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Regulation of cell polarity and invasion by TGF-β and BMP signaling

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Abstract

Shahidi Dadras, M. 2017. Regulation of cell polarity and invasion by TGF-β and BMP signaling. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1403. 53 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-0171-6.

Transforming growth factor β (TGF-β) and bone morphogenetic protein (BMP) signaling pathways are involved in many physiological processes during embryonic and adult life. TGF-β promotes epithelial to mesenchymal transition (EMT). We identified a gene target of TGF-β signaling, encoding the salt-inducible kinase 1 (SIK1). A potential substrate of this kinase, the polarity protein Par3, is an established regulator of tight junction assembly. SIK1 associates with Par3, can potentially phosphorylate Par3 and leads to its degradation, contributing to tight junction disassembly.

Glioblastoma multiforme (GBM) is a common malignancy in the central nervous system, characterized by high heterogeneity, invasiveness, and resistance to therapy. One of the causes of heterogeneity and therapy-resistance is the existence of glioblastoma stem cells (GSCs). TGF-β signaling promotes self-renewal while BMP signaling induces differentiation of GSCs. Snail is a potent inducer of the EMT in carcinomas. However, in the context of GBM, Snail induces BMP signaling and represses TGF-β signaling through interaction with SMADs, the signaling mediators of TGF-β and BMP. In conclusion, Snail differentially regulates the activity of the opposing BMP and TGF-β pathways, thus promoting an astrocytic fate switch and repressing stemness in GSCs.

Although profound changes in cell polarity is a hallmark of invasive malignancies, little is known about the role of the polarity machinery in tumor suppression. Patient transcriptomic data suggested low Par3 expression, correlating with poor survival of the GBM patients. Par3 silencing decreased the GSC self-renewal capacity and enhanced their invasiveness. Transcriptomic analysis indicates that loss of Par3 leads to downregulation of genes encoding mitochondrial enzymes that generate ATP. These results support a novel role of Par3 in GBM, beyond its contribution to junctional contacts between cells.

Another regulator of TGF-β and BMP signaling is the liver kinase B1 (LKB1). According to GBM patient mRNA analysis, high levels of LKB1 correlate with poor prognosis. Silencing of LKB1 in GSCs impairs invasion and self-renewal capacity due to downregulation of genes involved in these processes. Moreover, loss of LKB1 induces mitochondrial dysfunction, leading to decreased ATP levels. Collectively, this thesis has delivered a group of novel regulatory pathways that control critical aspects of cancer cell polarity, invasion and stemness.

Keywords: Cancer, cancer stem cells, invasion, metastasis, polarity, TGF-β signaling.

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To my parents and grandmother.
For your endless love and support.

List of Papers

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

I **Shahidi Dadras, M. ***, Vanlandewijck, M. *, Lomnytska, M., Mahzabin, T., Lee Miller, M., Busch, C., Brunak, S., Heldin, C-H., Moustakas, A. (2017) The protein kinase SIK downregulates the polarity protein Par3. *Oncotarget*, *Article in press*.

II Caja, L., **Shahidi Dadras, M. ***, Tzavlaki, K. *, Tan, E-J., Hatem, G., Maturi, P., Morén, A., Wik, L., Watanabe, Y., Savary, K., Kamali-Moghaddam, M., Uhrbom, L., Heldin, C-H., Moustakas, A. (2017) Snail regulates BMP and TGF-β pathways to control the differentiation status of glioma initiating cells . *Manuscript*.

III **Shahidi Dadras, M. ***, Caja, L.*, Heldin, C-H., Moustakas, A. (2017) Par3 promotes glioblastoma stem cell self-renewal while inhibiting cell invasion. *Manuscript*.

IV **Shahidi Dadras, M.**, Caja, L., Webb, A., Heldin, C-H., Moustakas, A. (2017) The protein kinase LKB1 takes oncogenic actions in glioblastoma promoting self-renewal and invasiveness. *Manuscript*.

* Authors contributed equally to the work.

The following paper, is not included in the thesis:

Kahata, K., **Shahidi Dadras, M.**, Moustakas, A. TGF-β family signaling in epithelial differentiation and epithelial-mesenchymal transition. *Cold Spring Harb Perspect Biol*. 2017 Feb 28. Pii:a022194.

Contents

Abbreviations

ActR-II	Activin type II receptor
ADAM	A disintegrin and metalloproteinase
ALK	Activin receptor-like kinase
AMH	Anti-Müllerian hormone
AMPK	Adenosine monophosphate-activated kinase
aPKC	Atypical protein kinase C
BHA	Butyl-hydroxyanisole
bHLH	Basic helix-loop-helix
BM	Basement membrane
BMP	Bone morphogenetic protein
BRSK	Brain-specific kinase
CBP	Creb binding protein
CDK	Cyclin-dependent kinase
CL	Classical
CNS	Central nervous system
Co-Smad	Common-mediator Smad
CSC	Cancer stem cell
CTGF	Connective tissue growth factor
DAP	Death associated protein kinase
DLG	Disc large
DSB	Double-stranded DNA break
ECM	Extracellular matrix
ELDA	Extreme limiting dilution assay
EMT	Epithelial to mesenchymal transition
ERK	Extracellular signal regulated kinases
GBM	Glioblastoma multiforme
GDF	Growth and differentiation factor
GSC	Glioma stem cell
GSK-3β	Glycogen synthase kinase-3β
HCC	Hepatocellular carcinoma
HIPK2	Homeodomain-interacting protein kinase 2
HSP90	Heat shock protein 90
I-Smad	Inhibitory Smad
ICD	Intracellular domain
ID	Inhibitor of differentiation
JAM	Junctional adhesion molecule
JNK	c-Jun N-terminal kinase
LAP	Latency-associated peptide
Lgl	Lethal giant larvae

LIF	Leukemia inhibitory factor
LIMK2	LIM domain kinase 2
LKB1	Liver kinase B1
LLC	Large latent component
LSCC	Lung squamous cell carcinoma
LTBP	Latent TGF-β binding protein
MES	Mesenchymal
MH	Mad homology
MMP	Matrix metalloproteinase
MRLC	Myosin regulatory light chain
MSK	Myocardial SNF1-like kinase
mTORC1	Mammalian target of rapamycin complex 1
NAC	N-acetyl-L-cysteine
NLS	Nuclear localization signal
OS	Overall survival
PAH	Pulmonary arterial hypertension
PAK1	P21 activated kinase-1
Par	Partitioning defective
PI3	Phosphatidylinositol
PJS	Peutz Jeghers syndrome
PKA	Protein kinase A
PKC	Protein kinase C
PN	Proneural
R-Smad	Receptor-activated Smad
ROS	Reactive oxygen species
SARA	Smad anchor for receptor activation
SBE	Smad binding element
SIK1	Salt inducible kinase 1
siRNA	Short interfering RNA
SLC	Small latency complex
STK11	Serine/threonine kinase 11
TACE	TNF-α converting enzyme
TAK1	TGF-β activated kinase 1
TGF-β	Transforming growth factor β
TIEG1	TGF-β inducible early gene 1
TIF1γ	Transcription intermediate factor 1 γ
TJ	Tight junction
TME	Tumor microenvironment
TORC	Transducer of CREB activity
TRAF	TNF-α receptor-associated factor
TSC2	Tuberous sclerosis complex 2
TTF1	Thyroid transcription factor 1
TβR	TGF-β receptor
UBA	Ubiquitin associated
VEGF	Vascular endothelial growth factor

Introduction

Cancer is the generic name given to a large group of diseases that can affect any part of the body and it is the second leading cause of mortality worldwide. The major cause of lethality in cancer is metastasis. During metastasis cancer cells gain invasive properties, leave the primary tumor, entering the circulation and form metastases in different organs. Within the tumor mass, a distinct population of cancer cells remains proliferative and undifferentiated. These cells are called cancer stem cells (CSCs) and are the main cause of relapse and metastasis by giving rise to new tumors. Increasing knowledge about the characteristics of CSCs is necessary for improvement of the survival and the quality of life of cancer patients.

Transforming growth factor β (TGF- β) and bone morphogenetic protein (BMP) signaling are two well-known signaling pathways playing important roles in the regulation of CSCs and metastasis. TGF-β promotes tumorigenesis by inducing cancer invasion and epithelial to mesenchymal transition (EMT). During EMT, cells lose their polarity and cell-cell junctions, and gain mesenchymal characteristics that are correlated with invasiveness. Additionally, TGF-β promotes self-renewal, while BMP induces differentiations in different sets of malignancies.

As a result, this thesis work is focused on the regulation of polarity and invasion by TGF-β and BMP signaling pathways and the mechanisms through which these two specific cytokines exert their functions on cancer cells.

1.Transforming growth factor beta (TGF-β) and bone morphogenetic protein (BMP) signaling pathways

1.1. TGF-β family ligands

The TGF-β family of ligands is a group of secreted and dimeric cytokines, encoded by 33 genes in human and mice [1]. These cytokines play a key role in normal development and disease by controlling cell physiology, proliferation, apoptosis, differentiation and migration [2, 3]. TGF-β family ligands are classified in two different subgroups according to their functions, TGF-β, activins, nodal, and some of the growth and differentiation factors (GDFs) as the TGF-β-like group; and the other group of ligands make the bone morphogenetic protein (BMP)-like group including BMPs, different GDFs and the anti-Müllerian hormone (AMH) [4].

All TGF-β ligands are produced as an inactive precursor molecule consisting of three domains: N-terminal signal peptide, amino-terminal prodomain (latency associated peptide, LAP) and a C-terminal mature domain [5, 6]. The inactive TGF-β precursor is a dimer, most often linked by a single disulfide bond (e.g. TGF-β) but sometimes lacking the covalent disulfide linkage. The inactive precursor protein is first processed in the Golgi apparatus by endoproteases [7]. The mature peptide and LAP remain non-covalently bound together, known as the TGF-β inactive complex or small latency complex (SLC) [5]. Furthermore, during processing, the SLC binds to the latent TGF-β binding proteins (LTBPs) and forms the large latent complex (LLC). Upon secretion, the LLC associates covalently to extracellular matrix (ECM) proteins, such as fibronectin and fibrillin-1, a mechanism that keeps TGF-β stored in an inactive form and makes it available for later action [5, 8]. Mature bioactive TGF-β that consists of the processed C-terminal homodimeric polypeptide binds directly to the protein core of a transmembrane proteoglycan receptor, β-glycan or TGF-β type III receptor. This co-receptor presents ligand to the signaling serine/threonine kinase receptors. β-glycan is not expressed in all cell types and it is not absolutely necessary for TGF-β signaling. In endothelial cells, the β-glycan function seems to be replaced by a related transmembrane glycoprotein named endoglin [9].

1.2. Smad-dependent signaling

There are eight Smad family members in vertebrates that can be divided into three distinct groups: receptor-activated Smads (R-Smads) including Smad1, 2, 3, 5, and 8, the common mediator Smad (Co-Smad) Smad4, and the inhibitory Smads (I-Smads) consisting of Smad6 and 7 [10]. The Smads have two folded domains, the N-terminal (MH1, mad homology 1) domain and the C-terminal MH2 domain, connected to each other by an extended and relatively unfolded linker region [3, 11] (Figure 1). The N-terminal MH1 domain is a DNA-binding domain that also contains a nuclear localization signal (NLS), whereas the MH2 domain is crucial to mediate the complex formation between R-Smads and Co-Smad. In addition, many regulatory proteins including transcription factors, and the co-activators p300 and Creb binding protein (CBP), bind selectively to the MH1 or MH2 domains [12-14].

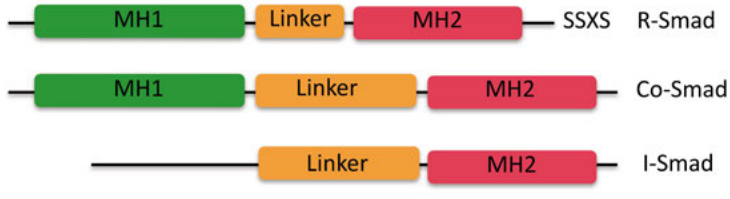

Figure 1. Illustration of the structure of Smad proteins with MH1 (green), linker (yellow) and MH2 (pink) domains.

The Smad signaling cascade initiates through binding of the TGF-β/BMP ligand to a heterotetrameric complex of serine/threonine kinase receptors known as type II and type I. The receptors are transmembrane proteins that transduce signals from the cell membrane to the nucleus through activation of the Smad proteins, which regulate gene expression (Figure 2)[3, 4, 15].

Members of this family of receptors have structural characteristics similar to both serine/threonine and tyrosine kinases. This receptor family is rather small in mammals, with only 12 members. In humans, there are seven type I receptors and five type II receptors through which individual members of the TGF-β family transduce their signals by binding to different pairwise combinations of the receptors [4, 16]. For instance, the TGF-β-like group of ligands signal via the type II receptors TβR-II, activin type II receptor (ActR-II) A and B together with the type I receptors, including, TβR-I (ALK5), activin receptor-like kinase 4 (ALK4), and ALK7. While the BMP-like group of ligands signal via type II receptors such as BMPRII, ActR-IIA and ActR-

IIB, in combination with the type I receptors ALK1, ALK2, ALK3 and ALK6 [4, 11].

TGF-β ligands bind TβRII with higher affinity than TβRI. Thus the binding of TGF-β ligands occurs first to TβRII [17]. On the contrary, BMP type I receptors have a higher affinitiy for the ligand than the type II receptor. Thus, the ligands for the BMP receptors bind to the receptor complex with lower affinity than TGF-β ligands to the corresponding receptors[16, 18, 19]. Upon ligand-induced oligomerization, the type II receptor phosphorylates the type I receptor at its glycine and serine (GS) rich segment on specific serine residues of the juxtamembrane domain, thus activating the kinase of type I receptor [11, 20]. The activated type I receptor kinase phosphorylates the receptor-activated (R) – Smads, including Smad2/3 in TGF-β signaling pathways and Smad1/5/8 in BMP signaling pathways, in their Ser-X-Ser motifs within the C-terminal of the MH2 domain [13, 21] (Figure 2). However, in endothelial cells TGF-β ligands can lead to phosphorylation of Smad1/5/8 through binding to a receptor complex containing the tissue selective ALK1 type I receptor [22, 23]. Some scaffolding cytoplasmic proteins associate with type I/II receptors and Smads, for instance, the Smad anchor for receptor activation (SARA) protein binds to non-activated Smad2 and the receptor complex facilitating presentation of Smad2, and to some extent Smad3 promoting their phosphorylation by the type I receptor. Moreover, binding of SARA to Smad2 inhibits its nuclear translocation [15, 24-26]. In an analogous manner, a SARA-like accessory function has been reported in BMP signaling, i.e. endofin [16, 27]. Once phosphorylated, R-Smads interact with Smad4 forming trimeric protein complexes, which can be translocated to the nucleus where they bind to DNA sequences called Smad-binding elements (SBE), 5´-GTCT-3´ or its reverse complement 5´-AGAC-3´, via the Smad MH1 domains. Smad complexes act as transcription factors regulating the expression of different genes involved in embryogenesis, differentiation, proliferation, apoptosis, migration and immune responses [20, 21]. The binding affinity of the Smads to SBE is relatively weak. For this reason, high affinity and high specificity recognition of DNA by Smad complexes depends on Smad-interacting transcription factors and co-activators and co-repressors, which contribute to the transcriptional responses induced by TGF-β family members [21]. Examples of Smad-interacting transcription factors include: CBP/p300, bHLH (basic helix-loop-helix), bZIP, forkhead family of transcription factors (for example FoxH1, FoxO1), nuclear receptors, and homeodomain proteins[28, 29]. A specific combination of these factors with the Smads and their expression and availability in different cell types determines the specific cellular response to TGF-β and BMP signaling[21]. The Smad pathway is evolutionary conserved and is regulated by posttranslational modifications, such as phosphorylation events, and ubiquitylation, as well as nucleocytoplasmic shuttling by inhibitory (I)-Smads [11, 30]. I-Smads are induced and accumulated in the nucleus by Smad signaling. After stimulation

of cells with TGF-β and BMP, I-Smads are exported to the cytoplasm where they bind to type I receptors and exert negative feedback by blocking R-Smad phosphorylation and R-Smad-Smad4 complex formation, stimulating receptor dephosphorylation by recruiting phosphatases, and promoting receptor poly-ubiquitylation and lysosomal degradation [30].

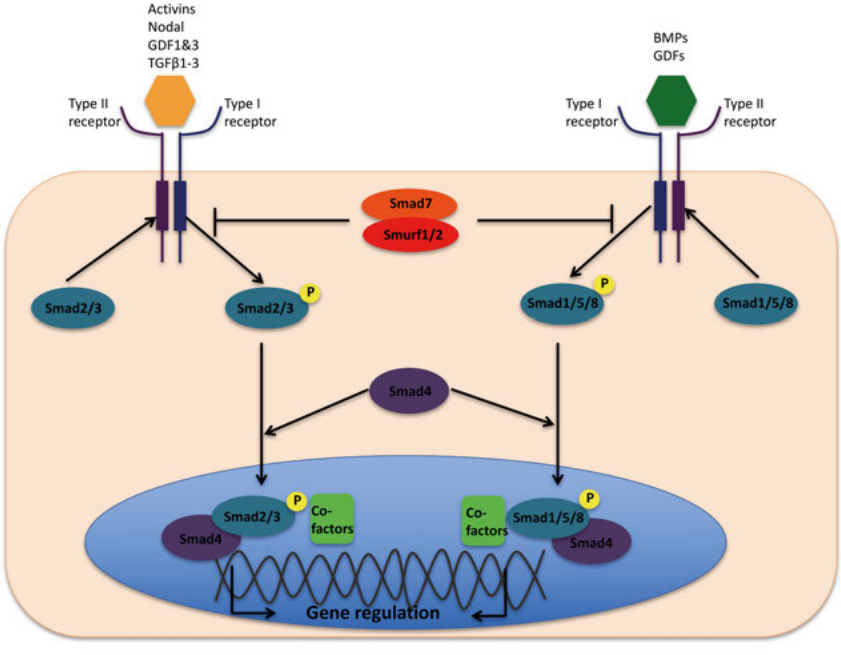

Figure 2. Smad-dependent pathway. TGF-β/BMP ligands dimerize and bind to their corresponding receptors. Upon phosphorylation, type I receptor phosphorylates the R-Smads. Phosphorylated R-Smads form complexes with Smad4 and translocate to the nucleus where they regulate the expression of different genes.

1.3. Smad-independent (non-canonical) signaling

Although Smads are the important signal transducers, an increasing amount of biochemical and developmental evidence has been recollected in favor of Smad-independent or non-canonical TGF-β signaling pathways, through which TGF-β controls a wide spectrum of cellular responses [31]. Non-Smad signaling proteins have three general mechanisms by which they contribute to physiological responses to TGF-β: (1) Direct modification (such as phosphorylation) of Smads by non-Smad signaling pathways, thus modulation of the activity of the central effectors; (2) Smads transmit signals to other pathways by direct interaction and modulation of other signaling proteins, such as protein kinases; (3) The TGF-β receptors directly interact with or

phosphorylate other proteins thus initiating parallel signaling that elicit physiological responses. These non-canonical pathways act as nodes of crosstalk between TGF-β and other signaling pathways, such as tyrosine kinase receptors, G protein-coupled receptors or cytokine receptors. This allows regulation of different cell processes, such as apoptosis, epithelial to mesenchymal transition (EMT), proliferation, matrix regulation and cell differentiation [30]. Early studies showed that TGF-β causes rapid activation of Ras and extracellular signal regulated kinases (ERK1/2) MAP kinases (MAPK) in normal epithelial and colon carcinoma cells [32-34]. Other non-canonical pathways include activation of other MAPK pathways i.e. p38, and c-Jun N-terminal kinases (JNK), phosphatidylinositol-3′ (PI3) kinase, Rho-like GTPases (Rho, Rac and Cdc42), protein kinase A (PKA), partitioning defective (Par) 6 and TGF-β activated kinase 1 (TAK 1) [10, 31, 35, 36] (Figure3). A characteristic mechanism involves the recruitment of ubiquitin ligases of the TNF-α receptor-associated factor (TRAF) family, such as TRAF4 and TRAF6 to the TGF-β receptor complex, which, via ubiquitylation activates the TAK1 protein kinase that then phosphorylates downstream kinases leading to p38 and JNK activation during EMT and apoptosis of epithelial cells[31]. One specific role of TβRI in tumor invasion is via TRAF6-mediated poly-ubiquitylation of this receptor, promoting cleavage of TβRI by TNF-α converting enzyme (TACE), in a PKC-ζ-dependent manner. The liberated intracellular domain (ICD) of TβRI is translocated to the nucleus where it associates with the transcriptional regulator p300 to activate genes involved in tumor cell invasiveness, such as Snail and MMP2 [37]. TRAF6 also has an important role in the activation of PI3-kinase (PI3K) and the downstream kinase Akt (Figure 3) [38]. The mechanism of activation of Par6 will be discussed later. In addition to activation of non-Smad proteins by the type I receptor in the non-canonical pathway, some studies indicate that the Smad proteins themselves can be regulated by other proteins different than type I receptors. For instance during hematopoiesis, transcription intermediate factor 1 γ (TIF1γ) can associate with Smad2-Smad3 and replace Smad4 in the complex [39]. Similar to TIF1γ, the thyroid transcription factor 1 (TTF1) can replace Smad4 in Smad2/3 complexes and regulate specific subsets of genes in response to TGF-β [40]. In some mammary and lung epithelial cells, Ras signaling can inhibit TGF-β signaling by activating the ERK1/2 MAP-kinases which are able to phosphorylate the R-Smads in the linker region and block their translocation in the nucleus [41]. Another example of Smad signaling regulation by non-Smad components is the protein kinase C (PKC), which is capable of phosphorylating Smad3 in the MH1 domain and inhibit its DNA binding [42].

Overall, there is a growing list of post-translational mechanisms that regulate Smad protein function and the concerted activity of non-canonical signaling proteins plus Smads mediates the cellular response to members of the TGF-β family [43, 44].

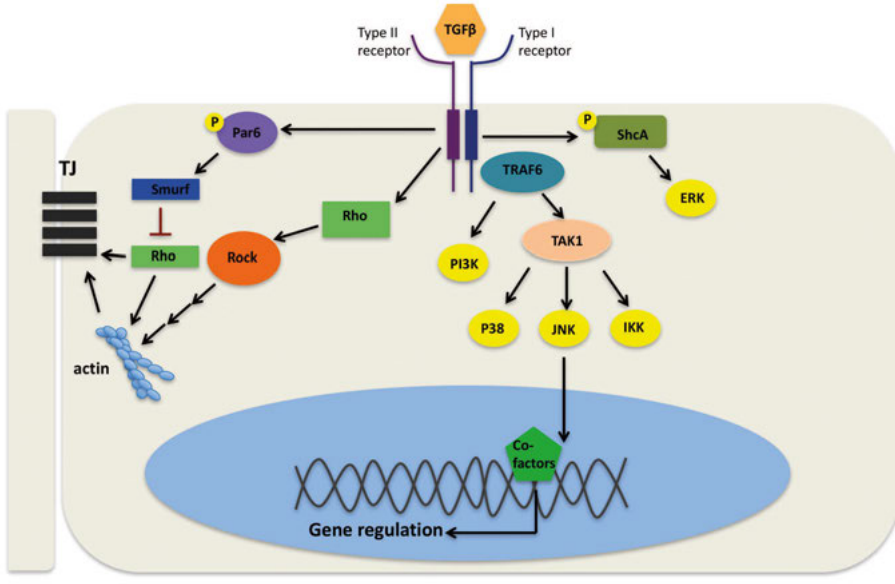

Figure 3. Smad-independent TGF-β signaling. The activated receptor complex activates non-Smad signaling pathways, including Erk, JNK and P38 MAPKs, IKK, Rho and Par6, leading to regulation of different genes, TJ and actin cytoskeleton rearrangement.

1.4. TGF-β physiological responses

The activities of members of the TGF-β family permeate every aspect of life in multicellular organisms. It is therefore impossible to summarize all important physiogical actions of the TGF-βs and the BMPs. In the following sections, I therefore present selective examples that illustrate major principles of function of ligands in the TGF-β family.

1.4.1. TGF-β and growth inhibition

TGF-β potently induces cell cycle arrest of several types of cells. Today it is well-established that TGF-β induces the growth inhibition in epithelial, endothelial and hematopoietic cells, as well as primary fibroblasts of embryonic origin [30, 45]. However, TGF-β has mitotic activity in certain transformed cells and immortalized fibroblasts [46]. The cytostatic effect of TGF-β signaling is very well studied in epithelial cells. TGF-β signaling inhibits cell cycle progression through transcriptional repression of *c-myc* and inhibitor of differentiation (*ID1, 2 and 3*) genes while it activates the expression of cyclin-dependent kinase *(CDK*) inhibitors, including *p15*, *p21*, and *p57*, which are induced by Smad proteins [9, 47, 48]. *P21* is induced rapidly by all TGF-β superfamily receptor complexes, but BMP7 induces higher expression of *p21* than

TGF-β in various epithelial cells [49]. Smad proteins cooperate with Sp1 and p53, leading to upregulation of p21 levels [50-52]. During TGF-β-induced growth inhibition, Smads cooperate with other transcription factors, such as Runx3, to promote *p21* gene expression in gastric epithelial cells [53]. In addition, TGF-β induces Smad3/4 to form a complex with FoxO transcription factors and activates *p21* and *p15*, a process that is negatively controlled by the PI3K pathway, which is a known inhibitor of FoxO in the nucleus [54, 55].

Some non-Smad pathways induced by TGF-β contribute to its cytostatic effects. For instances, TGF-β-induced activation of JNK and p38 leads to phosphorylation and stabilization of p21, therefore increasing its half-life [56].

1.4.2. Apoptosis

TGF-β is a well-known inducer of apoptosis mediating its pro-apoptotic effects through a set of genes regulated by Smads, including phospholipid phosphatase SHIP, death-associated protein kinase (DAPK) and TGF-β-inducible early response gene 1 (TIEG1) [45, 47]. TGF-β/Smad signaling suppresses development of a broad spectrum of cancer types by inhibiting cell proliferation and inducing apoptosis [32, 48, 57]. In hepatocellular carcinoma (HCC), CXXC5, a member of the CXXC-type zinc finger domain-containing protein family, associates with HDAC1 in competition with Smad2/3 and enhances transcriptional activity of Smad2/3 proteins, thus promoting TGF-β-induced cell cycle arrest and apoptosis [58]. Moreover, in gastric SNU-620 carcinoma cells, TGF-β induces expression and activation of the Fas receptor, leading to caspase-8 activation and apoptosis in a Smad3-dependent manner [59]. Additionally, MAPK p38 and JNK mediate apoptotic signals downstream of TGF-β receptors [30]. The pro-apoptotic adaptor protein Daxx has been shown to be required for TGF-β-induced apoptosis through interaction with TGF-β type II receptor and activation of JNK in epithelial cells and hepatocytes [60]. In the Daxx-JNK pathway, homeodomain-interacting kinase 2 (HIPK2) interacts and phosphorylates Daxx. Daxx phosphorylation activates MAPK kinases MKK4 and MKK7, which lead to activation of JNK and induction of apoptosis [61]. Another regulator of TGF-β-induced apoptosis is Smad7, whereby Smad7 acts as a scaffolding protein to facilitate the activation of p38 by interacting with TAK1 [35]. Moreover, TAK1 can form a complex with the BMP receptors through its binding partner TAB1 and the inhibitor of apoptotic caspases XIAP, an E3 ubiquitin ligase. Both TGF-β and BMP receptors activate TAK1, leading to MKK3, 4, 6 and 7 activation, and activation of the two apoptotic pathways, JNK and p38 MAPK, in various cell types [30, 62]. Another mechanism used by TGF-β to promote apoptosis involves mobilization of mitochondrial components. TGF-β induces localization of the septin-like mitochondrial protein ARTS in the cytoplasm, where it binds to and inactivates XIAP. Inactivation of XIAP leads to caspase-3 activation and apoptosis [63-65]. Finally, TGF-β antagonizes

pro-survival signaling through physical interaction of Smad3 with the survival kinase Akt. This interaction leads to inactivation of Akt, thus promoting apoptosis [66, 67].

1.4.3. TGF-β and EMT

TGF-β is abundant in the tumor microenvironment (TME), where it promotes various aggressive characteristics including invasive migration and epithelial to mesenchymal transition (EMT) [68]. In addition, the TGF-β family is implicated in multiple stages of early embryonic development; a prominent example is Nodal, which signals the generation of proximodistal polarity in the early embryo [69]. EMT plays a central role during embryonic development and tissue repair. However, it can adversely cause organ fibrosis and promote carcinoma progression through a variety of mechanisms [70]. During EMT, cells lose their epithelial identity and gain mesenchymal features. The molecular changes that characterize EMT include, transcription factor activation, expression of specific cell surface proteins, downregulation of many cell-cell contacts, reorganization of cytoskeletal components leading to the formation of actin stress fibers, upregulation of vimentin, changes in the expression of specific microRNAs, and production of extracellular matrix (ECM) proteins, such as fibronectin and degrading enzymes [71] (Figure 4).

In cancer cells, EMT promotes invasiveness and stem-cell like features [72]. TGF-β regulates EMT through Smad-dependent and Smad-independent pathways, such as the PI3K/Akt, ERK1/2, p38 and JNK MAPK [73]. Direct interaction between TGF-β receptors and Par6, a regulator of epithelial cell polarity and tight junction assembly, is required for TGF-β-dependent EMT. This interaction leads to phosphorylation of Par6 by TβRII, which in turn recruits Smurf1, an E3 ubiquitin ligase. Smurf1 then stimulates ubiquitylation and degradation of RhoA, thereby leading to loss and disorganization of tight junction and cell polarity [74] (Figure 3). In addition, TGF-β negatively regulates Rho-A through another mechanism by inducing the expression of miR-155 to disrupt tight junction assembly[75]. On the other hand, TGF-β mediates EMT through activation of RhoA and stress fiber formation[76]. Recent studies have shown that TGF-β induces the activity of mTORC2 during EMT and activation of mTORC2 contributes to migration and invasion. The mTORC2 is also required for TGF-β-induced RhoA activation [77].

TGF-β-induced EMT is driven by a transcriptional program that involves the zinc finger transcription factors Snail and Slug, the zinc finger E-box binding homeobox ZEB proteins (ZEB1 and 2), and the basic helix-loop-helix (bHLH) factors E47, E2-2 and Twist [71, 78, 79]. These transcriptional repressors recognize E-box DNA sequences near the transcription initiation site of the E-cadherin gene and other epithelial genes, repressing their expression [9]. Among the mentioned EMT transcription factors (EMT-TFs), Snail, ZEB and Twist control global plasticity programs affecting cell stem-

ness and fate. Aberrant activation of these factors in adult tumor cells is commonly seen, as they enable cancer cell plasticity and promote both tumor initiation and cancer metastasis [80]. Snail also participates in the activation of mesenchymal genes, such as fibronectin [81, 82]. In addition to TGF-β, various signaling pathways, including FGF, WNT and Notch pathways, can induce Snail expression [83-85]. The activity of Snail is regulated post-translationally through phosphorylation events via various kinases, including GSK3β and p21 activated kinase-1 (PAK1) [86, 87]. The high mobility group factor HMGA2 was identified as an upstream inducer of many major transcriptional repressors of E-cadherin during TGF-β-induced EMT. HMGA2 is induced by TGF-β through Smad3/4-dependent mechanism and induces expression of Snail and Twist during EMT of breast cancer cells [88, 89]. Silencing HMGA2 suppresses cell proliferation, migration, invasion and EMT via suppression of TGF-β/Smad and Wnt/ β-catenin pathways in bladder cancer [90]. Moreover, in mammary epithelial cells, HMGA2 suppresses E-Cadherin expression by inducing hypermethylation of the *Cdh1* gene promoter, thus promoting tumor cell invasion [91].

TGF-β also represses the expression of ID2 and ID3 during EMT. ID proteins antagonize bHLH proteins, such as Twist1. In addition, ID2 and ID3 are direct targets of BMP7 signaling pathway, which could explain how BMP7 can inhibit TGF-β-induced EMT [88, 92].

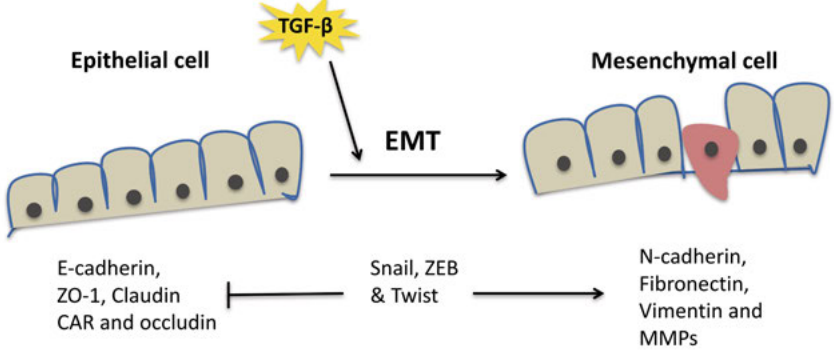

Figure 4. Epithelial to mesenchymal transition (EMT). Epithelial cells that undergo EMT upon TGF-β stimulation (red cell) acquire mesenchymal properties and dissociate from the neighboring cells. EMT-TFs induce EMT by downregulating epithelial and upregulating mesenchymal markers.

1.5. Aberrant TGF-β signaling in cancer

Due to widespread involvement of TGF-β signaling in multiple cellular processes, aberrant TGF-β signaling is associated with the development of many diseases, including cancer [39, 93]. TGF-β signaling plays a dual role in cancer

development. During the initial stages of cancer it acts as a tumor suppressor by inhibiting cell growth, while in the later stages of cancer it acts as a tumor promoter and promotes tumor invasiveness and metastasis [48]. Mutations of TGF-β type I and II receptors and Smads (most prominently Smad4) have been observed in many tumor types, including colorectal cancer, pancreatic cancer and hematopoietic malignancies, indicating the tumor suppressive role of TGF-β signaling in the early stages of certain malignancies. For instance, loss of functional cell surface TβRI correlates with insensitivity to TGF-β-mediated growth inhibition in the development and progression of human lymphoproliferative malignancies[94]. On the contrary, TGF-β signaling remains active and promotes invasion and metastasis of many cancer types, including breast cancer, prostate cancer and colon cancer [95]. These findings support a model, which is mainly corroborated by studies in mouse models of cancer progression, indicating that TGF-β gradually lose its tumor suppressive abilities and gain tumor promoting abilities during carcinogenesis [9, 48].

During tumor progression, cancer cells acquire motility by transforming to migratory cells through EMT. TGF-β is one of the main EMT inducers during cancer progression and metastasis. CULT1, also known as CDP, Cut or CUX-1, is a transcription factor known to be one of the critical TGF-β targets that enhances cancer cell motility and invasiveness [96]. Interactions of cancer cells with the primary tumor microenvironment are important determinants of cancer progression. For instance, direct contact of tumor cells with platelets primes metastasis and induces EMT via synergistic activation of both TGF-β and NF-κB pathways. Specific inhibition of platelet-derived TGF-β and NF-κB signaling in cancer cells prevents metastasis[97].

One of the major components of the defense mechanism against tumor cells is the immune system. T lymphocytes and natural killer cells are the two main immune cells for recognition and elimination of cancer cells. In order to promote metastasis, TGF-β signaling suppresses the immune response against tumor cells by downregulation of T lymphocytes, which, when absent, are not able to recognize and eliminate tumor cells, thus enabling cancer progression [95, 98]. TGF-β is a potent immunosuppressive cytokine in many biological contexts beyond cancer. This exciting topic is not discussed here, as it falls outside the scope of the thesis.

TGF-β signaling can also stimulate angiogenesis, which enables nutrient and oxygen delivery to the tumor cells. Angiogenesis in the tumor environment is achieved at least in part by induction of angiogenic mediators such as vascular endothelial growth factors (VEGFs) and connective tissue growth factor (CTGF), that are direct targets of the TGF-β signaling pathways [95, 99, 100].

1.6. Aberrant BMP signaling in diseases

The firs BMP was discovered in 1965 by Marshall R Urist as a cytokine inducing bone and cartilage formation in ectopic skeletal sites in vivo [101, 102]. BMPs play important roles in the development of many organs during embryogenesis, such as bone, cartilage, muscle, brain and kidney [103].

During bone and cartilage formation, BMP induces differentiation of mesenchymal progenitor cells to the mature osteoblasts through activation of the BMP type I receptors and Smad1/5/8 [104-106]. BMP-induced chemotaxis of mesenchymal progenitor cells plays a key role in development, disease and tissue repair. BMP2 induces the activation of PI3K leading to cortical actin rearrangement, planar cell polarity and chemotaxis[107]. Moreover, some studies have shown that BMPs are involved in the development of several cancers, and are sometimes linked to tumor progression while certain other BMPs act as tumor suppressors [108, 109]. BMPs are known to be important regulators throughout the body and defects in BMP production and functionality are linked to certain pathological conditions [110]. For instance, genetic mutations in the BMP signaling components, such as the BMPR2 gene, are linked to familial or sporadic cases of pulmonary arterial hypertension (PAH) [111]. BMP signaling plays a critical role in the development of the eye and neurogenesis. For example, BMPR1A is essential for lens and retinal growth. Some studies indicate that knockout of BMPR1A leads to formation of small lenses, due to increased apoptosis of lens epithelial cells [110, 112]. BMP13 and BMPR1B are implicated in maintaining cell survival in the eye and loss of either of them results in retinal apoptosis and smaller eye size [110, 113-115]. Another consequence of loss of BMP function is neural defects during the development of central nervous system (CNS). One of the highlights of BMP signaling in neurogenesis is the role of BMP7 signaling which regulates neural development in corticogenesis [116]. BMP7 deletion results in impaired neurogenesis and reduced cortical thickening. Moreover, BMPR1A is essential for the development of neurons involved in feeding behavior regulation [110, 116-118].

In different cancers, BMP proteins have a dual role suppressing and promoting tumorigenesis. In colorectal cancer and glioblastoma, BMPs mainly act as tumor suppressors, while in breast cancer, pancreatic cancer, prostate cancer and bone cancer BMPs promote tumorigenesis and metastasis [119-123].

2. Epithelial polarity and Par complex

The formation of functional epithelial tissues involves the coordinated action of the several protein complexes involved in polarity regulation. In epithelial cells these complexes are key players for the development of apico-basal polarity, asymmetric division and directed cell migration. The major polarity complexes in epithelial cells are the apical polarity complexes, including the Par3/Par6/aPKC (Par complex) and the Crumbs/PALS1/PATJ (Crumbs complex), which are conserved between species and along with a lateral complex, the Scribble/ lethal giant larvae (Lgl)/ Disc large (DLG) complex or Scribble complex. These are crucial to the formation of apical junctions, such as tight junctions in epithelial tissues [124, 125]. Although the Crumbs complex is restricted to epithelial cells, Par and Scribble complexes regulate cell polarity in a broader context, such as during axonal growth in neurons [126].

The Par complex is localized in the apical domain of epithelial cells and is linked to tight junctions (TJ) through interaction of junctional adhesion molecules (JAMs) with Par3 [127]. Par3 is an established central component of the apical-basal polarity system, which is a complex of Par3, Par6 and the atypical protein kinase C (aPKC), and downstream small GTPases of the Rho family, that determines the assembly of cell-cell junctions in epithelial cells, leading to compartmentalization of the epithelial plasma membrane [128]. Mammalian Par3 controls the epidermal barrier, keratinocyte differentiation and stem cell maintenance. Loss of epidermal Par3 leads to disturbed skin barrier, altered expression and localization of tight junction components and increased thickness of epidermis[129]. A tumor suppressor role of Par3 has been proposed in various types of cancer[130]. Par3 silencing promotes tumorigenesis and metastasis through induction of matrix metalloprotease (MMP) 9, destruction of extracellular matrix (ECM), all mediated by aPKC-dependent JAK/Stat3 activation. Par3 expression is significantly reduced in human breast cancer cells, which correlates with active aPKC and STAT3 [131]. Mutation of the Par3 gene has been observed in 8% of lung squamous cell carcinoma (LSCC). Par3 inactivation in LSCCs impairs STAT3 and promotes tumor invasion [132]. Par3 acts as a scaffold protein for the other members of the Par complex, Par6 and aPKC. As a result, any changes in the structure of Par3 that lead to the downregulation of this protein disrupt the Par complex and TJ assembly [125, 133].

Par (partitioning defective) proteins are evolutionary conserved in all the metazoans. For instance, in *Drosophila melanogaster* a Par3-related protein called Bazooka controls the orientation of mitotic spindles in epithelial cells [134]. In all types of eukaryotic cells, asymmetric cell division occurs and is essential for metazoan development. Several studies on asymmetric cell division in *Caenorahbditis elegans* have revealed that Par proteins are necessary for anterior/posterior polarity [135].

Par3 is a large protein with three PDZ (PSD-95/Dlg/Zo-1) domains. These domains mediate protein-protein interactions, usually with other PDZ domains or with specific carboxy-terminal motifs [133, 136, 137]. In addition to other members of the Par complex, Par3 interacts with T-cell lymphoma invasion and Rac activator Tiam1, LIM domain kinase (LIMK2) and the adaptor protein 14-3-3 [126, 138, 139]. 14-3-3ζ binds to Par3 via phosphorylated Ser144 preventing complex formation of Par3 with other members of the Par complex [139]. Overexpression of 14-3-3ζ results in a severe disruption of polarity, whereas overexpression of a 14-3-3ζ mutant that is unable to bind to phospho-proteins has no effect on cell polarity [139].

In addition to regulation of cell polarity, Par3 interacts with Ku70 and Ku80, two regulatory proteins that play essential roles in repairing double-stranded DNA breaks (DSBs). Nuclear association of Par3 with Ku70/Ku80 is enhanced after γ-irradiation proposing an unexpected role of cell polarity in the DSB repair mechanism [140].

Par6 and aPKC regulate polarization processes of normal cells. However, increasing evidence suggests that they also play a role in oncogenic progression [141]. In epithelial cells, TGF-β-induced Par6 phosphorylation results in dissolution of junctional complexes, cytoskeletal remodeling and increased metastatic potential [74, 141]. Some studies suggest that aPKC may be an attractive therapeutic target against tumor progression. For instance, loss of function experiments of aPKC in lung cancer cells reduces the invasive phenotype of cancer cells and tumor progression in vivo [142, 143].

3. Liver kinase B1 (LKB1)

The liver kinase B1 (LKB1), also known as serine/threonine kinase 11 (STK11), is a tumor suppressor kinase which is frequently mutated in sporadic lung adenocarcinoma and cervical cancer. Inactivating germline mutations of STK11 is linked to Peutz Jeghers syndrome (PJS) characterized by gastro-intestinal polyposis, mucocutaneous pigmentation and in severe cases a higher risk of developing malignancies [144, 145]. Increasing numbers of studies have contributed to the understanding of the molecular functions of LKB1 and have revealed its contribution to energy metabolism, cell death, cell proliferation and cellular polarity [144, 146]. Heterozygous $LKB1^{+/-}$ mice exhibit gastrointestinal polyposis identical to those seen in individuals affected with PJS. However, loss of LKB1 has been associated with resistance from oncogenic transformations such as Ras. These findings place LKB1 in a distinct class of tumor suppressors, suggesting that LKB1 loss of function may happen in early stages of tumorigenesis while LKB1 might be beneficial for the late stages of tumor progression [147]. In cells, LKB1 forms a heterotrimeric complex with the pseudokinase STRAD and the scaffolding protein MO25 at a 1:1:1 ratio. MO25, with the help of ATP, binds to STRAD and keeps STRAD in a closed and inactive conformation. STRAD in a closed conformation, typical of active protein kinases, binds to LKB1 as a pseudosubstrate and allows LKB1 to change to its active conformation. These findings indicate that STRAD is key factor in regulating LKB1 tumor suppressor activities [148-150]. The trimeric complex of LKB1 with STRAD and MO25 acts as an upstream kinase that activates AMPK (AMP-activated kinase) via phosphorylation at Thr172. Since AMPK inhibits lipid biosynthesis in the G1 phase of the cell cycle, activation of AMPK by LKB1 may explain the ability of LKB1 to act as a tumor suppressor [151]. In addition to interacting with STRAD and MO25, LKB1 associates with heat shock protein 90 (HSP90) chaperone and the Cdc37 kinase-specific targeting subunit for HSP90. Complex formation of LKB1 with HSP90 and Cdc37 is separate from the LKB1:STRAD:MO25 complex. HSP90 and Cdc37 stabilize LKB1 by preventing its degradation through proteasomes. [144, 152, 153].

The microRNA miR-451 regulates cell proliferation and migration in gliomas by targeting the LKB1 scaffolding partner MO25. Thus, miR-451 is a regulator of the LKB1/AMPK pathway, and this represents a fundamental mechanism that contributes to cellular adaptation in response to metabolic stress[154]. In addition, several reports support a miR-451 suppression of glio-

ma migration, suggesting a possible role of LKB1 in promoting cell migration and proliferation under metabolic stress conditions in gliomas [155, 156].

LKB1, in complex with STRAD and MO25, phosphorylates and activates 14 downstream human kinases including: AMPK1, AMPK2, NUAK1, NUAK2, BRSK1, BRSK2, QIK, QSK, SIK, MARK1, MARK2, MARK3, MARK4 and MELK. The catalytic activity of LKB1 and the presence of STRAD and MO25 are required for this activation. These kinases mediate the physiologic effects of LKB1, such as cell growth, cell polarity, cellular energy regulation, as well as LKB1 tumor suppressor activity [157].

3.1. LKB1 and cellular energy regulation

LKB1 plays a key role in controlling the energy charge through activation and phosphorylation of the AMPK family [158]. In normal cells AMPK activation results in inhibition of ATP-consuming processes, such as proliferation and biosynthesis of molecules through regulation of the mammalian target of rapamycin complex1 (mTORC1) pathway [159-161]. Moreover, loss of LKB1 leads to dysfunctional mitochondria and metabolic dysregulation [162]. Cells lacking LKB1 are resistant to cell transformation by oncogenes, and therefore, undergo apoptosis under metabolic stress [163]. Accordingly, LKB1 regulates starvation-induced autophagy as LKB1-deficient zebrafish larvae fail to activate autophagy and this autophagy defect can be partially rescued by inhibition of the mTOR signaling pathway [164]. Active AMPK leads to activation of tuberous sclerosis complex 2 (TSC2), which inactivates the Rheb GTPases and leads to inhibition of the mTOR pathway [144, 165]. However, TSC2-deficient cells remain responsive to energy stress. As a result, AMPK directly phosphorylates the mTOR binding partner Raptor that is required for the inhibition of mTORC1 and cell cycle arrest induced by energy stress. Therefore, during energy depletion, LKB1-activated AMPK is a metabolic checkpoint coordinating cell growth with energy status [166].

3.2. LKB1 and cellular polarity regulation

The link between LKB1 and cellular polarity was first established in 1988 by a set of studies in *C. elegans* mutants. Loss of LKB1 in worms disrupted the normal asymmetries established during embryogenesis. The identified genes in this study were Par-4 (the ortholog of LKB1), serine/threonine kinase Par-1 (MARK), a ring finger protein Par-2, two PDZ domain containing proteins Par-3 and Par-6, and Par-5 (the 14-3-3 protein). Interestingly, subcellular localization of Par-4/LKB1 was not affected by any other Par protein while mutations in Par-4/LKB1 disrupted the distribution of Par-3 and Par-6 [146, 167, 168]. Besides Par-1/MARKs, BRSKs and AMPKs are regulators of cellular

polarity. The brain-specific kinases (BRSKs) are mainly expressed in the nervous system and play a key role in polarization of neurons, as BRSK double knockout embryos have severely reduced axon growth and mislocalization of axon and dendritic markers [169].

AMPKs are involved in the regulation of epithelial tight junction assembly and cell polarization. During calcium-induced tight junction assembly and cell polarization, the level of AMPK phosphorylation is induced which depends on the kinase activity of LKB1[170]. In addition, LKB1-induced AMPK phosphorylation on Thr172 contributes to the formation of mitotic spindles during mitosis. Myosin regulatory light chain (MRLC) is a downstream target of AMPK, which mediates acto-myosin contractility. AMPK phosphorylates MRLC at the mitotic spindle poles and controls microtubule organization during mitosis [171, 172]. Finally, loss of LKB1 compromises epithelial integrity through mislocalization of cell polarity markers, disruption of basement membrane (BM) and lateralization of tight junctions in the mammary epithelium. Although loss of LKB1 is not a sole promoter of tumorigenesis, but the combination of LKB1 deficiency and oncogenic synergy with c-Myc leads to dramatic acceleration towards cell proliferation and tumor progression [173].

4. Salt inducible kinase 1 (SIK1)

Salt inducible kinase (SIK1) was discovered in 1994 in the myocardium of the developing mouse heart, called MSK (myocardial SNF1-like kinase) due to structural similarity with the SNF1 kinase in yeast [174, 175]. The name SIK refers to the observation of SIK1 induction in the adrenal glands of rats fed with high salt diet [175, 176]. SIK1 belongs to the AMPK family [157, 177]. The cloning of SIK1 led to a novel cDNA clone, encoding a polypeptide of 776 amino acids, similar to serine/threonine kinases in the SNF1/AMP kinase family [175, 178]. SIK1 has two close family members, SIK2 and SIK3. SIK2 is known to modulate the insulin-signaling cascade of adipocytes, and is as a consequence involved in the progression of insulin resistance [178].

The master kinase LKB1 activates SIK1 through phosphorylation of Thr182 within its T-loop [157]. Nevertheless, some studies indicate that LKB1 is not the sole kinase responsible for the activation of SIK1. Two other proteins including the 14-3-3 phospho-protein binding adaptor and glycogen synthase kinase-3β (GSK-3β) cooperate with LKB1 in order to phosphorylate and activate SIK1 [179 180]. SIK1 expression is induced during cardiogenesis and skeletal muscle differentiation[174], in adrenal glands, leading to steroidogenesis[181] and in the liver where it suppresses gluconeogenesis [182]. SIK1 may link LKB1 to the maintenance of epithelial junction stability by down-regulating EMT transcription factors, such as Snail, Zeb1 and Zeb2, which repress E-cadherin [183]. Moreover, SIK1 expression is induced by TGF-β/Smad signaling. SIK1 and Smad7 form a complex and cooperate to downregulate the activated TβRI receptor. The ubiquitin ligase Smurf2 and SIK1 form complex and cooperate to induce TβRI turnover. In addition, loss of endogenous SIK1 results in enhanced gene responses of the cytostatic and fibrotic programs of TGF-β. Thus, SIK is a negative regulator that mediates TGF-β receptor turnover and physiological signaling [184, 185].

The protein structure of SIK1 consists of two main domains, including an N-terminal serine/threonine kinase domain followed by a ubiquitin associated (UBA) domain of 43 amino acids, and finally a long C-terminal domain of not well-known function except for a nuclear localization site mapping around Ser577. The kinase domain is crucial for the catalytic activity of SIK1 and is also known to contain the LKB1-mediated phosphorylation site [186-188]. Recent studies disclose new substrates for SIK1. SIK1 phos-

phorylates transducer of CREB activity (TORC) through which it down-regulates its function as the co-activator of CREB (cAMP response-element binding protein), and as a result of this phosphorylation CREB localizes into the nucleus [189].

5. Glioblastoma multiforme

Glioblastoma multiforme (GBM) is one of the most frequent and lethal central nervous system (CNS) tumors characterized by a high rate of proliferation, tumor angiogenesis and chemoresistance [190, 191]. The prognosis of GBM patients is poor and the median overall survival (OS) in spite of all possible modern therapy treatments is only 12-18 months [191]. Standard treatment of GBM involves macroscopic complete surgical resection followed by combined radiation and chemotherapy with temozolomide [191, 192]. In the last decade, enormous genomic and transcriptomic analyses have led to a new classification and characterization of GBM genetic mutations, including gene copy number alterations and gene expression profiles. These studies classify GBM tumors to three subtypes, proneural (PN), classical (CL), and mesenchymal (MES) [193, 194]. One of the great challenges in therapy development is the large degree of heterogeneity of the GBM tumors [193]. Several advanced techniques, allowing detection of the single mutated DNA molecule confirm the high heterogeneity of GBM. Heterogeneity can affect GBM biology in different ways, such as creating chemoresistance microenvironment, acquiring new driver mutations, or mutations that confer resistance to therapy [195].

One of the most accepted theories to explain heterogeneity in cancer is the cancer stem cell (CSC) model. In tumors generated from cells with stem cell characteristics, these cells will maintain their population by asymmetric division and generate more differentiated daughter cells which establish the bulk of the tumor, while the more proliferative daughter cells, remain as a small subpopulation within the tumor mass and are known as CSCs [195, 196]. In this model, heterogeneity has a dualistic nature of CSCs daughters, tumorigenic and non-CSCs that are known to be non-tumorigenic. Glioma CSCs were defined by the expression of CD133 and cells not expressing this marker lack the tumorigenic potential [195, 197, 198]. In addition, recent studies have proposed that a subpopulation of glioma cells characterized by higher expression of CD44 and ID1 acquire stem-like phenotype. These markers can be used as stem cell markers depending on the GBM subtype. CD133, is believed to be mostly expressed in proneural glioma stem cells (GSC), while CD44 is highly expressed in tumors of the mesenchymal subtype [199, 200].

TGF-β is known to have a cytostatic effect in epithelial cells via inducing the expression of p21. However, in GSCs the levels of the transcription factor FoxG1 are higher leading to a direct interaction with FoxO3, therefore preventing FoxO3 from promoting the expression of TGF-β-induced p21. As

a result, GBMs can resist the cytostatic effects of TGF-β, thus promoting cell proliferation [55, 201]. Moreover, in GBMs higher levels of phospho-Smad2 correlate with increased proliferation leading to poor survival of the patients [202]. Autocrine TGF-β production maintains stemness in the GSCs. TGF-β up-regulates the expression level of the stem cell transcription factor Sox2 and the cytokine leukemia inhibitory factor (LIF) in a Smad-dependent manner [203, 204]. Interestingly, ID1, ID3, LIF, Sox2 and Sox4 are all required to maintain the $CD44^{high}$ population in GBMs, suggesting these molecular pathways are interconnected. However, not all GSCs respond potently to TGF-β, due to the different genetic background of the GBMs among different patients [201, 205]. Finally, TGF-β can maintain the proliferative characteristic of GSCs by inhibiting apoptosis via induction of Nodal expression levels [206].

In contrast with the stemness effects of TGF-β, BMPs exert an opposite function in the GBMs. For instance, BMP4 decreases the population of the $CD133^{+}$ GSCs, and promotes astrocytic differentiation [207]. BMP7 reduces GSCs cell proliferation and promotes differentiation via upregulation of transcription factor Snail [208]. All these observations are consistent with a tumor suppressor function of BMP in GBMs. However, another member of the BMP family, BMP2, promotes GBM proliferation, migration, enhanced GSCs self-renewal and enhanced tumor formation *in vivo*. The miR-656 impairs the BMP2 effects by targeting the BMPR1A receptor which is known to be downregulated in GBMs [209].

Present investigation

Aim

The aim of this thesis was to uncover mechanisms by which TGF-β and BMP signaling pathways regulate cell polarity, migration and invasion during cancer progression and metastasis.

Paper I. The protein kinase SIK downregulates the polarity protein Par3

This study describes a novel role of salt inducible kinase (SIK) in regulating the tight junction assembly by phosphorylation and degradation of the polarity complex protein Par3. We have performed an *in silico* screen for SIK phosphorylation substrates. Several novel substrates were uncovered, among which we have focused on Par3. We provided bioinformatic and biochemical evidence that SIK can phosphorylates Par3 on Ser885, a putative phosphorylation site of Par3. SIK association with Par3 leads to degradation of Par3 that can be prevented by proteasomal and lysosomal inhibitors. Ultimately, SIK-mediated phosphorylation of Par3 impacts epithelial tight junction assembly and contributes to the loss of epithelial polarity. Immunohistochemistry analysis displayed high levels of SIK expression in advanced and invasive epithelial tumor samples. In addition, SIK expression correlates with TGF-β/Smad signaling activity and low to undetectable levels of Par3. Finally, our model suggests that SIK can act directly on Par3 in order to regulate epithelial tight junction assembly.

Paper II. Snail regulates BMP and TGF-β pathways to control the differentiation status of glioma initiating cells

In this paper, we have shown that Snail displays tumor suppressor effects in GBM by inhibiting GSCs self-renewal and promoting a fate switch towards the astrocytic lineage, through induction of BMP and suppression of TGF-β. Blocking the BMP pathway using Noggin and DMH1, or knockdown of

BMP type I receptors or Smad1/5, abolish Snail-induced astrocytic fate. We have observed that Snail suppresses GSC stemness in a TGF-β-dependent manner. Therefore, suppression of TGF-β and its downstream signaling by Snail reduces GSC survival and self-renewal. In our GBM in vivo model, inhibiting TGF-β signaling reduced GBM proliferation, while in the same GBM model, TGF-β pretreatment enhanced tumor cell proliferation and stemness.

Our work suggests a novel role of Snail as a direct regulator of TGF-β through binding to the TGF-β1 promoter. Moreover, we observed Snail interaction with BMP and TGF-β R-Smad MH1-linker domains via its N- and C-terminal domains.

Altogether, our current work indicates that Snail inhibits the tumorigenic potential of GBM by blocking GSCs self-renewal capacity and promoting the astrocytic fate switch, by inducing BMP signaling and repressing TGF-β pathway.

Paper III. Par3 promotes glioblastoma stem cell self-renewal while inhibiting cell invasion

This work was inspired by recent studies that identified genetic mutations in the polarity protein Par3 gene, known as *PARD3*, in GBM. Taking advantage of patient-derived GSCs, we studied the role of Par3 in stem cell self-renewal, migration and invasion. According to patient transcriptomic data analysis, low levels of *PARD3* correlates with poor prognosis in GBM patients. Indeed, we could detect low or even undetectable Par3 expression levels in our patient derived GSCs. Unexpectedly, loss of Par3 decreased the neurosphere forming capacity of the GSCs as analyzed by extreme limiting dilution assay (ELDA) and enhanced their invasiveness. Genome-wide transcriptomic analysis confirmed low stemness and high invasive gene expression in GSCs after silencing the *PARD3* gene in GSCs. Moreover, according to our transcriptomic analysis we could identify another set of genes exhibiting low expression after silencing endogenous Par3. These genes encode mitochondrial enzymes that generate ATP; accordingly, loss of Par3 reduced ATP levels in the GSCs. This was accompanied by elevated levels of reactive oxygen species (ROS). Based on several studies increased ROS production is associated with aggressive phenotype in diverse types of cancers. In order to investigate whether increased level of ROS play any role in the invasion of the GSCs, we used two antioxidants, butyl-hydroxyanisole (BHA) and N-acetyl-L-cysteine (NAC). We indeed observed inhibition of invasion in GSCs after NAC or BHA treatment. Our results show that Par3 has both tumor suppressor and oncogenic function in GSCs. On one hand loss of Par3 enhances migration and invasion by increasing intracellular ROS levels, and

on the other hand it impairs GSC neurosphere forming capacity, self-renewal, mitochondrial membrane potential and ATP production.

Paper IV. The protein kinase LKB1 takes oncogenic actions in glioblastoma promoting self-renewal and invasiveness

This paper describes the significance of the liver kinase B1 (LKB1) in the maintenance of GSCs. An initial evaluation of LKB1 gene expression levels in GBM patients in various public datasets, have revealed that high LKB1 expression levels correlates with worse prognosis of GBM patients. However, we could not observe any significant changes of LKB1 mRNA expression in GBM patients compared to patients with other glioma subtypes or compared to the normal tissue in any available datasets. Taking advantage of our patient-derived GSCs, endogenous LKB1 was silenced with short interfering (si) RNA transfection technique to analyze how it affected gene expression using Ion torrent Ampliseq^{TM}. According to our transcriptomic analysis, silencing LKB1 strongly repressed genes related to endoplasmic stress and migration. Following our transcriptomic analysis, the effects of LKB1 knockdown were assessed in GSC self-renewal, proliferation, migratory and invasive capacity, and finally oxidative stress and mitochondrial function. Silencing LKB1 impairs invasion and stem cell self-renewal capacity of the GSCs. In agreement to the effects of LKB1 knockdown on impaired self-renewal and invasion in functional cell-based assays, we observed repression of the transcription of genes involved in these processes including, several members of the ADAM (a disintegrin and metalloproteinases) family, TGF-β ligands, and PDGF and EGF family ligands and receptors. In addition, loss of LKB1 leads to induction of mitochondrial dysfunction and decreased ATP levels, inhibiting GSC proliferation.

Taken all together, our data suggest that LKB1 is a good therapeutic target as it regulates several mechanisms that result in poor survival rate in GBM patients.

Future perspectives

Paper I. The protein kinase SIK downregulates the polarity protein Par3

SIK1 has been established as a negative regulator of TGF-β signaling through downregulation of type I receptor signaling in cooperation with the Smurf2 ubiquitin ligase [185]. In this work, we have explored new possible functions of this kinase. We have performed an *in silico* screen for further substrates of SIK1 and, interestingly, we found few candidates involved in EMT. Par3, the core member of the polarity complex Par, was shown to be phosphorylated by SIK1. Indeed, we have observed that SIK1 promotes phosphorylation of Par3 on Ser885, in a functionally important domain of Par3. However, the physiological relevance of Par3 phosphorylation on Ser885 requires further analysis using phospho-specific antibodies for this site, and performing a thorough screen against other possible protein kinases that could mediate a direct phosphorylation on this amino acid residue.

So far, our current results indicate that Par3 is degraded through proteasomes and lysosomes. However, attempts to prove SIK1-induced Par3 ubiquitylation have failed in our hands.

According to studies by other groups, Par3 is downregulated during TGF-β-induced EMT [210, 211]. Therefore, we continued our investigation discovering whether phosphorylation and downregulation of Par3 by SIK1 plays any role during TGF-β-induced EMT. Indeed, we observed that silencing SIK1 leads to stabilization of tight junction assembly during TGF-β-induced EMT. In agreement with these observations, loss of Par3 significantly elevated the effects of TGF-β during EMT. Therefore, to really establish a role for SIK1 in downregulation of epithelial markers during TGF-β-induced EMT, we need to perform experiments in which we replace endogenous Par3 with a Par3 Ser885 mutant construct to determine whether the non-phosphorylated Par3 mutant is unable to be downregulated during EMT.

Finally, our current data suggest that SIK1 expression is up-regulated in cancer and there is a correlation between SIK and phosphorylation level of Smad2. However, to validate these observations, we need to set a complete screen for the levels of SIK1 and phosoho-Smad2 by tissue microarray and check whether there is any correlation between high levels of SIK1 and phospho-Smad2, and low levels of Par3.

Paper II. Snail regulates BMP and TGF-β pathways to control the differentiation status of glioma initiating cells

In this paper, we obtained clear evidence that Snail inhibits the tumorigenic potential of GBM through blocking of GSCs self-renewal capacity and promoting the astrocytic fate switch by inducing BMP signaling and repressing the TGF-β pathway. We have demonstrated that Snail interacts with R-Smads and generates a positive feedback loop in the BMP signaling pathway, while it transcriptionally downregulates the *TGF-β* gene, leading to suppression of the TGF-β signaling arm. The antagonistic effects of BMP signaling against TGF-β are well-established in embryonic development and cancer [212]. However, whether BMPs promote Smad1/5 complexes with Snail to regulate additional target genes, which are important for astrocytic differentiation and suppression of GSC stemness, remains to be elucidated.

It would be interesting to perform a ChIP-sequence experiment in glioblastoma cells in response to TGF-β and BMP7, and compare the binding sites of Snail depending on the cytokine used to stimulate the cells. To further study the interaction of Snail and R-Smads, it would be important to compare the binding sites of Smad2/3 to the binding sites of Snail after TGF-β treatment, and those of Smad1/5 versus Snail after BMP7 treatment. An interesting question is whether TGF-β Smads and BMP Smads compete for binding to Snail in order to regulate gene expression, as both cytokines induce the expression of the transcription factor Snail.

Paper III. Par3 promotes glioblastoma stem cell self-renewal while inhibiting cell invasion

Loss of cellular polarity is an established hallmark of invasiveness in several malignancies. However, little is known about the role of the polarity machinery in tumor suppression of diverse tumors. In GBM, the role of polarity genes in the invasiveness of the tumors has not been excessively explored. The *PARD3* gene has been identified to carry several deletions in the exons 3 to 20 or exon 25 in GBM patients. These mutations result in disruption of astrocyte tight junctions, while reconstituting the wild-type Par3 expression restored tight junction formation [213]. In this study, we found that low levels of Par3 correlates with poor prognosis in GBM patients. Taken advantage of our patient-derived GSCs, we observed that loss of Par3 decreased the self-renewal capacity, cell proliferation and viability, while it increased the invasiveness of cells. In addition, loss of Par3 contributed to downregulation of self-renewal inducing factors, stem cell transcription factors, and PDGF and EGF family ligands and their receptors. This could be a possible explanation

for the effect of Par3 silencing on self-renewal capacity of the GSCs. It will be interesting to establish a specific mechanistic connection between the function of Par3 and possibly its associated polarity proteins and the regulation of such genes that drive the stemness and proliferative capacity of GSCs. In addition following further transcriptomic analysis, knockdown of Par3 in GSCs leads to significant repression of genes responsible for ATP synthesis and family members of mitochondrial membrane carriers and transporters. This correlates with low levels of ATP production and elevated levels of ROS after silencing Par3. However, it remains to be further investigated whether overexpression of the mentioned genes reverses the effects of Par3 knockdown on ATP and ROS levels, as well as restores the self-renewal capacity of GSCs. In our study, loss of Par3 leads to increased invasiveness of GSCs. In addition, mitochondrial ROS and different isoforms of NOX have been shown to amplify the tumorigenic phenotype and metastatic behavior in cancer cells [214, 215]. Indeed, using the antioxidants BHA and NAC, we could block the invasion induced by Par3 knockdown. Therefore, it would be interesting to examine the invasiveness of GSCs in an *in vivo* model, such as zebrafish, after silencing Par3.

Finally, generating stable knockout clones of Par3 in neural progenitor cells could be an interesting model in order to investigate whether loss of Par3 reproduce the same effects in the neural progenitor cells as it does in the GSCs and promotes their tumorigenesis (?).

Paper IV. The protein kinase LKB1 takes oncogenic actions in glioblastoma promoting self-renewal and invasiveness

In this paper we examined the role of tumor suppressor LKB1 in GSCs. So far the significance of LKB1 in the GSCs maintenance has not been fully elucidated. Certain previous studies suggested a tumor suppressor role of LKB1, while the others report its tumorigenic role. According to different available datasets high levels of LKB1 correlates with lower survival of GBM patients. Our transcriptomic analysis in our patient-derived GSCs revealed that silencing LKB1 strongly repressed genes related to endoplasmic reticulum stress and migration. Indeed, we observed low migratory capacity of GSCs after knocking down LKB1. However, the mechanisms in which loss of LKB1 leads to endoplasmic reticulum stress still remains unclear. We observed that LKB1 regulated GSC proliferation, viability and the ability of sphere formation. These observations can be explained by the fact that LKB1 silencing regulated key pathways and molecules that regulate GSCs' self-renewal capacity. In addition, we observed impaired mitochondrial function, reduction of ATP levels and increased levels of ROS. Further ex-

periments are needed to understand the signaling cascade downstream of LKB1 in order to further elucidate the mechanisms behind LKB1 regulation of self-renewal capacity, invasion and response to the metabolic stress. It would be interesting to uncover whether LKB1 exerts its effects by targeting AMPK/mTOR signaling or miR-451 or independent from these two well-known targets.

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