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Regulation and signal transduction of the platelet-derived growth factor receptor and the epidermal growth factor receptor

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Abstract

Wåhlén, E. 2024. Regulation and signal transduction of the platelet-derived growth factor receptor and the epidermal growth factor receptor. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy* 354. 57 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-2162-2.

Receptor tyrosine kinases (RTKs) are crucial regulators of cellular processes, including growth, differentiation, and survival. They function by transmitting extracellular signals through membrane receptors to intracellular signaling pathways. Among these RTKs, the platelet-derived growth factor receptors (PDGFRs) and the epidermal growth factor receptor (EGFR) are vital for maintaining cellular homeostasis and are implicated in various pathological conditions, including cancer. In addition to these pathways, nuclear receptors such as the vitamin D receptor (VDR) also play a significant role in modulating cellular functions. The VDR regulates gene expression in response to the active form of vitamin D, and its cross-talk with RTK pathways offers a complex layer of regulatory control that affects cellular proliferation and differentiation.

This thesis investigates the complex signaling mechanisms of PDGFRs and EGFR, emphasizing the influence of lipid rafts, Rho GTPases, and cross-talk with vitamin D receptor (VDR) signaling.

Paper I focuses on the consequences of lipid raft disruption on PDGFR-β signaling. The research highlights that disrupting lipid rafts alters the association of PDGFR-β with some of its downstream signaling components, reducing the activity of ERK1/2 and AKT in BJ-hTERT fibroblasts and AKT and Src in U2OS osteosarcoma cells.

Paper II explores the differential impact of cholesterol depletion on EGFR and PDGFR-β signaling. We found that EGFR and PDGFR-β internalize into distinct compartments after activation, converging only after prolonged stimulation. Cholesterol depletion enhanced EGFR dimerization and activation while reducing downstream AKT and ERK1/2 phosphorylation, suggesting distinct membrane microdomain dependencies for these receptors.

Paper III explores the impact of Rho GTPase depletion on the phosphorylation and internalization of PDGFR-α and -β. The findißngs suggest that the depletion of Cdc42, Rac1, or RhoA significantly diminishes PDGFR phosphorylation, and downstream stability of STAT1 and activation of STAT1 and STAT3 indicating that these Rho GTPases are integral for the optimal signaling of PDGFRs.

Paper IV examines the cross-talk between VDR and RTK signaling. We discovered that EGF, but not PDGF-BB, enhances VDR-mediated CYP24A1 expression, indicating a selective interaction between these pathways. Additionally, 1,25(OH)2-vitamin D_3 inhibited PDGF-BB-induced proliferation and PDGFR-β phosphorylation, revealing a complex interplay.

Collectively, these studies elucidate the nuanced regulatory mechanisms of PDGFRs and EGFR, emphasizing the roles of lipid rafts, Rho GTPases, and cross-talk with nuclear receptors.

Keywords: Receptor Tyrosine Kinases (RTKs), Platelet-Derived Growth Factor Receptors (PDGFR), Epidermal Growth Factor Receptor (EGFR), Signal Transduction, Lipid Rafts, Rho GTPases, Vitamin D Receptor (VDR) Signaling, Endocytosis, Cross-Talk, AKT Pathway, STAT pathway, ERK1/2 Pathway.

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List of Papers

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

I. **Wåhlén, E.**, Olsson, F., Söderberg, O., Lennartsson, J., Heldin, J. (2022) Differential impact of lipid raft depletion on platelet-derived growth factor (PDGF)-induced ERK1/2 MAP-kinase, SRC and AKT signaling. Cell signal, 22;98:110411

II. **Wåhlén, E.**, Olsson, F., Raykova, D., Söderberg, O., Heldin, J., Lennartsson, J. Activated EGFR and PDGFR internalize in separate vesicles and downstream AKT and ERK1/2 signaling are differentially impacted by cholesterol depletion. Biochem Biophys Res Commun, 12;665:195-201

III. **Wåhlén E.**, Lennartsson J., Heldin J. Silencing of Rho GTPases Cdc42, Rac1 or RhoA reduces PDGFRα and -β phosphorylation and downstream signaling of STAT1 and STAT3. (Manuscript).

IV. Olsson, F., **Wåhlén, E.**, Lennartsson, J., Maria, N. Unique signaling cross-talk between 1,25$(OH)_2$-vitamin D_3 and the growth factors EGF and PDGF. (Manuscript).

Contents

Abbreviations

7DHC	7-Dehydrocholesterol
AP2	Adaptor binding protein 2
AR	Amphiregulin
CCP	Clathrin-coated pits
CIE	Clathrin-independent endocytosis
CME	Clathrin-mediated endocytosis
CSFR	Colony stimulating factor 1 receptor
CUB	Complement subcomponents C1r/C1s, Urchin EGF-like protein, and Bone marrow protein 1
CYP	Cytochrome P450
DBD	DNA-binding domain
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ErbB	Avian erythroblastic leukemia viral oncogene B homolog
ERK	Extracellular signal-regulated kinase
FLT3	FMS-related receptor tyrosine kinase 3
GAP	GTPase-activating protein
GEF	Guanine nucleotide exchange factor
Grb2	Growth factor receptor-bound protein 2
GTP	Guanosine triphosphate
HER	Human epidermal growth factor receptor

JAK	Janus kinase
LDB	Ligand-binding domain
MAPK	Mitogen-activated protein kinase
MET	Mesenchymal-epithelial transition factor receptor
MβCD	Methyl-β-cyclodextrin
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKC	Protein kinase C
PLA	Proximity ligation assay
PLCγ	Phospholipase C - γ
PTP	Protein tyrosine phosphatases
RE	Response element
RTK	Tyrosine kinase receptor
STAT	Signal transducer and activator of transcription
SH2	Src homology 2
TGF-α	Transforming growth factor – alpha
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
WT	Wild-type

Introduction

Cellular signaling mechanisms are central to understanding both normal physiological processes and pathologies in human health. These mechanisms, governed by intricate networks of signaling pathways, ensure precise communication between cells and their environments, crucial for maintaining homeostasis. Among the diverse array of signaling molecules and receptors that orchestrate these processes, tyrosine kinase receptors (RTKs), e.g. platelet-derived growth factors (PDGFs), play pivotal roles. RTKs, with their significant influence over cellular functions such as growth, differentiation, and metabolism, are central to cellular communication and response mechanisms. Their dysregulation is often implicated in various diseases, most notably cancer, where aberrant signaling can promote uncontrolled cellular proliferation and tumor progression [1].

RTKs, first identified for their role in oncogenesis, have since been recognized as key players in a variety of biological processes. The discovery in the late 20th century that RTK mutations and malfunctions contribute to the pathogenesis of many types of cancer marked a turning point in cancer biology and treatment. This led to the development of tyrosine kinase inhibitors, which have revolutionized the treatment of cancers such as chronic myeloid leukemia and non-small cell lung cancer. Despite these advances, resistance to tyrosine kinase inhibitors remains a significant challenge, highlighting the need for continued research into RTK signaling pathways [2].

Other challenges include targeting specific pathway components without affecting normal cellular functions, as well as understanding the complex interplay between different signaling networks [3]. This thesis seeks to address these issues by delving deep into the molecular details of PDGFR pathways, and examining the cross-talk between different signaling molecules.

Furthermore, the modulation of these signaling pathways by factors such as Vitamin D, or through mechanisms involving cellular structures like membrane rafts and proteins like Rho GTPases, highlights the complexity and interconnectivity of cellular signaling networks.

This thesis explores these critical components and their interrelated pathways, offering insights into their roles in health and disease. By delving into the mechanisms of action and regulation, this work aims to contribute to a deeper understanding of cellular signaling and its implications in advancing medical science.

Tyrosine Kinase Receptors

Cellular signaling pathways orchestrate a myriad of essential processes governing cell behavior and function. At the heart of these intricate networks lie receptor tyrosine kinases (RTKs), a diverse group of transmembrane proteins intricately involved in transmitting signals both within and between cells. With 55 known members organized into 19 distinct subfamilies, RTKs exert profound influence over crucial cellular activities, including growth, survival, differentiation, and metabolism regulation [4]. Their pivotal role in mediating cell-signaling pathways positions RTKs as key players in the delicate balance of cellular homeostasis.

However, when the finely tuned signaling orchestrated by RTKs goes awry, the consequences can be dire. Dysregulation of RTK activity has been implicated in a spectrum of diseases, prominently among them, cancer [4]. The aberrant activation or expression of RTKs can fuel uncontrolled cell growth and proliferation, driving tumor initiation and progression.

Despite significant strides in understanding RTK biology, therapeutic interventions targeting these receptors often encounter a formidable obstacle: the emergence of drug resistance. Small molecular inhibitors designed to selectively target RTKs initially show promise in cancer treatment, only to be thwarted by the adaptive strategies of cancer cells [5].

In this context, elucidating the intricate mechanisms governing RTK signaling and regulation takes on paramount importance. A deeper understanding of the molecular intricacies underlying RTK function holds the key to developing more effective therapeutic strategies that can circumvent the development of drug resistance and improve patient outcomes [5].

Structurally, RTKs feature an amino-terminal extracellular ligand-binding domain, a single alpha-helix transmembrane domain spanning the plasma membrane, and a cytoplasmic domain comprising a juxtamembrane region, a tyrosine kinase domain, and a C-terminal domain [5, 6]. Activation of RTKs typically occurs upon ligand binding to the extracellular domain, prompting receptor oligomerization or dimerization and triggering a conformational change that leads to kinase domain transphosphorylation. This, in turn, ablates

inhibition of cis-autophosphorylation of the kinase domain, which can be mediated either via sequences of the C-terminal domain, the juxtamembrane region, or the activation loop, depending on the specific RTK. This in turn leads to the intracellular part of the receptor being further phosphorylated [4, 5]. Subsequent downstream signaling cascades are initiated through the recruitment of proteins containing Src homology (SH2) and phosphotyrosine-binding (PTB) domains, which bind to phosphorylated tyrosine residues on the RTK. This interaction amplifies kinase cascade reactions, facilitating the formation of larger signaling complexes and activating various pathways, including PI3K/AKT, MAPK/ERK, JAK/STAT, and PLCγ1/PKC [4, 7].

Platelet-derived growth factors

Growth factors play a pivotal role in regulating diverse biological processes essential for tissue development, homeostasis, and repair. Among these, platelet-derived growth factors (PDGFs) stand out as key regulators, exerting profound effects on cell proliferation, migration, and differentiation [8].

PDGFs comprise a family of polypeptide growth factors, consisting of four distinct isoforms: PDGF-A, PDGF-B, PDGF-C, and PDGF-D. These growth factors act as ligands for the platelet-derived growth factor receptors, initiating intracellular signaling cascades that drive cellular responses crucial for tissue maintenance and repair [8].

The PDGFs are a group of polypeptide cysteine-knot type growth factors, named this way for carrying the cysteine-knot structural motif, which is a structural motif of approximately 100 amino acids that is also found in the vascular endothelial growth factors (VEGFs) [9]. The evolutionary and functionally conserved growth factor domain within the cysteine-knot fold facilitates receptor recruitment and growth factor dimerization, essential processes for initiating downstream signaling cascades [8, 10, 11].

While PDGFs share this common structural motif, they exhibit variability in sequence and domain composition beyond the growth factor domains (Fig.1). Nonetheless, a secretion signal peptide sequence is conserved across all PDGFs, spanning between amino acid (aa) 1 and 21 for PDGF-A and -B, and between aa 1-22 and 1-18 for PDGF-C and -D, respectively [8].

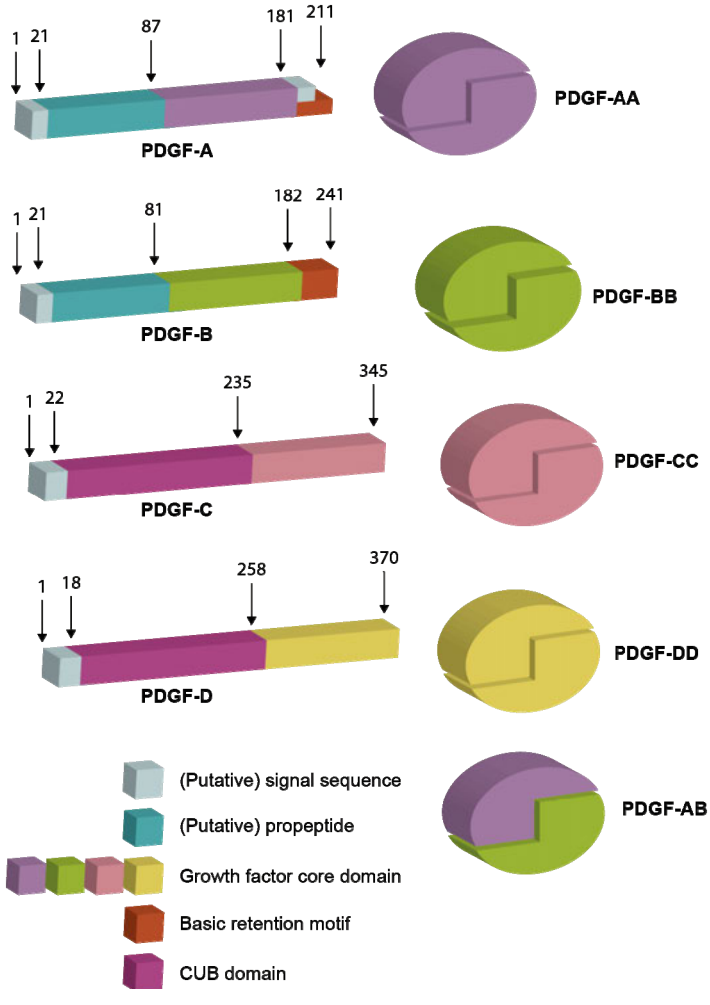

Figure 1. An illustrated and simplified cartoon of the different domains in PDGF-A, -B, -C and -D.

Encoded by four distinct genes located on different chromosomes, PDGF isoforms exhibit variations in size and structure. PDGF-A exists in two splice variants, and is located on chromosome 7, whereas PDGF-B, -C, and -D exhibit longer polypeptide chains, and are located on chromosome 22, 4 and 11, respectively. Both PDGF-A and PDGF-B are secreted from cells in their active state as they undergo proteolytic processing for their activation. This occurs during their transport via the exocytic pathway by either furin or other pro-protein convertases, whereupon a short N-terminal extension is cleaved from the structure [8]. In contrast, PDGF-C and PDGF-D are predominantly secreted in inactive forms, necessitating extracellular protease-mediated cleavage of their N-terminal Complement subcomponents C1r/C1s, Urchin EGF-like protein, and Bone marrow protein 1 (CUB)-domains away from

their growth factor domains by extracellular proteases [11]. PDGFR-C is enzymatically activated by either plasmin or tissue-type plasminogen activator, and PDGF-D by matripase or urokinase-type PA [12–14]. The CUB-domains of PDGF-C and -D are believed to, in part, have the function of inhibiting the binding of full-length PDGF-C and -D to its cognate receptor before proteolytic processing [11].

The tail section of PDGF-A and -B is rich in positively charged amino acids, such as lysine and arginine residues, which are involved in their adherence and distribution via their binding to heparin and heparin sulfate proteoglycans [15, 16]. Conversely, such a section is missing in PDGF-C and -D, and it is believed that their CUB-domains have an analogous function in their distribution, through interaction with other proteins or carbohydrates [8, 17].

Following proteolytic processing, PDGF isoforms form homo- or hetero-dimers stabilized by disulfide bonds, resulting in five distinct isoforms: PDGF-AA, -AB, -BB, -CC, and -DD. These isoforms exert potent mitogenic effects on cells of mesenchymal and neuroectodermal origins, including fibroblasts, smooth muscle cells, pericytes and oligodendrocytes [11].

In cancer, aberrant expression of PDGF isoforms contributes to tumor progression by promoting cell survival, motility, and proliferation. PDGFs can act in an autocrine manner on cancer cells or exert paracrine effects on stromal cells within the tumor microenvironment [18].

Platelet-derived growth factor receptor

PDGFR belongs to the class III type of tyrosine kinase receptors, along with CSF1R, KIT, and FLT3, which together are characterized by their five immunoglobulin-like domains in their extracellular region, and also with containing an intracellular split tyrosine kinase domain (Fig. 2) [19–21]. However, the KIT, FLT3 and CSF1R are considered distinct subgroups of the type III tyrosine kinase receptors to PDGFR, as they bind ligands with the four-helix bundle fold, rather than the cysteine-knot fold of the PDGFs [8, 22–27].

There are two isoforms of the PDGFR receptor, i.e. PDGFR-α and PDGFR-β, which only carry an overall 31 % amino acid similarity in their extracellular domain, but also have larger similarities specifically in their N-terminal and C-terminal domains, of 85 and 75 %, respectively, which consequently materializes in both differences and similarities in receptor activation and signal transduction [5,6].

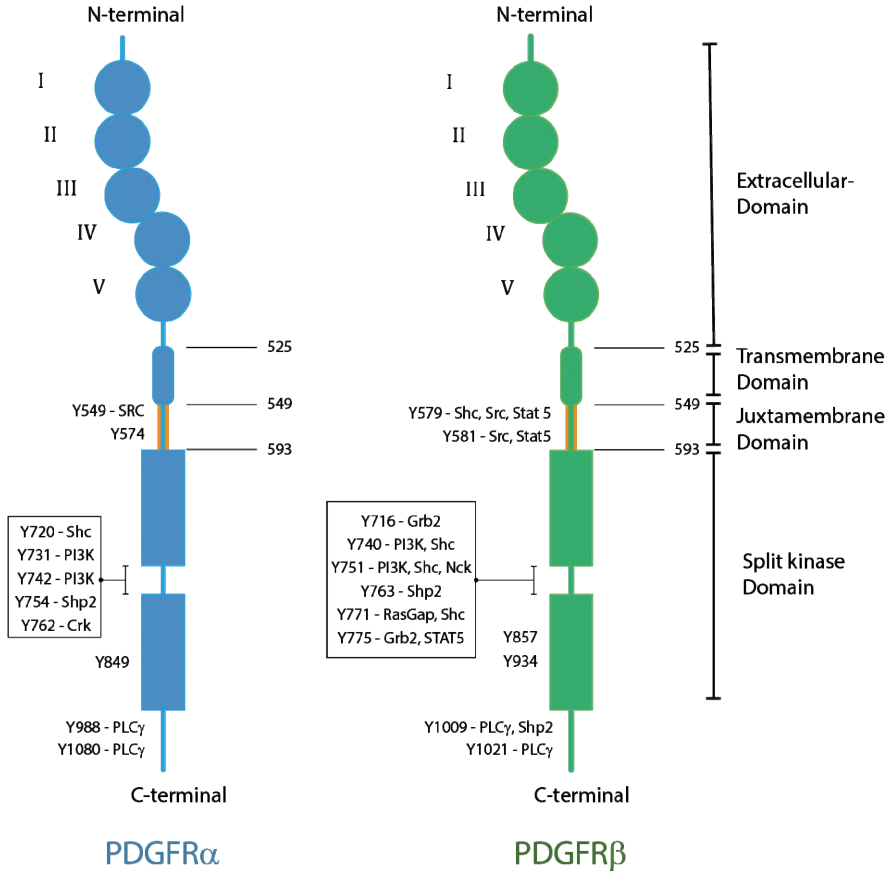

Figure 2. An illustration of a simplified cartoon of PDGFR-α (A) and -β (B), showing known phosphorylated tyrosine residues of the intracellular domain of the respective receptor isoform and proposed proteins interacting with the respective phosphorylated tyrosine.

The general composition of both isoforms of PDGFR-α and PDGFR-β is similar to the generalized description given in the *tyrosine kinase receptor* subchapter. The extracellular part of PDGFR is composed of five immunoglobulin-like domains (D1-D5), where the immunoglobulin-like domain closest to the N-terminal functions as the cap (D1); and the two immunoglobulin like domains below have ligand binding capacity (D2, D3); whereas the last two immunoglobulin-like domains (D4, D5), in concord with the single alpha helix transmembrane domain, stabilize the receptor dimerization process. This is followed by the cytosolic domain of the receptor, which is composed of the juxtamembrane-domain, the split-kinase domain, and the C-terminal domain. When no ligand is bound to the receptor, auto-inhibition is believed to occur through the juxtamembrane domain, and via a repeat motif of proline and glutamic acid in the C-terminal domain [21].

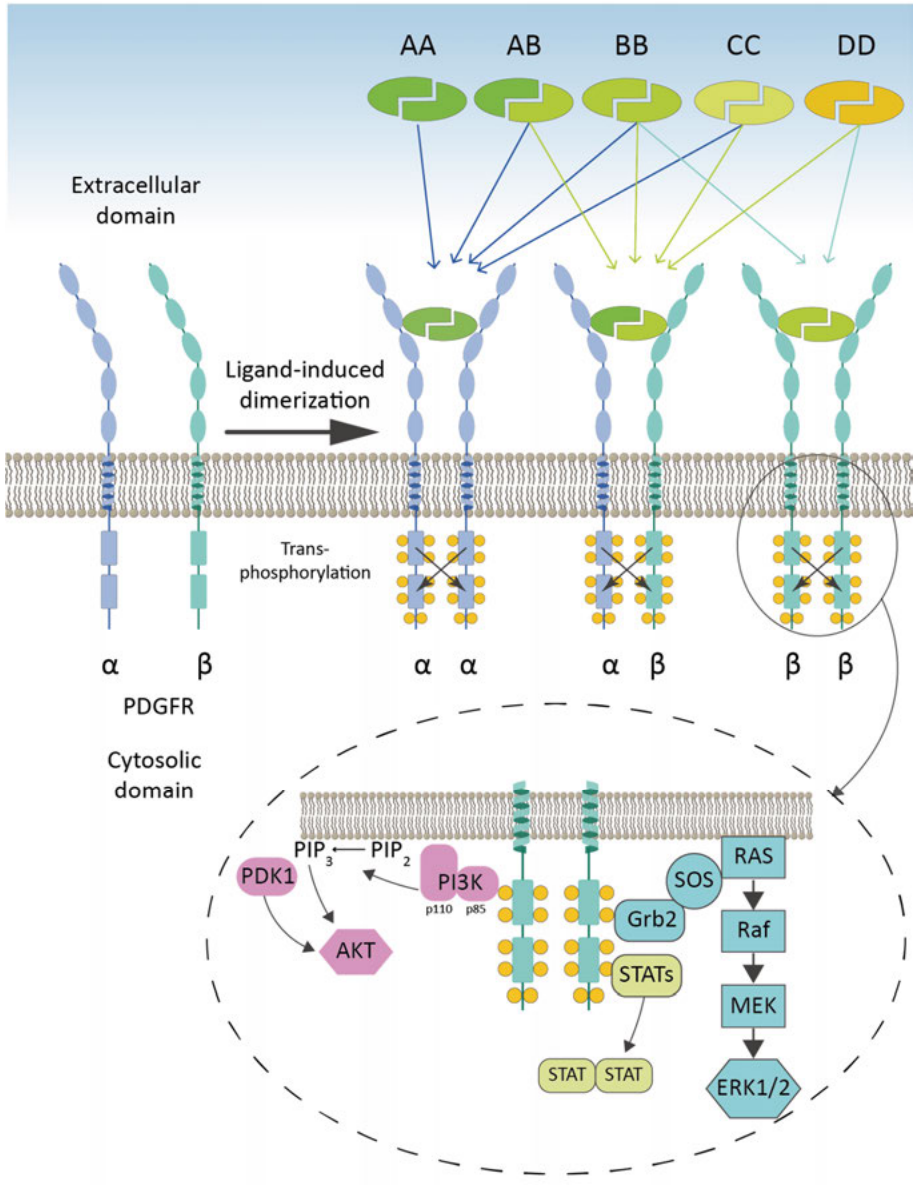

Figure 3. Shows a simplified illustration of the dimerization and subsequent transphosphorylation of PDGFRα and -β after ligand binding, as well as showing some examples of the activation pathways downstream of PDGFR. Namely, AKT, STAT and ERK1/2.

The homo- or heterodimeric PDGFs have been reported to bind with different selectivity to the two PDGFR isoforms, and through their binding causing the induction of homo- or heterodimerization of PDGFR (Fig. 3) [28]. PDGF-AA, -BB, -AB or CC induces PDGFR-αα homodimerization; whereas PDGF-AB,

-BB, -CC, -DD also can induce PDGFR-αβ heterodimerization; and lastly PDGF-BB and -DD induces PDGFR-ββ homodimerization [28–30]. Induction of hetero- and homodimerization induces both unique and overlapping signaling events [28]. For instance, both isoforms of PDGFRs stimulate actin filament restructuring, but it the β isoform is better at mobilizing Ca^{2+} ions than the α isoform, and PDGFR-β promotes the formation of circular ruffles, whereas PDGFR-α does not [30–32].

Upon ligand-induced dimerization, conformational changes in the receptor induce autophosphorylation of the cytosolic region of the receptor, causing the subsequent recruitment of effector molecules carrying the SH2 recognizing binding domains to the phosphorylated receptor and thus initiation of several downstream signaling cascades [28].

Function and expression of PDGFR and its ligands

PDGFR plays vital roles in embryonic development, including mesenchymal cell differentiation, and in adults, in maintaining tissue homeostasis and promoting wound healing. Dysregulation of PDGFR has been implicated in various diseases such as fibrosis, cancer, and atherosclerosis [28]. During embryogenesis, PDGFR-α regulates gastrulation and organogenesis, whereas PDGFR-β is essential for vascular development and early hematopoiesis [8, 30].

The expression patterns of the different PDGF isoforms and their receptors exhibit both unique and overlapping characteristics across various tissues and developmental stages [11]. PDGF-A, -B and -C is expressed in most cells and tissues, but in the case of PDGF–B it is mainly in cells such as endothelial, neurons and megakaryocytes, whereas PDGF-A and -C is mainly expressed in epithelial, neuronal progenitor and muscle cells [11, 30]. In addition, PDGF-C is expressed in most tissues, and there foremost in kidney, pancreas and heart, with lower levels found in the ovaries and liver, and no expression in peripheral blood leukocytes, colon and the spleen [11, 33]. Further, the expression of PDGF-D is not as well characterized, but have been found to be expressed in fibroblasts and smooth muscle cells, and been found in a majority of tissues, and there most abundantly in the heart, the ovaries, and the pancreas, whereas no expression is detected in the lung, skeletal muscle or in brain tissue [11, 30].

During embryonic development, PDGF-A and PDGF-B exhibit non-overlapping expression patterns with their receptors PDGFR-α and PDGFR-β, suggesting paracrine interactions essential for epithelial-mesenchymal interactions and vascular development. PDGF-C and PDGF-D also play critical roles

in embryonic development, contributing to organogenesis and tissue differentiation. PDGF-A is expressed in epithelial cells, whereas PDGFR-α is expressed in adjacent mesenchymal cells, allowing for epithelial-mesenchymal interaction [11, 34, 35]. During embryonal development, PDGF-B and PDGFR-β are observed to be mainly expressed and important for the formation of new vasculature, where PDGFR-β is expressed in mural cells, such as pericytes and vascular smooth muscle cells, and PDGF-B in adjacent endothelial cells [11, 36]. PDGF-C, on the other hand, is expressed by many cells during murine embryogenesis, such as vascular smooth muscle cells of arteries, somites, mesenchymal precursors. PDGF-D has also been shown to be important in murine development, for instance heart, kidney, muscles, and lungs [11].

Genetic knockout studies have revealed critical roles of the PDGF isoforms and their receptors in developmental processes. Knocking out either of the *pdgfb* or *pdgfr*-β has been observed to give a similar phenotype, impacting vascular development and causing embryonic lethality [28, 37, 38]. For instance, lessened recruitment of pericytes, as well as decreased integrity in vessel walls due to underdeveloped smooth muscle cells, leading to leaking vessels, was observed [28]. In addition, knock-out of either *pdgfb* or *pdgfr*-β was observed to negatively impact kidney function during embryonal development due to the underdevelopment of mesangial cells [28]. Knockouts of *pdgfd* lead to milder vascular defects, with slightly elevated blood pressure and disorganized pericyte coverage of vasculature [39]. This implies that PDGF-D also has an importance in the maintenance of arterial blood pressure and maintenance of vasculature homeostasis, but is not as vital as the other PDGFR-β ligand PDGF-B, in embryonal development [28, 39]. Knock-out of either *pdgfr*-β or *pdgfr*-α show a disrupted cardiac neural crest development, and displays an abnormal cardiac innervation [30].

In mouse models, knockout of PDGFA or PDGFC results in different phenotypes, with disruptions observed in intestinal development and mesenchymal derivatives. Knock-out of the *pdgfa* or *pdgfr*-α gene showed a phenotype of disturbed intestinal development, due to perturbed function and formation of vili [34]. These mice also showed severe impairment in mesenchymal derivatives in both embryonic and extraembryonic tissue, and died early around embryonic day 10.5, with some variability and seemingly dependent on genetic background [30, 40, 41]. The knock-out of PDGFA also gave an emphysema-like phenotype in mice due to underdevelopment of alveolar smooth muscle cells [42]. Knock-out of PDGFC gave different phenotypes, where in 128/Sv mice led to embryonic death and prior a complete cleft of the second palate, but in C57BL mice a more mild phenotype was observed, corresponding to

cerebrovascular vessel defects of the CNS, ventricular abberancies and reduced neuroependymal integrity [28, 43, 44]. Knocking out either PDGFR-α, PDGFA, or PDGFC in mice also showed a disturbed development of the axial skeleton and cranial neural crest [30].

Albeit, gene-targeting studies implicate specific roles of PDGFR-α and -β in embryonic development and function in adults, some of these differences could be explained through differing ligand affinities, restricted patterns of expression, and variations in mechanisms of downstream signaling activation. However, a study with generated knock-in mouse models with swapped intracellular signaling domains of the two isoforms showed a substantial rescue of normal development, but that the exchange of the intracellular PDGFR-β- to the intracellular -α-domain resulted in some degrees of vascular pathologies, while the opposite exchange gave a largely unaffected phenotype. Thus, implying that the distinct biological functions observed in the two PDGFRs arise from their variation in ligand affinities but also by variation in gene expression, while differences in unique signaling transduction contribute less [45].

Epidermal Growth Factor Receptor

The Epidermal Growth Factor Receptor (EGFR), also known as ErbB1 or HER1, stands as one of the most extensively studied proteins, renowned for its pivotal role in cellular signaling [4, 46, 47]. As a transmembrane tyrosine kinase receptor, EGFR has garnered significant attention in biomedical research, particularly for its implication in various pathologies, notably cancer [4]. Mutations in EGFR are prevalent, accounting for 60% of small cell lung cancers and approximately 40% of breast cancers or glioblastomas [48–50].

Belonging to the type I subfamily of Receptor Tyrosine Kinases (RTKs), EGFR shares its domain architecture with three other members: ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4) [51]. Its structure comprises a cysteine-rich ligand-binding extracellular domain, a single alpha-helix transmembrane domain, and intracellular juxtamembrane, tyrosine kinase, and C-terminal domains [4, 47, 51]. Other receptors of the ErbB/HER family of RTKs carry similarities in their kinase domains, but differ in extracellular C-terminal domains [46, 52].

EGFR activation is initiated by several ligands, including Epidermal Growth Factor (EGF), heparin-binding EGF-like growth factor, transforming growth factor alpha (TGF-α), betacellulin, and amphiregulin (AR) [46, 53, 54]. Upon ligand binding, EGFR forms homo- or heterodimers with HER2, HER3, or HER4 [55].

Conformational changes induced by ligand binding enable the removal of inhibitory interactions between specific regions of the extracellular domain, facilitating receptor dimerization. The extracellular part of EGFR is divided into four regions, of which, in a non-ligand bound state, region IV interacts with region II, blocking its protrusion. Region II can interact with the corresponding region of another HER receptor. Upon ligand binding, mediated through region I and III, conformational changes in the receptor lead to the removal of the region IV-mediated blockage of region II, thus enabling receptor homo- or heterdimerization of other ErbBs (Fig. 4) [4, 56].

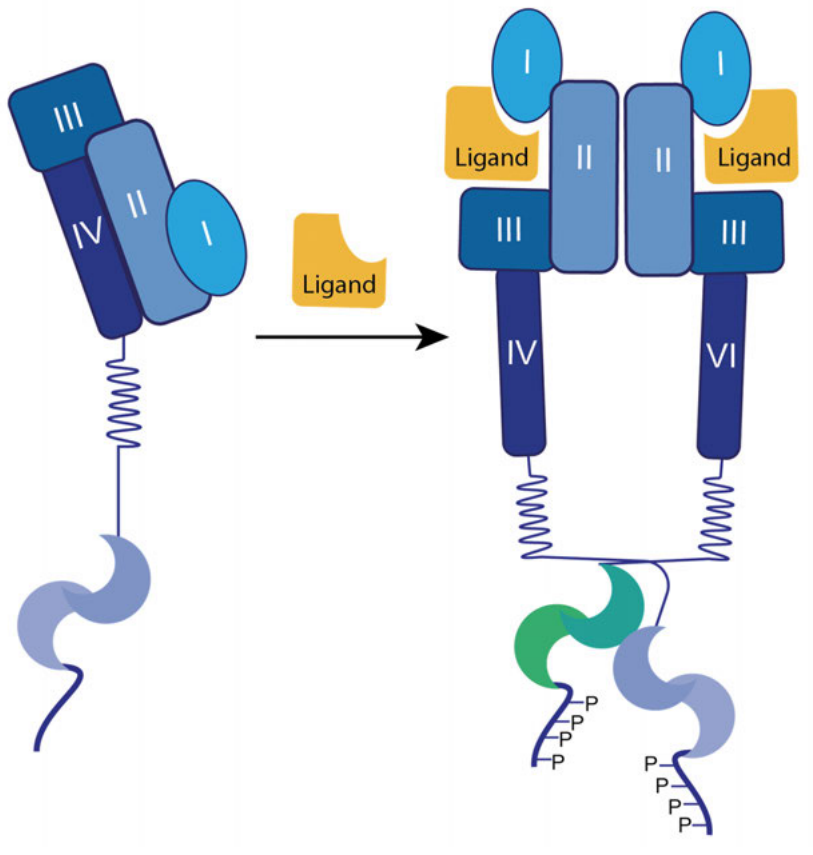

Figure 4. Simplified cartoon of EGFR, followed by ligand-induced dimerization and interaction between extracellular domain I-IV after ligand-binding.

Notably, in cancer cells EGFR has also been observed to form dimers with other RTKs, such as Platelet-Derived Growth Factor Receptor (PDGFR), Insulin-like Growth Factor 1 Receptor (IGF1R), and MET [4, 57–60]. Dimerization triggers receptor transphosphorylation, followed by cis-phosphorylation of tyrosine residues within the cytoplasmic domain of EGFR [4]. This initiates downstream signaling cascades, including the JAK/STAT3, MAPK-

ERK, and PI3K-AKT pathways, which regulate cell survival, proliferation, and apoptosis [47].

EGFR knock-out mice studies have revealed critical insights, showing that EGFR is important for embryonic development. The knock-out of EGFR typically results in embryonic or early postnatal lethality due to severe developmental defects in organs like the skin, gastrointestinal tract, lungs and the nervous system [61, 62]. These studies have also highlighted EGFR's roles in skin repair, hair follicle development, and reproductive functions [61, 63, 64].

Downstream activation pathways of PDGFR and EGFR

AKT Signaling Pathway:

The AKT signaling pathway, also known as the PI3K/AKT pathway, plays a crucial role in cell survival, growth, and proliferation. Upon activation of receptors such as EGFR and PDGFR, phosphatidylinositol 3-kinase (PI3K) is recruited to the receptor complex, leading to its activation [65, 66]. Activated PI3K catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which serves as a second messenger recruiting AKT to the plasma membrane. Once recruited, AKT is phosphorylated at Thr308 and Ser473 residues by phosphoinositide-dependent kinase 1 (PDK1) and mammalian target of rapamycin complex 2 (mTORC2), respectively [67]. Phosphorylated AKT then exerts its effects by phosphorylating downstream targets involved in cell survival and proliferation, such as BAD, caspase-9, and glycogen synthase kinase 3 (GSK3), ultimately promoting cell growth and inhibiting apoptosis [65, 68, 69].

MAPK Signaling Pathway:

The MAPK signaling pathway, also known as the Ras-Raf-Mek-ERK pathway, is pivotal in regulating cell proliferation, differentiation, and survival [70]. Upon activation of EGFR and PDGFR, the small GTPase Ras is activated, leading to the sequential activation of Raf, Mek, and ERK [71, 72]. Activated ERK translocates to the nucleus, where it phosphorylates transcription factors such as Elk-1, c-Myc, and c-Fos, thereby regulating gene expression involved in cell cycle progression, proliferation, and survival [73–75]. Additionally, ERK phosphorylates cytoplasmic targets such as ribosomal protein S6 kinase (p90RSK) and cytoskeletal proteins, influencing cellular processes such as protein synthesis and cytoskeletal rearrangement [70].

STAT Signaling Pathway:

The STAT signaling pathway is crucial for mediating cellular responses to cytokines and growth factors, including those activated by EGFR and PDGFR [76–78]. Upon ligand binding and receptor activation, Janus kinases (JAKs)

associated with the receptor are activated leading to the phosphorylation of STAT proteins [76]. Phosphorylated STAT proteins then form homo- or heterodimers, translocate to the nucleus, and act as transcription factors, regulating the expression of target genes involved in cell proliferation, differentiation, and apoptosis [79]. Notably, EGFR and PDGFR can activate STAT3 and STAT5, which have been implicated in promoting tumor cell survival and proliferation in various cancers [76, 79]. EGFR and PDGFR also activate STAT1 which has been observed to function as a tumor suppressor, but it has also been observed to promote tumorigenesis [80].

Cross-talk between Pathways:

It is important to note that these signaling pathways are interconnected and often exhibit cross-talk, amplifying cellular responses to extracellular stimuli. For example, AKT can phosphorylate and activate Raf, leading to the activation of the MAPK pathway [81]. Additionally, ERK can phosphorylate and activate transcription factors involved in the regulation of gene expression, including those involved in the AKT and STAT signaling pathways [82–84]. This intricate network of signaling pathways allows for the integration of diverse cellular signals and the coordination of complex cellular responses.

Down-regulation of tyrosine kinase receptor activity

The activation of RTKs is regulated via several mechanisms, where both stimulatory and inhibitory signals occur in parallel, such as negative feedback loops from several downstream pathways it activates, dephosphorylation by phosphatases, and through endocytosis with intracellular receptor sorting via the endosomal sorting system. After endocytosis the receptor is either fated for recycling back to the plasma membrane, lysosomal or proteasomal degradation, or further sorted into different subcellular compartments where the RTK may have further functions, such as ER, mitochondria or the nucleus [4, 30, 84, 85] The dysregulation of these processes can lead to aberrant RTK signaling, contributing to disease pathogenesis [30].

Simultaneously, tyrosine phosphatases such as protein tyrosine phosphatases (PTPs) and lipid phosphatases like PTEN deactivate RTKs by removing phosphate groups from key molecules, shutting down signaling cascades [86, 87]. Additionally, RTK pathways often incorporate negative feedback mechanisms; for instance, the ERK pathway inhibits its activator, SOS, and proteins like Sprouty interfere at various points in the Ras/MAPK pathway to modulate signaling [88]. Another critical control mechanism is ubiquitination, where enzymes like Cbl tag RTKs with ubiquitin on specific phosphorylated tyrosine residues, acting as a signal to recruiting endosomal machinery, and promoting proteasomal degradation, offering a rapid shutdown of signaling [21, 89].

These diverse mechanisms work in concert to finely tune tyrosine kinase signaling, ensuring that cellular communication remains precise and controlled, essential for cellular function and health.

Endocytosis of EGFR and PDGFR

The internalization of Receptor Tyrosine Kinases (RTKs) is a tightly regulated process aimed at modulating receptor signaling and maintaining cellular homeostasis. Upon activation, RTKs are rapidly internalized from the plasma membrane to attenuate signaling and facilitate receptor turnover. This internalization is thought to primarily be mediated by clathrin- and dynamin-dependent endocytosis, although clathrin-independent endocytosis (CIE) also contributes to receptor internalization [4, 21]. It has been reported that ligand type, concentration and cell localization appear to play an important role in which endocytic mechanism is activated [4].

Once internalized, the majority of PDGFR or EGFR receptors are directed towards either proteasomal or lysosomal degradation, while only a small fraction is recycled back to the plasma membrane [21, 28, 90]. CIE pathways, which can be dynamin-dependent or -independent, have been reported to be sensitive to ligand concentration and lipid raft-mediated, particularly at higher ligand concentrations [21, 91, 92]. For example, CIE pathways may be activated when clathrin-dependent endocytosis is saturated, such as with high concentrations of EGF (>2 nM) stimulation [4, 85].

Internalization of PDGFR and EGFR is facilitated by ubiquitination of the receptor by the Cbl family of E3 ubiquitin ligases, which promotes receptor endocytosis and subsequent endosomal sorting [4, 21, 93–97]. Ligand binding induces conformational changes in the receptor, exposing motifs that facilitate endocytosis. Adaptor proteins such as AP2 are then recruited to binding motifs or to ubiquitinated tyrosines of EGFR or PDGFR, which then recruit the receptors to clathrin-coated pits (CCPs), enabling clathrin-dependent endocytosis to proceed [4, 21, 90]. As the endosomal sorting process occurs, pH gradually lowers as the receptor moves from early to late endosomal compartment, leading to ligand-receptor dissociation and receptor dephosphorylation, thereby terminating receptor activation [28, 98].

Interestingly, signaling events continue even after internalization, as adaptor proteins and secondary kinases still bind to the phosphorylated receptor within the endosomal compartments [21, 98, 99]. This ongoing signaling suggests a dynamic interplay between endocytosis and downstream signaling pathways. Studies indicate that internalization of PDGFR not only regulates receptor activation but also promotes dimerization and promotes activation of down-

stream signaling cascades such as MAPK/ERK and STAT pathways, highlighting the intricate relationship between receptor internalization and signaling regulation [21, 91, 100].

Membrane Rafts

The compartmentalization of processes is one of the key features of life. From the epidermis functioning as a protective layer from the outside world, blood vessels carrying de-/oxygenated blood to diffuse to different tissues, or cellular membranes enclosing different organelles and cells. This vital biological function has also been proposed to occur also on a smaller scale within the plasma membrane, as first proposed by Simons and Ikonen, where highly ordered and more tightly packed sections of the plasma membrane purportedly form semi-distinct "zones" of so called membrane rafts, separate from the bulk plasma membrane [101, 102]. These membrane rafts are highly heterogeneous in composition, but have been found to be enriched in certain sterols, mainly cholesterol, and lipids, such as sphingolipids, e.g. sphingomyelin, and certain proteins. They form discrete domains in membranes, with distinct biological functions and physical properties, thus being able to compartmentalize certain cellular processes. Membrane rafts are also described as being small, below the limit of optical diffraction, i.e. 250 nm, and ranging in sizes from 10-200 nm, which may cluster and form larger structures [101–103].

Two major subtypes of membrane rafts have been identified with distinct structure and composition, i.e. caveolae and flat rafts. Cavolae are characterized by their invaginated structure and enrichment of the proteins caveolins and cavins, needed for the formation of these subdomains [104]. Flat rafts, on the other hand, lack morphological defining features, but have been found to be enriched in flotillin proteins [105].

Membrane rafts are believed to laterally compartmentalize molecules within plasma membranes, and thus function as signaling "hotspots", as it has been found that certain molecules involved in signaling have been isolated together with these rafts [102]. Among them, molecules involved in RTK signaling, such as EGFR, ephrin receptor B1, IGF1R, and components of the AKT and MAPK cascades, and these rafts are therefore thought to have an important modulatory role in signaling transduction [106]. Some reports also associate PDGFR with these microenvironments, but their exact role still remains unclear [107].

It is speculated that upon certain internal or external stimuli, a clustering of rafts and proteins with higher raft affinity can be triggered, and larger raft platforms can be formed [14, 102]. Thus, by their unique composition it is

postulated that certain molecules will have either a higher or lower affinity for these microenvironments, and certain molecules involved in signaling can either be included or excluded from these rafts, and thus be at a higher concentration and closer proximity to other signaling molecules, such as for instance phosphatases, kinases, GEFs, to facilitate and modulate certain signaling event [106]. Thus, it is proposed, that protein-lipid and protein-protein interactions can be favored, and the transduction of signaling cascades of RTKs through membrane rafts may either be suppressed or promoted [102].

Exactly what features are necessary for signaling proteins to preferentially translocate to raft subdomain is yet to be elucidated, but certain traits have been identified to be important for a higher raft affinity, such as greater transmembrane length, and lipid modifications, such as N-myrisoylation and, S-palmitoylation and Glycosylphosphatidylinositol-anchors [108].

Rho GTPase Proteins

Belonging to the RAS GTPase protein superfamily, Rho GTPases intricately regulate diverse cellular functions, including cell migration, polarity, trafficking, transformation, survival, and morphogenesis [109, 110]. Half of the 55 known tyrosine kinase receptors have been found to activate different or overlapping Rho GTPases, suggesting a link of interactivity [111].

Mammalian Rho GTPases encompass 20 distinct proteins, further categorized into eight subfamilies—Cdc42, Rac, Rho, RhoBTB, RhoD/F, RhoH, RhoU/V, and Rnd—based on their functional, structural, and sequence attributes. Of these Rho GTPases, the first characterized and most extensively studied are RhoA, Rac1 and Cdc42, who also belong to the canonical RhoGTPase:s, exhibiting biphasic transitions between active GTP-bound and inactive GDP-bound states. Conversely, non-canonical Rho GTPases persist in their constitutively active GTP-bound form (Fig. 5A) [110]. This dynamic switching is orchestrated by guanine nucleotide exchange factors (GEFs), promoting the transition to the GTP-bound state, while GTPase-activating proteins (GAPs) catalyze GTP hydrolysis, leading to Rho GTPase inactivation [110]. Additionally, inactive GTP-bound Rho GTPases may be sequestered by Rho GDP dissociation inhibitors (RhoGDIs), tethering them within the cytosol and modulating their spatial and temporal accessibility (Fig. 5B) [109, 110].

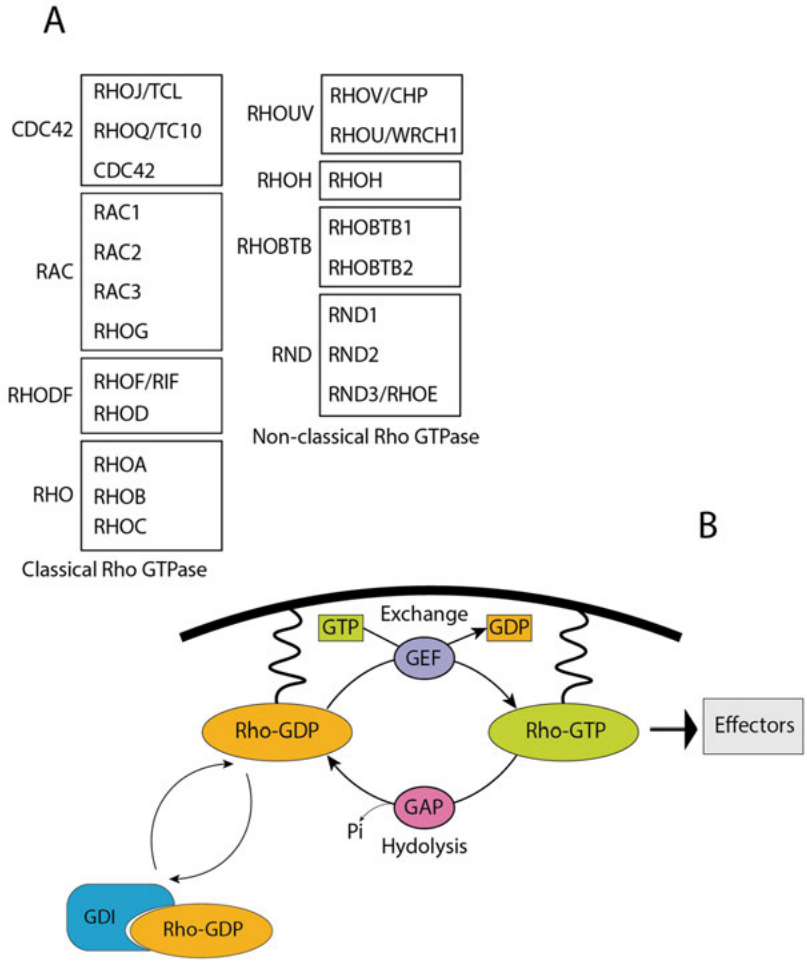

Fig. 5. An illustration of the 20 different Rho GTPases, and their respective subgroups (A). Show a simplified model of the activation of Rho GTPases by GEFs and inactivation by GAP, as well as their sequestration by Rho GDIs (B).

RhoGTPases have specific roles, but also overlapping ones, such as in regulating endocytosis. Emerging evidence underscores cross-talk between different Rho GTPases, although the precise mechanisms remain elusive, and instances when this occurs is therefore of interest. Cdc42, Rac1 and RhoA all have roles in cellular trafficking, mainly through their role in regulating the actin cytoskeleton, and regulating intracellular trafficking, as well as exo- and endocytosis, and through these mechanisms, when dysregulated, are often found to have a role in promoting tumorigenesis [109]. For instance, Cdc42 and Rac1 influence clathrin-mediated endocytosis, with inactive Cdc42 inhibiting this process in dendritic cells, whereas constitutively active Cdc42 promotes endocytosis. Similarly, Rac1 activation inhibits transferrin receptor en-

docytosis, potentially mediated by PI3K activation and subsequent inactivation of Rac1 inactivating GAPs or by direct interaction with synaptojanin 2, impeding clathrin-coated pit formation [109, 112, 113].

Moreover, Rho GTPases exert regulatory effects on the stability of certain RTKs. Activation of Cdc42 via EGFR, for example, stabilizes the receptor by indirectly sequestering the E3 ubiquitin ligase c-Cbl, via a shared interaction between Cdc42 and EGFR with Cool-1/β-PIX, preventing EGFR ubiquitination, endocytosis, and degradation [114]. Similarly, RhoA and its downstream effector Rho-associated coiled coil-containing protein kinase (ROCK) regulate EGFR stability by inhibiting receptor endocytosis through ROCK-induced phosphorylation of endophilin A1, a key regulator in clathrin-mediated endocytosis, thereby impeding its interaction with CIN85 and subsequent receptor internalization [109, 115].

PDGFR and Rho GTPases: Signaling Pathways, Disease Implications, and Therapeutic Targets

The interaction between platelet-derived growth factor receptor (PDGFR) and Rho GTPases is a pivotal element in cell signaling networks that regulate cytoskeletal dynamics, cell migration, and a variety of cellular processes with direct implications for human health [116].

PDGFR-induced pathways and cytoskeletal dynamics upon activation by its ligand, platelet-derived growth factor (PDGF), PDGFR initiates a series of downstream signaling events. One significant outcome of this activation is the modulation of Rho GTPases, including Rac1, Cdc42, and RhoA, which govern cytoskeletal organization and cell migration. PDGFR accomplishes this by engaging guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), which respectively activate and deactivate Rho GTPases [111, 117, 118].

Activated Rac1 and Cdc42 promote the formation of actin-based structures, such as lamellipodia and filopodia, at the leading edge of migrating cells [116]. This is crucial for cell motility, enabling cells to explore their environment and respond to external signals [119]. On the other hand, RhoA activation stimulates stress fiber formation and cell contraction, providing the necessary force for cell movement and maintaining cellular tension [120].

The PDGFR-Rho GTPases signaling axis plays a significant role in various pathological conditions, particularly in cancer and vascular disorders. In cancer, abnormal activation of PDGFR can lead to enhanced cell proliferation, migration, and invasion, contributing to tumor growth and metastasis. The

pathways connecting PDGFR and Rho GTPases are often altered in cancer cells, promoting their ability to invade surrounding tissues and metastasize to distant sites [30, 121].

Similarly, in the context of vascular biology, PDGFR signaling is crucial for angiogenesis—the formation of new blood vessels—which is essential for tumor growth and tissue repair [30]. The activation of Rho GTPases by PDGFR in endothelial cells leads to increased cell migration and vessel formation, illustrating its critical role in angiogenesis [122].

Vitamin D signaling

The systemic hormone vitamin D, also known as calcitriol or $1,25(OH)_2D_3$, plays a pivotal role in regulating calcium and phosphorus metabolism. Beyond its classical functions, it also exerts control over the development, growth, and differentiation of specific cells in various tissues, e.g. osteoblasts and osteoclasts in bone; and keratinocytes in the epidermis [123].

Vitamin D_2 is primarily obtained through plant-based diets or supplements, while vitamin D_3 is predominantly synthesized endogenously. Exposure to ultraviolet B radiation from sunlight triggers the conversion of 7-dehydroxycholesterol (7DHC) to vitamin D_3 in the epidermis [124, 125]. Although a small portion is obtained from animal-based sources like dairy products and fatty fish, vitamin D_3 itself lacks biological activity and requires enzymatic activation through hydroxylation by various cytochrome P450 (CYP) enzymes [126].

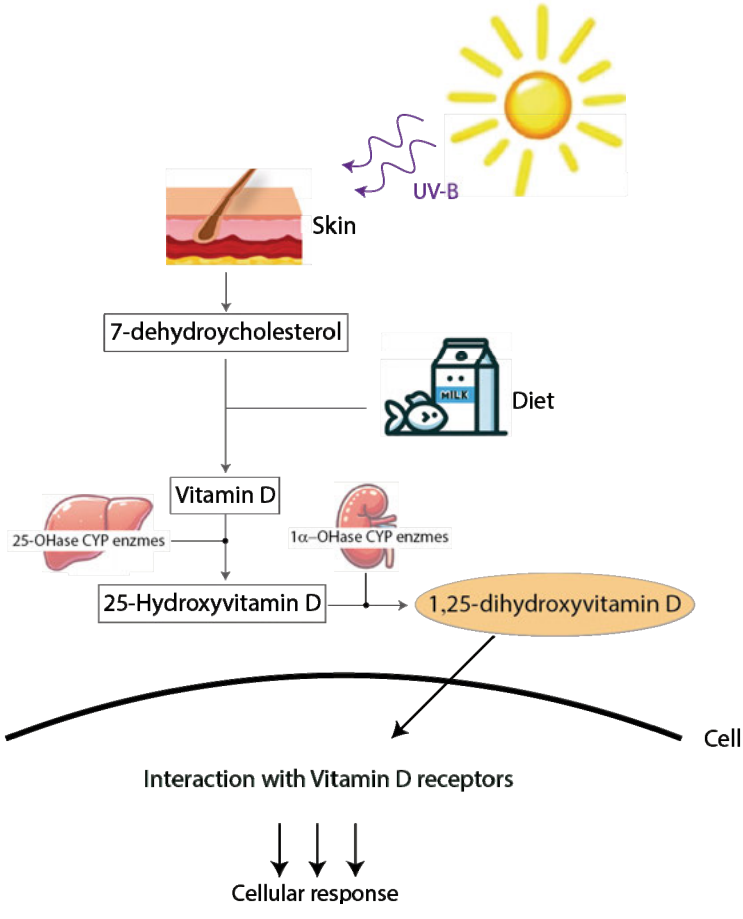

Fig. 6. Shows a simplified illustration of the steps required for the conversion of vitamin D into its active form: 1,25-dihydroxyvitamin D.

The initial activation of vitamin D_3 involves hydroxylation at the 25th position, yielding 25-hydroxyvitamin D_3, which serves as a major circulating metabolite in serum (Fig. 6). This metabolite is often utilized as a biological marker to assess an individual's vitamin D status and is primarily synthesized in the liver by microsomal and mitochondrial 25-hydroxylases [126, 127]. Notable hepatic and extrahepatic 25-hydroxylases involved in this process include CYP27A1 (for vitamin D_3 activity), CYP2R1 (for both vitamin D_2 and D_3 activity), CYP3A4 (for vitamin D_2 activity), and CYP2J2 (with greater activity on vitamin D_2 than vitamin D_3) [126, 128–130].

In a subsequent step, 25-hydroxyvitamin D_3 undergoes catalytic conversion, primarily mediated by the CYP27 subfamily, specifically CYP27B1, to form its biologically active form, 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). This conversion mainly occurs in the proximal convoluted tubule of the kidneys.

The activity of $1,25(OH)_2D_3$ is primarily endocrine, as it is transported via the bloodstream to various target tissues where it regulates, for instance, bone homeostasis and facilitates calcium absorption in the intestines [126].

Additionally, alternative hydroxylation reactions have been observed, including 20- and 22-hydroxylations of vitamin D derivatives, catalyzed by enzymes such as CYP11A1. Subsequent hydroxylations at positions 1, 24, 25, or 26 lead to the formation of metabolites with limited activity in calcium homeostasis regulation but potential involvement in other biological processes, such as inhibition of cellular proliferation [124, 126, 131, 132]. Notably, $1,25(OH)_2D_3$ has exhibited anti-proliferative effects in certain cancers and tumor-derived endothelial cells, highlighting its significance as a potential target for further anti-cancer research [133].

Nuclear receptor - Vitamin D receptor

Vitamin D receptor, the target receptor for active vitamin D, belongs to the superfamily of transcription factors called nuclear receptors, consisting of 48 different receptors, which are often the target and become activated by small and lipophilic molecules [134]. Nuclear receptors share conserved structures, featuring a DNA-binding domain (DBD) and a ligand-binding domain (LBD), enabling them to function as transcription factors upon ligand binding [135]. Typically, nuclear receptorss bind DNA hormone response elements, driving the transcriptional activation of target genes [135–138].

The DBD of VDR can bind no more than six nucleotides, generally recognizing a consensus region of RGKTSA (R: A/G; K: G/T; S: C/G), within the large groove of genomic DNA, also called response elements (REs) [139]. VDR itself is however unable to form a stable complex with DNA and further needs to form a complex with a partner protein to stably bind DNA [139, 140]. Upon ligand activation, VDR forms a complex, primarily with the retinoid X receptor, along with other partners like CEBPA, PU.1, and GABPA, binding to VDR-response elements (VDREs) on genomic DNA [141–144]. This complex recruits regulatory proteins, either negative (co-repressors, CoRs) or positive (co-activators, CoAs), ultimately modulating gene transcription via transrepression, via CoAs, or transactivation, via CoAs, of VDR target genes [139].

Vitamin D mediates its action through its hydroxylated forms, of which ($1,25(OH)_2D_3$) has been identified as the most biologically active metabolite, and observed to have the highest affinity for the transcription factor VDR [141]. However, as the serum levels are nearly a thousand times higher than $1,25(OH)_2D_3$ it can still function as an important ligand for VDR, and is not as previously believed, only a prohormone of $1,25(OH)_2D_3$ [126]. VDR, in

turn, regulates several cellular processes, such as energy metabolism, and the adaptive and the innate immune system, and is therefore more generally involved in the regulation of cellular survival, proliferation and differentiation, as immune cells are the most quickly proliferating cells in the body [141, 145]. Further, and most famously, is its associated role in maintaining calcium homeostasis for proper bone formation, which if dysregulated or with insufficient amount of vitamin D in the body, may lead to bone malformation and disease, such as rickets [141, 146]. As the VDR is expressed in more than half of human cells and tissues it had also been proposed that it has further functions beyond the regulation of the calcium homeostasis and the immune system, as more than 900 genes directly and interacts with VDR, and it is estimated that up to 5 % of the genome is regulated by vitamin D [141].

Apart from its transcriptional role, $1,25(OH)_2D_3$ also influences the genome indirectly by modulating the epigenome and chromatin remodeling [141, 147]. Additionally, rapid non-genomic effects mediated by $1,25(OH)_2D_3$ have been observed at cellular membranes or the cytosol, possibly involving VDR or alternative interacting proteins as for instance via the protein disulphide isomerase family A member 3 (PDIA3) [148, 149].

Cross-talk between EGFR or PDGFR with Nuclear Receptor $1,25(OH)_2D_3$-Mediated Signaling

A common convergence of RTK and nuclear receptor signaling lies in their innate ability to regulate transcriptional activation, thus modulating gene output and function. It is thus hypothesized that there is cross-talk between the signaling pathways that are regulated by the two groups of receptors. Both nuclear receptors and RTKs have been observed to regulate each other in genomic and non-genomic manners, either indirectly through the modulation of transcription factors or directly by affecting protein-protein interactions or post-translational modifications of molecules involved in their signaling pathways. Despite extensive research in their respective fields, limited investigation has been conducted on their interconnected signaling. Understanding this interplay is crucial for comprehending their normal physiological functions and the development and progression of different pathologies resulting from dysregulation [135].

For instance, the activity of RTK class I receptors has been observed to reduce the levels of sex hormones such as progesterone, estrogens, and androgens [135, 150–152]. Similarly, downstream effector molecules of RTKs, which modify post-translational modifications such as phosphorylation, ubiquitination, and lipid anchors, can influence the transcriptional activity of nuclear receptors [135, 137].

Nuclear receptors may also influence transcription factors downstream of RTKs through tethering, where nuclear receptors directly bind to other transcription factors, altering their activity and leading to either transrepression or transactivation [135, 153]. Tethering has been observed between nuclear receptors and several transcription factors activated by RTKs, such as STATs, ETSs, NF-κB, and SP1, resulting in both transrepression and transactivation [135].

In this thesis, particular focus will be on potential interactions between EGFR and VDR, as well as between PDGFR and VDR, and signaling mediated by VDR's ligand $1,25(OH)_2D_3$. Reports indicate that $1,25(OH)_2D_3$ affects signaling mediated from the plasma membrane by growth factors or cytokines. Growth factors such as EGF, transforming growth factor (TGF)- β, and Wnt have been observed to have their signaling affected by $1,25(OH)_2D_3$, either in influencing cytosolic pathways or the activity of target transcription factors. Moreover, $1,25D(OH)_2D_3$ can trigger the release of growth factors from neighboring cells by affecting gene expression involved in paracrine mechanisms, such as their synthesis or secretion [154].

Differential effects of cross-interaction between EGF and $1,25(OH)_2D_3$ have been observed in a cell-type-specific manner [154]. For instance, Tong *et al*. observed that EGF and $1,25(OH)_2D_3$ down-regulate the respective receptor of the other ligand, VDR and EGFR, in Caco-2 colon and T47D breast cancer cells [154–156]. In another study, $1,25(OH)_2D_3$ was found to down-regulate EGFR in human colon cancer cells by inhibiting the expression of the protein SPROUTY-2, which protects EGFR from ubiquitination and subsequent internalization and degradation. Additionally, $1,25(OH)_2D_3$ was observed to increase levels of E-cadherin that in turn downregulated EGFR levels [154, 157]. The increase of E-cadherin due to presence of $1,25(OH)_2D_3$ and subsequent decrease in EGFR activity has also been observed in further studies in colon cancer cells [158].

Reports of cross-talk between $1,25(OH)_2D_3$-mediated signaling and PDGF-mediated signaling are less extensive than those between EGF and $1,25(OH)_2D_3$-mediated cross-talk. However, some cross-talk between PDGF and vitamin D signaling has been reported. For instance, the PDGF-A gene has been found to be induced by VDR activation through VDR binding, together with RXRα, to a response element approximately 7 kb upstream of the PDGF-A gene's transcription start site. This may partially explain why $1,25(OH)_2D_3$, in some settings, promotes mitogenesis and growth while also exhibiting proapoptotic and anti-growth factor properties. However, induction of the PDGF-A gene by activated VDR is not observed in all VDR-expressing cell lines, suggesting additional mechanisms inhibiting this function in some

cells [159]. Additionally, $1,25(OH)_2D_3$ has been found to induce other growth factor genes such as TGF-β in human keratinocytes, nerve growth factor in mouse fibroblasts, and VEGF in several osteosarcoma cell lines [160–162].

Contrastingly, the addition of $1,25(OH)_2D_3$ has been observed to decrease PDGFR-β activity in human prostate cancer cells by reducing PDGFR-β expression levels, thereby inhibiting PDGF-BB-mediated and basal cell growth [163]. Furthermore, a general reduction in DNA synthesis has been observed in human and embryonic mice fibroblasts treated with $1,25(OH)_2D_3$ before stimulation with either serum or PDGF [164].

$1,25(OH)_2D_3$ also appears to play a crucial role in blood vessel formation and maintenance, partly mediated through PDGF-mediated signaling. In VDR knock-out mice, upregulation of angiogenic factors such as PDGF-BB and VEGF has been observed in tumor-infiltrating vessels compared to wild-type mice [133]. VDR knock-out mice exhibited less vascular pericyte coverage, more extensive vascular volume, greater vascular leakage, and larger tumors compared to wild-type mice. Treatment with $1,25(OH)_2D_3$ inhibited the growth of tumor-derived endothelial cells in the wild-type but not VDR knock-out mouse model [133].

Jamali et al. also reported an intricate cross-talk in the regulation of retinal pericytes during vascularization between $1,25(OH)_2D_3$, VEGFR-R2, and PDGFR-β. In wild-type *Vdr* (+/+) mice, $1,25(OH)_2D_3$ reduced pericyte proliferation and mobility, leading to G0/G1 cell-cycle arrest, and reduced pericyte attachment to ECM compared to knockout *Vdr* (-/-) mice. Additionally, $1,25(OH)_2D_3$ incubation induced an increase in VEGF expression and increased co-localization of VEGFR-2 with PDGFR-β in pericytes, resulting in attenuated VEGF-R2 and PDGFR-β signaling and a decrease in proangiogenic activity of retinal pericytes. This suggests that $1,25(OH)_2D_3$ also plays a crucial role in regulating retinal vascular integrity, at least partly through RTKs [165]. In another study, Brun et al. observed that upon abrogation of PDGFRs, $1,25(OH)_2D_3$-mediated osteoblastogenesis was prevented in osteoblasts [166].

Present investigation

Paper I. Differential impact of lipid raft depletion on platelet-derived growth factor (PDGF)-induced ERK1/2 MAP-kinase, SRC and AKT signaling.

This study explored the potential connection between membrane rafts and PDGFR-β signaling. Methyl-β-cyclodextrin (MβCD) was used to deplete cholesterol from human foreskin fibroblasts (BJ-hTERT) or human osteosarcoma (U2OS) cells, which purportedly disrupts membrane rafts and inhibits membrane raft-mediated signaling. We found that cholesterol depletion in these cells impacted PDGFR-dependent signaling in a cell-specific manner. In both U2OS and BJ-hTERT cells, PDGFR-dependent activation of AKT was negatively affected by depletion of cholesterol, whereas ERK1/2 activation was only decreased in BJ-hTERT cells, and autophosphorylation of PDGFR was decreased only in U2OS cells. In addition, we also observed a more severely affected morphology in two cancer cell lines (U2OS and T98G) as compared to two non-cancer fibroblast cell lines (HFL1 and BJ-hTERT). Possibly, this implies there could be differential membrane raft-dependent PDGFR-signaling between different cell types, which potentially could be a new avenue for cell-specific targeted therapeutics, and has interesting possibilities in further studies.

Paper II. Activated EGFR and PDGFR internalize in separate vesicles and downstream AKT and ERK1/2 signaling are differentially impacted by cholesterol depletion.

This study contrasts the RTK epidermal growth factor receptor (EGFR) potential membrane raft-dependent signaling with PDGFR-β, after cholesterol depletion of BJ-hTERT cell with Methyl-β-cyclodextrin. In addition, potential differences in ligand-induced EGFR and PDGFR-β receptor trafficking were explored. It was observed that ligand-induced autophosphorylation and dimerization of EGFR was increased upon cholesterol depletion, in contrast to PDGFR-β, which was unaffected by the process. Similarly, to PDGFR-β, EGFR-dependent downstream activation of AKT and ERK1/2 was negatively impacted by cholesterol removal. It was further observed that EGFR and PDGFR-β use overlapping and different internalization pathways, where both receptors are recruited to clathrin-coated pits, but could potentially differ in clathrin-independent internalization pathways. It was observed that EGFR is

recruited to and potentially internalized by caveolae upon receptor activation. In contrast, PDGFR had a high level of association with caveolae at the steady state, which gradually decreased upon receptor activation, likely corresponding to PDGFR leaving the caveolae as it is activated. These differences in trafficking could give a partial explanation for the selective effects of cholesterol depletion on their signaling.

Paper III. Silencing of Rho GTPases Cdc42, Rac1 or RhoA reduces PDGFRα and -β phosphorylation and downstream signaling of STAT1 and STAT3.

Paper III explores the relationship between PDGFR and three Rho GTPases: Cdc42, Rac1, and RhoA. To investigate the Rho GTPases role in regards to PDGFR activation, downstream signaling, and receptor internalization. We found that the knock-down of any of the three Rho GTPases caused reduced activation via phosphorylation of PDGFR, which was not caused by a reduction in receptor dimerization or receptor stability. In addition, the knockdown further reduced downstream activity of STAT1 and STAT3, and also STAT1 stability, whereas downstream ERK1/2 and AKT activation and stability were unaffected. Further, it was also observed that the three Rho GTPases have a role in the clearance of PDGFR from the plasma membrane, where RhoA was observed to have a role in maintaining steady-state levels of PDGFR on the plasma membrane, while Cdc42 and Rac1 were shown to be involved in ligand-induced internalization of PDGFR. Together, this unveils an interesting relationship between the Rho GTPases and PDGFR, and their role in modulating the receptor signaling. This is important both for deepening the understanding of proper and aberrant receptor function, as well as for the long-term goal of the identification of new potential pharmaceutical targets.

Paper IV. Unique signaling cross-talk between $1,25(OH)_2D_3$ and the growth factors EGF and PDGF.

The interplay between different and interconnected signaling pathways are highly complex. In this manuscript we explore potential interactions between EGFR or PDGFR signaling with that of $1,25(OH)_2D_3$ signaling. Although both PDGFR and EGFR activate similar downstream signaling pathways, this occurs with distinct and discreet differences, and perhaps due to this, this results in cross-talk with the $1,25(OH)_2D_3$ target nuclear receptor, vitamin D receptor (VDR), in specific and unique ways. For instance, we found that CYP24A1, a known positively induced target gene of VDR, was more greatly upregulated with the additional presence of the EGFR ligand EGF, over the solitary induction with just $1,25(OH)_2D_3$ in U2OS, T98G and U251 cells. This increase was found to be dependent of the activation of either Mek1/2 or PI3K. However, addition of PDGF-BB did not have this potentiating effect, as observed with the combinational addition of EGF and $1,25(OH)_2D_3$. Not all target genes of VDR were observed to have this synergistic cross-talk effect with

the combined addition of $1,25(OH)_2D_3$ and EGF however, as another target gene of VDR, CYP27B1, expression was only responsive to addition of $1,25(OH)_2$ vitamin D_3, and not further affected by presence of either PDGF-BB or EGF. Even though PDGF-BB was not found to effect expression of CYP24A1, conversely we found that $1,25(OH)_2D_3$ negatively impacted PDGF-BB induced phosphorylation of PDGFR, as well as proliferation in U2OS cells. In summation, cross-talk between $1,25(OH)_2D_3$-related signaling occurs in unique and specific ways with either EGF or PDGF-BB.

Future perspectives

Paper I

Identifying molecular mechanisms underlying reduced PDGFR and downstream activities

In Paper I, we discovered that the cholesterol-depleting agent MβCD disrupts PDGFR-β signaling pathways in human fibroblast BJ-hTERT cells, specifically affecting ERK1/2 and to a lesser extent, AKT pathways. In contrast, human osteosarcoma U2OS cells showed a broader disturbance, affecting PDGFR-β activation itself and leading to greater reductions in AKT and SRC activation, while not affecting ERK1/2. These results highlight cell-specific responses to MβCD, influenced by variations in lipid and sterol content across cell lines.

To further explore these findings, we plan to investigate the differential integration of Ras isoforms (H-Ras, K-Ras, N-Ras) with membrane rafts, which may explain the varied signaling responses [167, 168]. Using proximity ligation assays, we aim to examine the interaction between PI3K-AKT and Ras-Raf-Mek-ERK1/2 pathways and their modulation by MβCD. In addition, examining post-translational modifications of PDGFR-β and downstream effectors may reveal new regulatory mechanisms. In addition, RNA sequencing of cells treated with MβCD can identify changes in gene expression profiles, uncovering downstream targets and pathways regulated by lipid rafts. Employing other purported membrane raft disruptors like zaragozic acid and cholesterol oxidase will help further solidify the role of membrane rafts in PDGFR-β signaling [169].

Expanding this study to additional cell types, such as other cancer cell lines and primary cells from various tissues, could broaden the perspective on how lipid raft-dependent signaling varies. To also expand to in vivo studies using animal models would offer a more comprehensive insight into the therapeutic potential and side effects of targeting lipid rafts.

Investigating the therapeutic implications of these findings would also be beneficial. Such as, studying the effects of combination therapies with cholesterol-lowering drugs and other targeted therapies or chemotherapy agents to determine potential synergistic effects. Further, by assessing the expression

levels of lipid raft-associated proteins and PDGFR-β in patient samples one could correlate these findings with clinical outcomes, evaluating whether these components could function as biomarkers for prognosis or therapeutic response.

By integrating these strategies, we can advance our understanding and therapeutic exploitation of lipid raft-dependent signaling in PDGFR-β pathways.

Paper II

Delving deeper into characteristics of specific internalization for EGFR and PDGFR

In paper II, we explored the differential signaling and internalization patterns of EGFR and PDGFR-β in response to lipid and sterol depletion using MβCD in BJ-hTERT human foreskin fibroblasts. We observed that EGFR and PDGFR-β internalize into distinct compartments, likely early endosomes, following simultaneous receptor activation, but converge in perinuclear endosomes after prolonged activation. EGFR shows recruitment to caveolin-1 upon activation, while PDGFR-β dissociates from it, indicating distinct endocytic pathways, with both receptors associating with clathrin, as confirmed by proximity-ligation assays.

Our findings suggest that lipid rafts play a differential role in modulating EGFR and PDGFR-β signaling. Notably, sterol and lipid depletion increased EGFR dimerization and activation but did not affect PDGFR-β, indicating that membrane rafts might negatively modulate EGFR activation. This aligns with previous studies showing that EGFR translocates out of lipid rafts to become dimerized upon ligand binding [170–172].

Several avenues remain to be explored based on our findings. Firstly, the negative impact of MβCD-induced sterol and lipid depletion on the downstream activation of ERK1/2 and AKT pathways warrants further investigation. Specifically, understanding the distinct mechanisms by which PI3K-AKT pathway activation is perturbed in EGFR-dependent signaling, compared to PDGFR-β, and could provide deeper insights into raft-associated signaling and its variability across different RTKs.

Additionally, as observed with PDGFR-β in different cell lines (human foreskin fibroblasts and human osteosarcoma cells), it would be beneficial to expand the study to other cell models to determine if EGFR-dependent signaling also exhibits cell-type specific responses to cholesterol depletion.

The differential internalization routes of EGFR and PDGFR-β could explain the observed variations in downstream AKT and ERK1/2 activation. Given

that signaling can occur from internalized receptors, different internalization pathways might expose the receptors to unique regulatory effectors and trafficking dynamics, ultimately influencing signaling outcomes and sensitivity to lipid and sterol content [173].

Future research should aim to further delineate variations in internalization and sorting routes of EGFR and PDGFR-β. This could help in understanding how cells fine-tune signaling outcomes and why these differ among RTKs.

Paper III

Molecular mechanisms underlying reduction in STAT activities and total protein-levels

In paper III, we investigated the role of the three classical Rho GTPases—Cdc42, RhoA, and Rac1—in regulating the stability and signaling of PDGFR, in contrast to their known effects on EGFR and potentially VEGFR2 [114, 174]. Our initial hypothesis suggested that these GTPases might similarly influence PDGFR stability. However, our findings revealed that while these Rho GTPases play a role in regulating STAT1 stability and the activity of STAT1 and STAT3 in a PDGFR-activated manner, they do not significantly affect PDGFR internalization and stability.

Notably, the depletion of Cdc42, RhoA, and Rac1 did not markedly influence PDGFR-β internalization, but only to a small extent, contradicting the idea that STAT3 signaling is reduced due to disrupted receptor endocytosis. Instead, these GTPases appear to have a modest role in PDGFR activation, with more substantial effects observed on STAT1 stability and STAT3 activity, as has been previously suggested [175].

Several intriguing findings from this study warrant further exploration. Firstly, the regulation of STAT stability and activity by Rho GTPases, particularly STAT1, should be investigated in detail. Previous research has indicated that Rho GTPases can regulate protein stability via E3 ubiquitin ligases, suggesting a similar mechanism might be at play with STAT1 [114]. Investigating the impact of Rho GTPase depletion on STAT1 ubiquitination could provide insights into this regulatory pathway. Additionally, exploring whether the reduction in STAT1 levels is due to accelerated degradation via proteasomal, lysosomal, or autophagosomal pathways would be valuable. Examining whether the decreased levels of STAT1 are influenced at the transcriptional level should also be investigated.

Furthermore, the observed abrogation of STAT3 activation, independent of PDGFR internalization, suggests alternative regulatory mechanisms at play. Identifying the specific pathways through which Rho GTPases modulate

STAT3 activation could reveal novel therapeutic targets. Expanding this study to assess the role of Rho GTPases in regulating other members of the STAT family would provide a broader understanding of their function in RTK signaling.

Our data indicate that Rho GTPases contribute to the differential regulation of PDGFR and STAT signaling, distinct from their roles in EGFR and VEGFR2 pathways. Future studies should focus on delineating the precise mechanisms by which Rho GTPases influence these pathways.

Paper IV

Identifying molecular mechanisms behind $1,25(OH)_2D_3$ negative impact on PDGFR activation

From the insights revealed in paper IV study on the signaling cross-talk between $1,25(OH)_2D_3$ and growth factors EGF and PDGF, there is a possibility to further expand this research into broader and more detailed explorations. Here we found that activation of one of VDRs target genes, CYP24A1, was potentiated by co-stimulation with EGF and $1,25(OH)_2D_3$, while another target gene, CYP27B1, was unaffected by simultaneous EGF-stimulation. This potentiating effect was not observed with the co-stimulation of PDGF-BB together with $1,25(OH)_2D_3$, even though EGFR and PDGFR target overlapping downstream pathways. Indicating specific cross-talk between EGFR and VDR. On the other hand, $1,25(OH)_2D_3$ was in turn observed to modulate PDGF-BB-induced PDGFR phosphorylation and proliferation, indicating a role of $1,25(OH)_2D_3$ in modulating PDGFR-activity. The differential effects observed between the growth factors in modulating VDR-related signaling, or in $1,25(OH)_2D_3$-signaling in modulating PDGFR-mediated signaling pathways, suggest a complex regulatory mechanism that warrants further investigation.

By studying additional molecular pathways and receptors that participate in or are affected by this cross-talk, there is the potential to gain more detailed information on cellular responses and adaptations, which could have significant implications for understanding cellular behaviors in different contexts, including cancerous and non-cancerous conditions.

To further expand this study a more comprehensive analysis of the gene expression changes driven by the combinatory treatments of EGF, PDGF-BB, and $1,25(OH)_2D_3$ should be done. High-throughput genomic technologies could help in identifying which genes are regulated by the combinational treatments of growth factor and $1,25(OH)_2D_3$, thus providing a more comprehensive profile of how the genomic landscape is influenced by these interactions.

Extending the study to include various cell types and pathological states would also enhance our understanding of the universality and specificity of these signaling interactions. Collectively, these efforts could significantly enhance our understanding of cell signaling complexities.

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This thesis was partially created with the assistance of GPT-4, Open AI's large-scale language generation model. Upon generating draft language, I reviewed, edited, and refined the text to my own liking and retain full responsibility for the content presented in this publication.

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