TGF-β Family Signaling in the Control of Cell Proliferation and Survival

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The transforming growth factor β (TGF-β) family controls many fundamental aspects of cellular behavior. With advances in the molecular details of the TGF-β signaling cascade and its cross talk with other signaling pathways, we now have a more coherent understanding of the cytostatic program induced by TGF-β. However, the molecular mechanisms are still largely elusive for other cellular processes that are regulated by TGF-β and determine a cell's proliferation and survival, apoptosis, dormancy, autophagy, and senescence. The difficulty in defining TGF-β's roles partly stems from the context-dependent nature of TGF-β signaling. Here, we review our current understanding and recent progress on the biological effects of TGF-β at the cellular level, with the hope of providing a framework for understanding how cells respond to TGF-β signals in specific contexts, and why disruption of such mechanisms may result in different human diseases including cancer.

Since the discovery of the transforming growth factor β (TGF-β) family more than three decades ago, its biological activity has been a focal topic in the broad fields of cell proliferation and survival. TGF-β and other members of its family, which are evolutionarily conserved secreted proteins with widespread expression in both embryonic and adult tissues, control a variety of fundamental aspects of cellular behavior (Massagué 2000, 2012). In this review, we will focus on the biological effects of TGF-β at the cellular level, which represent an important example illustrating the molecular basis of how cells read extracellular signals to maintain their intrinsic balance and, as a result, tissue homeostasis.

For multicellular organisms, an individual cell's decision to survive and/or proliferate is not simply determined by the available nutrients in the surrounding environment but also controlled by a dense network of cell communication signals. These cell communication signals, mainly consisting of secreted polypeptides named cytokines, growth factors or hormones, play a central role in maintaining physiological tissue homeostasis. TGF-β and its family members—bone morphogenetic proteins (BMPs), nodal, activins, myostatin, and others, are particularly prominent among these cell communication signals and function as important regulators of cell proliferation and survival.

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At the cellular level, TGF-β stimulation induces cytostasis in almost all non-neoplastic epithelial cells, as well as in endothelial cells, hematopoietic cells, neuronal cells and certain types of mesenchymal cells (Siegel and Massagué 2003). However, this cytokine is able to promote proliferation of other mesenchymal cell types such as kidney fibroblasts and smooth muscle cells (Roberts et al. 1985; Battegay et al. 1990). In addition, regulating cell proliferation only represents one aspect of TGF-β's many effects at the cellular level. TGF-β has been reported to either induce or suppress programmed cell death in different cell types (Schuster and Krieglstein 2002), although a consensus is still lacking in terms of a coherent mechanism for TGF-β to regulate apoptosis. In addition, in recent years, several studies indicate that TGF-β plays a role in mediating cell dormancy (Salm et al. 2005; Yamazaki et al. 2011; Gao et al. 2012; Bragado et al. 2013) and autophagy (Kiyono et al. 2009; Ding et al. 2010; Koesters et al. 2010), two biological processes that regulate cell survival and are closely linked with tumor progression. Under certain conditions, TGF-β can also induce cellular senescence, an irreversible form of cell-cycle arrest that is usually associated with a specific cellular secretome (Katakura et al. 1999; Tremain et al. 2000).

The versatility of TGF-β signaling function in different cell types has drawn great attention from both scientists and clinicians during the past three decades. Although we have now accumulated a significant amount of knowledge on the molecular details of TGF-β signaling in certain cell types, it remains an essential question to illustrate all the context-dependent mechanisms that govern the specificity of TGF-β signaling in a given target cell. Answering this question is important for us to understand how TGF-β signaling orchestrates the growth and homeostasis of a whole tissue, in which multiple cell types organize together in a highly ordered manner. Here we will discuss the known molecular mechanisms by which TGF-β regulates cell proliferation and cell survival, with the hope of providing a framework to understand how different cells respond to TGF-β signals in their specific contexts, and why disruption of such mechanisms may result in different human diseases including cancer.

THE TGF-β SIGNALING PATHWAY AND ITS CONTEXT-DEPENDENT REGULATION

The main signal transduction pathway that conveys TGF-β inputs from the membrane receptor to its target genes has been well established. This signaling cascade and its cell-context-dependent regulation by cell-type-specific factors and other signaling pathways set the basis for understanding the different effects of TGF-β in controlling cell proliferation and survival in different individual cells.

As outlined in other recent reviews, the TGF-β signaling cascade is initiated when an activated extracellular TGF-β ligand brings together two pairs of receptor serine/threonine kinases, TGF-β type II receptor (TβRII) and TGF-β type I receptor (TβRI), to form a complex. Formation of this complex results in phosphorylation and activation of type I receptors, thereby allowing binding of receptor-regulated (R-) Smads. Subsequently, R-Smads are activated through phosphorylation at carboxy-terminal serines and released from the receptor complexes. Activated R-Smads then translocate into the nucleus and form transcriptional complexes with the common partner Smad4 and a variety of other DNA-binding cofactors, as well as non-DNA-binding coactivators and corepressors, which function together with Smad proteins to regulate the expression of particular target genes (Fig. 1A) (Derynck and Zhang 2003; Shi and Massagué 2003). Depending on the abundance and activities of extracellular TGF-β ligands, the composition of the activated receptor complexes, the intracellular expression pattern of receptor-interacting and Smad-interacting proteins, and the interactions with sequence-specific cotranscription factors, this signaling pathway can generate hundreds of cell-specific gene responses (Feng and Derynck 2005; Massagué 2012). In addition, activated TGF-β receptor complexes can also function through Smad-independent, noncanonical pathways in certain cell types, adding another layer of complexity to TGF-β signaling (Derynck and Zhang

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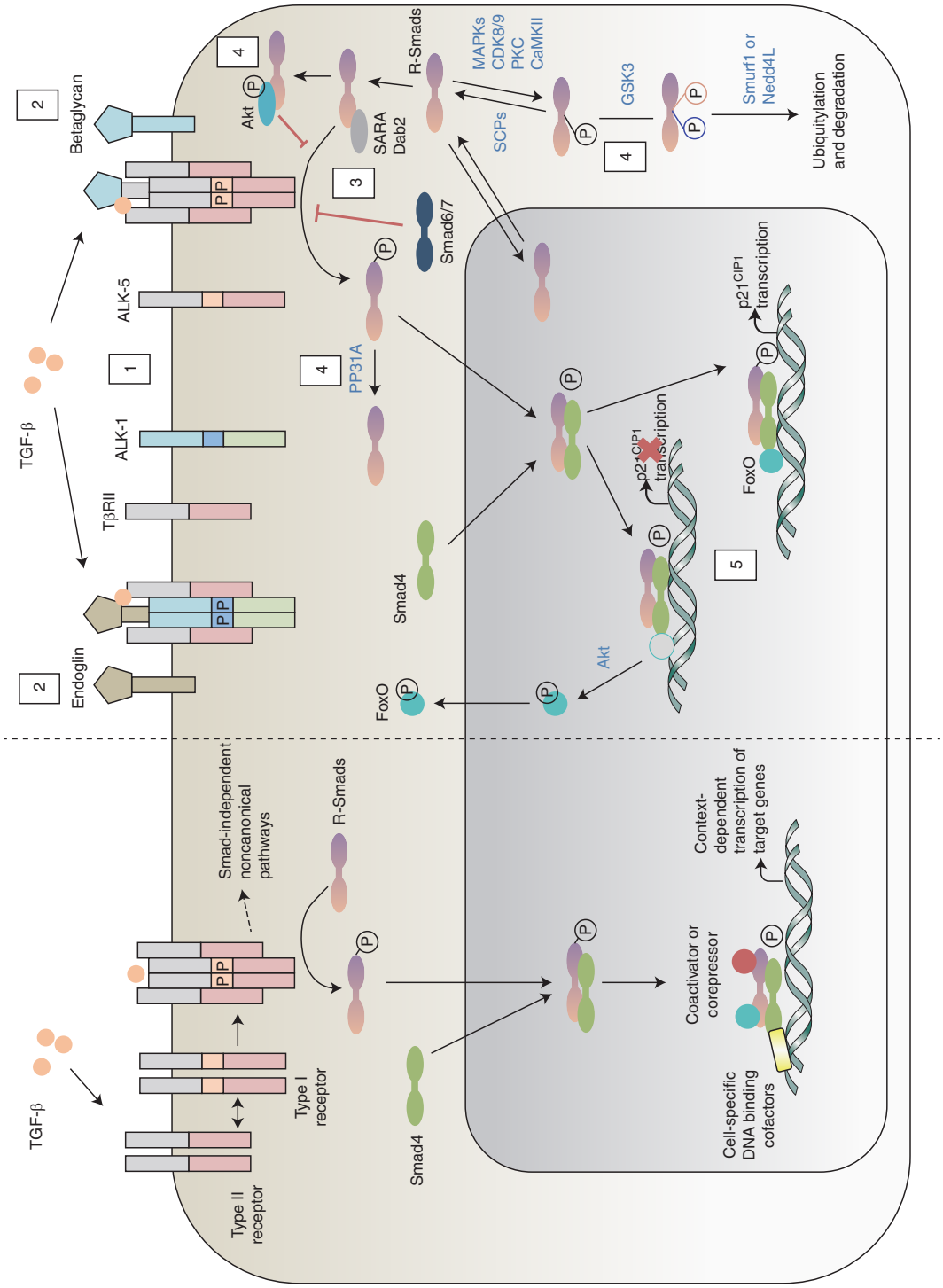

Figure 1. (*Legend on following page.*)

2003). Nevertheless, this context-dependent nature lays the foundation for understanding the perplexing observation that TGF-β can exert opposing effects in different cell types. In the following sections, we will summarize some of the context-dependent regulatory mechanisms of this signaling pathway and their correlations with the control of cell proliferation and survival (Fig. 1B).

Regulation of TGF-β Signaling through TGF-β Receptors

The human genome encodes seven type I TGF-β family receptors (activin receptor-like kinases 1-7 [ALKs 1-7]) and five type II receptors (ActRIIA, ActRIIB, BMPRII, AMHRII, and TβRII). Different combinations of paired type I and type II receptors allow for diverse ligand binding as well as intracellular signaling (Feng and Derynck 2005). As a result, the composition of the activated TGF-β receptor complexes represents an important level of regulation that determines the response of a cell. The promotion or suppression of endothelial cell proliferation by TGF-β is an example of how different receptor complexes establish different cellular responses. Although TGF-β induces growth inhibition on cultured endothelial cells (Takehara et al. 1987), TGF-β signaling is required for vasculogenesis and angiogenesis during development, and elicits an angiogenic response in vivo (Dickson et al. 1995; Larsson et al. 2001; Lebrin et al. 2005). Endothelial cells express two types of TGF-β type I receptors: ALK-1, which activates Smad1, 5, and 8, and ALK-5, also known as TβRI, which activates Smad2 and 3 (Oh et al. 2000). It has been shown that activated ALK-1 leads to increased proliferation and migration, whereas ALK-5 signaling suppresses these processes. The selection between these two type I receptors is likely because of different concentrations of TGF-β, with ALK-1-induced signaling through Smad1, 5, and 8 activated at higher doses of TGF-β (Goumans et al. 2002).

The expression of accessory proteins at the cell membrane that regulate the binding efficiency and specificity of TGF-β for their receptors can also influence the downstream responses. For example, in the case of endothelial cells, efficient activation of the ALK-1-Smad1/5/8 pathway requires the expression of a membrane glycoprotein endoglin. Increased expression of endoglin in endothelial cells shifts the TGF-β response from the ALK-5 pathway to the ALK-1 pathway, leading to the promotion of endothelial cell proliferation (Lebrin et al. 2004). Betaglycan, also known as TβRIII, is another transmembrane protein that functions as a reservoir of the TGF-β ligand for TGF-β receptors, and enhances cellular responsiveness to TGF-β when up-regulated at the cell surface by the PDZ protein GIPC (Gα interacting protein (GAIP) interacting protein carboxyl terminus) (Blobe et al. 2001). In addition, the cytoplasmic domain of betaglycan can be phosphorylated by TβRII, triggering the association of β-arrestin with betaglycan and subsequent internalization of both betaglycan and TβRII, which in turn results in suppressed TGF-β signaling (Chen et al. 2003).

Certain proteins are known to regulate the recruitment and access of R-Smads to TGF-β receptors. One of these, Smad anchor for receptor activation (SARA), regulates the subcellular localization of R-Smads and enhances their binding to receptors (Tsukazaki et al. 1998). In another example, Disabled-2 (Dab2) inter-

Figure 1. (*Figure on previous page.*) The transforming growth factor β (TGF-β) signaling pathway and its context-dependent regulation. (*Left*) A schematic diagram of TGF-β signaling. (*Right*) TGF-β signaling is regulated at several levels by context-dependent factors: (1) Different combinations of paired type I and type II receptors allow for diverse ligand binding as well as intracellular signaling. (2) Accessory proteins at the plasma membrane that regulate the binding efficiency and specificity of TGF-β to their receptors influence downstream responses. (3) Proteins that regulate the recruitment and access of R-Smads to TGF-β receptors. (4) Several proteins regulate TGF-β signaling by posttranslational modification of R-Smads or by preventing their association with TGF-β receptors. (5) A specific TGF-β response can be determined by the expression and activity of transcription cofactors.

acts with R-Smads and stabilizes the receptor-Smad interactions (Hocevar et al. 2001). On the other hand, inhibitory Smads (Smad6 and Smad7) compete with R-Smads for association with the receptor complexes and thus decrease R-Smad phosphorylation and activation (Hayashi et al. 1997; Imamura et al. 1997; Nakao et al. 1997). In addition to their competitive regulation of receptor-Smad interaction, inhibitory Smads also recruit Smurf E3 ubiquitin ligases to type I TGF-β receptors, leading to their proteasomal degradation and reduced TGF-β signaling (Kavsak et al. 2000; Ebisawa et al. 2001; Murakami et al. 2003). Interestingly, the expression of Smad6 and Smad7 is induced both by TGF-β signaling as a feedback mechanism and by other signaling pathways as nodes for signaling cross talk (Miyazono et al. 2000). For example, Smad7 expression can be induced by either the JAK-STAT pathway in response to interferon-γ (Ulloa et al. 1999) or by the NF-κB pathway in response to inflammatory cytokines (Bitzer et al. 2000).

Regulation of TGF-β Signaling by Intracellular Kinases and Phosphatases

Although phosphorylation at the carboxyl terminus by type I receptors is essential for R-Smad activation, these proteins can also be phosphorylated in their linker region that connects the DNA-binding domain and the transcriptional activation domain. Indeed, these modifications by various other kinases provide important mechanisms that integrate multiple cellular signaling pathways. For example, mitogen-activated protein kinases (MAPKs) are able to phosphorylate Smad1, Smad2, and Smad3 in their linker regions, leading to impaired nuclear translocation and reduced TGF-β responses (Kretzschmar et al. 1997). Interestingly, glycogen synthase kinase 3 (GSK3) can further phosphorylate the MAPK-primed Smad1 linker region, resulting in recognition of Smad1 by the E3 ubiquitylation ligases Smurf1 or Nedd4L, and subsequent degradation (Fuentealba et al. 2007; Gao et al. 2009). Activation of canonical Wnt signaling induces GSK3 inactivation, which augments the stability of activated Smad1, resulting in cooperation between Wnt and TGF-β family signaling pathways. In addition, the G_1 cyclin-dependent kinases CDK4 and CDK2 also phosphorylate Smad3 in the linker region and suppress its transcriptional activity (Matsuura et al. 2004). Similarly, protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent kinase II (CaMKII) can also phosphorylate Smad3 and Smad2, respectively, leading to attenuated TGF-β signaling (Wicks et al. 2000; Yakymovych et al. 2001). Additionally, R-Smads that are engaged in transcription become rapidly phosphorylated in the linker region by CDK8 and CDK9, two nuclear CDKs belonging to the transcriptional mediator and elongation complexes, respectively. This phosphorylation facilitates the recruitment of additional transcription cofactors but at the same time primes the linker region for GSK3-mediated phosphorylation and subsequent degradation. This process is considered an integral part of the TGF-β pathway to terminate signaling (Alarcon et al. 2009; Aragon et al. 2011).

On the other hand, several Smad phosphatases have been identified that reverse Smad phosphorylation. For example, protein phosphatase, Mg^{2+}/Mn^{2+}-dependent, 1A (PPM1A) can dephosphorylate Smad2 and Smad3 at their carboxyl-terminal sites, and ectopic PPM1A expression efficiently abolishes TGF-β-induced cytostatic effects (Lin et al. 2006). Conversely, the small carboxy-terminal domain (CTD) phosphatases, SCP1, SCP2, and SCP3, dephosphorylate the linker regions of R-Smads and thus prolong the duration of activation of Smad proteins in the transcriptional complex (Sapkota et al. 2006).

There are also kinases known to regulate Smad signaling independently of their kinase activity. An important example in this category is protein kinase B (PKB), also known as Akt, which does not phosphorylate, but instead directly interacts with Smad3 to inhibit its activation. Although the phosphorylation of Akt is not absolutely necessary for Akt-Smad3 binding, it enhances this interaction in vivo. It has been shown that the ratio of Akt to Smad3 regulates the sensitivity of cells to TGF-β-mediated apoptosis, demonstrating a mechanism of

phosphoinositide 3-kinase (PI3K)-Akt pathway cross talk with the TGF-β pathway (Conery et al. 2004; Remy et al. 2004).

Regulation of TGF-β Signaling by Cooperating Transcription Factors

By themselves, Smads only have a low affinity for DNA. Thus, the transcriptional regulation of TGF-β target genes requires additional transcription factors that have high affinity with DNA and cooperate with Smads. For example, FoxH1 or FAST1, a forkhead (Fox) transcription factor, was the first transcription factor identified to cooperate with Smads in mediating TGF-β signaling. Association of FoxH1 with Smads allows their recognition of activin response elements (AREs) in the promoter regions of genes regulating mesoderm differentiation (Chen et al. 1997). Similarly, forkhead subfamily FoxO proteins have been shown to associate with Smad3-Smad4 complexes in epithelial cells and induce the expression of the CDK inhibitors $p21^{CIP1}$ and $p15^{INK4B}$, and thus play an important role in determining the cytostatic effects of TGF-β (Seoane et al. 2004). Accumulating evidence indicates that Smads interact with a diverse group of transcription factors. A comprehensive review of these cooperating transcription factors can be found elsewhere (Feng and Derynck 2005).

The existence of many Smad-binding transcription partners not only results in a diverse set of biological effects of TGF-β signaling in different cellular contexts, but also provides another entry point for signaling cross talk. For example, Akt is able to phosphorylate FoxO proteins, resulting in FoxO translocation from the nucleus to the cytoplasm and effectively preventing their association with Smads to induce $p21^{CIP1}$ expression (Accili and Arden 2004). This molecular mechanism partially explains the observation that during augmented PI3K-Akt signaling a cell can lose responsiveness to TGF-β-mediated growth inhibition. Another example is the cooperation between TGF-β and Wnt signaling through the interaction of their downstream transcription factors. Wnt signaling is mediated by several transcription factors, such as LEF1 and TCF, and their coactivator β-catenin. At the *Xenopus* twin promoter, Smad3 and Smad4 associate with LEF1 to activate gene expression in a synergistic manner (Labbé et al. 2000; Nishita et al. 2000). At the *MYC* promoter of the gene encoding c-Myc, BMP is able to induce interaction of Smad1 with TCF4 and β-catenin to stimulate its transcription (Hu and Rosenblum 2005).

TGF-β EFFECTS ON CELL PROLIFERATION

Suppression of Cell Proliferation through TGF-β Signaling

One of the well-studied functions of TGF-β is its cytostatic effect. The earliest evidence demonstrating the growth inhibitory effect of TGF-β can be traced back to the mid-1980s when multiple groups reported that exposure to TGF-β led to the cell-cycle arrest of a variety of cultured cell types including epithelial cells, endothelial cells, hematopoietic cells, and skin keratinocytes (Tucker et al. 1984; Roberts et al. 1985; Takehara et al. 1987; Coffey et al. 1988). Shortly after, transgenic mouse models provided additional evidence to show the cytostatic effects of TGF-β on mammary gland epithelial cells and several types of T lymphocytes (Shull et al. 1992; Pierce et al. 1993). Although the cytostatic effect of TGF-β has been observed in multiple cell types, most knowledge on the molecular mechanisms was discovered using epithelial cells.

A typical eukaryotic cell cycle contains four major phases: nuclear division (M phase), DNA synthesis (S phase), a gap between M and S phases named G_1 phase, and another gap between S and M phases named G_2 phase. Studying the cytostatic effects of TGF-β signaling helped define a landmark in cell-cycle regulation, the restriction (R)-point, which occurs in the mid-late G_1 phase and represents a specific time window during which cells sense and interpret extracellular signals to decide whether or not to undergo cell division (Planas-Silva and Weinberg 1997). As revealed in early studies and later transcriptional profiling of TGF-β-treated epithelial cells, TGF-β causes cell-cycle arrest at

the R-point in the G_1 phase mainly through two interconnected processes, the repression of expression of certain growth-promoting transcription factors and the induction of expression of specific CDK inhibitors.

TGF-β suppresses the expression of several key transcription factors regulating growth control including the growth-promoting transcription factor c-Myc and cell differentiation inhibitors Id1, 2, and 3 (Fig. 2A) (Alexandrow and Moses 1995; Kang et al. 2003). Repression of *MYC* expression represents a central event in the TGF-β cytostatic response. Indeed, cells that fail to down-regulate c-Myc expression are usually resistant to the growth inhibition mediated by TGF-β (Chen et al. 2001). Mechanistically, this repression depends on direct Smad3 binding to a specific region of the *MYC* promoter, the TGF-β inhibitory element (TIE), which contains a repressive Smad-binding element (RSBE) and a consensus E2F-binding site. At this region, a repressor complex containing Smad3, E2F4/5, DP1, and corepressor p107, which is preassembled in the cytoplasm and translocated into the nucleus in response to TGF-β stimulation, can stably bind to DNA and thus repress *MYC* transcription (Chen et al. 2002; Yagi et al. 2002; Frederick et al. 2004). Interestingly, the decrease of c-Myc expression not only directly prevents cells from entering a growth-promoting state (Eisenman 2001), but also primes the promoters of several important CDK inhibitor genes so they become responsive to further transcriptional activation in response to the same TGF-β signals. The latter process will be discussed in the following sections.

Transcription factors belonging to the Id family are another example of growth-promoting transcription factors repressed by TGF-β. The Id proteins including Id1, Id2, and Id3, suppress the cell differentiation process and promote cell proliferation. Specifically, Id1 inhibits E2A-dependent expression of $p21^{CIP1}$ and, in turn, increases the phosphorylation of Rb protein by alleviating the inhibition of CDK2 expression (Ruzinova and Benezra 2003; Perk et al. 2005; Lasorella et al. 2014). In epithelial cells, the expression of activating transcription factor 3 (ATF3) can be induced by TGF-β, and ATF3 then forms a repressor complex consisting of Smad3, Smad4 and ATF3, which in turn silences the *Id1* promoter (Kang et al. 2003). Using a different mechanism, *Id2* expression is suppressed as a consequence of down-regulation of *c-Myc* expression by TGF-β (Siegel et al. 2003).

The induction of expression of CDK inhibitors is another important mechanism by which TGF-β causes cell-cycle arrest (Fig. 2B). It is known that the $G_1 \rightarrow S$ transition requires activated CDKs including CDK2, CDK4, and CDK6. To drive this progression, CDK2 must bind cyclin E to phosphorylate its substrates, whereas CDK4 or CDK6 needs to bind cyclin D. INK4 (inhibitor of cyclin-dependent kinase 4) protein family proteins, which include $p15^{INK4B}$, specifically bind to CDK4 and CDK6 proteins, inhibit their binding to cyclins, and thus inhibit cell-cycle progression. Alternatively, CDK inhibitors of the CIP/KIP family, including $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$, interact with cyclin-CDK complexes and inhibit their kinase activities (Morgan 1997; Murray 2004). At the core of TGF-β's cytostatic program is the transcriptional induction of expression of the CDK inhibitors $p21^{CIP1}$ (Datto et al. 1995; Reynisdottir et al. 1995) and $p15^{INK4B}$ (Hannon and Beach 1994). As mentioned previously, c-Myc inhibits the expression of $p15^{INK4B}$ and $p21^{CIP1}$ in proliferating cells by forming a complex with the zinc-finger protein Miz1, and binding to the promoters of these genes. Suppression of *MYC* expression by TGF-β limits the amount of c-Myc available to repress $p15^{INK4B}$ and $p21^{CIP1}$, and thus allows for enhanced transcriptional induction of these genes (Seoane et al. 2001, 2002; Staller et al. 2001).

To further induce the expression of $p21^{CIP1}$ and $p15^{INK4B}$, at least two Smad-containing transcription complexes have been identified. At the distal region of the *CDKN1A* promoter that drives $p21^{CIP1}$ expression, the Smad3/4 complex interacts with FoxO subfamily proteins and the resulting Smad-FoxO complex can efficiently induce the expression of $p21^{CIP1}$ (Seoane et al. 2004). At the proximal region of the *CDKN1A promoter*, however, Smad3 interacts with Sp1, a Zn finger transcription factor, to

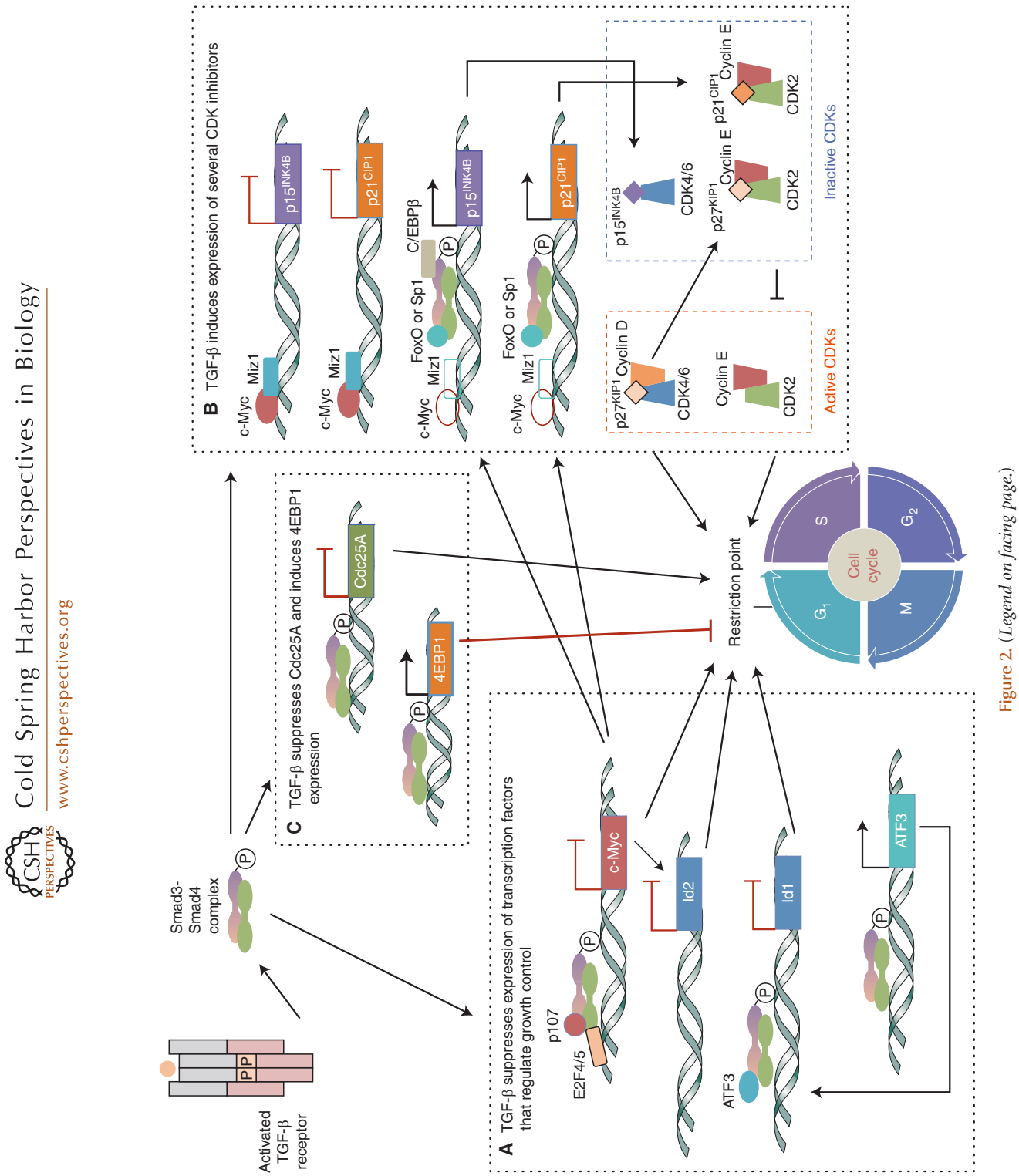

Figure 2. (*Legend on facing page.*)

activate $p21^{CIP1}$ expression (Pardali et al. 2000). Similarly, for the induction of the *CDKN2B* gene encoding $p15^{INK4B}$, a transcriptional complex containing Smads, FoxO proteins, and C/EBPβ are sufficient to up-regulate *CDKN2B* expression (Gomis et al. 2006). The interaction of Smads with Sp1 at the *CDKN2B* promoter also contributes to the induction of this gene in response of TGF-β (Feng et al. 2000). It remains to be determined whether these mechanisms operate concurrently to regulate $p21^{CIP1}$ and $p15^{INK4B}$ expression, or function in a cell-type-specific manner. In addition, TGF-β can increase the $p27^{KIP1}$ activity without changing its expression level (Polyak et al. 1994), partly because increased $p15^{INK4B}$ disrupts the existing $p27^{KIP1}$-CDK4/6-cyclin-D complexes and leads to $p27^{KIP1}$ redistribution. When bound to cyclin D in the $p27^{KIP1}$-CDK4/6-cyclin-D complexes, $p27^{KIP1}$ is inactive. However, free $p27^{KIP1}$ binds to cyclin E-CDK2 complexes and inhibits the kinase activity of CDK2 (Reynisdottir and Massagué 1997).

Other TGF-β-mediated cytostatic pathways exist in addition to the suppression of growth-promoting transcription factor expression and the induction of CDK inhibitors (Fig. 2C). For example, TGF-β represses the expression and activity of Cdc25A, the CDK tyrosine phosphatase that removes inhibitory phosphates on CDKs to promote G_1/S and G_2/M transitions. In keratinocytes, this process is mediated by a repressor complex consisting of E2F4-p130 complex and histone deacetylase 1 (Iavarone and Massagué 1997). In mammary epithelial cells, TGF-β stimulates $p160^{ROCK}$ translocation into the nucleus through a Smad-independent mechanism, which results in the inhibitory phosphorylation of Cdc25A (Bhowmick et al. 2003). TGF-β can also regulate the proteosomal degradation of Cdc25A by enhancing the association of Cdc25A with the ubiquitin ligase complex $SCF^{\beta-TrCP}$, consisting of Skp1, Cullin1 and an F-box $protein^{\beta-TrCP}$ (Ray et al. 2005). In addition to the regulation of Cdc25A, the translation initiation factor 4E-binding protein 1 (4EBP1), a translation-inhibitory protein that suppresses cell growth and proliferation by regulating the assembly of the multisubunit eukaryotic translation initiation factor-4F (eIF4F), has also been reported to mediate TGF-β-induced cytostasis in a Smad4-dependent manner (Azar et al. 2009).

It should be noted that the mechanisms discussed above are mainly delineated using epithelial cells. Less is known about the TGF-β cytostatic program in hematopoietic and glial cells, two cell types that show consistent cell-cycle arrest in response to TGF-β. In hematopoietic cells, it appears that the TGF-β-induced growth-inhibitory pathways generally parallel those in epithelial cells. However, the induction of $p57^{KIP2}$ expression in response to TGF-β is likely to be the most important downstream event to suppress hematopoietic cell proliferation (Scandura et al. 2004). In astrocytes, TGF-β suppresses cell proliferation by inducing $p15^{INK4B}$ expression in a Smad3-dependent manner (Rich et al. 1999).

The antiproliferative mechanisms mediated by TGF-β signaling provide a foundation for understanding many somatic mutations in human diseases, especially cancer. For example, the well-characterized proto-oncogenes *SKI* and *SKIL* (encoding SnoN) encode TGF-β antagonists that physically interact with R-Smads and disrupt their transcriptional complexes in carcinoma cells (Deheuninck and Luo 2009). However, some tumor suppressor genes, like *RUNX3*, cooperate with TGF-β for growth in-

Figure 2. (*Figure on facing page.*) The transforming growth factor β (TGF-β)-mediated cytostatic program in epithelial cells. (*A*) TGF-β suppresses the expression of transcription factors that regulate growth control including those encoding c-Myc and Id family members. (*B*) TGF-β induces the expression of several cyclin-dependent kinase (CDK) inhibitors, including $p15^{INK4B}$ and $p21^{CIP1}$. TGF-β also increases $p27^{KIP1}$ activity without changing the transcription of *CDKN1B* gene, partly because increased $p15^{INK4B}$ disrupts existing $p27^{KIP1}$-CDK4/6-cyclin D complexes. (*C*) TGF-β also suppresses cell-cycle progression by repressing the expression and activity of Cdc25A and inducing expression of 4EBP1.

hibition. *RUNX3* is frequently inactivated by allelic loss or promoter hypermethylation in human gastric cancers. At least in stomach epithelial cells, the RUNX3 protein is required for the TGF-β-induced expression of $p21^{CIP1}$ (Ito and Miyazono 2003; Chi et al. 2005). Genes for several other core components of the TGF-β signaling cascade are also mutated or deleted in tumors from cancer patients. Indeed, the original name of Smad4 was "deleted in pancreatic carcinoma, locus 4 (DPC4)" because *DPC4* is homozygously deleted in about 50% of pancreatic cancer patients (Hahn et al. 1996). *SMAD2* mutations have also been found in human lung and colorectal cancers (Eppert et al. 1996; Uchida et al. 1996).

Promotion of Cell Proliferation through TGF-β Signaling

Although TGF-β induces cytostasis in multiple cell types, under certain conditions, it stimulates proliferation of endothelial cells and several types of mesenchymal cells. As discussed previously, endothelial cells display both proliferative and antiproliferative responses to TGF-β, mainly depending on the concentration of this cytokine. For smooth muscle cells and certain fibroblasts, TGF-β functions as an indirect mitogen by inducing the expression of different growth-promoting factors. For example, TGF-β treatment of smooth muscle cells induces the expression of platelet-derived growth factor (PDGF) (Battegay et al. 1990). The autocrine stimulation of PDGF then promotes cell proliferation in this cell type. Similarly, TGF-β induces proliferation of renal fibroblasts by inducing the expression of basic fibroblast growth factor 2 (FGF-2) (Strutz et al. 2001). It is still not clear why these cells are not sensitive to TGF-β-induced cell-cycle arrest in the first place. However, TGF-β was found to induce a strong and prolonged expression of SnoN, a negative regulator of TGF-β signaling, in some fibroblasts. For example, prolonged SnoN expression can be induced by TGF-β in AKR2B and NRK fibroblasts but not in epithelial RIE-1 cells (Zhu et al. 2005). This result suggests that TGF-β may be able to induce opposing biological responses in fibroblasts versus epithelial cells by activating distinct downstream signaling.

It should be noted that a cell's decision to divide largely relies on a balance of all the signaling inputs from different growth factors and cytokines. Although certain cell types are naturally programmed to be insensitive to the TGF-β-induced cytostatic cascade, this antiproliferative effect might still dominate over opposing mitogenic signals when the abundance or activity of extracellular TGF-β ligands is changed. An interesting example delineating this transition comes from the observation that TGF-β's growth stimulatory effect on smooth muscle cells only occurs at low concentrations, whereas higher concentrations of TGF-β inhibit their proliferation (Battegay et al. 1990). The exact molecular mechanism underlying this phenomenon is still unclear but the inhibition of expression of PDGF receptor by high concentrations of TGF-β may play an important role.

TGF-β EFFECTS ON APOPTOSIS AND CELL SURVIVAL

TGF-β Signaling in Apoptosis

The effect of TGF-β on apoptosis is highly dependent on the cell type and context. Although TGF-β can either induce or suppress apoptosis, proapoptotic effects of TGF-β have been reported in most cases. TGF-β-induced apoptosis has been shown to play an important role in limb formation during development (Dunker et al. 2002; Schuster et al. 2002). In addition, TGF-β is also a potent inducer of apoptosis for hepatocytes in cirrhotic liver (Dooley and ten Dijke 2012), as well as for immature lymphocytes to maintain the homeostasis of the immune system (Li et al. 2006b). However, lack of a coherent molecular program for TGF-β-initiated apoptosis may suggest that the cell death decision requires an integrated interpretation of multiple signaling inputs. Indeed, TGF-β has been found to tightly link with numerous pathways that regulate the survival and death of a cell.

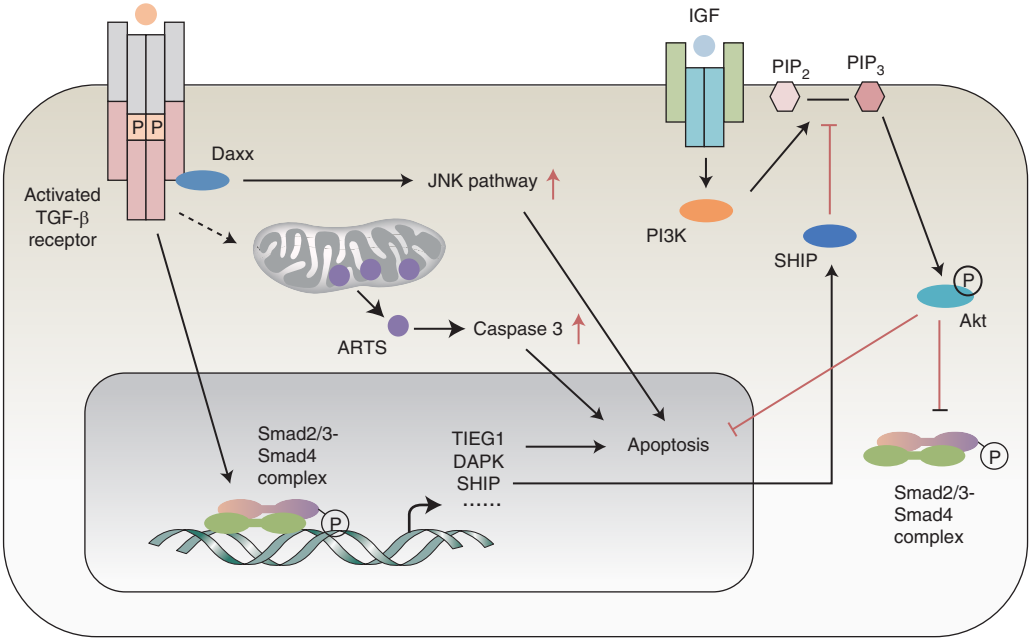

Figure 3. A schematic diagram illustrating possible mechanisms of transforming growth factor β (TGF-β)-induced apoptosis. The displayed mechanisms are described in distinct cell types. The balance between TGF-β and phosphoinositide 3-kinase (PI3K)-Akt signaling is essential for determining whether a cell will undergo apoptosis in response to TGF-β.

Several apoptotic target genes are known to be regulated by Smad transcriptional complexes (Fig. 3). TGF-β-inducible early response gene 1 (TIEG1) is a zinc-finger transcription factor that is among the TGF-β-inducible early response genes in pancreatic epithelial cells (Tachibana et al. 1997). Because TIEG1 is able to suppress the expression of Bcl2, a major inhibitory regulator of the intrinsic pathway of apoptosis in mammalian cells, its induction contributes to the proapoptotic effects of TGF-β (Chalaux et al. 1999). Using a different mechanism, the expression of the death-associated protein kinase (DAP-kinase) is induced by TGF-β in hepatocytes. Although the molecular details are still unclear, increased expression of this kinase has been connected with elevated caspase activity and apoptosis (Jang et al. 2002). In hematopoietic cells, however, TGF-β induces the expression of the inositol phosphatase SHIP (Src homology 2 (SH2) domain-containing 5′ inositol phosphatase) to induce apoptosis. Increased SHIP results in decreased Akt phosphorylation as well as impaired cell survival (Valderrama-Carvajal et al. 2002). The pool of identified proapoptotic genes that is transcriptionally regulated by TGF-β in different cell types continues expanding; recent examples include *GADD45B*, *BMF* and *BCL2L11* in hepatocytes (Yoo et al. 2003; Ramjaun et al. 2007), *CTGF* in breast cancer cells (Hishikawa et al. 1999), *BCL2L11* in gastric epithelial cells (Ohgushi et al. 2005), and *PDCD4* in hepatocellular carcinoma cells (Zhang et al. 2006). Additionally, TGF-β has been shown to induce apoptosis in Smad4-competent pancreatic cancer cells by the cooperation of TGF-β-induced expression of the epithelial-to-mesenchymal transition (EMT) transcription factor Snail with TGF-β-induced Sox4 expression. This proapoptosis effect of TGF-β is dysfunctional in Smad4 mutant pancreatic cancer cells because the induction of Snail requires the Smad4 containing transcriptional complex (David et al. 2016).

Study of the proapoptotic mechanisms mediated by TGF-β has led to the identification of

several proteins whose expression is not regulated by TGF-β but facilitates TGF-β-induced apoptosis (Fig. 3). For example, apoptosis-related protein in the TGF-β signaling pathway (ARTS) is a septin-like protein that is required for TGF-β-induced apoptosis. ARTS is released from mitochondria on TGF-β stimulation and this leads to increased caspase 3 activity (Larisch et al. 2000). Another example is the adaptor protein Daxx, which is known to bind the Fas receptor, activate the JNK pathway, and enhance Fas-mediated apoptosis (Yang et al. 1997). In both lymphocytes and hepatocytes, Daxx is found to directly interact with TβRII, leading to the activation of JNK and enhanced apoptosis, in a similar manner to Fas-induced apoptosis (Perlman et al. 2001). These results also indicate that regulating the expression of these molecules by other pathways can influence the sensitivity of TGF-β-induced apoptosis in these cell types.

Accumulating evidence indicates that cross talk between the PI3K-Akt and TGF-β pathways is essential to determining whether a cell will undergo apoptosis in response to TGF-β (Fig. 3). As discussed previously, TGF-β can induce the expression of SHIP to reduce Akt activation (Valderrama-Carvajal et al. 2002). On the other hand, activated Akt is able to form an Akt-Smad3 complex by direct interaction, and diminish phosphorylation and nuclear accumulation of Smad3 (Conery et al. 2004; Remy et al. 2004). One study indicates that the kinase activity of Akt and its downstream target mechanistic target of rapamycin (mTOR) are also important to suppress Smad3 function (Song et al. 2006). This finding is consistent with the observation that the PI3K-Akt pathway can suppress the activity of FoxO transcription factors and thus diminish the TGF-β cytostatic response (Seoane et al. 2004). The fact that the TGF-β and PI3K-Akt pathways can antagonize each other suggests that the ratio of activated R-Smads and activated Akt, rather than their absolute amounts, is the determining factor in a cell's choice between proliferation, cell-cycle arrest, and apoptosis.

A puzzle in understanding the role of TGF-β in programmed cell death is that, under certain circumstances, TGF-β signaling can act as a survival signal and prevent apoptosis. For example, in follicular dendritic cells, TGF-β has been found to down-regulate Fas and caspase-8 expression and thus prevent Fas-mediated programmed cell death (Park et al. 2005). In microglia, however, TGF-β induces the expression of FLIP, a protein that prevents Fas-induced activation of caspase-8 and caspase-3, and thus protects cells against apoptosis (Schlapbach et al. 2000). TGF-β has also been reported to induce the expression of Dec1, a transcription factor known to suppress apoptosis, thus promoting cell survival in mouse mammary carcinoma cells (Ehata et al. 2007). Interestingly, in some mammary gland epithelial cells such as NMuMG and 4T1 cells, TGF-β can activate PI3K-Akt signaling and thus enhance cell survival, although the exact molecular mechanism is largely unknown (Shin et al. 2001). A seemingly contradictory report suggests that PI3 kinase is only activated by TGF-β in fibroblasts but not in epithelial cells (Wilkes et al. 2005). However, it is interesting to note that 4T1 cells express both mesenchymal and epithelial markers and NMuMG cells undergo an EMT in response to TGF-β (Gal et al. 2008; Drasin et al. 2011). It is possible that the transition from an epithelial state to a mesenchymal state significantly changes the cellular response to TGF-β.

In short, the TGF-β apoptotic program consists of various components that link TGF-β signaling with the core machinery of programmed cell death. The molecular mechanism underlying this process seems to be highly context-dependent, determined by the epigenetic and transcriptional state of a given cell. Clearly, further studies are necessary to fully understand the molecular determinants governing these disparate effects of TGF-β on apoptotic cell death.

TGF-β Signaling in Cell Survival: Dormancy and Autophagy

Cell dormancy is a particular state in which cells cease dividing but survive in a quiescent state. For many stem cells and metastatic cancer cells, dormancy in a specific niche increases their

ability to survive in stressful environments and promotes their long-term repopulating activity (Aguirre-Ghiso 2007). TGF-β signaling has been shown to control the maintenance of cell dormancy in both normal stem cells and disseminated cancer cells. For example, high expression of TGF-β in the proximal regions of mouse prostatic ducts keeps prostatic stem cells in a dormant state. The proximal prostatic stem cells express high level of Bcl2, an antiapoptotic protein, to protect them from TGF-β-induced apoptosis. During castration-induced involution, TGF-β signaling decreases at the proximal region and thus primes the stem cells to respond to mitogenic factors (Salm et al. 2005). TGF-β is also responsible for maintaining the dormancy of hematopoietic stem cells residing in the bone marrow. Surprisingly, the active form of TGF-β maintaining HSC dormancy is mainly provided by nonmyelinating Schwann cells, a type of glia that wraps around nerve fibers inside the bone marrow, making this glial cell type a novel component of the HSC niche (Yamazaki et al. 2011). A similar effect has been observed in disseminated head and neck squamous cell carcinoma (HNSCC) and breast cancer cells. High levels of TGF-β in bone marrow have been shown to define a restrictive microenvironment for HNSCC cells that maintains disseminated tumor cells in a quiescent state in the niche (Bragado et al. 2013). BMPs also induce breast cancer dormancy at the metastatic target organ. Coco, also known as Dante or Cerberus-like 2, is a secreted antagonist of BMP ligands, and is reported to induce dormant breast cancer cells to reactivate in the lung, but not in the bone or brain, indicating an organ-specific regulation of cancer cell dormancy (Gao et al. 2012).

Autophagy is a cellular catabolic pathway for recycling intracellular components including macromolecules as well as whole organelles (Mizushima and Komatsu 2011; Jiang and Mizushima 2014). This evolutionarily conserved pathway is thought to occur in every cell at a basal level to maintain cellular homeostasis. In a stressful environment when nutrients are limited, increased autophagy promotes cell survival through this "self-eating" mechanism (Shintani and Klionsky 2004). Dysregulation of autophagy is associated with several human diseases including cancer. Although the evidence has just started to emerge, it is believed that autophagy plays a tumor suppressive role during the early stages of carcinogenesis, perhaps through its suppressive effect on reactive oxygen species production and proinflammatory cytokine secretion. Autophagy has also been shown to suppress the recruitment of regulatory T cells during early oncogenesis, and thus maintain active anticancer immunosurveillance (Rao et al. 2014). In contrast, autophagy has been reported to facilitate tumor growth and metastasis in established tumors, by increasing cancer cell survival in poorly oxygenated conditions (Maes et al. 2013). TGF-β has been shown to rapidly stimulate cellular autophagy in hepatocellular carcinoma cells and mammary carcinoma cells, although the precise mechanistic details underlying this phenotype are still elusive (Kiyono et al. 2009). The effect of TGF-β on autophagy is not limited to neoplastic cells; a similar correlation has also been reported in mesangial cells and renal epithelial cells (Ding et al. 2010; Koesters et al. 2010). The study of TGF-β and autophagy is still in its nascent stage. Given the importance of autophagy in the preservation of cellular energy homeostasis as well as in changing cells' signaling output, it will be interesting to further characterize the interactions between TGF-β signaling and autophagy in additional cellular contexts.

TGF-β SIGNALING IN CELLULAR SENESCENCE: AN IRREVERSIBLE CELL-CYCLE ARREST

Cellular senescence is a state of irreversible cell-cycle arrest known to occur in a variety of mammalian cell types. Senescence can be triggered by several known stresses including telomere shortening, DNA damage, and oncogene activation (Muñoz-Espín and Serrano 2014). Whereas stress-induced senescence is thought to be important to the pathogenesis of cancer and age-related tissue degeneration (Campisi 2013), programmed cellular senescence seems to occur naturally in certain tissues during normal embryonic development (Muñoz-Espín

et al. 2013; Storer et al. 2013). Molecularly, senescent cells often express a common set of markers including the cell-cycle inhibitors $p15^{INK4B}$, $p16^{INK4A}$, and $p21^{CIP1}$, the tumor suppressors p53 and Rb, and the lysosomal enzyme β-galactosidase (senescence-associated β-galactosidase, SA-βgal). Moreover, permanently nondividing cells often secrete a variety of proteins including interleukin 6 (IL-6), interleukin 8 (IL-8) and insulin-like growth factor-binding proteins (IGFBPs), among others (Coppé et al. 2008). This has been termed the senescence-associated secretory phenotype (SASP), and has been shown to have pleiotropic effects on neighboring cells (Rodier and Campisi 2011).

The first hint that TGF-β signaling might regulate cellular senescence came from the discoveries that this cytokine could induce transcriptional up-regulation of the genes encoding the cell-cycle inhibitors $p21^{CIP1}$ (Datto et al. 1995; Reynisdottir et al. 1995) and $p15^{INK4B}$ (Hannon and Beach 1994), which are known to promote some forms of senescence (Fig. 4). Shortly after these observations were published, more direct evidence for a functional role of TGF-β in senescence was obtained. Culturing several human lung cancer cell lines in the presence of recombinant TGF-β resulted in morphological alterations and elevated SA-βgal activity associated with stable cell-cycle arrest (Katakura et al. 1999). Along similar lines, both genetic and pharmacological suppression of TGF-β signaling was found to be sufficient to inhibit oncogene-induced senescence in primary mouse keratinocytes (Tremain et al. 2000). Mechanistically, TGF-β was shown to promote senescence via Smad3-dependent induction of $p15^{INK4B}$ expression, and, consistent with this, Smad3 activity could impede skin carcinoma progression in mouse models of this disease (Vijayachandra et al. 2003). Further work performed around this time showed that oxidative stress-induced fibroblast senescence could be blocked using neutralizing antibodies to either TGF-β1 or its receptor (Frippiat et al. 2001).

The p53 tumor suppressor is perhaps the most studied mediator of cellular senescence, and the p53 pathway is known to intersect with TGF-β signaling at several levels (Fig. 4). First, p53 can interact directly with both Smad2 and Smad3, resulting in the transcriptional activation of genes containing both p53-binding elements and TGF-β responsive elements, including the gene encoding $p21^{CIP1}$ (Dupont et al. 2004; Elston and Inman 2012). In addition to this, recent work has focused on p53- and Smad-mediated control of the expression of miRNAs, which are short RNA molecules that inhibit gene expression via target mRNA degradation or translational repression. Interestingly, Smad- and p53-mediated control of miRNA expression happens at both the transcriptional and posttranscriptional levels, the latter of which occurs using a unique molecular mechanism that has thus far only been identified for Smad2 and Smad3, and p53. In both cases this involves direct binding of the transcription factor to a pri-miRNA containing the appropriate response element, resulting in enhanced recruitment of DEAD-box RNA helicase p68 to the DROSHA complex and increased miRNA processing to the mature, active form (Suzuki et al. 2009; Davis et al. 2010). Among transcripts regulated in this manner, the antiproliferative miRNAs miR-34a, miR-215, and miR-192 might control the establishment of cellular senescence (Braun et al. 2008). Thus, the p53 and TGF-β pathways converge to regulate gene expression at different levels through multiple molecular mechanisms.

Another recurring theme in TGF-β-mediated control of cellular senescence concerns regulation of the telomerase (hTERT) gene, which is known to block forms of senescence associated with telomere shortening. For example, TGF-β signaling results in transcriptional down-regulation of hTERT expression in both colon and breast cancer cells (Yang et al. 2001; Katakura et al. 2003). Mechanistically, TGF-β regulates hTERT expression through both direct and indirect mechanisms (Fig. 4). First, hTERT transcription is known to be under the control of the oncogene c-Myc, and in many human cancers TGF-β seems to regulate hTERT expression indirectly by repressing c-Myc expression (Frederick et al. 2004). However, Smad3 can also interact directly with the hTERT promoter in certain contexts, leading to direct repression

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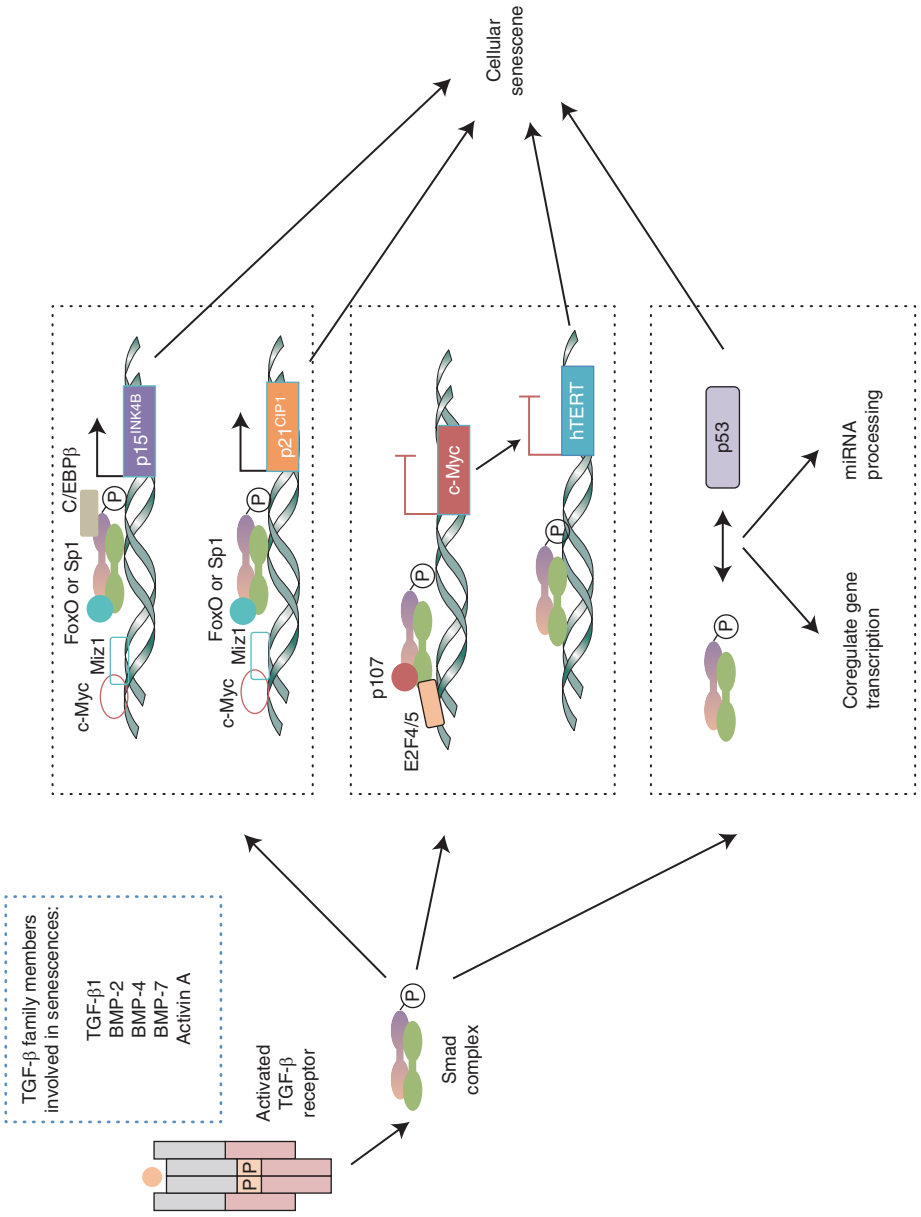

Figure 4. Regulation of cellular senescence by transforming growth factor β (TGF-β) signaling. TGF-β family proteins are able to promote cellular senescence in normal and tumor cells through multiple molecular mechanisms, including transcriptional induction of the expression of cell-cycle inhibitors, repression of telomerase expression, and convergent regulation with the p53 pathway.

(Li et al. 2006a). Finally, TGF-β has also been reported to regulate alternative splicing of the hTERT transcript to modulate enzyme activity (Cerezo et al. 2002), indicating complex levels of regulation.

Although TGF-β was not initially reported to be a key component of the SASP (Coppé et al. 2008), later studies have recognized a role for TGF-β signaling in this phenotype (Kuilman and Peeper 2009). In particular, TGF-β family ligands are capable of inducing paracrine senescence in both human diploid fibroblasts grown in culture and in mouse models, and in both cases senescence appears to depend on the induction of $p15^{INK4B}$ and $p21^{CIP1}$ expression (Acosta et al. 2013). Additional cellular contexts in which TGF-β has been shown to be capable of inducing senescence include normal fetal lung (Mv1Lu) cells, skin (AG04431) fibroblasts, human mammary epithelial (HME) cells, human bronchial epithelial (HBE) cells, as well as several types of transformed hepatocellular carcinoma cells (Debacq-Chainiaux et al. 2005; Yoon et al. 2005; Senturk et al. 2010; Cipriano et al. 2011; Minagawa et al. 2011; Lin et al. 2012). Conversely, TGF-β family ligands are not sufficient to induce prostate basal (Untergasser et al. 2003) or mesenchymal stromal cell (Walenda et al. 2013) senescence, indicating that this process is cell-type-specific with additional molecules likely playing important roles.

In addition to TGF-β itself, other TGF-β family members also play functional roles in various forms of cellular senescence (Fig. 4). Perhaps most prominently, BMP-4 is sufficient to induce lung cancer cell senescence (Buckley et al. 2004), a finding that might have clinical relevance in light of the observation that BMP-4 expression is up-regulated in lung cancer cells following doxorubicin treatment (Su et al. 2009). Interestingly, BMP-4-mediated activation of Smad1 and Smad5 leads to the induction of both $p16^{INK4A}$ and $p21^{CIP1}$ expression, and this is dependent on p38 MAPK activity (Su et al. 2011), implicating a role for noncanonical signaling in this process. BMP-7 has also been reported to induce breast and prostate cancer cell senescence (Cassar et al. 2009; Kobayashi et al. 2011), and in the latter case p38 MAPK also is involved. Moreover, BMP-2 is involved in oncogene-induced senescence in mouse embryonic fibroblasts (Kaneda et al. 2011).

Although most studies linking the TGF-β family to cellular senescence have been in the context of cancer, it should be noted that similar mechanisms might be involved in senescence associated with normal mammalian aging. For example, BMP-4 expression is increased in aged retinal pigment epithelial cells (Zhu et al. 2009), whereas activin A plays a role in age-related hepatocyte senescence by upregulating $p15^{INK4B}$ expression (Menthena et al. 2011). Collectively, these studies show roles for a variety of TGF-β family ligands in a diverse array of biological settings.

What might be the function of TGF-β-induced cellular senescence under normal, noncancerous physiological conditions? Two studies have identified the presence of significant numbers of senescent cells during normal mouse embryonic development (Muñoz-Espín et al. 2013; Storer et al. 2013). Intriguingly, this "developmentally programmed" senescence is specifically dependent on $p21^{CIP1}$, which is encoded by a known TGF-β target gene. Furthermore, elevated TGF-β expression and Smad2 phosphorylation in the mesonephros and endolymphatic sac of the inner ear, and treatment of pregnant mice with a TGF-β receptor antagonist led to diminished levels of senescence in these locations in the offspring of treated animals (Muñoz-Espín et al. 2013). Moreover, the identity of the other major signaling pathway that emerged from this study, the PI3K-Akt pathway, is intriguing given the cross talk known to exist between PI3K-Akt and TGF-β signaling, although the interplay between these pathways that occurs in senescence will await future study. Although these studies point to a functional role for TGF-β signaling in regulating senescence during normal mammalian development, further work is needed to understand this process in greater detail. It will also be important to determine whether TGF-β signaling is capable of inhibiting cellular senescence, perhaps by stimulating cell proliferation, which is known to occur in certain mesenchymal cell types.

CONCLUDING REMARKS

The biological effects of TGF-β at the cellular level, including inhibition or promotion of proliferation, apoptosis, and cell dormancy and autophagy as well as cellular senescence, are the basis for understanding TGF-β's physiological function in development and diseases. Advances in knowledge of the molecular details of the TGF-β signaling cascade and its cross talk with other signaling pathways, topics extensively discussed in other reviews, significantly facilitate our understanding of these cellular effects. However, much work remains to be performed to fully understand the precise molecular mechanisms by which TGF-β mediates these cellular behaviors in different contexts. Compared with the well-established TGF-β cytostatic program, the mechanistic links between TGF-β and other processes are still too limited to generate a coherent picture. This is partly because of the context-dependent nature of TGF-β signaling, the outcome of which differs dramatically in different cell types or cell states. Looking ahead, some of the more recently identified cellular processes regulated by TGF-β, including cell dormancy, autophagy and senescence, seem particularly worthy of future research. Such studies are not only important to further our understanding of the physiological function of TGF-β in development, but also have the potential to advance the development of targeted TGF-β-based treatments in certain human diseases including cancer.

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