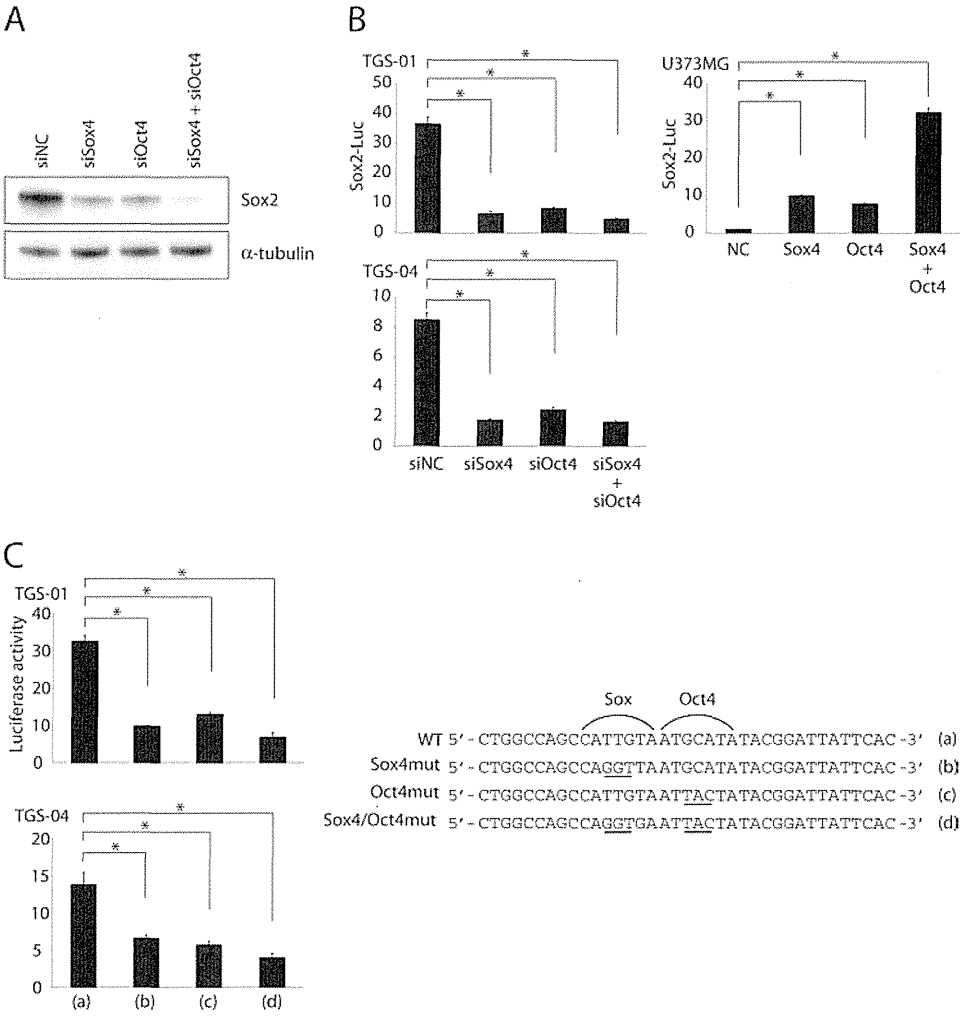

FIGURE 6. **Oct4 acts in concert with Sox4 to potentiate *SOX2* enhancer activity.** *A*, effects of Oct4 and/or Sox4 knockdown on expression of Sox2. Amount of Sox2 protein was determined after treatment with indicated siRNA duplex for 24 h. *B*, roles of Oct4 and Sox4 in activation of the *SOX2* enhancer region. Effects of Oct4 and/or Sox4 knockdown on *SOX2* enhancer activity were examined in TGS-01 and TGS-04 cells (*left graphs*). Effects of Oct4 and/or Sox4 overexpression on *SOX2* enhancer activity were examined in U373MG cells (*right graph*). *Error bars* represent ±S.E. *, $p < 0.001$. *C*, TGS-01 or TGS-04 cells were transfected with luciferase constructs containing wild-type or mutated *SOX2* enhancer region. The cells were collected 24 h after transfection, and luciferase activity was examined. *, $p < 0.001$. The *right panel* indicates the sequence of the *SOX2* enhancer region and corresponding mutations (*underlined*) used in this study.

activates the *SOX2* enhancer region to form a positive regulatory loop (25, 26). To determine whether this regulatory loop exists in neural progenitor cells and glioma-initiating cells, we examined recruitment of these transcription factors to the *SOX2* enhancer region in a ChIP assay (Fig. 7). Anti-Oct4 antibody enriched the DNA fragments of *SOX2* enhancer region equally well in fetal neural progenitor cells and glioma-initiating cells. In addition, anti-Sox2 antibody immunoprecipitated this region in neural progenitor cells. However, strong enrichment of the same region by anti-Sox2 antibody was not observed in glioma-initiating cells. These results indicate that transcription factor complex on the *SOX2* enhancer region does not contain Sox2 in glioma-initiating cells and that Sox2 expression in glioma-initiating cells is regulated by mechanisms different from those in fetal neural progenitor cells.

We next examined recruitment of Sox4 to the *SOX2* enhancer region in neural progenitor cells and glioma-initiating cells. In contrast to the experiment using anti-Sox2 antibody, anti-Sox4 antibody immunoprecipitated the DNA fragments of the *SOX2* enhancer region in glioma-initiating cells, whereas the enrichment observed in fetal neural progenitor cells was much weaker. These findings together indicate that Sox2 expression in glioma-initiating cells is potentiated by the Oct4-Sox4 complex acting on the *SOX2* enhancer region to maintain tumorigenic activity, whereas that in neural progenitor cells may be promoted by transcriptional complex containing Sox2 protein itself through a positive regulatory loop.

DISCUSSION

Although the origin of glioma stem cells (or glioma-initiating cells) is controversial (27), several studies have suggested that glioma-initiating cells share characteristics with neural or glial stem/progenitor cells (28, 29). Glioma stem cells express neural stem cell markers, including Nestin, Musashi, and Prominin-1 (CD133). Like normal neural stem cells, glioma stem cells are located in specific niches surrounding the tumor vasculature. A recent study has shown that the perivascular niches control self-renewal of glioma stem cells through endothelial cell-derived factors (30). However, in terms of transcription factor complexes, the similarities and differences between glioma

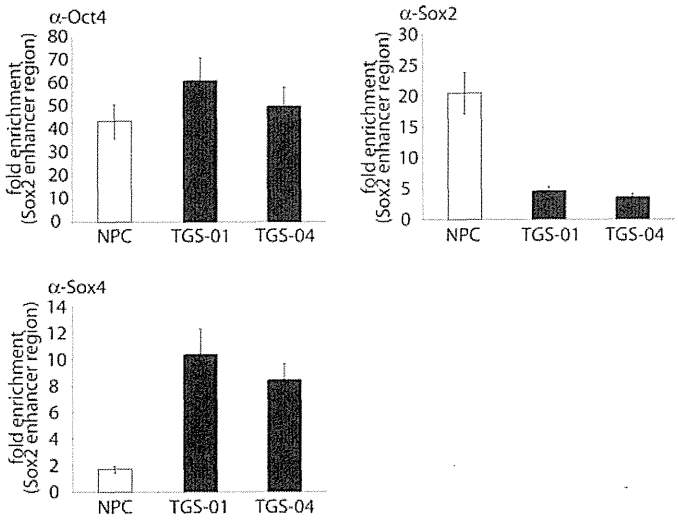

FIGURE 7. **The partner of Oct4 on *SOX2* enhancer region in glioma-initiating cells is distinct from that in neural progenitor cells.** Soluble chromatin was prepared from glioma-initiating cells (TGS-01 and TGS-04) and neural progenitor cells (*NPC*). ChIP analysis was performed using anti-Oct4, anti-Sox2, and anti-Sox4 antibodies. Eluted DNAs were subjected to quantitative real-time PCR analysis. Values were normalized to the amount of the first intron of HPRT1. *Error bars* represent ±S.E.

stem cells and neural stem cells have not been clearly determined.

Here we have shown that Oct4 expression is required for the maintenance of the self-renewal capacity of glioma-initiating cells. In addition, transient suppression of Oct4 by siRNA abolished the induction of Sox2 by TGF-β[4] and decreased the tumorigenic activity of glioma-initiating cells (Fig. 2), suggesting that impairment of stemness properties via Oct4 knockdown may be an irreversible process. We also demonstrated that Oct4 knockdown increases sensitivity to the chemotherapeutic alkylating agent, temozolomide.

Oct4 is essential for establishing and maintaining the pluripotent state of stem cells (14, 15). Moreover, Oct4 is one of the key factors in the generation of induced pluripotent stem cells (17, 18). However, the role of Oct4 in the development and progression of malignant tumors has not been fully determined. Our findings indicate that Oct4 is an essential factor for glioma-initiating cells and plays roles similar to those in embryonic stem cells.

One of the most intensively investigated topics in current cancer research is the identification of specific therapeutic compounds that can effectively eliminate cancer-initiating cells. Recent studies have identified factors essential for retention of cancer-initiating cells, including several growth factor signaling pathways such as Wnt, Hedgehog, Notch, PI3K-mTOR, TGF-β, and LIF (9, 31–36). Although new therapeutic targets have been intensively sought based on findings related to these pathways, one problem is that almost all of these signaling pathways are also indispensable for normal stem cells. Inhibitors of these signaling pathways may affect the characteristics of normal stem cells and impair maintenance of normal tissues. Thus, from a clinical standpoint, it is important to identify factors not only essential for the maintenance of cancer-initiating cells but also different from those present in normal stem cells.

Here, we have demonstrated that Oct4-Sox4 complex activates the enhancer region of *SOX2* genes to sustain stemness properties of glioma-initiating cells. Oct4 and Sox2 are also important for the maintenance of normal stem cells, and Oct4-Sox2 complex activates the *SOX2* enhancer region to form a positive regulatory loop. However, in glioma-initiating cells, Sox2 is not predominantly present in the transcription factor complex on the *SOX2* enhancer region. Instead, Sox4 forms a transcriptional complex with Oct4 in glioma-initiating cells to activate the enhancer region of *SOX2*, a gene essential for the maintenance of tumorigenicity of glioma-initiating cells. These findings suggest that Sox2 expression in glioma-initiating cells can be potentiated via up-regulation of Sox4, whereas Sox2 expression in neural progenitor cells is regulated by a self-reinforcing regulatory loop and is relatively self-contained (Fig. 7 and supplemental Fig. S5). In other words, the positive regulatory loop of Sox2 is not active in glioma-initiating cells, and alternatively, Oct4 acts with Sox4 to enhance Sox2 expression. We also confirmed that, in neural progenitor cells, Sox2 is only weakly induced by TGF-β stimulation (supplemental Fig. S6), whereas this cytokine activates the Sox4-Sox2 cascade in glioma-initiating cells (11). Loss of the regulatory loop of Sox2 expression may thus cause glioma-initiating cells to become susceptible to exogenous stimuli. However, we should bear in mind that our glioma-initiating cells were obtained from adult tumors, whereas neural progenitor cells were from a fetus. Further studies in neural progenitor cells from adults may be important to elucidate the differences between glioma-initiating cells and normal neural progenitor cells.

We examined combined effects of siRNAs against Sox4 and Oct4 in a limiting dilution assay but failed to observe any significant synergistic effects (supplemental Fig. S7). It may be because a defect of either factor in the Sox4-Oct4 complex results in significant inactivation of the *SOX2* enhancer and/or because a single effect of siSox4 or siOct4 is strong enough to reduce sphere-forming ability of glioma-initiating cells.

It remains to be determined why the common Oct4-binding sequence and Sox-binding elements are differently regulated in neural progenitor cells and glioma-initiating cells. Upon differentiation of erythroid precursors into mature erythrocytes, GATA-binding protein 2 (GATA2) on some promoter regions is replaced by GATA1 (37). This process is termed the "GATA switch" and is an essential step in the maturation of erythrocytes and the expression of α-globin. One of the crucial mediators of this switching is Friend of GATA1 (FOG-1, also known as Zfpm1), a multi-zinc-finger protein critical for the development of erythrocytes and megakaryocytes (38, 39), and GATA-FOG interaction is believed to be required for "GATA switch" (40). Like the "GATA switch," the Sox-binding element on *SOX2* enhancer region in glioma-initiating cells is differently regulated from that in neural progenitor cells, although the mechanism responsible for this remains to be determined.

Although Sox4 plays a crucial role in the retention of tumorigenicity of glioma-initiating cells through up-regulation of Sox2 expression (11), *Sox4*$^{-/-}$ mice exhibit no neurological defects (41). This finding suggests that the mechanism of action

[4] H. Ikushima and K. Miyazono, unpublished observation.

of Sox4 in glioma-initiating cells is distinct from that in neural stem/precursor cells. Because the self-renewal and proliferation of normal stem cells are likely strictly regulated, perhaps by genetic or epigenetic programs, the uncontrolled expansion of cancer-initiating cells may result from deregulation of such strict programs. In support of this conclusion, we found that the self-regulatory loop of Sox2 expression observed in neural progenitor cells was disrupted in glioma-initiating cells. This finding may enable the determination of a novel molecular target and eventually yield a therapeutic approach to eradication of glioblastoma without affecting the normal brain.

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REVIEW

TGF-β signal transduction spreading to a wider field: a broad variety of mechanisms for context-dependent effects of TGF-β

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Abstract Transforming growth factor (TGF)-β signaling is involved in almost all major cell behaviors under physiological and pathological conditions, and its regulatory system has therefore been vigorously investigated. The fundamental elements in TGF-β signaling are TGF-β ligands, their receptors, and intracellular Smad effectors. The TGF-β ligand induces the receptors directly to phosphorylate and activate Smad proteins, which then form transcriptional complexes to control target genes. One of the classical questions in the field of research on TGF-β signaling is how this cytokine induces multiple cell responses depending on cell type and cellular context. Possible answers to this question include cross-interaction with other signaling pathways, different repertoires of Smad-binding transcription factors, and genetic alterations, especially in cancer cells. In addition to these genetic paradigms, recent work has extended TGF-β research into new fields, including epigenetic regulation and non-coding RNAs. In this review, we first describe the basic machinery of TGF-β signaling and discuss several factors that comprise TGF-β signaling networks. We then address mechanisms by which TGF-β induces several responses in a cell-context-dependent fashion. In addition to classical frames, the interaction of TGF-β signaling with epigenetics and microRNA is discussed.

Keywords TGF-β · Smad · ALK5 · Epigenetics · microRNA

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Introduction

Cytokines are small secreted proteins that are produced by numerous types of cells and that play important roles in intercellular communication to maintain order in the organism. They elicit biological effects by binding to the extracellular domains of specific transmembrane receptors in the outer membrane of cells. Cytokines mediate intercellular communication via the regulation of cell growth and differentiation and are thus crucial for maintaining the homeostasis of multicellular organisms. Aberrant regulation of cytokine signaling can therefore result in various diseases.

The transforming growth factor (TGF)-β family is particularly prominent among these signals (Blobe et al. 2000; Feng and Derynck 2005; Massagué 2008). TGF-β signaling controls a diverse set of cellular processes, including cell growth, differentiation, apoptosis, survival, and specification of developmental fate, during embryogenesis and in mature tissues (Ikushima and Miyazono 2010a; Moustakas and Heldin 2009). To control TGF-β-induced cell responses, numerous factors tightly regulate this signaling pathway under physiological conditions (Ikushima and Miyazono 2010b; Bierie and Moses 2006). Loss of balance of TGF-β signaling thus leads to several pathological conditions, including malignant tumors, fibrotic diseases, and abnormal immune reactions (Levy and Hill 2006; Varga and Pasche 2009; Flavell et al.

2010). Indeed, studies of clinical samples indicate that a distortion of TGF-β signaling is one of the major causes of several disorders. Here, we first discuss the way that (1) cells translate TGF-β signaling into cellular responses, and (2) TGF-β signaling and TGF-β-induced cell responses are tightly controlled. Possible and/or established mechanisms of the context-dependent diversity of TGF-β-induced cell responses are also addressed. In addition, recent research on TGF-β signaling has spread into novel fields, including epigenetics and non-coding RNAs. Thus, we also mention the involvement of epigenetic regulation and non-coding RNAs in the classical TGF-β signaling pathway.

Extracellular regulation of TGF-β signaling

Effects of TGF-β are mediated by three TGF-β ligands: TGF-β1, TGF-β2, and TGF-β3 (Feng and Derynck 2005; Shi and Massagué 2003). Although each of these ligands is produced by distinct genes, they exhibit approximately 70%–80% sequence similarity. The TGF-β ligand is first synthesized as a dimeric pro-protein (pro-TGF-β), which is then cleaved to form the mature disulfide-bridged TGF-β dimer. The pro-peptide has high affinity for the cleaved mature TGF-β ligand, which is secreted from cells as a small latent complex (ten Dijke and Arthur 2007). Since TGF-β in this form does not have the ability to interact with its receptor, the pro-peptide is termed the latency-associated protein (LAP). The LAP dimer is also bound to the latent TGF-β binding proteins (LTBPs) by disulfide bonds, and the tri-molecular complex is termed the large latent complex (Rifkin 2005). The dissociation of TGF-β from the complex is a critical regulatory event and is achieved by integrin, shear force, thrombospondin-1 (TSP-1), some enzymes including plasmin, changes in pH, heat treatment, radiation, and other agents. Among the four different LTBPs, LTBP-1, 3, and 4 bind to small latent complexes and play key roles in targeting the large latent complex to the extracellular matrix, where active TGF-β is released by proteolytic cleavage. Although the synthesis of TGF-β is regulated by a variety of factors at the level of transcription and/or mRNA stability, the generation of active TGF-β from its latent form is also subject to regulation.

TGF-β receptors

Activated TGF-β ligands transduce their effects through TGF-β type I and II receptors (Ikushima and Miyazono 2010b; Wrana et al. 2008). The TGF-β type II receptor (TβRII) is the specific receptor for TGF-β ligands. Both type II and type I receptors are comprised of an N-terminal extracellular ligand-binding domain, a transmembrane region, and a C-terminal intracellular serine/threonine kinase domain. TGF-β has high affinity for TβRII, and upon binding the ligand, the type I receptor forms a heteromeric complex consisting of two of each receptor type and is activated by the type II receptor (Fig. 1). The type I, but not type II, receptors contain a characteristic GS domain, located N-terminal to the kinase domain. Activation of the type I receptor involves the phosphorylation of its GS domain by the type II receptor. Although activin receptor-like kinase 5 (ALK5), also known as TβRI, mediates TGF-β signal transduction in most types of cells, ALK1 and other type I receptors also transduce TGF-β signaling in certain cells, including endothelial cells (Goumans et al. 2003; Daly et al. 2008).

TGF-β is also able to interact with proteins called TGF-β type III receptors, which do not have intrinsic kinase activity (Bernabeu et al. 2009). Betaglycan is a membrane-anchored proteoglycan that facilitates binding of TGF-β2 to TβRII (Gatza et al. 2010). Endoglin, a glycoprotein expressed at high levels in endothelial cells, binds to TβRII and is thought to act as an accessory protein for the receptor complex (ten Dijke et al. 2008). Although the function of endoglin in TGF-β signaling is still controversial, mutations of it have been linked to hereditary hemorrhagic telangiectasia (McAllister et al. 1994; Abdalla and Letarte 2006). In addition, endoglin produced in a soluble form is associated with the pathogenesis of preeclampsia (Venkatesha et al. 2006). These findings indicate the central roles of endoglin in controlling vascular homeostasis.

Intracellular signal transduction through Smad proteins

Once the functional TGF-β receptor complex is formed, it regulates the activation of downstream signaling pathways. Although several substrates for the type I receptor kinases have been identified, the most important ones for the transduction of TGF-β stimulation are members of the Smad family proteins (Massagué et al. 2005; Schmierer and Hill 2007; Derynck and Zhang 2003). Phosphorylation and activation of the type I receptor enable the recruitment of receptor-regulated Smads (R-Smads). The type I receptor then phosphorylates R-Smads, allowing them to form hetero-oligomeric complexes with the common-partner Smad (Co-Smad) and to move into the nucleus. Of the five R-Smads in mammals, Smad2 and Smad3 are activated by the TβRII–ALK5 complex, whereas Smad1, Smad5, and Smad8 are activated by the TβRII–ALK1 complex. Interestingly, Liu et al. (2009) have recently reported that ALK5 can directly activate Smad1/5 in certain types of

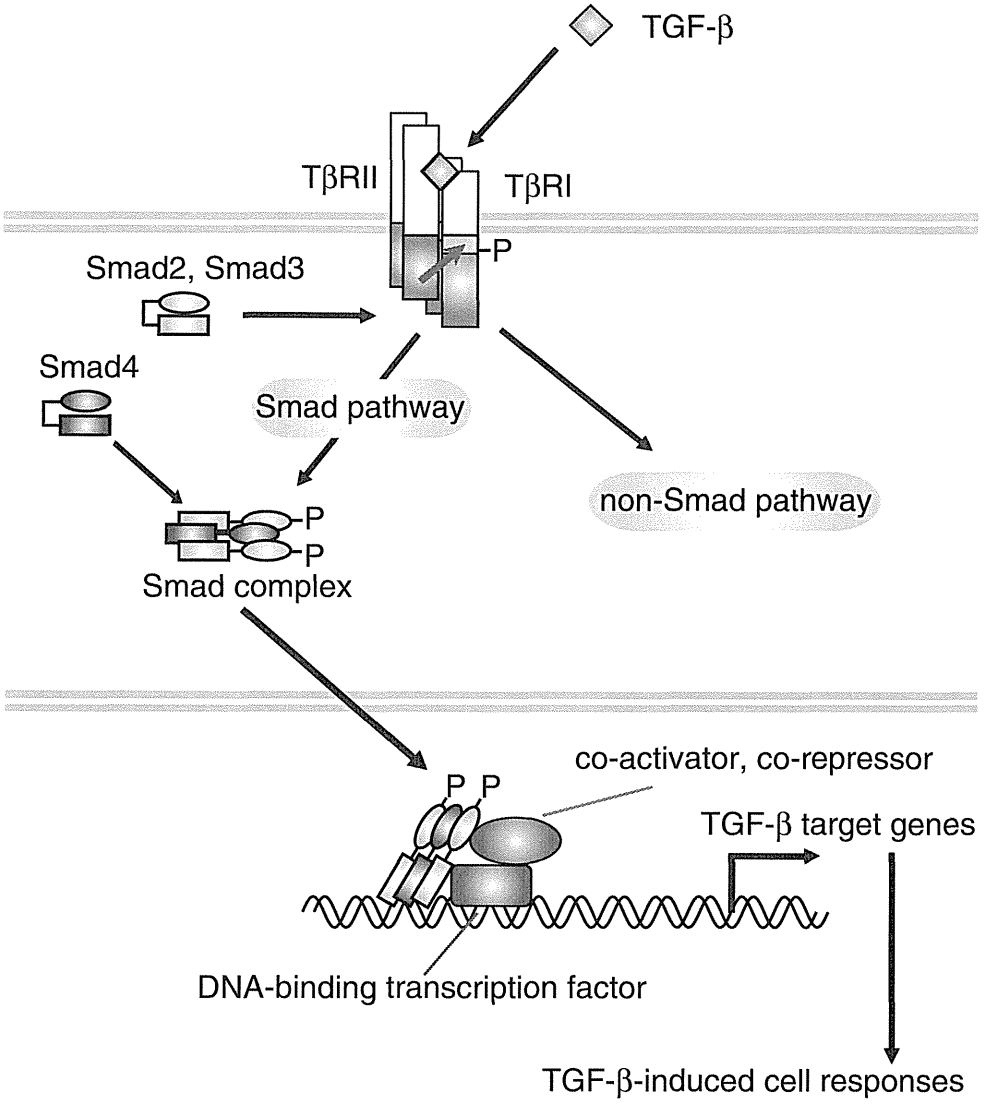

Fig. 1 Intracellular transforming growth factor-β (TGF-β) signal transduction. TGF-β signals are transduced by type II receptor (*TβRII*), type I receptor (*TβRI*), and their downstream Smad proteins (*Smad2-4*). Activated Smad complex interacts with DNA-binding transcription factors and co-activators/co-repressors and binds to the promoter regions of TGF-β target genes. Active TGF-β receptors also regulate signaling cascades other than the Smad pathway

cells. Smad4 is the only known Co-Smad in mammals. R-Smads consist of conserved Mad homology 1 (MH1) and MH2 domains, which are connected with a less-conserved linker region. The C-terminus of R-Smads has a characteristic SSXS (Ser-Ser-X-Ser) motif that is phosphorylated by active type I receptors. Smad4 contains MH1 and MH2 domains but lacks the C-terminal SSXS motif and, thus, is not phosphorylated by type I receptors. Smad complexes bind specific DNA sequences, namely 5′-AGAC-3′ or its reverse complement 5′-GTCT-3′, in the promoters or enhancers of target genes. They interact with other DNA-binding transcription factors, co-activators or co-repressors, and chromatin remodeling factors to the regulatory regions of target genes in order to regulate diverse TGF-β-induced cell responses. TGF-β stimulation also activates intracellular signals through non-Smad pathways, including mitogen-activated protein kinase, PI3K-Akt, and small GTPase pathways (Moustakas and Heldin 2005; Zhang 2009).

Context-dependent diversity of TGF-β-induced cell responses

At the core of this signaling pathway, TGF-β induces its membrane receptors directly to activate Smad proteins, which then form transcriptional complexes to control target genes. The aspect that makes this system complex is that these complexes activate or repress numerous target genes at the same time in a tightly regulated fashion. Furthermore, TGF-β stimulation induces numerous cell responses in a cellular context-dependent fashion (Roberts and Wakefield 2003; Bierie and Moses 2006). For example, TGF-β promotes cell proliferation in certain cellular contexts but inhibits it in most others (Ikushima and Miyazono 2010a). This cytokine plays crucial roles in the maintenance of the tumorigenic activities of some types of cancer stem cells (Ikushima et al. 2009; Peñuelas et al. 2009; Anido et al. 2010; Naka et al. 2010) but promotes the loss of tumorigenicity in others (Tang et al. 2007; Ehata et al.

2011). The cells making up one human body are all derived from a single cell, even if they are abnormal. However, they exhibit different responses to TGF-β because of slight but crucial differences. Moreover, even in the same type of cell, the cell responses mediated by TGF-β differ depending on environmental factors. Because of this inherent diversity, TGF-β-based therapeutic strategies are considered complex. Here, we discuss proposed or established mechanisms responsible for the chaotic diversity of TGF-β signaling.

Signal cross-talk

TGF-β is able to induce certain cell responses, under conditions including other types of signaling, but fails to induce the same responses without such signaling (Guo and Wang 2009). Cross-interaction with additional signaling is thus required for some TGF-β-induced cell responses (Fig. 2). Many signaling pathways have been reported to exhibit cross-talk with the TGF-β signaling pathway (Luo 2008; Zhang 2009); here, we discuss cross-talk with the Wnt, p53, and Ras signaling pathways.

Wnt signaling plays diverse roles in regulating numerous cell responses, including cell proliferation, differentiation, migration, and survival (Kestler and Kühl 2008; Logan and Nusse 2004). Canonical Wnt signaling is mediated by β-catenin, which functions as a transcription co-factor and is also essential for the formation of adherence junctions between cells through its interaction with cadherins. In the absence of Wnt, cytoplasmic β-catenin is degraded through glycogen synthase kinase (GSK)-3β-mediated serial phosphorylation and subsequent polyubiquitination, which keeps the Wnt pathway in an "OFF" state. The binding of Wnt ligand to its receptor Frizzled (Fz) and co-receptor LRP5/6 leads to GSK-3β inactivation and β-catenin stabilization. The cytoplasmic accumulation of β-catenin promotes its translocation into the nucleus, where it binds the lymphocyte enhancer factor/T-cell transcription factor (Lef/TCF) family of transcription factors and turns the Wnt pathway "ON". The most common format of cross-talk between the TGF-β and Wnt signaling pathways occurs in the nucleus, where the Smads and Lef/β-catenin synergistically regulate a set of shared target genes (Labbé et al. 2000, 2007; Hussein et al. 2003; Sasaki et al. 2003). These two pathways are also linked by protein interactions in the cytoplasm (Tang et al. 2008; Han et al. 2006; Liu et al. 2006; Edlund et al. 2005; Furuhashi et al. 2001).

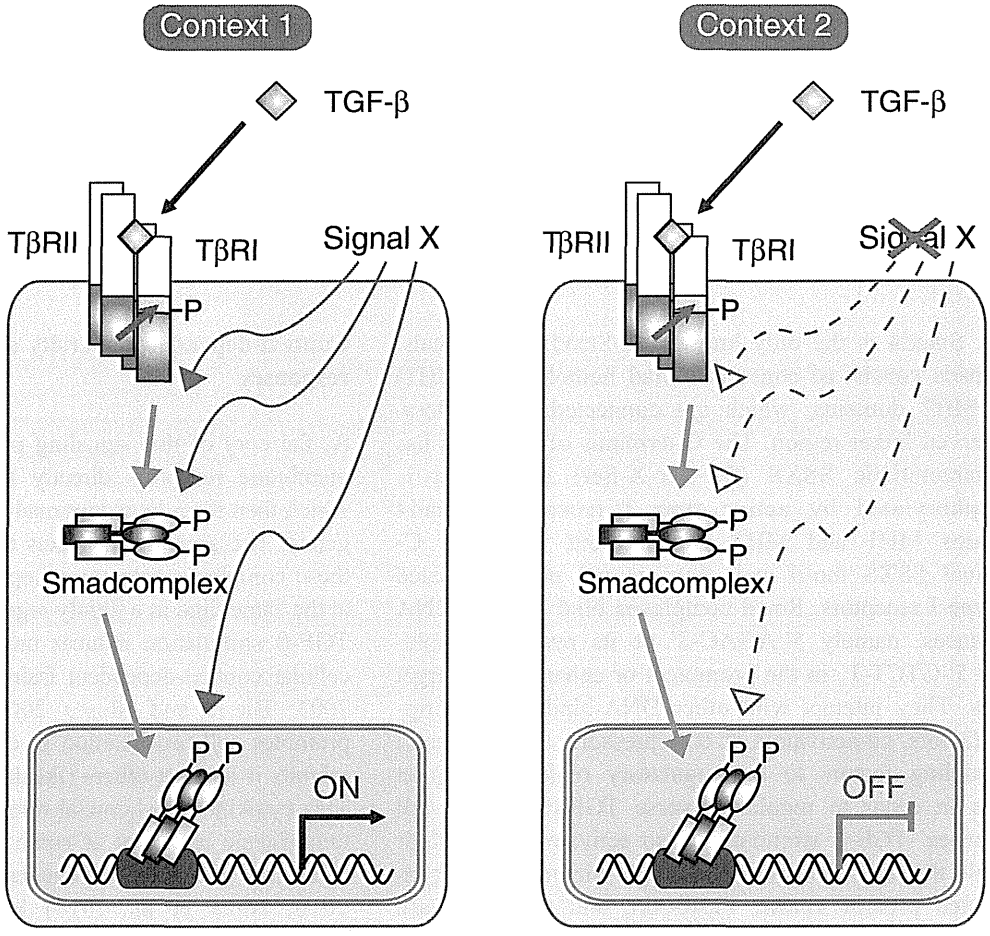

Fig. 2 "Signal cross-talk" model. In *Context 1*, but not in *Context 2*, *Signal X* is transduced in cells to modify downstream transducers of TGF-β signaling and induce a certain context-1-specific cell response

Perturbations of TGF-β signaling have been strongly implicated in cancer progression. TGF-β can play both tumor-suppressive and tumor-promoting roles and is now generally accepted to act as an anti-oncogenic factor in the early phase of tumorigenesis, although it can be converted to a pro-oncogenic factor during cancer progression (Roberts and Wakefield 2003; Bierie and Moses 2006). This switching of TGF-β from an anti-oncogenic factor to a pro-oncogenic factor might be induced by various mechanisms. Adorno et al. (2009) have reported that additional mutation of p53 plays a role in this switching. In the early stages of tumorigenesis, TGF-β inhibits the proliferation of tumor cells in concert with wild-type p53 as an anti-oncogenic factor. In contrast, in the later stages, Smad complexes function cooperatively with mutant p53 to abrogate the abilities of p63 to suppress sharp-1 and cyclin G2 expression and to inhibit metastasis. Indeed, the expression of mutant p53 in noninvasive tumor cells enhances the pro-invasive and migratory effects of TGF-β, whereas the suppression of mutant p53 expression in aggressive tumors impairs their ability to metastasize.

TGF-β induces epithelial-mesenchymal transition (EMT), in which epithelial cells acquire mesenchymal characteristics (Thiery et al. 2009). Some transcription factors, including Snail, Slug, Twist, δEF1/ZEB1, and SIP1/ZEB2, are induced by TGF-β signaling and regulate the expression of E-cadherin and other EMT-related genes. In certain cells, oncogenic Ras and TGF-β signaling pathways have been shown to induce EMT cooperatively (Oft et al. 1996, 2002). TGF-β alone can only weakly induce the expression of Snail and repress that of E-cadherin; however, oncogenic Ras signaling enhances the expression of Snail induced by TGF-β and synergistically induces EMT (Horiguchi et al. 2009).

In this fashion, TGF-β-induced cell responses can be determined by cooperatively acting signaling pathways.

Co-factors

Since the affinity of the activated Smad complex for the Smad-binding element (SBE) is insufficient to support an association with promoters of target genes, Smad complexes are associated with other DNA-binding transcription factors to regulate gene expression. Furthermore, the combination of the direct interactions of Smads with DNA and with sequence-specific DNA-binding transcription factors yields the selectivity of interaction between Smad complexes and the regulatory promoter sequences. Various families of transcription factors, such as the forkhead, homeobox, zinc-finger, activator protein 1, Ets, and basic helix-loop-helix (bHLH) families, serve as Smad partners (Ikushima et al. 2008; Koinuma et al. 2009a, b). The juxtaposition of an SBE at variable distances from the sequence, to which the Smad-interacting transcription factor binds, allows selection of a subset of promoter sequences to which the Smad transcription complexes bind with high affinity. Each Smad-cofactor combination targets a particular set of genes, which is determined by the presence of cognate binding sequence element combinations in the regulatory regions of target genes. Gene responses induced by TGF-β are thus classified by groups of genes that are simultaneously regulated by a common Smad-cofactor combination. A group of genes jointly controlled by a given Smad-cofactor complex is denoted a "synexpression group". Cells of different types or those exposed to different environments contain distinct repertoires of transcriptional partners for Smads and link their cellular context to their responses to TGF-β (Fig. 3).

A novel negative regulator of TGF-β signaling, human homolog of maternal Id-like molecule (HHM), has been demonstrated to suppress TGF-β signaling in a cell-response-selective fashion (Ikushima et al. 2008; Seto et al. 2009). Among the several cell responses induced by TGF-β, cell cycle arrest is repressed by HHM, but EMT is not. HHM bins to DNA-binding transcription factor Olig1 (oligodendrocyte transcription factor 1), a novel Smad-binding cofactor, and abrogates the binding of Olig1 to Smad proteins. Olig1 and R-Smads interact with each other on chromosomes and synergistically promote the expression of TGF-β target genes whose promoter regions have Olig1-binding sequence(s) and Smad-binding sequence(s) in close vicinity. HHM interferes with the interaction between Olig1 and the activated Smad complex and, as a consequence, inhibits the gene expression of the Olig1-Smad synexpression group at the transcriptional level. Since HHM interacts with some but not all Smad-binding transcription factors, HHM abrogates only a subset of Smad-cofactor complexes, including the Olig1-Smad complex. HHM thus inhibits TGF-β-induced cell responses, which are controlled by Smad-cofactor synexpression groups targeted by HHM, but fails to affect cell responses, which are regulated by Smad-cofactor synexpression groups not targeted by HHM.

The transcriptional cooperativity of Smad complexes with a variety of DNA-binding transcription factors thus creates marked complexity in the transcriptional regulation of target genes.

Genetic alterations

Although all cells except immune cells have nearly identical blueprints, or genomes, under physiological conditions, cancer cells have a variety of genetic alterations conferring survival advantage on them. Deletion or ampli-

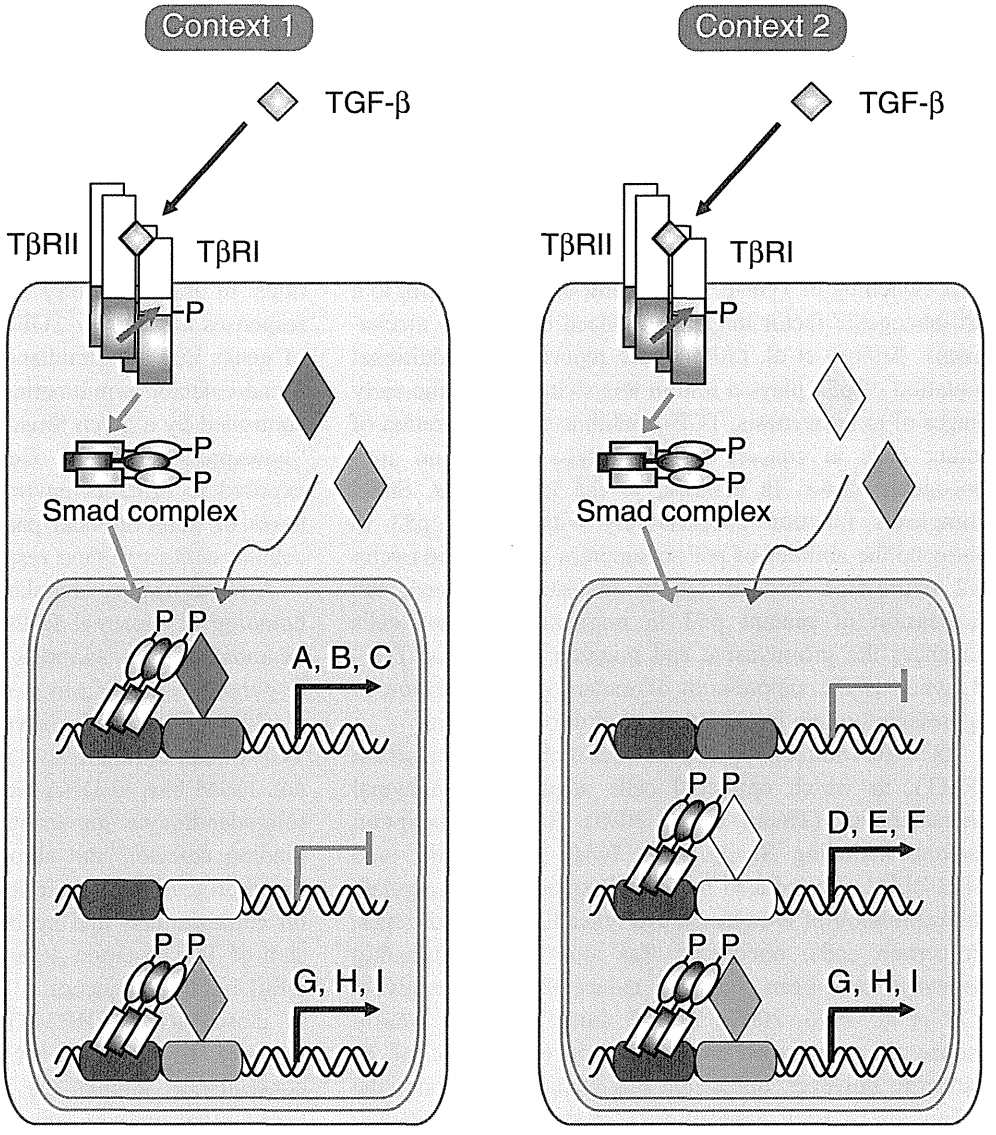

Fig. 3 "Cofactors" model. TGF-β target genes (*A–I*) are regulated by Smad proteins. Profiles of expression of cofactors of Smad proteins differ between *Context 1* and *Context 2*, resulting in different responses to TGF-β stimulation

fication of TGF-β target genes in cancer cells alters their responsiveness to TGF-β stimulation (Fig. 4). Although TGF-β up-regulates the expression of $p15^{Ink4b}$, one of the tumor suppressor genes, to inhibit cell proliferation (Hannon and Beach 1994), a subset of glioma cells sustains homozygous deletion of the $p15^{Ink4b}$ locus on chromosome 9p21 (Jen et al. 1994). Loss of $p15^{Ink4b}$ attenuates the anti-oncogenic effects of TGF-β, and glioma cells might benefit from host- and/or tumor-derived TGF-β stimulation.

Thus, genetic alterations of downstream genes modify the cell responses induced by TGF-β and contribute to the cellular context-specific plasticity of TGF-β signaling.

Epigenetics

Classical genetic processes are not sufficient to establish an organism. For proper development and cell functioning, epigenetic phenomena are absolutely required for the control of gene expression (Hirabayashi and Gotoh 2010; Ordovás and Smith 2010). In addition to genetic mechanisms, the gene expression and cell responses induced by TGF-β stimulation are regulated by epigenetic systems, including DNA methylation and post-translational histone modulation (Fig. 5).

DNA methylation is one of the most intensely studied epigenetic modifications in mammals and has a large impact on molecular pathophysiology and normal cell physiology (Esteller 2008; Suzuki and Bird 2008). Indeed, tumor cells are characterized by a different methylome from that of normal cells (Kulis and Esteller 2010). Interestingly, both hypo- and hypermethylation events can be observed in cancer. For instance, two cell-cycle-related genes, $p16^{INK4a}$ and $p15^{INK4b}$, undergo DNA methylation-mediated silencing in various types of cancer, leading to tumor development (Kulis and Esteller 2010).

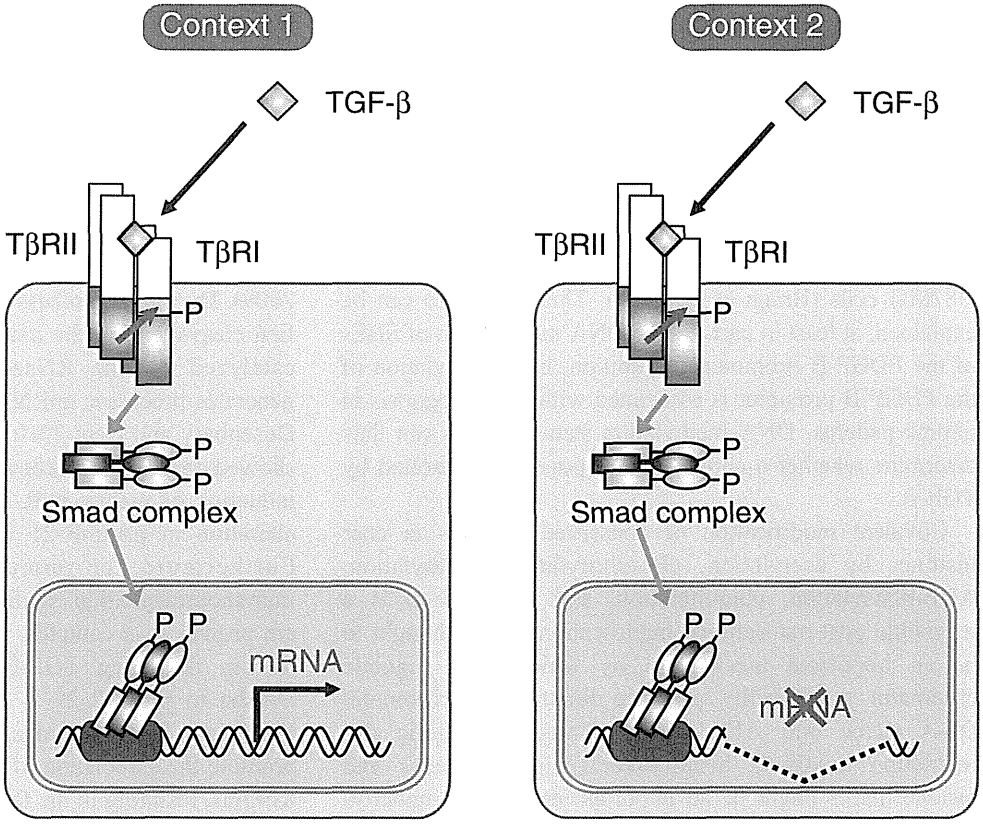

Fig. 4 "Genetic alterations" model. In *Context 1*, expression of a certain target gene is induced by TGF-β signaling. In *Context 2*, the gene is deleted at the chromosomal level, and TGF-β stimulation fails to induce its expression

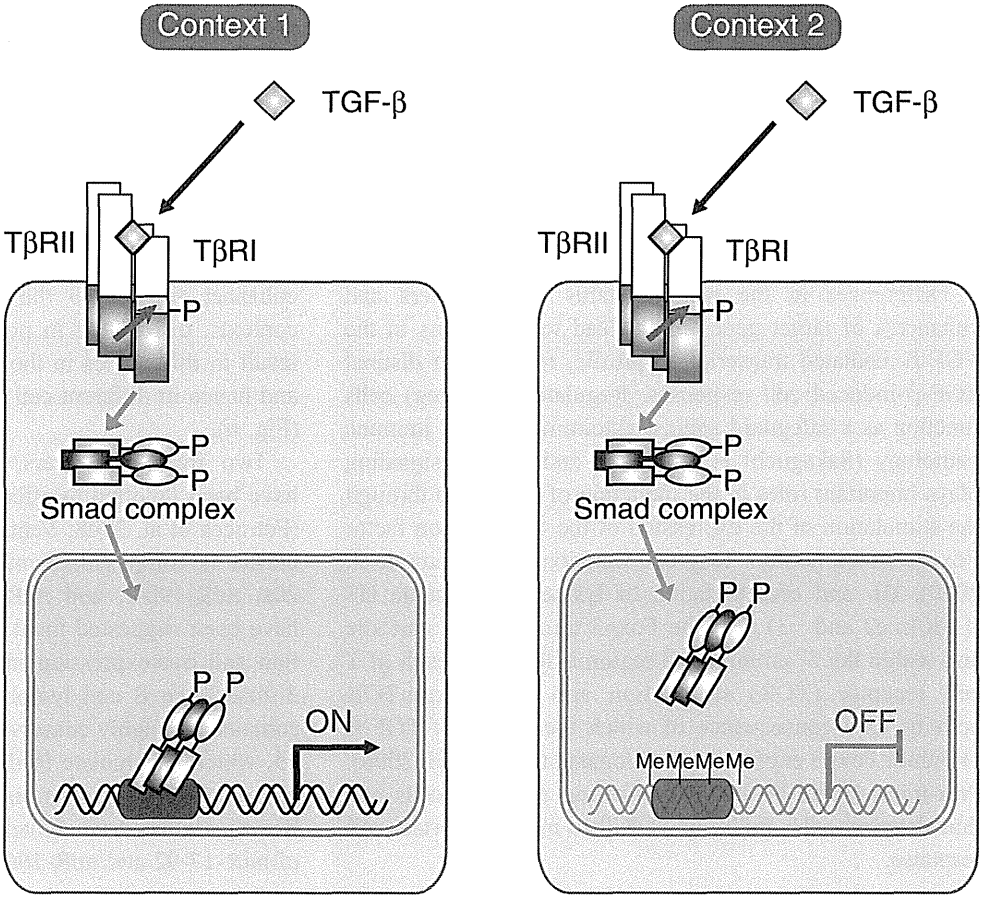

Fig. 5 "Epigenetics" model. In *Context 1*, promoter regions of certain TGF-β target genes adopt an "open conformation" and are exposed to the Smad complex. Conversely, in *Context 2*, promoter regions of the same target genes adopt a "closed conformation", and the Smad complex fails to access the Smad-binding elements. This difference results in differential responses to TGF-β stimulation

On the other hand, a global decrease in methylated CpG content contributes to genomic instability and to the activation of silenced oncogenes.

The regulation of gene expression by TGF-β can be affected by DNA methylation status. TGF-β induces platelet-derived growth factor-B (PDGF-B) expression in glioblastoma U373MG cells but fails to affect it in another glioblastoma cell line, U87MG cells. TGF-β thus induces the proliferation of U373MG cells but inhibits that of U87MG cells (Bruna et al. 2007). This difference can be explained, at least in part, by the DNA methylation of SBEs of the PDGF-B promoter. In addition, hypomethylation of the PDGF-B promoter is associated with poor prognosis in glioma patients. DNA methylation status in cells can thus determine whether a certain cell response is controlled by TGF-β.

Covalent modification of conserved residues in core histones by acetylation, phosphorylation, methylation, ADP-ribosylation, ubiquitination, and sumoylation is a reversible post-translational modification and is thought to be an important mechanism by which cells regulate chromatin accessibility and the function of chromatin DNA (Rice and Allis 2001). Thus, epigenetic de-regulation involving histone-modifying complexes and histone marks might be an important mechanism underlying the development and progression of diseases (Sawan and Herceg 2010). Furthermore, recent research has demonstrated that different types of cells might have specific patterns of histone modifications (histone modification signatures), which cause cellular context-dependent behaviors of cells (Lee et al. 2010). Indeed, the modification of histones varies drastically during tumorigenesis, and the disruption of many chromatin-modifying proteins is associated with the formation of various malignant tumors (Esteller 2007).

Differences in the histone status of promoters and enhancers of target genes might lead to alterations in the TGF-β-mediated transcription profile, resulting in distinct TGF-β-induced cell responses. Regulatory T (Treg) cells function as a safeguard against autoimmunity and immune pathology (Sakaguchi et al. 2010), and TGF-β signaling plays important roles in the induction of Treg cells through the stimulation of the expression of the transcription factor Foxp3, which confers Treg cell function (Yoshimura et al. 2010). Di- and trimethylation of lysine 4 of histone H3 (H3K4me2 and −3) near the Foxp3 transcription start site and within the 5' untranslated region is lost as a result of T cell receptor (TCR) stimulation and PI3K/Akt/mTOR activity, as a consequence of which the ability of TGF-β to induce Foxp3 expression is abrogated (Sauer et al. 2008). Post-translational histone modification status in cells can thus determine the ability of TGF-β to induce a certain cell response.

Non-coding RNA

Interactions of TGF-β signaling and non-coding RNA occur at various levels. microRNAs (miRNAs) are small non-coding RNAs that modulate diverse biological functions through the repression of target genes (Filipowicz et al. 2008; Winter et al. 2009). Recent studies have demonstrated that Smad complexes play a regulatory role in the processing of miRNA in the nucleus (Hata and Davis 2009). During the process of the maturation of miRNA, the first cleavage after the transcription of the miRNA gene is catalyzed by the RNase III enzyme Drosha, which generates precursor miRNA from primary miRNA (Davis-Dusenbery and Hata 2010). Davis et al. (2008, 2010) have showed that the knockdown of the R-Smads prevents the induction of mature miR-21 and pre-miR-21, although no alteration in pri-miR-21 transcription has been detected. Furthermore, co-immunoprecipitation and RNA-immunoprecipitation studies have confirmed that Smads are present in a complex with Drosha and the pri-miR-21 hairpin following TGF-β stimulation. The binding of Drosha to pri-miR-21 is also elevated following TGF-β treatment. These findings indicate that Smad complexes promote the association of Drosha with a subset of miRNA hairpins, resulting in the facilitation of the processing of the miRNAs, and that TGF-β can regulate gene expression not only through the direct transcriptional regulation of target genes, but also through miRNA processing.

Non-coding RNAs also contribute to the context-dependent diversity of TGF-β-induced cell responses (Singh and Settleman 2010). Cells of different cell types or cells exposed to different conditions express diverse repertoires of non-coding RNA (Lu et al. 2005), and TGF-β stimulation thus produces context-specific cell responses. Even when TGF-β stimulation activates promoter and/or enhancer regions to the same degree in two different contexts, differences in post-transcriptional regulation can result in differences in the levels of expression of proteins and hence in different cell responses to TGF-β stimulation (Fig. 6).

Two miRNA clusters, miR-17-92 and miR-106b-25, have been reported to affect the TGF-β signaling pathway (Petrocca et al. 2008; Ventura et al. 2009). The miR-17-92 cluster is composed of miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a-1. Tumor-promoting roles have been suggested for it based on its frequent amplification and overexpression in small-cell lung carcinoma and diffuse large B cell lymphoma. The miR-106b-25 cluster contains the highly conserved miR-106b, miR-93, and miR-25, which accumulate in different types of cancer, such as neuroblastoma, gastric cancer, and multiple myeloma. Recent studies have unveiled the functional involvement of miR-17-92 and miR-106b-25 clusters in TGF-β-induced

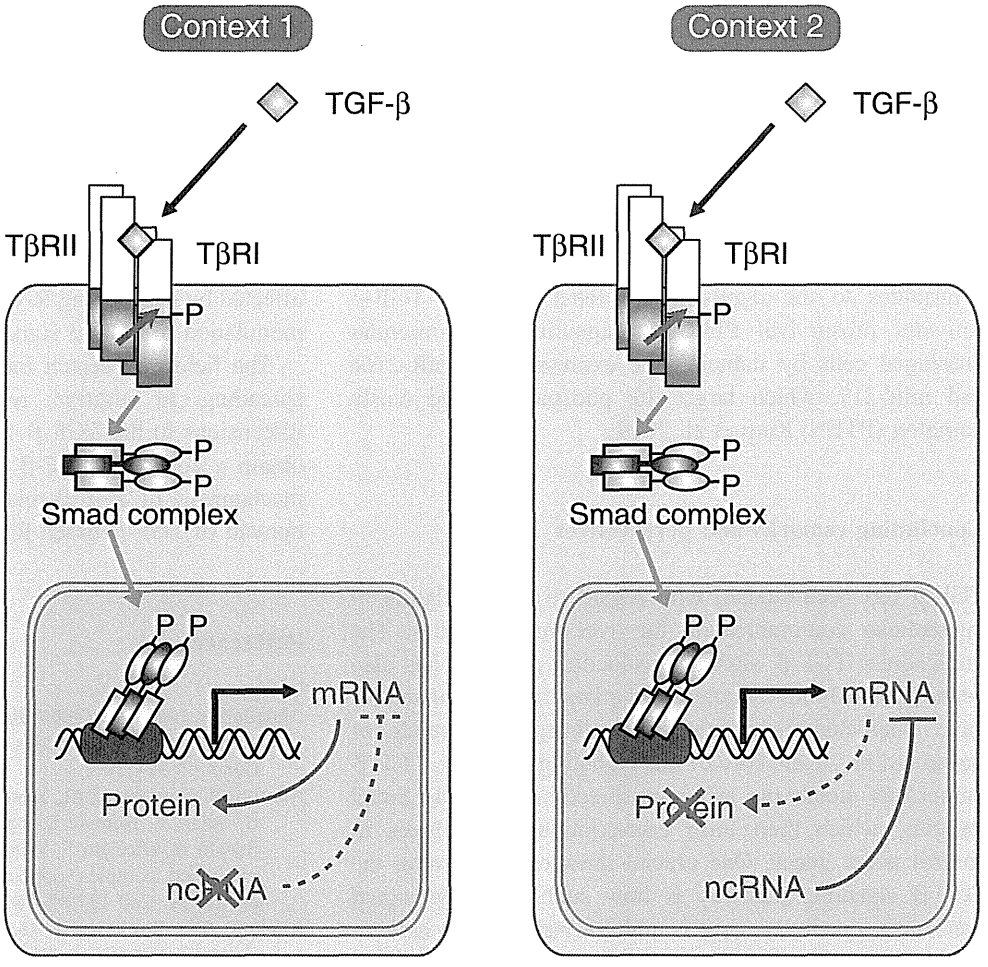

Fig. 6 "Non-coding RNA" model. In *Context 2*, transcribed mRNAs of TGF-β target genes are negatively regulated by non-coding RNA (*ncRNA*). In *Context 1*, such ncRNA is not expressed, resulting in the translation of the mRNAs

apoptosis and cell cycle arrest. They silence two main downstream effectors playing central roles in these cell responses: the pro-apoptotic gene Bim and the cyclin-dependent kinase inhibitor $p21^{Waf1}$. Furthermore, overexpression of miR-25 inhibits TGF-β-induced apoptosis, and overexpression of miR-106b and miR-93 prevents TGF-β-mediated cell cycle arrest. These reports indicate that the profiles of expression of miR-17-92 and miR-106b-25 clusters can determine whether TGF-β signaling has tumor-suppressive effects.

The miR-17-92 cluster is also involved in the post-transcriptional regulation of some of the regulatory components in TGF-β signaling. This cluster targets Smad4 and TβRII and, as a result, shuts down this signaling pathway (Dews et al. 2010; Mestdagh et al. 2010). In addition, enforced expression of miR-17-92 has been demonstrated to result in impaired gene activation by TGF-β in glioblastoma cells (Dews et al. 2010) and neuroblastoma cells (Mestdagh et al. 2010).

TGF-β-induced EMT, in which epithelial cells acquire mesenchymal characteristics, has been reported to be regulated by the miRNA-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429; Gregory et al. 2008; Korpal et al. 2008; Burk et al. 2008; Park et al. 2008). These miRNAs cooperatively interfere with expression of δEF1/ZEB1 and SIP1/ZEB2, which are transcriptional repressors of E-cadherin induced by TGF-β and involved in EMT. Manipulation of miR-200 family expression suppresses EMT and induces the opposite change, namely mesenchymal-epithelial transition. Since the levels of expression of the miR-200 family might vary from cell to cell, they determine, at least in part, at downstream gene levels whether TGF-β induces EMT. TGF-β has also been demonstrated to induce miR-155 expression through the Smad pathway, which in turn regulates epithelial plasticity by targeting RhoA and promotes TGF-β-mediated EMT as a result of the dissolution of tight junctions (Kong et al. 2008).

TGF-β-induced miRNAs also play important roles in cancer stem cells. TGF-β up-regulates miR-181 at the post-transcriptional level in breast cancer cells. miR-181 targets a tumor suppressor (ataxia telangiectasia mutaed, ATM) and maintains the breast cancer stem cell population (Wang et al. 2011).

PDGF-BB antagonizes the effects of TGF-β in certain cells, including smooth muscle cells, and Chan et al. (2010)

have reported that this antagonism is mediated in part via the function of miR-24. However, PDGF-BB induces the expression of miR-24, which in turn down-regulates Tribbles-like protein-3 (Trb3). Trb3 has been shown to induce the degradation of Smurf1 (Chan et al. 2007), and repression of Trb3 by miR-24 therefore results in the reduced expression of Smad proteins and the attenuation of TGF-β and bone morphogenetic protein signaling.

The interaction of TGF-β signaling and miRNAs also contributes to the regulation of renal function. TGF-β activates prosurvival PI3K-Akt signaling in glomerular mesangial cells by inducing the expression of miR-216a and miR-217, which target the phosphatase and tensin homolog (PTEN; Kato et al. 2009).

Concluding remarks and perspectives

TGF-β has been studied with regard to the regulation of intercellular communication for over three decades. The intracellular TGF-β signal transduction pathway has also been vigorously investigated, and a large number of studies have elucidated its simple but well-organized mode of transmission. At the core of this signaling pathway, TGF-β induces its membrane receptors directly to activate Smad proteins, which then form transcriptional complexes to control target genes. One crucial question concerning the TGF-β signaling pathway is how such a simple signal transduction pathway triggers multiple behaviors in cellular context-dependent fashion, i.e., how does TGF-β induce different responses in two different types of cells, despite their derivation from a single cell and possession of identical genetic makeup?

This question has been answered in part in terms of the classical frames: cross-interaction with other signaling pathways, different repertoires of Smad-binding transcription factors, and genetic alterations, especially in cancer cells. Nevertheless, the question remains largely unanswered, and recent research has added new frames to the field of intracellular TGF-β signal transduction.

The importance of epigenetic regulation in the development and maintenance of the human body is indicated by its disturbance in several types of diseases. Not surprisingly, gene expression and cell responses induced by TGF-β stimulation are regulated by epigenetic systems. Dynamic epigenetic changes determine an "open conformation" or "closed conformation" of chromatin status on TGF-β target genes; this is directly reflected in the induction of certain cell responses by TGF-β. Thus, differences in the epigenetic map can, at least in part, explain the cellular context-dependent diversity of TGF-β-induced cell responses.

Another new frame of intracellular signal transduction is its regulation by non-coding RNAs. The subtraction of transcribed mRNAs has added a novel paradigm to the regulation of TGF-β signal transduction, and recent research has demonstrated that interactions of TGF-β signaling and non-coding RNA occur at various levels. In addition to changes in non-coding RNA repertories by TGF-β stimulation at the transcriptional level, the TGF-β-Smad pathway is involved in the process of maturation of miRNAs. On the other hand, TGF-β-mediated cell responses, including cell proliferation and EMT, are affected by non-coding RNAs through direct and/or indirect modulation of TGF-β signaling.

The field of research into TGF-β signaling is thus still spreading. In addition, recent research has added new dimensions to the TGF-β field. Further work is needed to obtain a complete TGF-β map for the elucidation of the mechanisms of TGF-β-related diseases and for the development of TGF-β-based therapeutic strategies.

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REVIEW

Genome-wide mechanisms of Smad binding

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A dual role of transforming growth factor β (TGF-β), to both suppress and promote tumor progression and metastasis, has been well established, but its molecular basis has remained elusive. In this review, we focus on Smad proteins, which are central mediators of the signal transduction of TGF-β family members. We describe current knowledge of cell-type-specific binding patterns of Smad proteins and mechanisms of transcriptional regulation, obtained from recent studies on genome-wide binding sites of Smad molecules. We also discuss potential application of the genome-wide analyses for cancer research, which will allow clarification of the complex mechanisms occurring during cancer progression, and the identification of potential biomarkers for future cancer diagnosis, prognosis and therapy.

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INTRODUCTION

Members of the transforming growth factor β (TGF-β) family, which include three TGF-β isoforms, as well as activins, nodal and bone morphogenetic proteins (BMPs), regulate a variety of cellular processes including differentiation, proliferation, migration and cell death in cell-type-specific and context-dependent manners.[1–3] The biological effects of TGF-β family members are highly contextual, for example, their responses may differ in different tissues, local environments and stage of disease. Since TGF-β activates cytostatic and cell death processes that maintain homeostasis in mature tissues, it functions as a suppressor of epithelial cell tumorigenesis at early stages. Inactivation of the TGF-β signaling pathway through mutation and/or loss of heterozygosity of TGF-β receptors or Smad proteins has been found in certain types of cancer and is related to poor prognosis for the patients (reviewed in Levy and Hill[4]). However, TGF-β promotes tumor progression by enhancing migration, invasion and survival of tumor cells during the later stages of tumorigenesis, through stimulating extracellular matrix deposition and tissue fibrosis, perturbing immune and inflammatory function, stimulating angiogenesis and promoting epithelial–mesenchymal transition (reviewed in Yoshimura *et al.*[5], Roberts and Wakefield[6], Moustakas and Heldin[7] and Miyazono *et al.*[8]). Accumulating evidences also indicate critical roles of TGF-β/activin signaling in the maintenance of stem cell-like properties of certain cancer-initiating cells, such as glioma-initiating cells,[9,10] breast cancer-initiating cells,[11] pancreatic cancer-initiating cells,[12] and leukemia-initiating cells in chronic myeloid leukemia.[13] Intriguingly, small molecular inhibitors for type I receptors have therapeutic effects at least in animal models.[9,10,12,13] These observations suggest that targeting the TGF-β/activin signaling pathways could be an attractive therapy in certain advanced cancers, although it is possible that shutdown of these pathways in normal tissues will increase the risk for the development of other tumors. Thus, one of the major questions that remain to be addressed in this field is what defines the dual role of TGF-β in cancer biology.

Identification of the signaling components of TGF-β family members, including membrane receptor serine/threonine kinases and Smad transcription factors, has led to an understanding of the molecular mechanisms underlying this highly contextual process.[14,15] Genome-wide transcriptome analyses in various cell types have identified many target genes that are required for ligand-mediated cellular responses. Direct binding of Smad complexes was confirmed by *in vitro* binding assays, promoter assays and chromatin immunoprecipitation (ChIP) followed by polymerase chain reaction. Until recently, however, regulatory elements were mainly identified in the promoter regions of the target genes, especially 1–2 kb upstream of their transcription start sites.

ChIP with promoter array analysis (ChIP-chip) and ChIP followed by sequencing (ChIP-seq) have become powerful tools to analyze genome-wide mapping of protein-binding sites and epigenetic marks.[16,17] In this case, a DNA sample obtained after ChIP procedure is analyzed using promoter-tiling arrays, or massively parallel sequencing (Supplementary Figure 1), which provides a comprehensive chromatin-binding landscape of target transcription factors. Information obtained by these analyses has shed light on previously unrecognized mechanisms and sometimes challenged notions previously characterized in a specific situation. Recently, several groups have reported that Smad proteins tend to co-occupy target sites with cell-type-specific master transcription factors.[18–20] The results also indicate that co-occupied regions mainly overlap with enhancer elements, although previous studies have identified numerous Smad-responsive elements in the promoter regions of their target genes. In addition, recent ChIP-chip/ChIP-seq studies have identified a group of direct target genes, or target gene signatures, in specific cell types and cellular contexts. Intriguingly, Kennedy *et al.*[21] reported that the TGF-β/Smad4 target gene signature identified in ovarian cancer cell lines predicts patient survival.

In this review, we discuss current knowledge of cell-type-specific binding patterns of Smad proteins and mechanisms of transcriptional regulation obtained from recent ChIP-chip/

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ChIP-seq studies (Supplementary Table 1). We also highlight applications of the genome-wide analyses for cancer research. These insights contribute to the unraveling of the complex mechanisms of TGF-β signaling in cancer biology.

OVERVIEW OF SIGNALING PATHWAYS OF TGF-β FAMILY MEMBERS

The TGF-β family consists of 33 members in mammals. Two types of serine/threonine kinase transmembrane receptors, that is, type II and type I receptors, are required for intracellular signal transduction by the TGF-β family members.[14] Five type II receptors and seven type I receptors are present in mammals.[22] Ligand binding assembles specific type II and type I receptors into heterotetramers. Then the type II receptor transphosphorylates and activates the type I receptor, which subsequently transduces the signal by phosphorylating the carboxyl terminus of receptor-regulated (R)-Smad. In most cell types, TGF-β and activin induce phosphorylation of Smad2 and Smad3 (activin/TGF-β-specific R-Smads, or AR-Smads) and BMPs induce phosphorylation of Smad1, Smad5 and Smad8 (BMP-specific R-Smads, or BR-Smads). Activated R-Smads form heterooligomeric complexes with common-partner (co)-Smad (Smad4). The complexes translocate into the nucleus where they regulate the expression of target genes, such as the genes for *Serpine1* (plasminogen activator inhibitor-1), inhibitory (I)-Smads (Smad6 and Smad7) and *Id1* (inhibitor of differentiation-1 or inhibitor of DNA binding-1) (Figure 1). Because of their relatively low DNA-binding affinity, Smad complexes interact with a wide variety of DNA-binding proteins and cooperatively regulate a synexpression group of target genes (Figure 2a).[2] So far, several transcription factors, such as AP-1,[23] ETS,[24,25] basic helix-loop-helix proteins,[26,27] C/EBPβ,[28] FoxH1[29,30] and FoxO[31] have been identified and validated as important cofactors of TGF-β/BMP signaling pathways. In addition, Smad complexes recruit coactivators, such as p300 and CREB-binding protein,[32,33] or corepressors, such as ATF-3.[34] For example, TGF-β represses transcription of the *Id1* gene in epithelial cells through formation of a complex with ATF-3, while TGF-β induces Id1 in cells which do not express ATF-3, such as glioma-initiating cell-like cells.[35] Since ATF-3 is induced by tumor necrosis factor-α, signaling crosstalk between TGF-β and tumor necrosis factor-α pathways determines the transcriptional regulation of Id1. Thus, crosstalk with other signaling pathways and interaction with other DNA-binding cofactors define the specific binding patterns of Smads; in addition, interaction with coactivators/corepressors modulates their transcriptional activity (Figure 1).

Smad proteins are targets of protein modifications, such as phosphorylation, ubiquitination and ADP-ribosylation. The cyclin-dependent kinases (CDKs) CDK8 and CDK9, which are downstream effectors of extracellular-signal-regulated kinase (ERK) MAP kinase, phosphorylate the linker region of Smads in the nucleus.[36–39] Glycogen synthase kinase-3β (GSK3β) also phosphorylates the linker region of Smads, which requires priming phosphorylation by ERK MAP kinase.[40] These phosphorylations mark the proteins for polyubiquitination and promote proteasome-mediated degradation of Smad complexes. Several WW domain proteins have been reported to recognize the phosphorylated linker regions and interact with R-Smads.[41] Smurf1 is a member of the E3 ubiquitin ligase family, which can target BR-Smads for degradation,[42] while NEDD4L (also known as NEDD4-2) is an E3 ubiquitin ligase for AR-Smads.[43,44] Consequently, endogenous ERK MAPK and GSK3β signaling pathways are able to antagonize Smad activity through proteasome-mediated degradation. Recently, deubiquitinating enzymes (DUBs) for Smad proteins have been identified.[45,46] Monoubiquitination of the lysine-519 (K519) residue of Smad4 prevents its association with R-Smads and negatively regulates TGF-β/BMP signaling pathway. USP9x (also known as FAM) has been identified as a DUB that reverts this modification.[45] R-Smads are monoubiquitinated in their DNA-binding domains, which attenuates their affinity for DNA. This monoubiquitination is opposed by another DUB, USP15.[46] Recently, Lonn *et al.*[47] found that Smad proteins are targets of ADP-ribosylation. Poly(ADP-ribose) polymerase-1 (PARP-1) interacts with and ADP-ribosylates Smad3 and Smad4 in the nucleus, and affects the binding affinity of Smad complexes in a context-dependent manner.[47,48] Thus, posttranslational modifications of Smad proteins affect their signal transduction capacities; some of these modifications are regulated by other signaling pathways (Figure 1).

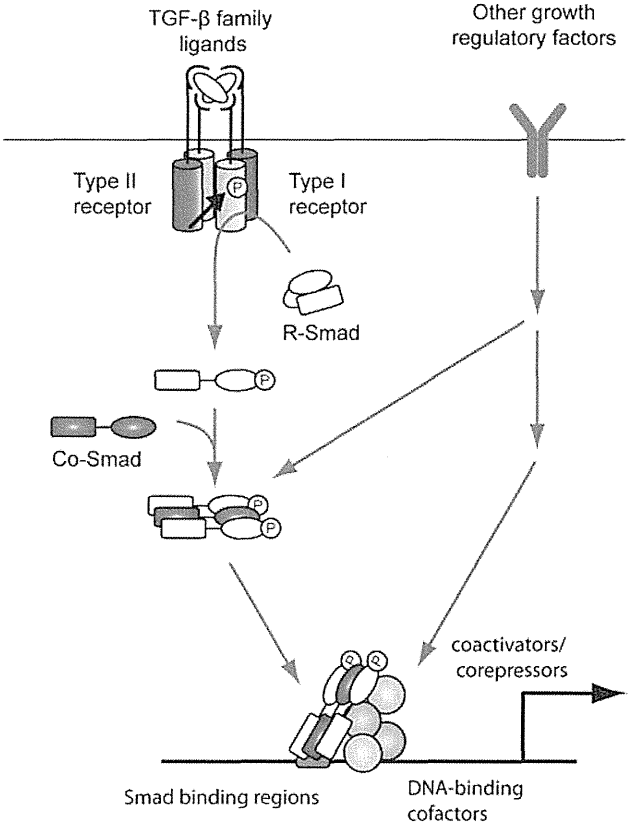

Figure 1. Signaling of TGF-β family members through Smad complexes. Smad proteins are central mediators of the signal transduction of TGF-β family members. Ligand binding assembles specific type II and type I receptors into heterotetramers. The type II receptor transphosphorylates (P) and activates the type I receptor, which subsequently activates receptor-regulated (R)-Smads. Activated R-Smads form heterooligomeric complexes with common-partner (co)-Smad. In the nucleus, Smad complexes interact with DNA-binding cofactors and cooperatively regulate a group of target genes. Crosstalk with other growth regulatory factors affects the specific binding patterns and transcriptional activity of Smads.

SMAD-BINDING MOTIFS

The R-Smads and Smad4 are composed of two evolutionally conserved domains named Mad Homology 1 and 2 (MH1 and MH2). The MH2 domain plays an important role for the formation of heterooligomeric Smad complexes and transcriptional activation, whereas the MH1 domain is responsible for sequence-specific DNA-binding activity. Using a polymerase chain reaction-based random-oligonucleotide selection process, an 8-bp palindromic DNA sequence, GTCTAGAC, was identified as a Smad3

and Smad4 binding motif.[49] In contrast to Smad3 and Smad4, Smad2 does not directly bind to DNA due to steric hindrance by an inserted sequence in the DNA-binding region.[50] The crystal structures of the MH1 domain of Smad1 and Smad3 have revealed that R-Smads recognize and directly bind to half of the palindrome, that is, GTCT or AGAC sequences, through an 11-amino-acid residue β-hairpin loop in the MH1 domain.[51–53] The amino-acid sequences of the loop are completely conserved among R-Smads and show a high level of similarity between R-Smads and Smad4. The half-site sequences are usually referred to as the CAGA box or Smad binding element (SBE). Recent ChIP-chip/ChIP-seq studies have confirmed that the SBE is enriched in the Smad2/3-binding regions.[18,24,26,54,55]

Although the MH1 domain of Smad1 has high affinity for SBE,[52,53] BR-Smads seem to prefer a GC-rich sequence, such as GCCGnCGC, which was originally identified in *Drosophila*.[56] In mammals, GC-rich sequences, such as GCCG and (T)GGCGCC, have been identified in the promoter regions of several BMP target genes. Using a *de novo* motif-finding method, we identified a Smad1/5-binding motif, which is consistent with the previously reported GC-rich sequences and thus named as GC-rich SBE (GC-SBE).[57] Importantly, both GC-SBE and SBE are enriched in the Smad1/5-binding sites identified in both endothelial cells (ECs) and pulmonary arterial smooth muscle cells (PASMCs).[57] Since binding motifs for R-Smads have been identified *in vitro* and *in vivo*, candidate Smad-binding sites can be predicted in the promoter regions of the target genes. However, these motifs are common throughout the genome, and the majority of them are not occupied by R-Smads when examined using ChIP-chip/ChIP-seq. Thus, additional mechanisms operate to determine the binding patterns of Smads.

FACTORS THAT DETERMINE THE BINDING PATTERNS OF SMADS

Recent studies have suggested that Smad complexes colocalize with master transcription factors that specify and maintain cell identities.[18–20] Chen *et al.*[20] pointed out that Smad1 colocalizes in the multiple transcription factor-binding loci with embryonic stem (ES) cell-specific transcription factors, such as Oct4 and Sox2 in mouse ES cells (mESCs). Mullen *et al.*[18] reported that binding regions of Smad3 also overlap with those of Oct4 in both human and mouse ES cells. Intriguingly, at least some of these co-occupied regions are still enriched after tandem ChIP-re-ChIP experiments, indicating that Oct4 and Smad3 bind to similar regions in mESCs simultaneously.[18] Moreover, Smad3 colocalizes with MyoD (encoded by *Myod1*) or PU.1, master transcription factors controlling muscle or hematopoietic differentiation, respectively, in specific cell types which express these genes; forced expression of MyoD in mESCs is sufficient to redirect Smad3 to muscle specific binding sites, where they colocalize.[18] In addition, Trompouki *et al.*[19] reported that induction of the myeloid lineage regulator C/EBPα shifted Smad1 to sites newly occupied by C/EBPα in the human erythroleukemia cell line K562. Overexpression of the erythroid regulator GATA1 restricts Smad1 binding to erythroid genes, while binding to genes expressed in other lineages is diminished.[19] These findings suggest that Smad complexes are passively recruited to cell-type-specific binding sites through the interaction with master transcription factors.

On the other hand, we recently found that HNF4α, one of the master regulators of hepatocyte differentiation and liver function, contributes to the hepatocyte-specific binding pattern of Smad2/3.[58] Interestingly, 32.5% of the Smad2/3-binding regions overlapped with those of HNF4α. This is against the simple model in which cell-type-specific master regulators recruit R-Smads to their binding sites and determine their function. In addition, through the analysis of the distances between the Oct4 peak and the peaks of Sox2 and Smad3 in mESCs, Mullen *et al.*[18] found that Oct4 sites are more closely associated with Sox2 sites than Smad3 sites, suggesting that Oct4 and Smad3 do not interact in a direct manner. They revealed that nucleosomes were relatively depleted at the sites co-occupied by cell-type-specific master transcription factors and Smad3, and hypothesized that master transcription factors increase the accessibility of SBEs and contribute to Smad3 binding. Intriguingly, MyoD binding has been reported to be associated with local histone acetylation.[59] PU.1 and C/EBPα binding has been reported to induce nucleosome remodeling, followed by monomethylation of H3K4.[60] John *et al.*[61] reported that cell-type-specific glucocorticoid receptor binding patterns are comprehensively predetermined by cell-specific differences in baseline chromatin accessibility patterns, with secondary contributions from local sequence features. Similarly, comparison of Smad1/5-binding patterns of ECs and PASMCs suggested that the endothelial-specific binding pattern of Smad1/5 is predetermined by baseline chromatin accessibility patterns.[57] Thus, these facts support the notion that Smad complexes determine their target sites together with other DNA-binding cofactors in two different ways: (1) cell-type- or lineage-specific transcription factors, or pioneer factors,[62] open up local chromatin structure to make SBE and GC-SBE accessible and (2) DNA-binding cofactors, induced and activated in context-dependent manner, strengthen the interaction between Smad and DNA (Figure 2b).

Intriguingly, it has been observed that different levels of activation of Smad signaling pathways cause different binding patterns of Smad complexes, possibly correlating to the amount of activated Smad complexes in the nucleus.[63] It has been well described that different concentrations of activin regulate the expression of distinct subsets of target genes.[64] Lee *et al.*[54] confirmed that phospho-Smad2 is dose-dependently able to bind to different subsets of target genes and regulate their transcription in mESCs. Comparing the ChIP-seq data of different BMP isoforms in ECs, we found that each binding site has different binding affinity for Smad complexes and that the strength of Smad1/5 signaling affects the number and distribution of Smad-binding sites over the genome.[57] Thus, these findings suggest that a distinct dose-dependency occurs in the regulation of different subsets of target genes, which may cause phenotypic change.

SMAD BINDING AND HISTONE MODIFICATION MARKERS

As discussed above, local chromatin structure or accessibility affects the binding patterns of Smads. Recent studies have emphasized the importance of enhancers for the precise regulation of expression of target genes.[18–20,54,57] On the other hand, several groups have found that most of the Smad-binding sites are located at promoters of known genes.[30,65,66] Kim *et al.*[30] reported that 50–60% of Smad2/3 binding occurs in exons and promoters in human ES cells (hESCs), while only 10–15% of Smad binding occurs in exons and promoters in derived endoderm. This finding suggests that the preference of binding pattern of Smads to either promoters or enhancers is modulated by the differentiation stages.

Smad proteins have also been shown to induce local chromatin remodeling and modification at their binding sites. Both Smad1/5 and Smad2/3 have been reported to physically interact with a histone demethylase, KDM6B (also known as JMJD3), to recruit it to the *NOG* (encoding noggin) and *NODAL* promoter regions, respectively, and to cause the loss of the repressive mark histone H3 lysine-27 trimethylation (H3K27me3) in mESCs.[67,68] Recently, Kim *et al.*[30] reported that Smad2/3 and KDM6B are simultaneously enriched in the *GSC* (encoding goosecoid) and *EOMES* (encoding eomesdermin) promoter of hESCs after activin treatment, followed by the loss of the H3K27me3 repressive mark (Figure 3a). Interestingly, Fei *et al.*[65] identified that KDM6B is one of

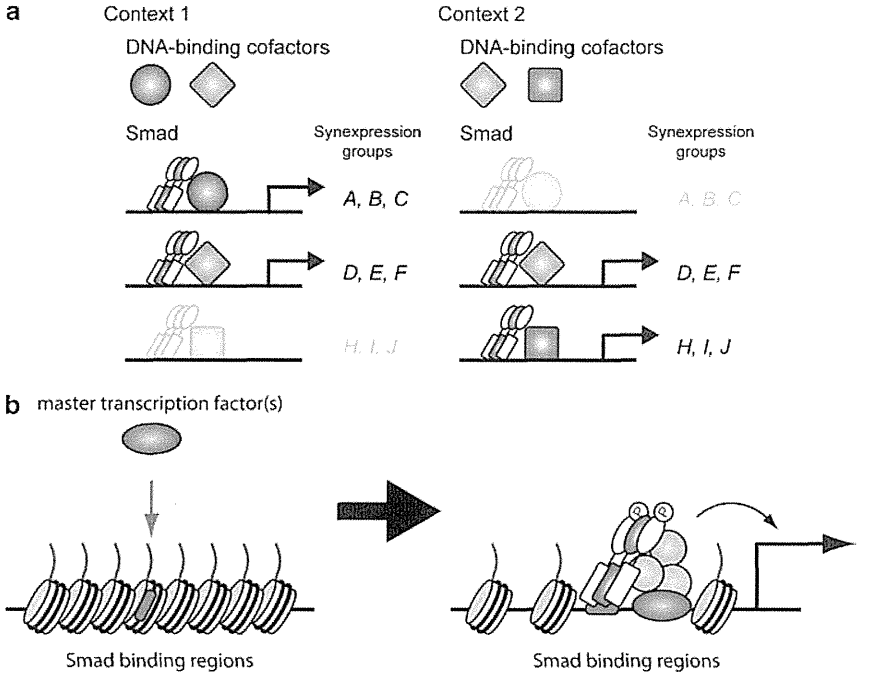

Figure 2. Factors that determine the binding patterns of Smads. (**a**) A group of genes that are simultaneously regulated by a specific Smad–cofactor complex is known as a synexpression group. Distinct combinations of DNA-binding cofactors in different contexts determine the set of genes regulated by Smad complexes. (**b**) Cell-type- or lineage-specific master transcription factors (purple) open up local chromatin structure to make Smad-binding regions (red) accessible. The master transcription factors also physically interact with Smads and, in some cases, recruit them to their binding sites. DNA-binding cofactors, induced and activated in context-dependent manner, strengthen the interaction between Smad and DNA. Interaction with coactivators/corepressors also affects the regulation of their target genes. A full colour version of this figure is available at the *Oncogene* journal online.

the BMP4-modulated early neural differentiation regulators, suggesting that loss of repressive histone marks through the Smad–KDM6B pathway explains the transcriptional regulation especially at later time points.

In addition to sequence-specific DNA-binding transcription factors, histone code reader proteins, which are recruited and bound to specific histone modifications, are reported to help to determine the binding sites of Smad proteins. Massagué and colleagues have reported that tripartite motif 33 (TRIM33, also known as TIF1γ or Ectodermin), physically interacts with Smad2 and Smad3, to make a TRIM33–Smad2/3 complex without Smad4.[69] The TRIM33 contains an N-terminal RING finger/B-box/coiled coil (RBCC) or TRIM domain, and a plant homeodomain (PHD) zinc finger and a Bromo domain in the C-terminus. They reported that the PHD-Bromo cassette recognized histone H3 lysine-9 trimethylation (H3K9me3) and H3 acetylation especially at lysine residues 18 and 23 (H3K18ac and H3K23ac). During mESC differentiation, nodal signaling triggered TRIM33–Smad2/3 complex formation. The TRIM33–Smad2/3 complex recognizes and binds to H3K9me3-K18ac dual histone marks and displaces the chromatin-compacting factor heterochromatin protein 1γ (HP1γ) in the *GSC* and *MIXL1* promoters, resulting in the remodeling of the local chromatin structure (Figure 3b).[70] Agricola *et al.*[71] also found that TRIM33 recognizes and binds to H3K18ac/K23ac. On the other hand, TRIM33 has been reported to bind Smad4 and function as a RING-type ubiquitin ligase for Smad4.[72] Consistent with this model, Agricola *et al.*[71] reported that TRIM33 inhibits Smad4 function through ubiquitin-mediated degradation of Smad4, and that its E3 ubiquitin ligase activity is induced after binding to histones. The detailed mechanisms have not been settled, but TRIM33 recognizes a specific histone code and modulates TGF-β/BMP signaling. Since the relationship between Smad proteins and histone modification marks has not been fully elucidated on a genome-wide scale, future analyses will address a possible mechanistic link between Smad proteins and epigenetic marks using ChIP-chip/ChIP-seq approach.

SMAD BINDING AND GENE REGULATION

Previous studies have indicated that binding of transcription factors detected by ChIP-chip/ChIP-seq experiments are not necessarily associated with transcriptional regulation of nearby genes (reviewed in Farnham[73]). It has frequently been observed that changing the level of a DNA-binding transcription factor alters the expression level of only 1–10% of its potential target genes. Most of the recent studies have confirmed that 1–20% of Smad-binding sites are associated with the regulation of expression of nearby genes. This discrepancy is in part due to the fact that mRNA levels do not only reflect transcriptional activities, since mRNA levels are also regulated by other biological processes, for example, degradation. Another explanation for the discrepancy is related to the definition of target genes. Although most studies assign binding sites to the nearest gene within 50 kb, this is not always the case. For example, Trompouki *et al.* revealed that several transcription factors, including Smad1, cooperatively regulate the expression of the hematopoietic gene *LMO2* through binding to the known enhancer region at 72 kb upstream of the transcription start site in K562 cells.[19,74] We also observed that Smad1/5 bound to a region 57 kb upstream of the transcription start site of *Smad6* in ECs, as well as the *LMO2* −72 kb enhancer.[57] This region has been reported to be associated with Smad6 expression in the heart, vasculature and hematopoietic organs,[75] suggesting that the binding to this region, as well as the promoter region, plays an important role in these cell types. Recently, methods that characterize the chromatin architecture have been developed. Chromosome conformation capture (3C) assays make