Perspectives of small molecule inhibitors of activin receptor-like kinase in anti-tumor treatment and stem cell differentiation (Review)

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Abstract. Activin receptor-like kinases (ALKs), members of the type I activin receptor family, belong to the serine/threonine kinase receptors of the transforming growth factor-β (TGF-β) superfamily. ALKs mediate the roles of activin/TGF-β in a wide variety of physiological and pathological processes, ranging from cell differentiation and proliferation to apoptosis. For example, the activities of ALKs are associated with an advanced tumor stage in prostate cancer and the chondrogenic differentiation of mesenchymal stem cells. Therefore, potent and selective small molecule inhibitors of ALKs would not only aid in investigating the function of activin/TGF-β, but also in developing treatments for these diseases via the disruption of activin/TGF-β. In recent studies, several ALK inhibitors, including LY-2157299, SB-431542 and A-83-01, have been identified and have been confirmed to affect stem cell differentiation and tumor progression in animal models. This review discusses the therapeutic perspective of small molecule inhibitors of ALKs as drug targets in tumor and stem cells.

Contents

1. Introduction
2. Activin signal transduction
3. Critical roles of activin in tumorigenesis
4. Effects of activin on stem cell differentiation
5. Activin receptor-like kinase as a target for anti-tumor therapy
6. Activin receptor-like kinase as a target for stem cell differentiation
7. Conclusions

1. Introduction

Activin receptor-like kinases (ALKs) belong to the type I activin receptor family. The activin receptor was first cloned in 1991, and subsequently named the type II activin receptor (ActRII) (1,2). In 1992, the second activin receptor was defined as the type I activin receptor (ActRI) and termed activin receptor-like kinase (ALK) (3,4). To date, seven ALKs, ALK1-7, have been identified in mammals. These ALKs are transmembrane proteins, known as serine/threonine kinase receptors belonging to the transforming growth factor-β (TGF-β) superfamily. The ALKs harbor a transmembrane domain, an extracellular binding domain and a glycine- and serine-rich sequence (GS) domain. The GS domain is a kinase site activated by the TGF-β superfamily type II receptor and can trigger downstream signal transduction. The ALKs elicit various downstream effects of activin/TGF-β, including cell differentiation, proliferation, apoptosis, migration and adhesion as critical modulators of these biological processes. ALKs are also involved in a variety of diseases that include tumorigenesis, skeletal malfunctions, hemorrhagic telangiectasia, renal and immune diseases (5-7). This review briefly discusses the therapeutic prospects of small molecule inhibitors of ALKs as drug targets in tumor and stem cells.

2. Activin signal transduction

In order to elucidate the biological actions of small molecule inhibitors of ALKs, an understating of the basic signal transduction of activin/TGF-β is important. The TGF-β superfamily includes several subfamilies, such as TGF-β, inhibin/activin, myostatin/GDF11 and bone morphogenetic protein (BMP) (8-11). As a member of the TGF-β superfamily, activin belongs to a group of multifunctional cytokines that possess numerous biological functions, including cell differentiation, proliferation and matrix formation (12-14). SMAD proteins, the *Drosophila* mothers against decapentaplegic

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gene homologs in mammals, are shared by activin and TGF-β in a classical signal transduction pathway (15-21). Members of the TGF-β superfamily bind to type II receptors that are subsequently stimulated by dimerization with their specific phosphorylated type I receptor to activate shared canonical and distinct non-canonical pathways (15-18). A total of 7 ALKs are designated as type I receptors of the TGF-β superfamily, ALK1-ALK7 (17,18). Ligands can bind multiple ALKs, albeit with different affinities. TGF-β interacts with ALK1 and ALK5 with high affinity. Activin binds to ALK2 and ALK4 with high affinity and with moderate affinity to ALK7. In addition, BMP interacts with ALK1, ALK3 and ALK6 with high affinity and with moderate affinity to ALK2. The activated ALKs phosphorylate and activate SMAD proteins and mediate intracellular signal transduction (19).

The mammalian SMAD protein family is a family of 8 members that serve as intracellular signaling mediators of the TGF-β superfamily (20,21). Smad2 and Smad3 mediate TGF-β and activin/inhibin signaling, while BMP signaling is mediated by Smad1, Smad5 and Smad8. On the other hand, Smad6 and Smad7 act as intracellular antagonists in the signaling pathway of the TGF-β superfamily. In the canonical pathway, the type I receptors (ALKs) phosphorylate Smad2 and/or Smad3, facilitating the formation of a protein complex with Smad4. The Smad2/3-Smad4 complex is translocated to the nucleus where a substantial number of genes are either transcriptionally activated or repressed. In addition, mitogen-activated protein kinase (MAPK)/ERK, PI3K/AKT, WNT and Notch are activated by the TGF-β superfamily, which in turn can transduce the signaling of the independent SMAD proteins; this cascade constitutes the non-canonical pathways (22-25). Moreover, ALK4 mediates activin signaling transduction in a SMAD-independent manner (26).

The recently identified activin receptor-interacting proteins (ARIPs) are present in the cytoplasm (27). These proteins contain the PDZ domain and mediate activin signaling in a SMAD-independent manner (27,28). PDZ proteins play a critical role in assembling the signaling molecules close to the sub-membranous regions and membranous receptors (29). ARIP1 has multiple protein-protein interacting domains that include 5 PDZ domains and 2 WW domains, and bind to ActRII through the fifth PDZ domain at the C-terminal (27,30). ARIP2 possesses only one PDZ domain that can also interact with ActRII and Ral binding protein 1 (RalBP1). The ternary complex of ARIP2, ActRII and RalBP1 is assembled near the sub-membranous regions (31). The overexpression of ARIP1 and ARIP2 suppresses the gene transcription induced by activin in a dose-dependent manner (27,31). The subsequently identified isoforms of ARIP2, ARIP2b and 2c (32), also harbor only one PDZ domain that binds specifically to ActRII. However, overexpression of ARIP2b and 2c enhances activin signaling transduction. Although structural homology is observed between ARIP2b, ARIP2c and ARIP2, the biological activities are different (31,32). Furthermore, current studies have revealed that ARIPs are not only functionally distinct but also exhibit differences in histological distribution (32-35).

3. Critical roles of activin in tumorigenesis

Activin shows pleiotropic functions in embryonic development, erythropoiesis, wound healing, inflammation, arterial pressure regulation, cancer initiation and progression (36-39). Also, it promotes the production of the extracellular matrix, which is the main factor causing liver, lung, heart and renal fibrosis (36,37). Furthermore, activin regulates the activities of macrophages, such as in promoting the activation of resting macrophages and in the polarization of M2 macrophages, while inhibiting the function of activated M1 macrophages in a dual-directional manner (38,39). Activin functions as a neuroprotective and neurotrophic factor in the survival of cultured neurons and promotes the neurite outgrowth of dorsal root ganglia neurons (40,41). Nevertheless, these studies suggest that activin plays a major role in the migration, proliferation and apoptosis of cancer cells (10,42).

Activin not only promotes the genesis and progression of certain tumors, but can also inhibit tumorigenesis, depending on the tumor type and signaling pathways involved (42,43). The overexpression of activin is associated with colorectal cancer, metastatic prostate cancer, lung cancer, hepatocellular carcinomas and pancreatic cancer (42-44), with poor patient prognosis and positive lymph node status in oral squamous cell carcinomas and lung adenocarcinoma. Additionally, a recombinant activin A promoted the proliferation of lung cancer cell lines SKLU1 and H460 and the invasion of ovarian cancer cell lines OCC1 and SKOV-3 without affecting proliferation (45). Recent evidence has implicated overactive activin signaling in breast cancer cell lines, including MCF7 and MDA-MB231, and higher levels of p-Smad2, p-Smad3 and activin A in advanced breast cancer (46,47). Activin A also promotes invasion, angiogenesis, epithelial-mesenchymal transition (EMT) and stemness in breast cancer cells (46).

A high level of activin is an independent prognostic factor for the survival of patients with cancer. In addition, it also serves as a marker for the severity of the neoplastic disease or the inflammatory process, and might be correlated with survival by effectuating the loss of skeletal muscle mass and the development of cachexia (43,48). Some studies have utilized adeno-associated viral vectors to increase the levels of circulating activin A, thereby inducing a rapid and profound body weight loss (49). Similarly, in cachectic patients the concentrations of activin A are higher than those in non-cachectic patients and are positively associated with weight loss (50,51). This phenomenon established a correlation between circulating activin A levels and anorexia/cachexia syndrome in patients with cancer.

Other studies have shown that activin also exerts a protective effect on patients with cancer. The T47D breast cancer cell model demonstrated that activin A treatment inhibits cell proliferation and induces cell cycle arrest (52). Additionally, in thyroid papillary carcinoma cells, activin exhibits an anti-proliferative mechanism, thereby regulating thyroid tumorigenesis (53). Consistently, activin A is an effective anti-angiogenic factor that inhibits the proliferation of endothelial cells and tube formation by downregulating the expression of cyclin D1 and retinoblastoma protein and enhancing the production of the cell cycle inhibitor p21 (54).

Other members of the TGF-β superfamily are also involved in tumor development (55-57). Previous studies indicated that TGF-β1 promotes cancer cell progression in a mechanism similar to that of activin, and induces EMT via the Smad-related canonical signaling pathway in various cancer cells. It has been reported that TGF-β1 suppresses the expression of E-cadherin by upregulating the expression of Twist related protein 1, zinc finger protein SNAI1 and zinc finger protein SNAI2 (Slug). Also, TGF-β1 induces EMT through non-canonical Smad signaling. Furthermore, a recent report stated that TGF-β1 acts as an inducer through TNF receptor-associated factor 6 to promote receptor cleavage of ALK5 (type 1 receptor of TGF-β) in a non-canonical signaling manner (58,59).

4. Effects of activin on stem cell differentiation

Stem cells have the ability to self-renew and generate differentiated cells that are retained in a specific tissue, and are defined as different types of embryonic stem cells (ESCs), somatic stem cells or induced pluripotent stem cells (iPSCs) (60-62). Several studies have demonstrated that activin A induces early embryonic development and plays a critical role in the differentiation of erythroblasts, including the generation and maturation of erythrocytes (63,64). Recent studies have demonstrated that activin enhances eye field formation from ESCs and promotes the generation of mature photoreceptors in primary rodent retina cultures; however, it inhibits the differentiation of pluripotent stem cells by influencing the expression of key genes, including Nanog and Oct4, in stem cells (65-68). During the embryonic development and differentiation of ESCs, high-intensity signals of Nodal/activin are required in pluripotent stem cell derivatives that give rise to the definitive endoderm (DE), compared with the posterior derivative that differentiates into the mesoderm (69,70). Furthermore, the effect of the length of stimulation with activin A plus Wnt3a on the development of hepatic and pancreatic progenitors from the DE cells derived from human pluripotent stem cells has also been investigated (71). Another study reported that activin upregulates the expression of developmental pluripotency associated 3 in early primordial germ cells (PGCs) and the tyrosine kinase receptor cKIT genes in both standard-derived and activin-derived human embryonic stem cells (hESCs), suggesting that activin induces differentiation by priming hESCs to become part of the PGC lineage (72). Interestingly, the treatment of human and mouse ESCs with high concentrations of activin A triggers stem cell differentiation via the activation of ALKs/Smads (73-75).

Additionally, marked changes in BMP and activin/Nodal signaling levels determine the specification of cardiomyocytes and the cardiac mesoderm. Human iPSC and ESC translational studies have indicated that the co-expression of platelet derived growth factor receptor A and kinase insert domain protein receptor directs the emergence of the cardiac mesoderm, and that this is dependent on the optimal levels of BMP and activin/Nodal signaling (70,76). Accumulating evidence has demonstrated that some cancer stem cells show stem cell properties, such as multipotency, self-renewal and the expression of stem cell markers in the tumor. Such cells have been identified in breast, brain and blood cancer (77-79). As the
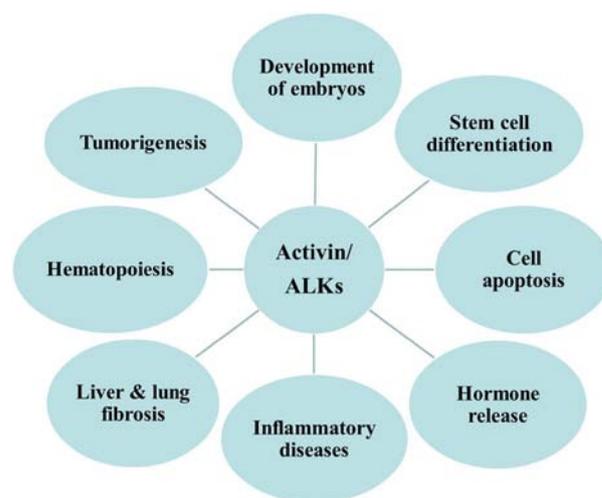

Figure 1. Biological functions of activin and activin receptor-like kinases. ALK, activin receptor-like kinase.

seeding cells, cancer stem cells are capable of forming a new tumor with the properties of the parental tumor. Activin/nodal signaling can control tumor metastasis and progression by influencing the differentiation and proliferation of cancer stem cells via the activin-related signaling pathway similar to that in somatic or embryonic stem cells, and activin signaling enhances the self-renewal of colorectal cancer stem cells and promotes the progression of colorectal cancer in vivo (80,81).

5. Activin receptor-like kinases as a target for anti-tumor therapy

Several ALKs exert regulatory roles in cell differentiation, proliferation, apoptosis, invasion and migration, and mutation of ALKs in various human cancer types has also been identified (5,82). The majority of tumors need a functional vascular network to maintain an environment conducive to tumor growth beyond local boundaries and to facilitate tumor metastasis; thus, a promising approach to cancer therapy is anti-angiogenic treatment. To date, a number of studies have investigated the anti-angiogenic and anti-tumor growth functions of ALK inhibitors with respect to anti-tumor therapy (5,83-85).

ALK1 has been found to exist widely in the tumor blood vessels of lymphoma and cancer of the skin, kidney, prostate, ovary, lung, thyroid, pancreas and liver (5). ALK1 has high affinity for BMP9 and is mainly expressed in endothelial cells, wherein it regulates angiogenesis (83). High ALK1 protein levels in blood vessels of tumor tissues may be a prognostic marker of tumor metastasis in patients with breast cancer. A total of two pharmacological inhibitors of ALK1, PF-03446962, a human antibody against the extracellular domain of ALK1 (84,85), and ACE-041/Dalantercept, a soluble ALK1-Fc fusion protein (84,86), have been used as anti-angiogenic drugs in clinical studies (84-86). ALK1-Fc (Dalantercept) is a chimeric protein consisting of the ligand-binding ALK1 extracellular domain fused to the Fc region of the antibody (84). The ALK1 inhibitor PF-03446962 can prevent the binding of TGF-β and BMP ligands to the extracellular domain of ALK1 (85). The inhibition of ALK1 results in a decrease in the phosphorylation
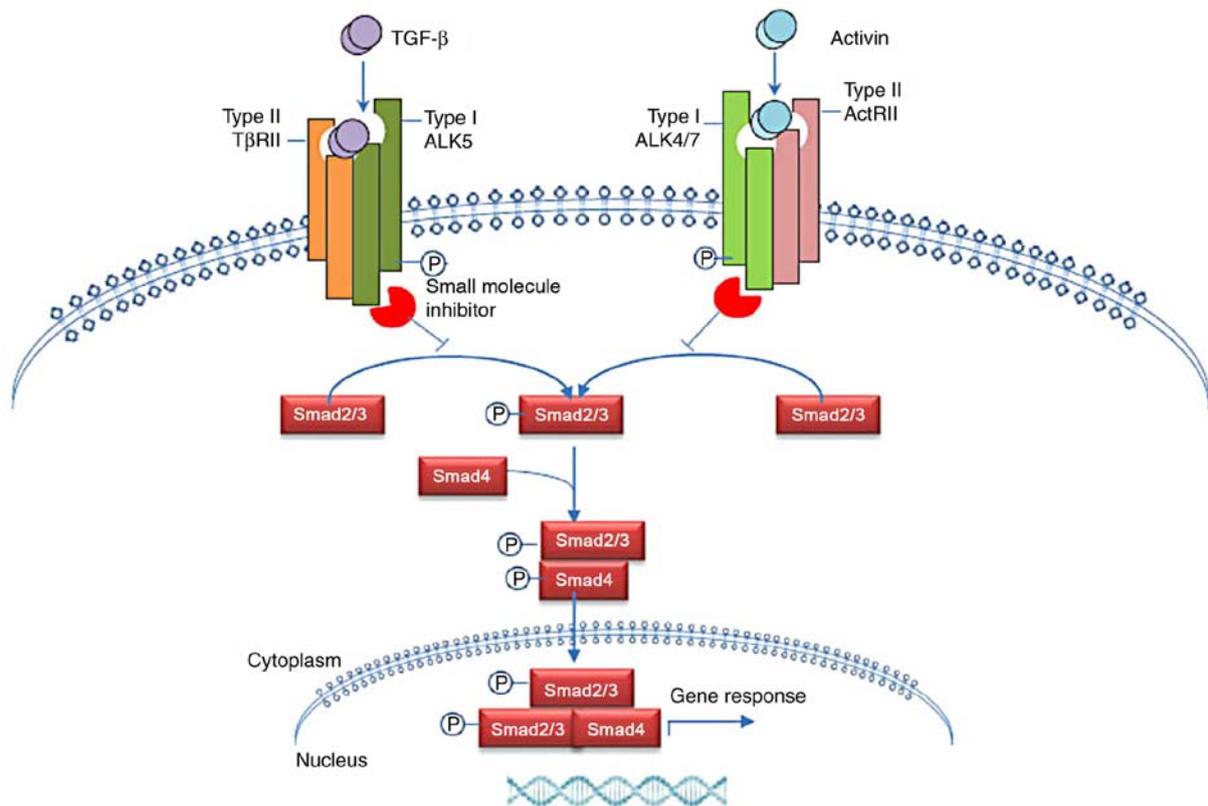
Figure 2. Small molecule inhibitors and the ALK-Smad signaling pathway. ALK, activin receptor-like kinase; TGF-β, transforming growth factor-β; ActRII, type II activin receptor; TβRII, transforming growth factor-β receptor type II.

of p38, a decline in the expression of EMT markers, including matrix metalloproteinase 1, vimentin, Slug and cadherin-2, and downregulation of the expression of the DNA binding 1 (ID1) and ID2 proteins. Pharmacological inhibition of ALK1 also prevents lung colonization and metastatic dissemination in endocrine pancreatic and mammary carcinoma mouse models (87).
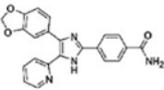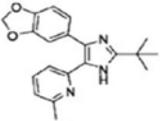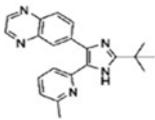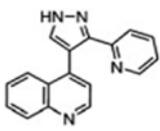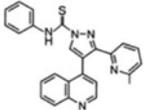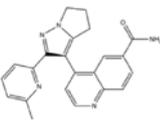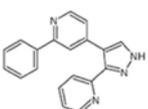
Recent studies have reported that BMP9 induces the phosphorylation of Smad1/5 through ALK1 and ALK2, which contributes to tumorigenesis by promoting the migration and proliferation of cancer cells (88-90). Abnormal expression of ALK2 is associated with a variety of diseases. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is required for the proliferation of TF-1 cells; however, BMP9 also promotes the proliferation of cultured TF-1 cells in the absence of GM-CSF. The overexpression of ALK2 triggers the autophosphorylation of Smad1/5 in TF-1 cells, leading to proliferation of the cells (91,92). KRC203 and KRC360, two specific ALK2 inhibitors, suppressed the BMP9 and ALK2-induced migration and proliferation of cancer cells. Compared to LDN193189, these two inhibitors were more specific and effective in inhibiting ALK2 and were considered as promising drug candidates for the treatment of cancers or diseases with abnormal BMP9 or ALK2 signaling (93,94).

In adult tissues, ALK4 mediates the anti-proliferative effect of activin in pituitary tumor cells. The truncated form of ALK4 functions as a dominant negative inhibitor of activin signaling and eliminates the growth suppression induced by activin (95), while the overexpression of wild-type ALK4 restores the anti-proliferative activity of activin in human pituitary tumor cells (96). The expression of ALK4 was decreased only slightly in breast cancer tissues when compared to normal breast tissues (97). Based on the abnormal activation of TGF-β signaling in hepatocellular carcinoma, LY2157299, a small molecule inhibitor targeting the serine/threonine kinases of TGF-βRI has been designed, which is currently under clinical investigation. LY2157299 exerts anti-tumor roles in patients with hepatocellular carcinoma and glioblastoma (98). LY2157299 blocked the migration and invasion of hepatocellular carcinoma cells, inhibited the growth of hepatocellular carcinoma growth, blocked the production of connective tissue growth factor and reduced stromal reactions (99,100).

The role of ALK5 in tumorigenesis has been studied extensively. In the prostate cancer cell line LNCaP, loss of the tumor suppressive effects of TGF-β was attributed to the rearrangement of the ALK5 gene; transfection with wild-type ALK5 restored the tumor suppressive effects of TGF-β (101). Furthermore, human prostate cancer displays a decrease in the mRNA and protein levels of ALK5, and the loss of ALK5 activity has been associated with an advanced tumor stage and poor 4-year survival (102). The critical role of ALK5 in the activity of TGF-β led to the development of drugs that target ALK5 for the inhibition of TGF-β signaling. Several ALK5 inhibitors have been identified and shown to affect fibrosis and tumor progression in animal models (103). CYLD lysine 63 deubiquitinase (CYLD), a deubiquitinating enzyme, is considered to be a potent tumor inhibitor. The knockdown of CYLD stimulates TGF-β signaling by maintaining ALK5

Table I. Characteristics of small molecule inhibitors of activin receptor-like kinase.

Authors, year	Name	Molecular structure	Target	Function	(Refs.)
Laping et al, 2002 Inman et al, 2002 Halder et al, 2005 Matsuyama et al, 2003 Sato et al, 2015	SB-431542		Selective inhibitor for ALK4, 5 and 7, and weak effect on ALK3	SB-431542 as a selective inhibitor of activin/TGF-β signaling can reduce nuclear accumulation of Smads and inhibit collagen I and fibronectin expression. Additionally, SB-431542 can also attenuate TGF-β tumor-promoting effects, EMT, cell motility, migration and invasion, and inhibit proliferation of osteosarcoma cells and lung metastasis of breast cancer.	(110,111,115-117)
Kim et al, 2017 Wang et al, 2018	SB-505124		ALK4, 5, 7	SB-505124 can offer protection of the neocortex, hippocampus, and thalamus with enhancing cerebral autophagy contributing to the decrease in the extent of progressive neuronal cell death, and up-regulate the expression of nephrin and synaptopodin, providing a novel therapeutic target for diabetic nephropathy.	(118,119)
Grygielko et al, 2005	SB-525334		ALK4, 5, 7	SB-525334 has been demonstrated to block phosphorylation and Smad2/3 nuclear translocation, and inhibits renal fibrosis by reducing procollagen and PAI-1 expression.	(120)
Xu et al, 2012 Gauger et al, 2012 Kimura-Kuroda et al, 2010	LY-364947		ALK4, 5, 7	LY-364947 has an anti-fibrotic role by inhibiting fibroblasts proliferation and epithelium cell transdifferentiation, and prevents proliferative vitreoretinopathy and subsequent tractional retinal detachment in vivo.	(121-123)
Tojo et al, 2005	A-83-01		Selective inhibitor for ALK4, 5 and 7, and weak effect on ALK1, 2, 3 and 6	A-83-01 can inhibit the activities of ALK4, 5 and 7. For other ALK receptors such as ALK1, 2, 3 and 6, A-83-01 showed weak inhibition. A-83-01 was proved to reduce EMT.	(112)
Giannelli et al, 2014 Bueno et al, 2008	LY-2157299		ALK4, 5, 7	LY-2157299 is now under phase II clinical studies of its anti-carcinoma activities against glioblastoma and hepatocellular carcinoma, and has also been reported that it inhibits the tumor growth in human lung anaplastic carcinoma cells and breast carcinoma cells.	(124,125)
de Gouville et al, 2005	GW-6604		ALK4, 5, 7	GW-6604 can prevent matrix deposition and promote hepatocyte regeneration as anti-cancer drug development.	(126)
Leung et al, 2006	SD-208		ALK4, 5, 7	SD-208 inhibits growth and invasiveness and enhances immunogenicity of murine and human glioma cells.	(127)

ALK, Activin receptor like kinases; EMT, epithelial to mesenchymal transition; PAI-1, plasminogen activator inhibitor-1.

stabilization in a cell-autonomous fashion. Moreover, the ALK5 inhibitor TGF-βRI kinase inhibitor II completely blocks the invasive phenotypes of cancer cells induced by CYLD knockdown (104).

6. Activin receptor-like kinase as a target for stem cell differentiation

The activities of ALKs are closely related to stem cell differentiation (105). Bone marrow-derived mesenchymal stem cells (BMSCs) are promising factors that regenerate the cartilage, as they are able to differentiate into chondrocytes in cartilage tissue. TGF-β is a critical inducer of chondrogenic differentiation in BMSCs. ALK1 and ALK5 are important factors for the chondrogenic differentiation of BMSCs induced by TGF-β. In addition, ALK1 and ALK5 mediate signal transduction in BMSCs (105,106).

BMP signaling is also a critical mediator of cartilage differentiation and endochondral ossification. The mutation of arginine 206 to histidine results in the abnormal activation of ALK2, and this mutation exists in 95% of patients with fibrodysplasia ossificans progressiva (FOP) (107). FOP is a rare autosomal genetic disease that results in the development of ectopic bone formation in muscle. Activation of 5'AMP activated protein kinase catalytic subunit α-2 promotes the expression of Smad6 and E3-ubiquitin protein ligase Smurf1, leading to increased interactions and inducing the proteasome-dependent degradation of ALK2 (108). Conversely, Smad6 or Smurf1 knockdown arrests metformin-induced ALK2 reduction. FOP fibroblasts were transformed into iPSCs, followed by osteogenic differentiation of iPSCs *in vitro*. Osteogenic differentiation of iPSCs was blocked by metformin, a pharmacological AMPK activator (108). Therefore, ALK2 acts as a potential therapeutic target during lesion-induced early chondrogenic stages to avoid the heterotopic bone formation in FOP and/or other pathological events (108,109).

4-(4-(Benzo(d)(1,3)dioxol-5-yl)-5-(pyridin-2-yl)-1H-imidazol-2-yl) benzamide, also known as SB-431542, is a selective and potent inhibitor of the type I receptors of ALK4, ALK5 and ALK7 in the activin/TGF-β family, specifically ALK5 (IC$_{50}$=94 nM) (105,109-111). On the other hand, divergent ALK family members, such as ALK3, ALK5 or ALK6 recognize TGF-β and BMP. This neither alters the components of JNK, ERK, or p38 MAPK pathways, nor does it influence their signal transduction. SB-431542 blocks the Smad signaling pathway activated by the TGF-β superfamily, promotes the differentiation of ESCs and iPSCs, and inhibits the renewal of ESCs and iPSCs. SB-431542 enhances the reprogramming efficiency when used together with PD0325901, a MEK inhibitor (109-111).

3-(6-Methylpyridin-2-yl)-N-phenyl-4-(quinolin-4-yl)-1H-pyrazole-1-carbothioamide, also known as A-83-01, is a selective ALK inhibitor; for example, it is a strong inhibitor of ALK4 (IC$_{50}$, 45 nM), ALK5 (IC$_{50}$, 12 nM) and ALK7 (IC$_{50}$, 7.5 nM), but only a weak inhibitor of ALK1, ALK2, ALK3 and ALK6. A-83-01 is a TGF-β/ALK inhibitor that can block TGF-β-induced EMT via the downregulation of Smad2 phosphorylation levels (112). Moreover, A-83-01 can maintain the pluripotency of rat iPSCs, leading to long-term and homogenous self-renewal and to the formation of ESC-like

colonies *in vitro*. It also can rapidly and uniformly alter the fate of mouse embryonic stem cells from the pluripotent to neuronal state (113,114).

7. Conclusions

Activin and ALKs exhibit a wide range of biological activities (Fig. 1). ALKs play a central role in the activin/TGF-β signaling pathway by propagating activin/TGF-β signals to intracellular signaling molecules, such as Smads (Fig. 2). Each ALK is capable of mediating signaling pathways induced via multiple ligands to form complexes with type II receptors. ALKs are essential for lineage determination, endoderm and mesoderm formation, body axis patterning during embryogenesis and for exerting regulatory effects on cell proliferation and apoptosis in tumorigenesis. In recent studies, several ALK inhibitors have been identified in the activin/TGF-β signaling pathway (Fig. 2 and Table I). These small molecule inhibitors of ALKs can affect stem cell differentiation, fibrosis and tumor progression in animal models (110,111,115-127). For example, a small molecule inhibitor, LY2157299, targeting the serine/threonine kinases of TGF-β type I receptors has been developed, which is now under clinical studies and has exhibited anti-tumor roles in patients with hepatocellular carcinoma and glioblastoma (Table I). While these receptors represent potential targets for cancer therapies, minimizing the side effects of these receptors on the tissue distribution and activities of several types of cells and tissues is still a challenge. Thus, additional studies are required to test these receptors in the targeted treatment of various diseases.

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Availability of data and materials

Data sharing is not applicable to this review article, as no datasets were generated or analyzed during the current study.

Authors' contributions

XC, YQ and ZL conceptualized and designed the study. XC, SS, XL and JZ collected, organized and drafted the information. XC and ZL wrote the manuscript. YQ and ZL revised the manuscript critically. All authors have read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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