MINIREVIEW

Regulation of Cell Proliferation, Apoptosis, and Carcinogenesis by Activin[1]

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The aim of this review is to provide insight into the molecular mechanisms by which activin A modulates cell proliferation, apoptosis, and carcinogenesis *in vitro* and *in vivo*. Activin A, a member of the TGFβ superfamily, has various effects on diverse biological systems, including cell growth inhibition in many cell types. However, the mechanism(s) by which activin exerts its inhibitory effects are not yet understood. This review highlights activin's effects on activin receptors and signaling pathway, modulation of activin signaling, and regulation of cell proliferation and apoptosis by activin. Based on the experiences of all the authors, we emphasized cell cycle inhibitors such as p16 and p21 and regulators of apoptosis such as p53 and members of the bcl-2 family. Aside from activin's inhibition of cell proliferation and enhancement of apoptosis, other newly developed methods for molecular studies of apoptosis by activin were briefly presented that support the role of activin as an inhibitor of carcinogenesis and cancer progression. These methods include subtractive hybridization based on covalent bonding, a simple and accurate means to determine molecular profile of as few as 20 cells based on an RNA-PCR approach, and a messenger RNA-antisense DNA interference phenomenon (D-RNAi), resulting in a long-term gene knockout effects.

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Key words: activin; activin receptors; Smad; activin-binding protein; signaling pathway; cell proliferation; apoptosis; carcinogenesis; subtractive hybridization; RNA-PCR; D-RNAi

Activins and inhibins are members of the transforming growth factor-β (TGF-β) superfamily, which consists of TGF-β, activins, bone morphogenic proteins (BMPs), anti-Mullerian hormone (AMH), and others. These structurally related growth factors have essential functions in regulating tissue development, homeostasis, cell proliferation, and apoptosis.

Activin and inhibin were originally isolated based on their activity in regulating follicle-stimulating hormone (FSH) release from the anterior pituitary: activin stimulates, whereas inhibin inhibits FSH secretion (1, 2). In addition to its endocrine function, activin has been found to possess various activities in different biological systems, e.g., erythroid differentiation, nerve cell survival, *Xenopus laevis* embryonic mesoderm induction, bone growth promotion, and somatostatin induction (3, 4). Subsequently, it was found that activin regulates a wide variety of cellular events, including cell proliferation, differentiation, and apoptosis. For example, in addition to its endocrine function in the pituitary, activin also controls the activity of hypothalamus and ovary (reviewed in Ref. 5), indicating that activin has profound paracrine and autocrine effects on the female reproductive system.

In addition, activin expression has been detected in cell lines derived from various human tissues, including prostate cancer (6–8), breast cancer (9, 10), retinoblastoma (11), retinal pigment epithelium (12) placenta (13), gut (14), and bone marrow stroma (15), as well as a number of *in vivo* human tissues, including the testis (16), ovary (17), endometrium (18), placenta (19), oocyte (20), adrenal

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gland (21), pancreas (22), and bone marrow stroma (23). Activin stimulates proliferation of cells of various origins, including lung fibroblast, keratinocyte, porcine thyroid cells, MC3T3-E1 osteoblasts, and spermatogonial cells (24, 25). Activin also regulates apoptosis of hepatocytes, B lineage cells, prostate cancer cells, and other cells (26–30). Recently, several studies have documented that activin and inhibin play critical roles in tumorigenesis in a variety of tissues (31–33). As these growth factors have profound effects on many tissues in both physiological and pathological conditions, activin and its related proteins have been intensively studied. This review mainly focuses on activin signaling and its mediators in regulating cell proliferation, apoptosis, and carcinogenesis.

Activin and Activin-Binding Proteins

Activins and inhibins are structurally related; they share common β subunits (1, 2), which have a nine-cysteine distribution pattern similar to TGF-β and other members of the TGF-β superfamily (34). A β subunit is about 14 kDa, and the most common β subunits are βA and βB. Activins are homodimers of β subunits linked by a disulfide bond. Depending on the combination of the subunits, there are three isoforms of activin, namely activin A (βAβA), activin B (βBβB), and activin AB (βAβB). Inhibins are heterodimers of an α subunit and a β subunit. α subunits, 18-kDa N-linked glycoproteins, can dimerize with either βA or βB to form inhibin A (αβA) or B (αβB), respectively. Additional β subunits have been identified, namely βC, βD, and βE. βC and βE were found in human and mouse, while βD was cloned from *Xenopus* (35–38). It has been shown that βC can dimerize to form activin C (39). Similar to other members of the TGF-β superfamily, both α and β subunits of activins are synthesized as large inactive precursors that undergo intracellular processing to release the carboxy-terminal bioactive mature forms. Dimerization between the subunits occurs inside the cell.

Several soluble ligand-binding proteins have been described. Follistatin can bind both activin and inhibin through the β subunit with a high affinity (40). Although it has no effect on inhibin, follistatin inhibits activin activity by preventing activin from interacting with its signaling receptors (41). Recent studies also suggest that follistatin can bind to BMP and inhibit BMP activity (42). α2-macroglobulin, an abundant plasma protein in the circulation, can bind to activin as well as inhibin, but the binding affinity is low, and this binding does not affect ligand functions (43). α2-macroglobulin also interacts with other growth factors such as TGF-β. It was proposed that α2-macroglobulin may function as a clearance mechanism to restrict the local action of TGF-β (44). α2-macroglobulin may have a similar function for activin and inhibin (45). Recently, a third activin-binding protein, ovine uterine milk protein, has been identified (46). Similar to α2-macroglobulin, ovine uterine milk protein does not neutralize activin actions.

Activin Receptors and Signaling Pathway

Activin Receptors and Their Activation. The signal transduction pathway is highly conserved for the TGF-β superfamily members, involving the receptor-Smad system. Similar to the TGF-β and BMP, activin needs two types of cell surface receptors (type I and type II receptors) for its signaling transduction (Fig. 1). Both receptors are transmembrane proteins with ligand-binding activity in the extracellular domain and serine/threonine kinase activity in the intracellular domain. The activin type II receptors, ActR-II and ActR-IIB, are the primary ligand-binding proteins and can bind ligand without type I receptors. However, the type I receptor, ActR-IB (also called ALK4), is unable to bind ligand in the absence of the type II receptors. This ligand-binding feature of activin receptors, similar to that of TGF-β receptors, is different from that of BMP receptors. Either BMP type I receptor or type II receptor can individually interact with BMP (47). Activin receptor expression has been detected in cell lines from various tissues, including prostate cancer (6–8), breast cancer (9), keratinocytes (48), erythroleukemia (49), osteoblasts (50), and teratocarcinoma (51), as well as in *in vivo* human prostate (52), ovary (53), brain tumor (54), oocyte (20), placenta (55, 56), and pancreatic cancer (37). Besides binding to activin, both ActR-II and ActR-IIB have shown to bind BMP in the presence of a BMP type I receptor (58, 59).

Another receptor, ALK2 (also known as ActR-I or Tsk7L), was originally identified as a type I receptor for activin (60). Subsequent studies, however, suggest that

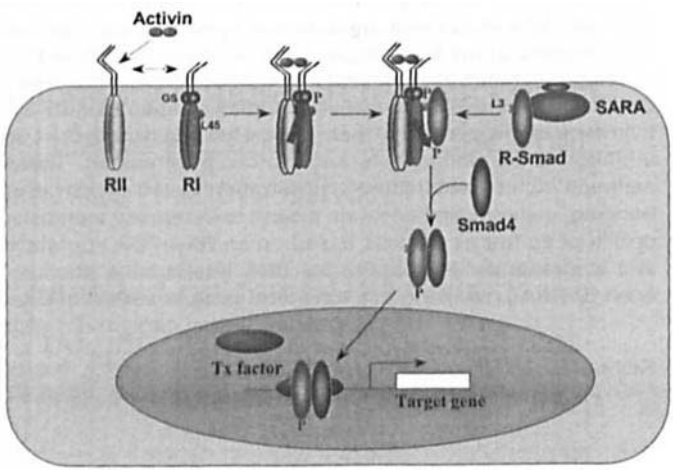

Figure 1. The activin sigaling pathway. Activin binding to the type II receptor (RII) leads to recruitment of the type I receptor (RI) and formation of the receptor heterocomplex. In the complex, the constitutively active type II receptor phosphorylates the type I receptor at the GS domain (GS) and activates it. The activated type I receptor is then able to bind and phosphorylate Smad2 and 3 (R-Smad). Smad2 activation is facilitated by SARA, which presents Smad2 to the receptor complex. After phosphorylation, the R-Smad dissociates from the receptor complex and associates with the co-Smad, Smad4. The resulting Smad complex will be accumulated in the nucleus and bind to DNA. Dependent on the transcriptional factors (Tx factor) they interact with, Smad proteins can stimulate or suppress target gene expression. The specific receptor-Smad interaction is determined by the L45 loop in the type I receptor and the L3 loop in the R-Smad.

ALK2 may serve as a type I receptor for BMP. Indeed, it binds to BMP7 and activates BMP-responsive reporters (61, 62). In *Xenopus* embryos, overexpression of ALK2 induces ventral mesoderm formation as BMP, whereas activin induces dorsal mesoderm formation (63). Furthermore, overexpression of ALK2 inhibited activin-induced cell growth arrest of B cell hybridoma, whereas ActR-IB facilitated this processing (64). Taken together, these data indicate that only ActR-IB is a *bona fide* type I receptor for activin, whereas ALK2 is not.

Upon activin binding, the type II receptor forms a tight complex with type I receptor. Activin binds to ActRII at the plasma membrane and activates its serine-threonine kinase. In the receptor complex, this constitutively active type II receptor kinase phosphorylates the type I receptor in the regulatory GS domain, a glycine- and serine-rich segment prior to the kinase domain, and this phosphorylation leads to activation of the type I receptor (65–67). The activation mechanism of type I receptor through phosphorylation in the GS domain by type II receptor is conserved for TGF-β, activin, and BMP signaling. The GS domain is a unique structure feature for the type I receptors of the TGF-β family and, to a high degree, is conserved in all type I receptors. In the crystal structure of the intracellular domain of the TGF-β type I receptor (TβR-I) in complex with FKBP12, a modulator of receptor activity (see below), the GS domain forms an α helix-loop-α helix structure (68). The loop of the GS domain acts as a wedge that maintains the receptor kinase in an inactive conformation. It was proposed that phosphorylation would release the inhibitory effect of the GS domain (68). In addition, phosphorylation of the GS domain could also provide a docking site for the receptor substrate Smad proteins (see below) (69).

Smad Proteins. Once activated, the type I receptor can bind and then phosphorylate a group of Smad proteins such as smad 2 for activin, called R-Smads, on the serine residues of the carboxyl-terminal tail. The phosphorylated R-Smads will form a heterocomplex with co-Smad such as Samd4 (70). The resulting Smad complex will translocate into the nucleus, bind to the promoter region of the target genes, and regulate their expression. Dependent on different types of transcription factors they interact with, Smads will either stimulate or suppress transcription of the target genes (71, 72). For instance, Smad3 and 4 interact directly and cooperate with AP1, Sp1, and p300/CBP transcription factors to positively regulate transcription (71). To date, at least three Smad corepressors have been identified: TGIF, c-Ski, and Sno-N (reviewed in Ref. 72). All of these Smad corepressors interact with the MH2 domain of Smad2 and 3 and repress TGF-β-mediated transcription by recruiting histone deacetylase. Since these repressors are associated with Smad2 and 3, they may be involved in activin-mediated transcription repression as well. Besides interacting with transcription activators and repressors, Smad proteins are also associated with other DNA-binding proteins. For example, FAST1 (forkhead activin signal transducer-1) was identified as a DNA-binding cofactor for Smad2 and required for activin-induced expression of Mix.2 gene in *Xenopus* embryos (73). In this model, Smad2 binds to FAST-1, which acts as a coactivator at its C-terminal domain termed Smad-interacting domain (SID) (74), and also Smad4. These three components make up the activin responsive factor (ARF), which binds to the activin response elements (AREs), the specific DNA sequence of the *Mix.2* promotor (75).

It was thought that phosphorylation by receptors is required for Smad nuclear translocation. However, a recent study with *in vitro* nuclear import assay demonstrated that both phosphorylated and unphosphorylated Smad2 have similar nuclear import ability (76). Hence, phosphorylation seems to be dispensable for the nuclear translocation, but is required for R-Smad proteins to dissociate from their retentional factors and to promote the heterocomplex formation between R-Smad and co-Smad.

Smad proteins consist of the amino-terminal Mad homology 1 (MH1) domain, the carboxyl-terminal MH2 domain, and the middle linker region. Both MH1 and MH2 domains are highly conserved in the amino acid level among the Smad proteins. The MH1 domain is mainly involved in DNA binding (except for Smad 2, which does not bind to DNA directly) and in interaction with other transcription factors. The MH2 domain contributes to transcriptional activation, Smad-Smad interaction, Smad-receptor interaction, and association with other binding proteins and transcription factors (47, 74, 77). The linker region provides a binding site for Smad ubiquitination regulatory factors (Smurfs), the ubiquitin E3 ligases, and thus participates in Smad ubiquitination and proteosome-mediated degradation (78–81). The linker region also contains phosphorylation sites for ERK and Ca^{2+}-calmodulin-dependent kinase II, and phosphorylation of the linker region by these kinases negatively regulate Smad activity (82, 83) (see below).

On the basis of their structure and function, Smad proteins can be divided into three groups: R-Smads, co-Smads, and anti-Smads. The R-Smads includes Smad1, 5, and 8, which mediate BMP signaling, and Smad2 and 3, which transduce signaling from TGF-β and activin (47, 74, 77, 84). The only co-Smad, Smad 4, is essential for all TGF-β, activin, and BMP signaling by forming a complex with phosphorylated R-smads. The anti-Smads, Smads6 and 7, do not mediate signaling. Instead, they inhibit signal transduction by binding to the activated type I receptors as a pseudosubstrate, or by associating with the activated Smad 1 as a decoy in the case of Smad 6 (85). Smad7 is able to interact with all type I receptors and functions as a general inhibitor for TGF-β, activin, and BMP signaling, whereas Smad6 is more specific for BMP signaling.

Specificity of Receptor-Smad Interaction. Smad2 and 3 bind to and are phosphorylated by the activated TGF-β and activin receptors, whereas Smad1, 5, and 8 are activated by BMP receptors. The specific receptor-Smad interaction is determined by the discrete structure motifs in the type I receptors and R-Smads. Mutagenesis

studies have identified the L45 loop in the kinase domain of the type I receptors and the L3 loop in the MH2 domain of the R-Smads as determinants for specific receptor-Smad interaction (62, 86–89). The amino acid sequence of the L45 loop is conserved among the type I receptors that have similar substrate specificity; that is, the TβR-I and ActR-IB have a similar L45 loop sequence, which is different from that of the BMP type I receptors (88). Interestingly, although another group of type I receptors, including ALK2 and ALK1 (also known as TSR-1), which have a distinct L45 loop sequence different from that of the BMP type I receptors, these type I receptors can also phosphorylate Smad 1 and related Smad 8 (61, 62, 90). Similarly, Smad2 and 3 share the same L3 loop sequence, which is distinct from that of Smad 1, 5, and 8 (62, 87).

Modulation of Activin Signaling

The physiological and pathological functions of activin are tightly modulated at different levels of the signaling transduction (85). Extracellularly, ligand activity is influenced by ligand-binding proteins or accessory receptors. On the plasma membrane, receptor activity is regulated by receptor-associated proteins, and receptor availability is controlled by degradation. In the cytoplasm, Smad activity is modulated via phosphorylation by receptors and other kinases and also by other post-translational modifications such as ubiquitination.

Regulation of Ligand Activity. As discussed above, follistatin neutralizes activin actions. It inhibits activin-induced FSH release from the pituitary (2). Follistatin also antagonizes activin on inducing dorsal mesoderm formation (91). Interestingly, activin increases the expression and secretion of follistatin in rat anterior pituitary cells (92–95). Thus, follistatin modulates activin activity via a negative feedback mechanism (Fig. 2).

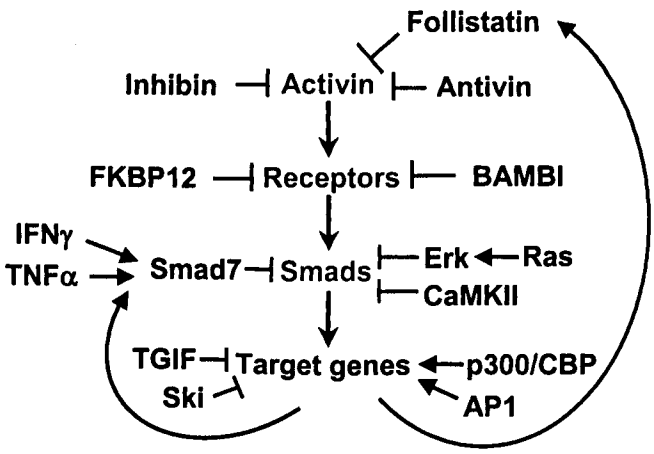

Figure 2. Regulators of activin signaling. The activin signaling is tightly controlled at a different level, from ligand, receptor, Smad, to the target genes. Both follistatin and Smad 7 regulate activin signaling via a negative feedback loop because their expression is stimulated by activin. The blue arrow donates the signaling flow, the red line donates the negative regulation, and the black arrow donates the positive regulation. See the text for details.

Although activin and inhibin are structurally related (sharing the same β subunit), their functions are very different. The nomenclature of activin and inhibin reflects their activity in regulating the secretion of FSH by the pituitary (2). In fact, inhibin antagonizes activin activity in multiple biological systems. In addition, activin promotes gonadotropin-releasing hormone-mediated luteinizing hormone secretion from the pituitary, induces androgen production in theca cells, and stimulates granulosa cell proliferation and follicle growth, whereas inhibin exerts opposite effects on these biological activities (2, 24). Furthermore, inhibin antagonizes activin activity on erythroid differentiation and chondrogenesis (96–98). To date, most data indicate that inhibin exerts its functions by inhibiting activin activity. However, several lines of evidence suggest that inhibin may have its own signaling pathways. There are specific inhibin-binding sites in the pituitary, testis, ovary, and in tumors derived from inhibin knockout mice (99). In addition, several studies have indicated the existence of specific inhibin-binding proteins (97, 100). However, the identity of inhibin-binding proteins remains to be clarified.

Since both activin and inhibin can bind to the activin type II receptors, it was proposed that inhibin blocks activin activity by competing for the binding sites of the signaling receptors (97, 101, 102). This hypothesis gained support from the recent studies by Vale and his colleagues (103). Inhibin has a low binding affinity to the ActR-II, and this binding is dramatically increased by β-glycan, a transmembrane cell surface proteoglycan (103). However, the binding of inhibin to ActR-II prevents the recruitment of ActR-IB. Consequently, inhibin sequesters the type II receptor and thus blocks activin signaling. Furthermore, β-glycan expression increases the inhibitory effect of inhibin in the cells that otherwise respond poorly to inhibin. β-Glycan, also known as a type III receptor for TGF-β, binds to TGF-β and brings it to its signaling receptors (104). This function of β-glycan becomes manifest in the case of TGF-β2, which has a low affinity to the TGF-β type II receptor, TβR-II. The binding of TGF-β2 to TβR-II is greatly enhanced by β-glycan. Therefore, β-glycan has dual functions: on one hand it promotes TGF-β signaling by facilitating TGF-β-receptor binding; and on the other hand, it mediates the antagonistic effect of inhibin on activin signaling. It is possible that β-glycan is the long-searched-for inhibin receptor.

Recently, a novel factor named antivin has been described from zebrafish (105). Antivin is structurally related to mouse Lefty, a member of the TGF-β superfamily. Overexpression of antivin diminished mesoderm formation at the blastula stage of embryos. This activity was mimicked by Lefty and was suppressed by activin or activin receptors. It was proposed that antivin may inhibit activin signals by competing for receptor binding (105). In this sense, the action of antivin is similar to that of inhibin.

Regulation of Receptor Activity. The next level of signaling modulation is at the receptors. Several receptor-associated proteins have been identified. FKBP12 is a ubiq-

uitous, abundant cytosolic protein that binds to immunosuppressants FK506 and rapamycin and is responsible for the immunosuppression effect of these drugs (106). Yeast two-hybrid screening identified FKBP12 as a binding protein for the type I receptors of the TGF-β family, including ActR-IB (107). The studies with the TGF-β receptors revealed that FKBP12 binds to the GS domain of the type I receptor (108, 109). This binding physically hinders phosphorylation in the GS domain of the type I receptor by the type II receptor, thereby inhibiting receptor activation (68, 108). Because FKBP12 dissociates from the TGF-β receptors upon ligand stimulation, the role of FKBP12 in TGF-β signaling pathway is thought to prevent leaky signaling in the absence of ligand (108). It is highly likely that FKBP12 plays a similar role in regulating the activity of the receptors for activin and BMP signaling.

Another group of proteins that play a role in signaling modulation at the receptor level is BAMBI (BMP and activin membrane-bound inhibitor). BAMBI was identified by its activity to inhibit both BMP and activin signaling during *Xenopus* embryogenesis (110). It is a transmembrane protein with the extracellular domain similar to that of BMP type I receptors. Its short intracellular domain shows no homology to other proteins. BAMBI is a general inhibitor for the TGF-β family: it inhibits activin and BMP signaling in *Xenopus* embryos and attenuates signaling of TGF-β, activin, and BMP in mouse embryonic carcinoma P19 cells. Biochemical studies revealed that BAMBI interacts with all the type I receptors except ALK2 and this interaction may lead to the formation of signaling-incompetent receptor complex (110). Interestingly, BAMBI is co-expressed with BMP4 in both *Xenopus* and mouse embryos and is controlled by BMP signaling (110, 114).

The third way to modulate the signaling transduction at the receptor level was shown by recent observations of degradation. The E3 ubiquitin ligases Smurf 1 and 2 can interact with TGF-β receptors via Smad7 and can enhance turnover of TβR-I and Smad7 (79, 112). As Smad 7 also interacts with activin and BMP type I receptors, it is possible that Smad7 targets Smurfs to those receptors and mediates their degradation.

Regulation of Smad Activity. Smad activity is controlled by multiple signal inputs. R-Smads are activated by receptor phosphorylation at the carboxyl-terminal tail, and this activation is modulated by Smad-interacting proteins. For example, Smad anchor for receptor activation (SARA) has been suggested to facilitate TGF-β receptor-mediated activation of Smad2 by bringing receptors and Smad together (69, 113). Since it specifically interacts with Smad2 and Smad3, but not with other Smads, SARA may also play a similar role in activin signaling.

The receptor activation of R-Smads is also regulated by anti-Smads. It has been demonstrated that Smad7, but not Smad6, inhibits activin-mediated Smad2 activation in B cell hybridoma HS-72 cells (114). Smad7 is able to bind tightly to the activated type I receptors and, thus, it prevents binding of R-Smads to the receptors (115, 116). By blocking R-Smad activation, Smad7 interferes with activin-mediated erythroid differentiation, growth arrest, and apoptosis of HS-72 cells (117, 118). Consistent with the notion that Smad7 participates in negative feedback regulation of activin signaling, the expression of Smad7 is induced by activin (115, 116) (Fig. 2). In addition, Smad activity is manipulated by cross-talk signals from other pathways.

As mentioned above, Smad2 and 3 are phosphorylated in the linker region by ERK protein kinase upon the activation of EGF receptor-Ras pathway (82) and by Ca^{2+}-calmodulin-dependent kinase II (83, 119, 120). Phosphorylation by these kinases imposes an inhibitory effect on Smads by attenuating their nuclear translocation. A recent study showed that protein kinase C (PKC) phosphorylates Smad2 and Smad 3 in the MH1 domain (121). PKC phosphorylation abrogates Smad3 binding to DNA and its transcriptional activity. Although Smad phosphorylation in the MH1 domain and the linker region has a negative influence on Smad activity, it was reported that hepatocyte growth factor activates Smad2, presumably via phosphorylation at the same serine residues phosphorylated by TGF-β and activin receptors (122).

The negative regulator Smad7 is also a site where signals from other pathways converge. Its expression is stimulated by interferon-γ via the Jak kinase-STAT pathway (123), as well as by tumor necrosis factor-α and interleukin-1 via the NF-κB/RelA transcription factors (124). Cross-talk between the Smad pathway and other signaling pathways also occurs in the nucleus. For instance, Smad3 has been found to interact with vitamin D receptor (125, 126). As discussed above, Smads also interact with a variety of transcriptional activators and repressors, and these transcription factors receive signaling inputs from many other pathways.

The protein amount of Smads is controlled by ubiquitination and proteosome-mediated degradation at both the basal and the activated levels. The basal protein level of Smad 1 is regulated by Smurf 1 and 2, whose overexpression leads to Smad 1 ubiquitination and subsequent degradation (78–81). Ubiquitination-mediated degradation also participates in signal turn-off. It has been demonstrated that upon TGF-β activation Smad 2 undergoes ubiquitination and then proteosome-mediated degradation (127). Because Smurf 2 has higher binding affinity to activated Smad 2, it may mediate Smad 2 turnover upon TGF-β stimulation (80). Thus, Smurfs appear to involve degradation of both receptor and Smad. Whether Smurf 2-mediated Smad 2 degradation is also involved in regulation of activin signaling remains unknown.

Regulation of Cell Proliferation and Apoptosis by Activin

Cell Proliferation. Although activin and TGF-β play very different roles in tissue development and in the reproduction system, they do share many similarities in signaling.

For instance, both of them share the same receptor-binding properties. Their type I receptors exhibit the same substrate specificity, namely they phosphorylate and activate Smad 2 and 3. Since they use the same set of Smads, it is conceivable that they may also share some of the regulatory mechanisms. In terms of cellular functions, both TGF-β and activin stimulate production of extracellular matrix proteins such as fibronectin and type I collagen (24). Increased expression of activin was associated with fibrosis of liver and lung, a pathological process known to be stimulated by TGF-β (128, 129). In addition, both activin and TGF-β have antiproliferative effects on many types of cells, including epithelial cells, lymphocytes, prostate cancer cells, and others.

TGF-β functions as a tumor suppressor in early stages of tumorigenesis. Many tumor cells escape the growth-inhibitory effect of TGF-β by acquiring mutations in the genes of TGF-β-signaling components, TGF-β receptors, and Smads (47). Some tumor cells have also developed a similar mechanism to elude the antiproliferative effect of activin.

Similarly, loss of function mutations of ActR-IB have been identified in human pituitary tumors (130). Subsequent studies showed that those mutations have a dominant negative effect on activin signaling: the derived ActR-IB mutants blocked activin-induced expression of junB, as well as a reporter in human chronic myeloid leukemia K562 cells (131). The receptor mutants also inhibited activin-mediated cell growth arrest.

Activin has demonstrated the ability to inhibit cell growth in many human cell types, including prostate cancer (132), breast (10, 133), B cell leukemia (134), vascular endothelial (135), vascular smooth muscle (136), peripheral blood granulocyte-macrophage colony-forming unit progenitors (137), and fetal adrenal (20), as well as HS-72 mouse B cell hybridoma (138), mouse plasmacytoma (139), BALB/c 3T3 mouse fibroblasts (140), rat liver (141), and rodent hepatocytes (142, 143). However, the mechanism(s) by which activin exerts its inhibitory effects are largely unknown. The limited information on the mechanism has been brought forth by studies in human prostate cancer LNCaP cells, human HepG2 hepatoma cells, HS-72 mouse B-cell hybridoma cells, and other cell models.

In B cell hybridoma, activin stimulates the expression of cyclin-dependent kinase inhibitor p21CIP1/WAF1 and suppresses cyclin D2 expression (117, 144), which lead to inhibition of cyclin-dependent kinase Cdk4 activity and consequently to the accumulation of hypophosphorylated Rb protein. The hypophosphorylated Rb exhibits a higher binding affinity to the transcription factor E2F and hence sequesters its activity. E2F controls expression of the genes required for cell cycle progression from G1 to S phase (145). Thus, activin causes cell growth arrest in the G1 phase. This antiproliferative effect of activin is abolished by the ectopic expression of Smad7, indicating that Smad proteins are involved in this process (117). Activin also inhibits growth of pituitary tumor cells by a similar mechanism: it increases the expression of p21CIP1/WAF1 (31).

Another of the important functions of activin is induction of erythroid differentiation. Activin stimulates the expression of α-, β- and ε-globin and hemoglobins in purified erythroid progenitor cells (146). Activin also induces hemoglobin accumulation and erythoid differentiation in human chronic myeloid leukemia K562 cells (147).

Apoptosis. Activin not only regulates cell growth and differentiation, but it also induces programmed cell death in several types of cells. It appears that activin induces apoptosis by activating caspases, the proteases responsible for cell death. Activin-induced apoptosis of hepatoma HepG2 cells is abrogated by dominant negative forms of ActR-IIB or Smad 2, whereas overexpression of either ActR-IB, ActR-IIB, Smad 2, or Smad 4 is sufficient to stimulate apoptosis in the absence of activin (29). Smad 7 expression abolishes activin-induced apoptosis in B cell hybridoma (117). Taken together, these data indicate that the receptor-Smad pathway plays a central role in relaying activin signal to programmed cell death.

In HepG2 hepatoma cells, activin (90 ng/ml) resulted in cell growth inhibition, with decreased [^{3}H]thymidine incorporation and cell number, which started at 24 h and continued for 5 days (148). Zauberman *et al.* (148) determined that the underphosphorylated form of pRb started to accumulate after a 9-hr incubation with activin, which suggested that activin's growth inhibitory effect was mediated at least in part by inhibition of pRb phosphorylation. Based on detection by Western blot, CDK4 levels decreased 3-fold following the 9-hr incubation with activin, whereas p21 protein increased starting at 3 hr and continued with time until levels reached 16-fold at 24 hr. A 2-fold increase in p21 mRNA was observed at 24 hr and was determined to be the result of transcriptional activation by the tumor suppressor protein p53. In fact, blocking p53 function by using a miniprotein, which oligermizes with p53 and prevents DNA binding, abrogated activin-induced transcription from the p21 promoter. These data indicate that in these cells, Rb hypophosphorylation via modulation of p53, p21, and CDK4 are involved in activin-mediated cell growth inhibition.

It was shown in our laboratory, and other laboratories, that activin treatment resulted in decreased human prostate cancer LNCaP cell growth, and that overexpression of activin inhibited proliferation, induced apoptosis, and decreased tumorigenecity in these cells (132). The growth inhibitory response to activin was dose and time dependent, with increasingly effective concentrations from 1 ng/ml (40% decrease) to 100 ng/ml (80% decrease) (149), and results observed from 24 hr to 5 days (149, 150). Coincubation with follistatin, an activin-binding protein, prevented the activin-induced cell growth inhibition (151). The tumor suppressor gene p53 was upregulated, whereas bcl-2 mRNA was downregulated in LNCaP cells 3 days following activin treatment (151). Whether bcl-2 downregulation was

a direct effect of p53 upregulation is unknown. It is a possibility because p53 was shown to downregulate *bcl-2* and upregulate *bax in vitro* and *in vivo* (152), and a p53-dependent negative response element was identified in the *bcl-2* gene (153). A recent study in our laboratory also determined that the p16 mRNA expression was also upregulated (154).

Yamato *et al.* (144) demonstrated that activin caused G1 cell cycle arrest followed by apoptosis in mouse B cell hybridoma cells. Activin (50 ng/ml) downregulated protein expression of cyclin D2, the only D-type cyclin expressed in the hybridoma cells, and upregulated p21 mRNA expression as early as 3 hr after activin treatment. This modulation resulted in decreased levels of cyclin D2/CDK4 complex and Rb phosphorylation. Expression of CDKs and p16 were not affected by activin treatment. However, overexpression of cyclin D2 partially removed activin's inhibition of Rb phosphorylation and G1 arrest. These results demonstrated that the mechanism of activin-mediated G1 arrest in these cells involves inhibition of CDK4-mediated Rb phosphorylation through combined modulation of cyclin D2 and p21 in hybridoma cells. Activin-induced apoptosis in hybridoma cells may be mediated through suppression of bcl-2 activity. Whereas activin upregulated bcl-x_s expression (155), upregulation of bcl-2 in these cells suppressed activin-mediated apoptosis (156).

Indeed, overexpression of the anti-apoptotic factor Bcl-2 suppressed activin-mediated apoptosis in B cell hybridoma (156). Furthermore, in the same cells, activin induces the expression of Bcl-Xs, a dominant negative repressor of Bcl-2 and Bcl-XL, both of which inhibit caspase activity (155). Further studies suggested that protein kinase C may be a downstream mediator in activin-induced apoptosis, as H7, a protein kinase C inhibitor, completely abolished activin-induced Bcl-Xs expression and apoptosis in B cell hybridoma (155). In human chronic myeloid leukemia KU812 cells, activin upregulates the expression of Bax, another repressor of Bcl-2, subsequently activates caspase-3 and caspase-9, and results in DNA fragmentation (147). Interestingly, continuous expression of Mcl-1, an anti-apoptotic member of the Bcl-2 family, changes cells response to activin. Instead of inducing cell death, activin treatment leads to hemoglobin accumulation and erythoid differentiation (147).

Carcinogenesis. Cancer growth is determined by the relationship between the rate of cell proliferation and the rate of cell death (157). Since cell cycle regulation depends largely on the Rb pathway, the importance of this regulatory mechanism in the development of human cancer cannot be overemphasized. Virtually all tumors have aberrant expression of at least one component of the Rb pathway (158). Examples include inactivation or loss of Rb in retinoblastomas, breast carcinoma, bladder carcinoma, osteosarcoma, and small-cell lung cancer (159); homozygous deletion or rearrangement of p16 in the majority of melanomas, gliomas, and leukemias (160); and cyclin D1 amplification in head and neck cancer, bladder cancer, small-cell lung cancer, breast cancer, and esophageal cancer (158).

Defects in the apoptosis mechanism also play an important role in tumor pathogenesis, allowing neoplastic cells to survive beyond their normal lifespan, thereby dismissing the need for exogenous survival factors and allowing the opportunity for genetic alterations to accumulate, resulting in deregulated cell proliferation, promoted angiogenesis, and increased cell motility and invasiveness during tumor progression (161). The anti-apoptotic effect of bcl-2 demonstrates its importance in cancer progression. Aberrations in the bcl-2 gene have been demonstrated in human cancers, including follicular non-Hodgkin's B cell lymphomas (162) and human breast cancer (163, 164). Also, bcl-2 expression was elevated in androgen-independent prostate cancers compared with normal prostate glands (165, 166). Bcl-2 has been implicated in resistance to therapy, and reductions in bcl-2 achieved by antisense method increased susceptibility of cancer cells to apoptosis induction by multiple chemotherapeutic drugs (161)

Because activin inhibits cell proliferation via modulation of Rb pathway components and enhancement of apoptosis via modulation of the bcl-2 family, it can be said to have an inhibitory function against cancer development. Activin may be involved in other aspects of cancer progression. As detected by mRNA differential display, activin exhibited mRNA expression in low-metastatic mouse melanoma cells, but not in high-metastatic melanoma cells (167), suggesting that the activin gene may be involved in metastasis.

The ability of a subset of cells within a primary tumor to metastasize is determined by a number of factors, including growth rate, adhesiveness, motility, secretion of degradative enzymes, and angiogenesis factors (158). The initial step of cancer metastasis is detachment of cells from the primary tumor mass. Development of increased metastatic capacity in glioma cells has been shown to correlate with decreased NCAM expression (168). Transfection of NCAM into the highly invasive MDA-MB-231 human breast cancer cell line resulted in a decreased capacity for penetration of the artificial basement membrane *in vitro*, longer tumor latency periods, and slower tumor growth *in vivo* (169). The addition of activin increased NCAM expression in embryonic chicken limb bud cells (170), suggesting that activin may help mediate cell-cell adhesion, thereby decreasing metastatic capacity.

Most tumors can induce angiogenesis for oxygen and nutrient supply and waste removal. Because angiogenesis also allows tumor cells to metastasize from the primary or secondary organ to distant organs, inhibition of angiogenesis may be an important approach for preventing tumor growth and metastasis (171). The possibility that activin may inhibit angiogenesis is based on activin's ability to inhibit growth in vascular endothelial cells (135) and is shown in a study whereby the activin-binding protein, follistatin, induced proliferation of human umbilical vein en-

dothelial cells and resulted in angiogenesis in the rabbit cornea (172). Because the transition of quiescent endothelial cells to the angiogenic phenotype enables the endothelial cells to engage in a series of activities, including proteolytic degradation of the surrounding extracellular matrix and invasion of the avascular tissue (97), activin's inhibitory effect on endothelial cell growth and angiogenesis further supports its possible preventive role against cancer progression and metastasis.

New Methods for Molecular Studies. Using the normal rat prostate gland and human prostate cancer cell lines, we have localized activin and activin receptors by immunohistochemistry, *in situ* hybridization, and RT-PCR, and we have conducted functional studies such as *in vitro* cell counting, BrdU incorporation, and apoptosis analysis, as well as *in vivo* tumorigenicity assay (6, 7, 149, 151). We have observed that activin βA and activin βB subunits and activin receptor I and II were expressed in normal rat prostatic epithelia (174), as well as in the three prostate cell lines (6, 7, 175). Furthermore, activin A inhibited growth and induced apoptosis specifically in the LNCaP cells, but not in the DU-145 and PC-3 cells. The induction of growth inhibition and apoptosis by activin was found to be associated with downregulation of bcl-2 and *c-myc*, and upregulation of p16 and p53, as well as several unknown genes (149, 151). In addition to growth change and apoptosis, activin A induced morphological changes in LNCaP cells as well. This was accompanied by upregulation of prostatic markers such as prostatic specific antigen (PSA), prostatic acidic phosphatase (PAP), and androgen receptor (149). The autocrine function of activin A was further demonstrated by overexpression of activin A in LNCaP cells, which confirmed observation that activin A inhibits growth and induces apoptosis. Furthermore, overexpression of activin in LNCaP cells reduced tumorigenicity *in vivo* (132).

In order to compare differential gene expression, we have further developed a method to generate full-length cDNA libraries from less than 20 LNCaP cells treated with or without activin (*in vitro*) (176), as well as from different stages of prostate cancer cells (*in vivo*) (177). This method is termed RNA-PCR (TCR) (Fig. 3) because the principle of the method relies upon the thermocycling steps of promoter-linked double-stranded cDNA synthesis and promoter-driven transcription to amplify mRNAs. Using a novel subtractive hybridization technique based on covalent bonding (176), we have identified differentially expressed genes in both *in vitro* (8) and *in vivo* systems (178). Recently, we have demonstrated that the mRNAs amplified from 20 LNCaP cells and total mRNAs prepared from the conventional method possess similar gene patterns as determined by the microarray technique, indicating that the RNA-PCR provides a simple and accurate way for clinicians and biologists to examine molecular profiling of gene expressions in small numbers of specific cell types of any specific biological system.

Using a novel antisense strategy, we have demonstrated

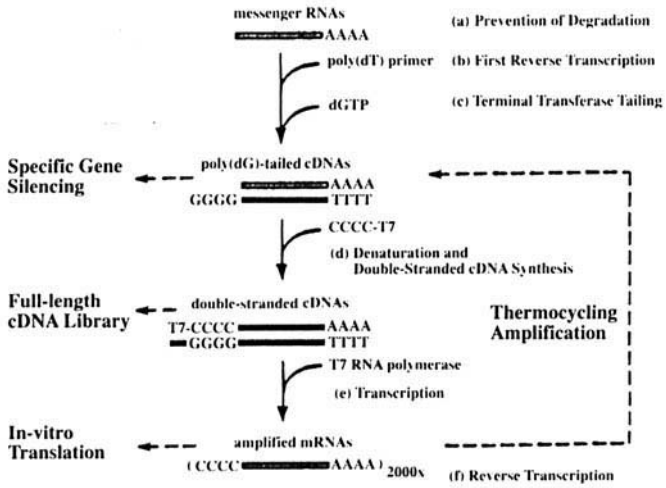

Figure 3. An illustration of the RNA-PCR thermocycling procedure. The cycling steps of d through f can be repeated at least once for the linear amplification of a mRNA library by *in vitro* transcription. Advantageously, the reactions of step a to f can be continuously performed in an RT & T buffer. The cycling of reverse and *in vitro* transcription reactions provides more flexibility for the enzymatic synthesis of single-stranded RNAs, RNA-DNA hybrids, and double-stranded DNAs that are ready for a variety of biochemical applications such as probe preparation for specific gene detection, full-length gene cloning, *in vitro* translation for protein synthesis, and gene knockout analysis through a post-transcriptional gene silencing mechanism.

the suppression of activin-induced apoptosis by the antisense to a differentially expressed gene, apoptosin, in human prostate cancer cells (179). Furthermore, we have developed a novel mRNA-cDNA interference phenomenon for silencing bcl-2 expression in human LNCaP cells (180). Our study demonstrates that ectopic transfection of a sequence-specific mRNA-cDNA hybrid (D-RNAi), rather than an antisense RNA (aRNA)-cDNA or a ds-RNA construct, induces specific intracellular gene silencing in human cells. We have successfully detected specific gene interference of bcl-2 expression by D-RNAi in human LNCaP prostate cancer cells. Therefore, the application of D-RNAi might have significant therapeutic potential in other varieties of cancer cells for preventing a specific gene expression such as bcl-2, increasing the susceptibility of these cancer cells to apoptotic stimuli, and thus reducing tumor growth. In addition, this method can also be used to determine the functional significance of DNA sequences that may be associated with chromosomes associated with urogenital diseases as identified by the genome project

Conclusions

Activin has a variety of biological functions, including inhibition of cell proliferation and enhancement of apoptosis in multiple *in vitro* and *in vivo* systems. Examination at the molecular level has revealed that these mechanisms involve activin receptors, Smad proteins, components of the cell cycle, and apoptosis. In addition to its effect on cell growth, activin's ability to increase cell adhesion protein levels and inhibit angiogenesis may be indicative of an in-

hibitory role against cancer progression and metastasis. Further investigation is needed to determine the molecular mechanisms whereby activin exerts its inhibitory effect on cell growth, apoptosis, and cancer progression. The RNA-PCR-derived molecular profile of specific cells, as well as D-RNAi, would provide useful tools.

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