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TGF-beta1, WNT, and SHH signaling in tumor progression and in fibrotic diseases

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1. ABSTRACT

Activation of resting fibroblasts to myofibroblasts characterizes several physiological and pathological conditions, from wound healing to aggressive metastatic cancers. In tissue damage, including wound healing, fibroblasts are activated in response to injury for a limited period of time to stimulate the healing process. Similar biological mechanisms are maintained in pathological conditions, e.g., scleroderma and cancer, where myofibroblasts persist in producing cytokines and growth factors to drive the development of fibrosis and the progression of disease. Studies characterizing the bi-directional signal transduction pathways between cancer cells and stromal cells have suggested novel druggable targets that may function in both the inhibition of fibrotic reactions in cancer stroma and in the inhibition of fibrotic diseases. In this review, we focus on transforming growth factor β (TGFβ), int/Wingless (WNT), and sonic hedgehog (SHH) signal transduction pathways and describe small molecule inhibitors that are used in phase I/II clinical trials to treat fibrosis or fibrotic cancers.

2. INTRODUCTION

Stroma is composed of cells of mesenchymal, hematopoietic, and epithelial origin, including fibroblasts, inflammatory cells, and vascular endothelial cells, which are embedded in the extracellular matrix (ECM). ECM is composed of collagen and elastin fibers, heparan sulfate, chondroitin sulfate, keratin sulfate proteoglycans, non-proteoglycan polysaccharides such as water absorbing anti-swelling hyaluronic acid, and other macromolecules, including fibronectin and laminin. The main physiological functions of ECM are to provide structural support for cells, mediate cell-to-cell communication, maintain cell adhesion, and serve as storage for factors needed for cellular growth, angiogenesis, differentiation, and, importantly, cell signaling (1). In tissue trauma and during carcinogenesis, fibroblasts activate to myofibroblasts, the large, spindle-shaped cells that initiate the tissue repair process. Characteristically, in tissue injury these cells are removed apoptotically or deactivated once the injury has healed (2, 3). In cancer and other pathological conditions, including scleroderma, myofibroblasts persist, as does their consequent continuous paracrine secretion of growth factors and cytokines (4, 5). Activated fibroblasts play a crucial role in injury healing and in tumor progression by influencing ECM structure and by interacting with epithelial cancer cells and immune cells. The presence of myofibroblasts in tumor stroma characterizes the desmoplastic reaction, initiating the fibrosis development that frequently correlates with invasion and poor outcome in cancers (5, 6). In general, fibrosis in skin, heart, liver, intestine, or kidney severely damages normal tissue function and architecture, thus representing an attractive therapeutic target to remove or reverse fibrotic development.

Tumor stroma is under constant development, creating a platform for migrating fibroblasts, inflammatory cells and transformed epithelial cells. Recent reports suggest that cancer stroma develops to respond the needs of the epithelial cells, creating a nurturing microenvironment for cancer cells. Stroma actively participates in carcinogenesis through paracrine

secretion of cytokines, growth factors, and proteins inducing epithelial-mesenchymal transition and metastasis. Additionally, stroma may directly serve as a source of nutrients for cancer cells, thereby increasing their aggressiveness (7-17). Apoptotic cell death in the hypoxic regions of tumors is well reported, although the fate of cellular debris has received less attention. In tissue trauma, cellular debris is removed by granulocytes that migrate into inflammatory sites. In cancer, epithelial cells can form a physical connection with damaged stromal cells to absorb cellular material, consequently increasing epithelial cancer cell aggression

3. TGF-β, WNT, AND SHH SIGNALING

The stromal signature is composed of hematopoietic, mesenchymal, and endothelial cell-derived factors that have paracrine effects on epithelial cancer cells. Transcriptional signature studies have suggested similarities between wound healing and carcinogenesis (18), indicating similarities in fibroblast function in different pathological conditions and corroborating observations suggesting the involvement of stromal cells in tumor development. This conclusion is further strengthened by comparing the fibroblast transcriptomes from different tissues, demonstrating that wound healing response genes (over 500 genes) are involved in cell cycling, cell migration, extracellular matrix remodeling, paracrine signaling, and fibroblast activation to myofibroblasts (18). Similar variations in gene expression observed in breast, lung, gastric, and prostate cancer stromal cells correlate with increased metastasis and unfavorable patient prognosis (18-20), corroborating the role of stroma-derived signaling in the development of highly aggressive, metastatic cancers.

A recent study reported activated macrophage tumor, monocyte tumor, and neutrophil tumor signal transduction pathways, revealing 22 macrophage-derived soluble factors that had 31 cognate receptors in epithelial cancer cells. These connections include transforming growth factor β (TGFβ)-SMAD signaling, which interacts with several signaling molecules and cascades, including WNT1, HIPPO, PI3K, ErbB, and HIF-1 (21). Another noteworthy stroma-originating soluble factor, WNT1 ligand, binds to Frizzled 5 (FZD5) and Frizzled 7 (FZD7) cancer cell receptors (21). In the colon, WNT expression, which activates JNK and *c-jun*, is absent in normal tissue but is detected in stage I-IV tumors and correlates with FZD7 expression. High WNT11-FZD7 expression has been observed in relapsed cancers and in incurable tumors (22).

3.1. TGF-β/SMAD signaling as tumor suppressor and tumor promoter

TGFβ signaling regulates a number of biological properties in cancer, including growth, apoptosis, differentiation, migration, invasion, angiogenesis, ECM production, and cancer cell interactions with the immune system. Recent studies have demonstrated that TGFβ suppresses growth in the early phases of tumor development by promoting cell cycle arrest related genes, including retinoblastoma-like 1 gene expression (23). At advanced stages of tumorigenesis, TGFβ promotes epithelial-mesenchymal transition (EMT), invasion, and metastasis. This switch is known as the TGFβ paradox. To enter the EMT phase, cellular mechanisms must genetically or epigenetically silence TGFβ-derived tumor suppressive effects (24). In advanced cancers, high levels of TGFβ induce EMT pro-tumorigenic effects, activating TGFβ-mediated modifications in the tumor microenvironment such as inhibiting the expression of E-cadherin and increasing the expression of N-cadherin, which are responsible for maintaining epithelial cell-cell connections at tight junctions and promoting cell migration, respectively (24-26). The switch from tumor suppressor signaling to pro-tumorigenic stimuli, including the activation of oncogenic mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), p38 MAPK, and cyclin-dependent kinase (CDK) pathways, occurs through TGFβ-mediated activation of non-canonical signal transduction (27, 28).

The TGFβ signal cascade is initiated by TGFβ ligand binding to the TGFβR2/TGFβR1 heterodimer and causing activation of TGFβR2, which then phosphorylates TGFβR1, thus inducing phosphorylation of SMAD2/SMAD3 and activation of downstream SMAD signaling. SMAD proteins contain MAD homology domains 1 (MH1) and 2 (MH2), which bind to DNA and to co-signaling molecules (SMAD4, receptor molecules, or transcriptional co-activators), respectively (29). Once SMAD4 has bound to the MH2 domain of phosphorylated SMAD2 and SMAD3, the SMAD complex translocates into the nucleus. In the nucleus, the SMAD complex MH1 domain binds to DNA, inducing target gene expression. The MH2 domain interacts with transcription factors/co-activators, receptors, or SMAD4 (Figure 1A). According to a number of recent studies, the abovementioned TGFβ paradox is caused by a change in linker region phosphorylation and by TGFβ-mediated activation of canonical non-SMAD pathways, most prominently RAS-ERK signaling (24, 27, 28, 30). This signaling activation increases ROS production (31, 32), causing DNA damage and activation of the DNA damage response (33). Mechanistically, TGFβ contributes to tissue fibrosis in chronic inflammation by inducing ROS production, thus creating a favorable microenvironment for cancer cell growth and tumor initiation (34). However, only approximately 15% of human cancers originate from existing chronic inflammatory diseases (35).

The growth inhibitory effect of TGFβ is enhanced by the direct binding of tumor suppressor p53 to the SMAD complex MH1 domain of SMAD2 and SMAD3 (Figure 1B). Importantly, binding occurs after phosphorylation of

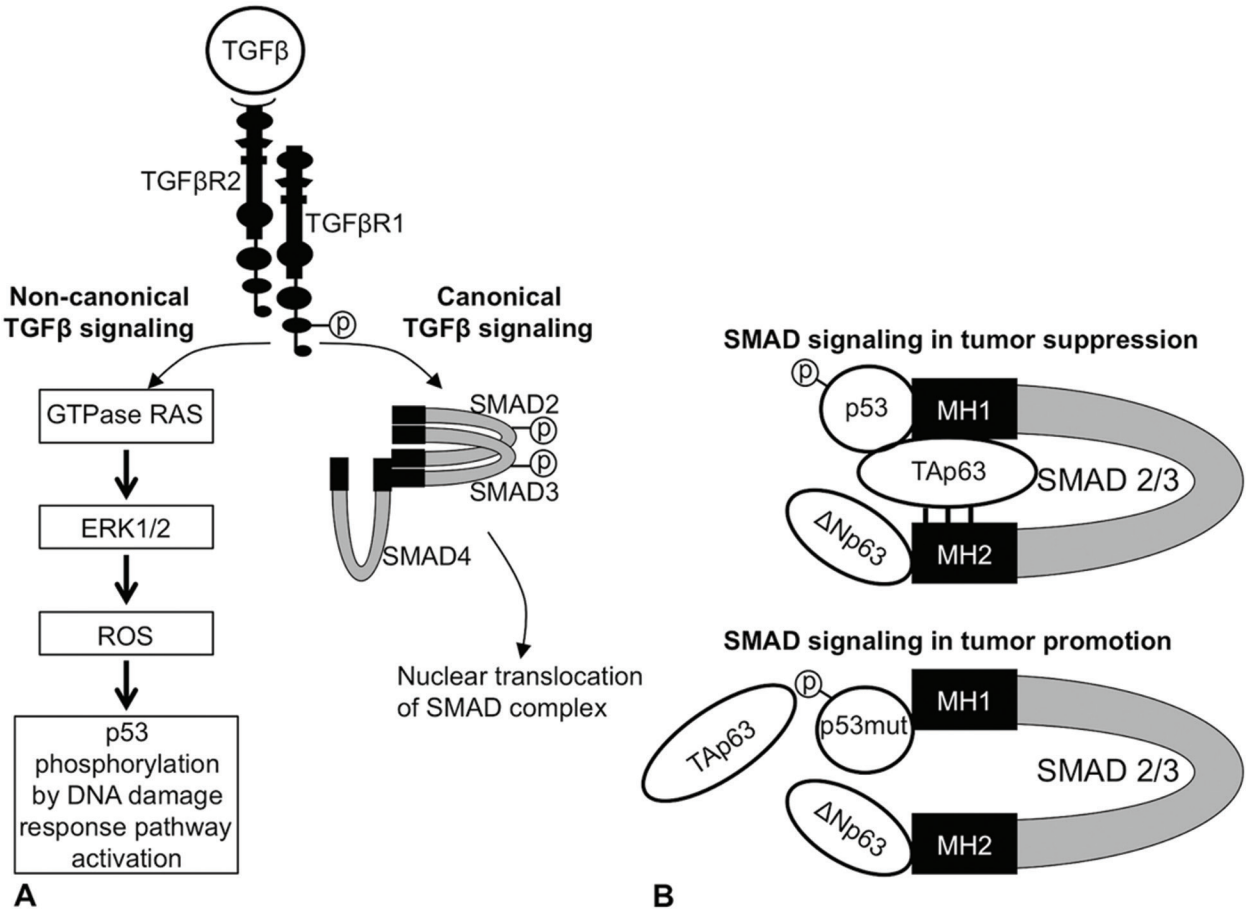

Figure 1. TGFβ signaling paradox. A. Canonical and non-canonical signal cascades. TGFβ binding induces canonical SMAD signal transduction through SMAD complex formation and non-canonical signal transduction activating MAP kinase pathways. In canonical signal transduction TGFβ ligand binds to TGFβR2 with subsequent phosphorylation of TGFβR1. Activated TGFβR1 phosphorylates SMAD2 and SMAD3 inducing SMAD4 binding to C-terminal MH2 domain to form SMAD complex that is translocated into the nucleus. Non-canonical pathway involves e.g. increased activation of small GTPase RAS that activate downstream MAP kinase p42/p44 causing increased reactive oxygen species formation, increase in DNA damages, DNA damage response pathway activation, and increased p53 signal transduction. Non-canonical mitogen pathway is required for SMAD complex p53 phoshorylation B. TGFβ signaling in tumor suppression and in tumor support. Only SMAD2 and SMAD3 are shown. TGFβ signaling inhibits tumor growth when tumor suppressor TAp63 and wild type phosphorylated p53 bind to SMAD complex N-terminal MH1 domain. Of note, TAp63 can interact also with MH2 domain. Abundantly present protumorigenic ΔNp63 binds to SMAD complex C-terminal MH2 domain. In the nucleus TAp63 and wild type p53 containing SMAD complex inhibits growth support transcription. The switch of TGFβ signaling from tumor suppressor to tumor promoter occurs after p53 mutation. Mutated p53 binds to SMAD complex N-terminal MH1 domain and hinders the binding of tumor suppressor TAp63. Mutated p53 and lack of tumor suppressor TAp63 in the nuclear SMAD complex results in a loss of growth inhibition. Importantly, phosphorylation of p53 by RAS-ERK1/2 signal transduction derived ROS is required for binding of wild type and mutated p53 to SMAD complex.

wild-type p53 by RAS-MEK-ERK signaling (36, 37), which is enhanced by ROS (38), DNA damage (39), and casein kinase 1 ε and δ (CK1ε/δ) (37). The wild-type p53-SMAD complex contributes to growth by activating the expression of tumor and metastasis suppressor genes (cyclin-dependent kinase inhibitors, *p21*, and *INK4B*), by downregulating the expression of oncogenes (MYC) and by decreasing the expression of genes initiating extracellular matrix degradation (plasminogen activator inhibitor 1 and matrix metalloproteinase 1 and 10) (40). In addition to wild-type p53 binding, SMAD proteins can affect the progression of tumorigenesis in co-operation with p63 splice variant-derived TA or ΔN proteins. TA proteins have tumor suppressor characteristics similar to p53 proteins, whereas ΔNp63 functions as a tumor promoter, controlling cell survival and self-renewal of stem cells (41). The anti-oncogenic characteristics of TAp63 prevent tumor development in p63 heterozygous mice with and without p53, inhibiting both spontaneous or chemically induced tumorigenesis (42).

Although pro-tumorigenic ΔNp63 is the most common isoform in poorly differentiated and metastatic cancers, the loss of tumor suppressor TAp63 binding to the SMAD complex is essential in TGFβ function (41). The TGFβ functional switch in tumorigenesis is related to two p63 SMAD binding sites. Tumorigenic ΔNp63 binds to the SMAD2/3 C-terminal MH2 domain, whereas tumor

suppressor TAp63 binds to the SMAD2/3 N-terminal MH1 domain. Interestingly, TAp63 has been demonstrated to interact also with MH2 domain (43). Another important mechanism in the TGFβ switch from tumor suppressor to cancer promoter is the mutation of p53. Mutated p53 can antagonize the tumor suppressor properties of TAp63 by inhibiting the binding of TAp63 to its cognate sites at the SMAD2/3 MH1 complex and/or at DNA (Figure 1B). Interestingly, binding of mutated p53 to the SMAD ternary complex occurs only in the presence of TGFβ (37, 43) and requires the activation of a tyrosine kinase receptor, such as fibroblast growth factor receptor (FGF-R), and RAS-RAF-MEK-ERK signaling through CK1ε/δ (37). The interaction of p63 with mutated p53 affects the expression of *SHARP-1*, *CYCLIN G2*, *ADAMTS9*, *FOLLISTATIN*, and *GRP87,* inducing the migration and metastasis programs of cancer cells (43).

3.2. WNT/β-catenin signaling

ΔNp63 signaling connects TGFβ pro-tumorigenic signaling to int/Wingless (WNT) signal transduction in cancer promotion. Nuclear ΔNp63 binds to the WNT receptor *Frizzled 7* (*FZD7*) enhancer region approximately 40 kb upstream of the *FZD7* coding sequence, upregulating *FZD7* mRNA production and the concentration of the receptor at the cell membrane (44). The FZD7 transmembrane family consists of ten members that bind 19 WNT ligands (45). The WNT/β-catenin pathway was first described in embryonic development, where it regulates cell polarity and body axis patterning, although this pathway has also been involved in the development and progression of tumorigenesis in different models. In colorectal cancer, loss of the destruction complex caused by adenomatous polyposis coli (APC) mutation and nuclear localization of β-catenin represent one of the initial tumorigenic events. Activation of the canonical WNT/β-catenin signaling pathway has been associated with fibroblast activation, fibrosis and tissue repair (46, 47).

WNT activates two alternative pathways: the β-catenin dependent (canonical) and the β-catenin independent (non-canonical) pathway (Figure 2) (48, 49). In the canonical pathway, secreted WNT ligands bind to transmembrane FRIZZLED receptors, inducing membrane recruitment of AXIN. Consequently, the β-catenin destruction complex, composed of AXIN, APC, casein kinase 1 (CK1) and serine-threonine glycogen synthase kinase 3β (GSK3β), is destabilized, allowing β-catenin cytoplasmic accumulation, entry to the nucleus, and activation of T-cell factor/lymphoid enhancer binding factor (TCF/LEF) target genes, e.g., *CYCLIN D1* and *c-MYC* (50). β-catenin independent non-canonical signaling is mediated through two parallel signaling cascades: the small GTPases RHO and RAC, which transmit the signal to the JNK pathway that regulates cell motility, and phospholipase C-protein kinase C-calcium 2^+ (PLC-PKC-Ca^{2+}), which controls cell migration (51, 52). Non-canonical signaling may also include the WNT-dependent activation of the PI3K/AKT pathway and the FZD-dependent activation of PKA/CREB or p38/ATF2 intracellular mediators (53).

3.3. Sonic hedgehog in tissue injury and cancer

The over-expression of ΔNp63 and TAp63 activates sonic hedgehog (SHH) production in mouse primary fibroblasts by directly binding to the SHH promoter region (54), linking the two pathways in stromal cells. Another recent study demonstrated the crosstalk between SHH and TGFβ in cancer cells, demonstrating increased SHH expression after long-term TGFβ exposure and suggesting that SHH is required for TGFβ-derived EMT (54), therefore supporting the coordinated action of TGFβ, p63, and morphogens in tissue injury response and tumorigenesis.

SHH signaling, which regulates embryonic development during ontogeny, is silenced in terminally differentiated adult tissues. However, recent reports demonstrate SHH pathway activation in a number of physiological and pathological conditions, including injury, inflammation and tumorigenesis (55, 56). The pathway is silenced by the protein patched homolog 1 (PTCH) receptor, which suppresses SMOOTHENED (SMO) receptor downstream signaling until SHH binds to PTCH and neutralizes the inhibitory action of PTCH. Consequently, SMO activates downstream canonical and non-canonical signaling, the latter mediated by RAC/RHO GTPases. Pathway activation disrupts the GLI phosphorylation complex (GLI-SUFU-FU-COS2), with consequent nuclear translocation of GLI transcription factors that stimulate the expression of genes regulating cell growth, survival, differentiation, angiogenesis, evasion of immune response, activation of invasion, and metastasis (e.g., *CYCLIN D1*, *MYC*, *BCL2*, *SNAIL*, and *NANOG*) (Figure 3) (57-59). The SHH pathway can be activated in an autocrine manner, i.e., SHH ligand and PTCH/SMO receptors are expressed in the same cells, or in a paracrine manner, when SHH ligand originates from neighboring cells. In tumors, paracrine signaling can be direct (SHH is secreted by cancer cells and acts on stromal cells) or inverse (SHH is secreted by stromal cells and acts on cancer cells) (58, 60, 61). The direct paracrine mechanism promotes angiogenesis, lymphangiogenesis and metastasis (58, 60-62), whereas inverse paracrine signaling activates cancer cell growth, survival and metastasis (63). In addition, it has been postulated that in injured/damaged cells, SHH ligand can induce PLATELET DERIVED GROWTH FACTOR (PDGF) and TGFβ production in myofibroblasts, inflammatory cells, and quiescent cells that can activate the GLI transcription factor independently of the SMO receptor. PDGF, on the other hand, has a feedback effect on SHH signaling by inducing SHH mRNA transcription and SMO membrane translocation (64). Hence, cancer cells may further

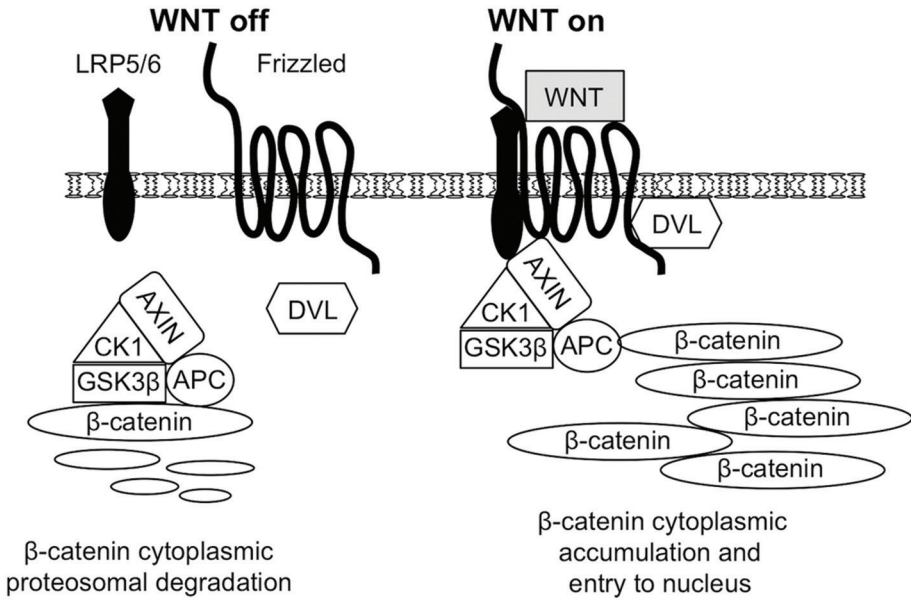

Figure 2. Schematic representation of canonical WNT signaling. In the absence of WNT ligand destruction complex formed of AXIN, GSK3β, CK1, and APC induces degradation of β-catenin. WNT ligand binding to FRIZZLED receptor inhibits destruction complex, induces DVL cell membrane localization, allows β-catenin cytoplasmic accumulation, and consequent nuclear entry.

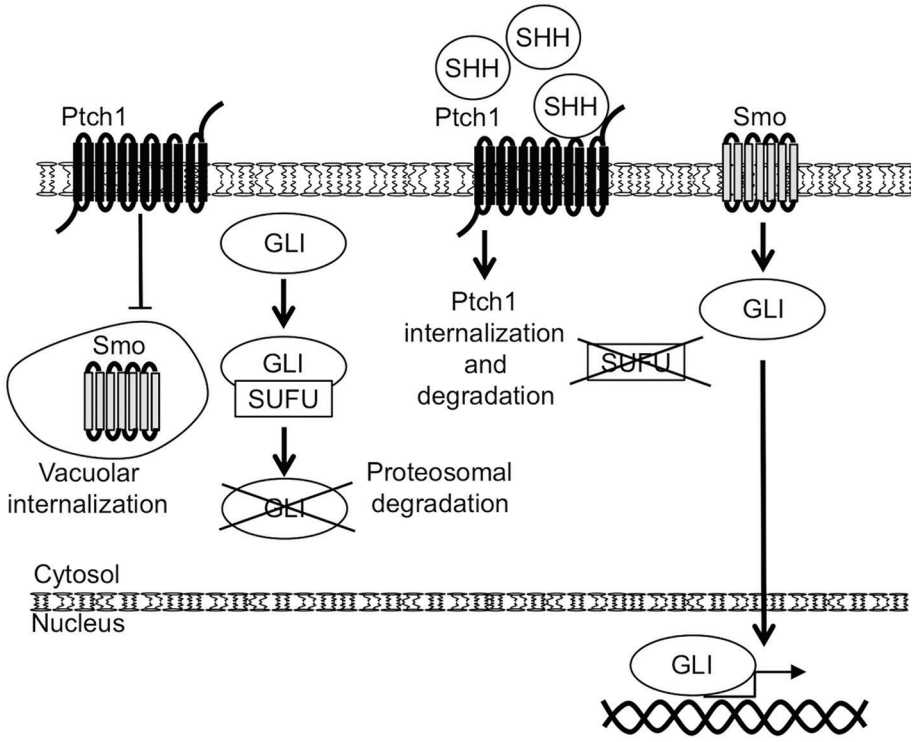

Figure 3. Schematic representation of SHH signaling. In the absence of SHH ligand signal transduction is inhibited at two levels: a) SMO receptor is inactivated by PTCH receptor and maintained in the cytosol in vacuoles. b) GLI transcription factor is bound to SUFU and degraded in the cytosol. Binding of SHH ligand to PTCH results in internalization and degradation of PTCH. Consequently, cell membrane translocated SMO receptor activates GLI-mediated signal transduction that enters to nucleus and promotes transcription.

enhance stromal paracrine signaling by autocrine SHH pathway activation to maintain transformed properties, including proliferation, epithelial-mesenchymal transition, induction of genomic instability inhibition, and apoptosis.

The importance of tumor stroma-derived SHH signaling has been demonstrated in medulloblastoma and in *K-RAS* transgenic pancreatic cancer mice, both models representing aggressive incurable cancers (65-67). Lack of GLI1 in the tumor microenvironment of *KRAS* mice markedly reduced cancer cell growth (68). Another indication of the importance of stroma-derived SHH ligand in carcinogenesis was observed in studies demonstrating that inhibition of SHH signal transduction reduced desmoplasia in pancreatic cancer tumors (69), highlighting the role of SHH in tumor stroma development. Indeed, it has been suggested that the reduced desmoplastic reaction caused by SHH inhibition may increase tumor vascularization, thus increasing cancer cell metabolism and progression of carcinogenesis (70-72). Although the role of SHH itself in carcinogenesis is not completely defined, pathway activation may have dual role in tumorigenesis by affecting the stromal organization and by regulating epithelial cancer cell proliferation and/or migration.

4. TGFβ, WNT, AND SHH IN NON-CARCINOGENIC FIBROTIC REACTION

Fibrotic reaction affects a number of non-carcinogenic diseases, including systemic sclerosis (SSc), pulmonary fibrosis, renal fibrosis and liver fibrosis. Fibrotic development is characterized by the presence of activated myofibroblasts that produce excess levels of ECM molecules, such as collagen. These molecules then form cross-link structures, leading to tissue remodeling and contraction, which can cause failure of organ function and even death of the patient (73). Although the main symptom of SSc is thickening of skin as a result of collagen accumulation, the disease affects internal organs including the gastrointestinal tract, kidneys, heart, and lungs. Lately, the coordinated action of TGFβ, WNT, and SHH was identified as a major driver of SSc-related resting fibroblast activation to collagen producing myofibroblasts (74-81).

TGFβ signaling is among the most intensively studied causes of fibrosis, employing SMAD-dependent and SMAD-independent pathways that induce the gene expression needed for resting fibroblast activation to myofibroblasts (82, 83). TGFβ-stimulated SMAD3 is highly active in fibrotic tissues. SMAD7, which inhibits SMAD complex action, is downregulated in a ubiquitin E3-ligase-dependent manner. Studies suggesting the correlation between SMAD4 downregulation and attenuated fibrosis development demonstrate the importance of SMAD4 in SMAD2/3 complex nuclear translocation (84). Consequently, SMAD3 promotes fibrotic development by binding directly to collagen gene DNA promoter region and inhibits ECM degradation by inhibiting matrix metalloprotease 1 activity (85-87).

A growing number of recent papers indicate a coordinated action of TGFβ/WNT signaling in the activation of fibrosis (74-77, 88, 89). TGFβ-stimulated activation of canonical WNT signaling enhances the nuclear accumulation of β-catenin, with consequently increased expression of *TCF/Lef* transcription factors, progressive skin fibrotic lesion development, increased dermal thickness, and elevated myofibroblast numbers (75, 90, 91). The exact mechanism underlying TGFβ-mediated WNT signal transduction activation is not completely understood. It has been suggested that TGFβ activates WNT by suppressing the expression of the endogenous WNT inhibitor DKK1 via TGFβ non-canonical p38 MAPK signaling. Another mechanism consists of TGFβ-mediated hypermethylation of DKK1 (74, 92). Both TGFβ and WNT increase the expression of *shh* mRNA in a murine skin fibrosis model that enhanced fibroblast activation and secretion of the extracellular matrix components collagen A1 and collagen A2. The stimulatory role of SHH in the initiation of dermal fibrosis was further supported by data showing SHH over-expression derived dermal fibrosis *in vivo* (80). SHH signal transduction pathway activation has been demonstrated in idiopathic pulmonary fibrosis (IPF), a progressive, lethal lung disease. Interestingly, SHH and GLI2 expression was observed in epithelial cells, whereas PTCH-1, SMO, and GLI1 expression was detected in stromal fibroblasts and inflammatory cells (79).

5. FIBROTIC PATHWAYS AS DRUG TARGETS

These functions of TGFβ, WNT1, and SHH signal transduction pathways in promoting fibrosis and the deleterious effect of fibrosis on normal tissue homeostasis have made them attractive drug targets. The clinical trials registry (https://clinicaltrials.gov) lists 103 clinical trials in which the effect of TGFβ has been studied in patients with different diseases. A recently terminated clinical phase I study (NCT01284322) aimed to treat diffuse systemic sclerosis using fresolimumab anti-TGFβ monoclonal antibody. The drug was administered intravenously in two different doses: 1 mg/kg twice at 1 week and 4 weeks (7 adult patients) or 5 mg/kg once at 1 week (8 adult patients). Fresolimumab treatment resulted in decreased gene expression of *THROMBOSPONDIN-1*, *CARTILAGE OLIGOMERIC PROTEIN*, *SERPINE 1*, and *CCCTC-BINDING FACTOR* (a zinc finger protein), which all are regulated by TGFβ. At the tissue level, there was a significant decrease in myofibroblast migration and in Rodnan skin core, referring to clinical skin disease. The authors concluded that the phase I clinical trial might be a safe alternative for currently used therapeutic protocols, although additional safety studies are needed for long-term treatments (93). In another phase I study, patients with myelofibrosis were treated with 1 mg/kg GC1008 (Fresolimumab). The dose was given every 28 days for a total of six cycles to study the safety, tolerability, clinical response, response to therapy, peripheral blood CD34 cell concentration, and JAK2V617F allele burden. Although only three patients were enrolled, and one died due to non-drug related reasons, the trial showed that

GC1008 at the given doses is a feasible strategy to treat myelofibrosis patients (94). The safety, tolerability, and pharmacokinetics of repeated treatment of patients with early stage diffuse scleroderma has been studied with human an anti-TGFβ1 neutralizing monoclonal antibody, CAT-192, in a clinical phase I/II study (NCT00043706). The study is completed, but the results have not yet been published. The first clinical human dose escalation study in solid tumor patients (NCT02160106) targeting TGFβR1 with TEW-7197 inhibitor causing downregulation of TGFβ signaling is currently is still recruiting patients. Hence, the clinical features of TGFβ1 inhibitors are not yet completely characterized.

The obtained data from TGFβ1 clinical studies have encouraged researchers to initiate more trials utilizing morphogen WNT- or SHH-targeting drugs. The clinical trials registry (https://clinicaltrials.gov) lists 43 studies in which the effect of WNT inhibitors has been characterized in patients. Porcupine WNT974 inhibitor LGK974 is an orally administered small molecule that prevents WNT ligand acetylation, consequently blocking their secretion and activation, reducing aberrant WNT signaling, and inhibition of WNT stimulated tumors. Currently, two ongoing clinical trials are recruiting patients to test the safety and patient tolerability of WNT974. The NCT01351103 clinical trial is a phase I, open-label, dose escalation study recruiting adult patients who have pancreatic adenocarcinoma, *BRAF* mutant colorectal cancer, and other WNT-driven tumors. The study does not include healthy volunteers. The aim of the trial is to evaluate the maximum/recommended dose tolerated by the patients to continue the studies with a larger patient population. Secondary aims include analysis of adverse events, evaluation of pharmacokinetics by determining absorption and plasma concentration of drug, screening of WNT pathway related biomarkers, and growth kinetics of the tumor. The estimated final data collection time point for the study is January 2017.

In another study currently recruiting patients (NCT02278133), WNT974 is studied in combination with LGX818 (encorafenib) and cetuximab (Erbitux, C225) in metastatic colorectal cancer patients with BRAF and WNT pathway mutations. The trial is phase I/II multi-center, open-label study for dose escalation of the combination of WNT974, LGX818, and cetuximab. LGX818 is an orally distributed V600E mutated BRAF-targeting small molecule, and cetuximab is a monoclonal antibody targeting EGFR. The main aims of the study are to characterize the dose limiting toxicities of the drugs and the overall response rate for the combination therapy. The secondary aims include analysis of overall survival, duration and time to response, progression free survival, disease control rate, plasma concentration of drugs, adverse effects, and WNT or RTK-MAPK pathway biomarker activation. The estimated closing date for the trial is June 2017.

Thus far, there are 43 (18 completed, 8 recruiting, and 8 studies with unknown status or not recruiting yet) clinical trials in which SHH inhibition has been studied in adult patients with advanced/metastatic solid tumor development. Trial number NCT01160250 resulted in an approved clinical treatment protocol for marketing. The aim of the phase IV clinical trial was to test the effect of vismodegib (GDC-0449, Erivedge), an antagonist of the SMO receptor, in 119 adult basal cell carcinoma patients with locally advanced disease (62 patients) and with metastasis (57 patients). The patients received 150 mg of vismodegib daily for 0.4.-19.6. months. Patients were assessed at treatment-associated clinical visits for adverse effects, concomitant medications, performance status, weight, blood values, metabolic values, and heart performance (electrocardiography). Most patients experienced mild adverse effects (grade 1 and 2), although 24 patients had grade 3, nine patients had grade 4, and 2 patients had grade 5 (death) effects. The listed common adverse effects include muscle spasm, dysgeusia, alopecia, diarrhea, nausea, fatigue, and weight loss. The outcome of the study was encouraging. Eight patients (6 with locally advanced disease and 2 with metastasis) showed complete response to therapy, 30 patients (20 with locally advanced disease and 10 with metastasis) had partial response, and 47 patients experienced stable disease (95).

Clinical phase I trial NCT01286467 was designed to clarify the role of the hedgehog inhibitor PF-04449913 administered orally. The aim of the work was to estimate the dose of the drug in 28-day cycles. No data have been published yet. The second completed phase I clinical trial, NCT00953758, with hedgehog inhibitor PF-04449913 recruited adult patients with hematologic malignancies that were refractory, resistant, or intolerant to prior therapies. The aims of the study were the same as in NCT01286467, to estimate the dose of the drug in 28-day cycles. The third completed SHH inhibition phase II trial NCT00980343 testing the SHH antagonist GDC-0449 was a multicenter study conducted in collaboration with eight institutes in the USA and was sponsored by NIH/NCI. The trial aimed to record progression free survival, survival times, response to therapy rate, toxicity incidence, appearance of CD133 neurospheres, and expression of SHH pathway biomarkers.

The effect of SHH inhibition with the combination of vismodegib (150 mg/day) and temozolomide (150-200 mg/m2) will be studied in a phase I/II clinical trial NCT01601184 that is currently recruiting adult patients with relapsing or refractory medulloblastoma. The aims of the phase I section include the evaluation of the safety of vismodegib-temozolomide combination with given doses and the evaluation of the response to therapy to initiate the phase II trial. The focus of the phase II portion of the trial is to register the response rate and length, progression free survival, and time to possible treatment

failure. The clinical trial is a multicenter study recruiting patients in nine hospitals in France, two hospitals in Italy, two hospitals in Switzerland, and one hospital in the United Kingdom.

6. CONCLUSIONS AND FUTURE ASPECTS

Cancer should no longer be viewed as the result of disorganized growth, but instead as a well-organized (organ-like) biological system. Therefore, therapeutic advances should be designed based on personalized medicine that targets both autocrine and paracrine tumor growth-supporting mechanisms. The role of the stroma in cancer development is still controversial and largely uncharacterized. The success/failure of the clinical trials targeting stroma may be linked to the idea of obtaining a global stromal depletion more than to a selective disruption of the desmoplastic components. Dense, poorly vascularized stroma may support cancer growth and progression and protect epithelial cancer cells from drug delivery. Therefore, its depletion would be beneficial in cancer therapy (96). On the other hand, vascularized stroma may enable immune cells to attack the tumor, and the depletion of the stroma could deteriorate the endogenous immune defense mechanisms (71, 97, 98).

Although fibrotic diseases arise from several different stimuli, the activation of resting fibroblasts to myofibroblasts with consequent cytokine secretion is a common aspect in the development of tumor stroma and in the progression of tissue fibrosis, which creates a platform to develop stroma-targeting drugs for incurable fibrotic diseases. Studies focusing on recently introduced “fibrotic signature” genes (99-105) have demonstrated novel connections and clarified the progression of fibrosis. Further studies are needed to fully characterize the function of the identified genes and microRNAs in preclinical models before proceeding to phase I/II clinical studies. The abovementioned TGFβ, WNT, and SHH signaling, first described in cancer, are currently being tested in clinical trials with fibrosis and fibrotic cancer patients. However, further clarifications of stromal mechanisms, fibroblast differentiation, and ECM modifications caused by aberrations in proteostasis (106) might reveal novel clinical diagnostic methods, druggable targets, and therapeutic protocols to improve the physiological functions of fibrosis-damaged tissues.

7. ACKNOWLEDGEMENTS

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Abbreviations: ADAMTS, A Disintegrin and Metalloproteinase with Thrombospondin motifs; AKT, v-akt murine thymoma viral oncogene homolog; APC, adenomatous polyposis coli; BCL2, B-cell CLL/lymphoma 2; Ca, calcium; CDK, cyclin-dependent kinase; c-Jun, jun Proto-Oncogene; CK1, casein kinase 1; COS, scaffold protein costal CREB, cAMP response element-binding protein; DKK1, dickkopf WNT signaling pathway inhibitor 1; ECM, extracellular matrix; EGFR; epidermal growth factor receptor; EMT, epithelial mesenchymal transition; ErbB, erythroblastic leukemia viral oncogene; ERK, extracellular signal-regulated kinase; FZD, frizzled; FGF-R, fibroblast growth factor receptor; Fu, fused; GLI, glioma-associated oncogene; GRP, G protein-coupled receptor; HIF, hypoxia inducible factor; JNK, jun N-terminal kinase; MAPK, mitogen activated protein kinase; MEK, MAPK/ERK kinase; MH, mad homology domain; MYC, myc Avian Myelocytomatosis Viral Oncogene Homolog; NANOG, nanog homeobox; p42/p44, ERK1/2; PDGF, platelet derived growth factor; PKA, protein kinase A; PKC, protein kinase C; PI3K, phosphoinositide-3-kinase; PLC, phospholipase C; PTCH, protein patch homolog RAF, raf proto-oncogene, serine/threonine kinase; RAS, rat sarcoma viral oncogene homolog; SHARP-1, Enhancer-of-split and hairy-related protein 1; SHH, sonic hedgehog; SMO, smoothened; SNAIL, snail homolog 1, a zinc finger protein; SSc, systemic sclerosis; SUFU, suppressor of fused homolog; TCF/LEF, T-cell factor/lymphoid enhancer-binding factor; TGFβ, transforming growth factor beta; TGFβR, transforming growth factor beta receptor; Wnt, int/Wingless;

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