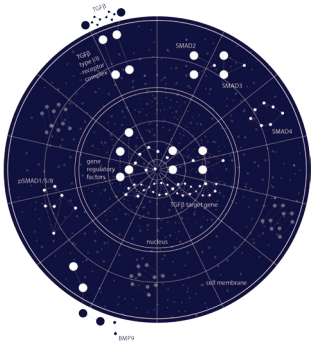
Targeting TGFβ signaling pathway in fibrosis and cancer

Karkampouna, S.



Chapter 1

Introduction

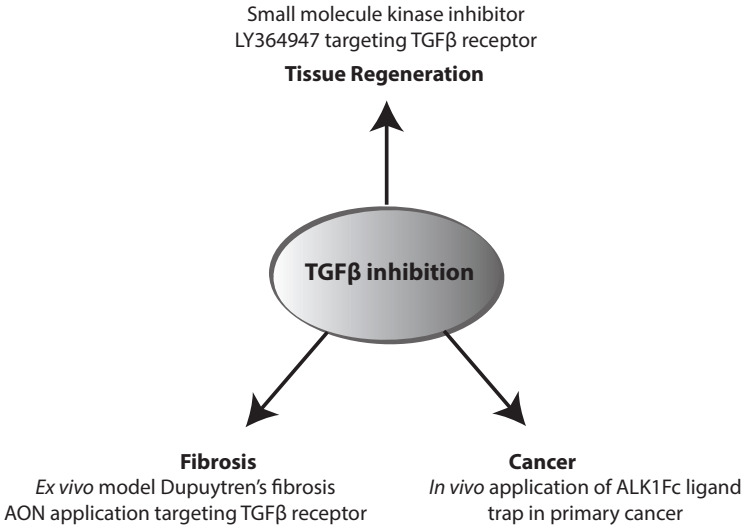
Introduction

1.1. Prologue

Survival of multicellular organisms requires resilience and regeneration of injured tissues due to damaging environmental or genetic factors. Virtually, all organisms from bacteria to primates have regeneration abilities at different extend (bacteria wall and flagellum regeneration[1], limb regrowth in reptiles, blood, nerve and liver regeneration in mammals). Regeneration is the process of cell repair, cell replacement and renewal leading to morphogenic changes and (re) growth of damaged tissue and organs. The ultimate aim is to preserve the physiological organ functionality (homeostasis) since repetitive exposure to damaging factors eventually leads to exhaustion of the regenerative properties of cells and development of pathological conditions such as malignancies or organ fibrosis.
Regenerating cells undergo cell duplication (proliferation) and phenotypic changes by gene expression modulation thus; they have increased need for DNA and protein synthesis. In order to precisely orchestrate these complex molecular processes, cells depend on mechanisms that allow communication between different parts of a single cell and among different cells. Cell communication during tissue injury occurs via signaling molecules that are being produced by injured cells (proteins, miRNAs, ions, chemical molecules). These signaling molecules cause a cascade of activation or inhibition of other molecules (signaling pathways) leading to a particular biological response in the same cell or in adjacent or further located healthy cells in different places in the body via blood circulation.
A major cell signaling pathway orchestrating homeostasis in many organ systems is the superfamily of proteins Transforming growth factor β (TGFβ). Its prototype TGFβ1 was identified over 3 decades ago. The TGFβ superfamily consists of 33 proteins encoded by the human genome, seven in Drosophila and four in C.elegans[2].
Its mechanisms of signal transduction are often deregulated in human diseases; thus, it is subject of vigorous investigation in various types of cancer and fibrosis.

1.2. Outline of the thesis

Dysregulation of gene function of TGFβ pathway components is a common cause of human diseases such as cancer and fibrosis. The scope of the research described in this thesis is to characterize the therapeutic potential of different inhibitory strategies of TGFβ protein signaling cascade. The objective is to target and potentially "correct" the expression and function of proteins encoding TGFβ signaling components using clinically applicable compounds such as antisense oligonucleotides, small molecule inhibitors or neutralizing antibodies. We focus on inhibition of TGFβ signaling pathway in *in vivo* and *ex vivo* models of human fibrosis (Dupuytren's, liver) and cancer (prostate, liver).



In **Chapter 1**, a general introduction on the TGFβ signaling pathway is presented and its role in normal conditions (homeostasis and tissue regeneration) as well as in pathological conditions (fibrosis, cancer) is described. An overview of different inhibitory strategies for molecular manipulation and ongoing clinical trials targeting TGFβ is provided.

In **Chapter 2**, a comprehensive review is presented about the pleiotropic role of TGFβ during liver development and regeneration with particular emphasis on regulation of epithelial cell (hepatocyte) and hepatic progenitor cell- induced regeneration. An overview of the current aspects of liver research with regards to cell replacement therapies is presented.

In **Chapter 3**, we show the *in vivo* use of a small molecule inhibitor (LY364947) targeting kinase activity of TGFβ type I receptor in a mouse model of injury-induced liver regeneration.

In **Chapter 4**, a novel *ex vivo* methodology is described for the study of human Dupuytren's fibrosis, a primarily TGFβ-driven disease. A step-by-step protocol of the tissue *ex vivo* culture system and its applications are described. This technique is used in combination with biochemical and imaging techniques that could be applicable for the study of various types of human fibrosis.

In **Chapter 5**, the *ex vivo* culture system of human fibrotic tissue is used as a platform for TGFβ pathway deactivation using small molecule inhibitor and antisense oligonucleotides. Both strategies aim to target the activin receptor-like kinase-5 (ALK5) activity (TGFβ type I receptor) either at the protein or messenger RNA level. Specifically, the data suggest that inhibition of ALK5 activity is applicable *ex vivo* and exhibits anti-fibrotic effects evident by reduction of extracellular matrix protein deposition.

Chapter 6 describes the use of anti-human ALK1 neutralizing antibody ACE-041 (ALK1Fc) as a tumor angiogenesis inhibitor in primary prostate cancer. This compound is currently being tested in clinical trials for solid tumor treatment. In this study, we show the *in vivo* effects of ALK1Fc in tumor burden and angiogenesis in a primary prostate cancer mouse model using orthotopic transplantation of human prostate cancer cells.

In **Chapter 7** (Discussion) the results presented in this thesis, implications deriving from this work and applicability of TGFβ targeting drugs are discussed.

In **Appendix I** we introduce preliminary data supporting a role for CRIPTO, a TGFβ superfamily type III receptor, in liver regeneration and human hepatocellular carcinoma. CRIPTO is normally expressed only during embryonic development. Our data indicate reactivation of CRIPTO in the mouse liver after toxin-induced acute injury and in human liver cancer specimens suggesting its use as a potential diagnostic biomarker for human hepatocellular carcinoma.

Summary (Appendix II) includes an overview of the key findings of this thesis.

1.3. TGFβ superfamily

The Transforming growth factors (TGFs) were firstly identified as secreted proteins from murine sarcoma virus-transformed fibroblasts; these proteins induced *in vitro* transformation to neoplastic-like phenotype as evidenced by cell division, morphological changes and anchorage-independent growth[3,4]. Two distinct classes of proteins were isolated, the type α and type β with different properties and synergistic effects. In fact, the original observations suggested that TGFβ *per se* does not stimulate cell growth unless combined with other growth factors such as TGFα and epidermal growth factor (EGF)[5]. Although initially TGFβ was found present in neoplastic cells [6,7] further studies showed its expression in various normal human cells and tissues such as platelets[8], placenta[9] and during embryonic development[10,11]. A physiological role in cell differentiation, wound healing, angiogenesis and as an inhibitor of cell growth was attributed to TGFβ in various tissues[12]. Despite the functional complexity of TGFβ the core signaling components and their interactions appear at first instance rather simple.

The TGFβ superfamily of ligands can be divided into subfamilies; TGFβ proteins, bone morphogenetic proteins (BMPs), the growth and differentiation factors (GDFs), ACTIVINs, MYOSTATINS, NODAL and Anti-Mullerian hormone (AMH).

The molecular skeleton of TGFβ pathway is comprised by the extracellular TGFβ-family ligands, which elicit their signals into the cell by binding to type II receptor (TβRII), forming heterodimer complexes with type I receptor TβRI/ACTIVIN receptor-like kinase (ALK5)[13]. This interaction activates the receptor's serine/threonine kinase activity to phosphorylate and activate SMAD transcription factors. Phosphorylation of receptor-activated SMADs (R-SMADs) by the activated type I receptor allows the R-SMADs to form heterodimers with partner SMAD (co-SMAD/ SMAD4) and translocate to the nucleus where, in collaboration with transcription factor complexes, they activate or inhibit the transcription of target genes[14] (**Fig.1**).

1.3.1. BMP subfamily

BMP factors were firstly identified as chondrogenic and osteogenic inducers. Further investigations revealed various roles for BMPs during embryogenesis; kidney, skin, hair, muscle, haematopoietic and neuronal development, as well as a role in iron metabolism and vascular maintenance[15-17]. Although BMPs typically activate BMP type I receptors and R-SMAD1, 5 and 8, they can be further classified into several subgroups, including BMP1/2/3/4 group, BMP5/6/7/8 group, growth and differentiation factor (GDF)5/6/7 group and BMP9/10 group[15,18]. BMP2, BMP6 and the most recently identified BMP9 are the most potent inducers of osteogenesis[19]. Although structurally different, other members of the BMP pathway are GDF8/MYOSTATIN and GDF9, having a role in muscle tissue and ovarian development, respectively[20,21].
Signal transduction of BMP subfamily is conducted in a similar manner as the TGFβ cascade. BMP ligands use BMPRII, ACVR2A or ACVR2B type II receptors and have affinity for ALK1, ALK2, ALK3 and ALK6[22]. SMAD1, 5 and 8 are the BMP-specific R-SMADs that translocate to the nucleus upon activation by the type I receptor and regulate BMP target gene transcription[15] (**Fig.1**).

1.3.2. TGFβ canonical pathway

Level 1. Ligand synthesis and secretion

There are three types of TGFβ ligand isoforms: TGFβ1, TGFβ2, and TGFβ3, all of which are highly conserved among species. Mature TGFβ1, TGFβ2 and TGFβ3, which are produced after proteolytic activation from cleavage precursor proteins, share high aminoacid sequence homology and functional similarity. However, it has been reported that TGFβ2 and TGFβ3 are biologically more active than TGFβ1[23] and their tissue expression follows a distinct pattern[24]. Following the identification of the three isoforms[25-28] novel information from transgenic mouse models added to our understanding of the crucial and specific role during embryonic development for all three isoforms. Genetic deletion of TGFβ1 in mice leads to general inflammation[29] and early death[30] due to vascular and hematopoietic abnormalities during development[31]. Mice lacking TGFβ3 exhibit lung and cleft palate defects due to abnormal epithelial-to mesenchymal transition (EMT) and die immediately after birth[32]. Homozygous deletion of TGFβ2 confirmed the previously suggested role of TGFβ2 in cardiogenesis[33] but also causes multiple defects (cardiac, lung, eye and skeletal tissues among others) that are not resembling the TGFβ1 or TGFβ3 knockout phenotype[34].

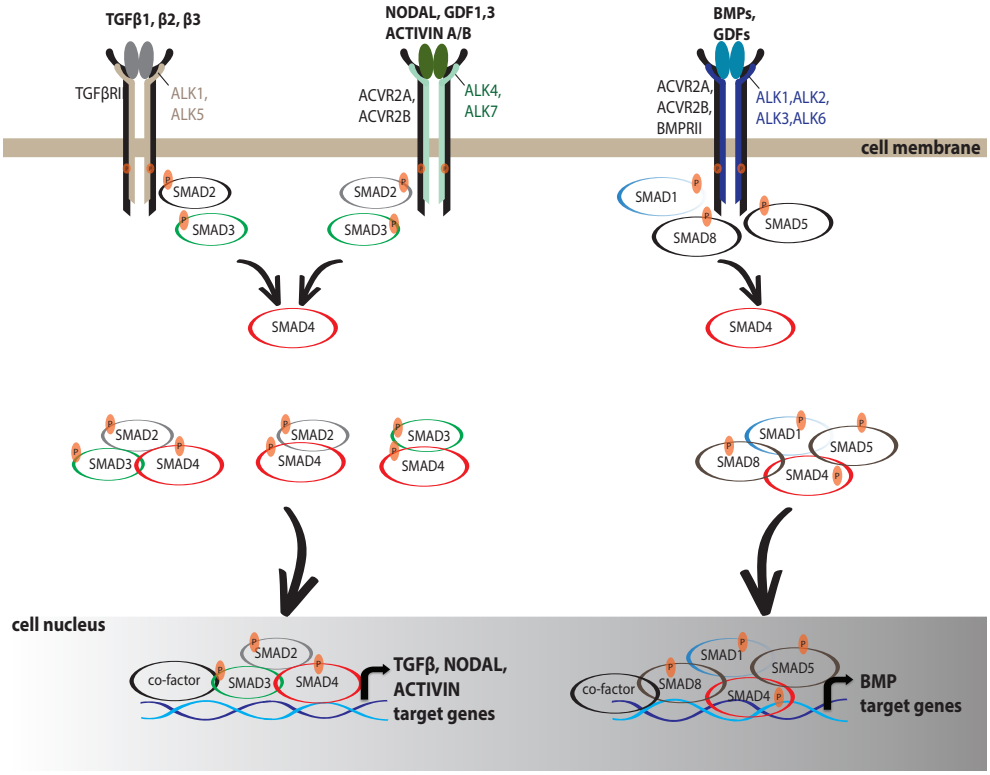


Fig.1. Overview of TGFβ signaling pathway
The core of the pathway is comprised by the SMAD effectors that mediate downstream signaling from extracellular ligands; TGFβ, ACTIVINS, NODAL, BMPs, GDFs. (left) TGFβ ligands bind to type II receptor TGFβRII, which recruits and phosphorylates the type I receptor ALK5 or ALK1 (endothelial cell-specific receptor). ALK5 phosphorylates R-SMADs (SMAD2 and SMAD3) that form complexes with phosphorylated SMAD4 and translocate into the nucleus. R-SMAD/SMAD4, together with co-factor proteins, bind to gene promoter regions and induce or repress target gene transcription. (middle) NODAL, GDF1, GDF3 and ACTIVIN-A, -B bind to ACVR2A, ACVR2B and activate ALK4 or ALK7. ALK4/7 transduce their signal via SMAD2 and SMAD3 effectors, similarly to the TGFβ cascade. (right) GDFs/ BMPs bind simultaneously to a combination of type II (BMPRII, ACVR2A or ACVR2B) and type I receptors (ALK1, ALK2, ALK3 or ALK6). This leads to phosphorylation of R-SMADs, SMAD1, SMAD5 and SMAD8 which couple to SMAD4 and as a complex enter the nucleus and regulate GDF/ BMP target gene expression.

TGFβ proteins are expressed as inactive pre-pro-peptides; latency is controlled by binding to the latency-associated peptide (LAP) and latent TGFβ binding protein (LTBP) and is reversed by proteolytic cleavage[35]. Briefly, after protein synthesis and signal peptide removal by endoplasmic reticulum proteases, the pro-TGFβ peptides assemble in inactive homodimers and are secreted in the extracellular space. The pro-TGFβ peptide is further cleaved in the secretory vesicles or in the extracellular matrix (ECM) by enzymes (convertases) into two fragments; a C-terminal immature TGFβ peptide and an N-terminal peptide (LAP). The LAP-TGFβ complex remains non-covalently attached (small latency complex, SLC) or binds further to LTBP forming the large latency complex (LLC)[36]. LTBP stabilizes the complex by binding to ECM components such as fibronectin, fibrillin1 and integrins[37].

Mechanical stress, cell contraction, extreme pH or temperatures are some of the factors inducing disintegration of LLC by degradation of fibrous matrix (proteases e.g. plasmin, elastase, thrombin). Cleavage of LTBP by BMP1 protease and matrix metalloprotease enzymes (MMP-2) leads to release of TGFβ from the LAP complex[38]. Additional mechanisms of activation of latent TGFβ include integrins [39], fibronectin[40] and the matricellular protein thrombospondin-1[41]. After dissociation from the LAP, the active TGFβ homodimer is then recognized by the TGFβ type I and type II receptors on recipient cells in a cell autonomous, paracrine and endocrine manner.

Level 2. Receptor binding

Receptors of TGFβ signaling are serine/threonine kinase receptors and are distinguished in two types (type I and type II)[12]. The co-receptor class (type III; β-GLYCAN, ENDOGLIN and CRIPTO) has auxiliary function. Active TGFβ ligands bind to the constitutively active TGFβ type II receptor[13]. The type II receptor transphosphorylates the type I ACTIVIN receptor-like kinase (ALK) thus leading to an interacting formation between a ligand homodimer and a heterotetramer of type I/ type II receptors[14]. Five type II receptors (TGFβRII, BMPRII, ACVR1A, ACVR1B, and seven type I receptors (ALK1-ALK7) have been identified[42]. Different combinations of receptor-ligand assemblies provide differential signaling specificity (**Table 1**). The predominant type I receptor of TGFβ ligands is the ALK5[43], with the exception of signaling via ALK1 in endothelial cells[44]. The ALKs contain an extracellular domain for ligand interaction, a glycine-serine residue rich (GS) domain; site of phosphorylation by the type II receptor occurs and the serine/threonine kinase domain[45-47]. Levels of activated receptors determine the levels and duration of activation of downstream signal mediators [48].

	Ligand	Type I receptor	Type II receptor
TGFβ	TGFβ1, TGFβ2, TGFβ3	TGFβRI (ALK5) ALK1	TGFβRII
BMPs	BMP2, 4 GDF5/6/7 BMP5/6/7/8 BMP9/10	ACVRL1 (ALK1) ACVR1 (ALK2) BMPR1A (ALK3) BMPR1B (ALK6)	ACVR2A ACVR2B BMPRII
ACTIVINs, NODAL	ACTIVIN A, AB, A Inhibin A, B NODAL GDF1/3	ACVR1B (ALK4) ACVR1C (ALK7)	ACVR2A ACVR2B
AMH	AMH	ACVR1 (ALK2) BMPR1A (ALK3)	AMHR2

Table 1. Combinations of receptor- ligand interactions of TGFβ subfamilies
Abbreviations: ACVR2A; ACTIVIN A receptor type IIA, ACVR2B; ACTIVIN A receptor type IIB, ACVRL (ALK); ACTIVIN A receptor type II-like, AMH; anti-mullerian hormone, AMHR2; anti-mullerian hormone receptor type II, BMPR; bone morphogenetic protein receptor, GDF; Growth and differentiation factor. TGFβR; transforming growth factor receptor. Adapted from[49].

Level 3. SMAD protein recruitment

The intracellular canonical TGFβ pathway effectors, which mediate signal transduction from the receptors towards the nucleus, are the SMAD proteins. Nomenclature of SMAD is based on the homology with the *C. elegans* and *Drosophila* mutants SMA and MAD, respectively, in whom they were firstly identified[50]. Three classes of proteins are distinguished; the receptor-associated SMADs (R-SMADs; SMAD1, 2, 3, 5, 8), the inhibitory SMADs (SMAD6, 7) and the common SMADs (co-SMAD4)[14]. Structurally, SMAD proteins have two globular domains (MH1 and MH2) associated by a regulatory linker region[2]. MH1 is required for DNA binding. The MH2 domain is required for interaction with membrane receptors, nucleoporins, other SMADs and transcription factors[51]. The linker region is a site for protein-protein interactions (positive and negative regulators of SMADs) and is regulated by phosphorylation e.g. by cyclin-dependent kinases (CDKs)[52], MAPK kinases[53]. Ligand binding of TGFβ1, β2, and β3 to the receptors leads to recruitment and activation of the SMAD2 and SMAD3 mediators. Instead, BMP ligands lead to recruitment of SMAD1, SMAD5 and SMAD8 to BMP receptors. In either case, R-SMADs are phosphorylated by the type I receptor at the Ser-X-Ser motif of the MH2 domain (X; any aminoacid) and form a complex with other R-SMADs and subsequently with the co-activating SMAD4 mediator[42]. The R-SMAD/SMAD4 protein complex relocates to the nucleus, where it interacts with DNA sequences by assembling with co-activators, transcription factors (TFs) and chromatin modifiers to regulate activation and repression of certain target genes.

Level 4. Transcriptional activity

Activated R-SMAD2 and -3 form heterodimeric or heterotrimeric complexes with SMAD4 complexes and bind to DNA in CAGA motifs namely SRE sites (SMAD-response element). BMP-related TFs (R-SMAD1, 5, and 8) bind to SRE sites but have higher affinity for GC-rich regions containing BMP-response-elements (BRE sites). An additional BMP binding site has been identified (GC-BRE) that confers cell type- specific gene transcription in endothelial versus smooth muscle cells[54]. However, R-SMADs DNA binding affinity is weak, thus they form complexes with other DNA binding TFs, which explains the multifunctionality of SMAD pathway[42]. Cell type- specific responses are determined by differential interaction with specific TFs that direct R-SMAD2 and -3 in certain binding sites; e.g. RUNX factors in hematopoietic cells, OCT4 and SOX2 in pluripotent cells, MYO-D to initiate the muscle program[55]. Interactions of R-SMADs with co-activators and co-repressors determine activation or inhibition of downstream target gene transcription. Identified co-activators of SMADs include adenovirus early gene1 (E1A)- binding protein (p300), CREB binding protein (CBP) and Specificity protein-1 (SP1). Co-repressor factors associating with R-SMADs are the SWItch/Sucrose Non-Fermentable (SWI/SNF) nucleosome positioning proteins, DNA demethylating complex (DNDM), Forkhead Box (FOX) and elongation factor-2 (E2F) factors[2]. The TGFβ target genes SKI and SNON are co-repressor factors associating with phosphorylated SMADs in the nucleus in order to repress SMAD transcriptional activity[56]. Multiple target genes are controlled by SMAD canonical pathway and can be classified based on the activating ligand (TGFβ/ACTIVIN/NODAL or BMPs) or depending on its function on a particular cellular process (angiogenesis, ECM, immunosuppression, apoptosis). **Table 2** indicates the most common target genes activated by TGFβ and BMP pathway.

	SMAD Target genes				
	Growth arrest	**Apoptosis**	**Angiogenesis**	**ECM**	**EMT**
TGFβ/ACTIVIN-Responsive	CDKN1A (p21CIP1), CDKN1B (p27KIP1), CDKN2B (p15INK2B), CMYC,	BAD (BCL-XL),-BIM,GADD45B	TSP-1	FN, COL1A1, COL1A2, DCN, PAI-1, PDGFβ, MMP2, TIMP1, CTGF, ACTA2,	SNAIL1/2, ZEB1/2, HMGA2
	Differentiation	**Osteogenesis**	**Inflammation**	**Cardiogenesis**	**EMT/ MET**
BMP-Responsive	ID1, ID2, ID3, SMAD6/7, GATA3	OSTEOCALCIN, RUNX2, OSX	JUNB, IKBα	NKX2.5	ZO-1, SNAIL1, ID2

Table 2. Common target genes of TGFβ/ BMP pathways classified in various cellular responses
Abbreviations. ACTA2; aorta smooth muscle actin α 2, BAD (BCL-XL); BCL-2 associated agonist of cell death, BIM; BCL-2-like 11, CDKN1A (p21, CIP1); cyclin-dependent kinase inhibitor 1A, CDKN1B (p27KIP1); cyclin-dependent kinase inhibitor 1B, CDKN2B (p15INK2B); cyclin-dependent kinase inhibitor 2B, CMYC; v-myc avian myelocytomatosis viral oncogene homolog, COL1A1; collagen type 1 α 1, COL1A2; collagen type 1 α 2, CTGF; connective tissue growth factor, DCN; decorin, ECM; extracellular matrix, EMT; epithelial-to-mesenchymal transition, GADD45B; growth arrest and DNA-damage-inducible 45 beta, FN; fibronectin, GATA3; GATA binding protein 3, HMGA2; high motility group AT-hook 2 protein, ID; inhibitor of differentiation, IKBα; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha, JUNB; jun B proto-oncogene, MET; mesenchymal-to-epithelial transition, MMP2; matrix metalloprotease 2, NKX2.5; NK 2 homeobox 5, OSX; osterix (Sp7 transcription factor), PAI-1; plasminogen activator inhibitor 1, PDGFβ; platelet derived growth factor β polypeptide, RUNX2; runt related transcription factor 2, SMAD; Sma-Mad family member, SNAIL; snail zinc finger protein, TIMP1; tissue inhibitor of metalloproteinase 1, TSP-1; thrombospondin-1, ZEB; zinc finger E-box binding homeobox, ZO-1; tight junction protein 1.

1.3.3. Regulation of the TGFβ pathway

Multiple inhibitory mechanisms are integrated within the network of TGFβ pathway to control the timing, duration and cell context- dependent activation of the signaling cascade. Induction of TGFβ pathway simultaneously elicits negative feedback mechanisms that span throughout the cell from the extracellular space to the nucleus (reviewed in[51,56-58]). Understanding these mechanisms is clinically relevant for treatment of human diseases, as the intrinsic inhibition of the pathway has been mimicked with drug compounds used in clinical trials. An overview of these mechanisms is discussed in this section, starting from the cell membrane level and progressing gradually towards the cytoplasmic and nuclear level (**Fig.2**).

1.3.3.a. Antagonists of ligands

Extracellular inhibitory mechanisms of the TGFβ signaling include the association with LAP and LTBP that keep ligands in inactive state, as discussed in section 1.3.2 (**level 1**), as well as additional molecules with similar function.
For example, DECORIN is a small leucine-rich proteoglycan produced by smooth muscle cells, fibroblasts and vascular endothelial cells with inhibitory role for TGFβ[59]. It forms a large network with matrix proteins, receptor tyrosine kinases and growth factors, in particular with components of the TGFβ pathway[60]. DECORIN inhibits TGFβ signaling by sequestering the ligands and preventing their binding to the type II receptor[61].
Alternatively, DECORIN employs the calmodulin-dependent kinase II to phosphorylate SMAD2 at the inhibitory Ser-240 site[62]. This phosphorylation does not prevent nuclear translocation of SMAD2 (due to activating Ser 465/467 phosphorylation by type I receptor) but blocks interaction of SMAD2 with SMAD3 and nuclear translocation of SMAD3[62].

Other antagonists of different branches of TGFβ signaling with similar function as DECORIN are NOGGIN, GREMLIN, SCLEROSTIN, CHORDIN, FOLLISTATIN, VENTROPTIN, Follistatin-related gene protein (FLRG), CERBERUS and LEFTY[18,22,63] that negatively regulate signal activation at the ligand level.

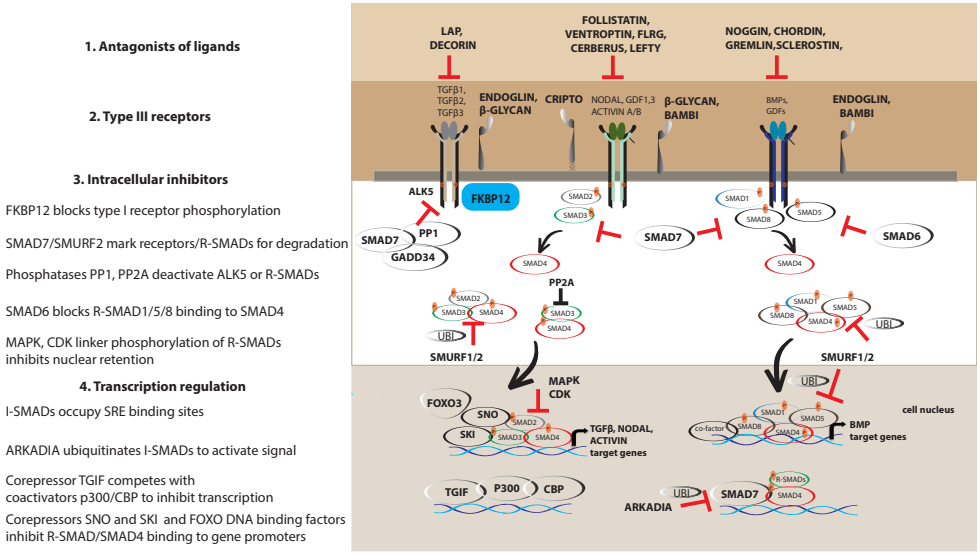


Fig.2. Regulation of TGFβ pathway from extracellular space to nucleus
Overview of the intrinsic TGFβ pathway mechanisms that control the duration of TGFβ signal activation at different subcellular localizations; (1) ligand antagonism in the extracellular space, (2) modulation of receptors and ligands at the membrane level by ENDOGLIN, CRIPTO, β-GLYCAN, pseudoreceptor bone morphogenetic protein and activin membrane-bound inhibitor BAMBI, FKBP12, (3) intracellular inhibitors I-SMADs (SMAD6 and SMAD7), E3 ligases SMURF1/2, phosphatases PP1/PP2A, MAPK and CDK kinases inhibit R-SMAD nuclear translocation via phosphorylation in the linker site of R-SMADs, (4) at the nucleus level, SMURF1/2 mark activated R-SMADs for degradation, SMAD7 and FOX0 factors directly inhibit gene transcription, co-repressors TGFβ-induced factor 1 TGIF, SNO and SKI antagonize for gene promoter binding either with the co-activators p300/ CBP or R-SMAD/ SMAD4 complexes and E3 ubiquitin ligase ARKADIA marks I-SMADs for degradation to elongate signal activation.

1.3.3.b. Type III receptors

A third group of TGFβ receptors (type III co-receptors) modulate the interactions between the type I/ II receptors and the ligands. To date, three such co-receptors have been identified; β-GLYCAN, ENDOGLIN (CD105) and CRIPTO (TDGF1). β-GLYCAN directs TGFβ2 to the type II receptor to facilitate signal activation[64] and enhances ALK3 and ALK6 signaling[65]. ENDOGLIN plays an important role in angiogenesis along with ALK1, TGFβ, BMP9 and BMP10[66,67]. ENDOGLIN is present in two isoforms (S and L form) as a membranous and secreted protein[68] and facilitates the binding of TGF ligands to the ALK1 in endothelial cells (activation of R-SMAD1, 5, 8), therefore preventing association of TGFβ with ALK5 and activation of R-SMAD2/3[69]. The TGFβ ligands NODAL and GDF1/3 require association with CRIPTO co-receptor in order to bind to the ALK4, ALK5 and ALK7 and induce activation of SMAD2 and SMAD3. Paradoxically, CRIPTO can inhibit SMAD2/3 phosphorylation mediated by TGFβ by binding to TGFβ ligands and preventing their binding to receptors[70,71]. Multiple functions are attributed to CRIPTO that are NODAL and TGFβ-independent, such as modulation of a network of signaling pathways (e.g. NOTCH, WNT, AKT) pathways in various tissues[72-74] and regulation of EMT in development and cancer[75]. The role of CRIPTO will be extensively discussed later in this chapter.
A distinct type of co-receptors are the glycoproteins of the repulsive guidance molecules (RGM) family, which inhibit specifically the ligands of the BMP branch[76-78], as opposed to other co-receptors that recognize various ligands. RGMa, RGMb (Dragon) and RGMc (hemojuvelin) particularly enhance signaling mediated by BMP2 and BMP4 or guide them to use alternative type II receptors e.g. ACVR2A instead of BMPRII[79].

1.3.3.c. Inhibition at the cell membrane level

Assembly of type I and II heterodimers is tightly regulated by a certain type of pseudoreceptor (BMP and ACTIVIN membrane-bound inhibitors; BAMBI) that consists of an extracellular domain of high structural similarity to serine/threonine receptors[80]. BAMBI binds BMPs and type I/ type II receptors but lacks cytoplasmic domain, thus preventing activation of type I receptor and downstream signaling[22].
TGFβ and BMPs directly induce BAMBI transcription[81,82] as a negative feedback mechanism to regulate the duration of signaling. To regulate the step of type II receptor-induced type I receptor transphosphorylation, the Ser/Thr residues of the type I receptor, that are phosphorylated by the type II kinase, are blocked by the inhibitor FK506-binding protein (FKBP12). In the inactive state (absence of ligand), type I receptor interacts via the GS domain with the inhibitor FKBP12 at the intracellular cell membrane site[83]. FKBP12 prevents type I phosphorylation rather than interaction with type II receptor and is a regulatory mechanism to prevent ligand-independent receptor activation for ALK5[84] as well as for BMP type I receptors[85]. Phosphorylation in the GS domain (adjacent to FKBP12 binding site) by the type II receptor releases FKBP12 and induces conformational change that facilitates binding of downstream effectors R-SMADs[84,86].
In homeostatic conditions, R-SMADs are actively retained in the cytoplasm while SMAD4 shuffles continuously from the cytoplasm to the nucleus[48].
The presence of the docking complex SMAD anchor for receptor activation (SARA) in the intracellular part of cell membrane keeps SMADs in the cytoplasm to facilitate receptor interaction and phosphorylation[87]. SARA binds SMAD2 and blocks the nuclear import signal

located in the MH2 domain; this process is reversible upon phosphorylation of R-SMADs by the type I receptor[88].

1.3.3.d. Post-translational modifications (PTMs) of receptors and R-SMADs

Post-translational modifications (phosphorylation, ubiquitination, sumoylation, glycosylation, fucosylation) affect protein folding and activity and as a result may increase or limit the bioavailability of the receptors and the activated R-SMADs during TGFβ response. In this paragraph an overview of the most common PTMs and their dynamic role in TGFβ pathway is discussed.

Phosphorylation promotes or inhibits kinase activity of both type I and type II receptors; the Ser/Thr kinase of TGFβRII is constantly active due to autophosphorylation[89], while Ser416 phosphorylation inhibits kinase activity[90]. Type I receptor exerts multiple functionalities by dual kinase activity; Ser/Thr phosphorylation as well as autophosphorylation of tyrosine (Tyr) residues. Lee et al., have demonstrated the way Tyr autophosphorylation of ALK5 activates ERK kinase which comprises a cell growth stimulus, counteracting the SMAD2/3 cytostatic pathway[91]. Deactivation of kinase domain and activated SMADs by dephosphorylation controls the duration and location of signal. Phosphatases are enzymes that remove the phosphate group from proteins and reverse phosphorylation, thus, switching the protein activity. There are three types of phosphatases, Ser/Thr, Tyr or of dual activity that contain catalytic and regulatory domains. Phosphatases PP1 and PP2A are established regulators of TGFβ member dephosphorylation. For instance, PP1 is recruited to dephosphorylate the type I receptor by a complex of SMAD7 and growth arrest and DNA damage protein GADD34, a regulatory subunit of PP1[92]. Phosphatase PP2A dephosphorylates SMAD3 but not type I receptor or SMAD2, indicating the specificity and regulatory role of these enzymes[93].

In addition to Ser/Thr and Tyr phosphorylation multiple other PTMs have been identified to positively or negatively regulate the function of the type I/ II receptors, such as sumoylation, ubiquitination[94] and possibly others. Such modifications also alter protein folding, protein localization, assembly with other proteins or target a protein for degradation[95]. For instance, sumoylation marks on unique sumoylation site Lys389 of the ALK5 modulates the kinase activity, recruits SMAD3 and potentiates signal activation[56,96,97].

Activated TGFβ/type I and type II complexes follow two intracellular routes; the clathrin-mediated endocytosis, that propagates the signal downstream, and the caveolae-associated cascade that interrupts the signal by degradation of the ligand/receptor complexes[57]. The degradation takes place either in lysosomes or in proteasomes; the latter requires ubiquitination by E3 ligases (ARKADIA, SMURF family)[98,99].

SMURF1 and SMURF2 often bind I-SMADs, such as SMAD7[100], and migrate from the nucleus to the cytoplasm to form complexes with activated receptors in the caveoli. This leads to polyubiquitination of the receptors, SMAD7 and SMURFs all of which are proteolytically degraded[57,80].

ARKADIA is a RINF-finger E3 ubiquitin ligase that marks SMAD7 for ubiquitin-mediated degradation thus aborting SMAD7 inhibitory function and enhancing SMAD signaling[101]. Inflammation-induced nuclear receptor NR4A1 is responsible for ARKADIA activation and SMAD7 degradation, a mechanism linking TGFβ hyperactivation with inflammation and tumor promoter activity[102,103].

Function of ARKADIA is highly determined by sumoylation[104,105]. Deletion of ARKADIA in mice leads to upregulation of SMAD6, SMAD7 and SKI and it has been shown that it

ubiquitinates SMAD6 and potentiates BMP signaling[106]. ARKADIA has dual functions at the transcriptional level where it interacts with chromatin remodelers such as Polycomb repressive proteins but has also proven to abort methylation-induced gene silencing of TGFβ target genes[107]. In addition, ARKADIA ubiquitinates SKI/ SNON associated with pSMAD2/3 complexes[108]. Degradation of this repressory network allows the formation of new pSMAD/ DNA complexes[109,110]
In addition, other PTMs of R-SMADs include phosphorylation in the linker region by GSK3, MAPK kinases, or cell cycle protein CDK4 which causes ubiquitination by E3 ligases (Lys11, Lys48) and proteasomal degradation[42]. Sumoulation of SMAD4 (Lys 159, 113) has been reported to regulate its function in renal and breast cancer cell lines[111,112]. Nuclear pSMAD4 is monoubiquitinated by USP9x to disrupt activated R-SMAD/SMAD4 complexes and release SMAD4 back to the cytoplasm[113].

1.3.3.e. Inhibitory SMADs (I-SMADs) and transcriptional repression

An intracellular negative feedback loop mechanism, directly induced by TGFβ[114], as well as BMPs[115], is the activation of I-SMADs, SMAD6 and SMAD7[116,117]. I-SMADs effectively limit or block completely the pathway by functioning in multiple subcellular localizations[57,80]. The intracellular circulation of I-SMADs is coupled to their function; (i) type I receptor blockers at the cell membrane[117,118], (ii) antagonists of SMAD4 in the cytoplasm[119,120] or (iii) occupying SRE binding sites to prevent SMAD- DNA functional complexes in the nucleus[121]. SMAD7 is primarily found in the nucleus during absence of ligand stimulation, while presence of TGFβ1 directly mediates the nuclear export of SMAD7 to induce inhibition of the type I receptor[122].
In addition, I-SMADs recruit E3 ubiquitin ligases SMURF1 and SMURF2 to direct them towards the phosphorylated type I receptors or R-SMADs for degradation[2,100]. SMAD7 inhibits both TGFβ and BMP signaling[123,124] while SMAD6 has BMP-specific action[15]. I-SMADs are subjected to functional restriction by other interacting proteins; e.g. BMPs activate an inhibitor of SMAD6 (associated molecule with the SH3 domain of signal transducing adaptor molecule, AMSH), which blocks SMAD6/SMAD1 complex formation and thereby SMAD1 phosphorylation is maintained[125].
Ultimately, the presence of activating and repressing TFs and complexes at a given gene promoter site determines whether SMADs exert a positive or negative transcriptional activity. Co-repressors play an important role in regulating the duration of signaling and proper target gene expression. Such co-repressors SKI, SNON, TGFβ-induced factor homeobox (TGIF) that interfere with SMAD signaling by repressing transcription of TGF/BMP target genes[126]. In turn, expression of these co-repressor proteins is induced by SMAD signaling. Another mechanism is the recruitment of HDAC co-repressor complexes to inhibit transcription which is mediated by I-SMADs; SKI co-repressor recruits HDACs and methylase complexes to repress the expression of SMAD7[127].

1.3.4. NODAL pathway

NODAL pathway has important functions during gastrulation, mesendoderm formation, induction of extraembryonic endoderm and left/ right asymmetry during embryonic development[63,128-131]. During adulthood NODAL pathway is quiescent and its reactivation is often associated with pathological situations[132].
Signaling is activated upon NODAL, GDF1 or GDF3 ligand binding to ALK4 or ALK7 and

ACTIVIN type II receptors. The accessory type III receptors CRIPTO (obligatory co-receptor for NODAL, GDF1/3) and CRYPTIC bind to the activated receptor heterotetramer and mediate SMAD2 activation[133]. CRIPTO and CRYPTIC belong to the epidermal growth factor-like, cysteine-rich CRIPTO-FRL1-CRYPTIC (EGF-CFC) protein domain family and have a dual role both as membranous and secreted proteins after cleavage of the glucophosphatidylinositol (GPI) link[134-136]. CRIPTO also functions as a chaperone of immature NODAL protein, directs it to the extracellular part of cell membrane where it is being subjected to proteolytic activation by convertases (FURIN and PACE-4)[137]. CRIPTO interacts with NODAL and TGFβ ligands via the EGF domain and with ALK4 via the CFC domain. CRIPTO interaction with NODAL is functionally dependent on PTM O-fucosylation on Thr88 residue which is characteristic of EGF domains[138].

Downstream signal transduction is primarily mediated by SMAD2/SMAD4 heterodimers, which associate with nuclear co-factors such as p53, FoxH1 to direct target gene transcription[139]. A role for SMAD3 during NODAL signaling remains to be further characterized[140]. NODAL target genes involve NODAL itself[63], CRIPTO[141] and the negative regulators LEFTY and CERBERUS[139,142]. LEFTY is an extracellular direct antagonist of NODAL ligand and the ACTIVIN receptors, while CERBERUS and CERBERUS-like (DAN protein family) bind to NODAL preventing its association with the receptors. Other negative regulators of NODAL signaling include; DAPPER2 (binds type I/II receptors for lysosomal degradation)[143], ECTODERMIN[144], TGIF1/2 proteins (co-repressors)[145], BMP3 and BMP7 (sequesters NODAL ligand)[133,146]. In turn, NODAL can also inhibit BMP signaling in a CRIPTO-independent manner[133].

In addition, CRIPTO has autonomous signaling functions that are NODAL and SMAD-independent[74]. In fact, CRIPTO individually regulates a large network of signaling pathways e.g. activating p38, ERK and c-SRC/MAPK/AKT pathways[130]. For this alternative function CRIPTO synergizes with glucose-related protein-78 (GRP-78)[70,72], GLYPICAN-1 signaling[147], caveolin[148], apelin[149], leucine-rich protein 5 (LRP5)[150] or NOTCH to modulate WNT and NOTCH signal transduction[75,151],

Aberrant CRIPTO pathway activity, particularly mediated via GRP78 by inhibiting TGFβ and activating and c-SRC/MAPK/AKT, is associated with human malignancies; breast, lung, prostate, ovarian, bladder, colon, liver, melanoma and glioblastoma[73,152-160]. Prognostic methods and strategies for *in vivo* inhibition of tumor-promoter role of CRIPTO/ GRP78 (peptides, monoclonal antibodies, tumor vaccines) are being studied preclinically[161-163] and in phase I clinical trials[164].

1.3.5. Non canonical SMAD pathways

In addition to the classical SMAD pathways TGFβ receptors exert their multifunctionality by activating non-SMAD pathways such as PI3K/Akt, Ras/ MAPK kinases ERK, p38 and JNK[165] (**Fig.3**). Both canonical and non-canonical branches have as starting point the TGFβ receptors, however, differential activities of the receptor complex due to PTMs, ligand-independent oligomerization or binding to different interaction partners determine which subpathway will be activated[56].

Cells circumvent the growth inhibition of TGFβ/SMAD signaling by using TGFβ to activate the growth stimulatory RAS/ RAF/ MEK/ ERK MAPK kinase pathway[42]. The MAPK kinase pathway is activated in response to mitogens such as EGF bound to receptor tyrosine kinases (RTKs)[51]. However, TGFβ elicits MAPK response due to the dual kinase activity of TGFβRI and TGFβRII to transautophosphorylate not only serine/ threonine but also tyrosine

residues (RTK function)[56]. Activation of RTK leads to activation of monomeric GTPase RAS, which acts as a scaffold between the RTK and RAF kinase (MAPKKK). Phosphorylation and activation cascade of downstream kinases MEK (MAPKK) and finally ERK (MAPK) propagates the signal to alter gene expression in favor of cell growth and proliferation[166]. ERK MAPK kinase propagates the signal via phosphorylation of target proteins in the cytoplasm (e.g. inhibition of SMADs by linker phosphorylation) and also translocates to the nucleus to activate gene regulatory proteins[166,167].

Another TGFβ/ non-SMAD mediated mechanism is the RTK function of TGFβ receptors, which activates the phosphoinositole-3 kinase (PI3K) pathway[168]. PI3K activation leads to recruitment of kinases PDK1 and Akt receptors in phosphorylated lipid docking sites where the two kinases phosphorylate each other leading to activation of Akt[169]. Akt phosphorylates other proteins such as cell survival complex mTOR 170 or inactivates the proapoptotic protein BAD via recruiting adaptor protein 14-3-3[171,172]. The net outcome is cell survival, growth and proliferation.

In addition, TGFβ regulates actin cytoskeleton formation and cell adhesion by interfering with the monomeric GTPase proteins of the RHO family (RHO, RAC and CDC42)[168]; TGFβRII phosphorylates polarity protein PAR6 that together with SMURF1 marks RHO for ubiquitin-mediated degradation[173]. The function of RHO signaling is to maintain the epithelial tight junctions, thus, TGFβ induces a mesenchymal transformation of epithelial cells[173].

Another example of non-SMAD activation by TGFβ receptor is the network of ubiquitin ligase TNFα-associated factor 6 (TRAF6) and TGFβ-associated kinase 1 (TAK1), key inducers of p38 and JNK MAPK pathway[56]. TGFβ activates TAK1 via TRAF6; TRAF6 is constitutively bound to ALK5 and upon oligodimerization due to TGFβ binding, TRAF6 molecules reach physical proximity, which facilitates their transautoubiquitination. Subsequently, TRAF6 ubiquitinates TAK1 and activates its kinase catalytic domain. TAK1 activates p38 and JNK MAPK by phosphorylation resulting in activation of transcription factors AP-1, c-JUN and c-FOS[174-176]. Furthermore, as TGFβ activates other non-SMAD pathways similarly members of other signaling pathways are modifying SMAD effectors[2]. Thus, it should be kept under consideration that crosstalk between pathways is usually bi- or multidirectional, increasing the complexity of cellular responses to extracellular stimuli.

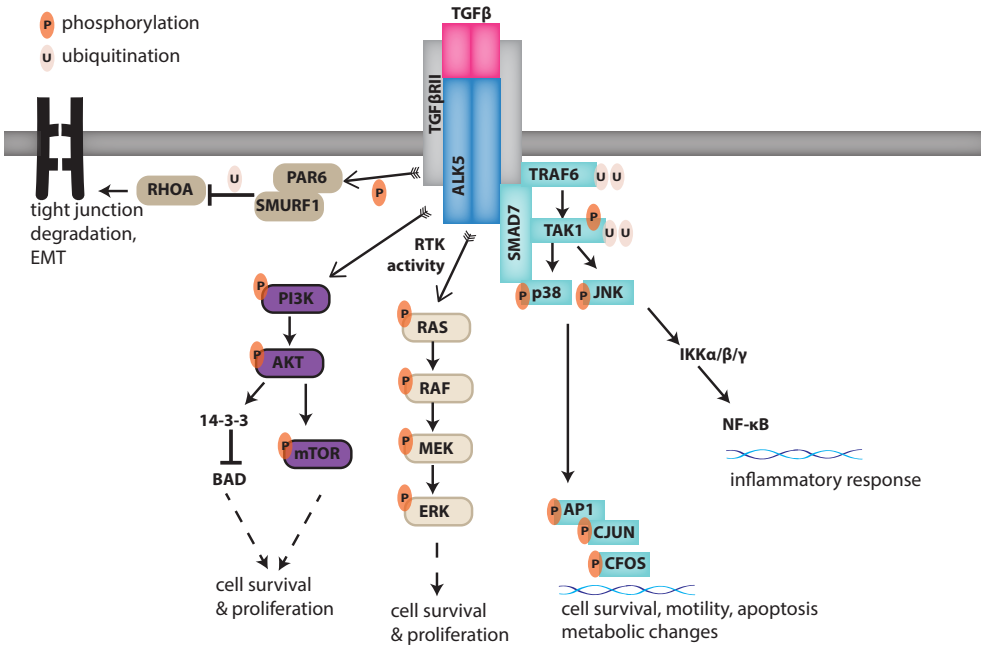


Fig.3. TGFβ/non-SMAD pathways and their biological effects
TGFβ receptors alter gene expression via the MAPK/ERK, TRAF6/TAK1/p38/JNK, NF-κB, PI3K/AKT/mTOR and PAR6/RHO signaling pathways that lead to multiple cellular responses.

1.4. TGFβ signaling pathway in homeostasis and disease- studies in liver, prostate and connective tissue

A plethora of biological processes are regulated by TGFβ cytokine in embryonic and adult tissues by means of growth arrest, cell differentiation, EMT, immune system regulation and angiogenesis. In fact, the description attributed to TGFβ as cytokine (growth factor) is a paradox since it promotes growth inhibition and halts cell proliferation (cytostasis) under nearly all physiological conditions. Transcriptomic analyses have revealed that TGFβ signaling controls the activation and repression of hundreds of genes in a single cell and leads to differential gene responses[177,178]. Thus, tight regulation of this pathway is crucial to guard signal specificity; the importance of growth inhibition is evident in human cancers where TGFβ-induced cytostasis is often disrupted.
The homeostatic role of TGFβ is cell type and microenvironment-dependent. In brief, anti-proliferative effects are exerted in epithelial tissues (for instance; skin, liver, breast, prostate, and lung)[58]. Mechanistically, TGFβ inhibits cell cycle progression via regulation of cyclin-dependent kinase inhibitors p15INK4B, p21CIP1, and p27KIP1[179,180], inhibits cell cycle promoters such as the proto-oncogene C-MYC and ID proteins. Apoptosis is induced through activation of caspase protein cascade[181]. Non-epithelial tissues are also under growth control e.g. endothelium[182,183], fibroblasts[184], neuronal tissues[185], cells of the immune and hematopoietic system[186]. In addition, TGFβ signaling orchestrates wound-healing response in most organ systems. If aberrantly regulated, it may lead to excess scar tissue formation, accumulation of collagen-producing cells and extracellular matrix (ECM) and eventually disrupt normal tissue structure and physiology.

In this thesis the homeostatic role of TGFβ is highlighted in three organ systems; liver, prostate epithelium and connective tissue (**Fig.4**).
Liver function is crucial for the homeostasis of the whole organism and is evident by the evolutionarily preserved regenerative capacity of mammalian liver. Under normal conditions, the liver is metabolically active but quiescent in terms of cell proliferation; cell division is minimal greatly due to cytostatic role of TGFβ among other factors. Overexpression of TGFβ1 in hepatocytes (liver epithelial cells) leads to increased apoptosis, fibrosis and reduced proliferative and regenerative response[187,188]. Liver fibrosis is associated with genetic polymorphisms of TGFβ gene leading to increased TGFβ1 serum levels[189]. The role of TGFβ in the liver is extensively discussed in Chapter 2.
Prostate tissue is divided in proximal, distal and intermediate ducts and androgen hormones are the main regulators of its physiology[190]. Androgens have functional convergence with TGFβ[191] which is expressed in a gradient form in prostate tissue. High levels of TGFβ signaling are present in the quiescent proximal region of ducts and androgen ablation reverses the proximal-distal TGFβ signaling gradient, leading to an increase in TGFβ signaling in the distal region[192]. Testosterone (5α-dihydro) decreases the level of TGFβ receptor II (TGFβRII) leading to suppression of the ability of TGFβ to down-regulate expression of Bcl-xL and cyclin D, activate caspase-3, and induce apoptosis[193]. Overexpression of dominant-negative form of TGFβ receptor type II in transgenic mice decreased apoptosis in the prostate epithelium[194]. Accordingly, *in vivo* injection of TGFβ1, in the ventral prostate, increases apoptotic events[195].
Connective tissue is an example of a non-epithelial system that is regulated by TGFβ signaling. Connective tissue is comprised of cells and ECM, and is found in different types in the body. ECM is composed by glycoproteins, fibrous proteins and glycosoaminoglycans, which are secreted by cells, mainly fibroblasts. Variations in the ECM composition determine the properties of the connective tissue (tendons, cartilage, eye cornea or if the matrix is calcified, it can form bone or teeth). Generally, connective tissue is either loose (adipose), or dense (tendons between muscles and bones), depending on the fiber arrangement. TGFβ plays an important role in the maintenance of the structural elements of ECM (collagen, elastin fibers) as well as the proliferation of fibroblasts and their transdifferentiation into myofibroblasts (MFBs). MFBs are crucial for wound healing as the main source of ECM proteins and maintain a vicious cycle of TGFβ production, responsiveness to TGFβ and ECM secretion. In fact, normal wound healing in adult animals is greatly regulated by TGFβ; initially TGFβ is secreted by platelets, which leads to recruitment of other immune cell types (neutrophils, macrophages) and fibroblasts[196]. Fibroblasts initially migrate into the wound area and secrete a collagen- and cellular fibronectin-rich ECM[197]. In fact, fibronectin cross-talks with TGFβ signaling influencing activation of latent TGFβ in the matrix. Wound closure is achieved by ECM remodeling and angiogenesis; both processes are orchestrated by the pro-fibrotic and pro-angiogenic actions of TGFβ.
Despite the plethora of biological processes that TGFβ signaling is involved in, from a clinical point of view, aberrant TGFβ expression/ downstream activation is often associated with connective tissue disorders[198]. Mutations in TGFβ receptors are linked with Marfan syndrome[199], Loeys-Dietz syndrome and others[200].

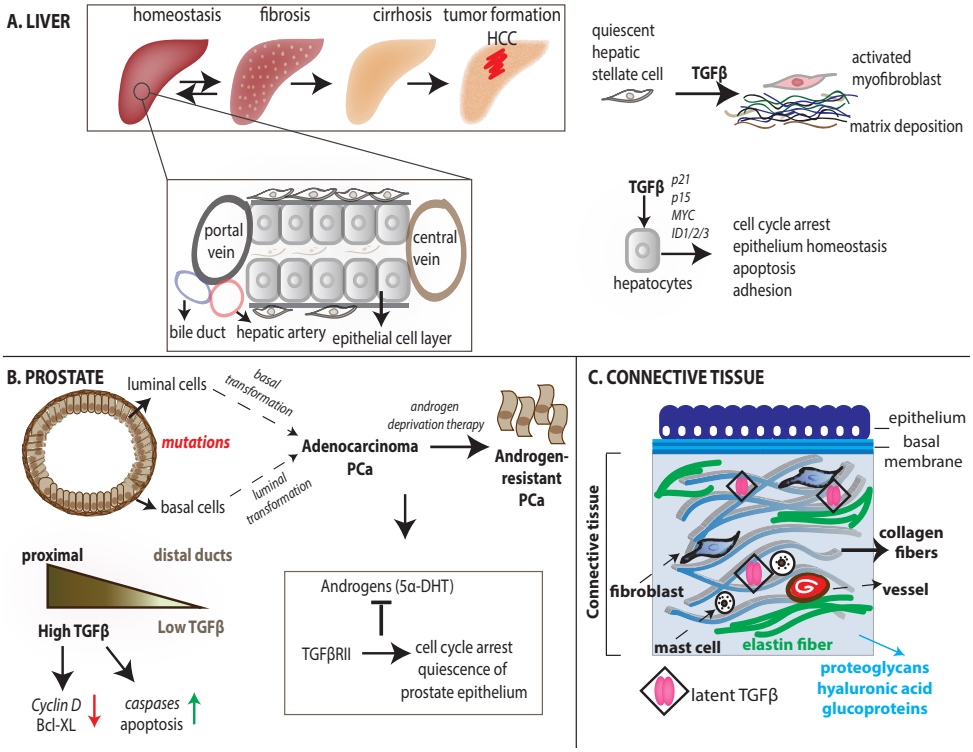


Fig.4. Homeostasis in distinct organ systems and their regulation by TGFβ signaling
(A). Liver disease progression from fibrosis to hepatocellular carcinoma (HCC), tissue cell types and morphology are depicted. Effects of TGFβ are summarized for the epithelial cells (hepatocytes and cholangiocytes) and the hepatic stellate cells (HSCs), precursors of myofibroblasts (MFBs) in the liver. (B). Prostate tissue morphology with proximal and distal ducts comprised by luminal and basal cells (neuroendocrine cells are not depicted here). TGFβ ligands exist in morphogenic pattern during homeostasis; highest concentration and signaling occurs in the proximal duct site (note that prostate stem cells reside in this area). Androgens promote cell proliferation by interfering with TGFβRII levels and androgen ablation therapy following prostate cancer detection leads to reversal of TGFβ distribution (distal instead of proximal). Transformed luminal cells cause adenocarcinoma development. Basal cells also contribute to prostate malignancy following a step of luminal differentiation. (C). Morphology and cell type distribution in the connective tissue underlying the epithelial barrier. Connective tissue is comprised by matrix (proteoglycans, hyaloronan, glycoproteins, elastin and collagen fibers) and a cellular component (endothelial cells, macrophages, mast cells and the most abundant, fibroblasts).

1.5. TGFβ signaling in fibrosis

We discussed the structural role of the matrix in connective tissues in the previous section. However, the matrix is not a static element made by cellular proteins but it mechanically and biochemically influences basic cellular processes[201]. Bissell *et al.,* firstly defined this phenomenon, as dynamic reciprocity between ECM, cell cytoskeleton and nuclear matrix[202]. This interplay of matrix and cells not only affects cell shape or motility but also actively alters signal transduction and gene expression pattern (mechanotransduction)[203-205]. TGFβ, a latent extracellular cytokine that regulates cellular processes by activating intracellular signaling is a key factor in the interface between cells and their ECM context[206]. For instance, the matrix can induce the expression of TGFβ1[207]. Moreover, the extracellular agonist of TGFβ ligands, DECORIN, is also a regulator of collagen maturation and assembly[208]. In this

section, we will discuss the implications of TGFβ in pathological fibrosis, in particular liver and Dupuytren's fibrosis (DD).
During the last decades fibrosis has accounted for up to 45% of deaths[209] and yet there are no approved antifibrotic therapies available. Fibrosis is a pathological state characterized by the excessive deposition of ECM proteins commonly occurring during wound healing and tissue regeneration. Excess deposition of collagen and proteoglycans is associated with reduced tissue epithelization and cell death, and eventually disrupted cell functionality and tissue architecture (**Fig.5**). Fibrosis may affect most organ systems and lead to a variety of diseases including liver cirrhosis, connective tissue fibrosis, pulmonary hypertension, systemic sclerosis and heart fibrosis representing a major medical challenge.
A complex set of genetic, immune response, epigenetic factors may lead to fibrosis by triggering constant activation of quiescent tissue fibroblasts to MFBs, the key pathogenic cells in fibrosis. The cellular and molecular phenotype of MFBs is highly dependent on TGFβ signaling pathway[210,211]. TGFβ stimulates ECM protein synthesis and secretion, decreases expression of proteases that cleave ECM (matrix metalloproteases, MMPs) and increases protease inhibitors (TIMPs)[212]. The outcome is a shift of balance towards ECM protein synthesis, secretion and deposition rather than degradation leading to scar tissue formation. TGFβ family members and target genes include ECM and cytoskeleton proteins that are often deregulated in fibrotic and other diseases, such as plasminogen activator inhibitor 1 (PAI-1)[213], collagen type1α1 (COL1A1), COL1A2, COL4A2, COL5A1, COL5A2, α-smooth muscle actin (α-SMA, ACTA2) and fibronectin[214-216].

1.5.1. Liver fibrosis

Liver fibrosis (cirrhosis) occurs in response to chronic liver injury due to alcohol intoxication or viral hepatitis B and C infections (HBV, HCV)[217]. TGFβ plays a role in all the stages of liver disease progression from inflammation, cirrhosis to cancer formation [218].
Cirrhosis often is a precursor to hepatocellular carcinoma (HCC), thus, the need for effective treatment is high. Collagen-depositing MFBs accumulate around the portal and central vein of the liver lobules. The source of MFBs in the liver is mainly the pericyte population of the liver, hepatic stellate cells (HSCs), transformed epithelial cells and fibrocytes from the bone marrow. The activation of HSCs and their transdifferentiation to MFBs is controlled by the pro-fibrogenic effect of TGFβ pathway, evident by multiple studies (reviewed extensively in[218-220]). Fibronectin modulates this response of HSCs to TGFβ during liver injury in a way that controls the extend of fibrosis[221]. *In vivo* deletion of SMAD3 results in improvement of liver fibrosis in mice[222].
Expression of fibrosis-related genes, such as collagens or PAI-1 in MFBs is induced by phosphorylation of SMAD2 and SMAD3 in the linker site by CDK4, p38 and JNK MAPK kinases[223]. In fact, the differential phosphorylation isoforms of SMAD2/3 (linker, cytoplasmic) may induce different levels of the inhibitor SMAD7[223]. SMAD2 and SMAD3 both are needed for induction of MMP2 and αSMA expression, however, it seems that SMAD2 mostly orchestrates the TGFβ-mediated cytostasis and maintains the epithelial phenotype while SMAD3 is indispensable for TGFβ-profibrogenic role[224,225]. SMAD7 blocks the fibrogenic response of HSCs in acute liver injury but not in chronic liver injury indicating that this negative feedback mechanism might be deactivated in liver fibrosis[226]. However, hepatocyte-specific deletion of SMAD7 in transgenic mice with chronic carbon tetrachloride (CCl_4)-induced fibrosis ameliorates the fibrotic phenotype[227].

TGFβ binds to both ALK1 and ALK5 in the liver, thus, a certain balance of ALK1/ ALK5 ratio is necessary to maintain the balance between protective, anti-fibrogenic action and pro-fibrogenic activity of TGFβ. TGFβ/ALK1 signaling appears to directly antagonize TGFβ/ALK5 signaling, while in other circumstances, the presence of ALK5 is an absolute requirement for efficient TGFβ/ALK1 signaling[228]. The role of BMPs in liver diseases is also being addressed by recent studies suggesting BMP9 as a pathological driver[229] and BMP7 as an anti-fibrotic factor that antagonizes TGFβ pathway[230-232].

1.5.2. Dupuytren's fibrosis (part of this section is published in[233])

Dupuytren's disease (DD) is one of the most common connective tissue disorders with a higher prevalence in Caucasians of northern Europe[234] and particularly in males[234-236]. DD is a fibroproliferative disease affecting the palmar fascia, and may lead to permanent flexion contracture of the digits[237] (**Fig.5**). Current treatment of DD is symptomatic; surgical removal of the fibrotic nodules and cords leads to immediate relief of the contractured digits. Injection of collagenase enzyme obtained from Clostridium Histolyticum, has been approved by FDA (Xiaflex, Pfizer) as alternative treatment for DD[238]. However, the recurrence rate of the disease using the current therapeutic approaches remains high.
Several environmental and genetic risks have been linked to DD supported by studies of familial cases, ethnicity and sex prevalence, occurrence in twins and postoperative recurrence[239]. However, the genetic mechanism is not fully understood and there is no evidence of a single genetic dysregulation as a cause of DD. Thus, the aetiology of DD remains unknown, although it is clear that TGFβ plays a role in the pathogenesis[210,237].
High TGFβ1, TGFβ2 mRNA and protein levels have been associated with DD fibrosis[210,215,240]. TGFβ2 shows intracellular localization within MFBs in the proliferative and involution stages of the disease[241,242]. In fact, transdifferentiation of quiescent fibroblasts into MFBs requires signaling by TGFβ[243,244]. Contractility of MFBs in increased after *in vitro* stimulation with low concentrations of exogenous TGFβ[245-247]. Presence of SMAD binding sites on the promoters of connective tissue growth factor (CTGF/CCN2), αSMA, fibronectin and collagens, Serpine-1 (PAI-1) show that TGFβ directly controls the expression of MFBs-associated proteins[248-253].
The high proliferative properties of MFBs contradict the TGFβ proapoptotic and growth inhibitory role. However, it has been proven that in DD cells TGFβ induces expression of other cytokines such as platelet-derived growth factor (PDGF); in turn, PDGF activates ERK MAPK kinase pathway, induces expression of proto-oncogene c-MYC[242] and promotes cell proliferation. Studies on BMP signaling have not proven a link between deregulated BMP pathway members and DD pathogenesis. BMP6 has a potential antagonistic role against TGFβ as shown by reduced *in vitro* contractility and SMAD/ ERK activation in fibroblasts treated with BMP6[254].
Although much work has attempted to unravel the complex mechanisms underlying fibrosis, the current state of the art in DD and generally in fibrosis research fails to meet the demanding need for treatment[255]. Cell culture models for studying fibrosis currently include primary cells and/or cell lines as well as the use of different culture matrices and co-culture models. It is now evident that two-dimensional (2D) cultures of fibroblasts have distinctly different properties and gene expression profile than the intact tissue[256,257]. This can be, in part, attributed to the *in vitro* protocols and adaptation to culture conditions. For experimental reasons, connective tissue obtained from carpal tunnel tissue operations is used for comparison to diseased DD palmar fascia, and arbitrarily considered "healthy

control" while it may be molecularly very similar to DD[258]. All these describe one of the biggest limitations of the field, i.e. the lack of an *in vitro/ex vivo* model that allows molecular and genetic manipulation. A recent study proposes xenograft transplantation of human DD fibroblasts in the subcutaneous layer of the skin of mice as an *in vivo* model for DD research, but yet this model poorly recapitulates the human disease.

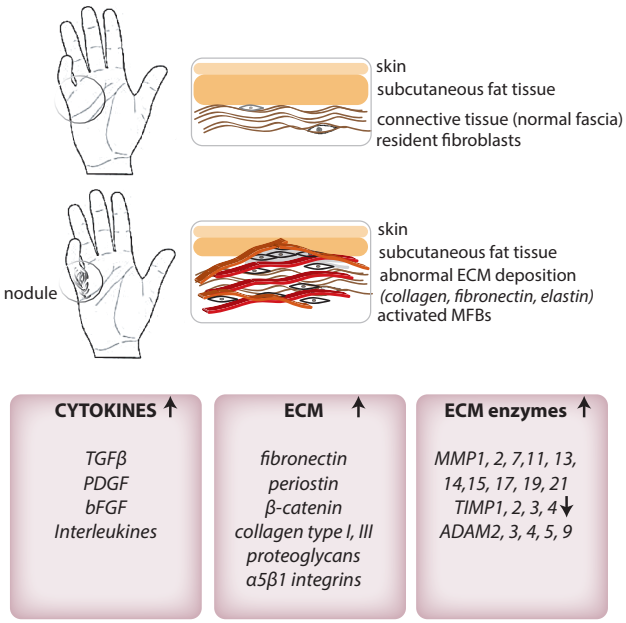


Fig.5. Overview of the disrupted tissue architecture and the most common molecular aberrations associated with Dupuytren's fibrosis

Fibrotic nodules consist of highly proliferative and contractile myofibroblasts (MFBs) that deposit matrix proteins. Normal matrix turnover and degradation are decreased due to molecular aberrations leading to fibrosis, tissue disfiguration and digit contracture. Adapted from [237].

1.6. TGFβ/ BMP signaling in cancer

In normal cells, growth inhibition mediated by TGFβ is usually dominant over growth stimulatory action of other factors. However, the situation is reversed in malignant situations that are characterized by hyperproliferation due to mitogens, action of mutated oncogenes and hyposensitivity to anti-proliferative action of TGFβ[198,259]. In normal conditions, TGFβ keeps normal epithelial tissues in a proliferation blockage, thus having tumor-suppressor role. Occurrence of oncogenic somatic mutations in epithelial cells leads to formation of primary carcinoma. Aberrant cell division without tight regulation of DNA synthesis and repair leads to additional accumulation of oncogenic mutations.
The primary carcinoma may remain spatially confined if the TGFβ-mediated cytostatic cues are intact. However, if mutations in TGFβ/ BMP ligands, receptors and SMADs occur, then the cells acquire proliferative and migratory properties that facilitate cancer metastasis[58].

Apart from its cytostatic role, TGFβ regulates many other biological processes that are hallmarks of cancer, such as EMT, suppression of cytotoxic T lymphocytes and angiogenesis. Thus, malignant cells hijack TGFβ to obtain phenotypic characteristics crucial for cancer progression such as mesenchymal cell shape, increased motility and invasion through basal membranes into extracellular space and blood vessels (**Fig.6**).

The tumor-promoting role of TGFβ in advanced carcinomas is mainly due to TGFβ signaling through the SMAD/1/5/8 machinery and inducing expression of ID proteins[260], via non-SMAD pathways (circumventing the Ser/Thr kinase activity of TGFβ receptors or PTM regulation of linker and C-terminal SMAD phosphorylation) to activate the growth-stimulatory pathways ERK and AKT[56,168,261]. The essential role of TGFβ in stimulating metastasis[262] and the high frequency of genetic mutations in TGFβ pathway leading to cancer[263] highlight the necessity for TGFβ-targeting therapies.

1.6.1. Epithelial-to-mesenchymal transition (EMT)

Polarized cells, positioned adjacent to each other via tight junctions, comprise epithelial tissues. Epithelial cells are stably in contact with the basal membrane forming a basal-apical polarity and have epithelial- specific gene expression pattern (E-CADHERIN, ZO-1, LAMININS)[264]. However, epithelial cells are quite plastic under certain conditions such as tissue morphogenesis during development and would healing[49]. Plasticity allows them to progressively switch on the genetic program of mesenchymal gene expression that leads to loss of epithelial phenotype and acquisition of a mesenchymal one. This cellular process of epithelial cells disintegrating from the basal membrane, losing cell-cell contacts and becoming motile is termed epithelial-to-mesenchymal transition (EMT) and is reversible (MET) (**Fig.6**). Both EMT and MET are mechanisms of cancer metastasis; cancer cells undergo EMT to extravasate from the primary tumor into blood circulation or from blood vessels into other epithelia and reverse to MET program to invade and colonize the new sites[259] (**Fig.6**). During EMT, epithelial proteins such as E-CADHERIN are downregulated and mesenchymal, cytoskeletal and ECM proteins are upregulated (FIBRONECTIN, VIMENTIN, NCADHERIN[265]). High motility group AT-hook 2 protein (HMGA2) via TGFβ/SMAD pathway regulates EMT master transcription factors SNAIL1/2, ZEB1/2 and TWIST, which repress epithelial genes and activate mesenchymal genes[266-268]. In addition, TGFβ signaling, via the SMAD1/5/8/

ID1 activation, is implicated in MET and promotes metastasis[260,269]. TGFβ promotes EMT by interfering with RHO complexes in epithelial cell junctions; this mechanism is TGFβRI and SMAD-independent (TGFBRII/PAR6/SMURF1/RHO)[173]. The reverse process (MET) is regulated by BMP signaling, in particular BMP7[270].

1.6.2. Tumor angiogenesis

Deregulation of TGFβ and BMP pathways lead to vascular defects, such as pulmonary hypertension, hereditary telangiectasia (HHT)[198,271]. Deletion of TGFβ ligands or ALK1 in transgenic mice results in embryonic lethality due to vasculogenesis defects[272,273]. Vascular homeostasis relies on endothelial cells, smooth muscle cells and pericytes[274]. The key angiogenesis-related members are TGFβ ligands, ENDOGLIN, ALK1 and its ligands BMP9 and BMP10, that synergize with proangiogenic factors such as vascular endothelial growth factor (VEGF), NOTCH pathway, PDGF, angiopoietins and basic fibroblast growth factor (bFGF)[271,275,276].

Angiogenesis determines normal and malignant tissue growth. The requirement for new vessels is high in primary tumors as the highly metabolic and proliferative cancer cells need oxygen, nutrients, and cytokines from the blood. As the epithelial cells cluster and proliferate forming a primary carcinoma, new vessels must be formed (**Fig.6**) in order for the blood flow to reach all the cells within the tumor (angiogenic switch). However, since tumor cells cannot perform *de novo* angiogenesis they have evolved to disrupt the existing normal vasculature and recruit endothelial cells into the tumor[277]. TGFβ promotes tumor angiogenesis by inducing expression of MMPs that degrade the basal membrane and assist endothelial cell migration[278]. TGFβ induces MMP2 and MMP9 in tumor cells[279]. Tumor angiogenesis is also influenced by MMP14 protease that releases membranous ENDOGLIN into its secreted form[280]. High TGFβ-expressing prostate cancer cells induce an angiogenic response when transplanted *in vivo*[281] and inhibition of TGFβ activity by neutralizing antibodies decreases tumor angiogenesis[282].

1.6.3. Prostate cancer

Prostate cancer (PCa) arises from precursor lesions, defined as prostatic intraepithelial neoplasia (PIN), gradually progresses to locally invasive disease and ultimately to metastasis. Disease progression from PIN lesions or organ-confined PCa towards metastatic PCa involves multiple genetic and epigenetic events to take place. Each stage of this disease is associated with characteristic morphological and histo-pathological alterations. Associated with the human disease are also genetic chromosomal alterations, which have led to the identification of several tumor suppressor genes (for example, TP53, CDKN1B and PTEN) and androgen related gene fusions (such as TMPRSS2-ERG) of key importance in the early stages of the disease[282, 283]. Furthermore, the androgen receptor (AR) is required for maintenance of the prostate epithelium during normal organogenesis as well as carcinogenesis, including hormone-independent cancer. The androgen refractory stage is the final and most aggressive stage of the cancer, characterized by bone and lymph node metastases. As in most cancer types, TGFβ has growth inhibitory effects on primary PCa, but tumor-promoting role during advanced stages and leads to metastasis formation. In addition, stromal TGFβ can activate AR signaling in absence of androgens, which might contribute to hormone-independent growth of tumor[284].

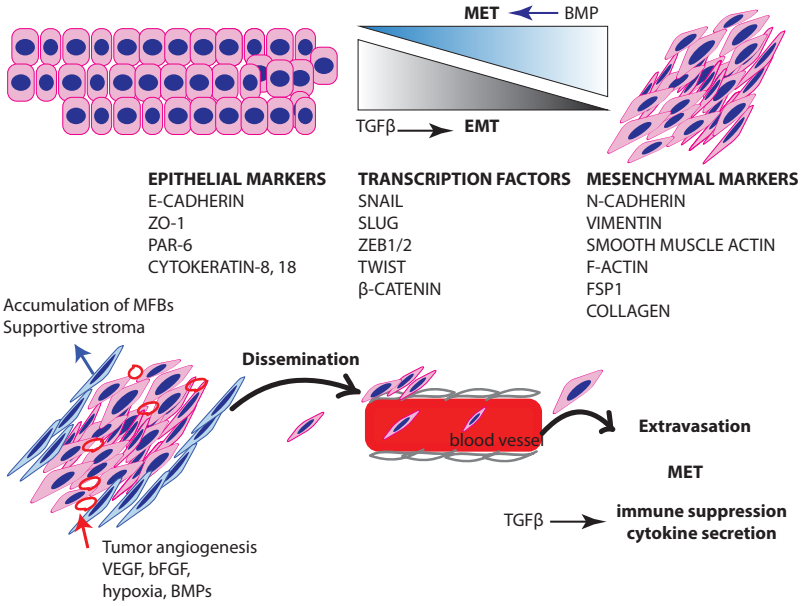


Fig.6. Stages of cancer progression and TGFβ/BMP signaling
Formation of primary carcinoma and phenotypic transition of epithelial cells into mesenchymal cells (EMT) are depicted. Tumor microenvironment (supportive stroma) consists of infiltrating immune cells and myofibroblasts (MFBs) derived by quiescent fibroblasts or by tumor epithelial cells via EMT. Crosstalk between the stroma and tumor cells, using cytokines and other signaling molecules, promotes acquisition of tumor vasculature (angiogenesis) which sustains tumor growth by delivery of nutrients and oxygenated blood. Tumor cells may disseminate from the primary tumor into the blood circulation, extravasate from the vessels through the perivascular and extracellular matrix and metastasize to secondary tissues (mesenchymal-to-epithelial transition, MET).

EMT and migration of PCa cells are induced by TGFβ via MAPK kinase ERK2 and c-MYC expression[285]. BMP ligands also play a role in PCa[286], in particular BMP2, BMP4, BMP7[287, 288]. Cell proliferation is increased in presence of BMP2 and BMP4, however, it not clear from the existing studies whether BMP7 is tumor promoter or suppressor in prostate[289]. BMP2 expressed by osteoclasts might act as chemotactic factor for PCa cells to metastasize to the bone[290]. BMP9, the primary ligand of ALK1 in endothelial cells, might also play a role in PCa, as in other types of cancer as we will discuss in the next section. BMP9 is expressed in prostate epithelium along with BMP receptors[291].

1.6.4. Hepatocellular carcinoma

Liver tissue can endure chronic damage and symptoms become evident in advanced disease stage making prognosis of liver diseases difficult. Hepatocellular carcinoma (HCC), one of the most frequent forms of cancer, is usually detected at a late stage, thus the current forms of therapy are often not curative e.g. tumor resection, treatment with sorafenib or eventually liver transplantation[292].
TGFβ plays a role in HCC development[218,293] and one of the first indications was the finding that circulating levels of TGFβ1 in plasma from HCC patients are significantly higher than in patients with cirrhosis or viral hepatitis[294]. SMAD7 overexpression has tumor-suppressing role[295]. Liver expresses high amounts of BMP9 that are also secreted in the circulation[296]. HSCs and potentially also hepatocytes, express ALK1 and ENDOGLIN, the interaction partners of BMP9, under normal and pathological conditions. Apart from a role in endothelial cells and HSCs, BMP9 has been associated with HCC[297].
NODAL expression is shut down after embryonic development, but it is re-expressed in adult tissues, along with its co-receptor CRIPTO during malignant conditions. NODAL and CRIPTO are involved in plasticity of tumor cells, cancer-stem cell maintenance and metastasis[298,49]. In the liver, stem cell renewal transcription factor NANOG is reactivated during HCC and it mediates EMT by activating NODAL/ CRIPTO and SMAD3 expression[158]. Moreover, the interaction partner of CRIPTO, GRP78, has been associated with liver cell stress response and HCC[299-301]. Thus, both the canonical and the non-canonical NODAL pathway have a contributing role in HCC.

1.7. Anti-TGFβ strategies (antisense oligonucleotides/ small molecule kinase inhibitors/ ligand traps)

Several components of the TGFβ pathway have been investigated for drug development; however, only a few compounds have proceeded into later stages of clinical trials[302,303]. TGFβ signaling is a key pathway for homeostasis and given its pleiotropic, cell-type and context-dependent role, therapeutic interventions are beneficial for a particular tissue or cell type, at a specific stage of the disease. During cancer progression, if tumor cells are insensitive to growth inhibition by TGFβ, therapeutic enhancement of signaling might be useful to constrain their proliferation if the tumor is detected at an early stage[304]. Nevertheless, malignant cells quickly adapt and start using TGFβ to promote their metastatic spread. Given that cancer diagnosis does not detect micrometastasis events, it is more logic to preventively inhibit rather than induce TGFβ signaling when a primary tumor is diagnosed in order to stop the lethal consequences of metastasis. Moreover, expression levels of TGFβ pathway members are often elevated during conditions such as organ fibrosis and cancer; thus, inhibitory strategies are required to block the fibroproliferative role of TGFβ in MFBs (fibrosis), cancer-associated fibroblasts (CAFs) and to suppress tumor cell metastasis.
A growing number of recent studies have tested TGFβ inhibitors as combination treatment with chemotherapeutics[305], immune stimulatory agents (interleukin-2)[303,306] or to minimize radiotherapy-induced carcinogenesis[307]. In the cancer field inhibition of TGFβ type I and type II receptors has been accomplished, while in the fibrosis field most of the anti-fibrotic drugs are designed to interfere at the ligand level of pathway transduction, therefore preventing their binding to the receptors[308]. The most successful TGFβ inhibitory strategies (**Fig.7**) used in experimental and clinical studies (**table 3**) are the antisense oligonucleotides

AONs targeting TGFβ ligand mRNA (Antisense Pharma), the competitive peptides against ligands (ligand traps, Digna Biotech), the neutralizing antibodies and the small molecule inhibitors of receptor kinase activity.

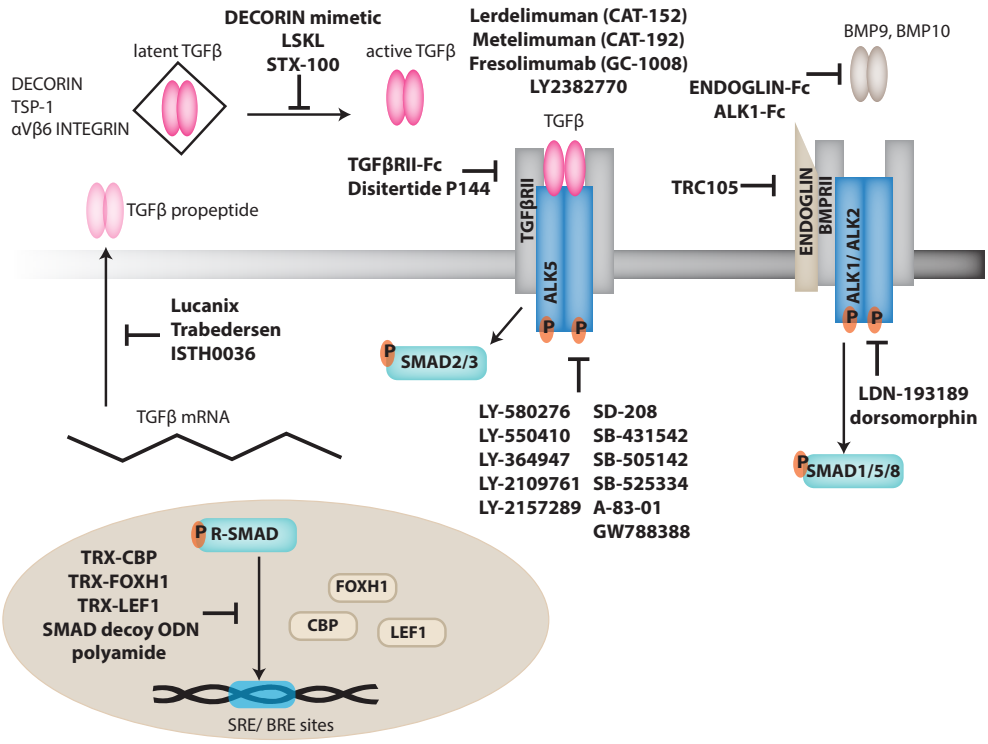


Fig.7. Schematic overview of different inhibitory strategies targeting TGFβ/BMP signaling activation
TGFβ pathway activation can be inhibited by different compounds, which target mRNA/ protein expression or protein function and act at different subcellular compartments; (1) compounds that inhibit activation of latent TGFβ (DECORIN mimetic, THROMBOSPONDIN and αvβ6 INTEGRIN antagonists), (2) soluble type II/ III receptor ectodomain peptides that trap ligands and prevent interaction with a functional receptor, (3) neutralizing antibodies against TGFβ, BMP ligands and receptors, (4) small molecule kinase inhibitors that inhibit activity of type I or type II receptors , (5), antisense oligonucleotides interfering with mRNA translation of TGFβ2 mRNA, oligonuleotides that bind to gene promoters mimicking SMAD binding (pyrrole-imidazole polyamide, SMAD decoy ODN), (6) thioredoxin peptide aptamers for SMAD binding to co-factors FOXH1,LEF and CBP (TRX).

1.7.1. Antisense oligonucleotides (AONs)

AON methodology is used to modulate gene expression in a sequence-specific way and has shown broad therapeutic applicability in many human diseases, particularly in the field of muscular dystrophies[309] with very promising results reported in clinical trials[310,311]. The use of AONs in basic and translational research aims to either "partially correct" a non-functional protein or to disrupt a protein of interest as a way to inhibit its expression or functionality. AONs can be designed to interfere at the molecular level either during messenger RNA (mRNA) splicing or mRNA translation into protein. Splicing involves the

removal of non-coding sequences (introns) and "stitching" of the remaining coding DNA (exons). Particular exon(s) encoding protein domains crucial for protein function can become excluded from the mature mRNA; specific AONs bind to sites involved in exon splicing in the exon-intron boundaries and interfere with the splice machinery. Thereby, the particular exon is not integrated as part of the mRNA[312]. The resulting mRNA has an intact open reading frame and is translated into a protein that lacks only the particular peptide sequence encoded by the skipped exon. The advantage of this system is that no genetic alterations are introduced, since interference occurs exclusively at the pre-mRNA splicing process. Translation-blocking AONs function by forming a complex with complementary mRNA sequence; RNA-RNA or RNA-DNA dimers are then recognized by RNAse helicase (H), which leads to mRNA degradation[313]. Alternatively, AON binding to mRNA in the 5' untranslated region might interfere with 5' cap formation, ribosome binding and recognition of the ATG starting codon, thus hindering mRNA translation into protein[314].

AON-mediated inhibition of TGFβ ligand expression has been proposed and attempted as a novel cancer therapy for various malignancies. Interference with TGFβ1 production at the mRNA level by AON AP11014, developed by Antisense Pharma/Isana Therapeutics, significantly reduces TGFβ1 in prostate, lung and colon cancer cell lines[315]. TGFβ2 cytokine plays a key role in glioblastoma and pancreatic cancer. Trabedersen, interferes with TGFβ2 mRNA translation and has reached the phase III of clinical trials for glioblastoma treatment[316]. The same company has developed TGFβ2-targeting AONs for glaucoma treatment (phase I trials). Another antisense TGFβ2 strategy has been developed for tumor vaccines (Lucanix, NovaRx)[317,318]. TGFβ2 AON sequence is transfected into lung cancer cells, which are used as anti-tumor vaccination. The vaccine has progressed into phase III clinical trials.

A distinct type of gene therapy is the nucleic acid or peptide-based strategies that inhibit SMAD transcriptional activity at the DNA level; (1) the SMAD transcription factor- "decoy" double stranded oligonucleotides (decoy ODN) that block SMAD binding to SRE binding sites[319,320], (2) pyrrole-imidazole polyamide compounds designed to bind to DNA minor groove in SRE binding sites, thus inhibiting SMADs to interact with gene promoters and other cis-regulatory elements[321].

1.7.2. Peptides that antagonize ligand function

Ligand traps are peptides engineered to have the extracellular domain (ECD) of receptors or receptor-associated proteins, fused to stable region of an antibody (Fc of IgG). The logic is that the soluble receptor ectodomains will bind with the same affinity its ligands in the extracellular space and sequester them from binding to a functioning receptor on the cell membrane [322,323]. Soluble receptor type II (TGFβRII-Fc) ligand trap was developed by Genzyme but did not progress into clinical trials[324].

Similar anti-TGFβ peptide mimicking the ECD of TGFβRIII receptor β-GLYCAN (Disitertide P144, Digma Biotech) is currently in phase II trials. P144 TGFβ1-inhibitor has been specifically designed to block the interaction of TGFβ1 with β-GLYCAN. It has shown significant anti-fibrotic activity when applied topically in mice receiving repeated subcutaneous injections of bleomycin, a widely accepted animal model of human scleroderma[325]. Anti-fibrotic effects have been reported for liver fibrosis[326], myocardial fibrosis[327] and as combination therapy with antitumor immunotherapy[328]. Another compound of the same company is an antagonist based on the structure of THROMBOSPONDIN-1 (TSP-1) for the treatment of diabetic nephropathy[329]. The LSKL peptide binds to LAP domain of the latent complex

via the motif LSKL (Leu-Ser-Lys-Leu) and blocks TGFβ activation. αvβ6 INTEGRIN mediates release of latent TGFβ from LAP complex; anti-αvβ6 INTEGRIN antibody (STX-100, Stromedix) inhibits tumor progression *in vivo* by blocking activation of latent TGFβ ligands[330]. A similar peptide has been developed that mimics DECORIN binding to latent TGFβ; by blocking release of latent TGFβ the compound enhanced anti-tumor immune response in glioma[331]. Treatment with soluble ENDOGLIN ECD (ENDOGLIN-Fc) has beneficial outcome in inhibiting BMP9/ALK1 signaling and VEGF-mediated tumor angiogenesis and reducing tumor size in preclinical studies[332]. The ALK1-Fc ligand trap for BMP9 and BMP10 has anti-angiogenic [333-335] effects with enhanced safety profile compared to VEGF inhibitors[336]. Treatment with ALK1Fc indicates anti-tumorigenic response of patients with solid tumors[336] and is currently being tested in phase I clinical trials for recurrent ovarian and endometrial cancer (ClinicalTrials.gov identifier; NCT01720173). Peptide aptamers (TRX-CBP, TRX-FOXH1, TRX-LEF1) have been engineered to inhibit SMAD binding to their transcription factor interacting partners CBP, FOXH1, LEF1[337].

1.7.3. Neutralizing monoclonal antibodies

Monoclonal antibodies are used for neutralization of excessive ligand or soluble receptors at the extracellular space. Due to their *in vivo* stability they can be administered less frequently however, administration is done intravenously, which remains a drawback.
Neutralizing antibodies have been developed for binding to an individual or all TGFβ ligands (pan-TGFβ). TGFβ1-specific neutralizing antibody LY2382770 (Eli Lilly) was tested in phase II clinical trials for kidney fibrosis and although it was safe, it proved not sufficiently effective in ameliorating disease progression (ClinicalTrials.gov identifier; NCT01113801). CAT-192 (Metelimumab) is a humanized antibody against TGFβ1. CAT-152 (Lerdelimumab) binds TGFβ2 (anti-scarring postoperative treatment of glaucoma)[338]. However, both antibodies did not show adequate efficacy and the studies did not proceed any further. A more effective neutralizing antibody is the pan-TGFβ compound Fresolimumab, which targets all three TGFβ ligands and showed promising anti-fibrotic and anti-cancinogenic potential. Single-shot treatment with Fresolimumab against glomeruloschelosis is in phase I[339] and is also being tested for malignant melanoma and renal cell carcinoma[340]. The antibody against human ENDOGLIN (TRC105) has anti-tumorigenic effects in advanced solid tumors[341] and is currently in phase II clinical trials for glioblastoma[342]. Currently, TRC105 is also tested as combination therapy with VEGF inhibitors for prostate, breast, ovarian, and liver cancer (HCC). Phase II trials of TRC105 in combination with chemotherapy agents such as bevacizumab for recurrent glioblastoma (ClinicalTrials.gov identifier; NCT01564914), or sorafenib (VEGFR, PDGFR, RAF kinase inhibitor) for HCC (ClinicalTrials.gov identifier; NCT01306058) have shown promising outcome.

1.7.4. Small molecule kinase inhibitors (SMIs)

SMIs is a class of receptor kinase inhibitors which bind into the ATP pocket of the kinase domain, thus, preventing ADP to ATP conversion (phosphorylation) and receptor activation. During this mode of inhibition, TGFβ ligands bind to non-functional receptors (SMI blockage of the type I or type II receptor); thereby the signal is not transmitted downstream. SMIs have the advantages that are cell permeable due to their small molecular weight (as opposed to antibodies), however, they are less specific for a particular receptor because of the high

structural similarity of the ATP pocket among kinases.
Several SMIs have been developed against TGF and BMP receptors with different selectivity for particular kinases or *in vitro* and *in vivo* performance[343]. A panel of frequently used SMIs (**Fig.7**) targeting the TGFβ branch (ALK4, ALK5 and ALK7) is the SB-431542, SB-505142, LY-364947 (selective for ALK5) and A-83-01. Potent BMP receptor inhibitors against ALK2, ALK3, ALK6) is the LDN-193189 and dorsomorphin (compound C).
Although several SMIs have shown promising results in *in vitro* and *in vivo* preclinical studies (SB-431542, SB-505124, GW788388, SD-208)[344] they did not meet the pharmacokinetic stability criteria of clinical trials[303]. Currently, the SMIs being tested in preclinical studies are the LY-580276[345], LY-550410[346,347], LY-364947[348] and LY-2109761[349] for the treatment of various types of cancer. A study for HCC treatment with sorafenib (VEGFR, PDGFR, RAF kinase inhibitor) combined with ALK5 kinase inhibitor (LY-2157299) in patients with HCC is currently in phase II of clinical trials (NCT01246986)[350].

Drug	Type	Target	Disease	Stage	Refs/Identifier
Trabedersen	Antisense oligo	TGFβ2	Glioblastoma, Pancreatic cancer	Phase II	*316*
Belagen-pumatucel-L (Lucanix)	Antisense oligo- mediated tumor cell vaccine	TGFβ2	Non-small-cell-lung-carcinoma	Phase III	*317,318*
ISTH0036	Antisense oligo	TGFβ2	Post-operative glaucoma treatment		NCT02406833
LY2382770	Neutralizing antibody	TGFβ1	Diabetic kidney fibrosis	Phase II	NCT01113801
Pirfenidone	Small molecule	TGFβ activity	Idiopathic pulmonary fibrosis	Clinic	*351*
P144	peptide	TGFβ1, β-GLYCAN	Skin fibrosis, Systemic sclerosis	Phase II	*325*
CAT-192	Neutralizing antibody	TGFβ1	Systemic sclerosis	Phase II	*352*
GC-1008/ Fresolimumab NCT00043706	Neutralizing antibody	TGFβ1,2 ,3	Melanoma, Renal fibrosis, glaucoma	Phase I	*339,340,* NCT01472731
STX-100/ Stromedix	Neutralizing antibody	αVβ6 INTEGRIN	Fibrosis	Phase II	NCT01371305
TRC105	Neutralizing antibody	ENDOGLIN	Glioblastoma, Liver cancer	Phase II	NCT01564914
ALK1Fc	Ligand trap	BMP9, BMP10	Endometrial cancer Ovarian cancer	Phase I	NCT01720173
LY2157299	Small molecule kinase inhibitor	ALK5	Hepatocellular carcinoma	Phase II	NCT01246986

Table 3. Ongoing clinical trials on compounds targeting TGFβ pathway members in fibrosis and cancer

References

1. Kerridge, D., The effect of inhibitors on the formation of flagella by Salmonella typhimurium. J Gen Microbiol, 1960. 23: p. 519-38.
2. Massagué, J., J. Seoane, and D. Wotton, Smad transcription factors. Genes Dev, 2005. 19(23): p. 2783-2810.
3. Roberts, A.B., et al., Transforming growth factors: isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extraction. Proc Natl Acad Sci U S A, 1980. 77(6): p. 3494-3498.
4. de Larco, J.E. and G.J. Todaro, Growth factors from murine sarcoma virus-transformed cells. Proc Natl Acad Sci U S A, 1978. 75(8): p. 4001-5.
5. Anzano, M.A., et al., Synergistic interaction of two classes of Transforming growth factors from murine sarcoma cells. Cancer Res, 1982. 42(11): p. 4776-4778.
6. Roberts, A.B., et al., Isolation from murine sarcoma cells of novel transforming growth factors potentiated by EGF. Nature, 1982. 295(5848): p. 417-9.
7. Moses, H.L., et al., Transforming growth factor production by chemically transformed cells. Cancer Res, 1981. 41(7): p. 2842-2848.
8. Assoian, R.K., et al., Transforming growth factor β in human platelets. Identification of a major storage site, purification, and characterization. J Biol Chem, 1983. 258(11): p. 7155-7160.
9. Frolik, C.A., et al., Purification and initial characterization of a type β transforming growth factor from human placenta. Proc Natl Acad Sci U S A, 1983. 80(12): p. 3676-3680.
10. Stromberg, K., et al., Human term placenta contains transforming growth factors. Biochem Biophys Res Commun, 1982. 106(2): p. 354-361.
11. Twardzik, D.R., J.E. Ranchalis, and G.J. Todaro, Mouse embryonic Transforming growth factors related to those isolated from tumor cells. Cancer Res, 1982. 42(2): p. 590-593.
12. Massague, J., et al., Affinity labeling of a transforming growth factor receptor that does not interact with epidermal growth factor. Proc Natl Acad Sci U S A, 1982. 79(22): p. 6822-6826.
13. ten Dijke, P., K. Miyazono, and C.H. Heldin, Signaling via hetero-oligomeric complexes of type I and type II serine/threonine kinase receptors. Curr Opin Cell Biol, 1996. 8(2): p. 139-45.
14. Heldin, C.-H., K. Miyazono, and P. ten Dijke, TGFβ signaling from cell membrane to nucleus through SMAD proteins. Nature, 1997. 390(6659): p. 465-471.
15. Miyazono, K., Y. Kamiya, and M. Morikawa, Bone morphogenetic protein receptors and signal transduction. J Biochem, 2010. 147(1): p. 35-51.
16. Steinbicker, A.U., et al., Perturbation of hepcidin expression by BMP type I receptor deletion induces iron overload in mice. Blood, 2011. 118(15): p. 4224-4230.
17. Cai, J., et al., BMP signaling in vascular diseases. FEBS Lett, 2012. 586(14): p. 1993-2002.
18. Canalis, E., A.N. Economides, and E. Gazzerro, Bone morphogenetic proteins, their antagonists, and the skeleton. Endocr Rev, 2003. 24(2): p. 218-235.
19. Yang, J., et al., Bone morphogenetic proteins: Relationship between molecular structure and their osteogenic activity. Food Sci Hum Welln, 2014. 3(3–4): p. 127-135.
20. Kawabata, M., T. Imamura, and K. Miyazono, Signal transduction by bone morphogenetic proteins. Cytokine Growth Factor Rev, 1998. 9(1): p. 49-61.
21. Elkasrawy, M.N. and M.W. Hamrick, Myostatin (GDF-8) as a key factor linking muscle mass and skeletal form. J Musculoskelet Neuronal Interact, 2010. 10(1): p. 56-63.
22. Bragdon, B., et al., Bone morphogenetic proteins: A critical review. Cell Signal, 2011. 23(4): p. 609-620.
23. Graycar, J.L., et al., Human transforming growth factor-β 3: recombinant expression, purification, and biological activities in comparison with transforming growth factors-β 1 and -β 2. Mol Endocrinol, 1989. 3(12): p. 1977-86.
24. Millan, F.A., et al., Embryonic gene expression patterns of TGFβ1, β2 and β3 suggest different

developmental functions in vivo. Development, 1991. 111(1): p. 131-143.
25. ten Dijke, P., et al., Identification of another member of the transforming growth factor type β gene family. Proc Natl Acad Sci U S A, 1988. 85(13): p. 4715-4719.
26. Derynck, R., et al., Human transforming growth factor-β complementary DNA sequence and expression in normal and transformed cells. Nature, 1985. 316(6030): p. 701-5.
27. Derynck, R., et al., A new type of transforming growth factor-β, TGF-β3. EMBO J, 1988. 7(12): p. 3737-3743.
28. Madisen, L., et al., Transforming growth factor-β 2: cDNA cloning and sequence analysis. DNA, 1988. 7(1): p. 1-8.
29. Shull, M.M., et al., Targeted disruption of the mouse transforming growth factor-β1 gene results in multifocal inflammatory disease. Nature, 1992. 359(6397): p. 693-699.
30. Kulkarni, A.B., et al., Transforming growth factor β 1 null mutation in mice causes excessive inflammatory response and early death. Proc Natl Acad Sci U S A, 1993. 90(2): p. 770-774.
31. Dickson, M.C., et al., Defective haematopoiesis and vasculogenesis in transforming growth factor-β 1 knock out mice. Development, 1995. 121(6): p. 1845-1854.
32. Kaartinen, V., et al., Abnormal lung development and cleft palate in mice lacking TGFβ3 indicates defects of epithelial-mesenchymal interaction. Nat Genet, 1995. 11(4): p. 415-421.
33. Dickson, M.C., et al., RNA and protein localisations of TGFβ 2 in the early mouse embryo suggest an involvement in cardiac development. Development, 1993. 117(2): p. 625-639.
34. Sanford, L.P., et al., TGFβ2 knockout mice have multiple developmental defects that are non-overlapping with other TGFβ knockout phenotypes. Development, 1997. 124(13): p. 2659-2670.
35. Miyazono, K., et al., Latent high molecular weight complex of transforming growth factor β 1. Purification from human platelets and structural characterization. J Biol Chem, 1988. 263(13): p. 6407-6415.
36. ten Dijke, P. and H.M. Arthur, Extracellular control of TGFβ signaling in vascular development and disease. Nat Rev Mol Cell Biol, 2007. 8(11): p. 857-869.
37. Munger, J.S. and D. Sheppard, Cross talk among TGF-β signaling pathways, integrins, and the extracellular matrix. Cold Spring Harb Perspect Biol, 2011. 3(11): p. a005017.
38. Pappano, W.N., et al., Use of Bmp1/Tll1 doubly homozygous null mice and proteomics to identify and validate in vivo substrates of Bone morphogenetic protein 1/Tolloid-like metalloproteinases. Mol Cell Biol, 2003. 23(13): p. 4428-4438.
39. Sheppard, D., Integrin-mediated activation of latent transforming growth factor β. Cancer Metastasis Rev, 2005. 24(3): p. 395-402.
40. Fontana, L., et al., Fibronectin is required for integrin αvβ6-mediated activation of latent TGF-β complexes containing LTBP-1. FASEB J, 2005. 19(13): p. 1798-1808.
41. Crawford, S.E., et al., Thrombospondin-1 is a major activator of TGF-β1 in vivo. Cell, 1998. 93(7): p. 1159-1170.
42. Massague, J., TGFβ signaling in context. Nat Rev Mol Cell Biol, 2012. 13(10): p. 616-630.
43. Franzén, P., et al., Cloning of a TGFβ type I receptor that forms a heteromeric complex with the TGFβ type II receptor. Cell, 1993. 75(4): p. 681-692.
44. Lux, A., L. Attisano, and D.A. Marchuk, Assignment of Transforming growth factor β1 and β3 and a third new ligand to the type I receptor ALK-1. J Biol Chem, 1999. 274(15): p. 9984-9992.
45. ten Dijke, P., et al., Activin receptor-like kinases: a novel subclass of cell-surface receptors with predicted serine/threonine kinase activity. Oncogene, 1993. 8(10): p. 2879-87.
46. ten Dijke, P., et al., Characterization of type I receptors for transforming growth factor-β and activin. Science, 1994. 264(5155): p. 101-4.
47. Vellucci, V.F. and M. Reiss, Cloning and genomic organization of the human Transforming growth

factor-β type I receptor gene. Genomics, 1997. 46(2): p. 278-283.
48. Inman, G.J., F.J. Nicolás, and C.S. Hill, Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-β receptor activity. Mol Cell, 2002. 10(2): p. 283-294.
49. Wakefield, L.M. and C.S. Hill, Beyond TGFβ: roles of other TGFβ superfamily members in cancer. Nat Rev Cancer, 2013. 13(5): p. 328-341.
50. Schmierer, B. and C.S. Hill, TGFβ-SMAD signal transduction: molecular specificity and functional flexibility. Nat Rev Mol Cell Biol, 2007. 8(12): p. 970-982.
51. Wrana, J.L., Crossing Smads. Sci STKE, 2000. 2000(23): p. re1.
52. Alarcón, C., et al., Nuclear CDKs drive Smad transcriptional activation and turnover in BMP and TGFβ pathways. Cell, 2009. 139(4): p. 757-769.
53. Sapkota, G., et al., Balancing BMP signaling through integrated inputs into the Smad1 linker. Mol Cell, 2007. 25(3): p. 441-454.
54. Morikawa, M., et al., ChIP-seq reveals cell type-specific binding patterns of BMP-specific Smads and a novel binding motif. Nucleic Acids Res, 2011. 39(20): p. 8712-8727.
55. Mullen, A.C., et al., Master transcription factors determine cell-type-specific responses to TGF-β signaling. Cell, 2011. 147(3): p. 565-576.
56. Heldin, C.-H., M. Landström, and A. Moustakas, Mechanism of TGF-β signaling to growth arrest, apoptosis, and epithelial–mesenchymal transition. Curr Opin Cell Biol, 2009. 21(2): p. 166-176.
57. Itoh, S. and P. ten Dijke, Negative regulation of TGF-β receptor/Smad signal transduction. Curr Opin Cell Biol, 2007. 19(2): p. 176-184.
58. Massagué, J., S.W. Blain, and R.S. Lo, TGFβ Signaling in growth control, cancer, and heritable disorders. Cell, 2000. 103(2): p. 295-309.
59. Iozzo, R.V. and L. Schaefer, Proteoglycans in health and disease: Novel regulatory signaling mechanisms evoked by the small leucine-rich proteoglycans. FEBS J, 2010. 277(19): p. 3864-3875.
60. Neill, T., L. Schaefer, and R.V. Iozzo, Decorin: A guardian from the matrix. Am J Pathol, 2012. 181(2): p. 380-387.
61. Yamaguchi, Y., D.M. Mann, and E. Ruoslahti, Negative regulation of transforming growth factor-β by the proteoglycan decorin. Nature, 1990. 346(6281): p. 281-284.
62. Abdel-Wahab, N., et al., Decorin suppresses transforming growth factor-β-induced expression of plasminogen activator inhibitor-1 in human mesangial cells through a mechanism that involves Ca2+-dependent phosphorylation of Smad2 at serine-240. Biochem J, 2002. 362(Pt 3): p. 643-649.
63. Schier, A.F. and M.M. Shen, Nodal signaling in vertebrate development. Nature, 2000. 403(6768): p. 385-389.
64. López-Casillas, F., J.L. Wrana, and J. Massagué, Betaglycan presents ligand to the TGFβ signaling receptor. Cell, 1993. 73(7): p. 1435-1444.
65. Lee, N.Y., et al., The Transforming growth factor-β type III receptor mediates distinct subcellular trafficking and downstream signaling of Activin-like kinase (ALK)3 and ALK6 receptors. Mol Biol Cell, 2009. 20(20): p. 4362-4370.
66. Blanco, F.J., et al., Interaction and functional interplay between endoglin and ALK-1, two components of the endothelial transforming growth factor-β receptor complex. J Cell Physiol, 2005. 204(2): p. 574-584.
67. Gougos, A. and M. Letarte, Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells. J Biol Chem, 1990. 265(15): p. 8361-4.
68. Bellón, T., et al., Identification and expression of two forms of the human transforming growth factor-β-binding protein endoglin with distinct cytoplasmic regions. Eur J Immunol., 1993. 23(9): p. 2340-2345.
69. Lebrin, F., et al., Endoglin promotes endothelial cell proliferation and TGF-β/ALK1 signal transduction.

EMBO J, 2004. 23(20): p. 4018-4028.
70. Shani, G., et al., GRP78 and Cripto form a complex at the cell surface and collaborate to inhibit Transforming growth factor β signaling and enhance cell growth. Mol Cell Biol, 2008. 28(2): p. 666-677.
71. Gray, P.C., et al., Cripto binds Transforming growth factor β (TGF-β) and inhibits TGF-β signaling. Mol Cell Biol, 2006. 26(24): p. 9268-9278.
72. Gray, P.C. and W. Vale, Cripto/GRP78 modulation of the TGF-β pathway in development and oncogenesis. FEBS Lett, 2012. 586(14): p. 1836-1845.
73. Spike, Benjamin T., et al., CRIPTO/GRP78 signaling maintains fetal and adult mammary stem cells ex vivo. Stem Cell Reports, 2014. 2(4): p. 427-439.
74. Nagaoka, T., et al., An evolving web of signaling networks regulated by Cripto-1. Growth Factors, 2012. 30(1): p. 13-21.
75. Klauzinska, M., et al., The multifaceted role of the embryonic gene Cripto-1 in cancer, stem cells and epithelial-mesenchymal transition. Semin Cancer Biol, 2014. 29(0): p. 51-58.
76. Healey, E.G., et al., Repulsive guidance molecule is a structural bridge between neogenin and bone morphogenetic protein. Nat Struct Mol Biol, 2015. 22(6): p. 458-465.
77. Samad, T.A., et al., DRAGON, a Bone morphogenetic protein co-receptor. J Biol Chem, 2005. 280(14): p. 14122-14129.
78. Babitt, J.L., et al., Repulsive guidance molecule (RGMa), a DRAGON homologue, is a Bone morphogenetic protein co-receptor. J Biol Chem, 2005. 280(33): p. 29820-29827.
79. Xia, Y., et al., Repulsive guidance molecule RGMa alters utilization of Bone morphogenetic protein (BMP) type II receptors by BMP2 and BMP4. J Biol Chem, 2007. 282(25): p. 18129-18140.
80. Miyazono, K., Positive and negative regulation of TGF-β signaling. J Cell Sci, 2000. 113(7): p. 1101-1109.
81. Sekiya, T., et al., Transcriptional regulation of the TGF-β pseudoreceptor BAMBI by TGF-β signaling. Biochem Biophys Res Commun, 2004. 320(3): p. 680-684.
82. Karaulanov, E., W. Knöchel, and C. Niehrs, Transcriptional regulation of BMP4 synexpression in transgenic Xenopus. EMBO J, 2004. 23(4): p. 844-856.
83. Okadome, T., et al., Characterization of the interaction of FKBP12 with the transforming growth factor-β type I receptor in vivo. J Biol Chem, 1996. 271(36): p. 21687-90.
84. Chen, Y.G., F. Liu, and J. Massague, Mechanism of TGFβ receptor inhibition by FKBP12. EMBO J, 1997. 16(13): p. 3866-3876.
85. Spiekerkoetter, E., et al., FK506 activates BMPR2, rescues endothelial dysfunction, and reverses pulmonary hypertension. J Clin Invest, 2013. 123(8): p. 3600-3613.
86. Wang, T., et al., The immunophilin FKBP12 functions as a common inhibitor of the TGFβ family type I receptors. Cell, 1996. 86(3): p. 435-444.
87. Tsukazaki, T., et al., SARA, a FYVE domain protein that recruits Smad2 to the TGFβ receptor. Cell, 1998. 95(6): p. 779-791.
88. Xu, L., Y.-G. Chen, and J. Massague, The nuclear import function of Smad2 is masked by SARA and unmasked by TGFβ-dependent phosphorylation. Nat Cell Biol, 2000. 2(8): p. 559-562.
89. Lin, H.Y., et al., Expression cloning of the TGF-β type II receptor, a functional transmembrane serine/threonine kinase. Cell, 1992. 68(4): p. 775-785.
90. Luo, K. and H.F. Lodish, Positive and negative regulation of type II TGFβ receptor signal transduction by autophosphorylation on multiple serine residues. EMBO J, 1997. 16(8): p. 1970-1981.
91. Lee, M.K., et al., TGF-β activates Erk MAP kinase signaling through direct phosphorylation of ShcA. EMBO J, 2007. 26(17): p. 3957-3967.
92. Shi, W., et al., GADD34–PP1c recruited by Smad7 dephosphorylates TGFβ type I receptor. J Cell Biol,

2004. 164(2): p. 291-300.
93. Heikkinen, P.T., et al., Hypoxia-activated Smad3-specific dephosphorylation by PP2A. J Biol Chem, 2010. 285(6): p. 3740-3749.
94. Geiss-Friedlander, R. and F. Melchior, Concepts in sumoylation: a decade on. Nat Rev Mol Cell Biol, 2007. 8(12): p. 947-956.
95. Dikic, I., S. Wakatsuki, and K.J. Walters, Ubiquitin-binding domains from structures to functions. Nat Rev Mol Cell Biol, 2009. 10(10): p. 659-671.
96. Cashman, R., et al., SENP5 mediates breast cancer invasion via a TGFβRI SUMOylation cascade. Oncotarget, 2014. 5(4).
97. Kang, J.S., et al., The type I TGF-β receptor is covalently modified and regulated by sumoylation. Nat Cell Biol, 2008. 10(6): p. 654-664.
98. Kavsak, P., et al., Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGFβ receptor for degradation. Mol Cell, 2000. 6(6): p. 1365-1375.
99. Ebisawa, T., et al., Smurf1 interacts with Transforming growth factor-β type I receptor through Smad7 and induces receptor degradation. J Biol Chem, 2001. 276(16): p. 12477-12480.
100. Cao, Y. and L. Zhang, A Smurf1 tale: function and regulation of an ubiquitin ligase in multiple cellular networks. Cell Mol Life Sci, 2013. 70(13): p. 2305-2317.
101. Liu, W., et al., Axin is a scaffold protein in TGF-β signaling that promotes degradation of Smad7 by Arkadia. EMBO J, 2006. 25(8): p. 1646-58.
102. Zhou, F., et al., Nuclear receptor NR4A1 promotes breast cancer invasion and metastasis by activating TGF-β signaling. Nat Commun, 2014. 5.
103. Sharma, V., et al., Enhancement of TGF-β signaling responses by the E3 ubiquitin ligase Arkadia provides tumor suppression in colorectal cancer. Cancer Res, 2011. 71(20): p. 6438-49.
104. Sun, H. and T. Hunter, PolySUMO-binding proteins identified through a string search. J Biol Chem, 2012.
105. Erker, Y., et al., Arkadia, a novel SUMO-targeted ubiquitin ligase involved in PML degradation. Mol Cell Biol, 2013. 33(11): p. 2163-2177.
106. Tsubakihara, Y., et al., Arkadia enhances BMP signaling through ubiquitylation and degradation of Smad6. J Biochem, 2015.
107. Sun, H., Y. Liu, and T. Hunter, Multiple Arkadia/RNF111 structures coordinate its Polycomb body association and transcriptional control. Mol Cell Biol, 2014. 34(16): p. 2981-2995.
108. Nagano, Y., et al., Arkadia induces degradation of SnoN and c-Ski to enhance Transforming growth factor-β signaling. J Biol Chem, 2007. 282(28): p. 20492-20501.
109. Levy, L., et al., Arkadia activates Smad3/Smad4-dependent transcription by triggering signal-induced SnoN degradation. Mol Cell Biol, 2007. 27(17): p. 6068-6083.
110. Mavrakis, K.J., et al., Arkadia enhances Nodal/TGF-β signaling by coupling phospho-Smad2/3 activity and turnover. PLoS Biol, 2007. 5(3): p. e67.
111. Lee, P.S.W., et al., Sumoylation of Smad4, the common Smad mediator of Transforming growth factor-β family signaling. J Biol Chem, 2003. 278(30): p. 27853-27863.
112. Zhou, X., et al., High glucose induces sumoylation of Smad4 via SUMO2/3 in mesangial cells. Biomed Res Int, 2014. 2014: p. 782625.
113. Dupont, S., et al., FAM/USP9x, a deubiquitinating enzyme essential for TGFβ signaling, controls Smad4 monoubiquitination. Cell, 2009. 136(1): p. 123-135.
114. Maeda, S., et al., Endogenous TGF-β signaling suppresses maturation of osteoblastic mesenchymal cells. EMBO J, 2004. 23(3): p. 552-563.
115. Ishida, W., et al., Smad6 is a Smad1/5-induced Smad inhibitor: Characterization of Bone morphogenetic protein-responsive element in the mouse Smad6 promoter J Biol Chem, 2000. 275(9): p. 6075-6079.

116. Afrakhte, M., et al., Induction of inhibitory Smad6 and Smad7 mRNA by TGFβ family members. Biochem Biophys Res Commun, 1998. 249(2): p. 505-511.
117. Nakao, A., et al., Identification of Smad7, a TGFβ-inducible antagonist of TGF-β signaling. Nature, 1997. 389(6651): p. 631-635.
118. Goto, K., et al., Selective inhibitory effects of Smad6 on Bone morphogenetic protein type I receptors. J Biol Chem, 2007. 282(28): p. 20603-20611.
119. Hata, A., et al., Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. Genes Dev, 1998. 12(2): p. 186-197.
120. Morén, A., et al., Degradation of the tumor suppressor Smad4 by WW and HECT domain ubiquitin ligases. J Biol Chem, 2005. 280(23): p. 22115-22123.
121. Zhang, S., et al., Smad7 antagonizes Transforming growth factor β signaling in the nucleus by interfering with functional Smad-DNA complex formation. Mol Cell Biol, 2007. 27(12): p. 4488-4499.
122. Itoh, S., et al., Transforming growth factor β1 induces nuclear export of inhibitory Smad7. J Biol Chem, 1998. 273(44): p. 29195-29201.
123. Valdimarsdottir, G., et al., Smad7 and protein phosphatase 1α are critical determinants in the duration of TGF-β/ALK1 signaling in endothelial cells. BMC Cell Biol, 2006. 7: p. 16-16.
124. Ishisaki, A., et al., Differential inhibition of Smad6 and Smad7 on bone morphogenetic protein- and activin-mediated growth arrest and apoptosis in B cells. J Biol Chem., 1999. 274(19): p. 13637-13642.
125. Itoh, F., et al., Promoting bone morphogenetic protein signaling through negative regulation of inhibitory Smads. EMBO J, 2001. 20(15): p. 4132-4142.
126. Yang, J., et al., Downregulation of Smad transcriptional corepressors SnoN and Ski in the fibrotic kidney: An amplification mechanism for TGF-β1 signaling. J Am Soc Nephrol, 2003. 14(12): p. 3167-3177.
127. Tabata, T., et al., Ski co-repressor complexes maintain the basal repressed state of the TGF-β target gene, SMAD7, via HDAC3 and PRMT5. Genes Cells, 2009. 14(1): p. 17-28.
128. Zhou, X., et al., Nodal is a novel TGF-β-like gene expressed in the mouse node during gastrulation. Nature, 1993. 361(6412): p. 543-547.
129. Shen, M.M., Nodal signaling: developmental roles and regulation. Development, 2007. 134(6): p. 1023-1034.
130. Kruithof-de Julio, M., et al., Regulation of extra-embryonic endoderm stem cell differentiation by Nodal and Cripto signaling. Development, 2011. 138(18): p. 3885-3895.
131. Sarkar, P., et al., Activin/Nodal signaling switches the terminal fate of human embryonic stem cell-derived trophoblasts. J Biol Chem, 2015. 290(14): p. 8834-8848.
132. Bianco, C., et al., Role of Cripto-1 in stem cell maintenance and malignant progression. Am J Pathol, 2010. 177(2): p. 532-540.
133. Yeo, C.-Y. and M. Whitman, Nodal signals to Smads through Cripto-dependent and Cripto-independent mechanisms. Mol Cell, 2001. 7(5): p. 949-957.
134. Rosa, F.M., Cripto, a multifunctional partner in signaling: molecular forms and activities. Sci Signal, 2002. 2002(158): p. pe47-pe47.
135. Yan, Y.-T., et al., Dual roles of Cripto as a ligand and coreceptor in the Nodal signaling pathway. Mol Cell Biol, 2002. 22(13): p. 4439-4449.
136. Chen, C., et al., The Vg1-related protein Gdf3 acts in a Nodal signaling pathway in the pre-gastrulation mouse embryo. Development, 2006. 133(2): p. 319-329.
137. Blanchet, M.-H., et al., Cripto localizes Nodal at the limiting membrane of early endosomes. Sci Signal, 2008. 1(45): p. ra13.
138. Schiffer, S.G., et al., Fucosylation of Cripto is required for its ability to facilitate Nodal signaling. J Biol Chem, 2001.

139. Schier, A.F., Nodal Morphogens. Cold Spring Harb Perspect Biol, 2009. 1(5): p. a003459.
140. Cruz, C.D., et al., Expression of Nodal, Cripto, SMAD3, phosphorylated SMAD3, and SMAD4 in the proliferative endometrium of women with endometriosis. Reprod Sci, 2015. 22(5): p. 527-533.
141. Loying, P., et al., Autoregulation and heterogeneity in expression of human Cripto-1. PLoS One, 2015. 10(2): p. e0116748.
142. Schier, A.F., NODAL signaling in vertebrate development Annu Rev Cell Dev Biol, 2003. 19(1): p. 589-621.
143. Su, Y., et al., The evolutionally conserved activity of Dapper2 in antagonizing TGF-β signaling. FASEB J, 2007. 21(3): p. 682-690.
144. Dupont, S., et al., Germ-layer specification and control of cell growth by ectodermin, a Smad4 ubiquitin ligase. Cell, 2005. 121(1): p. 87-99.
145. Powers, S.E., et al., Tgif1 and Tgif2 regulate Nodal signaling and are required for gastrulation. Development, 2010. 137(2): p. 249-259.
146. Cai, W., et al., Coordinate Nodal and BMP inhibition directs Baf60c-dependent cardiomyocyte commitment. Genes Dev, 2013. 27(21): p. 2332-2344.
147. Bianco, C., et al., A Nodal- and ALK4-independent signaling pathway activated by Cripto-1 through Glypican-1 and c-Src. Cancer Res, 2003. 63(6): p. 1192-1197.
148. Bianco, C., et al., Regulation of Cripto-1 signaling and biological activity by Caveolin-1 in mammary epithelial cells. Am J Pathol, 2008. 172(2): p. 345-357.
149. D'Aniello, C., et al., G Protein-coupled receptor APJ and its ligand apelin act downstream of Cripto to specify embryonic stem cells toward the cardiac lineage through extracellular signal-regulated kinase/p70S6 kinase Signaling Pathway. Circ Res, 2009. 105(3): p. 231-238.
150. Nagaoka, T., et al., Cripto-1 enhances the canonical Wnt/β-catenin signaling pathway by binding to LRP5 and LRP6 co-receptors. Cell Signal, 2013. 25(1): p. 178-189.
151. Watanabe, K., et al., Enhancement of Notch receptor maturation and signaling sensitivity by Cripto-1. J Cell Biol, 2009. 187(3): p. 343-353.
152. Xu, C.-H., et al., Elevated expression of Cripto-1 correlates with poor prognosis in non-small cell lung cancer. Tumour Biol, 2014. 35(9): p. 8673-8678.
153. Cocciadiferro, L., et al., Profiling cancer stem cells in androgen-responsive and refractory human prostate tumor cell lines. Ann N Y Acad Sci, 2009. 1155(1): p. 257-262.
154. Terry, S., et al., CRIPTO overexpression promotes mesenchymal differentiation in prostate carcinoma cells through parallel regulation of AKT and FGFR activities. Oncotarget, 2015. 6(14): p. 11994-2008.
155. D'Antonio, A., et al., Transforming growth factor alpha, amphiregulin and cripto-1 are frequently expressed in advanced human ovarian carcinomas. Int J Oncol, 2002. 21(5): p. 941-8.
156. Fujii, K., et al., Expression of CRIPTO in human gall bladder lesions J Pathol., 1996. 180(2): p. 166-168.
157. Giorgio, E., et al., Cripto haploinsufficiency affects in vivo colon tumor development. Int J Oncol, 2014. 45(1): p. 31-40.
158. Sun, C., et al., NANOG promotes liver cancer cell invasion by inducing epithelial–mesenchymal transition through NODAL/SMAD3 signaling pathway. Int J Biochem Cell Biol, 2013. 45(6): p. 1099-1108.
159. Strizzi, L., et al., The significance of a Cripto-1-positive subpopulation of human melanoma cells exhibiting stem cell-like characteristics. Cell Cycle, 2013. 12(9): p. 1450-1456.
160. Tysnes, B.B., et al., Age-dependent association between protein expression of the embryonic stem cell marker Cripto-1 and survival of glioblastoma patients. Transl Oncol, 2013. 6(6): p. 732-741.
161. Sato, M., et al., GRP78 signaling hub: A receptor for targeted tumor therapy. Adv Genet, 2010. Volume 69: p. 97-114.
162. Adkins, H.B., et al., Antibody blockade of the Cripto CFC domain suppresses tumor cell growth in vivo.

J Clin Invest, 2003. 112(4): p. 575-587.
163. Bianco, C. and D.S. Salomon, Targeting the embryonic gene Cripto-1 in cancer and beyond. Expert Opin Ther Pat, 2010. 20(12): p. 1739-1749.
164. Bianco, C. and D.S. Salomon, Human Cripto-1 as a target for a cancer vaccine: WO2008040759. Expert Opin Ther Pat, 2009. 19(2): p. 141-144.
165. Derynck, R. and Y.E. Zhang, Smad-dependent and Smad-independent pathways in TGF-β family signaling. Nature, 2003. 425(6958): p. 577-584.
166. Hackel, P.O., et al., Epidermal growth factor receptors: critical mediators of multiple receptor pathways. Curr Opin Cell Biol, 1999. 11(2): p. 184-189.
167. Chen, R.H., C. Sarnecki, and J. Blenis, Nuclear localization and regulation of ERK- and RSK-encoded protein kinases. Mol Cell Biol, 1992. 12(3): p. 915-927.
168. Mu, Y., S. Gudey, and M. Landström, Non-Smad signaling pathways. Cell Tissue Res, 2012. 347(1): p. 11-20.
169. Yi, J.Y., I. Shin, and C.L. Arteaga, Type I Transforming growth factor β receptor binds to and activates phosphatidylinositol 3-kinase. J Biol Chem, 2005. 280(11): p. 10870-10876.
170. Lamouille, S. and R. Derynck, Emergence of the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin axis in Transforming growth factor-β-induced epithelial-mesenchymal transition. Cells Tissues Organs, 2010. 193(1-2): p. 8-22.
171. Zhang, L., et al., USP4 is regulated by AKT phosphorylation and directly deubiquitylates TGF-β type I receptor. Nat Cell Biol, 2012. 14(7): p. 717-726.
172. Zhang, X., et al., Akt, FoxO and regulation of apoptosis. Biochim Biophys Acta, 2011. 1813(11): p. 1978-1986.
173. Ozdamar, B., et al., Regulation of the polarity protein Par6 by TGFβ receptors controls epithelial cell plasticity. Science, 2005. 307(5715): p. 1603-1609.
174. Yamaguchi, K., et al., Identification of a member of the MAPKKK family as a potential mediator of TGF-β signal transduction. Science, 1995. 270(5244): p. 2008-11.
175. Yamashita, M., et al., TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-β. Mol Cell, 2008. 31(6): p. 918-924.
176. Sorrentino, A., et al., The type I TGF-β receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner. Nat Cell Biol, 2008. 10(10): p. 1199-1207.
177. Zavadil, J., et al., Genetic programs of epithelial cell plasticity directed by transforming growth factor-β. Proc Natl Acad Sci U S A, 2001. 98(12): p. 6686-6691.
178. Kang, Y., C.-R. Chen, and J. Massagué, A self-enabling TGFβ response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial eells. Mol Cell, 2003. 11(4): p. 915-926.
179. Reynisdóttir, I., et al., Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-β. Genes Dev, 1995. 9(15): p. 1831-1845.
180. Hannon, G.J. and D. Beach, pI5INK4B is a potential effector of TGF-β-induced cell cycle arrest. Nature, 1994. 371(6494): p. 257-261.
181. Oberhammer, F.A., et al., Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor β 1. Proc Natl Acad Sci U S A, 1992. 89(12): p. 5408-5412.
182. Pardali, K. and A. Moustakas, Actions of TGF-β as tumor suppressor and pro-metastatic factor in human cancer. Biochim Biophys Acta, 2007. 1775(1): p. 21-62.
183. Takehara, K., E.C. LeRoy, and G.R. Grotendorst, TGF-β inhibition of endothelial cell proliferation: Alteration of EGF binding and EGF-induced growth-regulatory (competence) gene expression. Cell, 1987. 49(3): p. 415-422.
184. Leask, A. and D.J. Abraham, TGF-β signaling and the fibrotic response. FASEB J, 2004. 18(7): p. 816-827.

185. Böttner, M., K. Krieglstein, and K. Unsicker, The Transforming growth factor-βs. J Neurochem, 2000. 75(6): p. 2227-2240.
186. Blank, U. and S. Karlsson, TGFβ signaling in the control of hematopoietic stem cells. Blood, 2015. 125(23): p. 3542-50.
187. Karkampouna, S. and M. Kruithof-de Julio, Fibrosis: a novel approach for an old problem. Receptors Clin Investig, 2014. 1(5).
188. Sanderson, N., et al., Hepatic expression of mature transforming growth factor β 1 in transgenic mice results in multiple tissue lesions. Proc Natl Acad Sci U S A, 1995. 92(7): p. 2572-2576.
189. Böttinger, E.P., et al., The recombinant proregion of transforming growth factor β1 (latency-associated peptide) inhibits active transforming growth factor β1 in transgenic mice. Proc Natl Acad Sci U S A, 1996. 93(12): p. 5877-5882.
190. Gewaltig, J., et al., Association of polymorphisms of the transforming growth factor-β1 gene with the rate of progression of HCV-induced liver fibrosis. Clin Chim Acta, 2002. 316(1–2): p. 83-94.
191. Goto, K., et al., Proximal prostatic stem cells are programmed to regenerate a proximal-distal ductal axis. Stem Cells, 2006. 24(8): p. 1859-1868.
192. Kang, H.Y., et al., From transforming growth factor-β signaling to androgen action: Identification of Smad3 as an androgen receptor coregulator in prostate cancer cells. Proc Natl Acad Sci U S A, 2001. 98(6): p. 3018-3023.
193. Salm, S.N., et al., TGF-β maintains dormancy of prostatic stem cells in the proximal region of ducts. J Cell Biol, 2005. 170(1): p. 81-90.
194. Song, K., et al., Androgenic control of Transforming growth factor-β signaling in prostate epithelial cells through transcriptional suppression of Transforming growth factor-β receptor II. Cancer Res, 2008. 68(19): p. 8173-8182.
195. Kundu, S.D., et al., Absence of proximal duct apoptosis in the ventral prostate of transgenic mice carrying the C3(1)-TGF-β type II dominant negative receptor. Prostate, 2000. 43(2): p. 118-124.
196. Martikainen, P., N. Kyprianou, and J.T. Isaacs, Effect of transforming growth factor-β 1 on proliferation and death of rat prostatic cells. Endocrinology, 1990. 127(6): p. 2963-8.
197. Walraven, M., et al., Altered TGF-β signaling in fetal fibroblasts: What is known about the underlying mechanisms? Wound Repair Regen, 2014. 22(1): p. 3-13.
198. Tomasek, J.J., et al., Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat Rev Mol Cell Biol, 2002. 3(5): p. 349-363.
199. Akhurst, R.J., TGFβ signaling in health and disease. Nat Genet, 2004. 36(8): p. 790-792.
200. Mizuguchi, T., et al., Heterozygous TGFβR2 mutations in Marfan syndrome. Nat Genet, 2004. 36(8): p. 855-860.
201. Akutsu, K., et al., Phenotypic heterogeneity of Marfan-like connective tissue disorders associated with mutations in the TGFβ receptor genes. Circ J, 2007. 71(8): p. 1305-1309.
202. Schultz, G.S., et al., Dynamic reciprocity in the wound microenvironment. Wound Repair Regen, 2011. 19(2): p. 134-148.
203. Bissell, M.J., H.G. Hall, and G. Parry, How does the extracellular matrix direct gene expression? J Theor Biol, 1982. 99(1): p. 31-68.
204. Bornstein, P. and E.H. Sage, Matricellular proteins: extracellular modulators of cell function. Curr Opin Cell Biol., 2002. 14(5): p. 608-616.
205. Vogel, V. and M. Sheetz, Local force and geometry sensing regulate cell functions. Nat Rev Mol Cell Biol, 2006. 7(4): p. 265-275.
206. Xu, R., A. Boudreau, and M. Bissell, Tissue architecture and function: dynamic reciprocity via extra- and intra-cellular matrices. Cancer Metastasis Rev, 2009. 28(1-2): p. 167-176.
207. Maeda, T., et al., Conversion of mechanical force into TGF-β-mediated biochemical signals. Curr Biol,

2011. 21(11): p. 933-941.
208. Streuli, C.H., et al., Extracellular matrix regulates expression of the TGF-β1 gene. J Cell Biol, 1993. 120(1): p. 253-260.
209. Weber, I.T., R.W. Harrison, and R.V. Iozzo, Model structure of decorin and implications for collagen fibrillogenesis. J Biol Chem, 1996. 271(50): p. 31767-70.
210. Wynn, T.A., Cellular and molecular mechanisms of fibrosis. J Pathol, 2008. 214(2): p. 199-210.
211. Kloen, P., et al., Transforming growth factor-β: possible roles in Dupuytren's contracture. J Hand Surg Am, 1995. 20(1): p. 101-108.
212. Rehman, S., et al., Molecular phenotypic descriptors of Dupuytren's disease defined using Informatics analysis of the transcriptome. J Hand Surg Am, 2008. 33(3): p. 359-372.
213. Border, W.A. and N.A. Noble, Transforming growth factor β in tissue fibrosis. N Engl J Med, 1994. 331(19): p. 1286-92.
214. Westerhausen, D.R., Jr., W.E. Hopkins, and J.J. Billadello, Multiple transforming growth factor-β-inducible elements regulate expression of the plasminogen activator inhibitor type-1 gene in Hep G2 cells. J Biol Chem, 1991. 266(2): p. 1092-100.
215. Shih, B., S. Watson, and A. Bayat, Whole genome and global expression profiling of Dupuytren's disease: systematic review of current findings and future perspectives. Ann Rheum Dis, 2012. 71(9): p. 1440-1447.
216. Kraljevic Pavelic, S., et al., An integrated proteomics approach for studying the molecular pathogenesis of Dupuytren's disease. J Pathol, 2009. 217(4): p. 524-533.
217. Ignotz, R.A. and J. Massague, Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem, 1986. 261(9): p. 4337-45.
218. Su, T.H., J.H. Kao, and C.J. Liu, Molecular mechanism and treatment of viral hepatitis-related liver fibrosis. Int J Mol Sci, 2014. 15(6): p. 10578-604.
219. Dooley, S. and P. ten Dijke, TGF-β in progression of liver disease. Cell Tissue Res, 2012. 347(1): p. 245-56.
220. Meindl-Beinker, N.M. and S. Dooley, Transforming growth factor-β and hepatocyte transdifferentiation in liver fibrogenesis. J Gastroenterol Hepatol, 2008. 23 Suppl 1: p. S122-7.
221. Gabbiani, G., The myofibroblast in wound healing and fibrocontractive diseases. J Pathol, 2003. 200(4): p. 500-503.
222. Kawelke, N., et al., Fibronectin protects from excessive liver fibrosis by modulating the availability of and responsiveness of stellate cells to active TGF-β. PLoS One, 2011. 6(11): p. e28181.
223. Jeong, D.H., et al., Smad3 deficiency ameliorates hepatic fibrogenesis through the expression of senescence marker protein-30, an antioxidant-related protein. Int J Mol Sci, 2013. 14(12): p. 23700-23710.
224. Furukawa, F., et al., p38 MAPK mediates fibrogenic signal through Smad3 phosphorylation in rat myofibroblasts. Hepatology, 2003. 38(4): p. 879-89.
225. Phanish, Mysore K., et al., The differential role of Smad2 and Smad3 in the regulation of pro-fibrotic TGFβ1 responses in human proximal-tubule epithelial cells. Biochem J, 2006. 393(Pt 2): p. 601-607.
226. Ju, W., et al., Deletion of Smad2 in mouse liver reveals novel functions in hepatocyte growth and differentiation. Mol Cell Biol, 2006. 26(2): p. 654-667.
227. Tahashi, Y., et al., Differential regulation of TGF-β signal in hepatic stellate cells between acute and chronic rat liver injury. Hepatology, 2002. 35(1): p. 49-61.
228. Dooley, S., et al., Hepatocyte-specific Smad7 expression attenuates TGF-β-mediated fibrogenesis and protects against liver damage. Gastroenterology, 2008. 135(2): p. 642-59.
229. Goumans, M.J., et al., Balancing the activation state of the endothelium via two distinct TGF-β type I receptors. EMBO J, 2002. 21(7): p. 1743-53.

230. Herrera, B., S. Dooley, and K. Breitkopf-Heinlein, Potential roles of bone morphogenetic protein (BMP)-9 in human liver diseases. Int J Mol Sci, 2014. 15(4): p. 5199-220.
231. Zhong, L., et al., The anti-fibrotic effect of bone morphogenic protein-7(BMP-7) on liver fibrosis. Int J Med Sci, 2013. 10(4): p. 441-50.
232. Wang, L.P., et al., BMP-7 attenuates liver fibrosis via regulation of epidermal growth factor receptor. Int J Clin Exp Pathol, 2014. 7(7): p. 3537-47.
233. Kinoshita, K., et al., Adenovirus-mediated expression of BMP-7 suppresses the development of liver fibrosis in rats. Gut, 2007. 56(5): p. 706-14.
234. Flatt, A.E., The Vikings and Baron Dupuytren's disease. Proc (Bayl Univ Med Cent), 2001. 14(4): p. 378-84.
235. Gudmundsson, K.G., T. Jónsson, and R. Arngrímsson, Guillaume Dupuytren and finger contractures. Lancet, 2003. 362(9378): p. 165-168.
236. McFarlane, R.M., On the origin and spread of Dupuytren's disease. J Hand Surg Am, 2002. 27(3): p. 385-390.
237. Shih, B. and A. Bayat, Scientific understanding and clinical management of Dupuytren disease. Nat Rev Rheumatol, 2010. 6(12): p. 715-726.
238. Witthaut, J., et al., Efficacy and safety of Collagenase Clostridium Histolyticum injection for Dupuytren contracture: short-term results from 2 open-label studies. J Hand Surg Am, 2013. 38(1): p. 2-11.
239. Hindocha, S., et al., Dupuytren's diathesis revisited: evaluation of prognostic indicators for risk of disease recurrence. J Hand Surg Am, 2006. 31(10): p. 1626-1634.
240. Kloen, P., et al., Transforming growth factor-β: Possible roles in Dupuytren's contracture. J Hand Surg Am, 1995. 20(1): p. 101-108.
241. Badalamente, M.A., et al., The role of transforming growth factor β in Dupuytren's disease. J Hand Surg Am, 1996. 21(2): p. 210-215.
242. Kraljevic Pavelic, S., et al., An integrated proteomics approach for studying the molecular pathogenesis of Dupuytren's disease. J Pathol, 2009. 217(4): p. 524-533.
243. Zhang, A.Y., et al., Gene expression analysis of Dupuytren's disease: the role of TGF-β2. J Hand Surg Eur Vol, 2008. 33(6): p. 783-790.
244. Krause, C., P. Kloen, and P. ten Dijke, Elevated transforming growth factor β and mitogen-activated protein kinase pathways mediate fibrotic traits of Dupuytren's disease fibroblasts. Fibrogenesis Tissue Repair, 2011. 4(1): p. 14.
245. Hinz, B., et al., The myofibroblast: one function, multiple origins. Am J Pathol, 2007. 170(6): p. 1807-1816.
246. Vaughan, M.B., E.W. Howard, and J.J. Tomasek, Transforming growth factor-β1 promotes the morphological and functional differentiation of the myofibroblast. Exp Cell Res, 2000. 257(1): p. 180-9.
247. Wong, M. and V. Mudera, Feedback inhibition of high TGF-β1 concentrations on myofibroblast induction and contraction by Dupuytren's fibroblasts. J Hand Surg Br, 2006. 31(5): p. 473-83.
248. Bisson, M.A., et al., Transforming growth factor-β1 stimulation enhances Dupuytren's fibroblast contraction in response to uniaxial mechanical load within a 3-dimensional collagen gel. J Hand Surg Am, 2009. 34(6): p. 1102-10.
249. Tse, R., et al., Enhanced Dupuytren's disease fibroblast populated collagen lattice contraction is independent of endogenous active TGF-β2. BMC Musculoskelet Disord, 2004. 5(1): p. 41.
250. Vindevoghel, L., et al., SMAD3/4-dependent transcriptional activation of the human type VII collagen gene (COL7A1) promoter by transforming growth factor β. Proc Natl Acad Sci U S A, 1998. 95(25): p. 14769-74.
251. Deckers, M., et al., The tumor suppressor Smad4 is required for transforming growth factor β-induced epithelial to mesenchymal transition and bone metastasis of breast cancer cells. Cancer Res, 2006.

66(4): p. 2202-9.
252. Leask, A., Potential therapeutic targets for cardiac fibrosis: TGFβ, angiotensin, endothelin, CCN2, and PDGF, partners in fibroblast activation. Circ Res, 2010. 106(11): p. 1675-80.
253. Desmouliere, A., et al., Transforming growth factor-β 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. J Cell Biol, 1993. 122(1): p. 103-11.
254. Wynn, T.A. and T.R. Ramalingam, Mechanisms of fibrosis: therapeutic translation for fibrotic disease. Nat Med, 2012. 18(7): p. 1028-40.
255. Taura, K., et al., Hepatocytes do not undergo epithelial-mesenchymal transition in liver fibrosis in mice. Hepatology, 2010. 51(3): p. 1027-36.
256. Mosakhani, N., et al., Unique microRNA profile in Dupuytren's contracture supports deregulation of β-catenin pathway. Mod Pathol, 2010. 23(11): p. 1544-52.
257. Satish, L., et al., Fibroblasts from phenotypically normal palmar fascia exhibit molecular profiles highly similar to fibroblasts from active disease in Dupuytren's Contracture. BMC Med Genomics, 2012. 5: p. 15.
258. Massagué, J., TGFβ in Cancer. Cell, 2008. 134(2): p. 215-230.
259. Liu, I.M., et al., TGFβ-stimulated Smad1/5 phosphorylation requires the ALK5 L45 loop and mediates the pro-migratory TGFβ switch. EMBO J, 2009. 28(2): p. 88-98.
260. Massague, J., Integration of Smad and MAPK pathways: a link and a linker revisited. Genes Dev, 2003. 17(24): p. 2993-7.
261. Oft, M., R.J. Akhurst, and A. Balmain, Metastasis is driven by sequential elevation of H-ras and Smad2 levels. Nat Cell Biol, 2002. 4(7): p. 487-94.
262. Levy, L. and C.S. Hill, Alterations in components of the TGF-β superfamily signaling pathways in human cancer. Cytokine Growth Factor Rev, 2006. 17(1–2): p. 41-58.
263. Thiery, J.P., Epithelial-mesenchymal transitions in development and pathologies. Curr Opin Cell Biol, 2003. 15(6): p. 740-6.
264. Kalluri, R. and R.A. Weinberg, The basics of epithelial-mesenchymal transition. J Clin Invest, 2009. 119(6): p. 1420-1428.
265. Thuault, S., et al., Transforming growth factor-β employs HMGA2 to elicit epithelial–mesenchymal transition. J Cell Biol, 2006. 174(2): p. 175-183.
266. Yang, J., et al., Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell, 2004. 117(7): p. 927-39.
267. Batlle, E., et al., The transcription factor Snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol, 2000. 2(2): p. 84-89.
268. Stankic, M., et al., TGFβ-Id1 signaling opposes Twist1 and promotes metastatic colonization via a mesenchymal-to-epithelial transition. Cell Rep, 2013. 5(5): p. 1228-1242.
269. Buijs, J., et al., TGF-β and BMP7 interactions in tumour progression and bone metastasis. Clin Exp Metastasis, 2007. 24(8): p. 609-617.
270. Pardali, E., M.-J. Goumans, and P. ten Dijke, Signaling by members of the TGF-β family in vascular morphogenesis and disease. Trends Cell Biol, 2010. 20(9): p. 556-567.
271. ten Dijke, P. and H.M. Arthur, Extracellular control of TGFβ signaling in vascular development and disease. Nat Rev Mol Cell Biol, 2007. 8(11): p. 857-69.
272. Urness, L.D., L.K. Sorensen, and D.Y. Li, Arteriovenous malformations in mice lacking activin receptor-like kinase-1. Nat Genet, 2000. 26(3): p. 328-31.
273. Armulik, A., A. Abramsson, and C. Betsholtz, Endothelial/pericyte interactions. Circ Res, 2005. 97(6): p. 512-523.
274. Liu, Z., et al., ENDOGLIN is dispensable for vasculogenesis, but required for vascular endothelial

growth factor-induced angiogenesis. PLoS One, 2014. 9(1): p. e86273.
275. Risau, W., Mechanisms of angiogenesis. Nature, 1997. 386(6626): p. 671-674.
276. Siegel, P.M. and J. Massague, Cytostatic and apoptotic actions of TGF-β in homeostasis and cancer. Nat Rev Cancer, 2003. 3(11): p. 807-21.
277. Adams, R.H. and K. Alitalo, Molecular regulation of angiogenesis and lymphangiogenesis. Nat Rev Mol Cell Biol, 2007. 8(6): p. 464-478.
278. Kim, E.S., M.S. Kim, and A. Moon, TGF-β-induced upregulation of MMP-2 and MMP-9 depends on p38 MAPK, but not ERK signaling in MCF10A human breast epithelial cells. Int J Oncol, 2004. 25(5): p. 1375-82.
279. Hawinkels, L.J., et al., Matrix metalloproteinase-14 (MT1-MMP)-mediated endoglin shedding inhibits tumor angiogenesis. Cancer Res, 2010. 70(10): p. 4141-50.
280. Stearns, M.E., et al., Role of Interleukin 10 and Transforming growth factor β1 in the angiogenesis and metastasis of human prostate primary tumor lines from orthotopic implants in severe combined immunodeficiency mice. Clin Cancer Res, 1999. 5(3): p. 711-720.
281. Tuxhorn, J.A., et al., Inhibition of Transforming growth factor-β activity decreases angiogenesis in a human prostate cancer-reactive stroma xenograft model. Cancer Res, 2002. 62(21): p. 6021-6025.
282. Barlow, L.J. and M.M. Shen, SnapShot: Prostate cancer. Cancer Cell, 2013. 24(3): p. 400 e1.
283. Baca, S.C., et al., Punctuated evolution of prostate cancer genomes. Cell, 2013. 153(3): p. 666-677.
284. Yang, F., et al., Stromal TGF-β signaling induces AR activation in prostate cancer. Oncotarget, 2014. 5(21): p. 10854-10869.
285. Amatangelo, M.D., et al., c-Myc expression and MEK1-induced Erk2 nuclear localization are required for TGFβ induced epithelial–mesenchymal transition and invasion in prostate cancer. Carcinogenesis, 2012. 33(10): p. 1965-1975.
286. Nishimori, H., et al., Prostate cancer cells and bone stromal cells mutually interact with each other through Bone morphogenetic protein-mediated signals. J Biol Chem, 2012. 287(24): p. 20037-20046.
287. Yang, S., et al., A novel Bone morphogenetic protein signaling in heterotypic cell interactions in prostate cancer. Cancer Res, 2008. 68(1): p. 198-205.
288. Lee, Y.-C., et al., BMP4 promotes prostate tumor growth in bone through osteogenesis. Cancer Res, 2011. 71(15): p. 5194-5203.
289. Morrissey, C., et al., Bone morphogenetic protein 7 is expressed in prostate cancer metastases and its effects on prostate tumor cells depend on cell phenotype and the tumor microenvironment. Neoplasia, 2010. 12(2): p. 192-205.
290. Lai, T.-H., et al., Osteoblasts-derived BMP-2 enhances the motility of prostate cancer cells via activation of integrins. Prostate, 2008. 68(12): p. 1341-1353.
291. Ye, L., H. Kynaston, and W.G. Jiang, Bone morphogenetic protein-9 induces apoptosis in prostate cancer cells, the role of prostate apoptosis response-4. Mol Cancer Res, 2008. 6(10): p. 1594-1606.
292. Llovet, J.M., et al., Advances in targeted therapies for hepatocellular carcinoma in the genomic era. Nat Rev Clin Oncol, 2015.
293. Majumdar, A., et al., Hepatic stem cells and transforming growth factor β in hepatocellular carcinoma. Nat Rev Gastroenterol Hepatol, 2012. 9(9): p. 530-538.
294. Shirai, Y., et al., Plasma transforming growth factor-β 1 in patients with hepatocellular carcinoma. Comparison with chronic liver diseases. Cancer, 1994. 73(9): p. 2275-9.
295. Wang, J., et al., Inhibitory role of Smad7 in hepatocarcinogenesis in mice and in vitro. J Pathol, 2013. 230(4): p. 441-452.
296. Wiercinska, E., et al., Id1 is a critical mediator in TGF-β-induced transdifferentiation of rat hepatic stellate cells. Hepatology, 2006. 43(5): p. 1032-41.
297. Herrera, B., et al., BMP9 is a proliferative and survival factor for human hepatocellular carcinoma cells.

PLoS One, 2013. 8(7): p. e69535.
298. Lonardo, E., et al., Nodal/Activin signaling drives self-renewal and tumorigenicity of pancreatic cancer stem cells and provides a target for combined drug therapy. Cell Stem Cell, 2011. 9(5): p. 433-46.
299. Ji, C., et al., Liver-specific loss of GRP78 perturbs the global unfolded protein response and exacerbates a spectrum of acute and chronic liver diseases. Hepatology, 2011. 54(1): p. 229-239.
300. Kuo, T.-C., et al., A unique P-glycoprotein interacting agent displays anticancer activity against hepatocellular carcinoma through inhibition of GRP78 and mTOR pathways. Biochem Pharmacol, 2011. 81(9): p. 1136-1144.
301. Chen, W.-T., et al., GRP78 as a regulator of liver steatosis and cancer progression mediated by loss of the tumor suppressor PTEN. Oncogene, 2014. 33(42): p. 4997-5005.
302. Connolly, E.C., J. Freimuth, and R.J. Akhurst, Complexities of TGFβ targeted cancer therapy. Int J Biol Sci, 2012. 8(7): p. 964-978.
303. Akhurst, R.J. and A. Hata, Targeting the TGFβ signaling pathway in disease. Nat Rev Drug Discov, 2012. 11(10): p. 790-811.
304. Derynck, R., R.J. Akhurst, and A. Balmain, TGF-β signaling in tumor suppression and cancer progression. Nat Genet, 2001. 29(2): p. 117-29.
305. Bhola, N.E., et al., TGFβ inhibition enhances chemotherapy action against triple-negative breast cancer. J Clin Invest, 2013. 123(3): p. 1348-1358.
306. Park, J., et al., Combination delivery of TGF-β inhibitor and IL-2 by nanoscale liposomal polymeric gels enhances tumour immunotherapy. Nat Mater, 2012. 11(10): p. 895-905.
307. Biswas, S., et al., Inhibition of TGF-β with neutralizing antibodies prevents radiation-induced acceleration of metastatic cancer progression. J Clin Invest, 2007. 117(5): p. 1305-1313.
308. Katz, L.H., et al., Targeting TGF-β signaling in cancer. Expert Opin Ther Targets., 2013. 17(7): p. 743-760.
309. Aartsma-Rus, A., et al., Targeted exon skipping as a potential gene correction therapy for Duchenne muscular dystrophy. Neuromuscul Disord, 2002. 12(1): p. 71-77.
310. Goemans, N.M., et al., Systemic administration of PRO051 in Duchenne's muscular dystrophy. N Engl J Med, 2011. 364(16): p. 1513-1522.
311. Cirak, S., et al., Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study. Lancet, 2011. 378(9791): p. 595-605.
312. Aartsma-Rus, A., et al., Guidelines for antisense oligonucleotide design and insight into splice-modulating mechanisms. Mol Ther, 2008. 17(3): p. 548-553.
313. Kole, R., T. Williams, and L. Cohen, RNA modulation, repair and remodeling by splice switching oligonucleotides. Acta Biochim Pol, 2004. 51(2): p. 373-8.
314. Chan, J.H.P., S. Lim, and W.S.F. Wong, Antisense oligonucleotides: from design to therapeutic application. Clin Exp Pharmacol Physiol, 2006. 33(5-6): p. 533-540.
315. Saunier E.F., A.R.J., TGFβ inhibition for cancer therapy. Curr Cancer Drug Targets, 2006.
316. Bogdahn, U., et al., Targeted therapy for high-grade glioma with the TGF-β2 inhibitor trabedersen: results of a randomized and controlled phase IIb study. Neuro Oncol, 2011. 13(1): p. 132-142.
317. Nemunaitis, J., et al., Phase II trial of Belagenpumatucel-L, a TGF-β2 antisense gene modified allogeneic tumor vaccine in advanced non small cell lung cancer (NSCLC) patients. Cancer Gene Ther, 2009. 16(8): p. 620-624.
318. Nemunaitis, J., et al., Phase II study of belagenpumatucel-L, a Transforming growth factor β2 antisense gene-modified allogeneic tumor cell vaccine in non–small-cell lung cancer. J Clin Oncol, 2006. 24(29): p. 4721-4730.
319. An, H.J., et al., Effects of Smad decoy ODN on shear stress-induced atherosclerotic ApoE-/-mouse. Int J Clin Exp Pathol, 2015. 8(4): p. 3971-8.

320. Boros, D.L., et al., A novel nonsteroidal antifibrotic oligo decoy containing the TGF-β element found in the COL1A1 gene which regulates murine schistosomiasis liver fibrosis. J Cell Physiol, 2005. 204(2): p. 370-4.
321. Matsuda, H., et al., Development of gene silencing pyrrole-imidazole polyamide targeting the TGF-β1 promoter for treatment of progressive renal diseases. J Am Soc Nephrol, 2006. 17(2): p. 422-32.
322. George, J., et al., In vivo inhibition of rat stellate cell activation by soluble transforming growth factor β type II receptor: A potential new therapy for hepatic fibrosis. Proc Natl Acad Sci U S A, 1999. 96(22): p. 12719-12724.
323. Qi, Z., et al., Blockade of type β transforming growth factor signaling prevents liver fibrosis and dysfunction in the rat. Proc Natl Acad Sci U S A, 1999. 96(5): p. 2345-2349.
324. Muraoka, R.S., et al., Blockade of TGF-β inhibits mammary tumor cell viability, migration, and metastases. J Clin Invest, 2002. 109(12): p. 1551-9.
325. Santiago, B., et al., Topical application of a peptide inhibitor of Transforming growth factor-β1 ameliorates bleomycin-induced skin fibrosis. J Investig Dermatol, 2005. 125(3): p. 450-455.
326. Ezquerro, I.J., et al., A synthetic peptide from transforming growth factor β type III receptor inhibits liver fibrogenesis in rats with carbon tetrachloride liver injury. Cytokine, 2003. 22(1-2): p. 12-20.
327. Hermida, N., et al., A synthetic peptide from transforming growth factor-β1 type III receptor prevents myocardial fibrosis in spontaneously hypertensive rats. Cardiovasc Res, 2009. 81(3): p. 601-9.
328. Llopiz, D., et al., Peptide inhibitors of transforming growth factor-β enhance the efficacy of antitumor immunotherapy. Int J Cancer, 2009. 125(11): p. 2614-23.
329. Lu, A., et al., Blockade of TSP1-dependent TGFβ activity reduces renal injury and proteinuria in a murine model of diabetic nephropathy. Am J Pathol, 2011. 178(6): p. 2573-86.
330. Van Aarsen, L.A., et al., Antibody-mediated blockade of integrin alpha v β 6 inhibits tumor progression in vivo by a transforming growth factor-β-regulated mechanism. Cancer Res, 2008. 68(2): p. 561-70.
331. Stander, M., et al., Decorin gene transfer-mediated suppression of TGF-β synthesis abrogates experimental malignant glioma growth in vivo. Gene Ther, 1998. 5(9): p. 1187-94.
332. Castonguay, R., et al., Soluble endoglin specifically binds bone morphogenetic proteins 9 and 10 via its orphan domain, inhibits blood vessel formation, and suppresses tumor growth. J Biol Chem, 2011. 286(34): p. 30034-46.
333. Kerr, G., et al., A small molecule targeting ALK1 prevents Notch cooperativity and inhibits functional angiogenesis. Angiogenesis, 2015. 18(2): p. 209-17.
334. Mitchell, D., et al., ALK1-Fc inhibits multiple mediators of angiogenesis and suppresses tumor growth. Mol Cancer Ther, 2010. 9(2): p. 379-88.
335. Cunha, S.I., et al., Genetic and pharmacological targeting of activin receptor-like kinase 1 impairs tumor growth and angiogenesis. J Exp Med, 2010. 207(1): p. 85-100.
336. Bendell, J.C., et al., Safety, pharmacokinetics, pharmacodynamics, and antitumor activity of dalantercept, an activin receptor-like kinase-1 ligand trap, in patients with advanced cancer. Clin Cancer Res, 2014. 20(2): p. 480-9.
337. Cui, Q., et al., Selective inhibition of TGF-β responsive genes by Smad-interacting peptide aptamers from FoxH1, Lef1 and CBP. Oncogene, 2005. 24(24): p. 3864-74.
338. Mead, A.L., et al., Evaluation of anti-TGF-β2 antibody as a new postoperative anti-scarring agent in glaucoma surgery. Invest Ophthalmol Vis Sci, 2003. 44(8): p. 3394-3401.
339. Trachtman, H., et al., A phase 1, single-dose study of fresolimumab, an anti-TGF-β antibody, in treatment-resistant primary focal segmental glomerulosclerosis. Kidney Int, 2011. 79(11): p. 1236-1243.
340. Morris, J.C., et al., Phase I study of GC1008 (Fresolimumab): a human anti-Transforming growth factor-β (TGFβ) monoclonal antibody in patients with advanced malignant melanoma or renal cell

carcinoma. PLoS One, 2014. 9(3): p. e90353.
341. Seon, B.K., et al., Long-lasting complete inhibition of human solid tumors in SCID mice by targeting endothelial cells of tumor vasculature with antihuman endoglin immunotoxin. Clin Cancer Res, 1997. 3(7): p. 1031-44.
342. Rosen, L.S., et al., A phase I first-in-human study of TRC105 (anti-endoglin antibody) in patients with advanced cancer. Clin Cancer Res, 2012. 18(17): p. 4820-9.
343. Vogt, J., R. Traynor, and G.P. Sapkota, The specificities of small molecule inhibitors of the TGFß and BMP pathways. Cell Signal, 2011. 23(11): p. 1831-1842.
344. Hawinkels, L.J.A.C. and P. ten Dijke, Exploring anti-TGF-β therapies in cancer and fibrosis. Growth Factors, 2011. 29(4): p. 140-152.
345. Yingling, J.M., K.L. Blanchard, and J.S. Sawyer, Development of TGF-β signaling inhibitors for cancer therapy. Nat Rev Drug Discov, 2004. 3(12): p. 1011-1022.
346. Scott Sawyer, J., et al., Synthesis and activity of new aryl- and heteroaryl-substituted 5,6-dihydro-4H-pyrrolo[1,2-b]pyrazole inhibitors of the transforming growth factor-β type I receptor kinase domain. Bioorg Med Chem Lett, 2004. 14(13): p. 3581-3584.
347. Flavell, R.A., et al., The polarization of immune cells in the tumour environment by TGFβ. Nat Rev Immunol, 2010. 10(8): p. 554-567.
348. Kano, M.R., et al., Improvement of cancer-targeting therapy, using nanocarriers for intractable solid tumors by inhibition of TGF-β signaling. Proc Natl Acad Sci U S A, 2007. 104(9): p. 3460-3465.
349. Korpal, M., et al., Imaging transforming growth factor-β signaling dynamics and therapeutic response in breast cancer bone metastasis. Nat Med, 2009. 15(8): p. 960-966.
350. Gueorguieva, I., et al., Defining a therapeutic window for the novel TGF-β inhibitor LY2157299 monohydrate based on a pharmacokinetic/pharmacodynamic model. Br J Clin Pharmacol, 2014. 77(5): p. 796-807.
351. Noble, P.W., et al., Pirfenidone in patients with idiopathic pulmonary fibrosis (CAPACITY): two randomised trials. Lancet, 2011. 377(9779): p. 1760-1769.
352. Denton, C.P., et al., Recombinant human anti–transforming growth factor β1 antibody therapy in systemic sclerosis: A multicenter, randomized, placebo-controlled phase I/II trial of CAT-192. Arthritis Rheum, 2007. 56(1): p. 323-333.