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Review

TGF-β-dependent Cell Growth Arrest and Apoptosis

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Transforming growth factor beta (TGF-β) is a member of a large family of multifunctional secreted polypeptides that are potent regulators of cell growth, differentiation, and matrix production (Massague, 1998; Dijke *et al.*, 2000; Derynck *et al.*, 2001).

TGF-β was initially identified and named on the basis of its ability to stimulate fibroblast growth in soft agar, but it is now the best-studied growth inhibitory protein. Its molecular components at each step in the TGF-β signaling pathway have been identified as tumor suppressors (Massague, 1998). Over the last two decades, the TGF-β family has emerged as a major source of signals that control cell growth and differentiation (Massague *et al.*, 2000). Members of the TGF-β family produce different effects, depending on the type and state of the cell. The signals of TGF-β family members are transduced across the plasma membrane by the heteromeric interaction of two receptors, types I and II, which are serine/threonine kinases. The initiation of the signaling requires binding of TGF-β to the TGF-β type II receptor, a constitutively active serine/threonine kinase, which subsequently transphosphorylates the TGF-β type I receptor. The activated type I receptor initiates intracellular signaling through the activation of specific Smad proteins. Smads relay signals into the nucleus where they, together with other proteins, direct transcriptional responses (Massague, 2000).

In this review, we will focus on how TGF-β mediates cell growth arrest and apoptosis.

The Smad proteins are major TGF-β signal mediators

The Smad proteins are a family of transcription factors found in nematodes, insects, and vertebrates (Heldin *et al.*, 1997). They constitute the only well-known TGF-β signaling effectors (Derynck *et al.*, 1998; Piek *et al.*, 1999; Itoh *et al.*, 2000; Massague, 2000; Massague and Wotton, 2000; Miyazano *et al.*, 2000). Smad-related genes were first discovered through genetic screens in *Drosophila* and *Caenorhabditis elegans*. The name Smad is derived from the Sma and MAD gene homologues in *C. elegans* and *Drosophila* (Derynck *et al.*, 1996). To date, 10 Smad proteins have been identified, and can be classified into three groups (Massague, 1998). Receptor-regulated Smads (R-Smads) are phosphorylated by an activated type I receptor kinase on two serine residues in a SSXS motif at their extreme C-termini, after which they form heteromeric complexes with common partner Smads (Co-Smads) (Hoodless *et al.*, 1996; Lagna *et al.*, 1996; Macias-Silva *et al.*, 1996; Souchelnyskyi *et al.*, 1996; Zhang *et al.*, 1996; Kretzschmar *et al.*, 1997). The TGF-βs, activins, and nodals bind to receptors that phosphorylate Smad2 and Smad3. The BMPs and related GDFs, as well as AMH/MIS, engage receptors that signal through Smads 1, 5, and 8. One mammalian Co-Smad, Smad4, is known, and two distinct Co-Smads (Smad4a and Smad4b) have been identified in Xenopus (Howell *et al.*, 1999; Masuyama *et al.*, 1999). The inhibitory Smads (I-Smads: Smad6 and Smad7) act in opposition to R- and Co-Smads, forming stable associations with activated type I receptors, and preventing the phosphorylation of R-Smads. Smad7 is induced by TGF-β signaling, and provides a TGF-β-induced negative feedback loop (Hayashi *et al.*, 1997; Nakao *et al.*, 1997). In contrast, Smad6 is not induced, but is translocated from the nucleus to the cytoplasm by TGF-β signaling and functions as a negative regulator (Inamura *et al.*, 1997; Hata *et al.*, 1998).

Although the Smad protein family does not share homology with other known proteins, each member of the family contains highly conserved N- and C-terminal domains that are separated by a proline-rich linker of variable length and sequence. The N-terminal domain has been called the Mad-homology domain 1 (MH1), and the C-terminal domain has been designated the Mad-homology domain 2 (MH2). The MH2 domain is important for homo- and heteromeric complex formation, and for transcriptional activation and repression. The MH1 domain

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has intrinsic DNA-binding activities, and also associates with other transcription factors (Shi *et al.*, 1997; Shi *et al.*, 1998; Dennler *et al.*, 1999). I-Smads have conserved MH2 domains that are sufficient for type I receptor interaction and inhibitory activity. The N-termini of I-Smads might be responsible for their signaling specificity.

TGF-β-mediated cell growth arrest

TGF-β is a potent inhibitor of growth in most epithelial cells, including many carcinomas in culture, endothelial cells, hepatocytes, lymphocytes, and myeloid cells (Coffey *et al.*, 1988; Laiho *et al.*, 1990; Moses *et al.*, 1990; Newman, 1990; Massague *et al.*, 1992; Ewen, 1994). Conversely, TGF-β is generally mitogenic in mesenchymally-derived cells (Battegay *et al.*, 1990). TGF-β-mediated growth arrest occurs by blocking all cycle transits at mid- and late-G1 phase of the cell cycle; the mechanism of this arrest is cell-type specific. In most cases, this arrest is reversible, but in some cases it is associated with terminal differentiation or programmed cell death.

Two classes of anti-proliferation gene responses are involved in TGF-β-mediated growth arrest: inactivation of cyclin-dependent kinases (cdks) and down regulation of c-myc.

Inactivation of cyclin-dependent kinase

Cell cycle transitions are governed by a family of cdks, whose activity is up-regulated by association with positive regulators, the cyclins, and down-regulated by association with negative regulators, the cdk inhibitors. Passage through G1 into the S phase is regulated by the activities of cyclin D-, E-, and A-cdks (Sherr, 1993, 1996; Weinberg, 1995).

In the mammalian cell cycle, promotion of the transition from G1 to the S phase (initiation of DNA synthesis) by mitogens is cooperatively regulated by cyclin D-cdk4, cyclin D-cdk6, cyclin E-cdk2, and cyclin A-cdk2. Cdk activity is tightly regulated by diverse mechanisms, including changes in the levels of cyclin, cdk2 phosphorylation of positive and negative regulatory sites, and interaction with stoichiometric inhibitors. Cdk inhibitors (CKIs) that govern these events have been assigned to one of two families, based on their structures and cdk targets (Sherr and Roberts, 1995, 1999). The first class, which specifically inhibits the catalytic subunits of cdk4 and cdk6, includes the INK4 proteins (inhibitors of cdk4, including $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$) (Serrano *et al.*, 1993; Guan *et al.*, 1994; Hannon and Beach, 1994; Hirai *et al.*, 1995; Chan *et al.*, 1995). The other class includes the Cip/Kip family, whose members act more broadly. All members of the Cip/Kip family, including $p21^{Cip1/WAF1}$, $p27^{Kip1}$, and $p57^{Kip2}$, contain conserved sub-domains within their amino terminus that enable them to bind to cyclins and cdks (El-Deiry *et al.*, 1993; Gu *et al.*, 1993; Harper *et al.*, 1993; Dulic *et al.*, 1994; Noda *et al.*, 1994; Polyak *et al.*, 1994a, 1994b; Toyoshima and Hunter, 1994; Lee *et al.*, 1995; Matsuoka *et al.*, 1995).

Progression through the cell cycle involves the phosphorylation of the retinoblastoma tumor suppressor gene product pRB (Weinberg, 1995). The generally accepted view is that cyclin D-dependent kinases initiate the pRB phosphorylation in the mid-G1 phase, after which cyclin E-cdk2 becomes active and completes the phosphorylation of pRB on additional sites (Ewen *et al.*, 1994; Matsushime *et al.*, 1994; Meyerson and Harlow, 1994; Kitagawa *et al.*, 1996). Cyclin A- and B-dependent cdks that are activated later in the cell cycle, maintain pRB in a hyperphosphorylated state in the next G1 phase (Ludlow *et al.*, 1990, 1993). The pRB hyperphosphorylation in late G1 disrupts its association with various E2F family members. This allows the coordinated transcription of a bank of genes whose activities are necessary for DNA synthesis (Dyson, 1998; Nevins, 1998).

TGF-β specifically induces an increase in a subset of cdk inhibitors, including p15, p21, and p27 (Fig. 1A). TGF-β causes the up-regulation of $p15^{INK4B}$ mRNA, and increased binding of the gene product to cdk4 and cdk6 in epithelial cells (Hannon and Beach, 1994; Reynisdottir *et al.*, 1995). This results in the release of p21cip/p27kip from cdk4/cdk6, and facilitates the association of $p21^{Cip}$/$p27^{Kip1}$ with cyclin E-cdk2 complexes (Reynisdottir and Massague, 1997). Induction of the $p15^{INK4B}$ expression in response to TGF-β is brought about by Smad-mediated transcriptional activation. TGF-β-activated Smad2 or Smad3 forms a complex with Smad4 and induce transcription by interacting with Sp1 at the $p15^{INK4B}$ promoter (Sandhu *et al.*, 1997; Feng *et al.*, 2000). p21 is known to be a transcriptional target of p53, and plays a role in senescence and differentiation, apoptosis, and the coordination of DNA damage repair with cell-cycle arrest. TGF-β also induces $p21^{Cip1}$ though a p53-independent mechanism (Datta *et al.*, 1995). Like $p15^{INK4B}$, TGF-β induces $p21^{Cip1}$ through Smad-mediated transcription. Smads mediate the enhancement of Sp1 affinity for the p21 promoter, independent of a direct association between Smads and DNA; Smad2, Smad3, and Smad4 physically interact with Sp1 through their MH1 domain (Pardali *et al.*, 2000). $p27^{Kip1}$ was identified as having inhibitory activity in cells that are arrested by TGF-β (Polyak *et al.*, 1994). The $p27^{Kip1}$ activity is increased in TGF-β-arrested cells and in contact-inhibited cells. In cells progressing from G_0 to the S phase, this inhibitory activity is highest in G_0, and decreases as cells enter the G1-to-S phase transition. Most of the p27 protein in a proliferating cell is found in association with cyclin D-cdk4/6 (Sherr, 1996). p27 is associated with cdk4/6 in proliferating cells until TGF-β induces p15. p27 is released by p15 from cyclin D-cdk4/6 and shuttled to cyclin E-cdk2, inhibiting this kinase (Fig. 1B). Binding of p27 can occlude a cdk2 complex from phosphorylation by the cdk-activating kinase (CAK). This may explain why TGF-β-treated cells lack cdk2 phosphorylation. TGF-β appears to inhibit cell growth by causing the association of p27 with cyclin/cdk2 to block its kinase activity, and arrest cells in G1. However, it also has been shown in certain studies that p27 is not essential for the

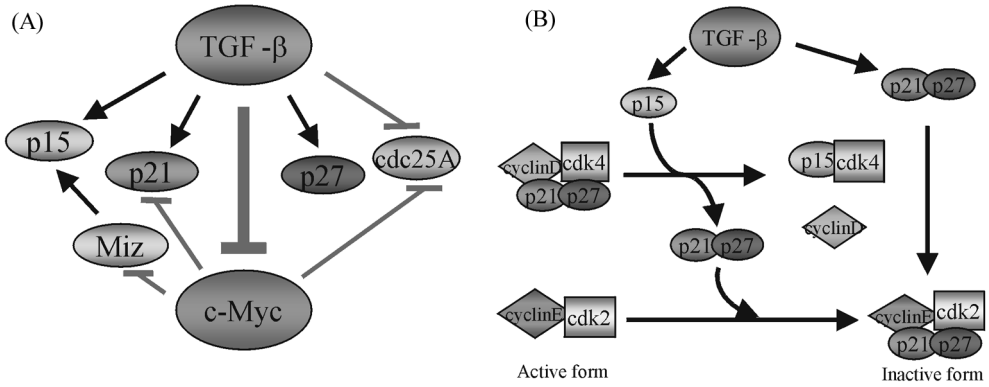

Fig. 1. The cell cycle arrest response to TGF-β A. Two classes of antiproliferative gene responses are known to be induced by TGF-β. The first is the cdk-inhibitory response that includes the induction of p15, p21, and p27, and the down-regulation of cdc25A. The second is the c-myc down-regulation that is observed in most cell types. B. The p15 binding to cyclin D-cdk4 leads to the shuttling of p27 from active cyclin D-cdk4-p27 to cyclin E-cdk2 complexes, resulting in their ultimate inhibition as well.

TGF-β-induced growth inhibition. TGF-β is still able to induce growth arrest in cells that are derived from mice with a homozygous deletion of p27; $p15^{-/-}$ or $p27^{-/-}$ mouse embryo fibroblasts also remain at least partly growth-inhibited by TGF-β (Nakayama *et al.*, 1996; Latres *et al.*, 2000), perhaps due to the presence of p21. Thus, TGF-β acts to arrest cell growth through multiple and probably compensatory mechanisms that are also cell type-dependent.

In addition, TGF-β prevents increases in cdk4 levels and down-regulates cdc25A. TGF-β induces suppression of cdk4 synthesis in a p53-dependent manner during G1 in mink lung epithelial cells (Ewen *et al.*, 1993) under the particular conditions of mitogen-deprived cell cultures that are replenished with serum. Regulation of cdk4 synthesis by both p53 and TGF-β is mediated by the 5-untranslated region (UTR) of the cdk4 mRNA (Ewen et al, 1995; Miller *et al.*, 2000). Cdc25A is a tyrosine phosphatase that removes inhibitory tyrosine phosphorylation from cdks. In keratinocytes and human mammary epithelial cells, TGF-β down-regulates cdc25A, which increases the level of tyrosine phosphorylation of cdk4/6 and ckd2, leading to the inactivation of these kinases (Iavarone and Massague, 1997, 1999).

TGF-β inhibits the expression of c-myc (Alexandrow and Moses, 1995). Cell growth, proliferation, and apoptosis is promoted by c-myc. Also, c-myc inhibits terminal differentiation; and when deregulated, is profoundly involved in the genesis of an extraordinarily wide range of cancers (Grandori and Eisenman, 1997; Facchini and Penn, 1998; Eisenman, 2001). The N-terminal fragment of Myc stimulates transcription when fused to a heterologous DNA binding domain, and the C-terminal basic-helix-loop-helix-zipper (bHLH-LZ) of Myc resembles those found in certain families of transcriptional factors. Myc heterodimerizes with another bHLH-LZ protein, Max, and interacts with the E-box sequence. The overexpression of c-myc blocks growth-inhibitory responses to TGF-β. The down-regulation of c-myc

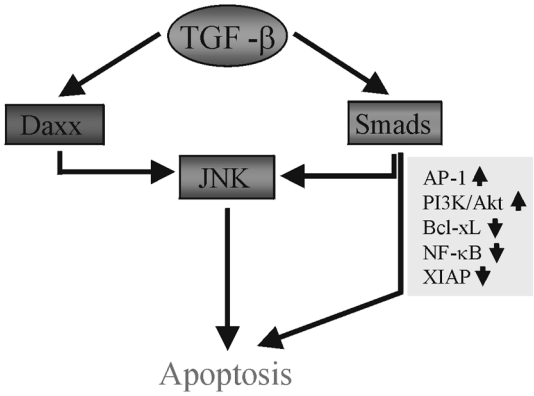

Fig. 2. Possible pathways of TGF-β induced apoptosis.

is required for the induction of $p15^{INK4B}$ and $p21^{Cip1}$ (Warner *et al.*, 1999; Claassen and Hann, 2000). The repression of the $p21^{Cip1}$ transcription by c-myc occurs at the promoter level in a region near the start site of transcriptional initiation, and it is independent of histone deacetylase activity. TGF-β prevents the recruitment of Myc to the $p15^{INK4B}$ transcriptional initiator by the Myc-interacting Zinc-finger protein 1 (Miz-1). Myc and Max form a complex with Miz-1 at the p15 initiator, and inhibit transcriptional activation by Miz-1. The repression relieves and enables transcriptional activation by a TGF-β-induced Smad protein complex that recognizes an upstream $p15^{INK4B}$ promoter region and contacts Miz-1. Thus, two separate TGF-β dependent pathways, Smad and Myc, keep tight control over the $p15^{INK4B}$ expression (Seoane *et al.*, 2001; Staller *et al.*, 2001) (Fig. 1A).

TGF-β induced apoptosis

TGF-β induces apoptosis in several cell types (Rotello *et al.*, 1991; Oberhammer *et al.*, 1992; Selvakumaran *et al.*, 1994; Chaouchi *et al.*, 1995; Landstorm et al, 2000; Larisch *et al.*,

2000; Patil *et al.*, 2000), including normal human B cells and a lymphoma B-cell line (Chaouchi *et al.*, 1995). The important role of TGF-β in orchestrating apoptosis in the liver is indicated by the hepatic fibrosis and apoptotic cell death of hepatocytes in transgenic mice that ectopically express TGF-β1 in the liver (McMahon *et al.*, 1986; Sanderson *et al.*, 1995; Arsora *et al.*, 1997). Current understanding of the mechanisms elicited by TGF-β is limited mostly to its effects on cell-cycle arrest. Thus, although TGF-β-induced apoptosis is a well-documented phenomenon in many different cell types, the biochemical mechanism responsible for mediating this death process is still poorly understood. Two independent pathways have been proposed for TGF-β-induced apoptosis, the Daxx adaptor pathway and the Smad pathway (Fig. 2). The Daxx adaptor protein is involved in TGF-β-induced apoptosis through its ability to interact with type II TGF-β receptors. The C-terminus of Daxx acts as a dominant negative inhibitor of TGF-β-induced apoptosis in B-cell lymphomas, and antisense oligonucleotides to Daxx inhibit TGF-β-induced apoptosis in mouse hepatocytes (Perlman *et al.*, 2001).

Smad4, an important mediator of TGF-β signaling, induces apoptosis through the c-Jun N-terminal kinase (JNK) signal pathway (Atfi *et al.*, 1997; Dai *et al.*, 1999). Smad7 can act as an effector of TGF-β-induced cell death, but can also protect cells from apoptosis, depending on the context (Ishisaki *et al.*, 1998; Landstorm *et al.*, 2000; Patil *et al.*, 2000; Lallemand *et al.*, 2001). Smad7 promotes apoptosis in prostate and keratinocyte cell lines, and inhibits the survival factor NF-κB and potentiates apoptosis in epithelial cells. However, Smad7 inhibits TGF-β-induced apoptosis in two B-cell lines, M1 and Hep3B. For example, Smad7 is induced by CD40 and protects $WEHI_{231}$ B-lymphocytes from the TGF-β-induced growth inhibition and apoptosis. The AP-1 complex appears to be involved in cell proliferation and survival. A role for this multi-component complex in apoptosis has also been suggested in some cell types (Karin *et al.*, 1997). Recent studies indicate that Smad3 directly binds c-Jun and c-Fos of the AP-1 complex (Zhang *et al.*, 1998). Both Smad3 and Smad4 bind all three Jun proteins-c-Jun, JunB, and JunD (Liberati *et al.*, 1999). Smad proteins and the AP-1 complex are involved in TGF-β1 signaling for apoptosis. The overexpression of a dominant negative Smad3 mutant or Smad7, both of which impair Smad-mediated signal transduction, inhibits TGF-β1-dependent apoptosis. Only the JunD-FosB form of the AP-1 complex is markedly activated during TGF-β1-induced apoptosis. FosB substantially enhances the Smad3-Smad4-dependent transcription, and dominant negative FosB blocks the TGF-β1-induced apoptosis, but not growth inhibition (Sanchez *et al.*, 1996, 1999; McDonald *et al.*, 1999; Yamamura *et al.*, 2000). These findings identify Smad proteins as key signal transducers in the TGF-β-dependent apoptosis. Pro-apoptotic gene targets of the Smads remain to be identified, although the $Bcl\text{-}X_L$ (Saltzman *et al.*, 1998; Chipuk *et al.*, 2001), caspase-8 (Chen and Chang, 1997; Shima *et al.*, 1999), and PI3K/Akt signaling pathways (Tanaka and Wands, 1996; Chen *et al.*, 1998; Chen *et al.*, 1999; Shih *et al.*, 2000**)** regulate apoptosis genes that are targets of Smad, and are activated by TGF-β.

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