# Inhibitors of the TGF-β Superfamily and their Clinical Applications

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**Abstract:** The transforming growth factor-β (TGF-β) superfamily includes TGF-βs, activin, myostatin and bone morphogenetic proteins. Misregulation of the activity of TGF-β family members is involved in pathogenesis of cancer, muscular dystrophy, obesity and bone and tooth remodeling. Natural inhibitors for the TGF-β superfamily regulate fine-tuning of activity of TGF-β family *in vivo*. In addition to natural inhibitors for the TGF-β family, soluble forms of receptors for the TGF-β family, blocking monoclonal antibodies and small chemical TGF-β inhibitors have been developed. In this review, we summarize recent advances in our understanding of inhibitors for the TGF-β superfamily and their medical applications.

**Key Words:** TGF-β, activin, BMP, cancer, fibrosis, follistatin, myostatin, muscular dystrophy.

## INTRODUCTION

The transforming growth factor-β (TGF-β) superfamily includes TGF-βs, activin, myostatin and bone morphogenetic proteins (BMPs) [1]. They are involved in many biological responses including growth and differentiation of various cell types. Misregulation of specific TGF-β family members is involved in pathogenesis of certain types of cancer, fibrosis, diseases affecting skeletal muscle, and osteoporosis [2]. Thus, proteins and chemicals that regulate TGF-β family members could be used as drugs for treatment of human diseases.

Members of the TGF-β superfamily bind to type I and type II serine/threonine kinase receptors and transduce intracellular signaling through Smad proteins. TGF-β/activin/myostatin activate Smad2/3, whereas the BMP subfamily activates Smad1/5/8. These pathway-restricted Smads associate with Co-Smad, Smad4 and translocate into the nucleus, and regulate transcription of target genes. Smad6/7 are inhibitory Smads that serve as negative regulators of signaling of the TGF-β family [1]. Signaling of the TGF-β family is regulated by extracellular ligand-binding proteins. Follistatin and related molecules regulate activin/myostatin [2]. Noggin, chordin, Cerberus and differential screening-selected gene aberrative in neuroblastoma (DAN) families are regulators of BMP family members.

Furthermore, TGF-β receptors are dynamically regulated by trafficking of receptors by clathrin-mediated endocytosis and the lipid raft-caveolar pathway [1].

As mentioned above, natural binding proteins for the TGF-β superfamily exist and play an important role in negative regulation of TGF-β signaling. In addition, soluble forms of receptors for the TGF-β superfamily, blocking monoclonal antibodies, and small molecule receptor kinase inhibitors have recently been developed.

Since TGF-β signaling is involved in pathogenesis and progression of various diseases, TGF-β inhibitors are promising as novel drugs for the treatment of cancer, muscular dystrophy, osteoporosis and fibrosis. Myostatin inhibitors such as monoclonal myostatin antibodies, follistatin and myostatin propeptide could be promising lead compounds in drug development for muscular dystrophy. BMP signaling is involved in osteogenesis and tooth development. Therefore, BMP inhibitors could be applicable for osteoporosis and tooth regeneration.

In this mini-review, we summarize recent advances in our understanding of TGF-β inhibitors and their potential medical applications.

## OUTLINE OF THE TGF-β SUPERFAMILY

TGF-β family members are pleiotropic cell signaling proteins that play essential roles in tissue homeostasis and development [1]. One of the most salient characteristics of TGF-β family members is inhibition of growth of various cell types. TGF-β is involved in inhibition of cancer cell growth. TGF-β induces cell cycle arrest through upregulation of cyclin-dependent kinase (CDK) inhibitors p21, p27 and p15, resulting in the inhibition of entry through the G1-phase into the S-phase [1,3]. This property of TGF-β has gained much attention for its role in tumor suppression in tumorigenesis. In the later phase of cancer progression, tumor cells become resistant to growth inhibition by TGF-β, and cancer cells secrete TGF-β. TGF-β secreted by cancer stimulates the neighboring epithelial cells and promotes cancer progression through epithelial–mesenchymal transdifferentiation, and promotes metastasis of malignant cells through mediating changes in cytoskeletal architecture [1,4]. Therefore, TGF-β signaling offers an attractive target for cancer therapy. TGF-βs signal through their own type II receptor

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(TβRII) and type I receptor (activin receptor-like kinase 5 (ALK5)), that are distinct from the type II and type I receptors which activins or BMPs utilize. Several strategies such as the use of antisense oligonucleotides for TGF-β, monoclonal TGF-β antibodies, dominant negative TβRII, and small drug molecules that inhibit TGF-β receptor I kinase have shown great promise in preclinical studies [4].

Activins are homo- or hetero-dimers composed of two β subunits and belong to the TGF-β superfamily. Follistatins are high affinity activin-binding proteins and efficiently neutralize activin functions [2,5]. Activin, inhibin and follistatin are well-recognized as endocrine hormones regulating gonadal functions [2]. However, recent investigation reveals that alteration of activin signaling, like TGF-β, is directly involved in tumor progression. Activins signal through heteromeric complexes of activin type II receptors (ActRIIA and ActRIIB) and type I receptor (ALK4). Two 8-bp polyadenine [(A)8] tracts of the ActRIIA gene were identified as targets for frameshift mutations in gastrointestinal cancers [6]. ALK4 (ACVR1B) gene mutations have also been found in pancreatic carcinoma [7]. Human genes for Smad2 and Smad4 (Dpc4), which act as downstream effectors of activins, TGF-β and myostatin, map to chromosome 18q and are mutated in colorectal and pancreatic carcinomas [8,9]. These findings indicate that alteration of the activin signaling pathway is involved in tumorigenesis.

Cripto, which is the founding member of the epidermal growth factor-Cripto, FRL-1, and Cryptic (EGF-CFC) family, has a role as a co-receptor for nodal [10]. In addition, Cripto binds directly to activin B and inhibits activin B signaling in breast cancer [10]. Blocking Cripto function by a monoclonal antibody suppresses tumor cell growth *in vivo*, most probably through augmentation of activin B signaling by sequestering Cripto from activin B [10]. Taken together, these findings indicate that, as with TGF-β, activins are important regulators of carcinogenesis and have dual roles as tumor suppressors and tumor promoters [11,12].

Myostatin is a recently discovered member of the TGF-β superfamily [13]. Mice lacking the myostatin gene have an ~30 % increase in skeletal muscle mass [13]. Both hyperplasia and hypertrophy are observed in the muscle fibers of myostatin-deficient mice [13]. Myostatin regulates skeletal muscle mass not only in mice but also in cattle and humans. Spontaneous mutations of the myostatin gene have been found in double muscled cattle, called Belgian blue and Piedmontese [14]. Recently, there was a report of a boy who had a splice site mutation in the myostatin gene and who displayed muscle hypertrophy [15]. Blockage of myostatin function, even in adult mice, by a myostatin antibody or conditional gene knockout technology results in an increase in skeletal muscle [16,17]. Thus, inhibition of myostatin is a promising therapeutic approach restoring muscle mass and strength in muscle wasting conditions, such as muscular dystrophy and aging [18].

## BINDING PROTEINS FOR THE TGF-β SUPERFAMILY

Activities of the TGF-β superfamily are regulated by their extracellular binding proteins. Several binding proteins such as type III TGF-β receptor facilitate ligand-binding to receptors [19]. However, the majority of TGF-β family binding proteins in the extracellular space sequester ligands to block ligand-binding to receptor serine kinase on the target cells. Research on the mechanism of extracellular regulation of TGF-β signaling by binding proteins provides clues for the treatment of diseases caused by misregulation of the TGF-β signaling pathway [20,21].

Follistatins are best characterized as activin-binding proteins [5,20,22]. Follistatin is a single-chain protein with a molecular mass of 31–39 kDa. Alternative splicing of a single gene and partial proteolysis produces multiple forms of follistatins. All forms of follistatins have an N-terminal domain and three cysteine-rich domains, called follistatin domains, and demonstrate high affinity binding to activins with estimated Kd values of 500–800 pM. Recently, the structures of the follistatin–activin complex were reported [23] (Fig. (**1**)). Two follistatin molecules encircle activin A, neutralizing the ligand by burying one-third of its residues and receptor binding sites. Both type I and II receptor binding sites of activin are blocked by follistatin binding to activin (Fig. (**1**)). Gene knockout studies have revealed that follistatin-deficient mice show numerous phenotypes including musculoskeletal and cutaneous abnormalities [24]. In particular, follistatin-deficient mice show a reduction in the muscle mass of the diaphragm and intercostal muscles. In addition, rib defects and a decreased number of lumbar vertebrae are observed in follistatin gene knockout mice. This finding suggests that follistatin serves as an inhibitor not only for activins but also for other growth and differentiation factor (GDF)/BMP subfamilies like myostatin and GDF11 [13,24,25]. In fact, recent characterization reveals that follistatin is an efficient inhibitor of myostatin and the closely related TGF-β family member, GDF11 [26]. Follistatin-related gene, called FLRG, was recently identified that has similar ligand binding specificity and structure as follistatin [27–29]. However, several differences between follistatin and FLRG should be noted. Follistatin has three follistatin domains, whereas FLRG only has two [20, 27–29]. Follistatin has a heparin-binding site in the first follistatin domain, whereas FLRG does not. In addition, FLRG immunolabeling was observed in the nuclei of several cell types [29,30]. Furthermore, transcriptional regulation of follistatin and FLRG is different [30,31]. Interestingly, myostatin and GDF11, whose affinity for follistatin is close to activin and higher than that of BMP ligands, shows activin-unique amino acid elements [23], indicating that myostatin and GDF11 are more closely related to activins. Proteomics analysis, to identify potential binding proteins for myostatin in human and mouse sera, yielded FLRG, myostatin propeptide, and a novel protein, GDF8-associating serum protein-1 (GASP-1), which also has a follistatin domain [32,33]. Thus, multiple proteins associate with myostatin *in vivo*.

Cerberus, DAN and gremlin have recently been identified as antagonists of BMP signaling [34]. Since BMP signaling plays a critical role in early embryogenesis, regulation of BMP signaling by the Cerberus/DAN family directly affects ventral/dorsal and anterior/posterior axis formation [35]. Noggin, unlike follistatin, forms an elongated homodimer (Fig. (**2**)). The crystal structure of noggin bound to BMP7 shows that noggin inhibits BMP7 signaling by blocking the molecular interfaces of the binding epitopes for both

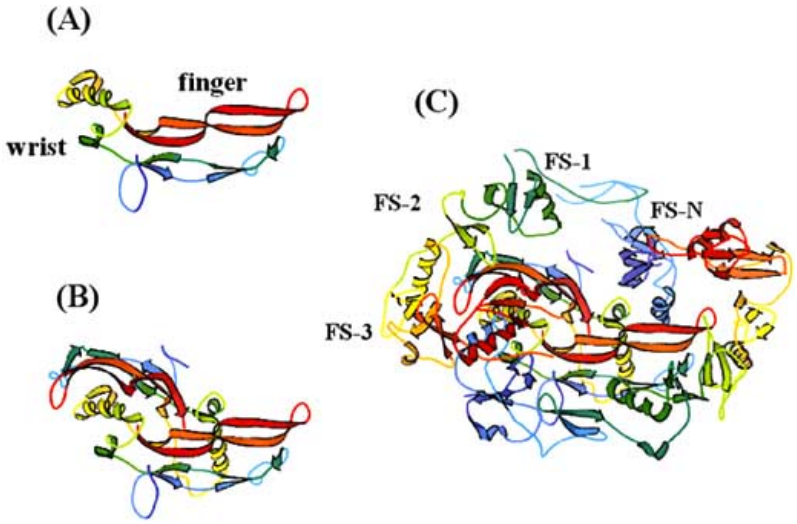


**Fig. (1).** Crystal structure of activin complexed with follistatin.

(A) Structure of an activin monomer is shown. The monomer shows a left-hand like structure including wrist and fingers. (B) Structure of activin dimer. The binding region for type I receptor is located near the wrist region, whereas the binding region for type II receptor is near the fingertips and knuckles. (C) Structure of activin dimer complexed with two molecules of follistatin. Follistatin adopts C-clamp-like conformations and encircles activin. Follistatin has four distinct domains, N-terminal domain (FS-N) and three follistatin (FS-1 to FS-3) domains. The follistatin N-terminal domain has a fold that occupies the type I receptor binding site. FS-1 and part of the FS-2 domains are involved in blocking the type II receptor binding site. The structure is from RCSB Protein Data Bank (PDB) 2B0U and displayed by using KiNG software. Alpha helix is shown in ribbon-like drawing, and beta strands are shown in specific colors.

type I and type II receptors [36] (Fig. (**2**)). Interestingly, the scaffold of noggin contains a cystine knot topology similar to that of the BMP family, suggesting that the ligand and its inhibitor may have evolved from a common ancestral gene by duplication [36] (Fig. (**2**)). The DAN family also has a cystine knot motif, indicating the importance of disulfide bondage to determine the three dimensional structure of BMP inhibitors.

Interestingly, ectodin, which belongs to the DAN/Cerberus family of BMP antagonists, is involved in mammalian tooth cusp patterning. Ectodin-deficient mice have enlarged enamel

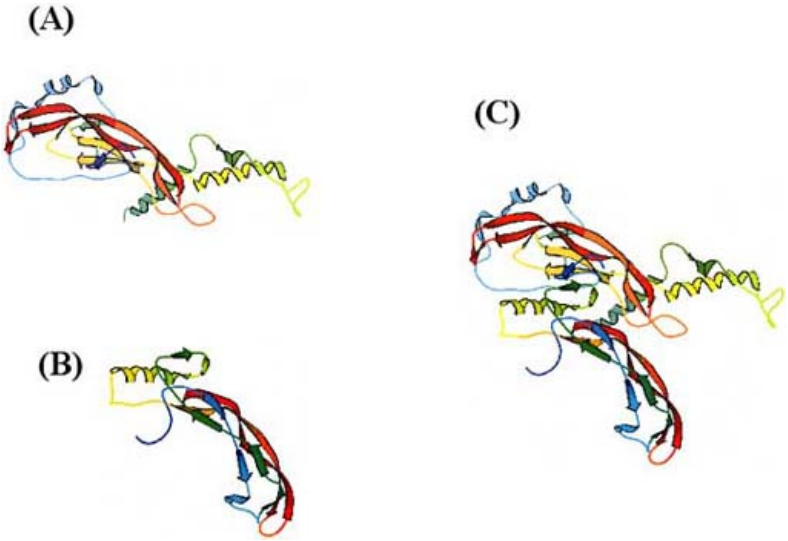


**Fig. (2).** Crystal structure of noggin and BMP7.

(A) Structure of noggin monomer. The scaffold of noggin has a cystine knot topology similar to BMP. Noggin forms a dimer with head-to-head contact, whereas BMP forms a dimer with head-to-tail interaction. (B) Structure of BMP7 monomer. (C) Structure of noggin monomer complexed with BMP7 monomer. Noggin inhibits BMP signaling by blocking the molecular interfaces of the binding epitopes for both type I and type II receptors. The structure is from PDB 1M4U and displayed by using KiNG software. Alpha helix is shown in ribbon-like drawing, and beta strands are shown in specific colors.

knots and extra teeth [37]. It is likely that excess BMP in ectodin-deficient teeth causes unchecked induction toward large enamel knots. Thus, modulation of ectodin would be useful for tooth regeneration.

Sclerostin is an osteocyte-derived negative regulator of bone formation [38]. The loss of sclerostin leads to sclerosteosis and Van Buchem disease characterized by high bone mass [39]. Sclerostin shows amino acid sequence similarity with the DAN family of BMP antagonists and was first hypothesized to act as a BMP antagonist [40]. However, recent characterization suggests that sclerostin binds low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/LRP6) and could be a canonical Wnt inhibitor as well as a BMP antagonist [41,42]. Sclerostin is regarded as an attractive target for the development of bone formation therapy for diseases such as osteoporosis.

## SMALL CHEMICAL TGF-β INHIBITORS

As a chemical TGF-β inhibitor, SB431542 was first reported as a selective inhibitor of TGF-β type I receptor kinase activity [43] (Fig. (**3**)). SB431542 is selective for inhibition of ALK4, 5 and 7 at low concentrations. Chemical TGF-β inhibitors may offer a novel option for cancer therapy by reducing cell proliferation, angiogenesis, motility, metastasis and fibrosis [44,45]. In addition to SB431542, SB505124 and A-83-01 have been developed [46,47]. A-83-01 was found to be more potent than SB431542 in the inhibition of TGF-β signaling [47]. The chemical inhibitors could be useful for preventing tumor progression and fibrosis in cases where TGF-β family members are involved. It is worthwhile noting that these compounds are not specific to TGF-βs, and inhibit activin, myostatin, GDF11 and nodal that signal through ALK4, 5 and 7.

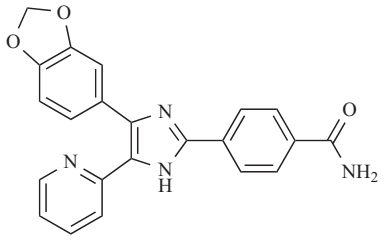


**Fig. (3).** Chemical structure of SB431542, 4-[4-(1,3-benzodioxol-5-yl)-5-(2-pyridinyl)-1H-imidazol-2-yl]benzamide, a specific inhibitor of TGF-β type I receptor kinase. Molecular formula of SB431542 is $C_{22}H_{16}N_4O_3$. Molecular weight of the compound is 384.39.

## CANCER AND TGF-β INHIBITORS

As mentioned above, TGF-β has a dual role in tumor progression, both as a tumor suppressor and tumor promoter. Targeting a TGF-β tumor promoting activity is attractive since tumors are often resistant to the growth-inhibitory effect of TGF-β at the time of tumor detection. In particular, TGF-β plays an important role in promoting metastasis. In fact, one report showed that lifetime exposure to a soluble TGF-β antagonist protects against metastasis without adverse side effects [48]. Thus, it is predicted that inhibitors of the TGF-β signaling pathway would result in delays in tumor progression and improved survival (Table **1**). Several clinical trials inhibiting TGF-β signaling by various strategies indicate that TGF-β inhibition may be a promising option for cancer therapy [2] (Table **1**). Initially, the dominant negative form of TβRII was used to block TGF-β activity, and has been shown to prevent epithelial–mesenchymal transition [49]. Dominant negative TβRII also suppresses tumorigenicity and metastasis of thymoma and mammary tumors [50,51]. Antisense oligonucleotides against TGF-β are good clinical candidates for treatment of cancer and fibrosis [52] (Table **1**). Monoclonal antibodies against TGF-β1 and β2, called lerdelimumab and metelimumab, respectively, have been developed and are now in phase II/III studies in nephropathy, fibrosis, glioblastoma, non-small cell lung carcinoma, and colorectal cancer [53,54] (Table **1**). RNA interference technology to suppress expression of TGF-β and its receptors may offer an additional option for cancer therapy in the future [55]. As mentioned above, small chemical TGF-β inhibitors also have promising therapeutic potential.

## MUSCULAR DISORDERS AND MYOSTATIN INHIBITORS

Skeletal muscle is affected in various muscle wasting conditions such as muscular dystrophy, neurogenic muscle atrophy, aging and disuse atrophy. Since skeletal muscle is the major target organ of insulin action, restoring skeletal muscle mass is favorable even in metabolic disorders, including diabetes mellitus and obesity [18]. Muscular dystrophies are intractable muscular diseases affecting skeletal muscles. Progressive muscle damage and muscle atrophy, infiltration of inflammatory cells in muscle and replacement of skeletal muscles with fibrous and fatty tissues are the hallmarks of the disease [18,56]. Although many of the genes responsible for the various types of muscular dystrophy have been investigated and identified by linkage analysis, effective therapies for muscular dystrophy have not yet become a reality. Theoretically, three major approaches i.e., gene therapy, cell therapy and drug therapy, have been tested with varying degrees of success. Myostatin, one of the TGF-β family of growth and differentiation factors, is a potent negative regulator of skeletal muscle growth and differentiation [13,57]. Regulation of myostatin activity determines skeletal muscle mass. Myostatin blockage is effective for an increase in muscle mass even in adults. Thus, myostatin is considered to be an excellent drug target for muscle wasting diseases such as muscular dystrophy. There are multiple strategies for inhibiting myostatin activity. Myostatin blocking antibody, myostatin propeptide, the soluble form of myostatin/activin receptor, and follistatin bind myostatin and block its action [18]. Indeed, antibody-mediated myostatin blockage in *mdx* mice, which is the model for Duchenne type muscular dystrophy, ameliorates the pathophysiology and muscle strength [58] (Table **1**). Similarly, myostatin blockage and amelioration of *mdx* mice by myostatin propeptide were also reported [59,60]. How myostatin inhibition favors recovery from muscular dystrophy is not clear. It is hypothesized that balancing the muscle loss and atrophy caused by muscle wasting with increased muscle mass by myostatin blockage is beneficial for dystrophic muscles and may overcome muscle wasting [61]. Since TGF-β is involved in fibro-

**Table 1. TGF-β Family Members and Their Inhibitors and Potential Clinical Applications**

| Ligand | Inhibitors | | | Applications |
|---|---|---|---|---|
| | **Proteins** | **Antibodies & Antisense** | **Chemicals** | |
| TGF-β | Decorin<br>Soluble<br>TGF-β receptor | lerdelimumab<br>metelimumab<br>AP-12009 | SB431542<br>SB505124<br>A-83-01<br>LY550410 | Cancer<br>Fibrosis |
| Activin | Follistatin<br>FLRG | | SB431542<br>SB505124<br>A-83-01<br>LY550410 | Cancer<br>Fibrosis |
| Myostatin | Follistatin<br>FLRG, GASP-1<br>GDF8 propeptide | MYO-029 | SB431542<br>SB505124<br>A-83-01<br>LY550410 | Muscular disorders |
| BMP | Noggin, chordin<br>Cer/Dan, ectodin<br>Sclerostin | | | Osteoporosis<br>Teeth regeneration |

sis in many organs, such as liver and kidney [45], and myostatin signaling is similar to that of TGF-β and activin, it is also likely that prevention of myostatin signaling is favorable for the prevention of fibrosis in muscular dystrophy. The biosafety and effectiveness of myostatin antibody MYO-029 is being evaluated in phase I/II studies in the United States in 108 patients suffering from muscular dystrophy [18]. In addition to myostatin antibody and myostatin propeptide, the soluble form of activin/myostatin receptor and follistatin are promising therapeutic tools for myostatin inhibition. The dominant negative form of activin type II receptor, ΔActRIIB, has a strong effect on increased skeletal muscle mass [26,62]. Also, skeletal muscle-targeted overexpression of follistatin causes strong increase of muscle mass [26]. Since ΔActRIIB and follistatin not only inhibit myostatin but also block the action of activin and GDF11, activin and GDF11 are also likely to control skeletal muscle differentiation and growth [4].

## POTENTIAL APPLICATIONS OF BMP INHIBITORS

One of the major actions of BMP is ectopic bone formation. However, the actions of BMP are not confined to osteogenesis. BMP is involved in development of neurons, chondrogenesis, induction of apoptosis, and axis and mesoderm formation in early development. Multiple BMP inhibitors, including noggin, chordin and the Cerberus/Dan family, have been identified and characterized. Some of the inhibitors could have useful medical applications. In the field of dentistry, GDF11 has been characterized as possessing the ability to induce dental pulp stem cell differentiation into odontocytes. Accordingly, GDF11 could enhance the healing potential of pulp tissue [63]. Furthermore, mice deficient in BMP antagonist, ectodin, have enlarged enamel knots and extra teeth [37]. Therefore, tooth regeneration and healing after cavity treatment could be accelerated by regulating BMP/ectodin activity (Table **1**).

Osteoporosis is an important disease affecting morbidity and mortality in an aging world population. Current therapeutics includes inhibitors of osteoclast bone resorption, estrogenic compounds, bisphosphonates and parathyroid hormone [64]. Sclerostin works as a negative regulator of bone formation [39]. Thus, sclerostin is a novel drug target of osteoporosis (Table **1**).

## PERSPECTIVES

Inhibitors for the TGF-β superfamily have great potential for multiple clinical applications. Inhibition of either TGF-β or activin could delay cancer progression and prevent fibrosis (Table **1**). Myostatin inhibition is a promising novel therapeutic strategy to treat muscular disorders, including muscular dystrophy (Table **1**). Targeting either BMP ligand or BMP inhibitors could be beneficial for osteoporosis and regeneration of bone and teeth (Table **1**).

Several molecules can inhibit TGF-β family members. Chemical inhibitors for the TGF-β family could be administered either intravenously or orally. Natural proteins and antibodies were intraperitoneally administered [10,58,60,62]. In the case of therapy of glioma, intracerebral and intrathecal infusion of antisense oligonucleotide has been trialed [65]. Since administered therapeutics degrade *in vivo*, frequent administration is needed. Conjugation/modification of antisense oligonucleotides, siRNA, chemicals and proteins with various compounds, such as polymers and nanoparticles, could prevent degradation before inhibitors for the TGF-β superfamily reach their target tissues and improve their delivery efficiency [66,67]. Even controlled and sustained release of potential therapeutics would be possible from microspheres embedded with hydrogels [67]. Development of TGF-β inhibitors and innovation of new technology for *in vivo* drug delivery will undoubtedly increase options for therapy against cancer and musculoskeletal disorders in which the TGF-β family plays a significant role.

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## ABBREVIATIONS

TGF-β = Transforming growth factor-β

BMP = Bone morphogenetic proteins

DAN = Differential screening-selected gene aberrative in neuroblastoma

CDK = Cyclin-dependent kinase

ActRII = Activin type II receptor

ALK = Activin receptor-like kinase

GDF = Growth and differentiation factor

TβRII = TGF-β type II receptor

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