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Regulation of Platelet-Derived Growth Factor Receptor Signaling and its Targeting in Cancer Therapy

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Abstract

Ma, H. 2015. Regulation of Platelet-Derived Growth Factor Receptor Signaling and its Targeting in Cancer Therapy. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1092. 64 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-554-9220-5.

Overactivity of platelet-derived growth factor receptor (PDGFR) is a frequent event in many types of solid tumors. Therefore, it is of great importance to uncover the mechanisms that regulate PDGF/PDGFR signalling, to develop efficient inhibitors targeting this pathway. The first step of downregulation of PDGFR activity upon ligand binding is internalization; thus we investigated how endocytosis pathways affect PDGFR signaling. We showed that in Ras-transformed fibroblasts, the internalization of PDGFR is shifted from the routine clathrin-dependent endocytosis to macropinocytosis, which results in enhanced PDGFR activity and subsequent downstream signalling, promoting anchorage-independent growth.

We were also interested in how intracellular trafficking regulates signalling attenuation of PDGFR. We found that His-domain containing protein tyrosine phosphatase (HD-PTP) positively regulates phosphorylation level of the ubiquitin-ligases c-Cbl and Cbl-b; consistently, silencing of HD-PTP led to a decreased level of PDGFR ubiquitination (paper II). Consequently, internalized PDGFR could not be sorted properly and escaped degradation. This resulted in enhanced activation of phospholipase C γ (PLCγ) and signal transducer and activator of transcription (STAT) 3 signalling, which further increased colony formation of HD-PTP silenced cells in soft agar, indicating a tumor suppressor role of HD-PTP.

Activation of PDGFR leads to stimulation of downstream pathways. We identified Fer kinase as a critical signal transducer downstream of PDGFR in a proteomic screen. We showed that Fer kinase is essential for PDGF-induced STAT3 activation; as a result (paper III), Fer depletion severely blunted the ability of PDGFR signalling to promote anchorage-independent growth in soft agar and delayed tumor initiation in a mouse model.

The crosstalk between host and tumor plays a critical role in tumor progression. At present most anti-cancer drugs are targeting tumor cells; we were interested in how targeting tumor host cells affects the efficacy of anti-tumor therapy. We found that selective PDGFRβ inhibition in host cells exerted tumor inhibitory effects on growth and vascularization of tumors with autocrine PDGF signaling, whereas tumors lacking such stimulation show only minor response on tumor growth (paper IV). Meanwhile, we demonstrated that PDGF/PDGFRβ signalling promotes expression of NG2, a marker for pericytes.

Keywords: PDGF, PDGFR, Ras, macropinocytosis, Fer, STAT3, HD-PTP, Cbl, ubiquitination, pericyte, vasculature, tumor, ASKA

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To my dear families

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numbers.

I Schmees, C., Villaseñor, R., Zheng, W., **Ma, H**., Zerial, M., Heldin, C.H., Hellberg, C. (2012). Macropinocytosis of the PDGFRβ promotes fibroblast transformation by H-RasG12V. *Mol Biol Cell. 23(13):2571-82.*

II **Ma, H**., Wardega, P., Mazaud, D., Wardega, A., Jurek, A., Engström, U., Lennartsson, J., Heldin, C.H. (2015). Histidine-domain-containing protein tyrosine phosphatase regulates platelet-derived growth factor receptor intracellular sorting and degradation. *Manuscript*

III Lennartsson, J*., **Ma, H***., Wardega, P., Pelka, K., Engström, U., Hellberg, C., Heldin, C.H. (2013). The Fer tyrosine kinase is important for platelet-derived growth factor-BB-induced signal transducer and activator of transcription 3 protein phosphorylation, colony formation in soft agar, and tumor growth *in vivo*. *J Biol Chem. 288(22):15736-44*

IV **Ma, H***., Cunha, S*., Tsioumpekou, M., Åhgren, A., Heldin, C.H., Lennartsson, J. (2015). Specific targeting of PDGFRβ kinase activity in host cells inhibits growth and angiogenesis of tumors with high PDGF-BB expression. *Manuscript*

*Indicates that the authors contributed equally to the work

Contents

Abbreviations

Abl	Abelson murine leukemia viral oncogene homologue
Alix	ALG-2-interacting protein
AP-2	Adaptor protein 2
ASKA	Analog-sensitive kinase allele
ATP	Adenosine triphosphate
α-SMA	α-smooth muscle actin
Cbl	Casitas B-lineage lymphoma
CDE	Clathrin-dependent endocytosis
CIE	Clathrin-independent endocytosis
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
EEA1	Early endosome antigen 1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Erk	Extracellular regulated kinase
ESCRT	Endosome sorting complex required for transport
FAK	Focal adhesion kinase
FCH	Fps/Fes/Fer/CIP4 homology
Fer	Fps/Fes related tyrosine kinase
FGF	Fibroblast growth factor
GEF	Guanine nucleotide exchange factor
GIST	Gastro-intestinal stromal tumor
HDAC	Histone deacetylase
HD-PTP	His-domain containing protein tyrosine phosphatase
Hrs	Hepatocyte growth factor-regulated tyrosine kinase substrate
JAK	Janus kinase
MAPK	Mitogen activated protein kinase
MVB	Multivesicular body
1-NaPP1	1-(1,1-dimethylethyl)-3-(1-naphthalenyl)-1H-pyrazolo[3,4-d] pyrimidin-4-amine
NG2	Neural/glial antigen 2
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PEST	Proline (P), glutamic acid (E), serine (S), and threonine (T)

PI3K	Phosphoinositide-3'-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PtdIns	Phosphatidylinositol
PTP	Protein tyrosine phosphatase
Rab	Ras superfamily of monomeric G proteins
Ras	Rat sarcoma
RasGAP	Ras GTPase activating protein
RING	Really interesting new gene
RTK	Receptor tyrosine kinase
SH2	Src homology 2
SH3	Src homology 3
Src	Ras sarcoma virus oncogene
STAT3	Signal transducer and activator of transcription 3
STAM	Signal transducing adaptor molecule
TC-PTP	T-cell protein tyrosine phosphatase
Tsg101	Tumor susceptibility gene 101
Ub	Ubiquitin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VPS	Vacuolar protein sorting

Introduction

Cells are the smallest units that are considered building blocks of life. Cells rely on a highly intricate but ordered signaling network to transduce inside and outside stimulus, thus regulating their own proliferation, division, differentiation and apoptosis. Finely tuned signaling and communication plays an important role in maintaining cellular homeostasis, otherwise diseases, such as cancer, may develop. One group of important molecules involved in cell signaling is growth factors, which function by activating their corresponding kinase-linked cell surface receptors. Among them, receptor tyrosine kinases are well studied. There are 58 genes encoding receptor tyrosine kinases among 90 tyrosine kinase genes present in the human genome (Robinson et al., 2000). It has been well known that receptor tyrosine kinases are not only critical regulators of physiological cellular functions, but also key drivers of pathological processes.

In present study, we focused on the mechanisms that regulate platelet-derived growth factor (PDGF)/ platelet-derived growth factor receptor (PDGFR) signaling; furthermore, we investigated the effect of targeting of PDGFRβ in cancer therapy.

Receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) are cell surface receptor-type enzymes, that upon ligand binding catalyze the transfer of the γ-phosphate group of high energy ATP to the hydroxyl groups of tyrosine residues on proteins (Carrasco-García et al., 2014), a process known as phosphorylation. The 58 receptor tyrosine kinases are classified into 20 subfamilies according to their structural characteristics, including epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR) families, which have been extensively studied. Generally RTKs contain an N-terminal extracellular domain for ligand binding, a single pass hydrophobic transmembrane domain, and a cytoplasmic kinase domain with a C-terminal tail.

Activation of RTK promotes cell survival, migration, differentiation and proliferation. Deregulated RTK signaling has been found in many types of

cancers and as such RTKs have become attractive targets for therapeutic intervention.

Platelet-derived growth factors and platelet-derived growth factor receptors

Platelet-derived growth factor (PDGF) was originally purified from the alpha-granules of platelets about 40 years ago and later found to be expressed by many different cell types, including epithelial, endothelial, inflammatory, glia and mesenchymal cells (Demoulin and Essaghir, 2014; Heldin, 1992; Heldin and Westermark, 1999; Westermark and Wasteson, 1976).

PDGF is a family of potent mitogens for connective tissue cells and other cells types that play important roles during embryonic development (Betsholtz, 2004) and wound healing (Cooper et al., 1994), as well as in regulation of interstitial fluid pressure (Rodt et al., 1996). The PDGF family is encoded by four different genes: *PDGF-A, -B, -C* and *-D* which translate into four PDGF homodimers (PDGF-AA, -BB, -CC and -DD) and one heterodimer (PDGF-AB) (Fredriksson et al., 2004a). PDGF-A and -B ligands are secreted as inactive forms but are cleaved by proteases during secretion from the cells. In contrast, PDGF-C and -D ligands are secreted as inactive precursors containing an N-terminal CUB (C1r/C1s, Uegf, Bmp1) domain, which binds to the extracellular matrix. Before binding to the corresponding receptors, the CUB domain needs to be cleaved off by proteases to activate the ligands (Betsholtz et al., 2001; Li and Eriksson, 2003). Studies showed that tissue plasminogen activator (tPA) specifically activates PDGF-CC, while plasmin can activate both PDGF-CC and -DD (Fredriksson et al., 2004a; Fredriksson et al., 2004b).

The PDGF dimers function by binding to two structurally related PDGF α and β tyrosine kinase receptors. Both PDGFRs contain an extracellular region composed of five Ig-like domains, a hydrophobic transmembrane domain, a cytoplasmic kinase domain containing a characteristic inserted sequence of about 100 amino acid residues without similarity to kinase domains, and a C-terminal tail. The PDGF isoforms have binding affinities for the receptors, i.e. PDGFRα binds PDGF-A, -B and -C chains, whereas PDGFRβ binds PDGF-B and -D chains. Thus, different homodimeric (PDGFR αα and ββ) and heterodimeric (PDGFR αβ) receptor complexes may form depending on which PDGF isoform the cell is exposed to and which receptor isoform the cell expresses **(Figure 1)** (Heldin, 2013). The αα and ββ receptor homodimers, as well as the αβ heterodimer, have overlapping, but to certain extent unique, signaling properties. Signaling differences include binding of the Crk family of adaptor proteins only to PDGFRα; while RasGAP selectively associate with PDGFRβ. Another difference is

that both PDGFRα and PDGFRβ can stimulate formation of edge ruffles and loss of stress fibres, but only PDGFRβ stimulates formation of circular actin ruffles on dorsal surface of cells (Heldin et al., 1998; Ostman and Heldin, 2007a; Rosenkranz and Kazlauskas, 1999).

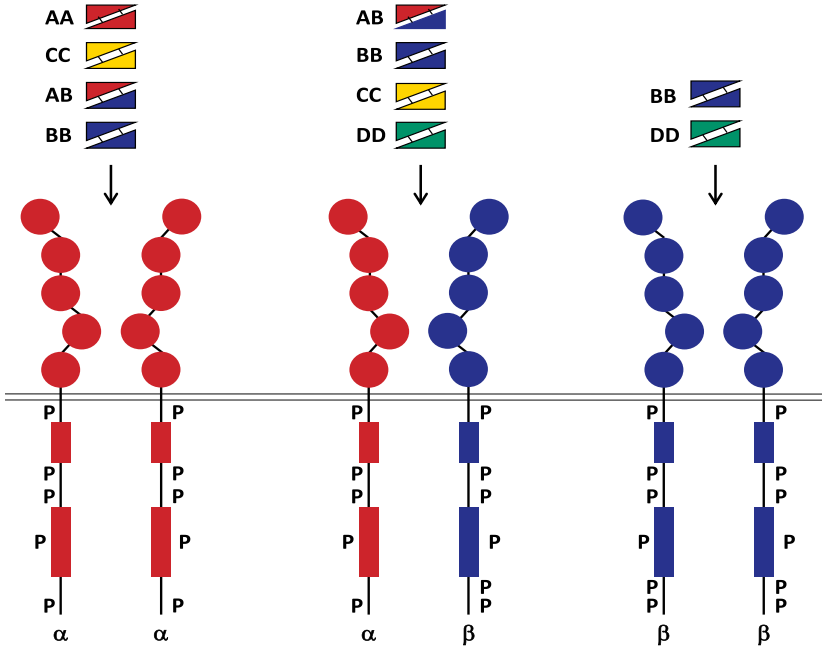

Figure 1. PDGF isoforms and their receptors. Four PDGF polypeptide chains forms five different PDGF homo- and heterodimers, PDGF-AA, -AB, -BB, -CC and -DD. The five PDGF isoforms bind the receptors with different affinities. PDGF-AA, -AB and -BB can activate PDGFRα homodimers, PDGF-BB and -DD promote formation of PDGFRβ homodimers and PDGF-BB induces PDGFRαβ heterodimer formation. In addition, PDGF-CC and -DD may induce PDGFRαβ heterodimers under some physiological conditions (Heldin, 2013). The picture is taken from the permission of (Journal) Cell Communication and Signaling, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

PDGFR activation and biological function

Upon ligand binding, PDGFR undergo dimerization and is activated by autophosphorylation in trans between the receptors. It has been shown that extracellular Ig-like domain 4 homotypic interactions are critical for PDGFRβ activation, but not essential for receptor dimerization (Li and Schlessinger, 1991). In total there are 11 and 12 autophosphorylation sites being identified in the PDGFRα and PDGFRβ, respectively (Heldin et al.,

1998). Phosphorylation of tyrosine residue 849 in PDGFRα and tyrosine residue 857 in PDGFRβ in the activation loops is considered to be critical for activation of the kinase (Fantl et al., 1989; Kazlauskas and Cooper, 1989; Kazlauskas et al., 1991). Overall phosphorylation level in vivo of a tyrosine 857 mutant of PDGFRβ (Y857F) has been found to be similar as the wildtype receptor; nevertheless, the mutant receptor displayed a decreased activation of Shp2, Erk1/2 and Akt, as well as PDGF-BB-induced cell proliferation (Wardega et al., 2010). Another study indicated that autophosphorylation of two juxtamembrane tyrosines at 579 and 581 in PDGFRβ is required for later phosphorylation of tyrosine 857 (Baxter et al., 1998), and that the C-terminal tail in PDGFRβ is also involved in regulation of the receptor kinase activity (Chiara et al., 2004).

Following tyrosine phosphorylation of the cytoplasmic part, the kinase activity is increased and the phosphorylated tyrosine residues provide docking sites for downstream Src homology 2 (SH2) domain-containing proteins, such as the adaptor molecules growth factor receptor binding 2 (Grb2) and CT10 regulator of kinase (Crk), Ras GTPase activating protein (RasGAP), Src family kinases, phosphoinositide-3'-kinase (PI3K), phospholipase Cγ (PLCγ) and signal transducer and activator of transcription factors (STATs) 1, 3 and 5 (Heldin and Westermark, 1999; Ostman and Heldin, 2007a). Docking of these proteins further activate the downstream pathways, including several kinases, such as extracellular regulated kinase (Erk) mitogen activated protein kinase (MAPK), c-Jun-N-terminal kinase (JNK) MAPK and the tyrosine kinase focal adhesion kinase (FAK), by which the signal is transduced within the cell promoting cell cycle progression, cell survival, migration and differentiation, depending on cell type and other external factors (Heldin and Lennartsson, 2013; Heldin et al., 1998; Heldin and Westermark, 1999). Activation of PKCα has been suggested to be essential for Rab4-dependent PDGFRβ recycling, but not for recycling of PDGFRα (Hellberg et al., 2009).

The physiological roles of PDGF and PDGF receptors have been investigated by gene knockout studies in mouse models. It was revealed that *PDGF-B* and *PDGFRβ* knockouts gave similar defects, including defects of vascular smooth muscle cells and pericyte recruitment to vessels, problems with heart and skeletal muscle development, and the defects of placenta vascular structure, as well as kidney mesiangial cell development. In contrast, knockout of *PDGFRα* gave a more severe phenotype than knockout of *PDGF-A* in mice; thus, whereas some *PDGF-A* knockouts die before E10, some can survive until birth; while *PDGFRα* knockouts die between E8 and E16. Phenotypic defects, including cleft face, spina bifida, skeletal and vascular defects of embryo development were seen in *PDGFRα* knockout mice, but not seen in *PDGF-A* knockouts (Betsholtz et al., 2001; Soriano, 1997). On the other hand, activation of PDGFRα by PDGF-AA is essential for for-

mation of alveolar smooth muscle cells, development of hair follicle and oligodendrocyte (Heuchel et al., 1999).

PI3K pathway

Phosphatidylinositol-4,5-bisphosphate 3'-kinases (PI3Ks) constitute a lipid kinase family involved in cell proliferation, migration, survival and intracellular trafficking, as well as carcinogenesis. PI3K(s) are able to phosphorylate inositol 3'-OH group in phosphatidylinositol-4,5-bisphosphate (PIP_2), thus creating a key lipid second-messenger phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P_3). Downstream effector molecules containing protein-lipid binding domains, such as FYVE domain (Fab 1, YOTB, Vac 1 and EEA1) and pleckstrin-homology (PH) domain, then are recruited to the PIP_3 to mediate cellular signaling (Mitsuuchi et al., 2000; Pawson and Nash, 2000). The recruited downstream effectors includes small GTPase families such as Rho and Ras families, certain members of the PKC family and serine/threonine kinases, including 3'-phosphoinositide-dependent kinase-1 (PDK1) and Akt/PKB (Fresno Vara et al., 2004), which have been profoundly studied. PH domain in Akt interacts with PIP_3, resulting conformational change of Akt/PKB, which allow heterodimerization of PDK1 with Akt; PDK1 then phosphorylates Akt at Thr308, a prerequisite for Akt activation. Further phosphorylation of Akt Ser473 in the C-terminal region by mammalian target of rapamycin complex 2 (mTORC2) is necessary for full activation of Akt (Jacinto et al., 2006; Sarbassov et al., 2005).

There are eight heterodimeric PI3K enzymes in mammals composed of eight distinct catalytic subunits and seven regulatory subunits, which have been grouped into three classes (Condliffe et al., 2005; Link et al., 2005; Maffucci et al., 2005; Vanhaesebroeck et al., 2005; Vanhaesebroeck et al., 1997). Class-I PI3K contains subclass IA and IB enzymes that are heterodimers composed of a catalytic subunit (p110) and a regulatory subunit (SH2 domain containing-p85). Subclass IA PI3K is activated by RTKs, while subclass IB PI3K is activated by G-protein coupled receptors (GPCRs) (Fresno Vara et al., 2004; Kondo et al., 2004). Previous studies of gene targeting of different classes of PI3K isoforms demonstrated that PI3K plays important roles in immune, metabolic and cardiac functions as well as in promoting cell survival (Deane and Fruman, 2004; Fruman, 2004; Hawkins et al., 2010; Okkenhaug and Fruman, 2010).

Most growth factor receptors contain a consensus binding motif (pYMXM) for the p85 subunit of PI3K (Songyang et al., 1993). Association of PI3K with PDGFRα at Tyr731 and Tyr742 (Yu et al., 1991) and PDGFRβ at Tyr740 and Tyr751 has been demonstrated after ligand stimulation {Kashishian, 1992 #388}. It has been shown that the p85 subunit of PI3K is phosphorylated at Tyr508 after binding to activated PDGFRβ upon ligand stimulation (Kavanaugh et al., 1994), which leads to activation of the cata-

lytic subunit of PI3K (Hunter, 2000). Earlier studies have demonstrated that the p110 catalytic subunit can act as a serine kinase and phosphorylate the p85 subunit at serine residues (Dhand et al., 1994a; Dhand et al., 1994b; Roche et al., 1994).

PI3K activation has been reported to be important for PDGF/PDGFRβ induced uptake of amino acids in vascular smooth muscle cells (Higaki and Shimokado, 1999), actin reorganization, cell proliferation, as well as migration. It has also been shown to mediate PDGF-BB-induced glycosaminoglycan synthesis and PDGFRα mediated nasal process development (He and Soriano, 2013; Liu et al., 1998). Most importantly, PI3K plays a key role in PDGF-induced cell survival.

PLCγ

Phospholipase C γ (PLCγ) cleaves $PI(4,5)P_2$ just before the phosphate group. The resulting product inositol-1,4,5 triphosphate (IP_3) is released in the cytosol and can diffuse through cytoplasm to activate IP_3 receptor in the endoplasmic reticulum (ER), resulting increased cytoplasmic Ca^{2+} concentration; moreover, the other hydrolysis product, diacyglycerol (DAG), together with Ca^{2+}, activate certain members of the PKC family and induce downstream signaling activity (Berridge, 1993).

PLCγ contains two SH2 domains, one SH3 domain and one PH domain. The two PLCγ isoforms, PLCγ1 and PLCγ2 are activated by PDGFR. It has been shown that PLCγ binds to activated PDGFRβ at phosphorylated Tyr1009 and Tyr1021 (Heldin, 1992; Valius and Bazenet, 1993), while it binds PDGFRα at Tyr988 and 1018 (Eriksson et al., 1995). Binding to PDGFR leads to PLCγ tyrosine phosphorylation, which is important for its activation and increased catalytic activity. Phosphorylation on Tyr783 *per se* is not sufficient to fully activate PLCγ (Sekiya et al., 2004), but enough to promotes reorganization of the actin filaments and membrane ruffles after PDGF stimulation (Yu et al., 1998). Another study showed that full activation of PLCγ is dependent on PI3K signaling from where the produced $PI(3,4,5)P_3$ binds the PH domain of PLCγ, thus targets PLCγ to the cell membrane (Falasca et al., 1998).

It has been shown that PLCγ1 is necessary for maximal PDGF-induced expression and induction of many immediate early genes, such as *FIC*, *COX-2*, *KC*, *JE*, and *c-fos* (Liao et al., 2001). Although PLCγ signaling through PDGF receptors is important for cell proliferation and migration in vitro, in vivo studies using a mouse model bearing mutant PDGFRβ unable to bind PLCγ did not show significant effect on mouse development, indicating overlapping or redundant signaling from different kinases or intracellular crosstalk and compensatory signaling involved (Tallquist et al., 2000).

Src

The Src family of kinases (SFKs) contains 8 non-receptor tyrosine kinases, including Src, Lck, Hck, Fyn, Blk, Lyn, Fgr and Yes. The ubiquitously expressed members are Src, Fyn and Yes. SFKs can be activated by various growth factors and cytokines as well as adhesion and antigen receptors that mediate diverse cellular responses, such as cell survival, proliferation, migration and invasion (Brown and Cooper, 1996; Thomas and Brugge, 1997). SFKs share a conserved domain structure that contains an N-terminal unique region, followed by SH3 and SH2 domains, as well as a C-terminal tyrosine kinase (also called SH1) domain (Koegl et al., 1994). From here the description will be focused only on Src, but other SFK members are believed to be regulated in an analogous manner.

Src plays an important role in regulating signaling from receptor tyrosine kinases. Src activity is regulated by intramolecular inhibitory tyrosine phosphorylation and SH2- and SH3-domain-mediated intermolecular interactions with tyrosine phosphorylated sequences and proline-rich sequences, respectively (Thomas and Brugge, 1997). Inactive Src contains an autoinhibitory phosphorylation of Tyr527 in C-terminal tail (Cooper et al., 1986); upon recruitment to stimulated cell surface receptors by SH2 domain binding, Src undergoes a conformational change where the autoinhibitory C-terminal Tyr527 is displaced, and Tyr416 in the activation loop is autophosphorylated, resulting in activation of the kinase (Smart et al., 1981). It has been suggested that Src-specific kinase Csk or its homolog Chk negatively regulates Src activity by phosphorylating the C-terminal Tyr527 of Src (Davidson et al., 1997; Hamaguchi et al., 1996; Nada et al., 1991).

Activated PDGFRβ recruits Src at phosphorylated Tyr579 or Tyr581 (Alonso et al., 1995) and PDGFRα at Tyr572 or Tyr574 (Gelderloos et al., 1998; Hooshmand-Rad et al., 1998). Another study showed that tyrosine phosphatase Shp2 is also involved in activation of Src by PDGF through dephosphorylating the phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), a Csk-binding protein which prevents access of Csk to Src, and hence preventing inhibitory phosphorylation of Src (Zhang et al., 2004). PDGF-induced Src activation has been shown to induce c-Myc production (Barone and Courtneidge, 1995), which is often seen in tumor progression and correlates to poor clinical prognosis. Src has also been shown to phosphorylate c-Cbl, which is a major E3-ubiquitin ligase for PDGFR; the catalytic activity of c-Cbl is enhanced by this phosphorylation, leading to PDGFR ubiquitination and downregulation (Blake et al., 2000; Galisteo et al., 1995).

Fer

Fer is a ubiquitously expressed cytoplasmic tyrosine kinase that contains a C-terminal kinase domain, a central SH2 domain, coiled-coil domains and an FCH (Fer/Fes/Fps/Cip4 homology) domain (**Figure 2**). The FCH domain promotes association of Fer and the related Fes/Fps to microtubules, and the coiled-coil domains seem to mediate homotrimer formation of Fer and oligomer formation of Fes/Fps. Fes/Fps belongs to the same kinase family as Fer, but has a more restricted and primarily hematopoietic expression. Knockin mice expressing either catalytically inactive *Fer* or *Fes/Fps* develop normally (Craig et al., 2001; Senis et al., 1999); mice with both *Fer* and *Fes/Fps* knockin display defects of fertility, increased levels of circulating neutrophils, erythrocytes, and platelets as well as myelomonocytic cells in bone marrow (Senis et al., 2003). The same study also showed that bone marrow-derived macrophages devoid of Fps and Fer displayed reduced STAT3 and STAT5 activation induced by GM-CSF (Senis et al., 2003). These studies indicated that Fer and Fps/Fes are not essential during embryogenesis and they play redundant biological roles.

Functionally, Fer and the related Fes/Fps has been shown to be able to directly phosphorylate certain downstream proteins, such as cortactin, p120 catenin, STAT3, STAT5 and Shp2, and are thus proposed to be involved in cell adhesion, migration and proliferation (Greer, 2002). Fer becomes tyrosine phosphorylated and associated with the activated receptor upon PDGF stimulation (Kim and Wong, 1995). But mouse embryo fibroblasts (MEFs) with kinase dead *Fer* showed equal activation of downstream Akt and Erk signaling after PDGF stimulation (Craig et al., 2001), indicating other kinases is involved in Fer phosphorylation. It has also been shown that PDGF treatment induces a complex formation between Fer and the p85 subunit of PI3K, suggesting that Fer may bind PDGFR also indirectly via the p85 subunit (Iwanishi et al., 2000). A recent phosphoproteome profiling study identified phosphorylated Fer to be associated with invasion and metastasis of hepatocellular carcinoma cells, suggesting an important role for Fer in tumor progression (Li et al., 2009).

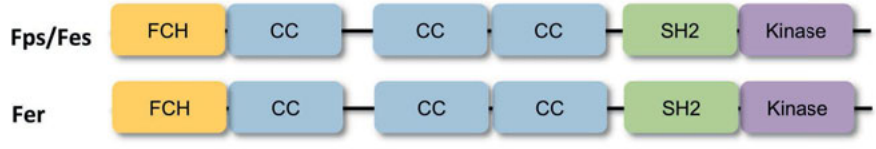

Figure 2. Domain structures of Fps/Fes family proteins. FCH: Fer/Fes/Fps/Cip4 homology domain; CC: coiled-coil domain; SH2: Src Homology 2 domain. The picture is adapted from (Greer, 2002).

MAPK pathway

An important pathway transducing signals from the cell surface to the nucleus is the mitogen activated protein kinase (MAPK) pathway composed of Raf, MEK and ERK MAPK (McKay and Morrison, 2007). The MAPK pathways contain three distinct kinases, named MAP kinase kinase kinase (MAP3K), MAP kinase kinase (MAP2K) and MAPK; for certain MAPK pathways, there is also another upstream kinase for MAPKKK, named MAP4K. Several MAPKs have been identified in mammals, including Erk1, Erk2, Erk3, Erk4, Erk5, Erk7, Erk8, JNK1, 2 and 3, and p38MAPKα, β, δ and γ (Erk6). The regulatory tripeptide motif -Thr-X-Tyr- is a common feature of all MAPKs (Bogoyevitch and Court, 2004; Zarubin and Han, 2005). Among these MAPKs, the Erk1/2 signaling pathway is the best studied.

Erk1/2 plays an important role in cell growth, survival and motility. It is believed that Erk1 and 2 have overlapping or redundant biological roles. The Erk1/2 signaling pathway is, e.g. activated by growth factors stimulation of RTKs. This leads to docking of a complex of the adapter protein Grb2 and Son of sevenless (Sos), which is a guanine exchange factor converting GDP-Ras to GTP-Ras, thus activating the Ras protein. Activated Ras then binds to Raf, a Ser/Thr kinase that function as a MAP3K. Kinase suppressor of Ras (KSR) is an Erk scaffold protein that translocates from the cytoplasm to the cell membrane upon RTK activation and facilitates downstream Raf interaction with Ras, followed by recruitment of MEK1 and MEK2, which are highly selective activators of Erk1/2 MAPK (McKay and Morrison, 2007; Stokoe et al., 1994). Raf phosphorylates MEK on two regulatory serine residues and MEK further phosphorylates Erk1/2 on tyrosine and serine residues. It has been suggested that conformational changes is also involved in Erk2 activation. Activated Erk1/2 thus translocate to the nucleus to regulate target transcription factor activity and gene expression, including Elk-1 and serum amyloid P component (SAP) that trigger a proliferative response (Garrington and Johnson, 1999; Huang et al., 2004; Pinzani, 2002).

STAT

Signal transducers and activators of transcription (STATs) that shuttle between the cytoplasm and the nucleus constitute another important group of signal regulators that transmit signals of growth factors (such as PDGF) from the cell surface to the nucleus. STAT family is composed of seven mammalian members, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 (Bromberg and Darnell, 2000; Kiu and Nicholson, 2012). STAT1 and STAT2 were discovered as downstream mediators of receptors for interferon and other cytokines that do not have intracellular kinase activity, but associates with members of the JAK family of tyrosine kinases. It has been suggested that interferon signaling through activation of STAT requires two different JAK kinases (Stark et al., 1998). So it was proposed that activation

of STAT proteins by RTKs, which harbor the intrinsic kinase activity, may not need activation of JAKs (Darnell, 1997; Velazquez et al., 1992). STAT family proteins share a similar structure, including an amino terminus responsible for STAT dimerization, a coiled-coil domain for interaction with other proteins, a central DNA binding domain and an SH2 domain. Upon growth factor stimulation, STAT proteins are recruited to the phosphorylated tyrosine residues in the RTK through SH2 domain binding and become phosphorylated on a C-terminal conserved tyrosine residue promoting their homo- or heterodimerization. The dimeric STAT complex then translocates to the nucleus where it regulates gene transcription.

The role of JAK kinases in PDGF-induced STAT3 activation is controversial since studies point in different directions, possibly indicating cell type dependency in the activation process (Leaman et al., 1996; Vignais and Gilman, 1999; Vignais et al., 1996). STAT1, 3 and 5 have been shown to bind both PDGFRα and PDGFRβ at several phosphorylated tyrosine sites. STAT3 has been shown to form a complex with v-Src and to be phosphorylated by v-Src (Cao et al., 1996; Wang et al., 2000); however, another study indicated that juxtamembrane tyrosine phosphorylation of PDGFR is sufficient to activate STAT1, 3 and 6 without involvement of Src (Sachsenmaier et al., 1999). Moreover, activation of STAT5 is dependent on PDGFR kinase activity, but not on the kinase activities of c-Src, Fyn, JAK1 or JAK2 (Paukku et al., 2000; Sorkin and von Zastrow, 2009).

It has been shown that the Fer kinase interacts with STAT3 via its N-terminal region, and induces tyrosine phosphorylation and activation of STAT3 (Priel-Halachmi et al., 2000). It has also been reported that Fer associates with STAT3 in interferon γ-treated colon carcinoma cells (Orlovsky et al., 2002). However, it was found that PDGF- or EGF-induced STAT3 phosphorylation was unaffected in embryonic fibroblasts expressing a mutant Fer devoid of kinase activity (Craig et al., 2001).

PDGFR downregulation

Ubiquitination

The activated PDGFRs are rapidly internalized from the cell surface and sorted toward degradation. A key post-translational modification regulating the downregulation process is ubiquitination. Ubiquitination occurs through sequential activation of a highly conserved cascade of reactions leading to covalent attachment of ubiquitin (Ub) to ε-groups of lysine residues in targeted proteins in an ATP-dependent manner.

Ubiquitin is a small protein of 76 amino acid residues and itself contains 7 lysine residues, thus the target proteins can either be monoubiquitinated, i.e. the attachment of one Ub moiety to a single lysine residue; multi-

ubiquitinated, i.e. several lysine residues of the protein are modified by a single Ub; or polyubiquitinated, i.e. poly-ubiquitin chain formation on one or more lysine residues in the target substrate (Kimura and Tanaka, 2010; Woelk et al., 2007). The ubiquitination of different lysine residues in proteins control their destination, thus controlling signaling outcome. Thus, polyubiquitination via Lys48 targets proteins for proteasome degradation (Komander and Rape, 2012), while polyubiquitination via Lys63 has been suggested to be involved in endocytosis and cell signaling. The precise functions of atypical ubiquitination on other lysine residues, such as Lys 6, 11, 27, 29 and 33 are less well defined (Kulathu and Komander, 2012).

Three classes of enzymes, E1, E2 and E3, catalyze the three-step reaction of ubiquitination. E1 is a ubiquitin activating enzyme that binds both ATP and ubiquitin and catalyzes the reaction of transfer of ubiquitin to an active cysteine residue, thus producing a thioester linkage between the C-terminal carboxyl group of ubiquitin and a cysteine in E1 (Schulman and Harper, 2009). E2 is an ubiquitin-conjugation enzyme that catalyzes the transfer of E1-bounded ubiquitin to a cysteine residue in the active site of an E2 enzyme (van Wijk and Timmers, 2010). The last step is catalyzed by E3 enzymes, a large family of ubiquitin ligases that are able to catalyze isopeptide bond formation between a C-terminal glycine of ubiquitin presented by E2 and lysine residues of substrate. The most common RING finger E3 ligases catalyze ubiquitin transfer directly from E2 to the substrate; while HECT type E3 ligases first transfer E2-bound ubiquitin to E3, then catalyzing transfer of ubiquitin to the substrate (**Figure 3**) (Metzger et al., 2012). E3 ubiquitin ligases interact with both E2 enzymes and substrates, thus function in substrate selection. In some cases, E3 ligases are responsible for activation of E2 enzymes.

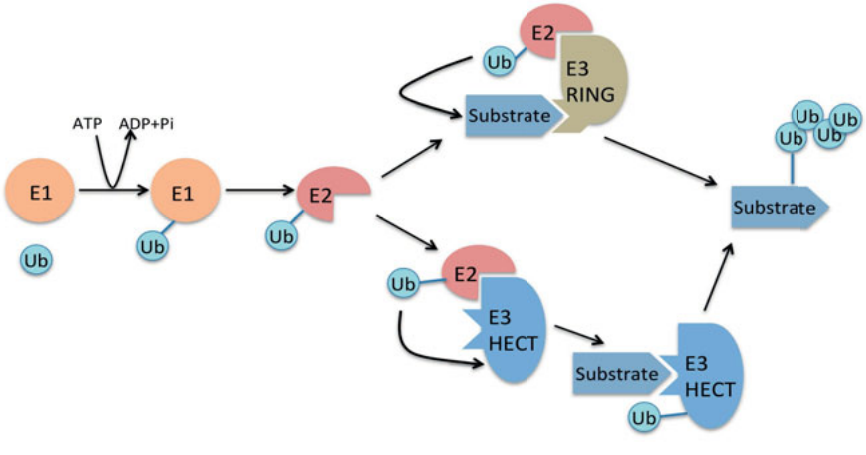

Figure 3. Illustration of ubiquitin conjugation catalyzed by E1, E2 and E3 enzymes. The picture is adapted from (Woelk et al., 2007).

E3 ubiquitin ligases are mainly categorized into four major classes according to their domain structure: homologous to the E6-AP carboxyl terminus (HECT) type, really interesting new gene domain (RING-finger) type, U-box-type and PHD-finger-type (Nakayama and Nakayama, 2006). Among various E3 ubiquitin ligases, the RING finger type Casitas B-lineage lymphoma (Cbl) proteins are well studied. There are three isoforms of Cbl proteins in mammals, including c-Cbl, Cbl-b and Cbl-c. c-Cbl and Cbl-b share similar domain structures; a tyrosine kinase binding domain (TKB), a RING figure domain, a proline rich region, Pro-Arg(PXXXPR) motifs and a C-terminal ubiquitin associate (UBA)/leucine-zipper motif (Lu and Hunter, 2009; Swaminathan and Tsygankov, 2006). Cbl-c is a short isoform, which lacks C-terminal UBA/LZ domain, and the function of Cbl-c is not well known (**Figure 4**). Mice with *c-Cbl* or *Cbl-b* knockout are born normal and fertile, but display hyperactivation of the immune system. Double knockout of *c-Cbl* and *Cbl-b* was found to be lethal to mice at E10 (Naramura et al., 2002; Rathinam et al., 2008). Cbl-c knockout mice did not show any phenotypic changes (Griffiths et al., 2003). Although ubiquitously expressed, high level of *Cbl-c* gene expression has been detected in the aerodigestive tract, prostate, adrenal gland, and salivary gland. Cbl-c has been found to negatively regulate EGF-induced MAPK signaling (Keane et al., 1999).

In normal conditions, ligand binding trigger dimerization and transphorylation of RTK, which allows Cbl association with activated RTK through its TKB domain. Cbl is activated through phosphorylation and initiates ubiquitination of receptors, providing a sorting signal for further RTK intracellular trafficking (Kassenbrock and Anderson, 2004). Previous findings showed that c-Cbl and Cbl-b homo- or heterodimerization is important for their activation and substrate ubiquitination (Kozlov et al., 2007; Peschard et al., 2007). It has been suggested that mutation in a conserved cysteine residue in the RING finger domain reduces c-Cbl-mediated EGFR ubiquitination and degradation (Waterman et al., 1999). It has also been shown that c-Cbl recruitment to PDGFRβ through Tyr1021, a binding site for PLCγ as well, is important for ligand-induced receptor ubiquitination and degradation. The same study also suggested that low density lipoprotein receptor-related protein 1 (LRP1) negatively regulates c-Cbl-mediated PDGFR ubiquitination and endocytosis (Reddi et al., 2007; Takayama et al., 2005). Cbl-b has also been found to participate in ligand-dependent PDGFR downregulation by interacting with the Cbl-interaction protein 85 (CIN85) (Szymkiewicz et al., 2002). Thus c-Cbl and Cbl-b may have partially redundant roles in mediating PDGFRβ ubiquitination and degradation.

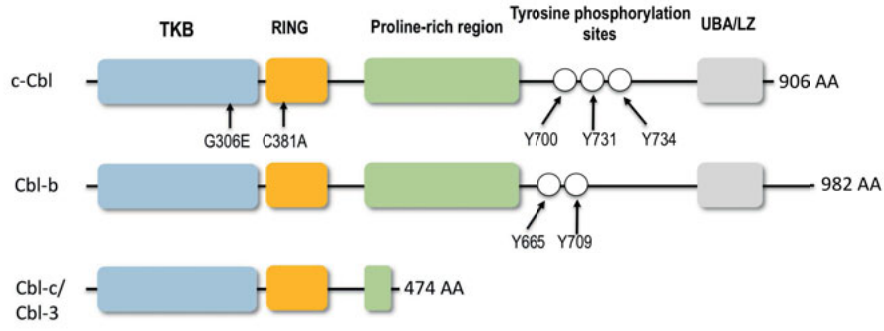

Figure 4. The domain structures of Cbl family proteins. TKB: tyrosine kinase binding domain; RING: RING domain; UBA/LZ: ubiquitin association/Leucien zipper motif. The picture is adapted from (Swaminathan and Tsygankov, 2006)

Endocytosis

Appropriate internalization, intracellular sorting and downregulation of activated ligand-RTK complexes are of importance to control the duration and the extent of receptor activity as well as signaling outcome, indicating a multi-layered control of RTK signaling by a trafficking machinery (Miaczynska, 2013). The first step in this complicated process is internalization of ligand-receptor complexes, which has been established as a major pathway of RTK attenuation. Endocytosis can occur in clathrin-dependent and clathrin-independent manners.

Clathrin-dependent endocytosis

As the name indicates, clathrin-dependent endocytosis (CDE) of RTK involves building coats of clathrin around receptor clusters induced by ligand binding, followed by membrane invagination and vesicle scission from the cell surface (Mosesson et al., 2008). Ligand binding triggers activation of Cbl proteins, followed by ubiquitination of RTK and the association of cytoplasmic adapter proteins, such as adaptor protein-2 (AP-2), CIN85 (Cbl-interacting protein 85KD) and EGFR pathway substrate 15 (EPS15) to clathrin-coated pits. After scission of the vesicles into the cytoplasm by GTPase dynamin, vesicles shed off the clathrin coat, which is mediated by synaptojanin, and then fuse with the endosome compartments through activity of Rab5 and its co-effectors. Receptors can either go back to the cell membrane by recycling through Rab4- or Rab11-dependent pathways, or be further transported to multivesicular bodies, late endosomes and subsequently to lysosomes for degradation. It has been suggested that the internalized receptors can either be dephosphorylated or continue signaling from these trafficking compartments. The endosomal sorting complex required for transport (ESCRT) machinery (ESCRT 0-III) is responsible for tightly control of this

intracellular trafficking process, thus controlling signaling output and cell homeostasis (**Figure 5**).

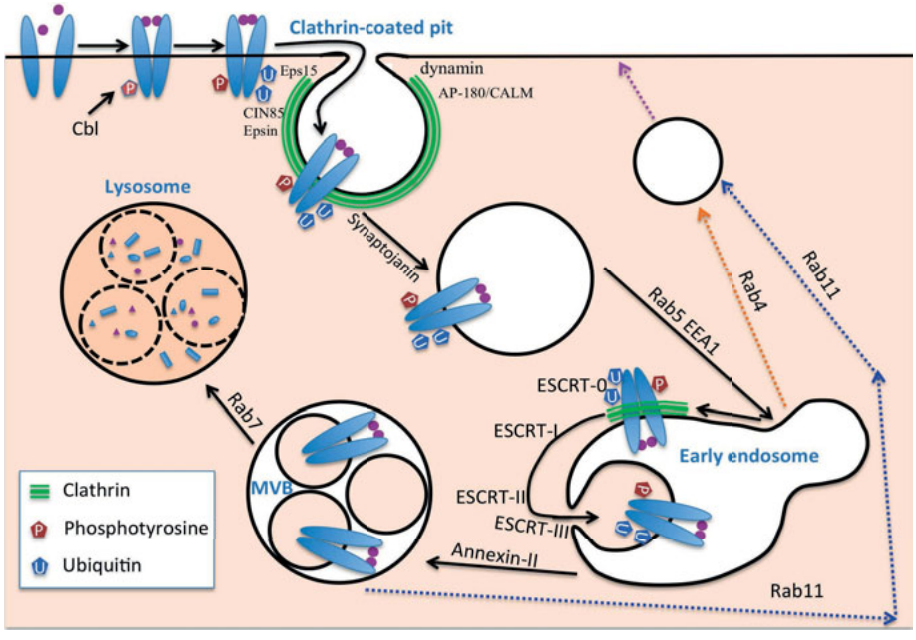

Figure 5. Clathrin-dependent endocytosis of receptor tyrosine kinases. RTKs are internalized upon ligand binding, followed by sorting to lysosome for degradation; or recycled back to plasma membrane through Rab4- or Rab11-dependent pathway. The picture is adapted from (Bache et al., 2004).

Clathrin-independent endocytosis

Clathrin-independent endocytosis (CIE) of RTK includes caveolae-dependent endocytosis and macropinocytosis. Caveolae are special lipid rafts generated by oligomerization of caveolin. Caveolae-dependent endocytosis pathway is similar to clathrin-mediated endocytosis, involving dynamin for inward budding, and targeting the vesicles directly to early endosome or indirectly through larger caveolae-enriched organelle, caveosome, followed by multivesicular bodies and late endosome as well as lysosome for receptor degradation (Parton and Simons, 2007; Pelkmans et al., 2004). Previous studies on EGF and TGFβ receptors showed that engagement of different endocytic pathway may determine signaling capacity and net signaling output; clathrin-mediated endocytosis regulates receptor recycling and sustained signaling, while clathrin-independent internalization targets the receptor for degradation and shut off the signaling (Di Guglielmo et al., 2003; Sigismund et al., 2008).

ESCRT machinery

The ESCRT protein complexes were initially discovered as genes required for vacuolar protein sorting (Vps) proteins in yeast, whose loss resulted in abnormal endosomal morphology and cytokinesis (Bankaitis et al., 1986; Rothman and Stevens, 1986). Later biological studies have shown that ESCRT machinery is also important in morphogenesis of multivesicular bodies by sorting transmembrane cargos from endosomal compartments (Hanson and Cashikar, 2012; Henne et al., 2013; McCullough et al., 2013).

More than 30 gene products have been found to be involved in assembly of the ESCRT machinery that consists of five distinct protein complexes: ESCRT 0, ESCRT I, ESCRT II, ESCRT III and the AAA-ATPase complex Vps4-Vta1 (sometimes referred to as ESCRT IV).

Hrs and STAM constitute the major components of the ESCRT 0 complex, and both contain an ubiquitin-interacting motif (UIM, **Figure 6**) for binding to ubiquitinated cargoes which are destined for lysosomal degradation. It has been claimed that Hrs plays the dominant role in binding to ubiquitinated cargoes; ESCRT 0 occurs as heterodimers or heterotetramers of Hrs and STAM. The coiled-coil domains in Hrs and STAM are needed for binding to each other (Bilodeau et al., 2003; Mayers et al., 2011). Hrs also contains a clathrin binding (CB) motif, an FYVE domain, a **V**PS-27, **H**rs and **S**TAM (VHS) domain and a PxxP motif. The CB motif is able to promote ESCRT 0 to associate with clathrin lattices assembled on early endosomes (Raiborg et al., 2002; Raiborg et al., 2001). The FYVE domain is capable of binding to PI3P enriched in early endosomes and targets Hrs to early endosome association with other effectors. As an UIM, the VHS domain enables ubiquitin binding as well. The PxxP motif is capable of associating with Tsg 101, a main component of ESCRT I complex, which mediates sorting of cargo to intraluminal vesicle of early endosomes (Burd and Emr, 1998; Katzmann et al., 2003; Stahelin et al., 2002).

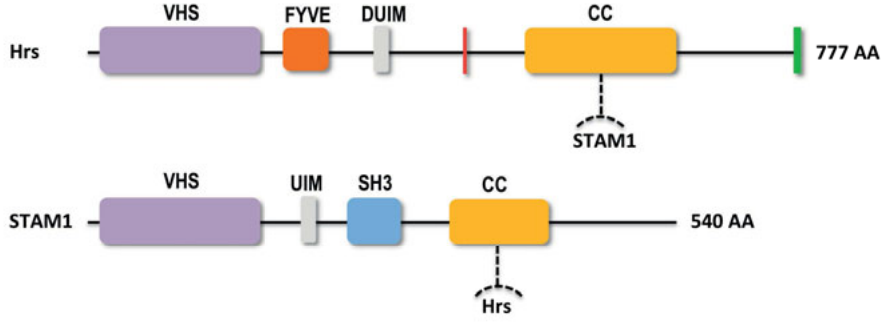

Figure 6. Schematic domain structures of subunits of ESCRT 0 complex. VHS: **V**PS-27, **H**rs and **S**TAM (VHS) domain; FYVE: Fab 1 (yeast orthologue of PIKfyve), YOTB, Vac 1 (vesicle transport protein), and EEA1; CC: coiled-coil domain; UIM: ubiquitin interacting motif. The picture is adapted from (Schuh and Audhya, 2014)

Earlier studies showed that loss of ESCRT 0 abolished ESCRT I recruitment to the early endosomes, suggesting a clear hierarchical assembly of ESCRT machinery in the sorting process (Bache et al., 2003). In mammals, the major subunits of ESCRT I complexes can connect to ubiquitin of ESCRT 0 and ESCRT II complexes to create a vesicle neck on early endosomes (Morita et al., 2007; Wollert and Hurley, 2010). However, the role of ESCRT II in endosome membrane budding is still unclear. Initiation of ESCRT III complex formation is started by interaction with ESCRT II and recruiting CHMP4/Snf7, a main component of ESCRT III, followed by assembly of other subunits of ESCRT III (Saksena et al., 2009; Teis et al., 2008). ESCRT III constitutes the major membrane scission machinery that can pinch off the intraluminal vesicles into the endosomal compartment (Wollert et al., 2009). The last step of the ESCRT cycle is disassemble the membrane-bound ESCRT III complex and recycle cytosolic pool of ESCRT III subunits. Vps4, a member of conformation-modifying AAA+ ATPases, together with its co-effector Vta-1 play a main role in disassembling protein complexes (Hurley, 2010).

Recent studies have shown that Bro-1-domain-containing proteins also have functions in intracellular trafficking mediated by the ESCRT machinery. It has been shown that Alix is able to bind to GPCR PAR-1 and mediate sorting of PAR-1 in an ESCRT III-dependent, but not ubiquitination-dependent pathway (Dores et al., 2012). The subunit of ESCRT I, ubiquitin-associated protein (UBAP) 1 has been suggested to bind Bro-1-containing protein HD-PTP which is necessary for correct EGFR sorting to MVB and cellular ubiquitin homeostasis (Stefani et al., 2011). A later study from the same lab found that deubiquitination enzyme UBPY promotes HD-PTP dissociation from ESCRT 0 complex, but enhanced its interaction with ESCRT III complex, which further facilitate EGFR sorting to MVBs (Ali et al., 2013).

Macropinocytosis

Macropinocytosis represents a distinct RTK endocytic pathway that is an actin-driven and PI3K-dependent process for uptake of fluid-phase material, nutrients and antigens from outside cells. Formed through macropinocytosis, the so-called macropinosomes range from 0.2 to 5 μm in diameters, and were originally described as a large heterogeneous phase-bright organelle emanating from the base of sheet-like extensions of membrane ruffles (Kerr and Teasdale, 2009; Swanson, 2008). Macropinocytosis plays an important role in antigen presentation by immune system and, on the other hand, enhancing cellular invasion and escape from immune surveillance by many pathogens.

It has been shown that v-Src and oncogenic K-Ras induce constitutive macropinocytosis in rat-1 fibroblast; moreover, pharmacological inhibition of PI3K and phosphatidylinositol-specific PLC (PI-PLC) in K-Ras and v-Src-transformed fibroblasts abolished macropinosome formation (Amyere et

al., 2000; Veithen et al., 1998; Veithen et al., 1996), indicating a requirement of PI3K and PLC in macropinocytosis. The Rab5 GTPase has been shown to have a role in macropinocytosis formation. Rab5 is activated after RTK activation and signals to the actin cytoskeleton through a Rab5-specific GTPase-activating protein (GAP), named RN-tre; through this pathway it participates in RTK-induced actin circular ruffling that is indispensible for macropinocytosis occurrence (Lanzetti et al., 2004). PDGFR activation activates PI3K and PLC pathway promoting actin oligomerization and rearrangement, induces membrane ruffling, and promotes macropinosome formation. A recent study showed that a cell-derived peptide induced macropinocytosis-like internalization of PDGFR, inhibited the degradation of PDGF-BB ligand when cells are deprived of nutrients, and therefore promoted cell survival signaling (Zhu et al., 2014).

Protein tyrosine phosphatases

Protein tyrosine phosphatases (PTPs) constitute a family of enzymes that remove phosphate groups from tyrosine residues in their substrates, thus reversing the phosphorylation catalyzed by kinases. For this reason, PTPs also play important roles in cellular signal transduction and regulate a variety of physiological and pathological processes. 107 genes encoding PTPs have been identified in the human genome and classified into 4 categories (Alonso et al., 2004): Classical PTPs, that are composed of non-receptor PTPs and receptor PTPs, constitute the largest family of PTPs; a single 18 kDa low molecular weight PTP (LMW-PTP); CDC25 phosphatases, including CDC25A, CDC25B and CDC25C, that are involved in cell cycle regulation and mainly dephosphorylate Cdks at their dually phosphorylated N-terminal Thr-Tyr motifs; and Asp-based PTPs, which has been shown to have Tyr/Ser phosphatase activity (Li et al., 2003; Rayapureddi et al., 2003; Tootle et al., 2003).

His-domain-containing protein tyrosine phosphatase

His-domain-containing protein tyrosine phosphatase (HD-PTP), also known as PTPN23, is classified as a non-receptor protein tyrosine phosphatase. HD-PTP is believed to be a tumor suppressor candidate since its gene is located within a putative tumor suppressor region on human chromosome 3 (3p21.3) which is often deleted in many types of tumors (Toyooka et al., 2000), such as lung, kidney, head and neck, breast, uterus and esophagus cancers (Kok et al., 1997).

Full-length HD-PTP has 1636 amino acid residues and contains a PTP domain, a Bro-1 domain, a PEST motif and a histidine domain that includes a coiled-coil domain and proline-rich regions. HD-PTP shares some structural similarity with the mammalian ALG-2 interacting protein X (Alix) which is an adaptor protein involved in cell apoptosis, receptor endocytosis

and intracellular sorting (Ichioka et al., 2007). Sequence blast also showed that the Bro-1 domain of HD-PTP shares similarity with other Bro-1 domain containing proteins, such as Rhophilin, a Rho-GTP binding protein involved in regulation of podocyte cytoskeleton (Lal et al., 2015) and yeast Bro-1 protein which has been shown to function as a ubiquitin receptor for protein intracellular trafficking into multivesicular bodies (Pashkova et al., 2013). Overall the sequence of HD-PTP shares a 94% similarity with its rat homologue, PTP-TD14, which has been shown to have tumor suppressor activity. HD-PTP has been claimed to be a catalytically inactive tyrosine phosphatase due to a conserved sequence divergence (VHCSSG to VHCSAG) in its phosphatase domain, where the serine to alanine change abolish the ability of phosphate binding and substrate recognization of the phosphatase (Gingras et al., 2009b).

At present not much is known about the biological function of HD-PTP. HD-PTP is ubiquitously expressed in early embryos and maintained in adult tissues, especially in epithelial cells of many organs. Knockout of the *HD-PTP* gene in mice led to death of the fetus at around E9.5 (Gingras et al., 2009a) indicating an essential role of HD-PTP in embryonic development. Grb2 and Grb2-related protein of the lymphoid system (GrpL), two important adaptor molecules involved several signaling transduction pathways, have been shown to bind HD-PTP (Tanase, 2010). Overexpression of the rat HD-PTP ortholog, PTP-TD14, was found to inhibit H-Ras-induced NIH3T3 cell transformation (Cao et al., 1998), which is consistent with the supposed tumor suppressor role of HD-PTP. Another study showed that HD-PTP expression inhibits colony formation in both ACHN and 786-0 renal cell carcinoma cell lines which further support the tumor suppressor function of HD-PTP (Mariotti et al., 2009b); overexpression of microRNA 142-3p counteract the tumor suppressive effect by HD-PTP (Tanaka et al., 2013). Silencing of HD-PTP in T24 bladder carcinoma cells induced cell migration in an Src-dependent manner (Mariotti et al., 2009b) and suppression of HD-PTP stimulated the endothelial cell migration by affecting the FAK tyrosine kinase (Mariotti et al., 2009a). Furthermore, HD-PTP depletion by shRNA increased the internalization of E-cadherin, impaired its intracellular trafficking and caused cell scattering. Moreover, HD-PTP suppression increased the activities of the Src and β-catenin pathways (Lin et al., 2011). HD-PTP has been found to be involved in endosomal cargo sorting and in the formation of MVBs. Depletion of HD-PTP inhibits EGFR lysosome degradation, presumably by blocking MVB formation, which is necessary for transport of cargo to the lysosome (Ali et al., 2013).

Recently, HD-PTP was shown to affect ESCRT machinery by binding to CHMP4, an important component of ESCRT III complex, to regulate neuron circuits pruning and EGR intracellular sorting (Ali et al., 2013; Loncle et al., 2015). A screening of regulators of the survival motor neuron (SMN) complex, which regulates small nuclear ribonucleic proteins (snRNP) assembly

involved in splicing of premRNA, showed that HD-PTP binds to SMN complex, keeps SMN complex in a highly phosphorylation state and shuttles between nucleus and cytoplasm (Husedzinovic et al., 2015). Taken together, these findings indicate a complicated role of HD-PTP in cellular trafficking and cell signaling regulation.

Tumor angiogenesis

Many factors promote genetic mutations and thereby cancer initiation and progression, including environmental pollution, radiation and microorganisms and their metabolites. Multiple cellular processes controlled by RTKs, including cell cycle progression, differentiation, migration and cell survival, are frequently malfunctioning in tumor cells. Consequently, it is not surprising that deregulated RTK signaling has been found in many diseases, including cancer.

Early in 1971, Folkman hypothesized that solid tumor growth is dependent on angiogenesis, a process defined as newly formed blood vessels from preexisting vessels to supply nutrients and oxygen to cells. Angiogenesis does not occur often in adults, only during pregnancy, wound healing, female reproductive cycles and heavy physical exercises (von Tell et al., 2006). In contrast, angiogenesis is necessary for growth of solid tumors. Abundant studies have suggested that the vasculature system in tumors is often functionally and structurally abnormal, characterized by poor blood flow and leakiness of vessels resulting in edema hindering chemotherapeutic drug uptake. As a result, anti-angiogenesis therapy by targeting tumor vasculature, aiming at shutting off the tumor energy supply and inhibiting trophic signals from tumor microenvironment, has attracted great attention during the past few decades.

Many growth factors are involved in positive regulation of angiogenesis, such as fibroblast growth factor (FGF), transforming growth factor β (TGFβ), tumor necrosis factor α (TNF), hypoxia inducible factor (HIF), angiopoitins and many others. In particular, vascular endothelial growth factor (VEGF) signaling is believed to have critical role in angiogenesis regulation (Karamysheva, 2008).

Endothelial cells and mural cells constitute the major cell types of the vasculature, where endothelial cell form the inner vessel wall and mural cells make close contact with and wrap around the vascular tube (Gaengel et al., 2009). Mural cells are further classified into vascular smooth muscle cells and pericytes according to their location, density, morphology and expression of specific markers. Pericytes are defined as those mural cells that wrap around the vessels of smallest diameter and share the basement membrane with the endothelium (Armulik et al., 2011). Early studies suggested that pericytes have a mesenchymal origin (Drake et al., 1998; Hungerford and

Little, 1999). It has been found that neural crest-derived mesenchymal cells are able to differentiate into mural cells in thymus, head and face regions, and CNS (Bergwerff et al., 1998; Etchevers et al., 2001; Foster et al., 2008). Pericytes are identified using different protein markers, including PDGFRβ, neuron/glial antigen 2 (NG2), desmin, α-smooth muscle actin (αSMA) and regulator of G-protein signaling-5 (RGS5). These markers are not only expressed on pericytes; hence combinations of them have been used in studies of pericytes.

Although not so much is known about the mechanisms that regulate expression of different subtypes of pericytes, it has been suggested that mice genetically deficient for *PDGF-B* or *PDGFRβ* display abnormal development and maturation of the vascular tree due to inefficient recruitment of pericytes and smooth muscle cells (Lindahl et al., 1997; Soriano, 1994). The function and homeostasis of pericytes is regulated to a large extent by the platelet-derived growth factor (PDGF/PDGFRβ) signaling pathway (Abramsson et al., 2003; Arimura et al., 2012; Keller et al., 2013; Pietras et al., 2003). It has also been shown that bone marrow-derived Sca1 and PDGFRβ positive cells are able to differentiate into NG2, desmin and αSMA positive pericytes in vivo (Lu et al., 2008). PDGFRβ positive progenitor cells isolated from tumors have also been proven to differentiate into mature NG2 positive or αSMA positive cells, but not desmin positive cells in tumors (Song et al., 2005). These observations further strengthen the critical role of PDGF/PDGFR signaling in pericyte development.

Targeted PDGFR cancer therapy

Tumor cells, fibroblasts, immune cells, capillary endothelial cells and pericytes, as well as extracellular matrix constitute a highly integrated ecosystem within a neoplasm, which promotes tumor growth (Folkman, 1971; Sudhakar and Boosani, 2008).

The past several decades witnessed an explosion of anti-angiogenesis drug development on solid tumors by targeting VEGF/VEGFR and related signaling pathways, either through neutralizing antibodies or tyrosine kinase inhibitors. The anti-angiogenesis monotherapy by targeting the VEGF/VEGFR pathway gave promising results in some animal models (Prewett et al., 1999), while studies in patients showed limited effects (Crawford et al., 2009; Shojaei et al., 2007). Since endothelial cells can express PDGF-BB and VEGFR, while pericytes secrete VEGF and express PDGFR, the crosstalk between endothelial cells and pericytes is believed to be important to support integrity and function vasculature (Gerhardt et al., 2003; Hellberg et al., 2010; Russin and Giannotta, 2011).

In recent years pericytes have been recognized as potentially important therapeutic targets in anti-cancer therapy. It has been shown that pericytes in

tumors protects tumor vasculature from inhibition of VEGF signaling; therefore, combined inhibition of PDGF receptors on pericytes and VEGF receptor inhibitors on endothelial cells enhances the anti-angiogenic effect (Hasumi et al., 2007). In addition, VEGF has been shown to be able to induce PDGFRβ phosphorylation in vitro, which may further increase tumor cell proliferation and migration (Pfister et al., 2012). Previous studies have demonstrated that tumor-derived PDGFRβ positive perivascular progenitor cells can only differentiate into desmin positive pericytes when they have close contact with endothelial cells, suggesting that crosstalk between pericytes and endothelial cells is important in the growth of certain pericyte subpopulations (Song et al., 2005). Therefore, PDGFR has become an important target in anti-cancer drug development.

All approved PDGFR tyrosine kinase inhibitors (TKI) are ATP-competitors that inhibit also other kinases. One of these inhibitors, imatinib (also named Glivec or STI 571), has been used clinically for treatment of chronic myeloid leukemia (CML), gastrointestitial stromal tumor (GIST), and gliomas (Ostman and Heldin, 2007b). Imatinib inhibits the kinase activities of PDGFR, c-kit, Bcr-Abl and c-Abl and was shown to inhibit the growth of dermatofibrosarcoma protuberans (DFSP) tumors by suppressing the activity of the PDGF receptor. Targeting PDGFRβ positive cancer-associated fibroblasts by imatinib was shown to dramatically reduce proliferation and angiogenesis of cervical carcinoma (Jain et al., 2008).

The multi-kinase targeting characteristic of imatinib makes it difficult to determine whether the effect seen is due to specific inhibition of PDGFR or to inhibition of other kinases. Therefore, more selective inhibitors specifically targeting PDGFR are urgently needed.

Another important aspect of anti-tumor therapy is the tumor-host interaction; recent studies have implicated that tumors not only respond to, but also actively perturb the host organs or tissues (McAllister and Weinberg, 2010). The bone marrow-derived cells constitute a group of active participants involved in tumor progression through their recruitment by tumor secreted cytokines or growth factors (Coussens and Werb, 2002; Ding et al., 2012; Zumsteg and Christofori, 2009). Studies on mouse tumor models have revealed that the mobilization and recruitment of specialized Gr1+/CD11b+ myloid cells from the bone marrow rendered tumors refractoriness to anti-VEGF treatment (Shojaei et al., 2007). Another animal study using sunitinib, a kinase inhibitor for both VEGFR and PDGFR, implicated that the condition of the host microenvironment is related to tumor progression. It was found that pretreatment of mice with sunitinib before tumor implantation accelerated tumor growth and metastases after tumor inoculation (Ebos et al., 2007; Ebos et al., 2009). Another recent study also indicated that host bone marrow microenvironment can be modified by crosstalk between host cell and tumor cells; osteoblast-derived receptor activator of nuclear factor kappa-B ligand (RANKL) stimulated secretion of interleukin-6 (IL-6) by

breast tumor cells, which in turn stimulated expression of RANKL by cancer cells, sensitizing the tumor to RANKL and significantly enhancing tumor growth and metastasis (Zheng et al., 2014).

Increasing evidence substantiates the important role of the host environment in tumor progression; therefore more efforts are needed to investigate systematically the crosstalk between host and tumor cells in order to find efficient therapy for the different cohorts of patients.

Present investigations

Fine-tuned receptor tyrosine kinase signaling plays an important role in maintaining cell homeostasis. Overactivation of PDGFR signaling is often detected in many types of malignancies. Thus, a deeper understanding of the mechanisms involved in activation and control of PDGF/PDGFR signaling is needed, in order to develop drugs that are able to normalize the signaling output. Tumor growth is highly dependent on the vascularization of tumors, and PDGF/PDGFR signaling has been found to be involved in tumor angiogenesis, therefore, the benefit of targeting PDGFR in tumor therapy needs to be investigated. The specific aims of the present project have been:

I. To explore how endocytosis pathways of PDGFRβ affect PDGFR signaling and cell transformation;

II. To elucidate the role of HD-PTP in PDGFRβ intracellular trafficking and degradation;

III. To explain how Fer tyrosine kinase regulates PDGF-BB induced STAT3 signaling;

IV. To investigate the effect of specific targeting of PDGFRβ kinase activity in host cells on tumor growth and angiogenesis.

Paper I

Schmees, C., Villaseñor, R., Zheng, W., **Ma, H**., Zerial, M., Heldin, C.H., Hellberg, C. (2012). Macropinocytosis of the PDGFR β promotes fibroblast transformation by H-RasG12V. *Mol Biol Cell. 23(13): 2571-82.*

The PDGFR is frequently overactivated in many types of human tumors as a result of point mutations or overexpression of ligands. These mutations often result in impaired internalization and degradation of the receptor. Ras mutations also occurs in ~20% of malignancies. It has been reported that oncogenic H-Ras and K-Ras induce macropinocytosis, a form of clathrin- and dynamin-independent endocytosis pathway. Macropinocytosis is PI3K-dependent and actin-driven plasma membrane ruffling mediates uptake of fluids and large molecules. Under physiological conditions, PDGFRs and EGFRs are mainly internalized through clathrin- or caveolin-mediated internalization. However, it was shown that Erb3 translocation to the nucleus in prostate carcinoma cells was abrogated by inhibition of macropinocytosis,

suggesting a role of macropinocytosis in tumor cell signaling (Koumakpayi et al., 2011).

In this paper, we showed that anchorage-independent growth of H-RasG12V-transformed human forskin fibroblasts (HFF) was dependent on PDGFR signaling. Meanwhile, PDGFR in H-RasG12V-transformed HFF internalized via macropinocytosis, which resulted in enhanced PDGFR activity and subsequent downstream signaling. The increased PDGFR activation was further enhanced by EGFR activation, suggesting that receptor tyrosine kinase crosstalk may occur during macropinocytosis. The main conclusion is that transformation by Ras occurs, at least in part, by shifting the receptor internalization route from the normal clathrin-dependent endocytosis to macropinocytosis.

Paper II

Ma, H., Wardega, P., Mazaud, D., Wardega, A., Jurek, A., Engström, U., Lennartsson, J., Heldin, C.H. (2015). Histidine-domain-containing protein tyrosine phosphatase regulates platelet-derived growth factor receptor intracellular sorting and degradation. Manuscript.

Overactive PDGF signaling has been observed in diseases, such as fibrosis, arthrosclerosis and certain types of malignancies. Several processes limit the signaling output from activated PDGF receptors. It has been proposed that receptor ubiquitination is essential for its internalization and intracellular sorting toward degradation. The ubiquitin ligases c-Cbl and Cbl-b have been implicated as particularly important for PDGFR ubiquitination and degradation. Beyond receptor degradation, receptor dephosphorylation with subsequent reduction in its kinase activity can attenuate signaling. The histidine-domain-containing protein tyrosine phosphatase (HD-PTP) plays an important role in the endosomal sorting of the EGFR into multivesicular bodies, and may thus affect the duration and magnitude of EGFR signaling.

In this study, we investigated the involvement of HD-PTP in PDGFR downregulation. We found that HD-PTP controlled the level of phosphorylation of the ubiquitin-ligases c-Cbl and Cbl-b; consistently HD-PTP depletion led to a decreased level of PDGFR ubiquitination. As a consequence, the internalized PDGFR was not sorted properly and therefore escaped degradation, which resulted in defective activation of signaling, in particular activation of STAT3 and PLCγ. Since STAT3 is involved in tumor growth, we investigated the ability of cells depleted of HD-PTP to grow in soft agar. We found that in cells driven by an autocrine PDGF-BB loop, HD-PTP depletion led to increased colony formation, whereas the opposite was true for cells driven by exogenously added PDGF. Thus, whether PDGFR is activated at

the cell surface or intracellularly (which is expected for the autocrine stimulation) determines if HD-PTP suppresses or promotes PDGF signaling.

Paper III

Lennartsson, J*., **Ma, H***., Wardega, P., Pelka, K., Engström, U., Hellberg, C., Heldin, C.H. (2013). The Fer tyrosine kinase is important for platelet-derived growth factor-BB-induced signal transducer and activator of transcription 3 protein phosphorylation, colony formation in soft agar, and tumor growth *in vivo*. *J Biol Chem*. *288(22):15736-44*

Fer is a ubiquitously expressed cytoplasmic tyrosine kinase that has been proposed to be involved in cell adhesion, migration, and proliferation. Previous reports have shown that upon acute PDGF stimulation, Fer becomes tyrosine-phosphorylated and associated with the activated receptor. In addition, PDGF treatment also induces the formation of a complex between Fer and the p85 subunit of phosphatidylinositol (PI) 3-kinase, suggesting that Fer may bind PDGFR also indirectly via p85.

In this study, we identified the Fer kinase a critical signal transducer downstream of PDGFRβ in a proteomic screen. PDGF-BB-induced Fer activation was dependent on PDGFRβ kinase activity and protein was essential for PDGF-induced STAT3 activation. Since STAT3 is a known oncogene product, we investigated the effect of Fer depletion on the ability of PDGF to promote tumorigenesis. We found that in the absence of Fer, PDGF was severely blunted in its abilities to promote anchorage-independent growth in soft agar and tumor growth in a mouse model. Our study suggested a critical role of Fer in PDGF-BB-induced STAT3 activation and cell transformation.

Paper IV

Ma, H*., Cunha, S*., Tsioumpekou, M., Åhgren, A., Heldin, C.H., Lennartsson, J. (2015). Specific targeting of PDGFRβ kinase activity in host cells inhibits growth and angiogenesis of tumors with high PDGF-BB expression. *Manuscript*

Tumor relies on blood vessels to supply nutrients and oxygen for growth and progression. PDGF signaling is of great importance in pericyte recruitment that has been suggested to have undergoing a protective role for tumors anti-angiogenesis therapy. So tumor therapy targeting PDGFR has recently attracted attention, and certain small molecule inhibitors, such as imatinib, which targets the activity of PDGFR and certain other kinases, have proved clinically efficient in certain types of malignancies.

In this study, we used a PDGFRβ kinase switch mouse model by analogue specific kinase alleles (ASKA) technology, in which the PDGFRβ kinase was mutated in the ATP-binding pocket; the mutant receptor can be specifically targeted by the inhibitor 1-NaPP1, without affecting the wild-type kinase or other kinases. Using this model system, we studied the impact of inhibiting host PDGFRβ on the growth of different tumor models, i.e. B16 and B16-BB melanoma, Lewis lung carcinoma and EO771 breast carcinoma. We found that selective PDGFRβ inhibition in host cells had inhibitory effects on growth of tumors with autocrine PDGF-BB signaling, whereas tumors lacking such a loop showed minor response on growth. Selective and unselective impairment of PDGFRβ kinase function rendered divergent effects on tumor pericyte populations in the ASKA mice. While PDGFRβ+ and α-SMA+ pericyte coverage seemed unaffected by specific PDGFRβ kinase activity impairment, NG2+ pericyte abundance and vessel coverage were substantially reduced. Unexpectedly, the tyrosine kinase inhibitor imatinib affected only PDGFRβ+ pericyte coverage.

An in vitro study on ASKA MEFs also showed that PDGF-BB induced NG2 and desmin expression, while inhibition of PDGFRβ kinase activity abolished expression of NG2, but not of desmin. Our observations suggested that PDGF promotes expression of certain pericyte markers, but not others.

Future Perspectives

Paper I and II

Our studies have established that upon Ras-transformation of fibroblasts, the routine clathrin-dependent endocytosis pathway of activated PDGFRβ is shifted to macropinocytosis, which contributes to enhanced PDGFRβ phosphorylation, PLCγ activation and Akt activation, consequently increasing anchorage-independent proliferation. Overexpression of Rabankyrin-5 in non-transformed cells can also directly enhance macropinocytosis and downstream signaling by PDGFRβ, indicating that macropinocytosis itself is sufficient to enhance PDGFRβ activity.

The mechanism of how macropinocytosis affects PDGFRβ activation is not clear, but may partially result from PDGFRβ recycling to cell surface or from decreased phosphatase activity. Another possibility is that macropinosomes may fuse with early endosomes which might be a consequence of increased PI3K activity, since the resulting products, PIP_2, PIP_3 are important for PKC activation and ESCRT complex formation. In addition, it has been suggested that internalized RTK can still signal from endosomal compartments, thus the fusion process may extend the duration of PDGFR signaling from early endosomes. Further investigations will focus on these aspects to uncover the underlying mechanisms.

Although we found that silencing of HD-PTP decreased c-Cbl/Cbl-b phosphorylation, the mechanism is not known. We would like to study if it is due to decreased binding of Cbl to PDGFRβ, or if HD-PTP functions as an adaptor protein binding to certain phosphatases; silencing of HD-PTP could release and activate such a phosphatase capable of dephosphorylating Cbl. Identification and purification of interacting partners of HD-PTP will also be explored. Another unsolved question is in which organell is the missorted PDGFRβ located. We did not observe any co-localization of PDGFRβ with early endosomes, recycling endosomes, multivesicular bodies or late endosomes. So, we would like to investigate other organells, such as class E compartment, which is formed when ESCRT machinery does not function properly.

Ras-transformation induced macropinocytosis of PDGFR and EGFR, while HD-PTP is involved in EGFR and PDGFR intracellular trafficking; thus, the role of HD-PTP in PDGFR internalization and sorting in Ras-transformed cells would be interesting to address. Since HD-PTP has been

shown to be tumor suppressive, the effect of overexpression of HD-PTP on signaling output of PDGFRβ in Ras-transformed cells will also be studied.

Paper III

We have found that Fer is activated upon PDGF stimulation and plays a critical role in the ability of tumor cells driven by an autocrine PDGF-loop (sis3T3 cells) to grow in an anchorage-independent manner in soft agar and in the establishment of tumors in nude mice. It would be interesting to see how Fer expression correlates to tumor progression in human tumor samples. As an extension of this work, we therefore investigated the expression of Fer in a cohort of high-grade glioma tumors and have observed that Fer expression impacts overall survival. These findings will be further explored using a panel of glioblastoma cell lines. We will use lentiviruses-mediated Fer shRNA to investigate how stable depletion of Fer influences tumorigenic properties (e.g. anchorage-independent growth) and response to radiation, since this patient group to a large extent is treated with radiotherapy. If we observe an effect of Fer expression on clonogenic survival, we will elucidate if Fer is involved in the regulation of apoptosis, autophagy or mitotic catastrophy upon cell division. Mitotic catastrophy and apoptosis may be due to defective DNA repair and this will also be addressed by measuring the rate of DNA repair in cells exposed to radiation in absence or presence of Fer expression. Next, we will connect the effect of Fer depletion in the response to radiotherapy with disturbances in signal transduction pathways, with the aim of identifying drugable targets or signaling activity that can be used to predict response to therapy. Initially this will be done by investigating the activity of selected pathways in cells in which Fer has been silenced with si/shRNA. Among the pathways to be studied are pathways well known to be activated by PDGF treatment, i.e. Erk1/2, Akt, p38, JNK and STAT3. To be more unbiased, we will use commercially available phospho-antibody/protein arrays to simultaneously study a large number of proteins/pathways in a given lysates (for example Kinexus or Pamgene methods). Once we have identified pathways, we will study the consequence of inhibiting those on the cellular response to irradiation, using low molecular weight inhibitors, if available, or siRNA depletion.

Paper IV

We found that targeting host PDGFRβ activity significantly inhibits growth and angiogenesis of tumors with an autocrine PDGF-BB loop. Since tumor-host interaction is critical for the tumor response to targeted therapy, we would like to perform treatment using spontaneous tumor models that repre-

sent the tumor development process in patients. We will crossbreed the ASKA mice with Riptag-2 mice and will identify mice with both ASKA PDGFRβ mutation and genotype of oncogenic SV40 large T antigen under the transcriptional control of the insulin promoter (named RipTag) by DNA genotyping. By using this tumor model, we can easily follow the whole process of tumor development and metastasis; meanwhile, we will compare the effects of specific targeting host PDGFRβ activity using 1-Napp1, and targeting tumor mass themselves using imatinib, on tumor growth and angiogenesis as well as metastasis. In addition, we will determine the effect of tumor therapy at different stages of tumor development.

We have found that PDGF-BB/PDGFR signaling is involved in NG2 positive pericyte differentiation and protein expression in primary ASKA MEFs. However, the molecular mechanism underlying this is not clear. A primary experiment using Erk5 inhibitor to treat ASKA MEFs, showed that inhibition of Erk5 also partially abolished NG2 expression; since PDGF is able to activate Erk5 and since Erk5 is also a downstream effector of VEGFR, the role of Erk5 and its kinase activity in PDGF-induced NG2 expression will be investigated.

We will also determine to which extent NG2 positive cells can be recruited to tumors from the host and how targeting of host PDGFRβ influences NG2 positive pericytes expression and recruitment to tumors. Furthermore, we will investigate how this contributes to tumor growth and angiogenesis. In this case we will crossbreed PDGFRβ ASKA mice with NG2DsRedBAC transgenic mice expressing an optimized red fluorescent protein variant under the control of the mouse NG2 promoter. Genotyping will be used to identify mice with PDGFRβ ASKA mutation and NG2 transgene; these mice will be used to investigate the role of different pericyte types in tumor progression.

Since different subgroups of pericytes expressing various markers play diversified roles in vasculature development and respond differently upon anti-tumor therapy, it is interesting to investigate the mechanisms that regulate pericyte marker expression. The understanding of these mechanisms may lead to the identification of new drug targets in tumor therapy.

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