulin family that is expressed in the testis of the human and rat." Biol Reprod **62**(6): 1593-9.

Lorenz, M., H. Yamaguchi, et al. (2004). "Imaging sites of N-wasp activity in lamellipodia and invadopodia of carcinoma cells." Curr Biol **14**(8): 697-703.

Louis, D. N., H. Ohgaki, et al. (2007). "The 2007 WHO classification of tumours of the central nervous system." Acta Neuropathol **114**(2): 97-109.

Lu, K. V., K. A. Jong, et al. (2004). "Upregulation of tissue inhibitor of metalloproteinases (TIMP)-2 promotes matrix metalloproteinase (MMP)-2 activation and cell invasion in a human glioblastoma cell line." Lab Invest **84**(1): 8-20.

Luna, J. J., A. Riesewijk, et al. (2004). "Gene expression pattern and immunoreactive protein localization of LGR7 receptor in human endometrium throughout the menstrual cycle." Mol Hum Reprod **10**(2): 85-90.

Madhra, M., E. Gay, et al. (2004). "Alternative splicing of the human luteal LH receptor during luteolysis and maternal recognition of pregnancy." Mol Hum Reprod **10**(8): 599-603.

Masini, E., S. Nistri, et al. (2004). "Relaxin inhibits the activation of human neutrophils: involvement of the nitric oxide pathway." Endocrinology **145**(3): 1106-12.

Mazella, J., M. Tang, et al. (2004). "Disparate effects of relaxin and TGFbeta1: relaxin increases, but TGFbeta1 inhibits, the relaxin receptor and the production of IGFBP-1 in human endometrial stromal/decidual cells." Hum Reprod **19**(7): 1513-8.

McCawley, L. J. and L. M. Matrisian (2001). "Matrix metalloproteinases: they're not just for matrix anymore!" Curr Opin Cell Biol **13**(5): 534-40.

McDonald, G. A., P. Sarkar, et al. (2003). "Relaxin increases ubiquitin-dependent degradation of fibronectin in vitro and ameliorates renal fibrosis in vivo." Am J Physiol Renal Physiol **285**(1): F59-67.

McGuane, J. T., J. E. Debrah, et al. (2011). "Relaxin induces rapid dilation of rodent small renal and human subcutaneous arteries via PI3 kinase and nitric oxide." Endocrinology **152**(7): 2786-96.

Mohamed, M. M. and B. F. Sloane (2006). "Cysteine cathepsins: multifunctional enzymes in cancer." Nat Rev Cancer **6**(10): 764-75.

Mookerjee, I., T. D. Hewitson, et al. (2009). "Relaxin inhibits renal myofibroblast differentiation via RXFP1, the nitric oxide pathway, and Smad2." FASEB J **23**(4): 1219-29.

Mookerjee, I., E. N. Unemori, et al. (2005). "Relaxin modulates fibroblast function, collagen production, and matrix metalloproteinase-2 expression by cardiac fibroblasts." Ann N Y Acad Sci **1041**: 190-3.

Moore, X. L., S. L. Tan, et al. (2007). "Relaxin antagonizes hypertrophy and apoptosis in neonatal rat cardiomyocytes." Endocrinology **148**(4): 1582-9.

Muda, M., C. He, et al. (2005). "Splice variants of the relaxin and INSL3 receptors reveal unanticipated molecular complexity." Mol Hum Reprod **11**(8): 591-600.

Nair, V. B., C. S. Samuel, et al. (2012). "Human relaxin-2: historical perspectives and role in cancer biology." Amino Acids **43**(3): 1131-40.

Nakamura, K., S. Yamashita, et al. (2004). "A splice variant of the human luteinizing hormone (LH) receptor modulates the expression of wild-type human LH receptor." Mol Endocrinol **18**(6): 1461-70.

Nistri, S. and D. Bani (2003). "Relaxin receptors and nitric oxide synthases: search for the missing link." Reprod Biol Endocrinol **1**: 5.

Nobes, C. D. and A. Hall (1999). "Rho GTPases control polarity, protrusion, and adhesion during cell movement." J Cell Biol **144**(6): 1235-44.

Noma, N., S. Simizu, et al. (2012). "Involvement of NF-kappaB-mediated expression of galectin-3-binding protein in TNF-alpha-induced breast cancer cell adhesion." Oncol Rep **27**(6): 2080-4.

O'Donnell, K. A., D. Yu, et al. (2006). "Activation of transferrin receptor 1 by c-Myc enhances cellular proliferation and tumorigenesis." Mol Cell Biol **26**(6): 2373-86.

Ohgaki, H. and P. Kleihues (2005). "Population-based studies on incidence, survival rates, and genetic alterations in astrocytic and oligodendroglial gliomas." J Neuropathol Exp Neurol **64**(6): 479-89.

Pajvani, U. B., X. Du, et al. (2003). "Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin. Implications fpr metabolic regulation and bioactivity." J Biol Chem **278**(11): 9073-85.

Park, J. I., J. Semyonov, et al. (2008). "Regulation of receptor signaling by relaxin A chain motifs: derivation of pan-specific and LGR7-specific human relaxin analogs." J Biol Chem **283**(46): 32099-109.

Parri, M. and P. Chiarugi (2010). "Rac and Rho GTPases in cancer cell motility control." Cell Commun Signal **8**: 23.

Parry, L. J. and L. A. Vodstrcil (2007). "Relaxin physiology in the female reproductive tract during pregnancy." Adv Exp Med Biol **612**: 34-48.

Parsell, D. A., J. Y. Mak, et al. (1996). "Relaxin binds to and elicits a response from cells of the human monocytic cell line, THP-1." J Biol Chem **271**(44): 27936-41.

Paulino, V. M., Z. Yang, et al. (2010). "TROY (TNFRSF19) is overexpressed in advanced glial tumors and promotes glioblastoma cell invasion via Pyk2-Rac1 signaling." Mol Cancer Res **8**(11): 1558-67.

Peluso, J. J., A. Gawkowska, et al. (2009). "Progesterone receptor membrane component-1 regulates the development and Cisplatin sensitivity of human ovarian tumors in athymic nude mice." Endocrinology **150**(11): 4846-54.

Peluso, J. J., X. Liu, et al. (2008). "Regulation of ovarian cancer cell viability and sensitivity to cisplatin by progesterone receptor membrane component-1." J Clin Endocrinol Metab **93**(5): 1592-9.

Peluso, J. J., J. Romak, et al. (2008). "Progesterone receptor membrane component-1 (PGRMC1) is the mediator of progesterone's antiapoptotic action in spontaneously immortalized granulosa cells as revealed by PGRMC1 small interfering ribonucleic acid treatment and functional analysis of PGRMC1 mutations." Endocrinology **149**(2): 534-43.

Peterson, J. M., Z. Wei, et al. (2009). "CTRP8 and CTRP9B are novel proteins that hetero-oligomerize with C1q/TNF family members." Biochem Biophys Res Commun **388**(2): 360-5.

Philippar, U., E. T. Roussos, et al. (2008). "A Mena invasion isoform potentiates EGF-induced carcinoma cell invasion and metastasis." Dev Cell **15**(6): 813-28.

Pichot, C. S., C. Arvanitis, et al. (2010). "Cdc42-interacting protein 4 promotes breast cancer cell invasion and formation of invadopodia through activation of N-WASp." Cancer Res **70**(21): 8347-56.

Pini, A., R. Shemesh, et al. (2010). "Prevention of bleomycin-induced pulmonary fibrosis by a novel antifibrotic peptide with relaxin-like activity." J Pharmacol Exp Ther **335**(3): 589-99.

Premzl, A., V. Zavasnik-Bergant, et al. (2003). "Intracellular and extracellular cathepsin B facilitate invasion of MCF-10A neoT cells through reconstituted extracellular matrix in vitro." Exp Cell Res **283**(2): 206-14.

Radestock, Y., C. Willing, et al. (2010). "Relaxin enhances S100A4 and promotes growth of human thyroid carcinoma cell xenografts." Mol Cancer Res **8**(4): 494-506.

Rao, J. S. (2003). "Molecular mechanisms of glioma invasiveness: the role of proteases." Nat Rev Cancer **3**(7): 489-501.

Rawlings, N. D. and A. J. Barrett (1999). "MEROPS: the peptidase database." Nucleic Acids Res **27**(1): 325-31.

Recht, L., C. O. Torres, et al. (1990). "Transferrin receptor in normal and neoplastic brain tissue: implications for brain-tumor immunotherapy." J Neurosurg **72**(6): 941-5.

Recklies, A. D., A. R. Poole, et al. (1982). "A cysteine proteinase secreted from human breast tumours is immunologically related to cathepsin B." Biochem J **207**(3): 633-6.

Richardson, D. R. and P. Ponka (1997). "The molecular mechanisms of the metabolism and transport of iron in normal and neoplastic cells." Biochim Biophys Acta **1331**(1): 1-40.

Ridley, A. J., M. A. Schwartz, et al. (2003). "Cell migration: integrating signals from front to back." Science **302**(5651): 1704-9.

Riemenschneider, M. J., J. W. Jeuken, et al. (2010). "Molecular diagnostics of gliomas: state of the art." Acta Neuropathol **120**(5): 567-84.

Roshy, S., B. F. Sloane, et al. (2003). "Pericellular cathepsin B and malignant progression." Cancer Metastasis Rev **22**(2-3): 271-86.

Rutka, J. T., G. Apodaca, et al. (1988). "The extracellular matrix of the central and peripheral nervous systems: structure and function." J Neurosurg **69**(2): 155-70.

Saitoh, O., W. C. Wang, et al. (1992). "Differential glycosylation and cell surface expression of lysosomal membrane glycoproteins in sublines of a human colon cancer exhibiting distinct metastatic potentials." J Biol Chem **267**(8): 5700-11.

Samuel, C. S., H. Tian, et al. (2003). "Relaxin is a key mediator of prostate growth and male reproductive tract development." Lab Invest **83**(7): 1055-67.

Sasaki, Y., T. Kohsaka, et al. (2001). "Immunoreactive relaxin in seminal plasma of fertile boars and its correlation with sperm motility characteristics determined by computer-assisted digital image analysis." Int J Androl **24**(1): 24-30.

Schaffler, A. and C. Buechler (2012). "CTRP family: linking immunity to metabolism." Trends Endocrinol Metab **23**(4): 194-204.

Schouten, L. J., J. Rutten, et al. (2002). "Incidence of brain metastases in a cohort of patients with carcinoma of the breast, colon, kidney, and lung and melanoma." Cancer **94**(10): 2698-705.

Schwabe, C. and J. K. McDonald (1977). "Primary structure of the B-chain of porcine relaxin." Biochem Biophys Res Commun **75**(2): 503-10.

Schwabe, C., J. K. McDonald, et al. (1976). "Primary structure of the A chain of porcine relaxin." Biochem Biophys Res Commun **70**(2): 397-405.

Schwartzbaum, J. A., J. L. Fisher, et al. (2006). "Epidemiology and molecular pathology of glioma." Nat Clin Pract Neurol **2**(9): 494-503; quiz 1 p following 516.

Scott, D. J., S. Layfield, et al. (2006). "Characterization of novel splice variants of LGR7 and LGR8 reveals that receptor signaling is mediated by their unique low density lipoprotein class A modules." J Biol Chem **281**(46): 34942-54.

Scott, D. J., G. W. Tregear, et al. (2005). "LGR7-truncate is a splice variant of the relaxin receptor LGR7 and is a relaxin antagonist in vitro." Ann N Y Acad Sci **1041**: 22-6.

Segal, M. S., L. Sautina, et al. (2012). "Relaxin increases human endothelial progenitor cell NO and migration and vasculogenesis in mice." Blood **119**(2): 629-36.

Shankar, J., A. Messenberg, et al. (2010). "Pseudopodial actin dynamics control epithelial-mesenchymal transition in metastatic cancer cells." Cancer Res **70**(9): 3780-90.

Shemesh, R., C. Hermesh, et al. (2009). "Activation of relaxin-related receptors by short, linear peptides derived from a collagen-containing precursor." Ann N Y Acad Sci **1160**: 78-86.

Shemesh, R., R. Shemesh, et al. (2008). "Discovery and validation of novel peptide agonists for G-protein-coupled receptors." J Biol Chem **283**(50): 34643-9.

Sherwood, O. D. (2004). "Relaxin's physiological roles and other diverse actions." Endocr Rev **25**(2): 205-34.

Shirota, K., K. Tateishi, et al. (2005). "Early human preantral follicles have relaxin and relaxin receptor (LGR7), and relaxin promotes their development." J Clin Endocrinol Metab **90**(1): 516-21.

Sibony-Benyamini, H. and H. Gil-Henn (2012). "Invadopodia: The leading force." Eur J Cell Biol.

Silvertown, J. D., B. J. Geddes, et al. (2003). "Adenovirus-mediated expression of human prorelaxin promotes the invasive potential of canine mammary cancer cells." Endocrinology **144**(8): 3683-91.

Silvertown, J. D., J. Ng, et al. (2006). "H2 relaxin overexpression increases in vivo prostate xenograft tumor growth and angiogenesis." Int J Cancer **118**(1): 62-73.

Silvertown, J. D., A. J. Summerlee, et al. (2003). "Relaxin-like peptides in cancer." Int J Cancer **107**(4): 513-9.

Singh, S. and R. G. Bennett (2009). "Relaxin family peptide receptor 1 activation stimulates peroxisome proliferator-activated receptor gamma." Ann N Y Acad Sci **1160**: 112-6.

Singh, S. and R. G. Bennett (2010). "Relaxin signaling activates peroxisome proliferator-activated receptor gamma." Mol Cell Endocrinol **315**(1-2): 239-45.

Smith, J. S. and R. B. Jenkins (2000). "Genetic alterations in adult diffuse glioma: occurrence, significance, and prognostic implications." Front Biosci **5**: D213-31.

Song, G. J., Y. S. Park, et al. (2002). "Alternatively spliced variants of the follicle-stimulating hormone receptor gene in the testis of infertile men." Fertil Steril **77**(3): 499-504.

Stewart, D. R. (2009). "Scar prevention and cosmetically enhanced wound healing using relaxin." Ann N Y Acad Sci **1160**: 336-41.

Strongin, A. Y., I. Collier, et al. (1995). "Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease." J Biol Chem **270**(10): 5331-8.

Stupp, R., W. P. Mason, et al. (2005). "Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma." N Engl J Med **352**(10): 987-96.

Stylli, S. S., A. H. Kaye, et al. (2008). "Invadopodia: at the cutting edge of tumour invasion." J Clin Neurosci **15**(7): 725-37.

Sudo, S., J. Kumagai, et al. (2003). "H3 relaxin is a specific ligand for LGR7 and activates the receptor by interacting with both the ectodomain and the exoloop 2." J Biol Chem **278**(10): 7855-62.

Sunn, N., M. Egli, et al. (2002). "Circulating relaxin acts on subfornical organ neurons to stimulate water drinking in the rat." Proc Natl Acad Sci U S A **99**(3): 1701-6.

Szpaderska, A. M. and A. Frankfater (2001). "An intracellular form of cathepsin B contributes to invasiveness in cancer." Cancer Res **61**(8): 3493-500.

Takeuchi, T., Y. Adachi, et al. (2011). "Expression of a secretory protein C1qTNF6, a C1qTNF family member, in hepatocellular carcinoma." Anal Cell Pathol (Amst) **34**(3): 113-21.

Tang, M. L., C. S. Samuel, et al. (2009). "Role of relaxin in regulation of fibrosis in the lung." Ann N Y Acad Sci **1160**: 342-7.

Tashima, L. S., G. Mazoujian, et al. (1994). "Human relaxins in normal, benign and neoplastic breast tissue." J Mol Endocrinol **12**(3): 351-64.

Teerlink, J. R., M. Metra, et al. (2009). "Relaxin for the treatment of patients with acute heart failure (Pre-RELAX-AHF): a multicentre, randomised, placebo-controlled, parallel-group, dose-finding phase IIb study." Lancet **373**(9673): 1429-39.

Teichman, S. L., E. Unemori, et al. (2009). "Relaxin, a pleiotropic vasodilator for the treatment of heart failure." Heart Fail Rev **14**(4): 321-9.

Teodorczyk, M. and A. Martin-Villalba (2010). "Sensing invasion: cell surface receptors driving spreading of glioblastoma." J Cell Physiol **222**(1): 1-10.

Thomas, P. D., M. J. Campbell, et al. (2003). "PANTHER: a library of protein families and subfamilies indexed by function." Genome Res **13**(9): 2129-41.

Tran, N. L., W. S. McDonough, et al. (2003). "The human Fn14 receptor gene is up-regulated in migrating glioma cells in vitro and overexpressed in advanced glial tumors." Am J Pathol **162**(4): 1313-21.

Unemori, E. N., L. B. Pickford, et al. (1996). "Relaxin induces an extracellular matrix-degrading phenotype in human lung fibroblasts in vitro and inhibits lung fibrosis in a murine model in vivo." J Clin Invest **98**(12): 2739-45.

van der Westhuizen, E. T., M. L. Halls, et al. (2008). "Relaxin family peptide receptors--from orphans to therapeutic targets." Drug Discov Today **13**(15-16): 640-51.

Villano, J. L., T. E. Seery, et al. (2009). "Temozolomide in malignant gliomas: current use and future targets." Cancer Chemother Pharmacol **64**(4): 647-55.

Vinall, R. L., C. M. Mahaffey, et al. (2011). "Dual blockade of PKA and NF-kappaB inhibits H2 relaxin-mediated castrate-resistant growth of prostate cancer sublines and induces apoptosis." Horm Cancer **2**(4): 224-38.

von Figura, K. and A. Hasilik (1986). "Lysosomal enzymes and their receptors." Annu Rev Biochem **55**: 167-93.

Wang, J., B. Liu, et al. (2012). "PTP1B expression contributes to gastric cancer progression." Med Oncol **29**(2): 948-56.

Wang, J., S. Tian, et al. (2010). "The complex role of multivalency in nanoparticles targeting the transferrin receptor for cancer therapies." J Am Chem Soc **132**(32): 11306-13.

Wang, Y., R. Hanley, et al. (2006). "Computational methods for comparison of large genomic and proteomic datasets reveal protein markers of metastatic cancer." J Proteome Res **5**(4): 907-15.

Washburn, M. P., D. Wolters, et al. (2001). "Large-scale analysis of the yeast proteome by multidimensional protein identification technology." Nat Biotechnol **19**(3): 242-7.

Weber, G. L., M. O. Parat, et al. (2011). "Abrogation of PIK3CA or PIK3R1 reduces proliferation, migration, and invasion in glioblastoma multiforme cells." Oncotarget **2**(11): 833-49.

Weiss, G. (1991). "The physiology of human relaxin." Contrib Gynecol Obstet **18**: 130-46.

Wilkinson, T. N., T. P. Speed, et al. (2005). "Evolution of the relaxin-like peptide family." BMC Evol Biol **5**: 14.

Winger, M. J., D. R. Macdonald, et al. (1989). "Supratentorial anaplastic gliomas in adults. The prognostic importance of extent of resection and prior low-grade glioma." J Neurosurg **71**(4): 487-93.

Winslow, J. W., A. Shih, et al. (1992). "Human seminal relaxin is a product of the same gene as human luteal relaxin." Endocrinology **130**(5): 2660-8.

Wong, G. W., J. Wang, et al. (2004). "A family of Acrp30/adiponectin structural and functional paralogs." Proc Natl Acad Sci U S A **101**(28): 10302-7.

Wreje, U., P. Kristiansson, et al. (1995). "Serum levels of relaxin during the menstrual cycle and oral contraceptive use." Gynecol Obstet Invest **39**(3): 197-200.

Wyatt, T. A., J. H. Sisson, et al. (2002). "Relaxin stimulates bronchial epithelial cell PKA activation, migration, and ciliary beating." Exp Biol Med (Maywood) **227**(11): 1047-53.

Yamaguchi, H. (2012). "Pathological roles of invadopodia in cancer invasion and metastasis." Eur J Cell Biol.

Yamaguchi, H., M. Lorenz, et al. (2005). "Molecular mechanisms of invadopodium formation: the role of the N-WASP-Arp2/3 complex pathway and cofilin." J Cell Biol **168**(3): 441-52.

Yamaguchi, H., J. Wyckoff, et al. (2005). "Cell migration in tumors." Curr Opin Cell Biol **17**(5): 559-64.

Yang, H., K. Zhao, et al. (2012). "Evaluation of plasma and tissue S100A4 protein and mRNA levels as potential markers of metastasis and prognosis in clear cell renal cell carcinoma." J Int Med Res **40**(2): 475-85.

Yao, L., A. I. Agoulnik, et al. (2008). "Relaxin acts on stromal cells to promote epithelial and stromal proliferation and inhibit apoptosis in the mouse cervix and vagina." Endocrinology **149**(5): 2072-9.

Yiin, J. J., B. Hu, et al. (2009). "Slit2 inhibits glioma cell invasion in the brain by suppression of Cdc42 activity." Neuro Oncol **11**(6): 779-89.

Zajc, I., I. Hreljac, et al. (2006). "Cathepsin L affects apoptosis of glioblastoma cells: a potential implication in the design of cancer therapeutics." Anticancer Res **26**(5A): 3357-64.

Zhang, Q., S. H. Liu, et al. (2002). "Relaxin activates the MAP kinase pathway in human endometrial stromal cells." J Cell Biochem **85**(3): 536-44.

Zhao, L., P. J. Roche, et al. (1999). "Mice without a functional relaxin gene are unable to deliver milk to their pups." Endocrinology **140**(1): 445-53.

Zheng, L. Y., D. X. Zhou, et al. (2012). "Down-regulated expression of the protein-tyrosine phosphatase 1B (PTP1B) is associated with aggressive clinicopathologic features and poor prognosis in hepatocellular carcinoma." Biochem Biophys Res Commun **420**(3): 680-4.

Zhong, H., H. SuYang, et al. (1997). "The transcriptional activity of NF-kappaB is regulated by the IkappaB-associated PKAc subunit through a cyclic AMP-independent mechanism." Cell **89**(3): 413-24.

Zuzga, D. S., J. Pelta-Heller, et al. (2012). "Phosphorylation of vasodilator-stimulated phosphoprotein Ser239 suppresses filopodia and invadopodia in colon cancer." Int J Cancer **130**(11): 2539-48.