THE LAR PROTEIN TYROSINE PHOSPHATASE ENABLES PDGF β-RECEPTOR ACTIVATION AND SIGNAL TRANSDUCTION

by

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ABSTRACT

Many cellular activities including cell survival, proliferation, migration and differentiation are controlled by growth factors and their corresponding tyrosine kinases receptor (RTKs). Growth factor receptor activation is strictly regulated by protein tyrosine phosphatases (PTPs). Here I investigated whether the receptor protein tyrosine phosphatase (RPTP) LAR, which is known to modify the activity of several RTKs, also regulates platelet derived growth factor (PDGF) receptor activity and signalling. Mouse embryonic fibroblasts (MEFs) expressing mutant LAR lacking its phosphatase domains (LARΔP) showed reduced phosphorylation of PDGFβ receptor (PDGFβR) compared with wild type (WT) cells. This was rescued by re-expression of WT LAR. The decreased phosphorylation of the PDGFβR was independent of ligand concentration and occurred on all tyrosine residues, suggesting that LAR is required for full PDGFβR kinase activation. The decreased kinase activity reduced the amplitude or duration of the different signalling pathways activated downstream of the PDGFβR, and resulted in reduced proliferation in response to PDGF-BB. These findings demonstrate, for the first time, that LAR activity is required for PDGF-induced fibroblast proliferation. The inhibition of PDGFβR kinase activity in LARΔP cells was exerted via increased basal activity of the tyrosine kinase c-Abl and its substrate protein kinase Cδ (PKCδ). Ligand-induced PDGFβR dimerization is defective in LARΔP cells, possibly due to the observed increase in the Nherf2 protein associating with the PDGFβR. In summary, I have identified LAR as a new regulator

of PDGFβR activity, and propose a novel mechanism where PDGF-induced activation of c-Abl serves as a negative feedback loop to terminate the PDGFβR kinase activity. This may occur via PKCδ activation promoting the association of Nherf2 with PDGFβR, thereby reducing ligand-induced receptor dimerization and kinase activation. In this model, LAR promotes PDGFβR activity by inactivating c-Abl.

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LIST OF ABBREVIATIONS

C-Abl	Abelson murine leukaemia viral oncoprotein
CKIγ2	Casein kinase Iγ2
DSPs	Dual specificity phosphatases
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
ESCRT I	Endosomal sorting complex required for transport I
GRK	G protein coupled receptor kinase
HGF	Hepatocyte growth factor
Hrs	Hepatocyte growth factor regulated tyrosine kinase substrate
LAR	Leukocyte common antigen related
MEF	Mouse embryonic fibroblast
MKP	MAP kinase phosphatase
MTOC	Microtubule organizing centre
Nherf	Na^+/H^+ exchanger regulatory factor
NPTP	Non-transmembrane PTPs
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDZ	PSD95/Drosophila discs large/ZO1
PIP3	Phosphatidylinositol (3,4,5) triphosphate
PI3K	Phosphoinositide-3'-kinase

PI(4,5)P2	Phosphatidylinositol 4,5 bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PTEN	Phosphatase and Tensin homolog
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
RasGAP	RasGTPase activating protein
RPTP	Receptor protein tyrosine phosphatase
RTK	Receptor tyrosine kinase
SH2	Src homology 2
STAT	Signal transducers and activators of transcription

CHAPTER 1 INTRODUCTION

Many of the cellular activities including cell survival, growth, proliferation, migration and differentiation are controlled by growth factors and their corresponding receptors. These growth factors act as signalling transduction molecules to bind to their specific receptors located on the cell membrane. These activated receptors then initiate a cascade of signal transduction through multiple downstream pathways to regulate the expression of a pattern of genes which results in the inhibition and promotion of protein production needed for cellular responses. The cellular response to growth factor stimulation is tightly controlled. This ensures that cells start and stop responding to stimulation at the correct time. Errors in the regulation of cellular signalling and response lead to cancer and metabolic diseases (Kadowaki *et al.* 1996; Heldin *et al.* 1998; Wells 1999).

Intracellular signal transduction involves many phosphorylation and dephosphorylation events. Reversible protein phosphorylation is an important regulatory mechanism in eukaryotic organisms. Kinases phosphorylate proteins by transferring a phosphate group from ATP to their substrates. In eukaryotic cells, phosphorylation usually occurs on serine, threonine and tyrosine residues (Thomason and Kay 2000; Cohen 2002; Fiedler *et al.* 2009). Phosphatases dephosphorylate

proteins by the hydrolysis of phosphoric acid monoesters into a phosphate ion and a residue with a free hydroxyl group (Tiganis and Bennett 2007). Reversible phosphorylation leads to conformational changes in the structure of many enzymes and receptors, causing them to become activated or inactivated, and therefore regulates the cellular signalling and responses these proteins are involved in (Krebs and Beavo 1979; Raju 2000).

1.1 PDGF and PDGFR

1.1.1 Introduction

Platelet-derived growth factor (PDGF) is a family of growth factors of importance for survival, proliferation and motility of several different cell types. This includes the connective tissue cells such as fibroblasts, smooth muscle cells and neurons (Kohler and Lipton 1974; Ross *et al.* 1974; Heldin *et al.* 1998). PDGF was originally isolated from human platelets (Ross 1986). PDGF and its corresponding receptor PDGFR (PDGF receptor) have important roles in regulating adult physiological functions. It was shown that PDGF causes the constriction of several types of blood vessel (Berk *et al.* 1986; Sachinidis *et al.* 1990). PDGF also regulates wound healing and the maintenance of interstitial fluid pressure (Robson *et al.* 1992; Rodt *et al.* 1996).

PDGF plays important roles in the development of alveoli in the lung by promoting the growth of alveolar myoblast cells (Bostrom *et al.* 1996; Soriano 1997). PDGF is

important for glomeruli development by allowing the growth of mesenglial cells and attracting pericytes to glomeruli blood vessels (Leveen *et al.* 1994; Lindahl *et al.* 1997; Soriano 1997). PDGF and PDGFR were shown to be highly expressed in testicular cells. They were suggested to be crucial for testis development by promoting the Leydig cell migration, proliferation and differentiation (Peltomaki *et al.* 1991; Carmona *et al.* 2009; Basciani *et al.* 2010). PDGFR has been reported to be expressed in the mouse migratory neural crest (Ho *et al.* 1994; Takakura *et al.* 1997). PDGF is required for neural crest development by stimulating neural crest cell migration and survival (Soriano 1997; Robbins *et al.* 1999). PDGF has been shown to stimulate the migration of the cells to the wound site of cartilage in combination with insulin growth factor (IGF) to mediate cartilage repair (Breinan *et al.* 2000; Schmidt *et al.* 2006). Because of the mitogenic and chemotactic responses caused by PDGF in osteoblast cells, PDGF has been shown recently to be important for bone regeneration, and therefore may serve as an effective and safer alternative for autogenous bone graft (DiGiovanni *et al.* 2012).

Studies on mice showed that PDGF plays important roles in regulating embryonic development. Knock out of PDGF and PDGFR leads to impaired development of alveoli in the lung (Bostrom *et al.* 1996; Soriano 1997), defective development of glomeruli and blood vessels with the absence of mesenglial in glomeruli and inability of the blood vessel to attract pericytes (Leveen *et al.* 1994; Lindahl *et al.* 1997; Soriano 1997), abnormal cerebral vascularization, loss of neuroependymal integrity

and ventricular abnormality (Fredriksson *et al.* 2012), leading to mice dying at the time around birth (Leveen *et al.* 1994; Lindahl *et al.* 1997; Soriano 1997).

Over-activation of PDGF and PDGFR leads to several physiological diseases and cancer. Up-regulation of PDGF and PDGFR were observed in almost all the human renal diseases (Zhang *et al.* 2005). Overactivation of PDGFR leads to renal fibrosis and glomerulosclerosis (Floege *et al.* 2008; Ostendorf *et al.* 2012). Overactivation of PDGFR in retinal cells causes proliferative vitreoretiopathy (Lei *et al.* 2007; Cui *et al.* 2009; Lei *et al.* 2010). Several observations suggest that overactivity of PDGF is involved in the development of various fibrotic conditions in the lung (Souza *et al.* 1996; Heldin and Westermark 1999).

Human glioma cells express both PDGF and PDGFR. The distribution of the ligand and the receptor indicates that the glial tumour cells are stimulated in PDGF autocrine and paracrine loops. Activation of PDGFR induces tumour blood vessel formation (Lokker *et al.* 2002; Martinho *et al.* 2009; Nazarenko *et al.* 2012). Overexpression of PDGFR is observed in breast cancer, and inhibition of PDGFR and c-kit with Imatinib suppressed the growth and invasion of breast cancer cells (Roussidis *et al.* 2007). PDGF overexpression also leads to autocrine stimulation in the rare skin tumour dermatofibrosarcoma protuberans (Sjoblom *et al.* 2001)

1.1.2 PDGF Ligand binding and PDGFR dimerization

PDGF is a dimeric molecule consisting of disulfide-bonded A, B, C and D polypeptide chains. All four types of polypeptides form homodimers, while A and B can also form heterodimers (Heldin *et al.* 1998; Li *et al.* 2000; Bergsten *et al.* 2001). These PDGF isoforms have their effects on the target cells by binding to two types of structurally related tyrosine kinase receptors called the PDGF α-receptor (PDGFαR) and PDGF β-receptor (PDGFβR) with different specificities.

Many of the tyrosine kinase receptors are activated by ligand binding induced receptor dimerization or oligomerization (Heldin and Ostman 1996). All PDGF isoforms are dimers and therefore have two receptor binding epitopes, thus each PDGF dimer binds to two receptors simultaneously (Duan *et al.* 1991; Fretto *et al.* 1993; Herren *et al.* 1993). PDGFαR binds to the A, B and C chain of the ligand, while PDGFβR binds only to the B and D chain with high affinity, therefore different ligand molecules will induce the dimerization of different types of PDGFRs. PDGF AA leads to the dimerization of PDGFαα receptor, AB leads to the αα and αβ receptor dimer, BB induces the formation of all three types of receptor dimer *i.e.* αα, αβ and ββ. PDGF CC and DD induce the αα and ββ dimerization respectively (Bishayee *et al.* 1989; Heldin *et al.* 1989; Seifert *et al.* 1989; Kanakaraj *et al.* 1991)(Figure A).

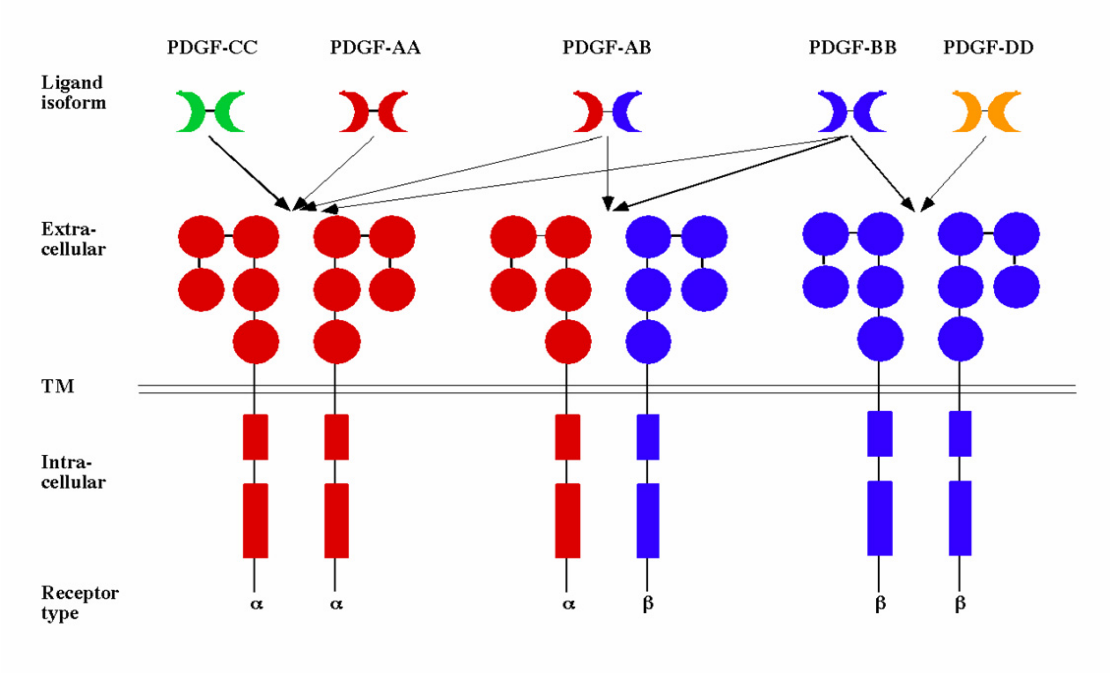

Figure A Ligand-induced dimerization of PDGF receptors

The ability of different isoforms of PDGF dimer to induce the formation of homo- and heteromeric receptor complexes is shown (From a self-made PowerPoint slide given by Dr. Carina Hellberg).

So the PDGF ligand serves as a bridge holding two PDGF receptors together to form a dimer. The link between the two PDGF receptor molecules was found to be further stabilized by the interaction between the immunoglobulin domain 4 from the N terminus of the dimerized receptors (Heldin *et al.* 1998). There is a low affinity interaction between the PDGF receptors in the absence of ligand, which allows the receptors to be dimerized and activated without ligand when the expression of receptor is high. Studies showed that when PDGF receptors are highly expressed in the cell, receptor dimerization and autophosphorylation were observed (Jensen *et al.* 1992; Herren *et al.* 1993).

1.1.3 PDGF receptor autophosphorylation and activation of its downstream signalling pathways

Dimerization of PDGF receptors brings the intracellular domains of the two receptors to a very close distance which then leads to the autophosphorylation of the receptors in trans (Kelly *et al.* 1991). Autophosphorylation plays important roles in elevating the receptor kinase activity and creating binding sites for the downstream signalling molecules (Heldin 1997). Tyr857 is one of the autophosphorylation sites, which is located in the catalytic part of the PDGFβR, mutation of this tyrosine to phenylalanine leads to a reduction of PDGFβR kinase activity (Fantl *et al.* 1989; Kazlauskas and Cooper 1989; Wardega *et al.* 2010). This tyrosine residue is conserved in PDGFαR and many other tyrosine kinase receptors, and evidence suggests that it has an important role in regulating the kinase activity (White *et al.* 1988; Naldini *et al.* 1991; Guiton *et al.* 1994; Mohammadi *et al.* 1996). Researchers (Chiara *et al.* 2004) later showed that allosteric auto inhibition of PDGFβR by its C terminal tail is one of the mechanisms keeping the receptor inactive in the absence of ligands. There are 11 autophosphorylation sites out of 15 tyrosine residues located in the intracellular non-catalytic part of the PDGFβR, these autophosphorylated tyrosine residues provide docking sites for the downstream signal transduction molecules and induce multiple signalling pathways (Claesson-Welsh 1994).

Src homology 2 (SH2) domains of proteins bind to phosphorylated tyrosine residues in specific environments with certain surrounding amino acids. SH2 domain

containing proteins have been shown to bind to the autophosphorylated tyrosine residues on the PDGF receptor, and this leads to the activation of the corresponding downstream signalling pathways. These include signal transduction molecules with enzyme activity e.g. phosphatidylinositol 3' kinase (PI3K), phospholipase C-γ (PLCγ), Src kinase, GTPase activating protein for Ras (RasGAP), as well as adaptor proteins such as Grb2, Shc, Nck, Grb7, Crk and signal transducer transcription activator STATS (Figure B). SH2 binding specificity is determined by the 3 to 6 amino acids C terminal to the phosphorylated tyrosine (Songyang *et al.* 1993).

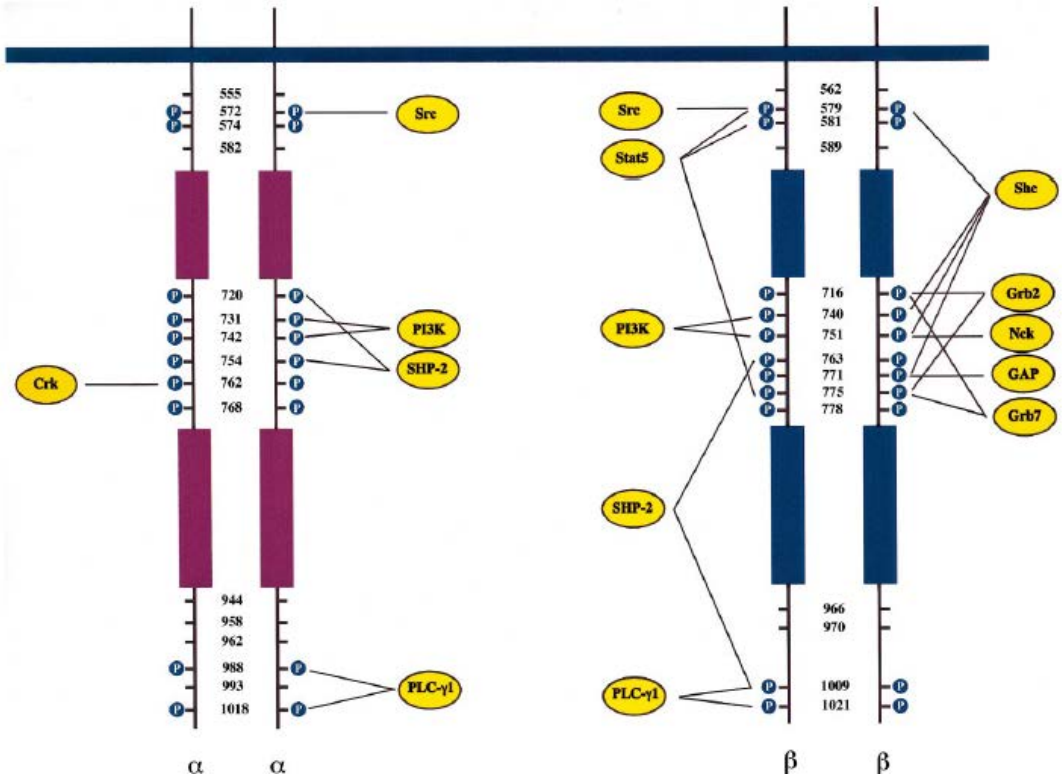

Figure B Interaction between PDGFαR and PDGFβR with SH2 domain containing signal transduction molecules

The intracellular parts of homodimerized PDGFαR and PDGFβR are drawn. All tyrosine residues outside the PDGFR catalytic domains are indicated; known autophosphorylation sites are indicated by an encircled P. The known interactions between phosphorylated tyrosine residues in PDGFR and different SH2 domain containing signalling molecules are shown (reproduced from Heldin *et al.* 1998).

1.1.3.1 PI3K pathway

PI3K is a family of kinases that phosphorylate phosphatidylinositol on its 3' position of the inositol ring (G. Panayotou 1996). Class IA PI3K contains a catalytic subunit p110 and a regulatory subunit p85. The SH2 domains on the p85 subunit allow it to bind to the phosphorylated tyrosine residue on PDGFR (Auger *et al.* 1989; Coughlin *et al.* 1989). Two binding sites of PI3K have been identified on PDGFβR including Tyr740 and Tyr751 (Fantl *et al.* 1992; Kashishian *et al.* 1992; Kazlauskas *et al.* 1992). It has been shown that the binding of the p85 subunit to the phosphorylated proteins leads to the conformational change of P110 catalytic subunit and increases its enzyme activity (Yu *et al.* 1991).

PDGFβR activation leads to the activation of PI3K. The activated PI3K generates PI(3,4,5)P_3 which plays a role in recruiting the downstream serine/threonine kinase Akt to the plasma membrane (Burgering and Coffer 1995). PI(3,4,5)P_3 binds to the PH (Pleckstrin homology) domain of Akt, leading to its conformational change, allowing the subsequent activation of Akt by the phosphorylation of its Ser473 residue by mTORC2 (Alessi *et al.* 1997; Persad *et al.* 2001; Feng *et al.* 2004; Sarbassov *et al.* 2005) and the phosphorylation of its Thr308 residue by phosphoinositide dependent kinase 1 (PDK1) (Franke *et al.* 1997; Klippel *et al.* 1997; Stokoe *et al.* 1997). Activated Akt then phosphorylates BAD. In its dephosphorylated form, BAD interacts with BLC_{XL} and leads to cell apoptosis (Zha *et al.* 1996), however, phosphorylation of BAD by Akt prevents its binding to BLC_{XL} thereby protecting the cell from apoptosis

(Datta *et al.* 1997). PI3K has also been shown to be important for the PDGF induced actin reorganization, cell migration and proliferation (Heldin *et al.* 1998).

1.1.3.2 PLCγ pathway

PLCγ carries out the hydrolysis of $PI(4,5)P_2$ leading to the production of inositol(1,4,5)trisphosphate (IP3) and diacylglycerol (DAG). IP3 causes Ca^{2+} to be released from the intracellular Ca^{2+} stores and increases cytoplasmic Ca^{2+} concentration. DAG can activate certain PKC family members (Berridge 1993). PLCγ1 and PLCγ2 were both shown to be activated by PDGF stimulation (Sultzman *et al.* 1991). PLCγ1 has been more thoroughly studied in the PDGF signalling system and binds to the autophosphorylation sites Tyr1009 and Tyr1021 on PDGFβR (Ronnstrand *et al.* 1992; Kashishian and Cooper 1993; Valius *et al.* 1993). The binding of PLCγ1 to the PDGFβR and its subsequent phosphorylation of Tyr783 (Kumjian *et al.* 1991) are involved in its activation (Meisenhelder *et al.* 1989). The activation of PLCγ by PDGF stimulation leads to several cellular effects including proliferation, cytoskeletal rearrangement and activation of ion channels (Ma *et al.* 1994; Krawczyk and Matuszyk 2011).

1.1.3.3 Ras pathway

The adaptor molecule Grb2 can form complex with the Ras Guanine nucleotide exchange factor (GEF) Sos1, which plays a role in converting Ras from its inactive

GDP bound form to its GTP bound active form (Schlessinger 1993). Upon simulation of PDGFR, Ras is activated by Sos1, the GTP bound Ras interacts with the regulatory N terminus of Raf1, causing activation of this serine threonine kinase. Activated Raf1 then phosphorylates and activates the downstream MEK, which in turn phosphorylates and activates MAP kinases Erk1 and Erk2. After the activation of Erk1 and Erk2, they can be translocated into the nucleus and phosphorylate certain transcription factors and regulate their activity to affect gene transcription (Nanberg and Westermark 1993; Satoh *et al.* 1993).

Grb2 has been shown to bind to the phosphorylated Tyr716 and Tyr775 on PDGFβR (Arvidsson *et al.* 1994; Ruusala *et al.* 1998). Grb2 can also bind to the PDGFβR indirectly through binding to its binding sites on other proteins such as the adaptor protein Shc and tyrosine phosphatase SHP2, after these proteins have bound to the PDGFβR and have had their specific tyrosine residues phosphorylated (Benjamin and Jones 1994; Yokote *et al.* 1994). The direct and indirect binding of Grb2 and Sos1 respectively with PDGFR allows this complex to interact with and activate the Ras, which is located at the inner leaflet of the plasma membrane. The activation of Ras can then lead to physiological changes such as cell growth, differentiation and cell migration (Arvidsson *et al.* 1994; Li *et al.* 1994; Yokote *et al.* 1994; Ruusala *et al.* 1998).

1.1.4 PDGFR downstream signalling leads to cell proliferation

Ras, PI3K and PLCγ pathways have all been shown to be involved in PDGFβR induced cell proliferation. The Ras MAPK signalling pathway was shown to be the most important pathway in cell proliferation downstream PDGFβR (Mulcahy *et al.* 1985; Marshall 1994). ERK1/2 downstream of Ras MAP kinase activation can phosphorylate and activate p90rsk kinase which in turn phosphorylates and activates transcription factors such as c-fos and CREB and leads to proliferation (Seger and Krebs 1995). ERK1/2 may be involved in the regulation of MTOC (microtubule-organizing center) which regulates the assembly of cytosolic microtubules during interphase and cell mitotic spindle during cell division (Verlhac *et al.* 1993).

PI3K and PLCγ also play roles in promoting the responses involved in cell proliferation (Klippel *et al.* 1996). Microinjection of an activating antibody against the N-terminus SH2 domain of P85 subunit of the PI3K, led to the activation of DNA synthesis in CHO cells (McIlroy *et al.* 1997). In experiments where the tyrosine residues were mutated on the PDGFβR, adding back the PI3K binding sites Tyr740 and Tyr751 or the PLCγ binding sites Tyr1021, partly restored the PDGF induced cell proliferation (Valius and Kazlauskas 1993). Microinjection of dominant negative PLCγ with the catalytic domain gone causes the blocking of the S phase entry upon PDGF stimulation (Wang *et al.* 1998). Homologous disruption of PLCγ gene leads to embryonic lethal phenotype due to the attenuation of proliferation and growth of all

parts of embryo (Ji *et al.* 1997).

1.1.5 Downregulation of PDGFR signalling

As described above, PDGFR plays an important role in regulating normal physiological responses, but overactivation of PDGFR causes transformation. It is therefore necessary for the cell to carefully control the strength and duration of PDGFR signalling. There are several mechanisms acting in parallel to downregulate and desensitize PDGFR to maintain its normal function. This section will discuss receptor ubiquitination, internalization, degradation and dephosphorylation.

1.1.5.1 Receptor internalization and degradation

Inactivation of PDGFR signalling is generally accomplished by receptor ubiquitination, internalization (also called endocytosis) and subsequent lysosomal degradation. The internalization and intracellular sorting of the cell surface receptors have been extensively studied (Maxfield and McGraw 2004), but comparatively little is known about PDGFR. After ligand stimulation, the activated receptors mainly udergo clathrin-mediated endocytosis, the endocytic vesicles containing the receptors then fuse with early endosomes (Gorvel *et al.* 1991; Bucci *et al.* 1992). At the early endosome, some receptors such as the transferrin receptor rapidly recycle back to the cell surface via a Rab4a GTPase dependent short recycling loop (Maxfield and McGraw 2004). Other receptors are either sorted through a Rab 11 dependent

endocytic recycling compartment to enter a long recycling loop to the cell surface, or through late endosomes to lysosomal degradation (van Ijzendoorn 2006). EGFR (epidermal growth factor receptor) has been shown to recycle through the Rab 11 positive recycling compartment, while PDGFR does not normally recycle (Karlsson *et al.* 2006; Chi *et al.* 2011). However, later studies have shown that loss of T cell protein tyrosine phosphatase (TC-PTP) induced Rab4a dependent PDGFβR recycling and PKC activity was necessary for this recycling (Hellberg *et al.* 2009).

Cbl mediated multiple monoubiquitination has been shown to be a sorting signal for the degradation of EGFR and PDGFR (Raucher *et al.* 2000; Schmidt and Dikic 2005). Ubiquitinated receptors interact with the hepatocyte growth factor regulated tyrosine kinase substrate (Hrs) which is a key player of the sorting mechanism, since it further interacts with the endosomal sorting complex required for transport I (ESCRT I). Hrs and ESCRT I mediate the sorting of ubiquitinated proteins into the late endosome (Bache *et al.* 2003; Bache *et al.* 2003). It was shown that Hrs binding to signal transducer adaptor molecule (STAM2) is important for the proper sorting of PDGFR towards degradation (Takata *et al.* 2000).

1.1.5.2 PDGFβR dephosphorylation by PTPs

The PDGFR is also inactivated through dephosphorylation. Several protein tyrosine phosphatases (PTPs) interact with and dephosphorylate selective tyrosine residues on

PDGFβR, thereby controlling the duration of PDGFβR signalling and modulating the pattern of PDGFβR downstream signal transduction. Previous studies have shown that TC-PTP site selectively dephophorylates the PLCγ binding site Tyr1021 on PDGFβR to reduce the migratory response to PDGF-BB (Persson *et al.* 2004). Low molecular weight protein tyrosine phosphatase (LMW-PTP) dephosphorylates Tyr857 on PDGFβR thereby reducing the receptor kinase activity (Chiarugi *et al.* 2002). SHP2 dephosphorylates the Ras-GAP binding site Tyr771 thereby promoting activation of the Ras/MAPK pathway (Ekman *et al.* 2002). PTP1B dephosphorylates the Src and Shc binding site Tyr579 (Ekman *et al.* 2002; Persson *et al.* 2004). DEP1 dephosphorylates several of the autophosphorylation sites on PDGFβR, but not Tyr857. Other PTPs such as SHP1, PTP-PEST and CD45 have all been shown to control PDGFβR phosphorylation, but their preferred substrate sites and effects on downstream signalling have not been studied thoroughly (Mooney *et al.* 1992; Yu *et al.* 1998; Kovalenko *et al.* 2000; Markova *et al.* 2003).

1.1.6 Serine phosphorylation reduces PDGFβR tyrosine phosphorylation

Serine phosphorylation of the receptor cytoplasmic domain is one of the common mechanisms to desensitize receptor activity after stimulation. This has been shown for growth factor receptors such as the insulin receptor and EGFR (Takayama *et al.* 1988; Countaway *et al.* 1992). Since it was described in 1999 (Bioukar *et al.* 1999), several serine threonine kinases have been found to serine phosphorylate PDGFβR and attenuate its autophosphorylation and signalling.

Casein kinase Iγ2 (CKIγ2) is the first serine threonine kinases that was shown to phosphorylate on the serine residues of PDGFβR, and thus negatively regulate its autophosphorylation activity (Bioukar *et al.* 1999). Researchers showed that CKIγ2 was activated 5 minutes after PDGFβR activation and stayed active for more than 30 minutes in rat 2 fibroblast cells. Treating cells with specific CKIγ2 inhibitor led to a considerable reduction in PDGFβR serine phosphorylation. Transfection of constitutively active CKIγ2 greatly reduced PDGFβR tyrosine phosphorylation upon stimulation (Bioukar *et al.* 1999).

Later studies found G protein coupled receptor (GPCR) kinase family member GRK2 and GRK5, which initiate the desensitization of GPCR, can both serine phosphorylate PDGFβR and lead to decreased PDGFβR autophosphorylation (Freedman *et al.* 2002; Wu *et al.* 2006). GRK2 was shown to be tyrosine phosphorylated and activated by PDGFβR upon PDGF stimulation, therefore initiating a negative feedback loop by phosphorylating Ser1104 of PDGFβR. Nherf1 (Na+/H+ exchanger regulatory factor 1) is known to normally facilitate PDGFβR dimerization and activation by binding to the receptor C terminus and forming oligomers. Phosphorylation of this C terminal serine 1104 blocks the binding site of Nherf 1 and thus prevents Nherf1 association with PDGFβR. This leads to the reduction of receptor dimerization, activation and autophosphorylation (Freedman *et al.* 2002; Hildreth *et al.* 2004).

1.1.7 Src phosphorylates PDGFβR

Src family proteins including Src, Fyn, Yes, Hck, Lck and Lyn play important roles in regulating many cellular events including cell survival, proliferation and migration (Cance *et al.* 1994; Oberg-Welsh and Welsh 1995; Thuveson *et al.* 1995; Brown and Cooper 1996). Src protein tyrosine kinase is a 60 kDa protein composed of six different parts including SH2 domain, SH3 domain, kinase domain, unique region, SH4 domain and a C terminus negative regulatory tail (Brown and Cooper 1996). The tyrosine residue Tyr527 is located on the C terminal tail, and phosphorylation of this tyrosine leads to the intramolecular interaction between the SH2 domain and the C terminus which mediates the autoinhibition of Src kinase activity (Brown and Cooper 1996). Mutation of Tyr527 of Src leads to constitutive activation of Src protein (Cartwright *et al.* 1987).

Upon PDGFβR activation, Src is recruited to the phosphorylated receptor (Mori *et al.* 1993). This leads to the displacement of Tyr527 from the Src SH2 domain, dephosphorylation of Tyr527 by protein tyrosine phosphatases (PTPs) and phosphorylation of the Tyr416 on the activation loop of Src which upregulates Src kinase activity (Hunter 1987). Several PTPs have been shown to dephosphorylate Tyr527 of Src, these include PTPα, SHP1, SHP2, PTP1B and LAR (Peng and Cartwright 1995; Tsujikawa *et al.* 2002; Roskoski 2005)

After the activation by PDGFβR, Src was shown to phosphorylate PDGFβR.

Researchers found that Src kinase can phosphorylate Tyr934 in the PDGFβR kinase domain. Mutation of this tyrosine to phenylalanine led to a reduction in mitogenic response and an increase in migration upon PDGF stimulation. In other words, Src activation provides positive feedback for PDGF induced proliferation by phosphorylating Tyr934 and negative feedback for PDGFβR downstream motility (Hansen *et al.* 1996).

1.1.8 Caveola localization of PDGFβR affects its signalling

Caveolae are generally defined as invaginations of the plasma membrane composed of cholesterol and sphingolipids (Bruns and Palade 1968; Scherer *et al.* 1994). The cholesterol and sphingolipids form a liquid-ordered phase which makes caveolae resistant to detergent solubilisation (Bruns and Palade 1968; Scherer *et al.* 1994; Brown and London 1997). Caveolae are not soluble in TritonX100 at 4°C, such as that when cells were fixed with paraformaldehyde and extracted with cold TritonX100 and examined under the microscope, the remaining insoluble membranefractions were found to be caveolae (Moldovan *et al.* 1995). Caveolin is the most abundant protein present in Caveolae. The caveolin protein family has three members, caveolin1, caveolin2 and caveolin3 (Glenney and Soppet 1992; Scherer *et al.* 1995). These caveolins form oligomers with each other and bind to cholesterol and sphinolipids, these interactions serve as a driving force of caveolae formation (Fra *et al.* 1995; Monier *et al.* 1995; Murata *et al.* 1995; Sargiacomo *et al.* 1995; Scherer *et al.* 1997;

Li *et al.* 1998). Several different important signal transduction molecules have been found to be localized in caveolae including receptor tyrosine kinases, G protein coupled receptors, src family kinases and protein kinase C family members. Therefore caveolae serve as preassembled signalling complexes to play a role in signal integration and rapid transduction (Smart *et al.* 1999).

In 1996, researchers showed that most of the PDGFβRs are located in caveolae in normal human fibroblast cells by immunofluorescence experiments (Liu *et al.* 1996). Multiple proteins in the caveolae fraction were shown to be phosphorylated upon the stimulation of PDGFβR, while the non-caveolae fraction showed little phosphorylation. The researchers suggested that PDGFβR initiates its signalling from caveolae (Liu *et al.* 1996). Later studies showed that PDGFβR interacts with caveolin1 and caveolin3 in NIH3T3 fibroblast cells. Experiments using recombinant PDGFβR and caveolin scaffolding domains showed that caveolin 1 and caveolin 3 interact with the receptor and inhibit PDGFβR kinase activity (Yamamoto *et al.* 1999). These studies gave conflicting conclusions on how caveolin affects PDGFR signalling. If PDGFβR kinase activity is inhibited by caveolin, how could it activate downstream signalling proteins and result in their phosphorylation within caveolae? Mattveev *et al.* 2004 later showed that PDGFβR is located in lipid rafts in the absence of ligand stimulation. With the presence of ligand, PDGFβR transiently associates with caveola region where the receptor is sequestered and kept inactive by interaction with caveolin (Matveev and Smart 2002). Thus it is possible that PDGFβR is transported into the

caveolae to interact with and activate some downstream signalling proteins. After this signal transduction process, PDGFβR activity is rapidly inhibited by the caveolin. The receptor is kept inactive in the caveolae for certain period of time before it is internalized for degradation or dephosphorylated and transported back to the lipid raft to be activated again. This would provide a short term reversible attenuation of PDGFβR signalling which may be required by the cell in certain conditions.

1.2 Protein tyrosine phosphatase (PTP)

1.2.1 Introduction of PTP

As discussed above, protein phosphorylation on tyrosine residues is one of the most important mechanisms of eukaryotic signalling; it plays an important role in regulating normal cellular processes such as cell proliferation, migration and differentiation. The cellular equilibrium of protein tyrosine phosphorylation is controlled by the balanced action of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Following the purification and sequencing of the first protein tyrosine phosphatase PTP1B, and the subsequently identified receptor-like PTP (RPTP) CD45, it became clear that the PTPs family is diverse and has many members with different substrate specificities. They are regulated in different ways and expressed in different patterns inside cells.

The members of the PTP superfamily are coded by about 100 genes; they can be

divided into two main categories, classical PTPs, which specifically dephosphorylate tyrosine residues in proteins, and dual specificity phosphatases (DSPs), which dephosphorylate phosphoserine/threonine and phosphotyrosine. The well-known tumour suppressor phosphatase and Tensin homolog (PTEN), which can dephosphorylate phosphatidylinositol (3,4,5) triphosphate (PIP3), is structurally related to PTPs (Ostman *et al.* 2006; den Hertog *et al.* 2008). Classical PTPs, DSPs and PTEN all catalyse dephosphorylation with a cysteine based mechanism, and possess a conserved $HC(X_5)R$ signature motif in the catalytic site (Ostman *et al.* 2011). It has been shown that PTPs have the capacity to regulate cellular signal transduction both positively and negatively.

1.2.2 Classical PTPs

There are currently 38 classical PTPs, which are further subdivided into receptor like transmembrane PTPs (RPTP) and non-transmembrane PTPs (NPTPs). Many RPTPs have features of cell adhesion molecules in their extracellular parts; they often have immunoglobulin domains and fibronectin type III domains. This implicates an involvement in the processes of cell-cell and cell-matrix adhesion (Ostman *et al.* 2006). 12 of the 22 RPTPs have a tandem arrangement of the phosphatase domains in the intracellular part. Studies have shown that all of the phosphatase activity resides in the membrane proximal phosphatase domain (D1), with the membrane distal phosphatase domain (D2) inactive. However, the D2 domain has been shown to be important for the activity, specificity and stability of the whole RPTP, and plays roles

in protein-protein interactions that regulate RPTP dimerization (Streuli *et al.* 1990; Felberg and Johnson 1998; Jiang *et al.* 2000; Blanchetot *et al.* 2002) (Figure C).

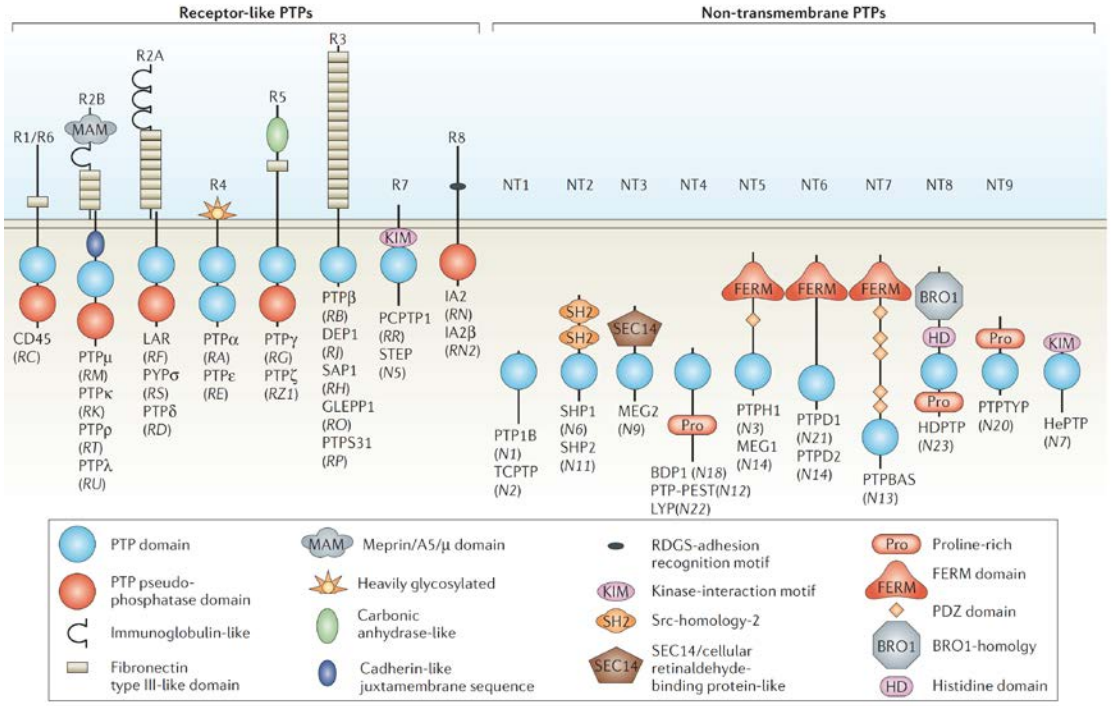

Figure C The classical PTPs

The classical protein tyrosine phosphatases (PTPs) can be divided into receptor-like (RPTP) or non-transmembrane (NPTP) proteins (reproduced from Tonks 2006)

The non-transmembrane cytoplasmic PTPs (NPTPs) generally contain specific sequences located around the catalytic domain. These sequences directly control the PTP activity by interaction with the PTP active sites and modulate their activity. The sequences flanking the PTP catalytic sites can also control the PTP specificity by enabling them to bind to specific target proteins. These sequences also control the PTP subcellular distribution; therefore restricting access to particular substrates located in certain parts of the cell (Garton *et al.* 1997; Pulido *et al.* 1998; Tonks 2006) (Figure C).

1.2.3 The mechanism of PTP mediated protein tyrosine dephosphorylation

The classical PTP catalytic domain contains a highly conserved active site that is required for its catalytic activity. The (I/V)HCXXGXXR(S/T) signature motif in the catalytic site contains the cysteine residue that forms an essential part of the active site cleft which recognizes the phosphate group of the target substrates. Dephosphorylation occurs through a two-step mechanism involving the production of a PTP-phosphate intermediate. In the first step, the sulphur atom of the essential cysteine residue nucleophilically attacks the phosphate group of the substrate, this leads to the protonation of the tyrosyl leaving group by the conserved aspartic acid residue on the PTP. The second step involves the hydrolysis of the PTP-phosphate intermediate. During this process, a conserved glutamine residue coordinates a water molecule; aspartic acid then acts as a base accepting protons from the water molecule, and the remaining hydroxyl group attacks the phosphate group causing the release of the phosphate group from the PTP cysteine residue (Tiganis and Bennett 2007) (Figure D).

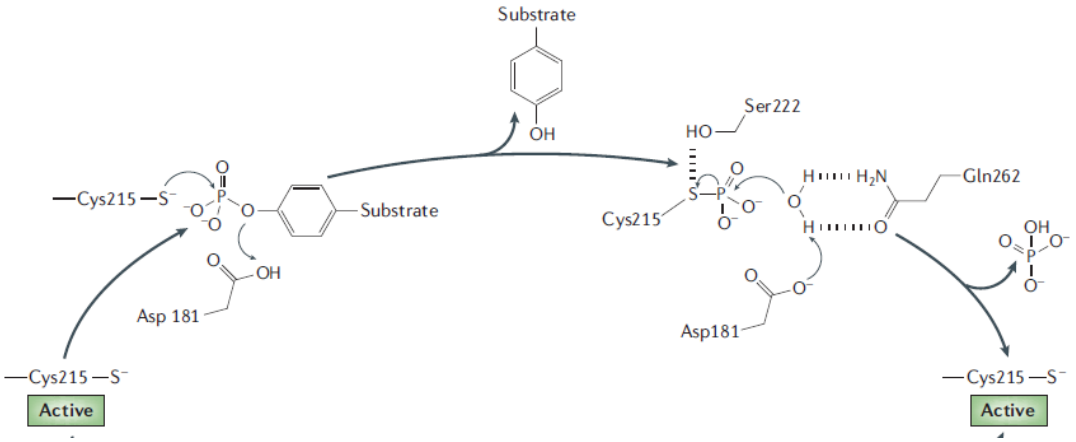

Figure D Catalytic mechanism for dephosphorylation of phosphotyrosine residue

The sulphur atom of the essential cysteine residue nucleophilically attacks the phosphate group of the substrate, this leads to the protonation of the tyrosyl leaving group by the conserved aspartic acid residue on the PTP. The conserved glutamine residue coordinates a water molecule; and aspartic acid acts as a base accepting a proton from the water molecule, and the remaining hydroxyl group attacks the phosphate group causing the release of the phosphate group from the cysteine (reproduced from Tonks 2006).

1.3 LAR RPTP

1.3.1 Introduction of LAR

The Leukocyte common Antigen-Related (LAR) subfamily of RPTPs, also known as type IIA PTPs, is one of the most studied RPTP families. It is composed of three vertebrate homologs which are LAR, RPTPσ and RPTPδ (Hasegawa *et al.* 1993; Harder *et al.* 1995; Wagner *et al.* 1996; Andersen *et al.* 2001). LAR has been shown to be widely expressed in the human neuronal system, lung, bladder, colon, heart and many other tissues (Homo sapiens PTPRF in Expressed Sequence Tag (EST) Profile http://bioinfo.wilmer.jhu.edu/tiger/db_gene/PTPRF-index.html accessed January 2003). This family of LAR RPTPs also includes invertebrate members Dlar and

DPTP69D in *Drosophila*, PTP3 in *caenorhabbditis elegan*s and HmLAR1 and HmLAR2 in *Hirudo medcinalis* (Pulido *et al.* 1995). In addition to regulating downstream signal transduction by dephosphorylating its substrates, LAR was also shown to interact with several cell surface and cytoplasmic proteins playing important roles in promoting neural development, neuronal growth and regeneration (Dunah *et al.* 2005; Yang *et al.* 2006; Sethi *et al.* 2010). Disregulation of LAR can lead obesity, diabetes and cancer (Zabolotny *et al.* 2001; Andersen *et al.* 2004).

LAR has a large extracellular domain which is very similar to the structure of cell adhesion molecule (CAM). It generally contains three immunoglobulin domains and four to eight fibronectin type III domains. Its hydrophobic transmembrane stretch is followed by two intracellular PTP domains D1 and D2 (Figure E). LAR is first expressed as a 200kDa protein before its proteolytic cleavage by furin like endoprotease. This cleavage produces the mature LAR protein consisting of a 150 kDa extracellular subunit (E subunit) non-covalently bonded to the 85 kDa intracellular PTP subunit (P subunit) (Serra-Pages *et al.* 1994; Pulido *et al.* 1995; Aicher *et al.* 1997) (Figure E). The D1 domain of LAR has robust catalytic activity, and D2 domain has weak catalytic activity. The LAR D2 domain was suggested to play a regulatory role on D1 domain activity (Streuli *et al.* 1990; Guan and Dixon 1991; Yu *et al.* 1992; Tsujikawa *et al.* 2002).

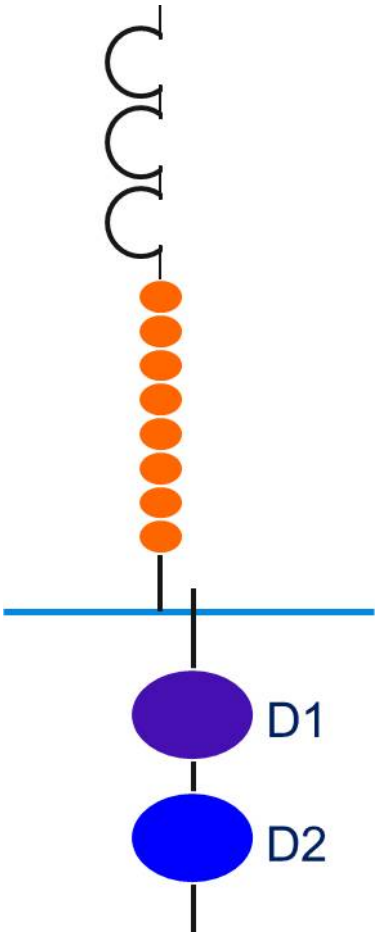

Figure E LAR RPTP structure

LAR generally contains three immunoglobulin domains and four to eight fibronectin type III domains. Its hydrophobic transmembrane stretch is followed by two intracellular PTP domains D1 and D2.

1.3.2 Regulation of LAR activity

In several of the previously published studies, researchers demonstrated that the activities of several RPTPs are negatively regulated by dimerization, these include CD45 (Majeti *et al.* 1998), RPTPα (Blanchetot and den Hertog 2000) and RPTPβ (Meng *et al.* 2000). Crystalographic studies showed that the catalytic sites of RPTPα homodimerize, and the wedge structure at the N terminus of D1 inserts itself into the D1 catalytic site of its partner and vice versa, and this leads to the inactivation of both RPTPα molecules (Bilwes *et al.* 1996). Since the wedge domain is also conserved in

LAR, it could also be a way of regulation for LAR activity (Chagnon *et al.* 2004). However, in the crystallographic studies, unlike RPTPα, the intracellular part of LAR did not crystalize into dimer (Nam *et al.* 1999). In the crystal structure of LAR, D1 sits on top of D2 and prevents the dimerization of D1 domains by steric hindrance. But if there were flexibility between D1 and D2 domain, it is possible that LAR might dimerize if D2 moves out of the way of the wedge insertion between D1 domains. Later on, researchers demonstrated that oxidation led to conformational changes of the LAR intracellular subunit, which suggested there is flexibility in the cytoplasmic part of LAR (Groen *et al.* 2008). Using tagged full length LAR, Groen *et al.* 2008 showed that LAR dimerizes constitutively inside cells.

Other researchers showed that treating cells with nickel ions elevated the phosphatase activity of LAR. Their experiments showed strong induction of liprin α4 in A549 cells at the presence of nickel ions, and it was suggested that nickel induced liprin α4 expression is necessary for the induced LAR phosphatase activity (Kiok *et al.* 2011).

1.3.3 LAR mediated interaction and signalling

Several ligands of LAR have been identified so far to bind to this RPTP and induce different physiological effects of the cell. The first identified ligand of LAR was the extracellular complex laminin nidogen which was shown to bind to the finbronectin type III domain 5 of LAR's extracellular domain and induce the formation of longer

cellular processes (O'Grady *et al.* 1998). Later on, researchers discovered an 11 kDa ectodomain isoform of LAR (named LARFN5C) which contains the 5th fibronectin type III domain of LAR at its C terminus. Substratum bound LARFN5C potently elicits neurite outgrowth response of LAR expressing COS cells, and this potency was reduced by 5 folds in the LAR knock out cells (Yang *et al.* 2003). In 2005, a new ligand of LAR, heparan sulfate proteoglycan syndecan (Sdc) was demonstrated to interact with Drosophila LAR. Sdc on muscle cells binds to neuronal LAR *in vivo* and positively regulates LAR mediated neurite outgrowth. Experiments using LAR and Sdc loss of function mutants showed that Sdc promotes LAR mediated axon guidance (Fox and Zinn 2005). Later on, synaptic protein Dallylike (Dlp) was demonstrated to bind to Drosophila LAR to regulate synapse morphogenesis and function (Johnson *et al.* 2006). Netrin G ligand 3 (NGL3) was more recently shown to form trans-synaptic adhesion with LAR to regulate the excitatory synapse formation (Woo *et al.* 2009; Kwon *et al.* 2010).

Little is known about the downstream targets of LAR, the existing evidence points to LAR promoting actin cytoskeleton remodelling. Downstream signalling of LAR leads to axon extension and guidance; this suggests that RhoGTPases and cytoskeletal rearrangement may be involved in this process. A large multi-domain protein, Trio ， was found to interact with LAR using a yeast two hybrid method (Debant *et al.* 1996). Trio contains a serine threonine kinase domain and two guanine nucleotide exchange

factor domains specific for Rac-1 and RhoA. In Drosophila, Trio was shown to regulate axon guidance. Since Trio is phosphorylated by focal adhesion kinase (Medley *et al.* 2003), LAR could regulate the tyrosine phosphorylation of Trio, thereby possibly regulating the activity of Rac1 and RhoA after being recruited to the focal adhesions by liprins (LAR interaction protein). Recent studies identified EphA2 as a substrate of LAR, researchers demonstrated that dephosphorylation of EphA2 by LAR uncouples EphA2 from Nck1 and thereby attenuating EphA2 mediated cell migration (Lee and Bennett 2013).

Many PTPs, including LAR, were shown to interact with and regulate different protein tyrosine kinases. LAR was coimmunoprecipitated with insulin receptor (IR) with both D1 and D2 domains important for this interaction (Tsujikawa *et al.* 2001). Autophosphorylation of IR increases when cells are transfected with Cysteine to Serine catalytic inactive LAR mutant, suggesting that IR is a substrate of LAR. Other protein tyrosine kinases such as EGFR (Suarez Pestana *et al.* 1999), HGFR (Kulas *et al.* 1996), RET (Qiao *et al.* 2001) and Src, Lck and Fyn (Tsujikawa *et al.* 2002) have been shown to be substrates of LAR RPTP.

1.3.4 Triangular interaction of LAR, Abl and Ena plays an important role in regulating axon guidance

LAR is highly enriched in axon growth cones which suggests that there may be

protein tyrosine kinase activity at this location. A screening study in Drosophila identified that Abl protein tyrosine kinase, which is also expressed in developing axons, acts as a regulator of Drosophila LAR (Dlar) signalling. Although Abl has been extensively studied in the context of cell proliferation, DNA damage response and cancer biology, relatively little is known about its role in controlling cell motility and shape. More and more evidence suggests that Abl is involved in rearrangement of the actin cytoskeleton (Wills *et al.* 1999; Sirvent *et al.* 2008).

Both mammalian Abl and Drosophila Abl were shown to phosphorylate LAR on its D2 domain *in vitro*. Conversely, Abl is a good substrate of LAR *in vitro*. Abl and Dlar show a potent antagonistic relationship *in vivo*. Reduction of Abl suppresses the axon guidance phenotype of Dlar loss of function. Conversely, overexpression of Abl in postmitotic neurons mimics the Dlar loss of function phenotype, and this effect is dependent on the kinase activity of Abl. Coexpression of Dlar reverses this effect of Abl, suggesting Dlar is able to dephosphorylate protein that are phosphorylated by Abl. Loss of Abl alone disrupts axon pathway formation, indicating both Abl and Dlar are important for axon development. These findings suggest LAR and Abl mediate a phosphorylation dependent switch that controls the axon guidance process (Wills *et al.* 1999; Chagnon *et al.* 2004). Although it was not shown directly, since LAR and Abl serve as substrates for each other and antagonize the function of each other, it is possible that LAR inhibits Abl activity by dephosphorylating Abl, and Abl may inactivate LAR by phosphorylating LAR (Figure F).

A screening study of the downstream effectors of Abl identified the protein Enabled (Ena) which is phosphorylated and regulated by Abl (Wills *et al.* 1999; Sirvent *et al.* 2008). This makes Ena a prime candidate for further investigation. It was found that Ena is a key regulator of actin cytoskeleton assembly and cell motility, it promotes actin assembly by protecting the growing tips from the capping proteins that terminate actin polymerization (Wills *et al.* 1999). Later studies showed that Ena mutation leads to the axon guidance phenotype of Dlar mutant in the developing axon cone. Like Abl, Ena can bind to the D2 domain of Dlar. It is phosphorylated by Abl and dephosphorylated by Dlar *in vitro.* Abl kinase activity antagonizes the action of Dlar and Ena, presumably by phosphorylating these proteins (Sirvent *et al.* 2008). It appears that Ena serves as a molecular switch which itegrates the signals from Dlar and Abl, and maintains a highly accurate axon guidance process (Figure F) (Wills *et al.* 1999; Sirvent *et al.* 2008).

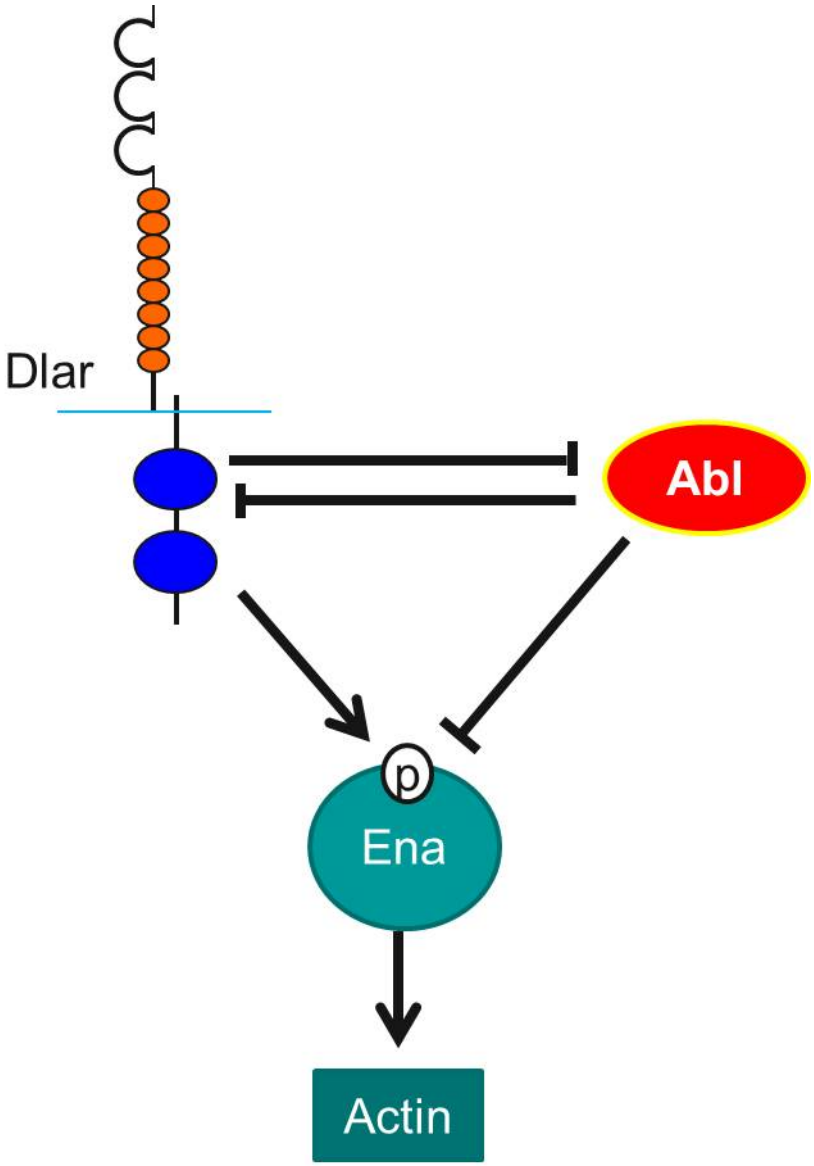

Figure F Dlar and Abl antagonize each other in regulating actin remodelling

Ena plays a role in promoting actin polymerization when unphosphorylated. Dlar dephosphorylates Ena to promote its function while Abl phosphorylates it to inhibit its activity. Abl and Dlar are also substrates for each other (Wills *et al*. 1999).

1.3.5 LAR related physiological functions and diseases

From vertebrate to invertebrate, LAR is mostly expressed in the developing nervous system. Immunohistochemistry studies showed that LAR is localized at the axon and growth cone of Drosophila, Leech, chick and in mammals. This suggests the role of

LAR in the nervous system is conserved through evolution (Tian *et al.* 1991; Desai *et al.* 1994; Sommer *et al.* 1997; Gershon *et al.* 1998; Schaapveld *et al.* 1998; Thompson *et al.* 2003).

In Drosophila, Dlar is expressed in CNS roots, developing motor nerves and the visual system (Tian *et al.* 1991). In mutant embryos lacking Dlar, the segmental nerve b motor axon stopped growing before reaching its muscle targets, or it followed incorrect pathways and bypassed the muscle targets. (Tian *et al.* 1991; Desai *et al.* 1994; Desai *et al.* 1996; Garrity *et al.* 1999; Clandinin *et al.* 2001; Maurel-Zaffran *et al.* 2001; Tayler and Garrity 2003). LAR is important for controlling R7 photoreceptor axon targeting. Although this function does not require the phosphatase activity of LAR, phosphatase dimerization is necessary for this process. This is possibly due to that dimerization of LAR may play a role in assembling downstream effectors (Hofmeyer and Treisman 2009).

In mammals, absence of LAR delays axon regeneration following injury to the sciatic nerve in mice. The upregulation for LAR expression in dorsal root ganglial neurons after sciatic nerve injury suggests a role of LAR in promoting neurite outgrowth in sensory neurons (Xie *et al.* 2001; Van der Zee *et al.* 2003). LAR was reported to be highly expressed in the synapses of rat hippocampus neurons and play important roles in neuron development and maintenance of excitatory synapses (Dunah *et al.* 2005). In the mice with the LAR cytoplasmic phosphatase domains deleted, the number of

cholinergic neuronal cells in the forebrain was greatly reduced. Cholinergic neuron innervation was also diminished in brain dentate gyrus. This indicates that LAR phosphatase activity is crucial for central nervous system development (Van Lieshout *et al.* 2001).

Several lines of evidence have suggested the involvement of LAR in various diseases. Some of the LAR loci are amplified or mutated in different human cancers such as small cell lung cancer and colon cancer (Harder *et al.* 1995; Andersen *et al.* 2004). Expression level of LAR is significantly increased in thyroid carcinomas (Konishi *et al.* 2003) and breast cancer (Yang *et al.* 1999). Expression of LAR may correlate with metastatic breast cancer prognosis (Levea *et al.* 2000). The role of LAR in regulating insulin receptor signalling has been extensively studied, and LAR was shown to suppress signalling downstream of the insulin receptor (Zabolotny *et al.* 2001). Transgenic mice overexpressing LAR maintained normal level of blood glucose. However, they showed a 2.5 fold increase in the fasting insulin level and reduced glucose uptake in the skeletal muscle cells, this indicates that LAR negatively regulate insulin receptor signalling and confers insulin resistance to cells (Zabolotny *et al.* 2004). The fact that LAR is highly expressed in adipose tissue makes it a possible prime drug target for tissue specific regulation of insulin resistance and adiposity (Norris *et al.* 1997).

1.4 C-Abl

C-Abl is a non-receptor tyrosine kinase which is located at the nucleus, cytosol, plasma membrane and actin cytoskeleton. The c-Abl protein is consists of a SH3 domain, a SH2 domain, a tyrosine kinase domain, a DNA binding domain and an actin binding domain. In addition, c-Abl also contains a NES (nuclear export sequence) and three NLSs (nuclear localization signals) which are important for c-Abl shuttling between the nucleus and cytoplasm. C-Abl plays important roles in coordinating actin remodelling in response to cell stimulation by phosphorylating the cytoskeleton regulating proteins (Hernandez *et al.* 2004). C-Abl regulates cell motility by phosphrylating CrkII, and disrupts CrkII induced cell migration (Takino *et al.* 2003). C-Abl was also shown to regulate the internalization of cell surface growth factor receptors with its cytoskeleton remodelling capacity and by phosphorylating these receptors (Kaksonen *et al.* 2005; Yarar *et al.* 2005). C-Abl overexpression leads to cell cycle arrest and cell apoptosis suggesting its role in DNA damage repair and cell death (Sawyers *et al.* 1994; Yuan *et al.* 1997). C-Abl was identified as part of the mutationally activated fusion oncoprotein, BCR-Abl which was commonly found in chronic myeloid leukemia (CML) patients (Ben-Neriah *et al.* 1986)

There have been several reports showing that c-Abl interacts with PDGFβR and is involved in PDGFβR downstream signaling. In 1999, Plattner *et al.* demonstrated that c-Abl is phosphorylated and activated by Src downstream PDGFβR upon ligand stimulation (Plattner *et al.* 1999). Later on, researchers further showed that c-Abl

phosphorylates PDGFβR after being activated and serves as a positive feedback loop to increase PDGFβR mediated cell proliferation, and at the same time reduceing PDGFβR downstream chemotaxis (Plattner *et al.* 2004; Srinivasan *et al.* 2009).

1.5 PKCδ

PKCδ (protein kinase C δ) is a member of the PKC family, and was shown to play important roles in regulating cell proliferation, apoptosis and tumour growth (Basu and Pal 2010). PKCδ was the first identified PKC isoform, and was found to be ubiquitously expressed in different cell types and tissues (Ono *et al.* 1988; Altman and Villalba 2002). It can be activated by the binding of diacylglycerol (Michell 1975) and tumour promoting phorbol ester (Castagna *et al.* 1982). Several tyrosine kinases have been shown to phosphorylate PKCδ including Src family kinases, c-Abl and growth factor receptors (Zrachia *et al.* 2002; Okhrimenko *et al.* 2005; Lu *et al.* 2007; Yoshida 2007; Lomonaco *et al.* 2008), depending on the site of phosphorylation, these tyrosine kinases can either activate or inactivate PKCδ. Caspase 3 was shown to proteolytically cleave PKCδ and generate a constitutively active catalytic fragment which largely enhances the apoptosis signal and leads to cell death (Wilson *et al.* 2004).

In 2000, Sun *et al.* showed that hydrogen peroxide induced oxidative stress induces

the binding of cellular PKCδ to c-Abl. As a substrate of c-Abl, PKCδ is tyrosine phosphorylated and activated by c-Abl. At the same time, PKCδ phosphorylates and activates c-Abl which establishes a positive feedback loop between c-Abl and PKCδ (Sun *et al.* 2000). Later on, researchers showed that phosphorylation of GRK2 Ser29 residue by PKCδ abolishes its inhibition by calmodulin and activates GRK2 (Krasel *et al.* 2001). Thus there may be an activation pathway from c-Abl to PKCδ and the GRK2 which acts as a signalling pathway to induce downstream cellular responses.

1.6 Nherf

The Na^+/H^+ exchanger regulatory factor (Nherf 1, also called EBP50) and its relative NHE3 kinase A regulatory protein (Nherf 2, also called E3KARP, SIP 1 and TKA 1) represent a family of adaptor proteins. Both Nherf 1 and Nherf 2 contain two tandem PSD95/Drosophila discs large/ZO1 (PDZ) protein interaction domains, and a C terminal domain which binds to ezrin-radixin-moesin-merlin (ERM) family of cytoskeletal proteins. Nherf 1 and Nherf 2 form homo- and heterodimers with each other via their PDZ domains. They also bind to other PDZ proteins to form a membrane scaffold which is consist of an array of transmembrane or intracellular proteins including transporters, ion channels and signalling proteins. Nherf proteins also interact with certain growth factor tyrosine kinase receptors such as PDGFR and EGFR to modulate their signalling and downstream responses (Weinman *et al.* 1993; Voltz *et al.* 2001; Lazar *et al.* 2004).

Studies have shown that Nherf 1 PDZ I domain binds to the DSFL motif at the C terminus of PDGFβR with high affinity and potentiates the PDGFβR autophosphorylation and downstream signalling. Nherf 1 oligomerizes when it is bound to the PDGFβR C terminus and the capacity of Nherf 1 to potentiate PDGFβR signalling depends on its oligomerization. It is suggested that Nherf 1 potentiates PDGFβR autophosphorylation by creating oligomeric complexes that keep individual PDGFβR monomers in close proximity to each other and therefore aiding the formation and stabilization of PDGFβR dimers. This explains the mechanism of the previous observation that a small truncation of PDGFβR C terminus strongly impairs PDGFβR signalling. Nherf 2 was also shown to bind to the carboxyl terminal tail with a specificity similar to Nherf 1, and therefore may also be an *in vivo* binding partner of PDGFβR (Maudsley *et al.* 2000). Later studies showed Nherf 2 binds to the PDGFβR C terminal tail at least as well as Nherf 1 (Lau and Hall 2001). However, no study has yet been carried out to investigate the effect of Nherf 2 on PDGFR autophosphorylation.

Since oligomerization of Nherf1 determines its capacity to potentiate PDGFβR signalling, later studies were carried out to investigate oligomerization of Nherf 1 and Nherf 2. It was shown Nherf1 and Nherf2 oligomerize with their PDZ domains. Purified Nherf 1 PDZ domains associate with each other only weakly when they are tested alone. Oligomerization of Nherf1 PDZ domains was greatly facilitated when

they are bound to the C terminus of PDGFβR. Nherf1 oligomerization is elevated by mutation of Serine 289 on Nherf1 to aspartate which mimics the phosphorylation state of this serine residue, suggesting serine phosphorylation on Ser289 of Nherf1 increases its oligomerization. On the other hand, the Nherf2 PDZ domain was shown to oligomerize robustly in the absence of any associated proteins (Lau and Hall 2001). However, other studies showed treating cells with the phosphatase inhibitor okadaic acid and calyculin A enhanced Nherf1 phosphorylation and inhibited its oligomerization. Researchers determined that serine phosphorylation of Nherf 1 PDZ domain inhibited its binding to its targets including PDGFR, and the phosphorylation was later mapped to Ser77 on Nherf 1 PDZ I domain (Voltz *et al.* 2007).

More recent studies showed that the interaction between Nherf1 and PDGFR can be disrupted by -2 position serine phosphorylation of PDGFR by G protein coupled receptor kinase 2 (GRK2). Phosphorylation at this position prevents Nherf 1 from binding to PDGFR, and therefore desensitizes PDGFR and reduces its activity (Hildreth *et al.* 2004).

Aims and Objectives

Aim: To investigate the role of LAR RPTP in regulating PDGFβR phosphorylation and downstream signal transduction.

Objectives:

1) To test and compare the ligand stimulated PDGFβR tyrosine phosphorylation and kinase activity in the WT and LARΔP cells

2) To investigate PDGFβR downstream signalling and proliferation response in the two cell types

3) To compare the ubiquitination, internalization and degradation rates of PDGFβR in the two cell types

4) To investigate if LAR deletion affects the activities of Src family proteins and PDGFβR.caveola localization

5) To investigate the expression and activity levels of c-Abl in the two cell types, and to test if inhibition of c-Abl restores PDGFβR tyrosine phosphorylation

6) To investigate if PKCδ and Nherf2 are involved in the LAR deletion mediated reduction in PDGFβR phosphorylation and to compare the PDGFβR dimerization in the two cell types

CHAPTER 2 MATERIALS AND METHODS

2.1 Reagents and antibodies

Recombinant human PDGF-BB was provided by Amgen (Thousand Oaks, CA). The Src kinase inhibitor SU6656 was from Calbiochem (San Diego, CA). Thiazolyl blue (MTT), enolase, the c-Abl inhibitor AG957, myelin basic protein (MBP), rottlerin and BS^3 were purchased from Sigma-Aldrich (St Louis, MO). $\gamma[^{32}P]ATP$ and $[^3H]$thymidine were from Perkin Elmer (Shelton, CT). Control and c-Abl siRNA were obtained from Dharmacon (Lafayette, CO). The pEGFP-N2-flmLAR is a construct with full length LAR linked with a C terminal GFP tag, a gift from Dr. Wiljan Hendriks in Department of Cell Biology, Radboud University Nijmegen Medical Centre, Nijmegen of the Netherlands. The secondary sheep anti-mouse IgG HRP conjugated antibody and the secondary donkey anti-rabbit IgG HRP conjugated antibody were from GE Healthcare (Uppsala, Sweden). The GFP antibody was from Invitrogen (Carlsbad, CA). The CrkII, the ubiquitin, Nherf2 and the PY99 monoclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The Akt, pThr308 Akt, Erk42/44 (Erk1/2), pErk42/44 (pERK1/2)(Thr202/Thr204), c-Abl and caveolin 1 antibodies were from Cell Signalling Technologies (Beverly, MA), and the PY20 antibody and PKCδ antibody were from BD Transduction Laboratories (San Jose,

CA). Rabbit polyclonal antibodies against the C-terminus of the PDGF β-receptor (Karlsson *et al.* 2006) and its site-selective phosphotyrosine antibodies have been described (Chiara *et al.* 2004; Persson *et al.* 2004; Toffalini *et al.* 2010). Antisera against PLCγ (Arteaga *et al.* 1991), Alix (Lennartsson *et al.* 2006) and Src, Yes and Fyn (Kypta *et al.* 1990), have been previously described. The concentration of the commercial antibodies used for western blot and Immunoprecipitation was according to the manufacturers' product information. 2μg/ml and 10μg/ml PDGF antibody was used for western blot and immunoprecipitation (IP) respectively. 5μg/ml site-selective PDGFβR phosphotyrosine antibodies were used for western blot. 1:500 and 1:200 dilution of PLCγ antiserum was used for western blot and IP respectively. 1:1000 Alix antibody was used for western blot, and 1:300 dilution of Src, Yes and Fyn antibody was used for IP.

2.2. Tissue culture and transfection

Mouse embryonic fibroblasts (MEF) were obtained from mice where the LAR cytoplasmic phosphatase domains had been deleted (LARΔP) and from littermate wild-type (WT) mice (Schaapveld *et al.* 1997) according to standard procedures (Manipulating the mouse embryo Cold Spring Harbor, Cold Spring Harbor Laboratory Press, NY, 1994, pp. 260–261). Both of the WT and LARΔP MEFs are gifts from Dr. Wiljan Hendriks in Department of Cell Biology, Radboud University Nijmegen Medical Centre, Nijmegen of the Netherlands. The cells were grown in

DMEM supplemented with 10% foetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml Fungizone and 5 μg/ml plasmocin. MEF cells at approximately 60% confluence were transfected using 1.5μg/ml pEGFP-N2-flmLAR DNA and 2.5μl/ml jetPEI transfection reagent (Polyplus-transfection SA, Illkirch, France) for 48 hours, according to the manufacturer's protocol.

2.3. PDGFR activation, cell lysis, receptor precipitation and immunoblotting analysis

Cells were grown to approximately 90% confluence and starved overnight in DMEM medium supplemented with 1 mg/ml BSA. When indicated, the cells were incubated with 2 μM SU6656 for one hour, 10 μM AG957 for two hours, 10 μM Rottlerin for two hours or DMSO (0.1%, 0.2% and 0.2%, respectively) as controls. Cells were stimulated with the indicated concentration of PDGF-BB for the indicated time periods at 37°C, cells were put on ice and rinsed with ice cold PBS (135 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, and 8 mM K2HPO4, pH 7.4) once and lysed in SDS-PAGE 1Xsample buffer (1.0M Tris-HCl pH 8.8, 0.5% Bromophenol blue, 43.5% glycerol, 10% SDS, 1.3% β-mercaptoethanol) followed by sonication. Alternatively, the cells were lysed in our standard lysis buffer (20 mM Tris–HCl, pH 7.5, 0.5% Triton X-100, 0.5% deoxycholate, 150 mM NaCl, 10 mM EDTA, 0.5 mM Na_3VO_4, and 1% Trasylol) for 15 minutes on ice. The lysed cells were scraped and collected into eppendorf tubes. After centrifugation at 11337g for 15 minutes, the supernatant

was collected and the protein concentration in each sample was determined using the BCA protein assay (Thermal Scientific, IL) according to the manufacturer's protocol. Lysates containing equal amounts of total protein in each sample was used for the PDGF β-receptor precipitation by incubation with wheat germ agglutinin (WGA, GE Healthcare, Little Chalfont, UK) on the end-over-end rotator for one hour at 4°C. Alternatively, lysates were incubated with the indicated antibody for three hours and then protein A agarose (GE Healthcare, Little Chalfont, UK) for one hour on the rotator at 4°C. The beads were washed three times with standard lysis buffer by vigorous shaking in hands, centrifugation at 11337g for 30 seconds and aspirating the supernatant. Equal volumes of the 2Xsample buffer (twice the concentration of 1Xsample buffer) were added to the beads or total cell lysate, and the mixture was boiled at 95°C for 5 minutes. The precipitated proteins or the total cell lysate samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. The transferred membrane was blocked with 5% Bovine serum albumin (BSA) at 37°C for 30 minutes on the shaker. The membrane was then rinsed in TBS-T (10 mM Tris-HCl 75mM NaCl, 0.1% Tween 20) once and put into primary antibody solution in TBS-T containing 1% BSA and incubated at 4°C overnight on the shaker. The membrane was then washed 6 times for 5 minutes with TBS-T and incubated with the secondary antibody solution in TBS-T for one hour. The membrane was again washed 6 times for 5 minutes with TBS-T and proteins was visualized by enhanced chemiluminescence (GE Healthcare, Little Chalfont, UK) using a LAS-100plus

CCD-camera (Fujifilm). Densitometrical analysis of the bands were performed using AIDA advanced image data analyser software (Fujifilm).

2.4 Isolation of plasmid DNA

A small pipette tip was used to take a little bit of the bacteria transformed with the pEGFP-N2-flmLAR construct and was put into 200ml of LB containing kanamycin at 100μg/ml. The culture was incubated at 37°C with strong shaking overnight. The bacteria suspension was centrifuged at 2350g for 15 minutes at 4°C. The DNA was then purified with the QIAGEN Maxi kit according to the manufacture's protocol. The concentration of the DNA isolated was determined by measuring the absorbance of the DNA solution at 260nm. An optical density of 1 was considered to represent 50μg/ml plasmid DNA.

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2.5 Detection of PDGFβR Ubiquitination

After SDS-PAGE, membranes for ubiquitin analysis were pre-incubated with denaturing buffer (6M guanidine-HCl, 20 mM Tris-HCl pH7.5, 5mM beta-mercaptoethanol and 1mM PMSF) for 30 minutes at 4°C. The membranes were washed extensively with PBS for 6X10 minutes, and then blocked with 5% BSA in TBS for 30 minutes at 37°C before incubated with ubiquitin antibody overnight at

4°C.

2.6 *In vitro* kinase assay

PDGFβR, Src family kinases or c-Abl kinase were immunoprecipitated, and *in vitro* kinase assays were performed using MBP as a substrate for PDGFβR and acid-denatured enolase as the substrate for Src and c-Abl kinase (Kypta *et al.* 1990). 1mM Dithiothreitol (DTT) was added into the lysis buffer during the IP and washing steps to prevent the formation of disulphide bonds between protein molecules. The precipitated proteins on protein A agarose were washed three times with lysis buffer followed by one wash using kinase buffer (20mM Hepes pH 7.5, 10mM $MnCl_2$). Samples were then resuspended in kinase buffer containing 90μM ATP, 125ng/μl MBP or enolase and 300nCi/μl [^{32}P]γATP and incubated at 30°C for 10 minutes with regular mixing. The reaction was stopped by adding sample buffer into each sample and boiling at 95°C for 5 minutes. In the case of c-Abl, the assay was done in the presence of 1% DMSO or 100 μM AG957. Samples were then separated using SDS-PAGE and transferred onto PVDF membranes. Membranes were exposed to Fuji film. The films were visualized with Fuji FLA3000 Bioimager, and quantification of radioactivity incorporated into PDGFβR, MBP and enolase was done with the quantification program coupled with the Bioimager.

2.7 MTT assay

Cells were seeded in 96-well plates at a density of 3000 cells/well followed by serum starvation, and grown for the indicated time periods in DMEM medium containing 50 ng/ml PDGF-BB or 10% FBS. The cells were incubated with 0.65 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for four hours at 37 °C followed by lysis in DMSO/ethanol (1:1). The formation of water-insoluble formazan was determined by measuring OD at 600 nm to analyse the relative cellular metabolic activity via NAD(P)H-dependent cellular oxidoreductase enzymes, and then the relative increase in cell density was calculated.

2.8 PDGFR internalization assay

Cells were stimulated with 20ng/ml PDGF-BB for different time periods as indicated. Cells were put on ice after stimulation and washed twice with PBS pH 7.3. Cell surface proteins were biotinylated by incubating with 0.5mg/ml sulfo-NHS-SS-biotin (Pierce Chemical, Rockford, IL) in PBS pH 8 for 1 hour on ice with gentle shaking. Unbound biotin was quenched by incubation with 50mM Tris pH 8 for 10 minutes on ice. The cells were washed with PBS pH 7.3 and lyzed. Cell lysate was incubated with streptavidin sepharose (GE healthcare, Uppsala, Sweden) for 1 hour end over end at 4°C. The precipitated cell surface receptors were separated using SDS-PAGE and visualized with the indicated antibodies.

2.9 Cell fraction separation

After the cells were stimulated for the indicated time, cells were lyzed with 50mM Na2HPO4, 1mM sodium pyrophosphate, 2mM EDTA, 2mM EGTA, 1% TritonX100, 0.5 mM Na_3VO_4 and 1% Trasylol, for 15 minutes on ice. Cell lysates were sonicated for 2 seconds and centrifuged at 11337 g for 30 seconds. The supernatants were saved as the TritonX100 fraction. The remaining pellets were dissolved in 1% SDS and heated at 95°C for 10 minutes. Sample buffer was added to the TritonX100 fraction and 1% SDS fraction and both were boiled at 95°C for 5 minutes before the samples being analysed by western blotting.

2.10 C-Abl knockdown by siRNA

Cells were transfected with 100 nM of control siRNA, or siRNA targeting c-Abl (RNA sequence: GAACCACCAUUCUACAUAA; Dharmacon, Lafayette, CO). For every experiment performed, Non-Targeting siRNA from the same company was used as a control. Transfection of siRNA was done for 72 hours using 10µl/ml INTERFERin (Polyplus-transfection SA, Illkirch, France) according to the manufacturer's protocol.

2.11 Crosslinking assay

After stimulation of the cells, PBS instead of lysis buffer was added on to the cells, and the cells were scraped and collected. The cell suspension was centrifuged at 2415g for 10 minutes. Pellets collected were added to 3mg/ml cross linker BS_3 in 1% TritonX100. After incubating for 15 minutes, the mixture was centrifuged at 11337g for 15 minutes, and sample buffer was added to the supernatant. The samples were boiled at 95°C for 5 minutes and separated by SDS-PAGE using a 4–20% precast linear gradient polyacrylamide gel (BioRAD, Solna, Sweden) and analysed.

2.12 Statistics

Where applicable, results were analysed using the Student's t-test, a P value of less than 0.05 was considered to represent a significant difference.

CHAPTER 3 LAR POTENTIATES PDGFβR TYROSINE PHOSPHORYLATION

3.1 Introduction

Many different protein tyrosine phosphatases have been shown to regulate PDGFβR phosphorylation by specifically dephosphorylating individual sites on the receptor. As described before, TC-PTP dephosphorylates the PDGFβR autophosphorylation site Tyr1021 to terminate downstream PLCγ activation and thereby reducing cell migration. PTP-1B specifically dephosphorylates Src binding site Tyr579, whereas SHP2 dephosphorylates at RasGAP binding site Tyr771 potentiating downstream activation of the Ras and MAPK pathway (Ekman *et al.* 2002; Persson *et al.* 2004). Regulation of PDGFβR phosphorylation by PTPs plays an important part in maintaining normal strength and pattern of PDGFβR signalling and its functions. LAR was previously shown to dephosphorylate tyrosine kinase receptors such as the insulin receptor, hepatocyte growth factor receptor and EGFR and reduce their autophosphorylation (Kulas *et al.* 1996; Falet *et al.* 1998; Suarez Pestana *et al.* 1999). On the other hand, LAR positively regulates tyrosine kinase neurotrophin receptor TrkB by dephosphorylating the negative regulatory tyrosine on Src which was shown to increase TrkB activity (Yang *et al.* 2006). In this study, I am interested in how LAR

phosphatase activity affects PDGFβR signal transduction and the underlying mechanism.

In this chapter, mouse embryonic fibroblasts with the LAR phosphatase domain deleted (LARΔP) and its wild type littermate (WT) cells were used to investigate tyrosine phosphorylation of PDGFβR and its autophosphorylation sites after different time points of stimulation and at different PDGF-BB concentrations.

3.2 Results

3.2.1 LARΔP cells show decreased PDGF β-receptor phosphorylation

The ligand induced tyrosine phosphorylation levels of PDGFβR in WT and LARΔP cells were investigated by stimulating cells for different time periods. Cells were stimulated with 20 ng/ml PDGF-BB and lyzed. Receptors were precipitated with WGA agarose and subjected to SDS-PAGE, transferred onto nitrocellulose membranes and blotted with antibodies raised against the tyrosine phosphorylation PY99 and PDGFβR protein antibody. The results showed that deletion of LAR phosphatase domains did not result in the induction of detectable tyrosine phosphorylation of the PDGF β-receptor in serum starved cells (Figure 1A). In both cell types, PDGF-BB induced tyrosine phosphorylation of the PDGF β-receptor, but the receptor tyrosine phosphorylation in LARΔP cells was consistently lower at all the investigated time points (Figure 1A). Densitometric analysis showed the average

receptor tyrosine phosphorylation in LARΔP MEFs stimulated for 10 minutes with PDGF-BB was only 18% of the receptor phosphorylation in WT cells. Retransfection of LAR-EGFP into LARΔP cells reverted this phenotype (Figure1A), showing that the LAR phosphatase domains are required for PDGF β-receptor activation. Immunoprecipitation of PDGFβR with the PDGFβR antibody instead of WGA agarose after cell stimulation gave similar results for PDGFβR phosphorylation level, confirming PDGFβR tyrosine phosphorylation is reduced in the absence of LAR (Figure 1B). This experiment was repeated twice and showed a similar pattern of receptor phosphorylation. A representative result blot is shown in Figure 1.

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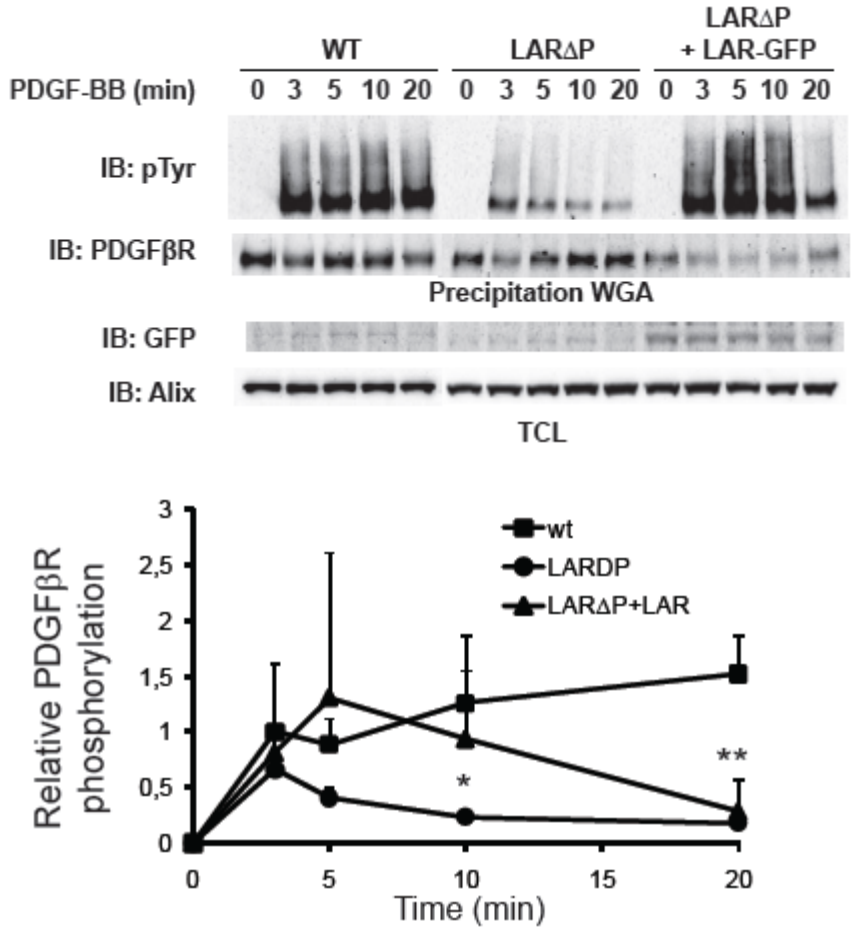

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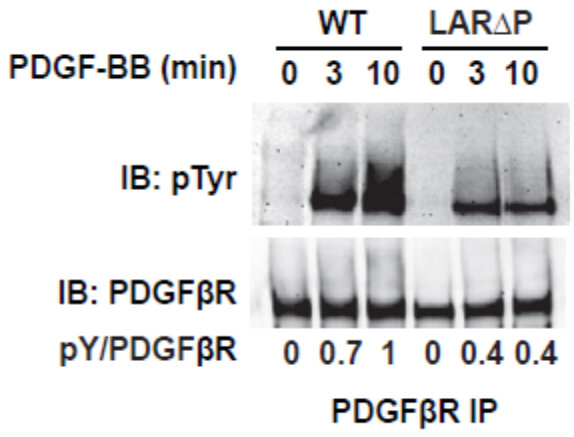

Figure 1 LAR promotes ligand induced PDGFβR phosphorylation

A. WT, LARΔP and LARΔP cells transiently expressing LAR-EGFP were starved for 16 hours and stimulated with 20ng/ml PDGF-BB for the time periods indicated. Cells were lyzed and incubated with WGA agarose for 1 hour to precipitate glycosylated receptors. Samples were separated by SDS-PAGE, and transferred onto nitrocellulose

membranes. The amount of tyrosine phosphorylation on PDGFβR was determined with PY99 phosphotyrosine antibody followed by immunoblotting with PDGFβR antibody. GFP expression in the corresponding total cell lysates was determined by blotting with GFP antibody， western blotting of Alix was a loading control. Antibodies were visualized with ECL and LAS-100 plus CCD camera. Densitometry analysis was performed with AIDA advance image data analyser software. This experiment was repeated three times and statistical analysis was carried out using student's t-test. The mean relative receptor phosphorylation +/−SEM for three experiments is plotted. Statistical significant differences compared to WT are indicated by *, $p<0.05$ and **, $p<0.01$. **B**. Cells were stimulated with 20ng/ml PDGF-BB for the indicated time period, cells were lyzed and PDGFβR were immunoprecipitated with PDGFβR antibody, and analysed as described above.

3.2.2 Decreased PDGFβR phosphorylation in LARΔP cells cannot be overcome by increasing ligand concentrations

Knowing that PDGFβR phosphorylation is lower in LARΔP cells, I wanted to investigate whether the phosphorylation level could be restored by increasing ligand concentration, as this would reveal whether PDGFβR in LARΔP cells can be fully activated. Cells were stimulated with concentrations ranging from 0 to 150 ng/ml PDGF-BB for 10 minutes and then lyzed. The PDGFβR was precipitated with WGA agarose. Receptor phosphorylation levels were assessed by western blot and quantified with image data analyser software. The relative PDGFβR phosphorylation was plotted (Figure 2).

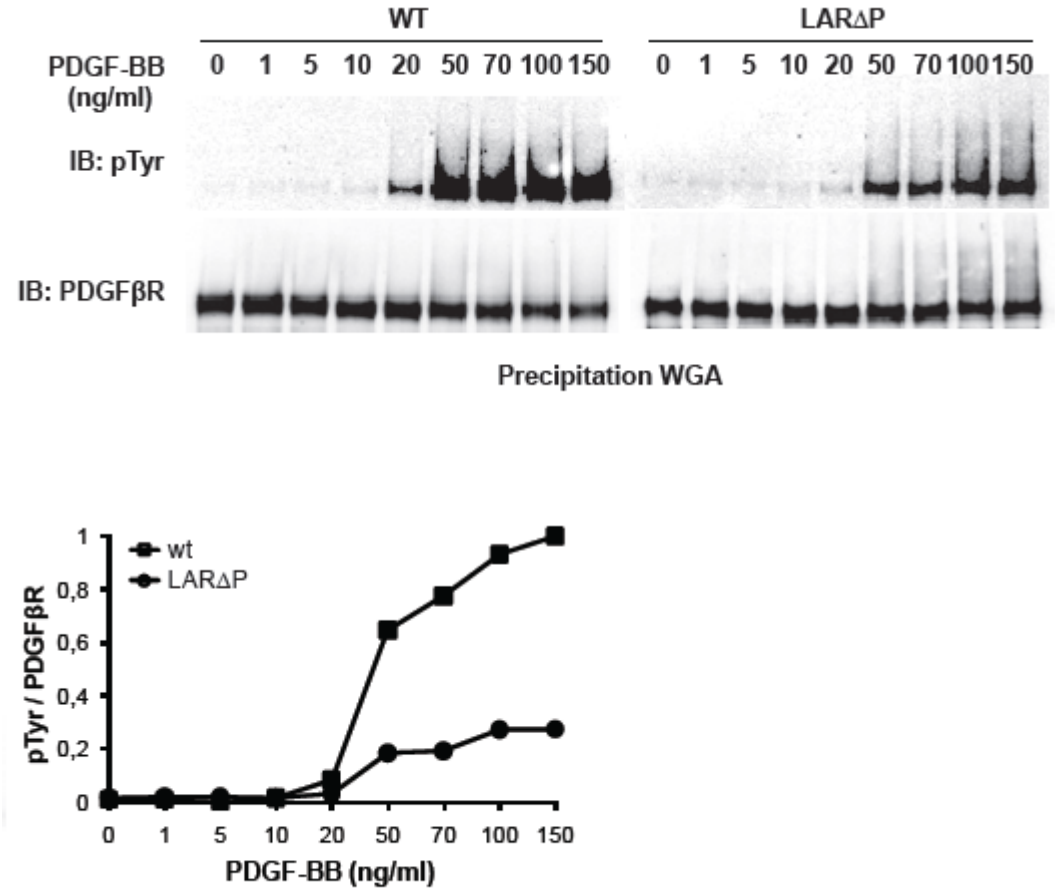

Figure 2 LAR deletion reduces ligand induced PDGFβR tyrosine phosphorylation at all PDGF-BB concentrations

After starvation for 16 hours, cells were stimulated with the indicated concentration of ligand for 10 minutes. Cells were lyzed and PDGFβR were precipitated with WGA agarose. Samples were run in SDS-PAGE and transferred to nitrocellulose membranes. The amount of tyrosine phosphorylation on PDGFβR was determined with PY99 phosphotyrosine blotting followed by immunoblotting with PDGFβR antibody. Antibodies were visualized with ECL and LAS-100 plus CCD camera. Densitometry analysis was performed with AIDA advance image data analyser software. The relative PDGFβR phosphorylation is plotted. These data are representative of three experiments.

As the ligand concentration increases, the PDGFβR phosphorylation in WT cells increased with the phosphorylation level still increasing at the highest concentration tested, i.e. 150ng/ml. In LARΔP cells, the phosphorylation peaked at 100 ng/ml PDGF-BB and did not increase any further, with PDGFβR tyrosine phosphorylation remaining clearly lower in LARΔP cells at all ligand concentration tested compared to WT (Figure 2). This result suggests that there might be a non-competitive inhibition of ligand stimulated PDGFβR activation in the absence of LAR, so that the PDGFβR kinase could not be fully activated in LARΔP cells.

3.2.3 Decreased phosphorylation of all PDGFβR autophosphorylation sites in LARΔP

Western blotting analysis with antibodies raised against the PDGFβR phosphorylated autophosphorylation sites and the general phosphotyrosine antibody PY99, were employed to test which sites were affected by loss of LAR activity. Phosphorylation levels of the PDGFβR autophosphorylation sites in LARΔP cells ranged from 30% to 46% of the phosphorylation of corresponding sites in WT cells. No statistically significant differences were detected between the phosphorylation of any autophosphorylation sites and general tyrosine phosphorylation (Figure 3). The finding that all sites showed lower phosphorylation levels is consistent with the concentration response experiment result shown above (Figure 2), and this suggests that LAR deletion decreases PDGFβR kinase activity.

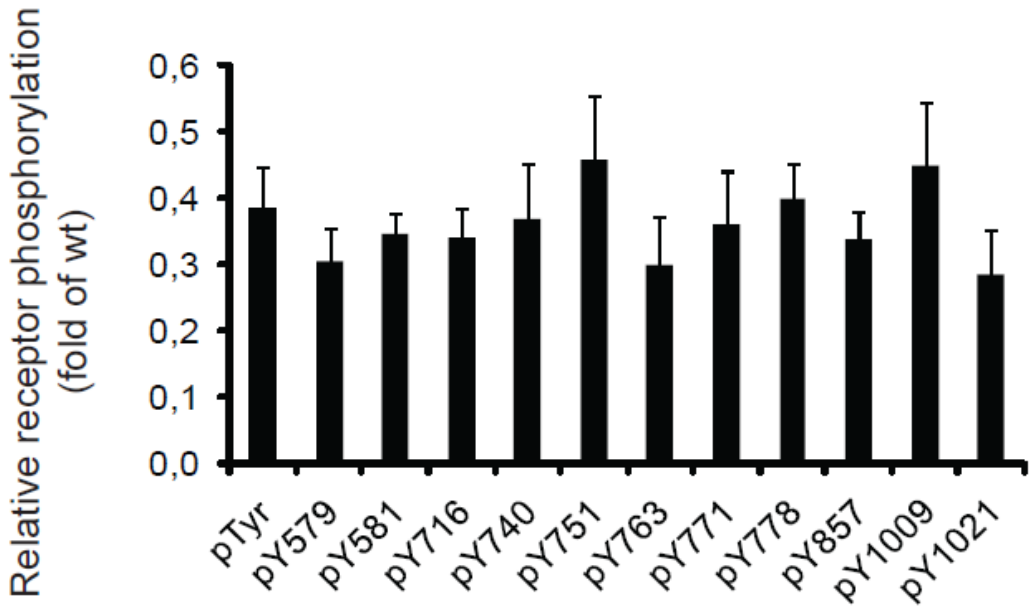

Figure 3 Relative tyrosine phosphorylation of PDGFβR in LARΔP cells

WT and LARΔP cells were stimulated for 10 minutes with 20ng/ml PDGF-BB, cells were then lyzed and PDGFβR was precipitated with WGA agarose. Samples were run on SDS-PAGE and transferred to nitrocellulose membranes. PDGFβR phosphorylation was determined with PY99 phosphotyrosine antibody followed by immunoblotting with antibodies selective for individual tyrosine phosphorylation sites and PDGFβR protein antibody. Blots were visualized with ECL and LAS-100 plus CCD camera. Densitometry analysis was performed with AIDA advance image data analyser software. The PDGFβR phosphorylation in LARΔP cells was compared with WT cells, and the relative tyrosine phosphorylation is plotted. Data from three separate experiments +/- SEM are shown; using student's t-test, no two phosphorylation sites showed significant differences from each other.

3.2.4 Reduced PDGFβR kinase activity in LARΔP cells

The finding that PDGFβR phosphorylation cannot be restored by increasing ligand concentrations in LARΔP cells suggests that PDGFβR kinase activity is decreased. To confirm this, an *in vitro* kinase assay was carried out to compare the PDGFβR kinase activity between the two cell lines. Preliminary result showed PDGFβR kinase activities were very low in the absence of ligand in both WT and LARΔP cells. PDGF-BB stimulation for 3 minutes induced PDGFβR activity in WT fibroblasts. This was demonstrated for both PDGFβR autophosphorylation and phosphorylation of the exogenous substrate MBP (Figure 4). After stimulation, PDGFβR activity in LARΔP increased compared to the un-stimulated sample. However, PDGFβR activity in LARΔP cells is much lower than in WT cells (Figure 4). Taken together, these results indicate that the kinase activity of PDGFβR is higher in WT than in LARΔP cells.

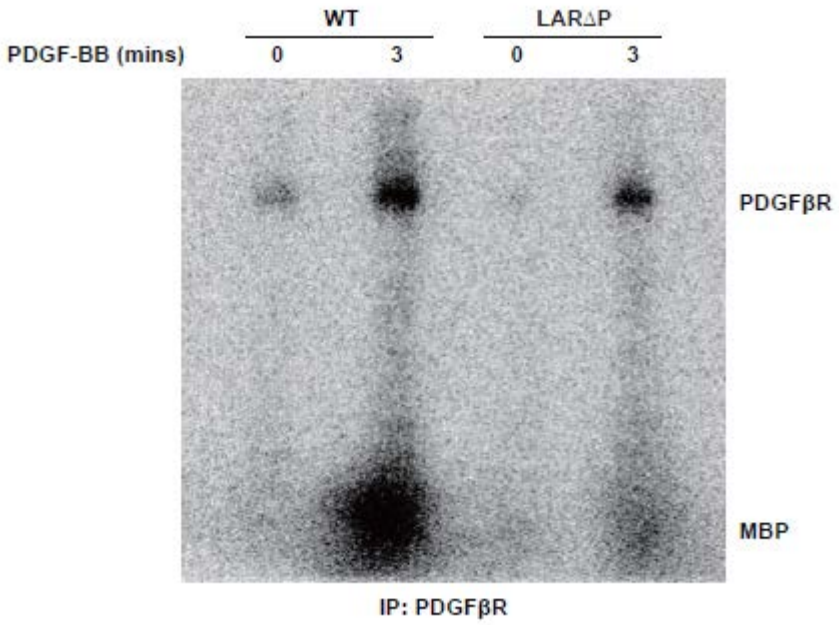

Figure 4 *In vitro* kinase assay for PDGFβR

Cells were starved for 16 hours and stimulated with 20ng/ml PDGF-BB for the indicated time periods. Cells were then lyzed, and PDGFβR was immunoprecipitated and incubated with kinase substrate MBP and γ[^{32}P]ATP at 37°C for 10 minutes with frequent mixing. Samples were run on SDS-PAGE and transferred onto PVDF membranes. The membranes were incubated under radioactive sensitive film. Incorporated radioactivity on the PDGFβR and MBP were analysed and quantified with a Fuji FLA3000 Bioimager. Similar results were observed in more than three experiments.

3.3 Discussion

Autophosphorylation of growth factor receptors such as PDGFβR generally plays important roles in regulating cell survival, growth and migration by providing docking sites for downstream signalling molecules and activating downstream signalling pathways. It is well known that many PTPs negatively regulate PDGFβR phosphorylation and its downstream signalling by dephosphorylating specific autophosphorylation sites. Interestingly, the results in this study indicate that LAR strongly potentiates PDGFβR activity and autophosphorylation. Deletion of LAR phosphatase domains considerably reduced PDGFβR tyrosine phosphorylation at all time points and ligand concentrations (Figure 1 and Figure 2). In LARΔP cells, phosphorylation of the autophosphorylation sites was reduced to 30% - 46% compared with the corresponding sites in WT cells (Figure 3). These results, together with the preliminary *in vitro* kinase assay (Figure 4), suggest that PDGFβR kinase activity is reduced in a non-competitive manner in LARΔP cells. This finding promoted further experiments to determine how knock out of LAR phosphatase could reduce the kinase activity of PDGFβR.

The regulation of phosphorylation of tyrosine 857 in the kinase domain of PDGFβR was shown to be important for the kinase activity of PDGFβR, sense mutation of PDGFβR Tyr857 led to lower kinase activity (Baxter *et al.* 1998). To confirm that PDGFβR activity is lower in LARΔP cells, one could blot with phosphortyrosine 857 antibody to see if the Tyr857 residue is less phosphorylated upon stimulation in

LARΔP cells. Immunofluorescence microscopy using PDGFβR phosphotyrosine specific antibodies could also be used to monitor PDGFβR activation at different time points and at different ligand concentrations. This would allow the localisation of the activated PDGFβR to be assessed in the LARΔP cells.

CHAPTER 4 LOSS OF LAR ACTIVITY REDUCES PDGFβR DOWNSTREAM SIGNALLING AND PROLIFERATION

4.1 Introduction

After the activation and autophosphorylation of PDGFβR, phosphorylated autophosphorylation sites serve as docking sites for downstream signalling molecules and allow the initiation of multiple downstream signalling pathways. The adaptor protein Grb2 binds to the phosphorylated Tyr716 and Tyr775 and leads to activation of the downstream Ras-MAPK pathway. Phosphorylated Tyr1009 and Tyr1021 recruit PLCγ1 to the PDGFβR and lead to the activation of downstream PKC family proteins. PI3K binds to phosphotyrosine 740 and 751 to activate downstream Akt signalling. Activation of Ras, PLCγ and PI3K pathways stimulate cell growth, survival and migration (Heldin *et al.* 1998). It was previously demonstrated that enhanced PDGFβR phosphorylation does not always result in enhanced downstream signalling (Persson *et al.* 2004). In this chapter, PDGFβR downstream signalling was investigated by analysing the signal strength at multiple downstream signalling pathways. Akt, PLCγ and ERK1/2 phosphorylation was analysed in WT and LARΔP cells.

4.2 Results

4.2.1 PDGFβR downstream signalling is reduced in LARΔP

To understand the consequences of LAR deletion for PDGFβR signalling, phosphorylation of downstream signalling molecules was investigated. Cells were stimulated with 20ng/ml PDGF-BB and lyzed. Total cell lysates and immunoprecipitated PLCγ samples were analysed. Phosphorylation of Akt on Thr308 in LARΔP cells occurred with similar kinetics as in WT cells, but had a lower amplitude (Figure 5A), whereas the PLCγ phosphorylation was more transient (Figure 5B). Re-expression of WT full length LAR partially restored the Akt and PLCγ phosphorylation (Figure5A and Figure 5B). However, there were no differences in phosphorylation of Erk1/2 following short term ligand stimulation (Figure 5C upper panel). When the cells were stimulated for longer time periods, sustained Erk1/2 phosphorylation was diminished in LARΔP cells was observed (Figure 5C lower panel). Re-expression of LAR partially restored Erk1/2 phosphorylation in LARΔP cells.

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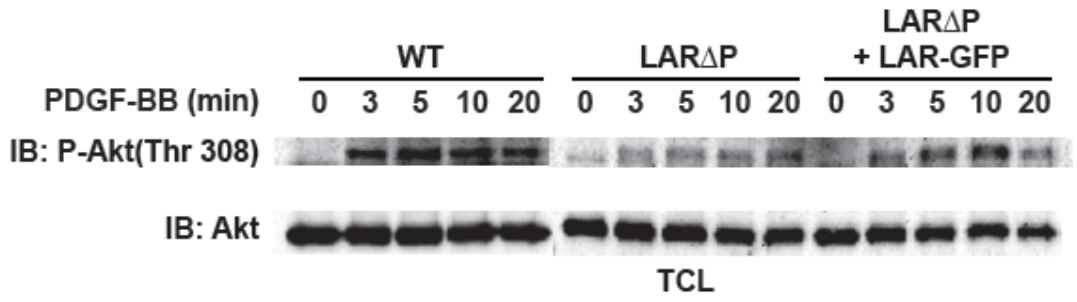

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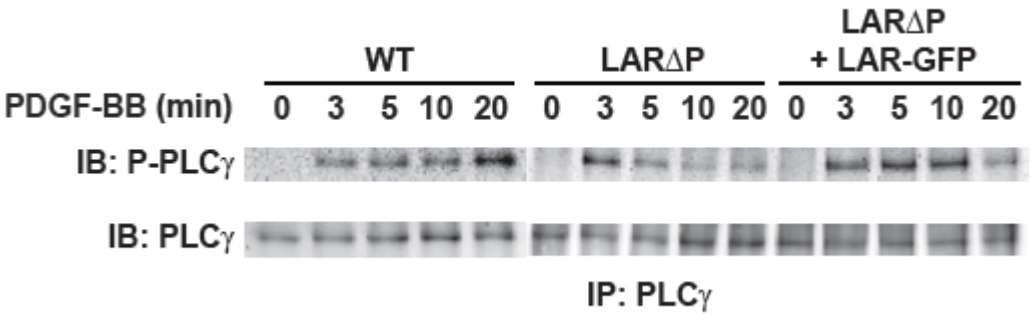

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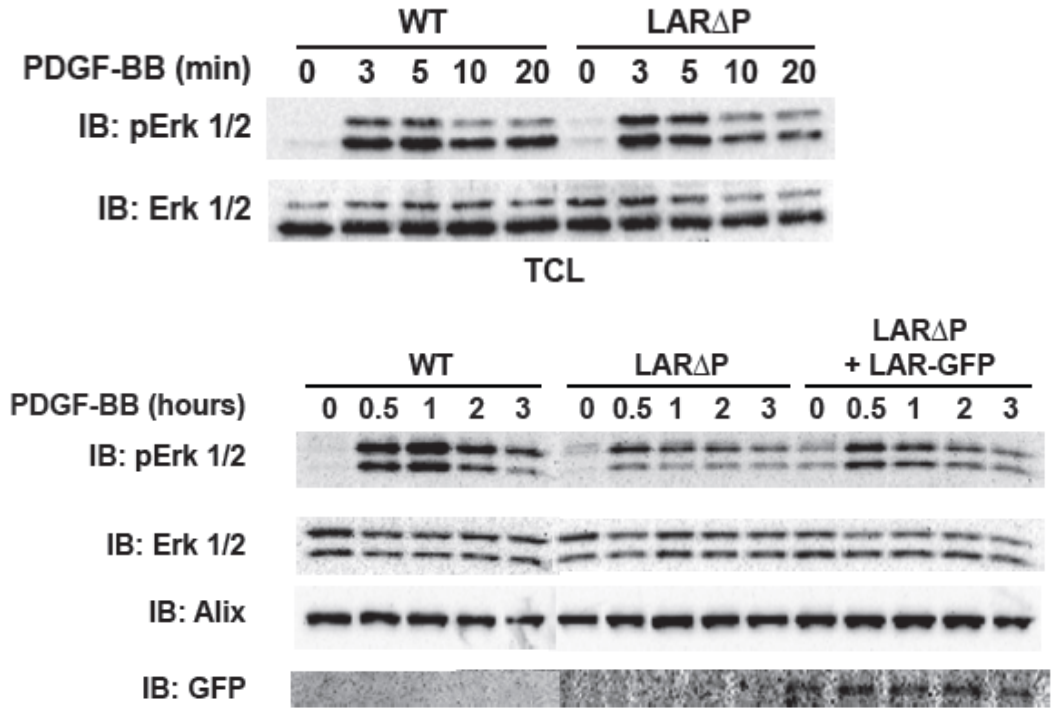

Figure 5 LAR deletion reduces signalling of PDGFβR downstream pathways

WT, LARΔP and LARΔP cells transfected with LAR-EGFP construct for 48 hours were starved for 16 hours and stimulated with 20ng/ml PDGF-BB for the time periods indicated. Cells were lyzed, PLCγ protein was immunoprecipitated and total cell lysate samples were collected. Samples were separated by SDS-PAGE, and

transferred onto nitrocellulose membranes. A. Akt activation was determined using P-Akt (Thr 308) antibody followed by immunoblotting with Akt antibody. B. Immunoprecipitated PLCγ phosphorylation was determined by blotting with PY99 phosphotyrosine antibody followed by PLCγ antibody. C. Erk 1/2 activation was analysed using P-Erk 1/2 (Thr202 Thr204) antibody followed by blotting with Erk 1/2 antibody. GFP protein amount in the corresponding total cell lysate sample was determined with GFP antibody followed by Alix blotting as the loading control. Antibodies were visualized with ECL and LAS-100 plus CCD camera. These experiments were repeated three times; one representative set of data is shown.

4.2.2 LAR deletion reduces PDGF induced cell proliferation

Cells were starved for 16 hours and then were either left untreated, or stimulated with 50 ng/ml PDGF-BB or 10% FBS for 48 h. After incubation with MTT, the formation of formazan was determined by measuring the OD at 600 nm, and the relative proliferation was calculated. The result shows higher proliferation rate of WT cells compared to LARΔP upon PDGF-BB stimulation, which is consistent with the stronger PDGFβR signalling in WT fibroblasts. On the other hand, foetal bovine serum stimulated enhanced proliferation in LARΔP cells compared with WT cells (Figure 6). These results demonstrate that LAR is selectively required for PDGF induced proliferation.

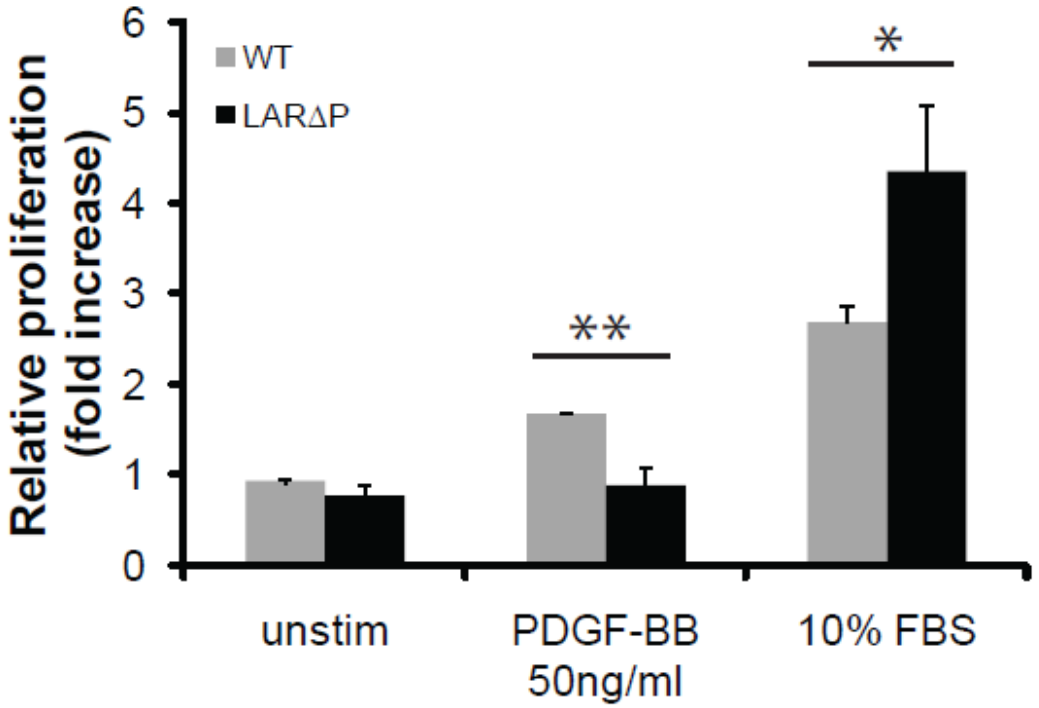

Figure 6 Decreased proliferation in response to PDGF-BB in LARΔP cells

Cells were starved for 16 hours and then were either left untreated or stimulated with 50 ng/ml PDGF-BB or 10% serum for 48 hours. Following incubation with MTT, the formation of formazan was determined by measuring OD at 600 nm, and the relative proliferation was calculated. Data from three separate experiments, each performed in quadruplicates, +/− SEM are shown. Statistical significant differences (Students T-test) are indicated by *, $p < 0.05$ and **, $p < 0.01$.

4.3 Discussion

To follow up on the finding that LAR is required for full activation of PDGFβR kinase, the activities of downstream signalling molecules were investigated. Downstream signalling of PDGFβR is clearly weaker in the absence of LAR (Figure 5), which is consistent with the reduced receptor phosphorylation.

Jurek *et al*, 2008, demonstrated that PDGF induced Erk activation depends on the degradation of MAPK phosphatase 3 (MKP3) which is a phosphatase highly selective for Erk (Jurek *et al.* 2009). PDGFβR activation induces the serine174 phosphorylation on MKP3 which leads MKP3 to degradation. It is possible that when LAR is deleted, PDGFβR phosphorylation is reduced. However, this phosphorylation is still enough to induce normal level of Erk1/2 phosphorylation through the Raf, MEK pathway. However, this phosphorylation strength might not be enough to cause enough phosphorylation of Ser174 on MKP3 somehow, and MKP3 degradation does not proceed as much as normal and lead to the dephosphorylation of Erk1/2 after longer periods of stimulation.

In line with the long term Erk1/2 activation, the PDGF-BB stimulated proliferation was significantly reduced in LARΔP cells. However LARΔP cells proliferated faster than WT cells in response to FBS (Figure 6), demonstrating that LAR does not regulate the proliferative machinery directly, but is selective for PDGF-induced signalling. FBS contains many types of growth factors including PDGF, EGF, FGF

and LPA. LAR has been shown to negatively regulate the signalling of other tyrosine kinase growth factor receptors including EGFR and HGFR (Mooney *et al.* 1997). Thus, it is conceivable that proliferative signals from other receptors are increased in LARΔP cells, which contribute to the faster proliferation in response to FBS.

To confirm the lower signalling strength in the Akt pathway in LARΔP cells compared to WT cells, *in vitro* kinase assay could be used to measure the kinase activity of PI3K which is an upstream signalling molecule responsible for Akt activation. As the upstream effector of ERK, Ras activity could be directly measured to investigate the Ras signalling pathway strength in the two cell types.

To determine that the MTT assay is reflecting proliferation of cells rather than changes in the activity of cellular NAD(P)H-dependent cellular oxidoreductase, proliferation could also be assessed using [^{3}H]Thymidine incorporation assay. In this assay radioactive thymidine and PDGF ligand can be incubated with the cells overnight. The incorporated thymidine can be quantified using a scintillation counter as a measure of cell proliferation.

CHAPTER 5 PDGFβR DOWNREGULATION IS AFFECTED BY LAR

5.1 Introduction

After PDGF receptor stimulation, it is important to desensitize the cells from PDGF stimulation in order to prevent over-activation and uncontrolled growth. Receptor degradation is one of the endogenous mechanisms to attenuate PDGF signalling. After ligand stimulation, ligand receptor complexes are internalized and removed from the cell surface. Ubiquitination of PDGFR will lead to proteolysis and receptor degradation inside cells. PDGFR internalization is one of the earliest responses elicited by PDGF stimulation. Internalization is mediated by clathrin coated vesicle formation and intracellular transport of the receptor away from the cell surface. After un-coating, the vesicles fuse with the existing early endosomes. Receptors are then sorted into the late endosome and further sorted towards lysosomal degradation (Maxfield and McGraw 2004). C-Cbl is one of the SH2 domain containing proteins which is recruited to PDGFβR upon stimulation. It has been identified as an E3 ubiquitin ligase that is involved in PDGFβ receptor ubiquitination and thereby targeting the receptor to degradation (Miyake *et al.* 1998). Both the ligand induced internalization and c-Cbl mediated ubiquitination require PDGFR tyrosine phosphorylation, and constitute downregulation mechanisms that terminate receptor

signalling.

In this chapter, PDGFβR internalisation was investigated in WT and LARΔP cells. PDGFβR degradation and ubiquitination were also assessed to obtain further information about the effect of LAR on PDGFβR function.

5.2 Results

5.2.1 PDGFβR ubiquitination

To analyse the receptor ubiquitination in the two cell types after stimulation, PDGFβR were immunoprecipitated. After SDS-PAGE and membrane transferring, membranes were probed with Ub antibody to visualize ubiquitination. The data showed higher ubiquitination levels in WT cells compared to LARΔP cells upon PDGF-BB stimulation with ubiquitination peaking at 20 minutes (Figure 7). This was expected, since ubiquitination of PDGFβR is suggested to be dependent on receptor phosphorylation.

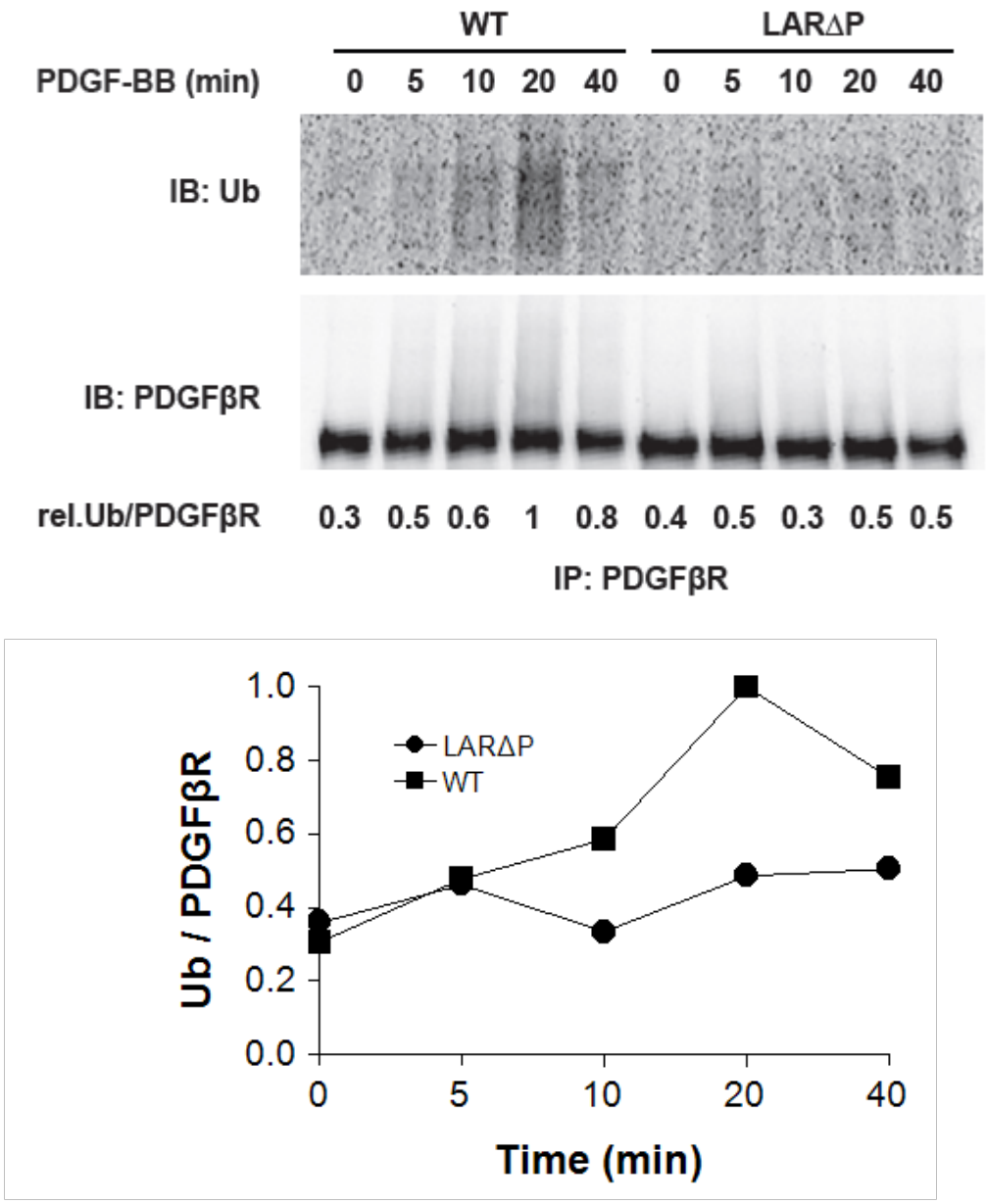

Figure 7 Ubiquitination of PDGFβR

Cells were starved for 16 hours and stimulated with 20ng/ml PDGF-BB for the indicated time periods. PDGFβR were immunoprecipitated and separated by SDS-PAGE. Proteins were transferred onto nitrocellulose membranes. Ubiquitination of PDGFβR was visualized with Ub antibody. Antibodies were visualized with ECL and LAS-100 plus CCD camera. Densitometry analysis was performed with AIDA advance image data analyser software. The relative ubiquitination of PDGFβR was plotted. This experiment was repeated three times, results from one representative experiment are shown.

5.2.2 PDGFβR internalization

After stimulation, ubiquitination of PDGFβR would lead to the receptor's degradation which is initiated by receptor internalization. Cells were starved overnight, stimulated and then put on ice to stop receptor internalization. Cell surface proteins were biotinylated by incubating with 0.5mg/ml sulfo-NHS-SS-biotin. Unbound biotin was quenched by incubation with 50mM Tris pH 8. The cells were lyzed and incubated with the streptavidin sepharose to precipitate the biotinylated cell surface proteins, immunoprecipitated PDGFβR was detected by western blotting. Although there are no significant differences between WT and LARΔP cells, the mean PDGFβR internalization rate was faster in the WT compared to LARΔP cells, with about 50% of the cell surface PDGFβR internalized in WT while only around 5% in LARΔP after 10 minutes stimulation (Figure 8). This is consistent with the ubiquitination result. In WT cells, PDGFβR are more phosphorylated, more ubiquitinated and internalized faster compared to LARΔP cells.

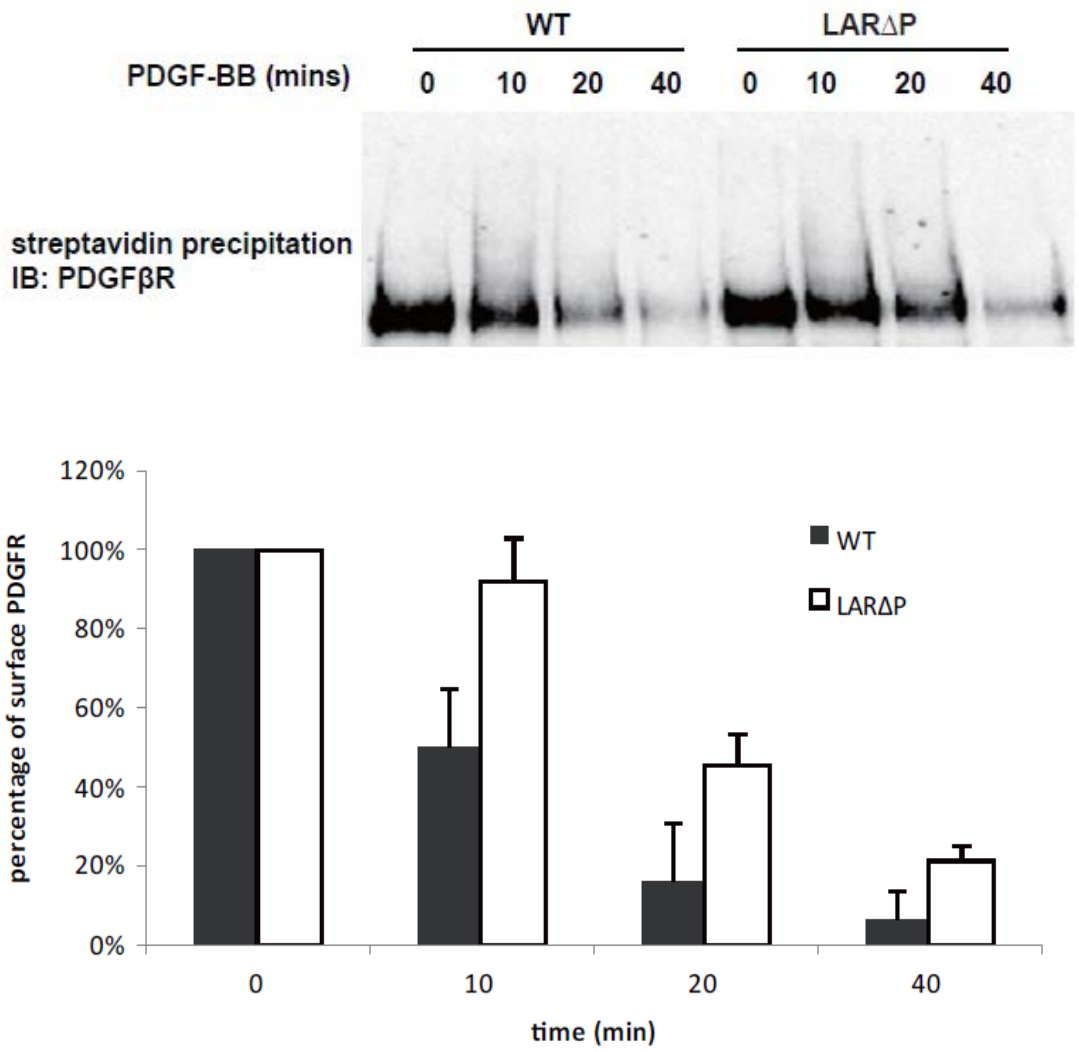

Figure 8 Ligand induced internalization of PDGFβR

Cells were starved for 16 hours and stimulated with 20ng/ml PDGF-BB for the indicated time periods. Cell surface proteins were biotinylated by incubating with 0.5mg/ml sulfo-NHS-SS-biotin, and cell surface proteins were precipitated using streptavidin agarose. Samples were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. Cell surface receptors were detected with PDGFβR antibody. Antibodies were visualized with ECL and LAS-100 plus CCD camera. Densitometry analysis was performed with AIDA advance image data analyser software. This experiment was repeate three times, and the mean relative receptor phosphorylation +/−SEM is plotted. No statistically significant differences were found between WT and LARΔP internalization at any time points

5.2.3 PDGFβR degradation

The degradation rate of PDGFβR of the two cell types was then investigated. Cells were stimulated and lyzed. Total cell lysate was separated on SDS-PAGE, and transferred onto nitrocellulose membrane. The amount of PDGFβR remaining in the cells was determined with PDGFβR antibody. The results show that around 80% of the PDGFβRs are degraded in both cell types with no significant differences between the two cell types after 3 hours stimulation (Figure 9). As a control, Erk phosphorylation was assessed to confirm that there was more sustained signalling in WT cells (Figure 9).

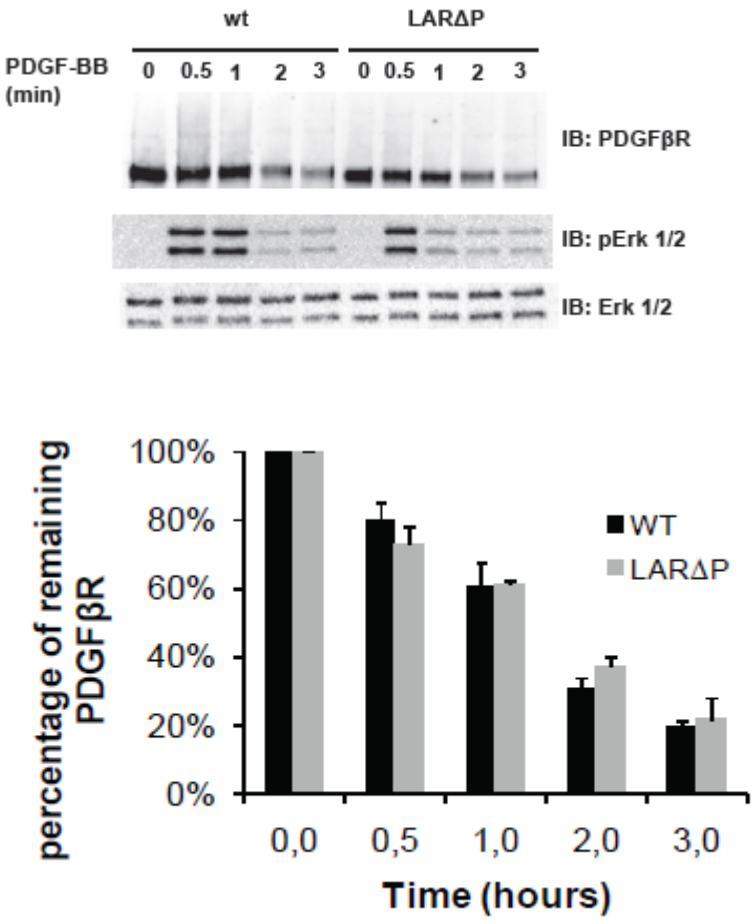

Figure 9 Ligand induced PDGFβR degradation

Cells were starved for 16 hours before they were stimulated with 20 ng/ml PDGF-BB for the indicated time periods. Cells were lyzed, and total cell lysate were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The amount of PDGFβR was determined with PDGFβR antibody, and phosphorylation of Erk 1/2 was determined with P-Erk 1/2 antibody followed by Erk protein blotting, and visualization with ECL and LAS-100 plus CCD camera. Densitometry analysis was performed with AIDA advance image data analyser software. This experiment was repeated three times, and statistical analysis was performed using student's t-test. The mean relative receptor phosphorylation +/−SEM is plotted. There are no significant differences between the two cell types for their PDGFβR degradation.

5.3 Discussion

PDGFβR activation leads to its down regulation in the form of dephosphorylation, followed by ubiquitination, internalization and degradation. In this chapter, PDGFβR downregulation was investigated in the presence and absence of LAR phosphatase activity. In LARΔP cells, the ubiquitination level is lower compared to WT, which is consistent with the tyrosine phosphorylation of PDGFβR (Figure 7). The internalization experiments showed no significant differences between WT and LARΔP internalization. However, there is a trend shown by the average values in the internalization experiments that WT cells internalized PDGFβR faster than in LARΔP cells (Figure 8). Interestingly, although the receptors in LARΔP are much less phosphorylated, after 20 minutes stimulation, around 50% receptors are internalized and after 40 minutes stimulation only 20% receptors are left on the cell surface (Figure 8). The decreased kinase activity, as demonstrated in chapter 3, would result in the same number of PDGFβR being phosphorylated in LARΔP and WT cells, but the PDGFβR would be less phosphorylated. This would result in lower c-Cbl recruitment and less receptor ubiquitination. This will make it slower for the activated receptor to be found by the endocytosis machinery and thus slow down the internalization process. However, since the same population of receptor was phosphorylated in LARΔP as in WT, receptors would eventually be internalized.

The receptor degradation rate showed no significant differences between the two cell types. With faster ubiquitination and internalization rates, one would expect the

degradation to be faster in WT cells. Although PDGFβR get internalized faster into the endosome in WT cells, the receptors may stay inside these vesicles longer before they are sent to lysosome for degradation. One of the possibilities is that the deletion of LAR phosphatase activity somehow accelerates the transportation of the receptors from late endosome to lysosome, or some shortcut is created in the absence of LAR activity from early endosomes to lysosomes. Several proteins in the ESCRT systems that sort the activated receptors, e.g. STAM and Hrs, are substrates for PDGFβR (Row *et al.* 2005). The role of ESCRT protein phosphorylation has not been determined, but it is possible that it regulates the time spent in endosomes allowing for signalling to occur. If so, reduced PDGFβR kinase activity would enhance the degradation rate.

Another possibility is that the degradation rate inside the lysosome is already saturated in LARΔP cells. Thus although the PDGFβR receptors could be sent to the lysosomes at a higher speed in WT cells, the lysosomal proteases degrade these internalized receptors at the same rate in both cell types at the same maximum capacity. The same PDGFβR degradation rate in the LARΔP and WT cells also supports the notion that the same sized population of PDGFβR molecules are activated during PDGF stimulation in the two cell types, thus the differences in receptor phosphorylation is caused by the lower PDGFβR kinase activity in LARΔP cells rather than a smaller population of receptors being activated.

To confirm the internalization and degradation results shown in Figure 8 and Figure 9,

immunofluorescent microscopy experiments could be carried out to look at the internalization and degradation of PDGFβR using PDGFβR specific antibody in the two cell types at different time points after stimulation. By doing this, one could visualise the receptor moving from the cell surface inside the cell after stimulation, followed by degradation of the receptor which would be marked by the disappearance of the fluorescent signal. Levels of PDGFβR remaining on the cell surface at different time points could be quantified and the internalization rate compared between the two cell types. This would provide an alternative method to measure the PDGFβR degradation rate.

CHAPTER 6 SEARCHING FOR THE UNDERLYING MECHANISM

6.1 Introduction

After observing that LAR promotes PDGFβR signalling and downregulation, I wanted to investigate the underlying reason. In most of the previously investigated cases, PTPs were reported to negatively regulate tyrosine kinase receptor signalling by dephosphorylating either the receptor or major downstream components. LAR was also reported to negatively regulate EGFR and HGFR signalling. However, LAR was once shown to positively regulate the neurotrophin tyrosine kinase receptor TrkB signalling and downstream physiological effects by dephosphorylating the negative regulatory tyrosine residue Tyr527 on Src (Yang *et al.* 2006). Researchers described that Src is activated after dephosphorylation of Tyr527 (Tsujikawa *et al.* 2002), the activated Src then binds to TrkB receptor and phosphorylates it to elevate the receptor signalling. Src has been shown to phosphorylate PDGFβR as well (Hansen *et al.* 1996). In the absence of LAR, the Src kinase activity could be suppressed which may lead to lower PDGFβR phosphorylation.

Other studies suggested that PDGF receptor activity is affected by its localization in

the plasma membrane. Liu *et al.*, 1996 demonstrated caveolae to be the principle location of PDGF receptor at the cell surface, and that PDGF receptor initiates signal transduction from caveolae. Proteins known to interact with the phosphorylated PDGF receptors are highly enriched in caveolae. Liu *et al.* 1997 showed that PDGF receptor phosphorylation and activation of MAPK occured in caveolae (Liu *et al.* 1996; Liu *et al.* 1997). It seems that caveolae are the place that PDGF receptors get activated, and the localization of PDGF receptor in caveolae affects PDGF receptor activity and function. However, a later study showed that PDGF receptor colocalized with the major component of caveolae, caveolin. Caveolin binds directly to PDGF receptor and inhibits its autophosphorylation and kinase activity (Yamamoto *et al.* 1999). The findings of Sergey *et al*, 2001 could explain these contradictory results described above. They showed that the PDGF receptor is associated with the non-caveolae lipid rafts, and after ligand stimulation the PDGF receptor transiently associated with caveolae which led to the phosphorylation of downstream signalling molecules. PDGF receptor was also shown to be sequestered in the caveolae region and desensitized from later activation (Matveev and Smart 2002). In summary, there have been several reports demonstrating that caveola localization of PDGFR may act as a major regulator of receptor kinase activity and signalling.

In this chapter, I looked at the possible involvement of Src kinase in the LAR related reduction in PDGFβR phosphorylation in LARΔP cells. The caveola localization of PDGFβR was also investigated in the two cell types.

6.2 Results

6.2.1 Src inhibition does not change PDGFβR phosphorylation in either WT or LARΔP cells

It is possible that Src is activated by LAR by its dephosphorylation at the Src Tyr527 residue in WT cells; activated Src could then phosphorylate PDGFβR and lead to higher phosphorylation. Therefore, the Src inhibitor SU6656 was used to inhibit Src activity in both cell types to see if this would affect PDGFβR phosphorylation. Cells were incubated with 2μM SU6656, stimulated and lyzed. Receptors were precipitated with WGA agarose beads, and subjected to western blotting. The result of one experiment is shown in Figure 10. Src inhibition did not alter PDGFβR phosphorylation in either the WT or LARΔP cells (Figure 10). This indicates that lack of Src activation by LAR is not the reason for the lower PDGFβR phosphorylation in LARΔP cells, since one would expect to see the PDGFβR phosphorylation dropping in the WT cells after the inhibitor treatment, if Src phosphorylates the receptor.

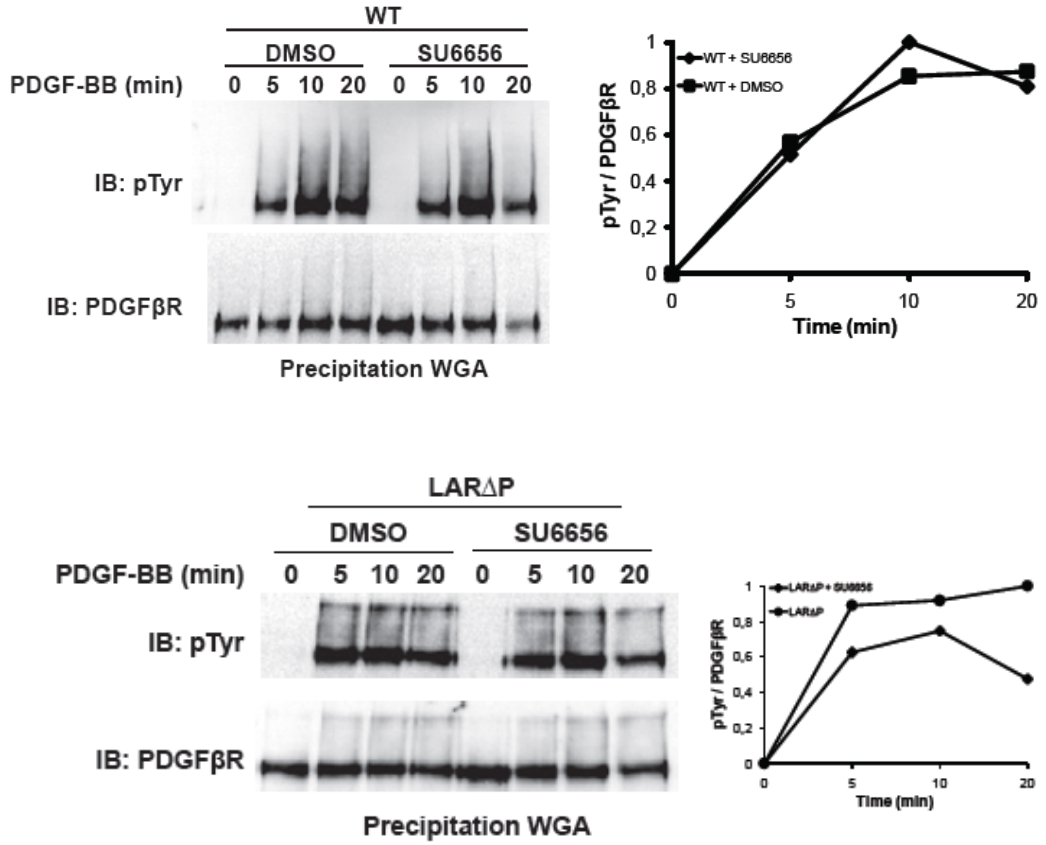

Figure 10 Src inhibitor SU6656 does not change PDGFβR phosphorylation in WT or LARΔP cells

Cells were starved for 16 hours before they were incubated with 2μM SU6656 or 0.1% DMSO for 1 hour. Cells were then stimulated with 20 ng/ml PDGF-BB for the indicated time periods and lyzed. Receptors were precipitated with WGA agarose beads, and western blotted. The PY99 antibody was used to determine the tyrosine phosphorylation level of PDGFβR followed by blotting with PDGFβR antibody. Antibodies were visualized with ECL and LAS-100 plus CCD camera. Densitometry analysis was performed with AIDA advance image data analyser software. The phosphorylation of PDGFβR was plotted. This experiment was repeated twice with similar results.

6.2.2 Src kinase activity is similar in WT and LARΔP

To further confirm that reduced Src activation does not cause the reduced tyrosine phosphorylation of PDGFβR in LARΔP cells, an *in vitro* kinase assay was utilized to compare Src kinase activity in the two cell types. Starved cells were stimulated with 20ng/ml PDGF-BB for the indicated time periods. Src family kinases were precipitated and incubated with the exogenous substrate enolase and γ[^{32}P]ATP at 37°C for 10 minutes with frequent mixing. Samples were run on SDS-PAGE and transferred onto PVDF membranes. The membranes were incubated under radioactive sensitive film. Radioactive signals on the phosphorylated enolase was detected and quantified with a Fuji FLA3000 Bioimager. The result of one experiment is shown in Figure 11. This shows that Src kinases were activated to a similar extent in the two cell types, confirming that Src is not the intermediate player here.

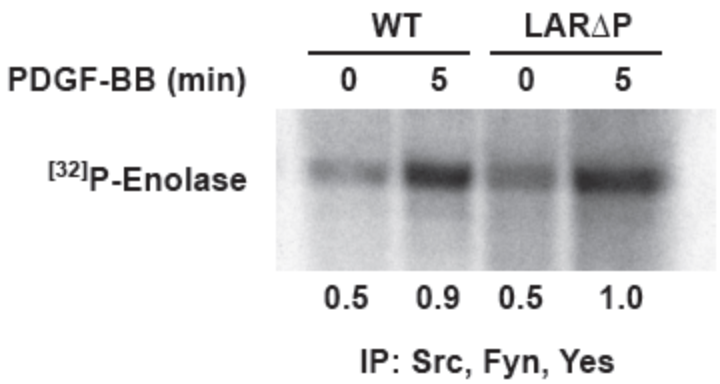

Figure 11 *In vitro* kinase assay for Src family kinases

Cells were stimulated with 20 ng/ml PDGF-BB for the indicated time periods. Src family kinases were precipitated with Src family kinase antibody, and incubated with enolase and γ[^{32}P]ATP at 37°C for 10 minutes with frequent mixing. Samples were run on SDS-PAGE and transferred onto PVDF membranes. The membranes were incubated under radioactive sensitive film. Radioactive signals on the phosphorylated enolase were detected and quantified with a Fuji FLA3000 Bioimager. This experiment was repeated twice.

6.2.3 A very small fraction of PDGFβR is present in the caveolae region

There have been several publications showing that PDGFβR phosphorylation and its signalling is affected by its localization in caveolae or lipid rafts. To investigate whether there are differences for the amount of PDGFβR localized in caveolae in the presence and absence of LAR activity, the caveola localization of PDGFβR in the WT and LARΔP cells were compared. Starved cells were stimulated and lyzed. PDGFβR was immunoprecipitated and caveolin 1 co-precipitation was analysed. Result shows a higher amount of caveolin 1 associating with PDGFβR in WT compared to LARΔP (Figure 12A). This is in accordance with the study that suggested activated PDGF receptors are sent to caveolae to be sequestered and kept inactive. WT cells are activated more than LARΔP cells, so the receptor association with caveolin 1 would be higher in WT cells. It is well known that caveolae do not dissolve in the TritonX100 at 4 °C (Moldovan *et al.* 1995). To further study the proportion of PDGFβR that is localized in caveolae region before and after ligand stimulation, cells were lyzed with buffer containing 1% TritonX100 for 15 minutes on ice. The cell lysates were sonicated for 2 seconds and centrifuged and the supernatants were saved as the TritonX100 soluble fractions. The remaining pellets were dissolved in 1% SDS with heating at 95°C for 10 minutes. Both fractions were boiled in sample buffer and analysed with western blot. The vast majority of both total PDGFβR and phosphorylated PDGFβR were located in the TritonX100 soluble non-caveolae fraction before and after stimulation in both cell types. There was no detectable PDGFβR located in the Triton insoluble fraction (Figure 12B). In combination with

the immunoprecipitation results, it seems that very small fraction of PDGFβR is located in the caveolar region in these two cell types. It is possible that the presence of caveolar structures is very limited in the mouse embryonic fibroblasts used for this study. The big difference in PDGFβR phosphorylation between WT and LARΔP cells did not appear to be caused by the different caveolar localization of PDGFβR in the two cell types.

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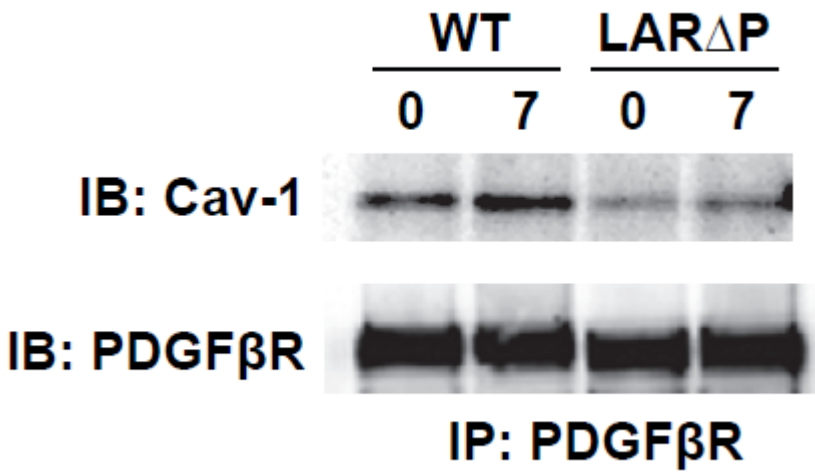

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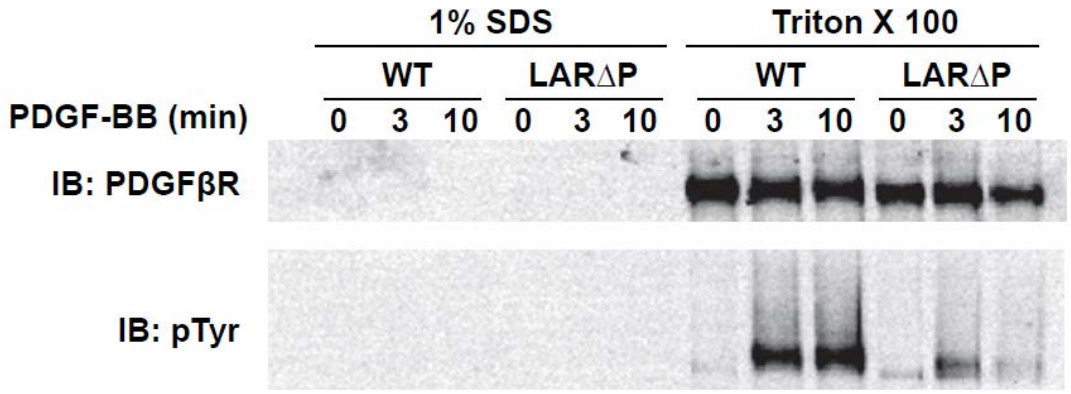

Figure 12 PDGFβR localization in different fractions

A. Cells were starved for 16 hours and stimulated with 20 ng/ml PDGF-BB for the time periods indicated. PDGFβR was immunoprecipitated and separated by SDS-PAGE and transferred onto nitrocellulose membranes. Caveolin 1 was visualized by using Cav-1 antibody, followed by blotting with PDGFβR antibody. This experiment was repeated twice, one representative result is shown. B. After stimulation for the indicated time periods, cells were lyzed with buffer containing 1% TritonX100 for 15 minutes on ice. Cell lysates were sonicated for 2 seconds and centrifuged. The supernatant was saved as the TritonX100 soluble fraction. Pellets were dissolved in 1% SDS with heating at 95°C for 10 minutes. Sample buffer was added to the TritonX100 fraction and 1% SDS fraction and both were boiled at 95°C for 5 minutes and analysed by western blot. This experiment was repeated twice with similar results.

6.3 Discussion

PDGF receptor phosphorylation can be affected by several different cellular components, so its signalling can be well regulated. Possible reasons for the reduced PDGFβR phosphorylation observed in LARΔP cells were investigated in this chapter. The Src negative regulatory tyrosine Tyr527 is a known target of LAR, and therefore Src could be activated by LAR to phosphorylate PDGFβR in WT cells. In the absence of LAR phosphatase activity, one would expect Src activity to be reduced, which in turn would decrease the PDGFβR phosphorylation in LARΔP cells. However, Src inhibitor treatment did not cause any reduction in PDGFβR phosphorylation in WT fibroblasts (Figure 10), demonstrating that no additional phosphorylation in WT compared to LARΔP was due to Src. Src kinase activity was similar in unstimulated MEFs, and PDGF-BB activated Src in both cell lines (Figure 11), demonstrating that loss of LAR activity did not reduce the Src kinase activity in LARΔP cells. The Src expression levels in the two cell types were checked to be the same with western blot (data not shown).

Several previous studies suggested that caveolar localization of PDGF receptor affects its kinase activity and function. If the caveolar PDGF receptor localization in WT and LARΔP showed some differences, it could be an explanation for the observed difference in receptor phosphorylation. However, the TritonX100 soluble non-caveolae fraction contained all the receptors detected in both cell types (Figure 12B). Although immunoprecipitation study shows caveolin 1 association with

PDGFβR in both cell types with more association in WT cells (Figure 12A), no phosphorylated receptors were detected in the caveolar fraction (Figure 12B). The altered membrane localization clearly cannot explain the large difference in PDGFβR phosphorylation we observed. Further experiments could be carried out to confirm if there are equal amounts of caveolin 1 present in both cell types. This would further show that caveolin 1 associates with PDGFβR more in the WT than in LARΔP cells (Figure 12A). The caveolin 1 amount should be checked in the Triton X 100 soluble fraction and in the Triton insoluble fraction to confirm that caveolin 1 is being isolated from the insoluble fraction (Figure 12B). Colocaisation of PDGFβR with caveolin in the different WT and LARΔP cell types could also be investigated with fluorescence microscopy.

CHAPTER 7 HIGHER C-ABL ACTIVITY IN LARΔP LEADS TO LOWER PDGFβR PHOSPHORYLATION

7.1 Introduction

C-Abl is a non-receptor tyrosine kinase that is located in both the cytoplasm and nucleus. It interacts with a large variety of cellular proteins including signalling adaptors, kinases, phosphatases, cytoskeleton proteins and transcription factors (Sirvent *et al.* 2008). C-Abl is a substrate for LAR, and LAR was shown to play a role in antagonizing Abl function in drosophila neuronal cells. The large variety of substrates for c-Abl could provide a link between LAR and PDGFβR activity (Chagnon *et al.* 2004). C-Abl phosphorylates kinases such as Protein kinase C and phosphatases such as SHP2, both of which could directly or indirectly regulate tyrosine phosphorylation of PDGFβR (Yuan *et al.* 1998; Mitra *et al.* 2008). Moreover, c-Abl localizes to and participates in the regulation of focal contacts which could also change integrin mediated adhesion and signalling. This could in turn affect PDGF receptor signalling as well (DeMali *et al.* 1999). Srinivasan *et al.* demonstrated that c-Abl is phosphorylated by PDGFβR at Tyr245 and Tyr412, the phosphorylation of which are required for c-Abl activation (Srinivasan *et al.* 2009). On the other hand, c-Abl was shown to phosphorylate PDGFβR and potentiate its kinase activity and

PDGF-BB induced proliferation. This would provide a positive feedback loop between c-Abl and PDGFβR. Therefore c-Abl could either phosphorylate the PDGFβR or activate other kinases that regulate PDGFR kinase activity. C-Abl expression and activity were compared in WT and LARΔP cells, and its role in PDGFβR activation was investigated in this chapter.

7.2 Results

7.2.1 Higher cytoplasmic c-Abl localization in LARΔP cells

As one of the major downstream targets of LAR, c-Abl expression, subcellular localization and activity were analysed in the two cell types. Unstimulated WT, LARΔP and LARΔP cells transiently expressing LAR-EGFP were lyzed with standard lysis buffer. The cell lysates were blotted and the c-Abl protein amount in the solubilized fraction was determined and densitometry analysis was performed. These data show that more than twice the amount of c-Abl was recovered in LARΔP compared to WT cells. LARΔP cells transfected with full length LAR-EGFP construct show a lower level of c-Abl than untransfected LARΔP cells but higher than in WT cells (Figure 13A). This suggests that the presence of LAR phosphatase activity suppresses either the expression or the cytoplasmic localization of c-Abl expression. To distinguish between these possibilities, cells were lyzed either with normal lysis buffer, which does not solubilize nuclear proteins, or with sample buffer which lyzes both fractions. A very similar level of c-Abl was observed in the total cell lysate of

WT and LARΔP cells (0.9 and 1 according to quantification using AIDA software (Figure 13B). 80% of the total c-Abl proteins were present in the cytoplasm in LARΔP, while only 56% of the c-Abl were located in the cytoplasmic fraction in WT cells. There are no changes in the amount of c-Abl protein in the cytoplasm after stimulation in the two cell types. These results indicate that the higher amount of c-Abl protein in cytoplasm of LARΔP cells is due to a larger proportion of total cell c-Abl located in the cytoplasm rather than enhanced protein expression. This is presumably caused by c-Abl exportation from the nucleus to the cytoplasm in the absence of LAR phosphatase activity.

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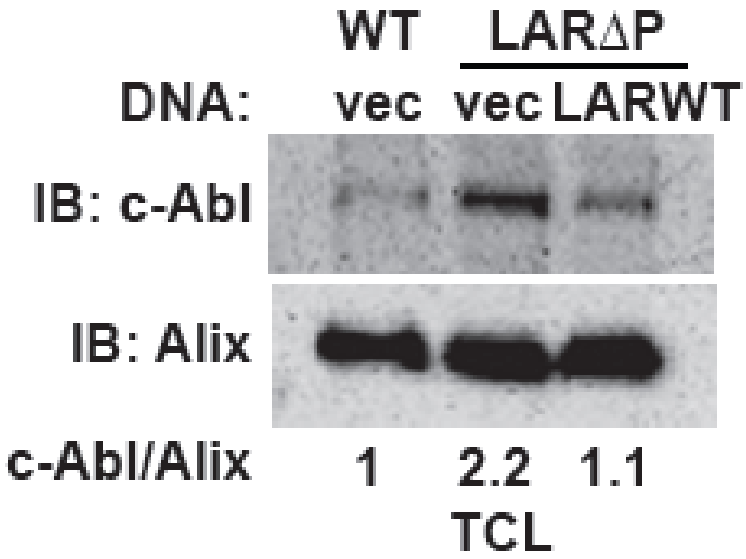

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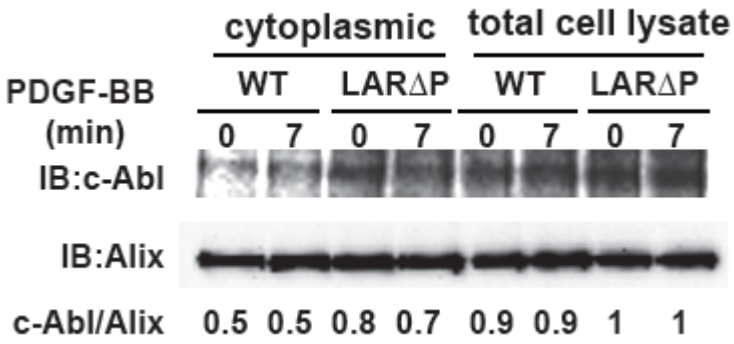


Figure 13 C-Abl exists in different fractions within WT and LARΔP cells

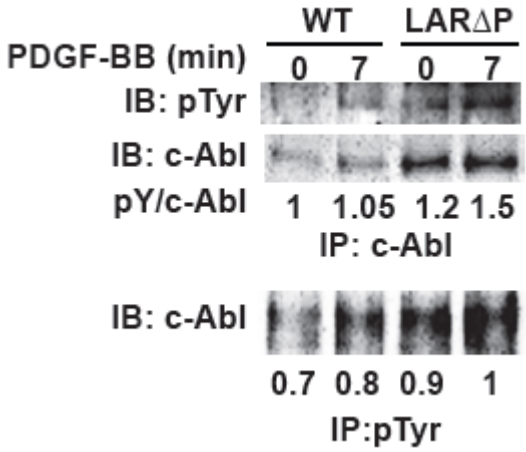
A. Unstimulated WT, LARΔP and LARΔP cells transfected with LAR-EGFP construct were lyzed with the lysis buffer described before to release the cytoplasmic proteins, the c-Abl protein amount in the cytoplasmic fraction was determined using c-Abl antibody followed with reblotting with Alix antibody as the loading control. This is a representative result of three experiments. B. cells were starved for 16 hours and stimulated with 20 ng/ml PDGF-BB for the time periods indicated. Cells were either lyzed with standard lysis buffer or sample buffer, the cell lysates obtained with lysis buffer were centrifuged at 11337g for 15 minutes. The supernatant was saved as the "cytoplasmic" fraction, sample buffer was then added to the sample and boiled at 95°C for 5 minutes. Lysates obtained with sample buffer were sonicated for 2 seconds and centrifuged at 11337g for 30 seconds and boiled at 95°C for 5 minutes and saved as "total cell lysate". Samples were subjected to western blot and the c-Abl protein amount was determined with c-Abl antibody followed by reblotting with Alix antibody as the loading control. Densitometry analysis was performed with AIDA advance image data analyser software. This experiment was repeated for three times, and results showed similar pattern. The result is representative of three experiments.

7.2.2 Higher c-Abl tyrosine phosphorylation in LARΔP cells

As mentioned before, c-Abl is a known substrate of LAR, so without LAR, c-Abl is expected to be hyperphosphorylated. Since c-Abl phosphorylation increases its kinase activity, c-Abl phosphorylation and kinase activity were analysed in WT and LARΔP cells. Starved cells were stimulated and lyzed, and c-Abl was immunoprecipitated and tyrosine phosphorylation of c-Abl protein was determined by western blot using PY20 antibody followed by reblotting with c-Abl antibody (Figure 14A upper panel). Tyrosine phosphorylated proteins were also immunoprecipitated with the phosphotyrosine antibody PY20 and the amount of precipitated c-Abl was determined using western blotting (Figure 14A lower panel). There is initial c-Abl phosphorylation in the absence of ligand in both cell types. C-Abl phosphorylation was increased after PDGF-BB stimulation in both cell types. However, c-Abl phosphorylation was consistently higher in LARΔP than in WT (Figure 14A upper panel). Likewise, phosphotyrosine immunoprecipitation pulled out more c-Abl proteins in LARΔP cells than WT as well (Figure 14B lower panel). These results suggest that c-Abl is active in the absence of PDGF in these cells, and that ligand addition increases c-Abl activity further, particularly in LARΔP cells. To confirm this, the phosphorylation of CrkII, a well-known downstream substrate of c-Abl, was analysed in the two cell types. Unstimulated cells were lyzed, and CrkII was immunoprecipitated with CrkII antibody. Samples were western blotted with phosphotyrosine antibody followed by CrkII antibody, Densitometry analysis showed CrkII phosphorylation in LARΔP cells and no detectable phosphorylation of CrkII in

WT, which strengthens the notion that the c-Abl activity in unstimulated LARΔP cells is much higher than in WT cells (Figure 14B).

A



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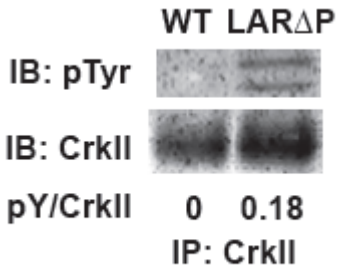


Figure 14 Higher tyrosine phosphorylation of c-Abl and its substrate CrkII in LARΔP cells compared to WT

A. Cells were starved for 16 hours and stimulated with 20 ng/ml PDGF-BB for the indicated time periods and lyzed. Samples were immunoprecipitated with c-Abl antibody and western blotted. Phosphorylation of c-Abl protein was determined with PY20 antibody followed by reblotting with c-Abl antibody (upper panel). Phosphorylated proteins in total cell lysate were also immunoprecipitated with phosphotyrosine antibody PY20, and the c-Abl protein amount in the IPs was detected using c-Abl antibody (lower panel). This is representative of three experiments. B. Unstimulated cells were lyzed and immunoprecipitated with CrkII antibody, samples were western blotted with phosphotyrosine antibody followed by CrkII antibody, Densitometry analysis was performed with AIDA advance image data analyser software, and the phosphorylation of c-Abl and CrkII were calculated and are indicated under the blot. Similar results were observed in three experiments.

7.2.3 C-Abl kinase activity in LARΔP is higher than in WT

In vitro kinase assays were carried out to confirm that the c-Abl kinase activity was higher in LARΔP cells. Cells were transiently transfected with the DNA indicated in Figure 15. After 48 hours, the cells were lysed and c-Abl was immunoprecipitated. The immunoprecipitates were divided into two parts; one part was subjected to an *in vitro* kinase assay, in the presence of solvent (DMSO) or 100 μM of the c-Abl inhibitor AG957, using enolase as the substrate. The other part was separated by SDS-PAGE, and c-Abl was detected by immunoblotting. The GFP protein amount within the total cell lysate was analysed, using Alix as the loading control. Results show higher c-Abl kinase activity in serum starved LARΔP cells compared to the WT. Transfecting LARΔP cells with full length LAR construct reduced the c-Abl kinase activity demonstrating that LAR activity suppresses c-Abl activity in MEFs. The kinase activity measured is due to c-Abl, since the addition of c-Abl inhibitor AG957 effectively reduced the activity measured in the samples (Figure 15).

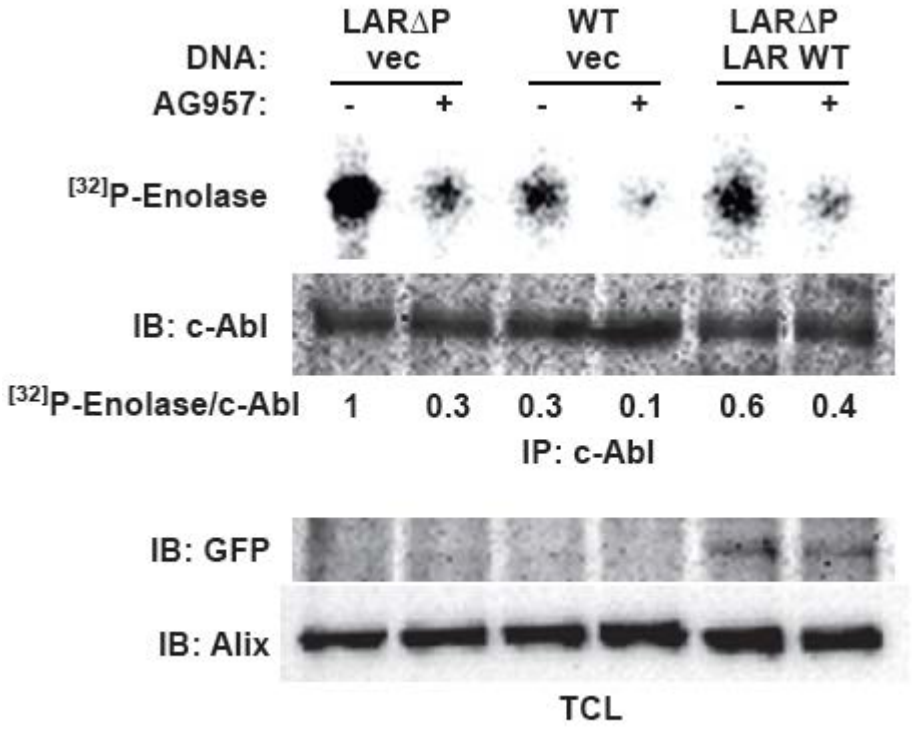
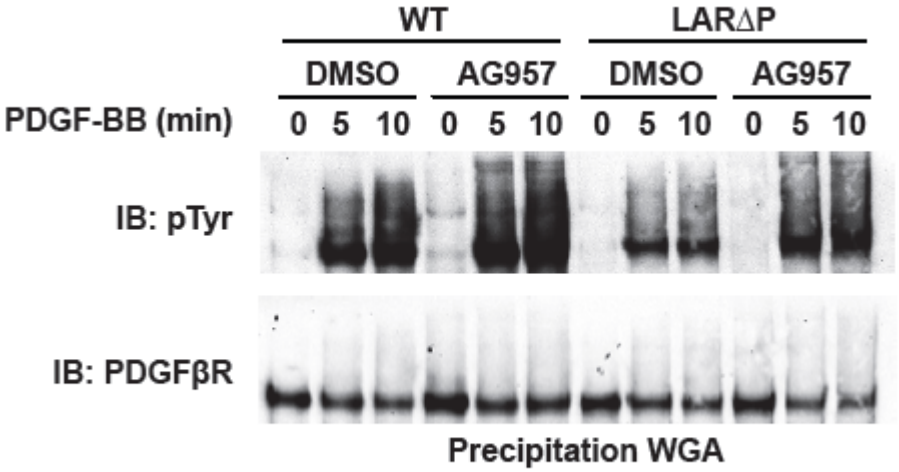


Figure 15 Higher c-Abl kinase activity in LARΔP cells compared with WT cells

WT and LARΔP cells were transiently transfected with the indicated DNA. After 48 h, the cells were lysed and c-Abl was immunoprecipitated. The immunoprecipitates were divided in two; one part was subjected to *in vitro* kinase assay in the presence of solvent (DMSO) or 100 μM of the c-Abl inhibitor AG957 using enolase as the substrate. The other part was separated by SDS-PAGE, and the immunoprecipitated c-Abl was detected by immunoblotting. The GFP protein amount within the total cell lysate was analysed, using Alix as loading control. This is representative of three experiments.

7.2.4 C-Abl inhibition restores PDGFβR phosphorylation in LARΔP cells

From the above experiments, we noticed that deletion of the LAR phosphatase domains resulted in a clear increase of c-Abl kinase activity. To investigate the possibility that the increased c-Abl activity reduces PDGFβR phosphorylation, I analysed the effect of the specific c-Abl inhibitor AG957 on PDGFβR phosphorylation. Cells were starved and treated with 10μM AG957 or 0.1% DMSO for two hours followed by stimulation with 20 ng/ml PDGF-BB for the indicated time periods. There was a considerable increase in PDGFβR phosphorylation after c-Abl inhibition with AG957 in both cell types. Incubation of LARΔP with AG957 brings the PDGFβR phosphorylation to a comparable level with that in the untreated WT (Figure 16). This suggests that the increased c-Abl kinase activity is the reason for the reduced PDGFβR phosphorylation in LARΔP cells. By reducing c-Abl kinase activity, PDGFβR phosphorylation can be restored to a similar level to that in WT cells, demonstrating that c-Abl is one of the intermediate players connecting LAR and PDGFβR.



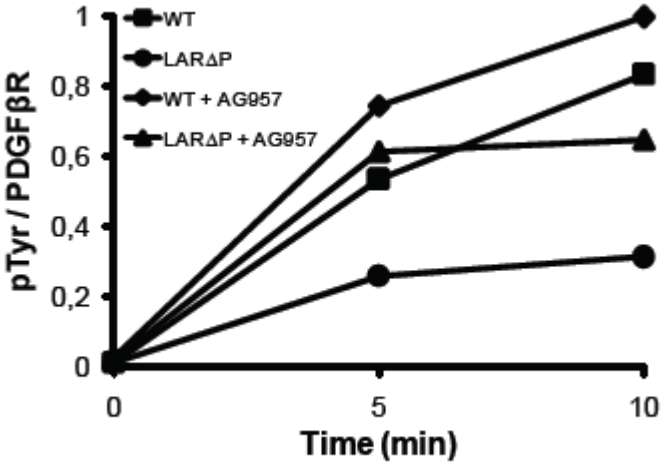


Figure 16 c-Abl inhibition restores PDGFβR phosphorylation in LARΔP cells

Cells were starved for 16 hours, and then treated with 10μM AG957 or 0.1% solvent DMSO for two hours. Cells were then stimulated with 20 ng/ml PDGF-BB for the indicated time periods. Cells were lyzed, and receptors were precipitated with WGA agarose beads and western blotted. Phosphorylation of PDGFβR was visualized by blotting with phosphotyrosine antibody PY99 followed by reblotting with PDGFβR antibody as loading control. Densitometry analysis was performed with AIDA advance image data analyser software, and phosphorylation of PDGFβR was plotted. These data are representative of three experiments.

7.2.5 Suppression of c-Abl expression restores PDGFβR phosphorylation in LARΔP cells

To confirm that inhibition of c-Abl restores the PDGFβR phosphorylation in LARΔP cells, c-Abl siRNA was used to knock down c-Abl expression, and the PDGFβR phosphorylation was analysed. Cells were transfected with control siRNA or c-Abl siRNA and then stimulated with 20 ng/ml PDGF-BB for the indicated time periods and the PDGF β-receptor was precipitated using WGA agarose. C-Abl downregulation in total cell lysates was confirmed by immunoblotting with c-Abl antibody, followed by immunoblotting with Alix antiserum as a loading control. C-Abl expression level was decreased after the treatment of c-Abl siRNA in both of the two cell types. Knocking down c-Abl considerably increased PDGFβR phosphorylation in LARΔP cells upon ligand stimulation (Figure 17), confirming that c-Abl kinase inhibition restores PDGFβR activity in LARΔP cells.

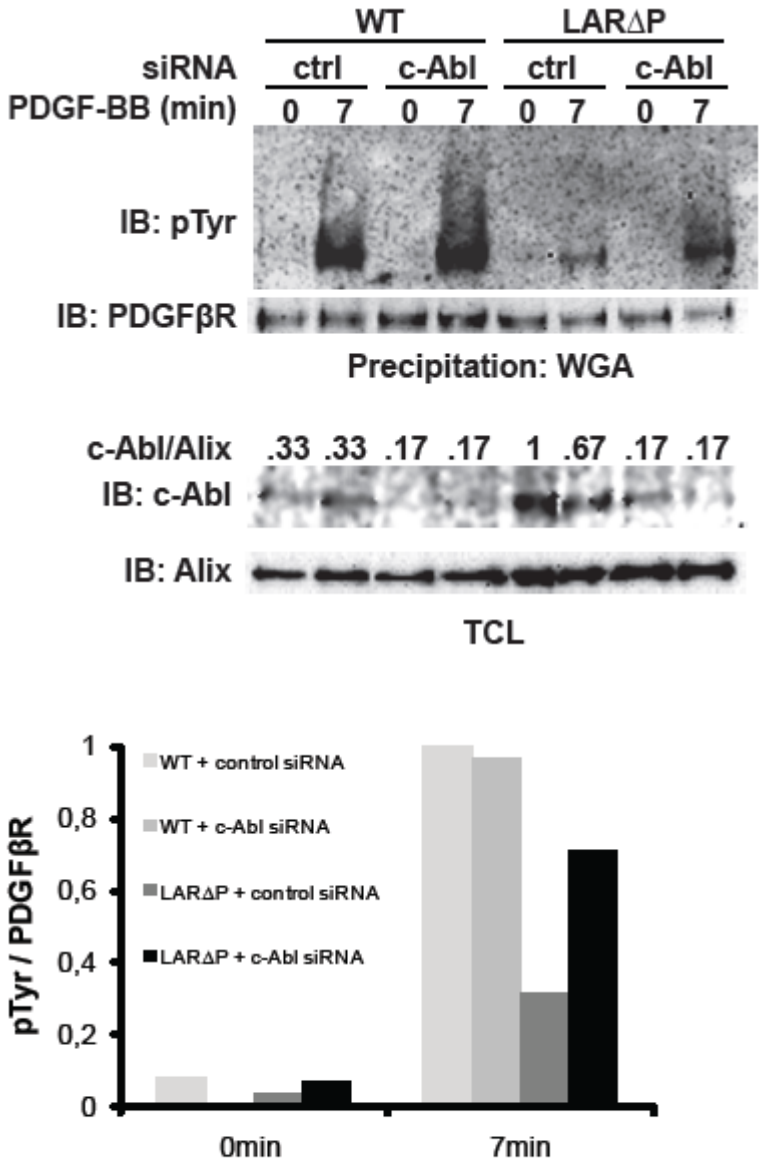


Figure 17 Knocking down c-Abl protein level restores PDGFβR phosphorylation in LARΔP cells.

Cells were transfected with control siRNA or c-Abl siRNA. The cells were stimulated with 20 ng/ml PDGF-BB for the indicated time periods and the PDGF β-receptor was precipitated using WGA agarose. The precipitated receptors were separated by SDS-PAGE and transferred to nitrocellulose membranes, and the amount of receptor phosphorylation was determined by immunoblotting using the PY99 phosphotyrosine antibody, followed by immunoblotting with PDGF β-receptor antibody. C-Abl knock down in total cell lysates was confirmed by immunoblotting with c-Abl antibody, followed by immunoblotting with Alix antiserum as a loading control. Shown are densitometric analyses of c-Abl protein compared to Alix protein. The relative PDGFβR phosphorylation is plotted. These data are representative of three experiments

7.3 Discussion

C-Abl non receptor tyrosine kinase shuttles freely between the nucleus and cytoplasm due to a nuclear export sequence (NES) at its C terminus and the presence of three nuclear localization signals (NLS). In the nucleus, c-Abl modulates cellular responses induced by DNA damage, and was therefore suggested to play a role in cell growth inhibition and apoptosis promotion (Pendergast 2002; Sirvent *et al.* 2008). In the cytoplasm, c-Abl plays important role in morphogenesis and actin filament dynamics (Woodring *et al.* 2003). Cytoplasmic c-Abl also has a role in signalling induced by extracellular stimulation. Integrin engagement has been reported to activate the cytoplasmic pool of c-Abl (Lewis *et al.* 1996). C-Abl in the cytoplasm is reported to be a downstream target of activated PDGF receptor and Src tyrosine kinase, and its activity is increased upon PDGF stimulation and dependent on the Src kinase activation (Plattner *et al.* 1999).

In my experiments, I found that the c-Abl level in the cytoplasm was clearly higher in the LARΔP cells compared to WT, while there were similar levels of c-Abl protein in the whole cell (Figure 13). C-Abl protein seems to be exported from the nucleus into the cytoplasm in the absence of LAR phosphatase activity. The regulation of nuclear importation and exportation of c-Abl are not yet well characterized. There has been a report showing that there is export of c-Abl from nucleus to cytoplasm and recruitment of c-Abl to early focal contacts upon cell adhesion (Lewis *et al.* 1996). C-Abl localization was later reported to be regulated by P300 histone

acetyltransferase (di Bari *et al.* 2006). P300 acetylates c-Abl on Lys730 located on its second NLS and drives c-Abl to accumulate in the cytoplasm. It is possible that the deletion of LAR phosphatase activity enhances the ability of cell adhesion mediated recruitment of c-Abl to the cytoplasm, or alternatively enhances the activity of P300. At present, it is not clear if these events are causatively linked, or if other yet to be identified mechanisms regulate c-Abl localization. The LAR phosphatase domain deletion leads to the increased tyrosine phosphorylation on c-Abl which positively regulates its kinase activity. In addition, it causes a shift of c-Abl protein from the nucleus to the cytoplasm in LARΔP cells, which plays a part in increasing c-Abl activity in cytoplasm.

There have not been any reports showing that increased c-Abl cytoplasmic activity inhibits PDGFβR activity before. One explanation for this effect would be that c-Abl activates serine threonine kinases which in turn phosphorylate PDGFβR. It was reported that serine phosphorylation of PDGFβR is an important mechanism for receptor signalling attenuation by inhibiting tyrosine kinase activity. Several serine/threonine kinases are downstream substrates of c-Abl including PKCδ, GRK2 and GRK5 (Yuan *et al.* 1998; Wu *et al.* 2005; Wu *et al.* 2006). The overactivation of c-Abl in LARΔP may lead to the higher activity of its downstream serine threonine kinases that could directly or indirectly lead to the serine phosphorylation on PDGFβR and downregulation of its activity.

On the other hand, it is possible that c-Abl could suppress integrin signalling, which is known to potentiate PDGFβR signalling. LAR and c-Abl were shown to have opposite effects in regulating actin filament development. Protein enabled (Ena) is known to promote actin polymerization, and is a substrate of LAR and c-Abl. Dephosphorylation of Ena by LAR increases its activity, while tyrosine phosphorylation of Ena by c-Abl inhibits its function (Wills *et al.* 1999). When LAR phosphatase activity is lost in LARΔP cells, the inhibitory effect of LAR on c-Abl and stimulatory effect on Ena would be gone. This would lead to the disruption of normal cytoskeletal regulation inside the cell, which could disrupt normal cell adhesion. The integrin signalling which assists ligand stimulated PDGF receptor phosphorylation would be expected to be disturbed when cells cannot adhere properly (Sundberg and Rubin 1996; DeMali *et al.* 1999).

To confirm the nuclear and cytoplasmic localization of c-Abl in the two cell types, cell fractionation experiment can be carried out to isolate nuclear fraction and cytoplasmic fraction of the cell. Each fraction could then be analysed for its c-Abl content using western blot analysis.

CHAPTER 8 C-ABL DOWNSTREAM PROTEIN KINASE C DELTA AND NHERF2 ARE INVOLVED IN LAR RELATED REDUCTION OF PDGFβR PHOSPHORYLATION IN LARΔP

8.1 Introduction

After identifying c-Abl as a mediator connecting LAR and PDGFβR phosphorylation, I continued to investigate the mechanisms of how c-Abl overactivation led to decreased PDGFβR signalling. It has been shown that c-Abl can directly phosphorylate PDGFβR (Plattner *et al.* 2004), but no report has demonstrated that c-Abl leads to reduction of PDGFR phosphorylation. The higher c-Abl kinase activity and lower PDGFβR activity in LARΔP cells suggests that the overactivated c-Abl either activates negative regulatory machinery for PDGFβR and/or inactivate proteins which potentiate PDGFβR signalling. One of the general mechanisms for tyrosine kinase receptor desensitization is the serine phosphorylation of the receptors. It has been shown that serine phosphorylation of PDGFβR reduces its kinase activity and tyrosine autophosphorylation (Bioukar *et al.* 1999; Freedman *et al.* 2002; Cai *et al.* 2009). Studies showed that PDGFβR serine phosphorylation was increased after 5 minutes of PDGF stimulation and remained elevated after 30 minutes. The serine

threonine kinase casein kinase CKI-γ2 is activated and translocated to PDGFβR where it then phosphorylates the receptors on serine residues. This negatively regulates PDGFβR tyrosine kinase activity leading to the receptor inactivation (Bioukar *et al.* 1999). G protein coupled receptor kinases (GRKs) were first identified to be the serine threonine kinases that phosphorylate activated G protein coupled receptor (GPCR) on its intracellular domain after their associated G protein has been released. The phosphorylated serine threonine residues on GPCR serve as binding sites for arresting proteins which prevent the reassociation of G proteins and target the receptor to internalization (Singh *et al.* 2008). Later, certain GRKs that were shown to serine phosphorylate the PDGF receptor, also were shown to promote receptor inactivation. Two members of the GRK family, GRK2 and GRK5 were shown to be activated by PDGFβR mediated tyrosine phosphorylation, and serve in a negative feedback loop by phosphorylating PDGFβR serine residues and thereby reducing the receptor kinase activity (Freedman *et al.* 2002; Cai *et al.* 2009). It was later shown that overexpression of GRK2 reduced the association of PDGFβR with Nherf1 by 60%, and at the same time reduced PDGFR autophophorylation and increased receptor serine phosphorylation. Nherf1 was reported to bind to the C terminus of PDGFβR through its PDZ domain. The ability of Nherf1 to oligomerize makes it possible that Nherf1 may serve as an adaptor protein that facilitates PDGFβR dimerization and activation (Voltz *et al.* 2001). Nherf1 recognizes the PDGFβR C terminus motif DSFL as a binding site for its PDZ domain, and mutation of leucine residue 1106 to alanine on PDGFβR prevents Nherf1 from binding. GRK2 was shown

to phosphorylate S1104 on PDGFβR where the Nherf1 binding motif is located, and this led to Nherf1 dissociation and PDGFβR desensitization (Hildreth *et al.* 2004).

The activities of both GRK2 and GRK5 have been shown to be regulated by the serine threonine kinase protein kinase C delta (PKCδ). PKCδ phosphorylates both kinases, this activates GRK2 but leads to the inactivation of GRK5 (Pronin and Benovic 1997; Krasel *et al.* 2001). C-Abl tyrosine phosphorylates and activates PKCδ, thus, in LARΔP cells, it is possible that the overactivated c-Abl activates PKCδ, which in turn activates GRK2. GRK2 then phosphorylates the serine residue S1104 on the PDGFβR C terminus and leads to the dissociation of Nherf1, therefore preventing receptor dimerization and full activation. In this chapter, I investigated the involvement of PKCδ and Nherf family members, Nherf1 and Nherf2, in PDGFβR phosphorylation and dimerization downstream of c-Abl.

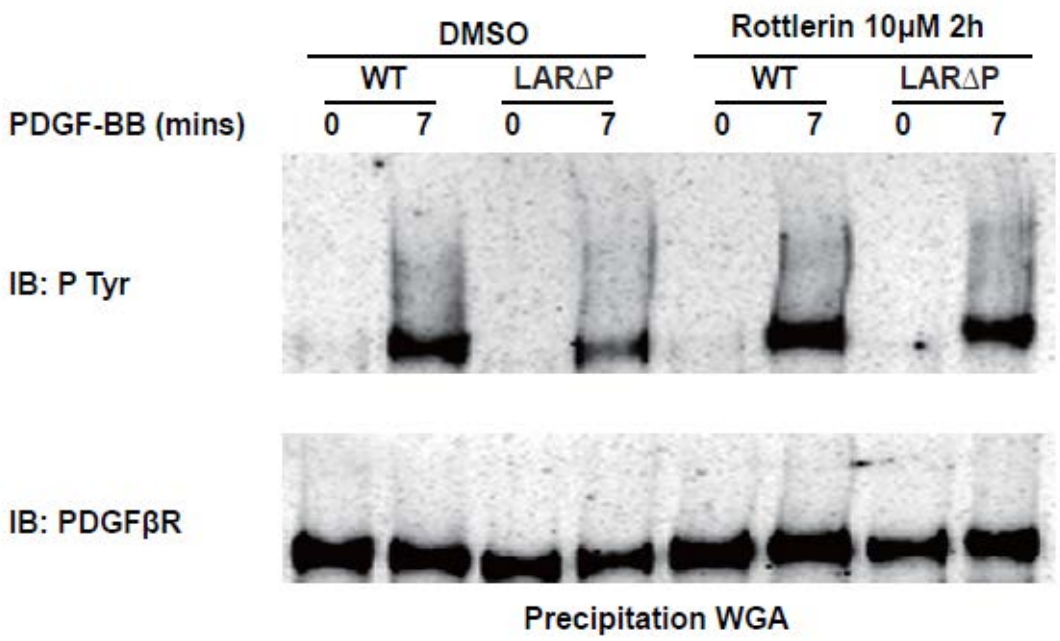
8.2 Results

8.2.1 PKCδ inhibition increases PDGFβR phosphorylation in LARΔP cells

To analyse whether the serine threonine kinase PKCδ activated downstream of c-Abl is involved in the reduced PDGFβR activation in LARΔP cells, cells were incubated with the PKCδ inhibitor Rottlerin. Serum starved cells were incubated with 10μM Rottlerin or vehicle (DMSO) for 2 hours. Cells were then stimulated, lyzed, and PDGFβR phosphorylation was determined. There was increased PDGFβR

phosphorylation in LARΔP cells after the Rottlerin treatment supporting the notion that PKCδ promotes inactivation of PDGFβR in these cells (Figure 18A). As a c-Abl downstream target, PKCδ is expected to be overactivated in LARΔP cells. It is known that after its activation, PKC is generally degraded for desensitization (Kang *et al.* 2000). If the PKCδ is overactivated downstream c-Abl in LARΔP cells, one would expect the PKCδ level to be lower in LARΔP cells due to degradation. To confirm this, experiments were carried out to compare the PKCδ levels in the two cell types. This experiment was carried out for two times. There were lower amounts of PKCδ in LARΔP cells compared to WT cells, suggesting that PKCδ is more activated in LARΔP cells (Figure 18B).

A



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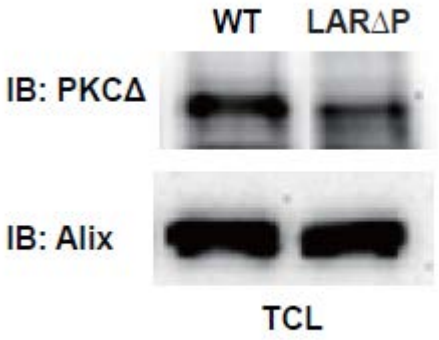

Figure 18 PKCΔ inhibition restores PDGFβR phosphorylation in LARΔP cells.

A. WT and LARΔP cells were starved for 16 hours before they were incubated with 10μM PKCδ inhibitor Rottlerin or the carrying solvent DMSO for 2 hours. Cells were then stimulated, lyzed, and PDGFβR were precipitated with WGA agarose. Receptor phosphorylation was determined with phosphotyrosine antibody PY99 followed by reblotting with PDGFβR antibody. This experiment was carried out twice with similar results. B. Cells were lyzed, and total cell lysates were western blotted using PKCδ protein amount was determined using PKCδ antibody followed by reblotting with Alix antibody as the loading control. This was repeated twice with similar results.

8.2.2 More Nherf2 associates with PDGFβR in LARΔP cells than WT

Thus, in LARΔP cells, it appears as if the increased c-Abl activation increases the PKCδ activity, which may result in increased activity of GRK2 and subsequent phosphorylation of the serine residue on the PDGFβR C terminus that causes Nherf1 dissociation from the receptor. I tried to co-immunoprecipitate Nherf1 with PDGFβR in my MEFs, but these experiments were not successful (data not shown). However, the other member in the Nherf family, Nherf2, is also known to bind to PDGFβR, but it is not known if this would facilitate PDGFβR dimerization and potentiate receptor autophosphorylation. To determine if Nherf2 regulates PDGFβR activity, I first performed experiments to analyse the amount of Nherf2 associated with PDGFβR. Cells were starved and stimulated with 20 ng/ml PDGF-BB. Cells were lyzed and immunoprecipitated with PDGFβR antibody, and Nherf 2 associated with PDGFβR was detected. Nherf2 associated with PDGFβR before stimulation in LARΔP cells, while no detectable Nherf2 was seen in association with the receptor in the WT cells. Nherf2 started to associate with the receptor in WT cells after stimulation, although the amount of associated Nherf2 in WT cells was lower than in LARΔP cells after stimulation (Figure 19).

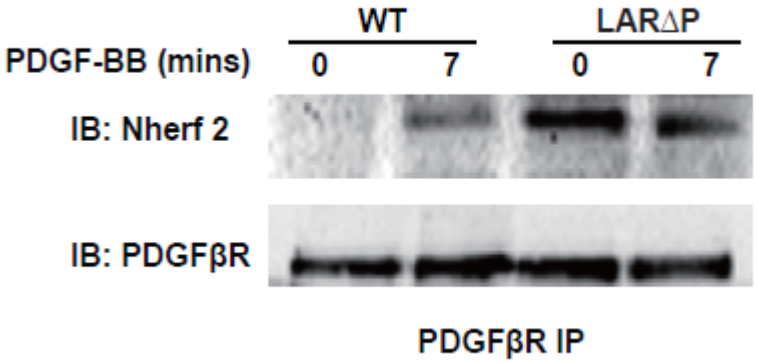

Figure 19 Nherf 2 association with PDGFβR

Cells were starved for 16 hours and stimulated with 20 ng/ml PDGF-BB for the indicated time periods. Cells were lyzed and immunoprecipitated with PDGFβR antibody, Nherf 2 associated with PDGFβR was shown by blotting with Nherf 2 antibody followed by reblotting with PDGFβR antibody. This experiment was repeated three times with similar results.

8.2.3 Impaired PDGFβR dimerization and receptor binding proteins interaction in LARΔP cells

Nherf1 association with the PDGF receptor has been shown to assist PDGF receptor dimerization and increase receptor autophosphorylation. Although Nherf2 was shown to bind to PDGF receptor (Maudsley *et al.* 2000; Lau and Hall 2001), it is not known if it affects PDGF receptor kinase activity. Since Nherf2 rather than Nherf1 was associated with PDGFβR in the MEF cells I used (data not shown), the dimerization of PDGFβR in these cells was analysed to determine if Nherf2 association affects receptor dimerization. Cells were starved overnight and stimulated with 20 ng/ml PDGF-BB. Cells were collected and centrifuged at 2415g for 10 minutes, and 3mg/ml cross linker BS_3, 1% TritonX100 in PBS was added to the pellet. After 15 minutes

incubation, the mixture was centrifuged at 11337g for 15 minutes, and sample buffer was added to the supernatant and boiled at 95°C for 5 minutes. Samples were separated on SDS-PAGE and analysed. PDGFβR was visualized with PDGFβR antibody followed by reblotting with Nherf 2 antibody. In WT cells, the PDGFβR largely existed as 190kDa monomers before stimulation (Figure 20 upper panel). After 7 minutes of stimulation with PDGF-BB, the PDGFβR shifted almost completely from being a 190kDa monomers to a higher molecular weight band. This demonstrates the presence of PDGFβR monomer before stimulation and the formation of PDGFβR dimers, possibly with associated signalling proteins after ligand stimulation in WT cells. The stimulation on ice sample showed an intermediate state where a portion of the PDGFβRs were still not stimulated and remained in the monomeric form as a 190kDa band, while the remainder of the population had dimerized. In unstimulated LARΔP cells, PDGFβR was detected as smeared bands of a higher molecular weight instead of just the sharp band at 190kDa that represents the monomeric form. This suggests that only a subpopulation of PDGFβR exists as monomers in LARΔP in the absence of ligand. This suggests that there might be several different types of proteins, or oligomers of the same protein, associating with the PDGFβR in this cell type even before stimulation. In the stimulated samples from LARΔP cells, there are four bands that are visible including the PDGFβR monomers and the higher molecular weight band observed in WT cells which presumably represents active dimers. However the constitution of the other two bands is unknown (Figure 20 upper panel).

For the Nherf2 blot, results showed no Nherf2 signal in the WT unstimulated samples, and there was very faint signal of Nherf2 in the WT sample stimulated for 7 minutes at around the dimer position (Figure 20 lower panel). When the WT samples were stimulated for 60 minutes on ice, there was more Nherf2 association. In LARΔP cells, the unstimulated and stimulated samples showed a sharp Nherf2 band at approximately 310kDa, and some smeary bands with higher molecular weights, stimulation on ice for 60 minutes gave a much denser signal in these two regions (Figure 20 lower panel). Nherf2 seems to be colocalized with PDGFβR at the higher molecular weight positions but not with the PDGFβR monomer at 190kDa in both of the cell types. However, the amount of Nherf2 associated was much higher in LARΔP cells. All the PDGFβR bands detected at higher molecular weights have corresponding Nherf2 bands suggesting that the complexes formed at approximately 310kDa and the higher molecular weights, exclusively observed in LARΔP cells, were the complexes of PDGFβR and Nherf2 oligomers.

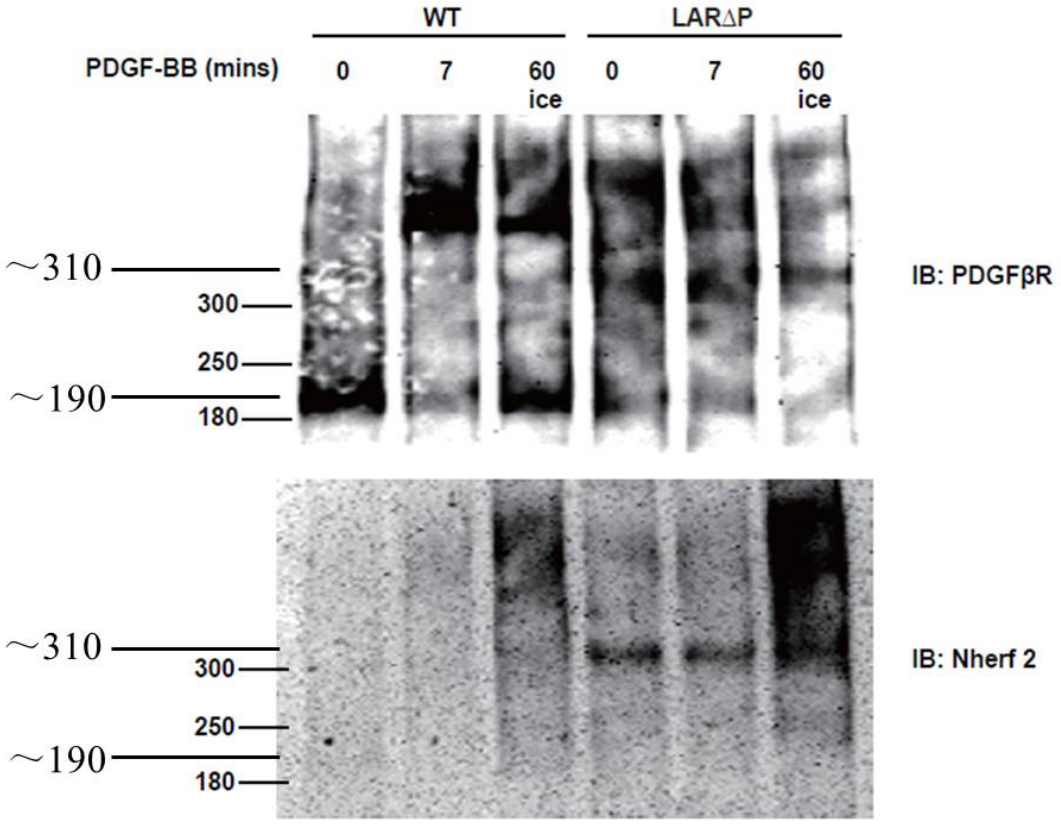

Figure 20 LARΔP cells show defective PDGFβR dimerization and more PDGFβR-Nherf2 interaction

Cells were starved overnight for 16 hours and stimulated with 20 ng/ml PDGF-BB for the time periods indicated. Cells were collected and centrifuged at 2415g for 10 minutes. The pellets collected were added to 3mg/ml cross linker BS_3 in 1% TritonX100. After 15 minutes incubation, the mixture was centrifuged at 11337g for 15 minutes, and sample buffer was added to the supernatant and boiled at 95°C for 5 minutes. Sample was run in SDS-PAGE and analysed. PDGFβR was visualized with PDGFβR antibody followed by reblotting with Nherf 2 antibody. This experiment was carried out for several times. The cleanest blot is shown.

8.3 Discussion

In this part of the study, it was found that PKCδ, a downstream target of c-Abl was at a decreased level in LARΔP cells, which suggests that PKCδ could have been degraded following its overactivation due to high c-Abl activity (Figure 18B). PKCδ inhibition by Rottlerin restored PDGFβR phosphorylation in LARΔP cells indicating that the overactivation of PKCδ by c-Abl leads to the reduction of PDGFβR activation (Figure 18A). However, it has not been reported before that PKC causes reduction in PDGF receptor phosphorylation.

Serine phosphorylation of PDGF receptor has long been known as one mechanism to desensitize the activated PDGF receptor. Serine threonine kinases CKI-γ2, GRK2 and GRK5 have been shown to directly phosphorylate PDGF receptor on serine residues leading to receptor inactivation (Pronin and Benovic 1997; Bioukar *et al.* 1999; Krasel *et al.* 2001). It is known from the literature that PKCδ plays a role in activating GRK2 via phosphorylation (Krasel *et al.* 2001). Thus, it would be possible that PKCδ activated GRK2 phosphorylates serine residues at the C terminus of PDGFβR in the absence of ligand stimulation. This would prevent the association of Nherf1 with PDGFβR. Without Nherf1, PDGFβR cannot dimerize properly. This would prevent the normal activation and autophosphorylation of PDGFβR.

Many cellular proteins are known to interact with the two tandem PDZ domains in Nherf1 and its homologue Nherf2. Previous reports suggested that where the two

Nherf proteins are present together, they generally perform overlapping function to regulate transmembrane receptors, transporters and other proteins located in or near the plasma membrane (Voltz *et al.* 2001). Nherf1 was reported to bind to the C terminus of PDGFβR with its first PDZ domain. The binding of Nherf1 to PDGFβR increases its oligomerization ability, therefore leading to the formation of Nherf1 oligomers. These Nherf1 oligomers serve as adaptors that bring PDGF receptors in close proximity which facilitates their dimerization and kinase activation (Maudsley *et al.* 2000). In our study, we analysed the association of Nherf1 to PDGFβR in the MEF cells with immunoprecipitation of PDGFβR. Experiments found no detectable Nherf1 associating with the PDGFβR in the MEF cells. However we cannot rule out that the Nherf1 antibody we use may not be sensitive enough to detect the Nherf1 signal associating with the PDGFβR.

Nherf2 was found to avidly associate with PDGFβR in LARΔP cells even before ligand stimulation, but not in the WT cells (Figure 19). It is important to compare expression levels of Nherf2 in the two cell types to determine whether higher levels of Nherf2 in LARΔP cells might contribute to its increased association with PDGFβR in this cell type. Nherf2 was reported to bind to the C terminus of PDGF receptor at least as well as Nherf1 when the two are directly compared in single-concentration overlay experiments (Maudsley *et al.* 2000). However, Nherf2 was not shown to assist the PDGF receptor dimerization and activation like Nherf1. One of the reasons that Nherf1 can facilitate receptor activation could be that Nherf1 oligomerization is

highly regulated. The PDZ domain of Nherf1 oligomerizes with fairly low affinity. The Nherf1 affinity of oligomerization is enhanced by more than 10 fold after binding to a ligand such as the C terminus of β2 adrenergic receptor or PDGF receptor. This property allows Nherf1 to form oligomers which act as a scaffold only when appropriately associated with the proteins it acts to regulate. In comparison to Nherf1, oligomerization is a constitutive property of Nherf2. The PDZ domains of Nherf2 show robust hetero- and homo-oligomerization in the absence of any other associated proteins (Vaillancourt *et al.* 1995). This constitutive robust oligomerization property of Nherf2 may allow it to form large multimers. The function and regulation of Nherf2 has not been extensively studied, probably because of the assumption that its properties and roles are similar to those of Nherf1. However in our study, we found that the association of Nherf2 correlated with the reduced PDGF receptor autophosphorylation and signalling.

One possibility is that in WT cells, small amounts of Nherf1 protein associate with PDGFβR monomer. This association increases the Nherf1 oligomerization ability allowing them to bind other Nherf1 associated PDGFβR monomers. This process would be facilitated by ligand bridging and would help promote ligand induced PDGFβR activation and desensitization. Nherf1 not bound to any PDGF receptor monomers would have a much lower capacity to bind to other Nherf1, therefore a lower chance of forming oligomers with Nherf1 already bound to the receptor monomer, and thus not disturbing the PDGF receptor dimer formation. Since Nherf1

is associating with the PDGFβR C terminus, no space is left for Nherf2 to bind any more. However, in LARΔP cells, after the phosphorylation of serine residues on PDGFβR C terminus by PKCδ activated GRK2, a change in the structure of the C terminus might prevent the binding of Nherf1. On the other hand, this structural change may potentiate the binding of Nherf2 which is also known to bind to the C terminus of PDGF receptor and has a similar PDZ domain structure to Nherf1. Nherf1 dissociation would also leave the C terminal space clear for Nherf2 to bind. Since the Nherf2 PDZ domain can constitutively form robust oligomers without binding to any ligand, it is possible that Nherf2 could form multimers around the PDGF receptor monomers thereby preventing the normal ligand induced dimerization of the receptors. This would also explain the large amount of Nherf2 present in the PDGFβR immunoprecipitation samples and crosslinked PDGFβR complexes in LARΔP both before and after ligand stimulation (Figure 19 and Figure 20). The smear of PDGFβR blot in LARΔP in the crosslinking experiments could be caused by the association of different amounts of Nherf2 linked to the receptor (Figure 20).

Although it has not been reported before, the findings in this chapter open up the possibility that Nherf2 binding could serve as a desensitization mechanism of PDGF receptor after its activation. In WT cells, I observed no detectable Nherf2 associated with PDGF receptor before stimulation, while a small amount of Nherf2 associated with the receptor after 7 minutes stimulation. Thus, Nherf2 may normally serve as a component that is recruited to the receptor after its activation to form an insulator to

disrupt receptor activity and facilitate receptor inactivation or internalization. This notion is supported by the findings in Chapter 7 where I observed increased PDGFβR activation in WT cells after c-Abl inhibition (Figure 16, 17).

In vitro kinase assays could be used to measure the kinase activity of PKCδ in both cell types to confirm that PKCδ is over-activated downstream of c-Abl in LARΔP cells. PKCδ siRNA could be used to knock down PKCδ expression level in LARΔP cells to confirm if PDGFβR tyrosine phosphorylation can be restored upon PKCδ inhibition.

The association of Nherf1 and Nherf2 with PDGFβR could also be investigated using immunofluorescent microscopy colocalization experiments. Such studies could reinforce the finding from the immunoprecipitation experiment (Figure 19) and cross linking experiment (Figure 20).

CHAPTER 9 DISCUSSION

PDGF is known to be a potent stimulator of growth and motility of different types of cells. Ligand stimulation of the PDGF receptor has important roles in embryonic development and angiogenesis. In adults, PDGFR activation induces wound healing and regulates interstitial fluid pressure. PDGFR is implicated in the pathogenesis of a number of tumour types. Tumour growth can be promoted by PDGF through autocrine stimulation of malignant cells, overexpression and overactivation of PDGFRs, and stimulation of angiogenesis and recruitment of cancer-associated fibroblasts within the tumour (Heldin *et al.* 1998; Board and Jayson 2005). PDGF drives pathological mesenchymal responses in vascular disorders such as atherosclerosis and retinal diseases (Boucher and Gotthardt 2004; Kernt *et al.* 2010). Abnormal PDGF stimulation also leads to fibrotic diseases including pulmonary fibrosis and liver cirrhosis (Martin *et al.* 2013; McGowan and McCoy 2013). Understanding the mechanism of endogenous potentiation and desensitization of PDGFR signalling would provide us possible therapeutic targets in treating these different diseases and tumour.

Several endogenous intracellular mechanisms potentiate PDGFR signalling; integrin signalling assists PDGF receptor activation (DeMali *et al.* 1999), association of the adaptor protein Nherf1 to PDGF receptor facilitates receptor dimerization and kinase

activation (Maudsley *et al.* 2000), and the non-receptor tyrosine kinase Src phosphorylates PDGF receptor which increases its signalling (Hansen *et al.* 1997). On the other hand, PDGF receptor is dephosphorylated and inactivated by several different protein tyrosine phosphatases (PTPs) (Persson *et al.* 2004; Karlsson *et al.* 2006). Serine phosphorylation of PDGF receptor induced by serine threonine kinases such as CKI-γ2, GRK2 and GRK5, reduces PDGFR kinase activity and tyrosine phosphorylation (Bioukar *et al.* 1999; Wu *et al.* 2005; Wu *et al.* 2006). Activated PDGF receptor is downregulated by internalization and degradation to prevent uncontrolled receptor signalling. In this thesis, I have described a novel mechanism of PDGFβR desensitization.

The data presented in this thesis suggest a mechanism where deletion of the phosphatase domains of LAR removes LAR mediated suppression of c-Abl leading to c-Abl overactivation. C-Abl then phosphorylates and activates downstream PKCδ which in turn serine phosphorylates and activates GRK2. In the absence of the LAR phosphatase domains, the constitutively active c-Abl results in the continuous activation and then degradation of PKCδ. This would reduce the inhibitory effect of PKCδ on histone acetyltransferase P300. Acetylation of c-Abl by P300 on its nuclear localization signal location would lead to the accumulation of c-Abl in cytoplasm and further enhance cytoplasmic c-Abl activity. PKCδ activated GRK2 phosphorylates a serine residue on the PDGFβR C terminus and prevents the association of Nherf1. Without the facilitation of Nherf1, PDGFβR cannot dimerize and be activated

properly upon ligand stimulation. Nherf1 dissociation from the receptor C terminus leaves the space clear for Nherf2 to bind. Nherf2 robustly oligomerizes and forms large complexes around PDGFβR monomers prior to stimulation and upon ligand stimulation prevents receptor dimerization, activation, autophosphorylatin and downstream signalling (Figure 21).

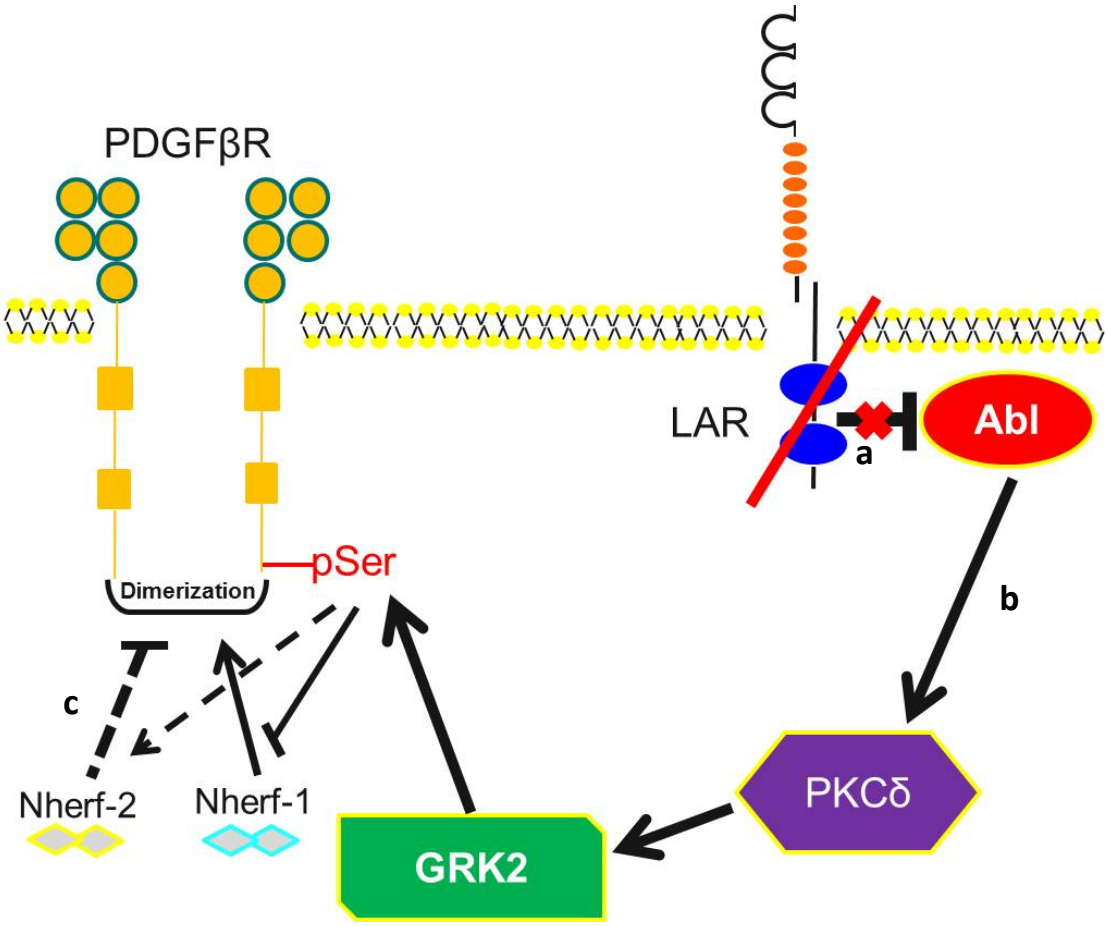

Figure 21 Suggested mechanism of LAR deletion mediated reduction in PDGFβR autophosphorylation.

The full lines indicate that the relationships between proteins were shown by previous publications. The dash lines indicate the proposed relationships between proteins. Arrowed headed lines mean activation. Flat headed lines mean inhibition. LAR knock out mediated reduction of PDGFβR autophosphorylation is surported by Figure 1, 2, 3 and 4. a, Ihibition of c-Abl by LAR is shown by Figure 13, 14 and 15. C-Abl mediated reduction of PDGFβR tyrosine phosphorylation is surported by Figure 16 and 17. Supressesion of PDGFβR autophosphorylation by PKCδ activation is surported by Figure 18A. b, PKCδ activation by c-Abl in the absence of LAR is surportted by Figure 18B. c, Nherf2 mediated reduction of PDGFβR dimerization is shown by Figure 19 and 20.

This project was continued by a Masters student Daniel Blakeway in our group under my supervision. We used siRNA for PKCδ, Nherf1 and Nherf2 to further analyse the effect of these proteins on PDGFβR phosphorylation and its downstream ERK signalling in LARΔP cells. His results showed reduction of PDGFβR and downstream ERK phosphorylation after transfection with Nherf1 siRNA and a clear increase of receptor and ERK phosphorylation after PKCδ or Nherf2 knock down. This supports the hypothesis presented in this thesis and suggests that Nherf1 and Nherf2 play opposite roles in the regulation of PDGF receptor phosphorylation and downstream signalling: Nherf1 association with PDGF receptor potentiates receptor phosphorylation and Nherf2 association impairs receptor activation and reduces its phosphorylation. Thus PKCδ activity, Nherf1 and Nherf2 are probably involved in the the regulation of PDGFβR activity.

It is known that c-Abl is activated by Src upon PDGFR stimulation (Plattner *et al.* 1999). This makes it possible that c-Abl activation downstream of PDGFR plays a role in initiating a negative feedback loop via activation of PKCδ and GRK2 to increase the binding of Nherf2 to PDGFR, thereby attenuating PDGFR signalling. This is also supported by the increased amount of Nherf2 associating with PDGFβR and increased c-Abl phosphorylation after ligand stimulation in WT cells (Figure19, Figure20 and Figure14A), and increased PDGFβR phosphorylation after treatment with c-Abl inhibitor AG957 and c-Abl siRNA in the stimulated WT cells (Figure16, Figure17).

In summary, I have demonstrated that LAR activity controls PDGFβR activity. The data presented in this thesis show that deletion of LAR activity potentiates cytoplasmic c-Abl activity. C-Abl overactivation continuously activates PKCδ and leads to PKCδ degradation, robust Nherf2 oligomerization and association with PDGFβR and reduction in PDGFβR activity and downstream signalling. In addition to identifying LAR as a critical regulator for PDGFβR activation, my data also suggest a negative feedback mechanism whereby PDGFβR/Src activation of c-Abl promotes Nherf2 association with PDGFβR and leads to attenuation of signalling via this receptor.

Most of the previous studies concerning LAR were focussed on its role in neuronal cells, and LAR has been shown to be crucial for axon guidance and nerve regeneration (Sethi *et al.* 2010; Wang *et al.* 2012). However, the mechanism of how LAR mediates nerve regeneration and outgrowth is still unclear. PDGF and PDGFR are also expressed in neuronal cells, and PDGFR was shown be required for neural crest development by promoting the survival and migration of neural crest cells (Smith and Tallquist 2010). However, no previous studies have ever shown any link between the function of LAR and PDGFR in any cell types including neuronal cells. The study described in this thesis established such a link between these two proteins by showing that PDGFR activation and its downstream signalling are strongly dependent on LAR. LAR enabled PDGFR signalling by attenuating a series of

proteins which are involved in PDGFR desensitization including c-Abl, PKCδ, GRK2 and Nherf2. Thus it is possible that in neuronal cells, LAR and PDGFR regulate each other's activity during nerve development, nerve regeneration and axon guidance. While LAR mediates the synapse formation between the neuronal axon cone and target cells, the binding of presynaptic LAR with its postsynaptic ligands such as Sdc may promote LAR's activity or function (Fox and Zinn 2005; Johnson *et al.* 2006). As a result, LAR could elicit an even stronger suppression on c-Abl activity and expression. This would in turn inactivate downstream PKCδ and GRK2, and lead to the potentiation of PDGFR signalling. In neuronal cells, PDGFR downstream signalling could play a role in neuron survival and stimulating the expression of certain proteins which serve as the building blocks for neuron growth and axon extension. Therefore, in the growing neuron, LAR may serve as the vanguard in the axon growth cone promoting correct axon guidance. At the same time, LAR would regulate PDGFR signalling via c-Abl, enabling the appropriate levels of PDGF mediated expression of proteins required for nerve growth and axon extension.

FUTURE DIRECTIONS

To further elucidate and confirm the mechanism of LAR deletion mediated reduction in PDGFβR phosphorylation, these experiments are designed to test the mechanism shown in Figure 21. To test if PKCδ and GRK2 are activated by c-Abl, PKCδ and GRK2 activities could be tested in WT and LARΔP cells using *in vitro* kinase assays. Higher PKCδ and GRK2 activities in LARΔP cells would be consistent with the hypothesis that PKCδ and GRK2 are activated by c-Abl.

To test if Nherf1 associates with PDGFβR more in the WT than in LARΔP cells, alternative Nherf1 antibodies with a higher affinity for Nherf1 might enable the investigation of the amount of Nherf1 associated with PDGFβR in the two cell types. Mass spectrometry could be used to test if PDGFβR phosphorylation at the C terminal residue serine 1104 is higher in LARΔP than in WT cells. To test if the phosphorylation at serine 1104 prevents the association of Nherf1 and promotes the association of Nherf2, tagged PDGFβR with the C terminus Serine 1104 mutated to alanine which mimics the unphosphorylated serine could be transfected to LARΔP cells, and the amount of Nherf1 and Nherf2 associated with the mutated receptor can be compared with the WT receptors. More Nherf1 binding and less Nherf2 binding to the serine to alanine mutated PDGFβR would indicate that phosphorylation at Serine

1104 prevents Nherf1 association and promotes Nherf2 association. Alternatively, tagged PDGFβR with the C terminus Serine 1104 mutated to aspartic acid which mimics the phosphorylated serine could be transfected to WT cells, and the amount of Nherf1 and Nherf2 associated with the mutated receptor can be compared with the WT receptors. Less Nherf1 binding and more Nherf2 binding to the serine to aspartic acid mutated PDGFβR would indicate that phosphorylation at Serine 1104 prevents Nherf1 association and promotes Nherf2 association. Nherf binding and oligomerization ability is known to be regulated by serine phosphorylation; elevated c-Abl activity could possibly affect downstream serine threonine kinase activity which could change the activity of Nherf proteins. The level of Nherf1 and Nherf2 phosphorylation in WT and LARΔP cells could be analysed.

To confirm the relationships between the component proteins in the proposed signalling pathway shown in figure 21 and their regulatory roles in PDGFβR signalling, specific kinase or phosphatase inhibitors and siRNAs can be used to inactivate or knockdown these proteins respectively and the activities of the other proteins in the pathway could be compared with the untreated cells.

If increased GRK2 activity and PDGFβR serine phosphorylation in LARΔP cells is not found, kinase incubator library could be used to screen for kinases involved in reducing PDGFβR activity in LARΔP cells. One such library from Cayman Chemical contains more than 150 kinase inhibitors which would be tested to determine if any

restore the PDGFβR phosphorylation in LARΔP cells upon stimulation with PDGF. Any active inhibitors could be targeting the mediators of the LAR deletion related PDGFβR signalling reduction. Further experiments could be done to check if those kinases are over-activated in LARΔP cells. WT cells could also be treated with the inhibitors in the library to test which inhibitors reduce PDGFβR phosphorylation in WT cells. This may allow the identification of other kinases which play a role in facilitating PDGFβR signalling. Experiments could be done to check if those kinases are inactivated in LARΔP cells, if they are, they could be the mediators involved in the mechanism. Further experiments can be carried out to confirm this.

To test if LAR is important for PDGFR signalling *in vivo*, experiments could be done to see if LAR knock out animals show deficiencies in the physiological functions that are essentially regulated by PDGFR. It has been shown that mice lacking the LAR phosphatase domains (LARΔP) grow normally and did not show any defect in the major organs by histological examination. However, upon breeding, female LARΔP mouse was unable to deliver milk due to an impairment of the mammary gland terminal alveoli differentiation. This indicates that LAR is important for mammary gland development and function (Schaapveld *et al.* 1997). PDGFR has been shown to be expressed in the mammary epithelial cells, and autocrine PDGFR signalling in these cells is required for the oncogenic metastasis (Jechlinger *et al.* 2006). These studies suggest that LAR may potentiate PDGFR signalling which in turn stimulates the differentiation of mammary gland cells and allows the normal development of the

mammary gland. PDGFR mediated recruitment of pericytes may support the formation of new vessels during mammary gland development. Experiments could be done to look at the blood vessel formation in the mammary gland site of these LARΔP mice, less blood vessel formation may suggest less PDGFR signalling in these mice. Alternatively, PDGF ligand could be administered to the LARΔP mice with mammary gland defects to test if this could restore the normal function and milk delivery.

In the LARΔP mice, the number of cholinergic neuron cells in the brain vertical diagonal band was significantly reduced. Cholinergic innervation of the supragranular layer of the dentate gyrus was also diminished. These indicate that LAR is important for the growth, proliferation and innervation function of the basal forebrain neuronal cells (Van Lieshout *et al.* 2001). PDGFR has also been shown to be important for neuronal cell survival, development and migration (Smith and Tallquist 2010). The PDGFβR phosphorylation level could be tested in cholinergic cells from the LARΔP and WT mice to see if it is lower in the cells from LARΔP mice.

Novel and less toxic LAR inhibitors are being generated by researchers in order to treat the LAR over-activation related diseases including diabetes, obesity and cancer (Ajay and Sobhia 2012). Tumours cells which are highly dependent on PDGFR stimulation to proliferate can be injected into mice to generate tumours, LAR inhibitors could be administered to the tumour area regularly, and the growth rate of the tumour in the treated mice can be compared to the control treated tumours. This

could tell us if inhibition of LAR could reduce PDGFR mediated tumour cell proliferation *in vivo*.

PKCδ has been shown to mediate albumin induced renal tubular cell apoptosis, treatment with PKCδ inhibitor rottlerin supressed apoptosis induced by albumin. PKCδ knock out mice also showed less severe apoptosis in renal tubular cells (Li *et al.* 2010). It is known that PDGFβR activation leads to renal tubular regeneration after ischemic renal injury (Takikita-Suzuki *et al.* 2003). PDGFβR signalling is important for maintaining cell survival (Heldin *et al.* 1998). If PKCδ activates GRK2 which mediates the serine phosphorylation and attenuation of PDGFβR, renal tubular cell apoptosis led by PKCδ activation could be the result of the suppression of PDGFβR signalling. PDGFβR could be conditionally knocked out from the kidney of the PKCδ knock out mice to test if renal tubular cell apoptosis is increased in this double mutant compared to the PKCδ single mutant. If the double mutant shows more apoptosis in the renal tubule, PKCδ may mediate mice renal tubular cell apoptosis by attenuating PDGFβR signalling.

These experiments could further confirm the signalling pathway between LAR and PDGFβR and provide us with more information about the importance of this signal transduction pathway in animal physiological function and disease treatment.

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Appendix 1: Relevant Published Paper

The LAR protein tyrosine phosphatase enables PDGF β-receptor activation through attenuation of the c-Abl kinase activity. Zheng W, Lennartsson J, Hendriks W, Heldin CH, Hellberg C. Ludwig Institute for Cancer Research, Uppsala University, Uppsala, Sweden. Cell Signal. 2011 Jun;23(6):1050-6. doi: 10.1016/j.cellsig.2011.01.024. Epub 2011 Feb 12.