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HOMEOSTASIS OF EXTRACELLULAR MATRIX BY TGF-BETA AND LEFTY

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1. ABSTRACT

Both normal and neoplastic tissues have a stroma comprised of fibroblasts which deposit an extracellular matrix (ECM) enriched in collagen. In most normal tissues, the synthesis and breakdown of the ECM is maintained at a low level. However, in normal adult tissues such as endometrium, and in rapidly growing embryonic and neoplastic tissues, there is a significant increase in the synthesis and/or breakdown of ECM. The homeostasis of the ECM is maintained by a molecular repertoire which appears to consist of TGF-beta and lefty. TGF-beta acts as a pro-fibrogenic cytokine by increasing the synthesis of collagen and decreasing the degradation of ECM. Physiologic levels of TGF-beta maintains tissue homeostasis and aberrant over-expression of TGF-beta leads to tissue fibrosis. TGF-beta acts through a core Smad signaling pathway which is initiated by the binding of homo-dimeric TGF-beta protein to two type I and II receptors. The constitutively active receptor type II leads to phosphorylation of receptor type I which, in turn, causes the R-Smads to get phosphorylated. The downstream gene transcriptional activity of this event includes significant increase in connective tissue growth factor (CTGF) and collagen mRNA synthesis which leads to deposition of collagen in tissues. Lefty inhibits Smad2 phosphorylation initiated by TGF-beta or its receptor and prevents CTGF promoter activity driven by TGF-beta. Moreover, lefty inhibits CTGF and collagen mRNA synthesis and increases collagenolysis and elastolysis and as a result of these actions, lefty significantly reduces the amount of collagen deposited in tissues. Thus, TGF-beta and lefty might coordinately participate in the homeostasis of ECM in tissues.

2. INTRODUCTION

All tissues are composed of two distinct but interdependent compartments, the parenchymal cells and a supporting fibrovascular meshwork. In the adult tissues, fibroblasts are quiescent but during tissue injury, in response to a number of pro-fibrogenic cytokines, fibroblasts become activated, grow, divide and lay down a collagen rich stroma that contributes to the healing process of the damaged tissues. This type of activation is self limiting and after the integrity of the damaged tissues is restored, the fibroblasts return to their quiescent state. However, under some diseased conditions, for reasons that are not yet clear, the fibroblasts become activated and continue to divide and synthesize collagen. This type of unabated activation of fibroblasts causes a number of diseases of unknown etiology which are collectively called fibromatosis. The fibroblasts also contribute to tumor growth by supporting the formation of its stroma. Fibrotic diseases, fibromatosis, and

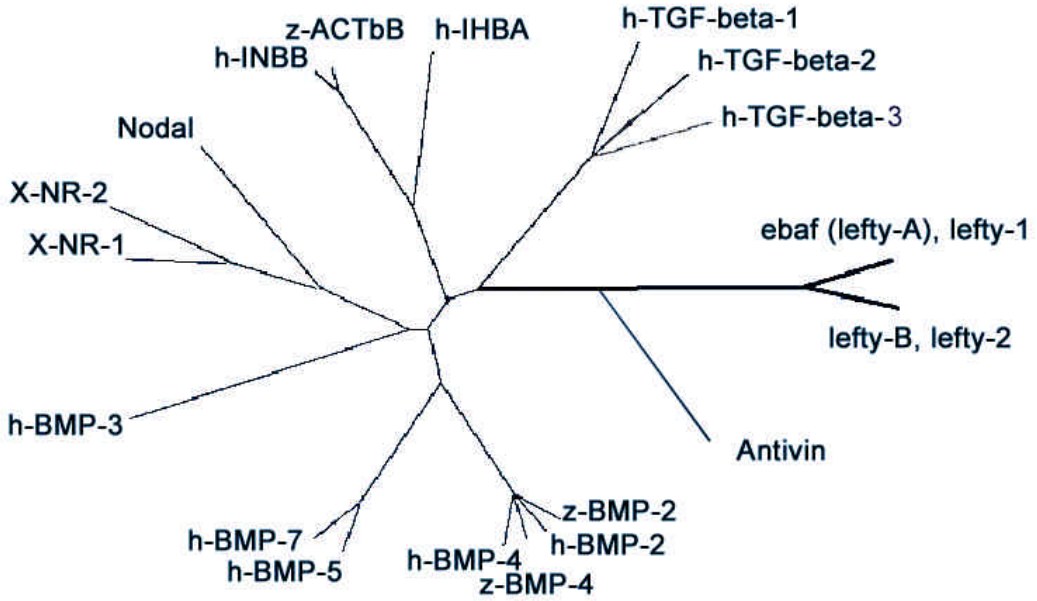

Figure 1. Dendrogram of TGF-beta superfamily. Major families of TGF-beta superfamily include TGF-beta, Activin-inhibin, Nodal, BMP and lefty.

neoplasias cause significant morbidity and mortality and as yet, there is no effective treatment for these diseases. For this reason, there is a great interest in identifying factors that control fibroblast growth and activation with the hope that fibromatosis, other type of undesirable fibrosis and neoplasias can all be effectively controlled.

3. TGF-BETA SUPERFAMILY

Transforming growth factor beta is the prototype of a superfamily of molecules which is comprised of more than 30 related proteins (Figure 1). In mammals, these include three isoforms (beta1, beta 2, beta 3) of TGF-beta, three isotypes of activins, nearly 20 isoforms of bone morphogenetic proteins (BMP), and the newly discovered lefty family (1-4). Lefty family consists of lefty-1 and lefty 2 in mouse, lefty A and lefty B in humans and antivin in *Xenopus*. Lefty genes have been localized to the chromosome 1 (1q42.1) both in mouse and man (1-4).

The deduced amino acid sequence of lefty shows a great amount of identity and similarity with the known members of the TGF-beta?superfamily (1). A motif search revealed that the predicted lefty protein contains most of the conserved cysteine residues of the TGF-beta related proteins (1) which are necessary for the formation of the cysteine knot structure (5-6). The lefty sequence contains an additional cysteine residue, 12 amino acids upstream from the first conserved cysteine residue. The only other family members, known to contain an additional cysteine residue, are TGF-beta, inhibins and GDF-3 (1). For simplicity, in this manuscript, we will refer to lefty A (ebaf) as lefty. Most of these proteins are produced as precursors which are cleaved to release the C-terminus monomeric proteins. The pro-form of TGF-beta, is cleaved intracellularly by the endopeptidase, furin of the convertase family of molecules at a single RXXR site. This enzymatic digestion releases a 12.5 kD C-terminus protein and a 75 kD N-terminal portion (latency-associated peptide, LAP) (Figure 2). In contrast to TGF-beta, lefty has two RXXR cleavage sites which leads to the secretion of two cleaved products of 28 and 34 kD proteins and is not cleaved by furin (7). Cleavage at the RGKR cleavage site appears to be mediated by PC5A but the enzyme that cleaves lefty at its second cleavage site, RHGR, has not yet been identified (7) (Figure 2).

TGF-beta family members have a characteristic signature motif which is comprised of a series of seven cysteine residues (Figure 3). All of these residues are used for the formation of intra-peptide bonds with the exception of the third cysteine residue from the C-terminus of mature protein. Active ligands are formed by the formation of disulfide linked bonds between this third cysteine residues at the C-terminus of the proteins (8-9)(Figure 4). Lefty, similar to GDF-3/Vgr2 and GDF-9, lacks the cysteine residue necessary for the formation of intermolecular disulfide bond (1) (Figure 4). Whereas the carboxy terminus of the TGF-beta family is usually CX1CX1, lefty has a longer C terminal sequence, CX1CX19 (1). Therefore, lefty appears to be an additional member of the TGF-beta superfamily with an unpaired cysteine residue which not exist as a dimer.

4. ROLE OF TGF-BETA IN HOMEOSTASIS OF ECM

Growth regulatory molecules of the transforming growth factor-beta family (TGF-beta) are one of the few classes of proteins that provide the necessary signals that

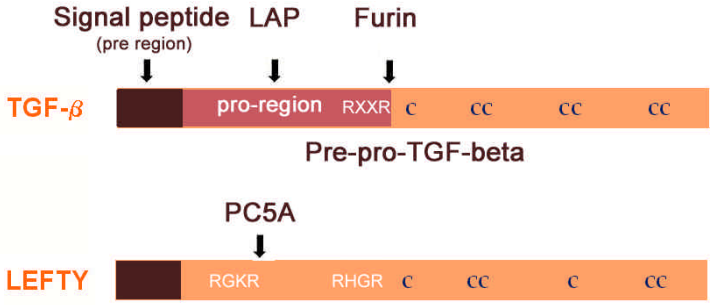

Figure 2. RXXR cleavage sites in TGF-beta and lefty. Both TGF-beta and lefty have signal peptide sequences and are secreted proteins. The processed form of TGF-beta is released by cleavage at a single cleavage site (RXXR). Furin is the convertase that cleaves TGF-beta at this cleavage site. In contrast, there are two such cleavage sites (RGKR and RHGR) in the lefty proteins, which causes release of two secreted proteins of 28 and 34 kD. PC5A cleaves lefty at RGKR site. The enzyme that cleaves lefty at the RHGR site has not yet been identified.

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TGF-β1          CCVRQLYIDFRKDLGW--KWIHEPKGYHANFCLGPCPYI---WSLDTQYSKVLALYNQH-----NPGASAAPCCVPQALEPLPIVYYVGRKPKV-EQL---SNMIVRSCKC--S
MIS             CALRELSVDLRAE-----RSVLIPETYQANNCQGVCGWPQSDR----NPRYGNHVVLLLKMQARGAALARPPCCVPTAYAG-KLLISLSEERISAHHV---PNMVATECGC--R
BMP-7           CKKHELYVSFR-DLGW-QDWIIAPEGYAAYYCEGECAFPLNSYMNATNHAIVQTL-----VHFINPETVPKPCCAPTQLNAISVLYFDDSSNVILKKY---RNMVVRACGC--H
INH-β A         CCKKQFFVSFK-DIGW-NDWIIAPSGYHANYCEGECPSHIAG-TSGSSLSFHSTVINHYRMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNIIKKDI---QNMIVEECGC--S
GDF-3           CHRHQLFINFQ-DLGW-HKWVIAPKGFMANYCHGECPFSMTTYLNSSNYAFMQAL-----MHMADPK-VPKAVCVPTKLSPISMLYQDSDKNVILRHY---EDMVVDECGC--G
GDF-9           CELHDFRLSFS-QLKW-DNWIVAPHRYNPRYCKGDCPRAVRHRYGSPVHTMVQNII--Y--EKLDP-SVPRPSCVPGKYSPLSVLTIEPDGSIAYKEY---EDMIATRCTC--R
lefty-1         CCRQEMYLDLQ-GMKWAENWILEPPGLFTYECVGSC----------------LQLPESLTSRWPFLGPRQ-CVASEMTSLPMIVSVKEGGRTRPQVVSLPNMRVQTCSCASD
ebaf (lefty-A)  CCRQEMYIDLQ-GMKWAKNWVLEPPGFLAYECVGTC----------------QQPPEALAFNWPFLGPRQ-CIASETASLPMIVSIKEGGRTRPQVVSLPNMRVQKCSCASD
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Figure 3. Signature motif of TGF-beta superfamily. The signature motif of TGF-beta family is comprised of a series of seven cysteine residues (boxed). Lefty and GDF are the only protein members that lack the third cysteine residue from the C terminus which leads to a dimerized form of the protein.

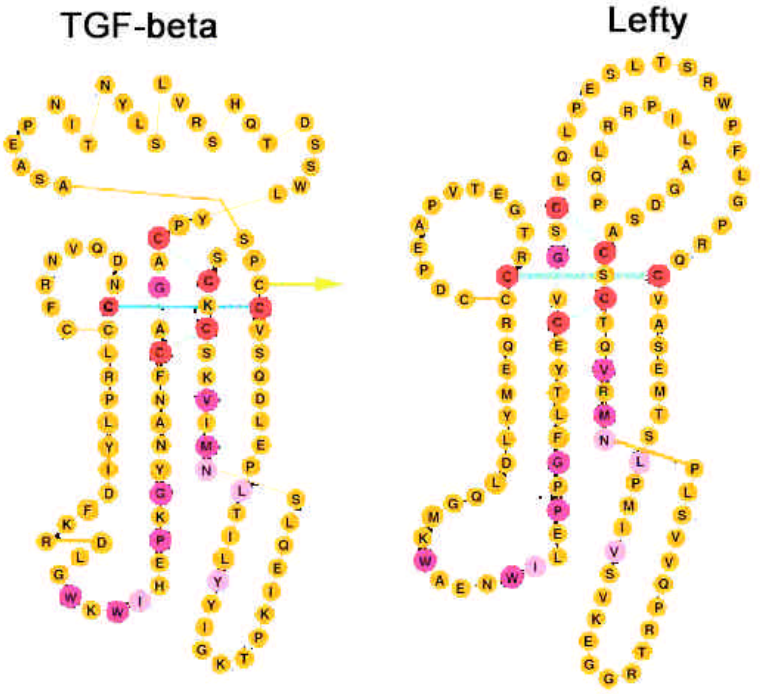

Figure 4. Dimerization of TGF-beta. The two dimensional structure of TGF-beta and a hypothetical 2-D structure of lefty are displayed. TGF-beta monomers dimerize by formation of a disulfide linked bond formed between the third cysteine residues at the C-termini of two monomeric proteins (arrow). Lefty lacks this residue and therefore remains as a monomeric protein.

Table 1. Implication of TGF-beta in normal wound healing and pathologic fibrosis

Tissue/Condition	Reference
Lung	10-14
Pancreas	15
Kidney	16
Liver	17
Wound healing-Scar	18-23
Hypertrophic scar, Keloid	24-27
Body burn	28
Radiation	29
Drugs	30-31
Transplantation	32
Scleroderma	33
Sarcoidosis	34
Fibromatosis	35-36
Idiopathic pulmonary fibrosis	37
Liver fibrosis	38-39

support the formation of a fibrovascular stroma. The available evidence shows that generally, there is a marked increase in the expression of TGF-beta mRNA and protein during physiologic wound repair and in fibrotic disorders, fibromatosis and neoplastic diseases (Table 1)(10-41). TGF-beta supports growth of stroma by increased proliferation of fibroblasts and enhancement of collagen deposition. This view is supported by the strong evidence that TGF-beta increases collagen mRNA synthesis and prevents the degradation of collagen by suppression of specific matrix metalloproteases (MMP) that digest collagen (42-43).

5. TGF-BETA AS A PRIME MEDIATOR OF FIBROSIS

TGF-beta is considered a fibrogenic cytokine that contributes to fibrosis. A large number of studies, performed in different experimental conditions, support the idea that TGF-beta increases fibroblast proliferation and deposition of extracellular matrix both in physiologic settings and pathologic conditions (40, 44-50). Essentially, there are three lines of evidence that incriminate TGF-beta in fibrosis. These include, increased expression of TGF-beta in fibrotic tissues, demonstration that direct administration of TGF-beta or its over-expression leads to fibrosis and the evidence that inhibition of TGF-beta actions inhibit fibrosis.

TGF-beta has been implicated *in vitro* (51), and in *in vivo* in normal wound healing, as well as pathologic conditions shown in table 1. A number of studies show a correlation between the expression of TGF-beta, its downstream factor, CTGF, and the expression of extracellular matrix components in conditions associated with fibrotic reactions (48-52). Elevated levels of TGF-beta are found in patients with fibrotic diseases and experimental animals with hepatic fibrosis and cirrhosis. In the liver, the activation of hepatic stellate cells, which are akin to fibroblasts, is the key initiating event in hepatic fibrogenesis (17). A high level of expression of TGF-beta is observed in the liver in congenital liver fibrosis, as well as in *in vivo* models of fibrosis induced by alcohol, iron and allylalcohol (37-38). In normal liver, low levels of TGF beta transcripts are present in some portal tract stromal cells. In fibrotic livers, high TGF beta RNA levels are maintained in hepatic stellate cells, mesenchymal liver cells, inflammatory cells, and in bile duct epithelial cells (53-55).

In a wound repair model, administration of TGF-beta, led to the enhanced mRNA content of collagen types I and III and fibronectin in the granulation tissue but decreased the expression of stromelysin mRNA which degrades collagen (56). Injection of TGF-beta or over-expression of TGF-beta in transgenic mice is associated with fibroplasia and deposition of extracellular matrix in major body organs including liver, kidney, heart and pancreas (50, 57-60). Adenovector-mediated gene transfer of active transforming growth factor-beta 1 induces prolonged severe fibrosis in the rat lung (61) and co-injection of CTGF or bFGF with TGF-beta enhances and prolongs the fibrotic response *in vivo* (59,62).

Inhibition of the TGF-beta activities by a number of experimental approaches such as administration of soluble betaglycan, anti-sense TGF-beta, and antibody to TGF-beta, all lead to decreases accumulation of extracellular matrix both *in vitro* and *in vivo* in kidney (63-66). The role of TGF-beta in fibrosis has been demonstrated in experimental forms of fibrosis including those induced by bleomycin and carbon tetrachloride (CCl_4) (67-78).

5.1. Bleomycin induced lung fibrosis

TGF-beta plays a prime role in the fibrotic response in bleomycin induced lung fibrosis (67-68). TGF-beta levels increase by 2.6- to 4.5-fold in bleomycin treated lungs (69) and this event is associated with increased expression of CTGF, a mediator of collagen synthesis (70). The level of TGF-beta mRNA increases rapidly, peaks on day 5, and precedes the five to sevenfold increase in levels of mRNAs for pro-collagens alpha 1(I) and alpha 1(III) which peak 10 days after bleomycin administration. The peak levels of these mRNAs in bleomycin-treated animals are higher than those of the control. In normal animals, TGF-beta is elaborated at high levels during the development of fibroproliferative lung disease but its expression is reduced in TNF-alpha receptor knockout mice which are protected from the fibrogenic effect of bleomycin. Overexpression of TGF-beta by means of a replication-deficient adenovirus vector induces fibrogenesis in the lungs of the fibrogenic-resistant TNF-alpha receptor knockout mice (71). The extent of fibrosis of the lung induced by bleomycin can be significantly reduced by Smad7, an antagonist of TGF-beta signaling (72), soluble TGF-beta receptor (73), antibodies to TGF-beta (74-75), anti-fibrotic drug, pirfenidone, which down-regulates the bleomycin induced overexpression of TGF-beta gene in the lungs (69), and by a single-stranded 27-mer phosphorothioate oligodeoxynucleotide (ssPT) containing the TGF-beta response element (67). Collectively, these data show that TGF-beta is a prime mediator of fibrosis in bleomycin induced lung fibrosis.

5.2. Carbon tetrachloride induced liver fibrosis

Plasma level of TGF-beta increase as early as 24 hours after administration of carbon tetrachloride and are maximal by 48 hours after CCl_4 administration (76). The

collagen alpha1(I) mRNA is increased 10-fold in CCl_4 treated wild-type mice compared to the untreated controls. The increase in collagen mRNA expression is reduced by about 80% in the TGF-beta knockout mice (77). Moreover, over-expression of TGF-beta by an adenoviral vector leads to 14-fold higher hepatic TGF-beta protein levels. This is associated with 15-fold higher collagen alpha1(I) mRNA levels than the levels reached in the control mice (77). Induction of expression of collagen type I is mediated by Smad3, a prime mediator of TGF-beta signaling (78). Seventy two hours after receiving a single intragastric administration of CCl_4, in Smad3 heterozygous or Smad3 homozygous knockout mice, the hepatic collagen mRNA levels are only 42% and 64%, of those in wild type control mice (78).

Collectively, these data demonstrate that TGF-beta and its signaling pathway play a prime role in fibrosis induced by bleomycin and CCl_4 as well as other fibrotic diseases and that inhibition of TGF-beta activity is expected to lead to decreased fibroplasia and desmoplasia *in vivo*.

6. ROLE OF TGF-BETA IN TUMOR PROGRESSION

It is currently thought that the cancer cell genotype is the manifestation of six essential alterations that collectively dictate malignancy; self sufficiency in growth, insensitivity to growth inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (79). However, strategies to treat cancer by curbing or perturbing one or several of these cancer cell attributes have failed to induce dramatic improvement in the treatment of human cancers. It is becoming increasingly clear that cancer treatment requires a better understanding of the contribution of ancillary cells such as fibroblasts, endothelial cells and tumor infiltrating leukocytes in the tumor stroma.

Solid tumors are composed of two distinct but interdependent compartments, the malignant cells and a supporting fibrovascular meshwork. Fibroblasts in the stroma, provide the physical framework for tumor growth and the supporting vasculature provides the necessary nutrients, gas exchange and waste disposal. The formation of tumor stroma is essential to tumor growth. The tumor stroma, does not simply provide a scaffold for growth of the tumor, rather it relays important signals to cancer cells. By anchoring themselves to extracellular matrix components, cancer cells can switch the type of the extracellular matrix receptors (integrins) that they display, favoring ones that convey pro-growth signals (79). The failure of the integrins to establish these extracellular links, impairs cell motility, induces cell cycle arrest or leads to apoptosis (79). It has been shown that fibroblasts in the stroma convey signals that modulate the growth and morphogenesis of tumors (80-81). Moreover, co-injection of human fibroblasts with human carcinoma cells into athymic nude mice, results in an increased rate of take and growth of tumors (81). Thus, impairing the formation of stroma might dramatically reduce the ability of tumors to reach a significant size.

Tumor stroma is comprised of a number of extracellular matrix (ECM) proteins. Collagen is one of the most major structural components of the tumor stroma. Formation of the tumor stroma is invariably associated with deposition of collagen. In some tumors, such as breast, ovarian, liver, pancreas, and gastric cancers, de-regulated collagen synthesis leads to a stroma rich in collagen, a process known as desmoplasia (82-86). In some desmoplastic tumors, such as breast and gastric carcinomas, up to 80% of the total tumor mass consists of tumor stroma (87).

The induction of the tumor stroma is regulated by a number of stimulatory and inhibitory factors. However, under normal circumstances, the effect of stimulators supersedes the effect of negative signals leading to progressive formation of the tumor stroma. Growth regulatory proteins of the TGF-beta are one of the few classes of proteins that provide the signal required both for formation of fibro-collagenous framework of the tumor and its vascular network. TGF-beta plays diverse and paradoxical roles in carcinogenesis and tumor progression. The current operative hypothesis is that, due to their potent inhibitory influence on epithelial cell growth and immunosuppressive activities, TGF-beta and molecules of the Smad mediated signaling pathway act as anti-cancer defense mechanisms (40, 44, 47). Despite this apparent potent tumor suppressor activity at early stages of carcinogenesis, TGF-beta acts as a tumor promoter at late stages of tumor development (88). It is increasingly becoming clear that, in later stages of tumorigenesis, the response of the tumor cells to TGF-beta is perturbed due to mutational inactivation of the signaling molecules of the TGF-beta or over-expression of the proteins that actively suppress TGF-beta responses such as Smad6 and Smad7 (89-90). These mutations have been described in a large number of human cancers arising from ovary, endometrium, prostate, GI tract malignancies as well as hematological, hepatic and thyroid cancers (40).

The contribution of TGF-beta to tumor aggressiveness has been attributed mainly to its action in providing support to the development of tumor stroma by inducing fibroblast proliferation and deposition of extracellular matrix components, to its ability to promote angiogenesis and to act as a potent immunosuppressor (40, 44 45, 47). TGF-beta and its receptors (TGF-beta RI, RII and endoglin) have recognized roles in vasculogenesis, vascular assembly and in the establishment and maintenance of vessel wall integrity (91-92). Moreover, TGF-beta leads to the proliferation of fibroblasts and collagen deposition as well as angiogenesis, both required for the formation of tumor stroma (44 31, 51, 93). These effects of TGF-beta appear to depend on induction of two factors that are essential to fibroblast proliferation and collagen formation, fibroblast growth factor (FGF) and connective tissue growth factor (CTGF) (94-96). By virtue of these functions, TGF-beta promotes late stage tumor development (40,44,47). Moreover, TGF-beta acts as an immunosuppressor and enhances tumor invasion and metastasis (40,44,47).

The available evidence shows that generally, there is a marked increase in the expression of TGF-beta

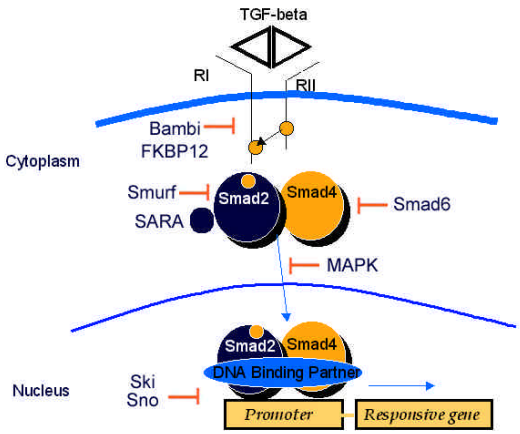

Figure 5. TGF-beta signaling pathway. Binding of a TGF-beta family member to the type II and type I receptor leads to phosphorylation of the type I receptor. The activated type I receptor phosphorylates a receptor-regulated SMAD (R-Smad). Following this phosphorylation, the R-Smad dissociates from the receptor and binds to a common Smad (Smad4). This heterodimer then moves into the nucleus where it associates with a DNA-binding partner, such as Fast1 activating transcription.

mRNA and protein in human cancers including those derived from pancreas, colon, prostate, stomach, lung, endometrium, breast, brain, and bone (40,44,47). The underlying basis for up-regulation of TGF-beta in human tumors remains unknown. However, this over-expression appears to have several major effects including fibrosis of stroma, as well as enhancement of invasion and metastasis. There is a close correlation between the expression of TGF-beta and the extent of fibrosis in human cancers that exhibit central fibrosis (52). Experimental evidence shows that the enhanced TGF-beta expression in cancer cells accounts for the stromal fibrosis. For example, over-expression of TGF-beta in PANC-1 pancreatic carcinoma cells leads to desmoplasia (over-proliferation of fibrotic tissue) (97) of the tumor stroma and accelerates the tumor growth (40).

Progressive loss of growth inhibitory response to TGF-beta correlates well with the malignant stage of certain forms of cancer such as colon carcinoma and glioblastoma multiforme (40). In many cancers, high expression of TGF-beta is seen in the more advanced stages of malignancy and is associated with decreased survival. Expression of TGF-beta isoforms in cancers of breast (98-99), colon (100), bladder (101) and prostate (102) carcinomas as well as melanomas (103) have all been associated with tumor progression.

It appears that when the tumor cells lose their sensitivity to TGF-beta growth inhibition, the excess TGF-beta that results facilitates invasion and metastasis (40,44,47). Various experimental evidence support the idea that TGF-beta promotes tumorigenesis and tumor aggressiveness and enhances late stage tumor progression in a number of model systems (40,44,47). Constitutive expression of mature transforming growth factor beta1 in the liver accelerates hepatocarcinogenesis in transgenic mice (104-105) and enhances invasion and metastasis of TGF-beta resistant tumor cells (47, 106). Since the anti-proliferative action of TGF-beta on the tumor cells is commonly abrogated by mutation or dysfunction of its signaling molecules, it is thought that TGF-beta produced by the tumor cells acts in a paracrine fashion on the tumor stroma to enhance tumorigenesis (50, 107). In some instances, however, the tumor stroma is the source of TGF-beta (108). Because of these functions, inhibition of TGF-beta, which is produced in large quantities in various human cancers, might perturb the formation of the tumor stroma to the extent that it can suppress or can even stop further tumor growth and prevent invasion and metastasis.

7. ROLE OF CTGF IN HOMEOSTASIS OF ECM

Among the positive signals, TGF-beta increases the extracellular matrix accumulation by signaling along the Smad pathway and by inducing connective tissue growth factor (CTGF).

CTGF, a member of the CCN (CTGF/Cyr61/NOV) family of growth regulators, is a secreted cysteine-rich, heparin binding, 38 kD protein. CTGF is considered to be an immediate early growth responsive gene and a downstream mediator of TGF-beta actions in fibroblasts (109). CTGF induces chemotaxis in mesenchymal cells and promotes proliferation of fibroblasts and collagen synthesis in a number of *in vitro* and *in vivo* models (110-12). Moreover, because of over-expression in human cancers, CTGF is considered to be part of the molecular pathways that lead to the formation of tumor stroma by TGF-beta (113-117). CTGF appears to be a mediator of fibrotic reactions in a host of fibro-proliferative diseases and in the paraneoplastic condition, pseudo-scleroderma, which develops in some patients with lung cancer (115). CTGF induces proliferation of fibroblasts and collagen synthesis, in a number of *in vitro* and *in vivo* models (110-112,117-118). Thus, expression of CTGF in wound healing and fibrotic diseases is considered to be part of the molecular pathways that lead to the formation of stroma by TGF-beta (119).

8. TGF-BETA SIGNALING

To exert its functions, TGF-beta brings together two transmembrane serine/threonine kinases, the type I and II receptors. The assembly and oligomerization of TGF-beta receptors leads to phosphorylation of receptor-regulated Smads (R-Smad), heterodimerization of the R-Smads with Smad4 and subsequent nuclear accumulation of these complexes (Figure 5). Activated Smad complexes interact with other transcription factors in the nucleus, bind to DNA by their NH2-terminal Mad homology-1 (MH1) domains, and activate transcription of TGF-beta responsive genes through the COOH- terminal MH2 domains (120).

9. REGULATION OF TGF-BETA ACTIONS

The actions of TGF-beta are regulated by feedback mechanisms since the TGF-beta expression and collagen

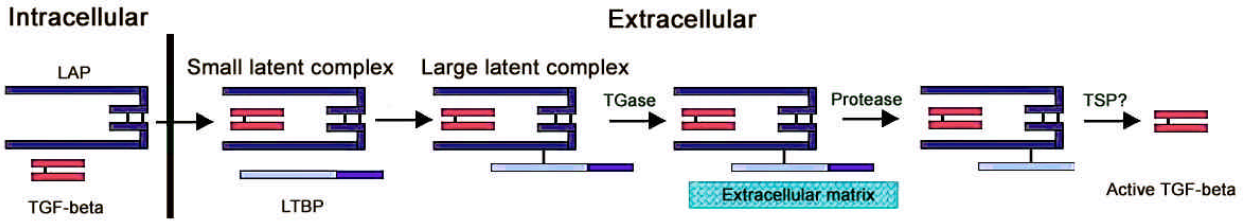

Figure 6. Mechanisms regulating biologic activity of TGF-beta. During secretion, a small latent, biologically inactive TGF-beta complex is formed by the non-covalent association of LAP and mature TGF-beta. This complex, binds to LTBPs. This association facilitates TGF-beta secretion and promotes its fixation to the ECM by a transglutaminase (TG) dependent mechanism.

deposition ultimately cease after the wound is healed and tissue damage is repaired. However, in a number conditions such as keloid, fibromatosis, idiopathic pulmonary fibrosis, and scleroderma, collagen synthesis is de-regulated. In such conditions, the de-regulated collagen formation leads to a stroma rich in collagen (121). For this reason, there is a great interest in identifying the controlling feedback mechanisms for TGF-beta action that selectively inhibit the action of TGF-beta on accumulation of extracellular matrix proteins. Such knowledge would be important in understanding how to control the formation of stroma during wound healing and in fibrotic conditions.

TGF-beta is a potent pleiotropic cytokine. For this reason, its actions are exquisitely controlled at multiple levels. The first level of control is exerted as TGF-beta is secreted. During secretion, a small latent, biologically inactive TGF-ß complex is formed by the non-covalent association of LAP and mature TGF-beta (Figure 6)(2). This complex, in turn, gets linked by disulfide bonds to one of four isoforms of latent TGF-beta binding proteins (LTBP). LTBPs, facilitate TGF-beta secretion and promote its fixation to the ECM by a transglutaminase (TG) dependent mechanism (Figure 6). This form of TGF- beta acts as a reservoir. TGF-beta can be released from the ECM by proteinases, most notably the plasminogen-plasminogen activator-plasminogen activator inhibitor system. The function of the Smad transcriptional regulators of TGF-beta function is controlled by a number of factors that either enhance or suppress the transcriptional output (Figure 5)(120). The membrane-anchored proteoglycan betaglycan which is also known as the TGF-beta type III receptor by binding to TGF-beta increases its affinity for the signaling receptors. The protein, *S*mad *A*nchor for *R*eceptor *A*ctivation (SARA), binds Smad2 and Smad3, and facilitates their interaction with TGF-beta receptors.

Among the negative regulators are Smad partners which are required to prevent the inappropriate activation of TGF-beta signaling, or turn off the signaling pathway following normal activation. Two Smads, Smad6 and Smad7, inhibit TGF-beta signaling. Smad6 by binding to receptor-activated Smad1, forms Smad1-Smad6 complex which appears to be inactive while a second inhibitory Smad, Smad7, stably binds to activated TGF-beta type I receptor, blocking the association, phosphorylation, and activation of Smad2 (120). The immunophilin, FKBP12, binds to the GS domain of the receptor I, and prevent it from ligand-independent receptor phosphorylation. BAMBI acts as a pseudo-receptor and prevents signaling by forming inactive dimers with type I receptor. TGF-beta dependent interaction of Smad2, 3, and 4 with Ski and Sno results in transcriptional repression of several different Smad responsive promoters (122-126). Moreover, calmodulin, the primary mediator of calcium signaling physically interacts with R-Smads and Co-Smads *in vitro*, and inhibits Smad mediated trans-activation of multiple TGF-beta responsive promoters. The classical mitogen-activated protein kinase (MAPK) pathway has also been implicated in both positive and negative regulation of TGF-beta signaling (127-129). Erk kinases which are activated via the Ras pathway phosphorylate R-Smads in the linker region, and inhibit nuclear accumulation of the Smad complex. The Smad1 ubiquitin ligase, Smurf1, regulates the basal levels of Smad1. The function of Smad2 is terminated by ubiquitylation which leads effectively to the degradation of the protein. The cross-talk among different signaling pathways also either specify, enhance, or inhibit TGF-beta responses. For example, IFN-gamma inhibits TGF-beta signaling by direct STAT mediated transcriptional induction of Smad7 (130).

10. LEFTY IS AN INHIBITOR OF TGF-BETA SIGNALING

Lefty and its related proteins are poised to act as inhibitors of TGF-beta family members (3, 131-133). Thus, it can be hypothesized that lefty might cause extracellular matrix remodeling by inhibiting TGF-beta actions. A number of carefully executed studies have shown the validity of this hypothesis (134). Lefty perturbs the TGF-beta signaling by inhibiting the phosphorylation of Smad2 following activation of the TGF-beta receptor (134). Moreover, lefty inhibits the events which lie downstream from R-Smad phosphorylation including hetero-dimerization of R-Smads with Smad4 and nuclear translocation of R-Smad-Smad4 complex. Lefty opposes the effect of TGF-beta on the expression of reporter genes for major cell cycle factors p21, and Cdc25. Smad3 and Smad4, both have domains that bind the 5'-TCTGAGAC-3' termed Smad Binding Element or (SBE). Lefty inhibits the TGF-beta induced promoter acitivity driven by SBE or promoter activity of a constitutively active TGF-beta RI (134). Moreover, it was recently shown that the expression of CTGF that induces proliferation of fibroblasts and collagen synthesis is mediated by Smad3 and Smad4 (135). Lefty is also capable of inhibiting the promoter activity of CTGF mediated by TGF-beta (134). Thus, lefty provides a repressed state of TGF-beta responsive genes and participates in negative

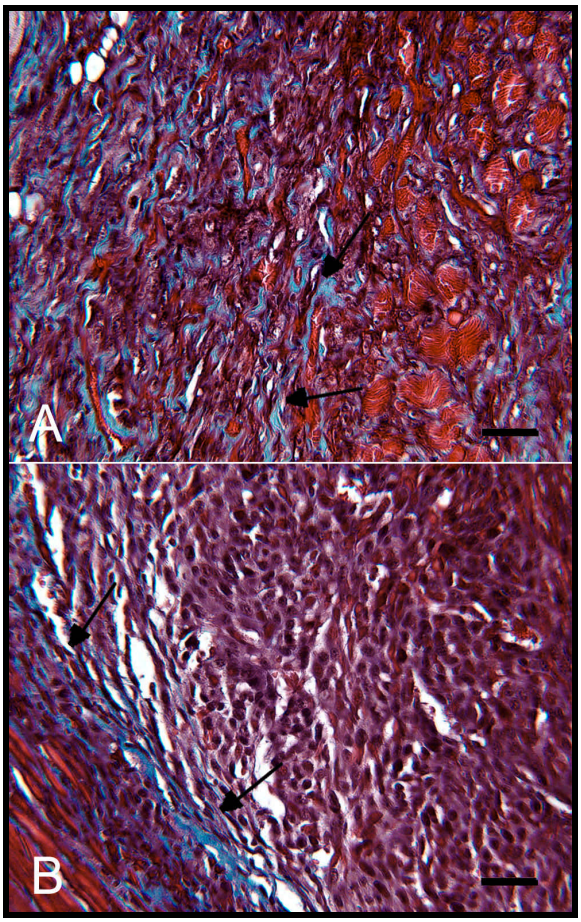

Figure 7. Reduced collagen deposition in stroma by lefty visualized in trichrome stained sections. A: The control transformed GP+E86 fibroblasts transduced with LG retroviral particles have extensively laid down collagen fibers (arrows) at the periphery of the tumor and have invaded surrounding muscle tissue. B: The LEIG transduced fibroblasts have no discernible stroma and show no evidence of collagen fibers. Collagen fibers are seen at the tissues surrounding the tumor (arrows). Bar=250 micrometers.

modulation of TGF-beta signaling by inhibition of phosphorylation of R-Smads (134).

11. LEFTY INHIBITS DEPOSITION OF COLLAGEN *IN VIVO*

The gene coding for the alpha2 chain of the type I collagen (Coll1A2) is one of the best characterized TGF-beta induced genes studied so far. Careful analysis of the promoter of the gene has shown that stimulation of the alpha2(I)-collagen transcription is a result of synergistic cooperation between Sp1 and Smad3-Smad4 transcription factors (136-137). Action of lefty as an inhibitor of TGF-beta signaling suggests that lefty is able to inhibit induction of collagen by TGF-beta for several reasons. First, lefty inhibits the phosphorylation of Smad2 and therefore, is a good candidate to inhibit the TGF-beta induced Smad3 phosphorylation since receptor mediated phosphorylation occurs, at serine residues in the C terminal motif SS(V/M)S of Smad2, which is shared by Smad2 and Smad3 (120). Second, lefty inhibits the activity of CTGF-promoter which is transcriptionally up-regulated by TGF-beta by a pathway which involves Smad3 and Smad4 combination). Third, lefty inhibits the TGF-beta mediated reporter activity of a *S*mad *B*inding *E*lement (SBE). Since R-Smads have the same affinity to SBE, lefty is likely to also inhibit the transcriptional activities that are driven by Smad3 (120). Consistent with this thesis, lefty inhibits the TGF-beta mediated promoter activity driven by SBE (134).

TGF-beta is a pro-fibrogenic cytokine that its actions are mediated by induction of collagen synthesis and suppression of MMP expression. Since lefty inhibits TGF-beta signaling, the possibility exists that lefty might oppose the TGF-beta actions *in vivo* and lead to the loss of ECM. Lefty is markedly expressed in endometrial stromal fibroblasts around the time of menstrual bleeding (1). To simulate this *in vivo* expression for characterizing the lefty actions, two retroviral vectors, a control vector (LG) enabling cells to express GFP and the vector, LEIG, that transduces the expression of both GFP and lefty-A have been used (138). The success of these transduction experiments was assessed by analysis of GFP fluorescence, immunostaining and demonstrating that lefty is secreted by the LEIG transduced and not LG transduced cells (138). These cells were then subcutaneously introduced into athymic mice and the amount of collagen deposited in the stroma of the tumors developed from the fibroblasts was quantitated. As expected, the LG tumors contained abundant collagen fibers. In contrast, in the LEIG tumors, there was little intervening stroma containing collagen (Figure 7). To further validate that the extracellular matrix observed in these tumors was collagen, sections were stained with trichrome, which in view of its affinity, casts a blue color onto collagen fibers. While collagen fibers were detected in large amounts both at the center and periphery of the LG tumors, the LEIG tumors exhibited a paucity of these fibers. These findings show that lefty suppresses collagen synthesis and prevents collagen deposition.

11.1. Lefty inhibits collagen and CTGF mRNA expression *in vivo*

The RNA from LG and LEIG transduced cultured cells and tumors derived from them were subjected to reverse transcription (RT) followed by polymerase chain reaction for mouse collagen type I (138). Although the pro-collagen type I mRNA was detected in both LG and LEIG transduced cells *in vitro*, the LG tumors had more collagen mRNA that found in the LEIG tumors (138). The lack of effect of lefty *in vitro*, might be attributable to absence of any stimulating signal such as TGF-beta that lefty is able to inhibit. The inhibitory actions of lefty can be observed in presence of a stimulating signal such as TGF-beta which is ubiquitously expressed *in vivo*. Real time quantitative RT-PCR confirmed the results of RT-PCR and showed 4.7 fold reduction in the expression of collagen type I mRNA in the tumors derived from lefty$^+$ cells. Moreover, there was 2.8 fold reduction of CTGF mRNA expression in the same tumors as compared with the control tumors. These results are consistent with the histologic data on reduced

Table 2. Potential therapeutic strategies based on antagonism of TGF-beta

Mechanisms of action	Factor	References
Suppression of TGF-beta expression/Synthesis	Perindopril (ACE inhibitor), and Camostat mesilate (Serine protease inhibitor)	155,156
	Transduction of hepatocyte growth factor (HGF) and antisense against TGF-beta	18,64,148-150,157,158
	Pirfenidone	69,159
	Single-stranded 27-mer phosphorothioate oligodeoxynucleotide (ssPT) containing the TGF-beta response element	67,160
TGF-beta binding proteins	alpha2-Macroglobulin, decorin	161-162
	Dominant-negative TβR-II	141,163
	Soluble TGF-beta receptor	73,140,164
	Soluble betaglycan	63
	TGF-beta antibody	66,74-75,154
Suppression of plasmin activity	Serine protease inhibitor, camostat mesilate	154-155
Antioxidants	Glutathione, alpha-tocopherol, Resveratrol, Quercetin, N-acetylcysteine	165-167
TGF-beta signal inhibitor	Smad7	72
Inhibition of R-Smad phosphorylation	Lefty	138

deposition of collagen in tissue sections of lefty+ tumors and show that such reduction is the result of reduced CTGF and collagen mRNA transcription. Collectively, the available data support the hypothesis that lefty inhibits collagen deposition *in vivo*. The findings show that lefty, by acting on the CTGF promoter, inhibits expression of CTGF and consequently reduces the deposition of collagen by a mechanism which includes transcriptional control of collagen type I mRNA expression.

11.2. Lefty induces collagenolytic and elastolytic activities *in vivo*

A critical step in building the hypothesis that lefty is involved in tissue dissolution would be induction of collagenolytic activity by lefty. Proteolytic activities, was not significantly different in the tissue culture media of LG and LEIG transduced cells. However, *in vivo*, there was a significant increase in the proteolytic activities in the LEIG transduced tumors. This difference was noted using collagen, gelatin or elastin as substrates (138). At least five different species of enzyme, induced by lefty causing collagenolysis and caseinolysis was seen by zymography. These findings show that lefty actively participates in the dissolution of ECM by inducing collagenolysis and elastolysis.

12. LEFTY IS OVER-EXPRESSED IN HUMAN CANCERS

Lefty is expressed at a low level in endometrium and other tissues but its expression is markedly increased in endometrium immediately prior to menstrual shedding or during menstrual bleeding and abnormal uterine bleeding (1). Despite the low expression of lefty in normal tissues, its expression is increased in certain forms of human cancers including cancers of colon, ovary, testis and pancreas (139). The biologic consequence of this over-expression remains to be determined. However, in an experimental form of fibrosarcoma, over-expression of lefty was associated with suppression of tumor growth (unpublished data) suggesting that lefty might be part of the molecular repertoire that regulates tumor growth presumably by its actions on the stroma.

13. POTENTIAL TREATMENT STRATEGIES

The negative regulators of the TGF-beta that perturb production of extracellular matrix including collagen might be utilized to cause a negative balance in production of ECM proteins. Many different strategies are being used to antagonize the effects of TGF-beta *in vivo* (table 2). Inhibition of the TGF-beta activities by a number of experimental approaches such as administration of soluble betaglycan, anti-sense TGF-beta, and antibody to TGF-beta all led to decreased accumulation of extracellular matrix *in vitro* and *in vivo* (64-66). In line with the role of TGF-beta in liver fibrosis, blockade of the TGF-beta actions by soluble TGF-beta receptor type II (140), or dominant negative type II receptor (141) prevented liver fibrosis.

Several lines of evidence show that the tumor promoting effects of TGF-beta can be halted by restoring the TGF-beta signaling in tumor cells. Over-expression of TGF-beta receptor I, in a highly malignant rat bladder carcinoma, abolished their malignant phenotype (142) and induction of expression of TGF-beta receptor type II restored the TGF-beta mediated growth inhibition in hepatoma cells (143), reduced malignancy in MCF-7 human breast cancer cells (144) and decreased the tumorigenecity of human gastric cancer cells (145). Moreover, expression of soluble TGF-beta type III receptor (sRIII) in human breast cancer, MDA-MB-231 cells, which antagonizes the tumor-promoting activity of TGF-beta by sequestering active TGF-beta isoforms produced by the cancer cells led to the formation of tumors that grew slowly and lost their ability to metastasize. (146). These findings make it clear that the tumor promoting activity of TGF-beta is due to its effects on stroma rather on cancer cells.

For treatment of cancer, in principle, targeting the tumor stroma circumvents the need to tailor the therapy to the unique genetic makeup of an individual cancer type. Moreover, the tumor stroma is comprised of genetically

stable normal cells which are less likely than tumor cells to become drug resistant. Thus, it might become possible to control tumor growth by regulating the formation of tumor stroma.

Novel approaches of this kind would be expected to substitute or significantly augment the efficacy of traditional therapies. The importance of cancer-stroma interaction is being realized and the understanding that targeting tumor stroma is a valid and reasonable treatment approach is gaining a considerable support. The strategy to inhibit endothelial cell proliferation of the tumor stroma by factors such as angiostatin and endostatin have already shown great promise in the treatment of cancers and three drugs that block VEGF receptor signaling and angiostatin have found their way to clinical trials (147).

Since formation of tumor stroma is required for tumor growth, elimination of the TGF-beta response in tumors should subside the tumor growth, invasion and metastasis. Blocking the TGF-beta action, should lead to tumor suppression by inhibition of the formation of tumor stroma since all solid tumors, regardless of their cellular lineage, require stroma to grow beyond a minimal size of 1-2 mm. Consistent with this thesis, the tumor growth, invasion and metastasis are inhibited when the effect of TGF-beta is interrupted by inhibition of endogenous TGF-beta, expression of soluble TGF-beta type III receptor (sRIII) which sequesters active TGF-beta isoforms, transfection of tumor cells with dominant negative mutant type II TGF-beta receptor (TβRII? cyt), or by administration of antibody to TGF-beta (146, 148-154). These findings show that inhibition of TGF-beta activity can be used as a means for reducing the malignant potential of the tumor cells in terms of growth, invasion and metastasis.

14. CONCLUSIONS

Virtually all normal tissues have a stroma that physically and bio-chemically supports the parenchyma. Under normal circumstances, fibroblasts are quiescent but become active upon tissue injury to restore the stroma of damaged tissues. This form of activation is self limiting. In other instances, however, for unknown reasons, fibroblasts become active and they continue to deposit a collagen rich stroma in a number of fibrotic conditions, and fibromatosis. Formation of stroma is also essential to tumor growth. Growth regulatory molecules of the transforming growth factor-beta family (TGF-beta) are one of the few classes of proteins that provide the necessary signals that support the formation of a fibrovascular stroma. The available evidence shows that generally, there is a marked increase in the expression of TGF-beta mRNA and protein during tissue repair and in fibrotic diseases, fibromatosis and in neoplasms. TGF-beta supports growth of stroma by increased proliferation of fibroblasts and enhancement of collagen deposition. This view is supported by the evidence that TGF-beta increases collagen mRNA synthesis and prevents the degradation of collagen by suppression of specific matrix metalloproteases that digest collagen. Factors such as lefty, that specifically perturbs the TGF-beta mediated growth of fibroblasts, inhibits the synthesis of collagen and enhances degradation of a collagenous stroma, would be an ideal candidate as a therapeutic target.

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Abbreviations: ECM: extracellular matrix, Ebaf: endometrial bleeding associated factor, TGF: transforming growth factor, latent LTBP: TGF-beta binding protein, LAP: Latency associated peptide, BAMBI: BMP and activin membrane-bound inhibitor, CTGF: connective tissue growth factor, GDF: growth and differentiation factor, BMP: bone morphogenetic protein, INB: inhibin, ACT: activin, MIS: Mullerian inhibiting substance, TG: transglutaminase, ECM: extracellular matrix, MMP: matrix metalloportease, MAPK: mitogen activated protein kinase, HGF: Hepatocyte growth factor, Smad: SMA/MAD related, STAT: , SBE: Smad binding element, CCl_4: Carbon tetrachloride, ACE: Angiotensin-I-converting enzyme, TSP: Thrombospondin

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