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TGFβ Signaling in Growth Control, Cancer, and Heritable Disorders

Review

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The transforming growth factor β (TGFβ) pathway occupies a central position in the signaling networks that control the growth, differentiation, and final fate of metazoan cells. Over the past few years, remarkable progress has been made in identifying the central components of this pathway, defining their interactions, and deciphering how a cell interprets its signals. Along the way, genetic alterations have been discovered in this pathway that provide answers to long-standing questions about the molecular basis of certain common somatic disorders as well as rare inherited ones. Recent reviews have covered TGFβ signal transduction (Heldin et al., 1997; Massagué, 1998; Whitman, 1998), transcriptional control (Derynck et al., 1998; Massagué and Wotton, 2000), and the regulation of these processes (Massagué and Chen, 2000). The present review focuses on the control of cell growth and differentiation by the TGFβ family, and the human disorders that result from genetic alterations in these pathways.

The Basics of TGFβ Signaling

Nearly thirty members of the TGFβ family have been described in human, and many orthologs are known in mouse, *Xenopus*, and other vertebrates (Hogan, 1996; Massagué, 1998). Four are present in *Caenorhabditis elegans* (Padgett et al., 1998), and seven in *Drosophila melanogaster* (Raftery and Sutherland, 1999). The family is divided into two general branches (the BMP/GDF and TGFβ/Activin/Nodal branches) whose members have diverse, albeit often complementary, effects. Additional members such as inhibin-α act as ligand antagonists. Some family members are expressed only in a few cell types or for limited periods of time during development, whereas others are widespread during embryogenesis and in adult tissues. AMH/MIS (Anti-Müllerian hormone or Müllerian inhibiting substance) and GDF8/myostatin are examples of the former; TGFβ1 and BMP4 of the latter.

TGFβ factors initiate signaling by assembling receptor complexes that activate Smad transcription factors (Figure 1) (Massagué, 1998). The ligand brings together members from two families of receptor serine/theonine kinases, known as the type I and type II receptors. The only known function of the type II receptors is to activate the type I receptors. The type I receptors propagate the signal by phosphorylating the Smads (Figure 1). Each ligand may have a choice of several type I and/or type II receptors (Figure 2), and a given cell may express different receptor forms. However, the various type I receptors funnel their activities through one of two groups of Smad proteins (Figure 1).

Several key events in the TGFβ receptor activation process are now understood. Adjacent to the kinase domain of the type I receptors is a conserved 30 amino acid segment known as the GS region (for a GSGS sequence it contains). In the basal state, the GS region forms a wedge that presses against the catalytic center (Huse et al., 1999). The immunophilins FKBP12 and FKBP12.6 bind to the GS domain and stabilize this inactive conformation. Activation occurs when the type II receptors phosphorylate the GS domain. To achieve this, the ligand must bring together type I and type II receptors, forming a heteromeric complex. The ligands themselves are dimers (most often homodimers held together by disulfide bonds), and each monomer has contact sites for type I and type II receptors, as defined using BMP2 (Kirsch et al., 2000a). The extracellular region of the receptors is formed by a small, tightly folded globular domain (Greenwald et al., 1999; Kirsch et al., 2000b) and the cytoplasmic region by a short juxtamembrane segment, a protein kinase domain, and often little else (Huse et al., 1999). In several cases, the extracellular or cytoplasmic regions contain alternatively spliced extensions of unknown function (Massagué, 1998, and references therein). One of these extensions, on the carboxy-terminus of the BMP type II receptor BMPR-II, is the target of mutations that cause familial primary pulmonary hypertension in humans (see below).

Smad Transcription Factors

Smad proteins are the only known TGFβ receptor substrates capable of signal transduction. They consist of two conserved globular domains known as the MH1 (*M*ad *h*omology 1) and MH2 domains coupled by a linker region (Figure 3) (Shi et al., 1997, 1998). The MH1 domain recognizes the DNA sequence CAGAC (Kim et al., 1997; Shi et al., 1998; Zawel et al., 1998) whereas the MH2 domain binds the transcriptional coactivators p300 and CBP in competition with the corepressors TGIF, Ski, and SnoN (reviewed in Massagué and Wotton, 2000).

Smads 2 and 3 (and perhaps the other Smads as well) have intrinsic nuclear import activity in the MH2 domain (Xu et al., 2000a). In the cell, however, most Smads are kept in the cytoplasm in the basal state, which ensures their prompt exposure to activated receptors. Cytoplasmic retention of Smads 2 and 3 is achieved in part by binding to the protein SARA (Smad anchor for receptor activation) (Tsukazaki et al., 1998). SARA plays three roles: it tethers Smads in the cytoplasm, it occludes a nuclear import signal on the MH2 domain (Xu et al., 2000a), and it facilitates Smad presentation to the activated receptors (Tsukazaki et al., 1998). Besides the Smad binding region, SARA contains a FYVE domain, a structure that in other proteins mediates binding to phosphatidylinositol 3-phosphate on endosome membranes.

The Smads that serve as receptor substrates (R-Smads) fall into two groups, each serving one branch

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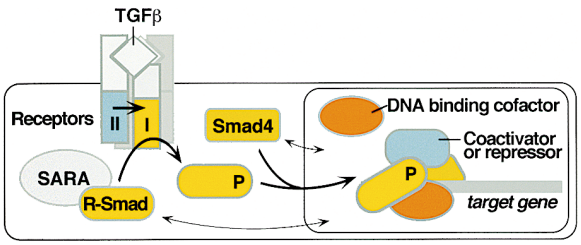

Figure 1. The Basics of TGFβ Signaling

A ligand-induced receptor complex phosphorylates a member of the R-Smad class (Smads1, 2, 3, 5, or 8), enabling its association with Smad4 and accumulation in the nucleus. In the nucleus, the activated Smad complex associates with two classes of proteins: DNA binding cofactors that will help select target genes, and coactivators or corepressors that will determine the transcriptional effect on the target genes. Smads have intrinsic nuclear import activity, but, at least in the case of Smad2/3, these proteins are retained in the cytoplasm by binding to SARA. Receptor-mediated phosphorylation of a R-Smad decreases its affinity for SARA and increases its affinity for Smad4.

of the TGFβ family (Figure 2). Structural elements have been identified that determine the specificity of the receptor–Smad interaction (Chen et al., 1998a) (Figure 3). Receptor phosphorylation of R-Smads, which occurs at the the carboxy-terminal end sequence SXS, diminishes the affinity of Smad2 for SARA, exposing the nuclear import signal. At the same time, phosphorylation increases the affinity of R-Smads for a second group, called Co-Smads, that are essential for the assembly of transcriptional complexes (Xu et al., 2000a). Only one Co-Smad (Smad4) is known in human and mouse, and it is shared by all R-Smads (Figure 1). A second Co-Smad (Smad4β) has been identified in *Xenopus* (Howell et al., 1999; Masuyama et al., 1999). Smad4 contains a nuclear export signal (NES) that keeps it out of the nucleus in the absence of agonist stimulation (Watanabe et al., 2000). Smad4β lacks this NES and is constitutively nuclear (Masuyama et al., 1999). Beyond this, the role of nuclear export of Smad4 and the functional differences between Smad4 and 4β remain unknown.

Alternative Pathways

Smad function is involved in most actions of the TGFβ family, which is not to say that the TGFβ receptors could not act on other substrates and activate other pathways. Several Smad4-defective cell lines from human or mouse retain some level of reponsiveness to TGFβ, suggesting that, if R-Smads are involved in these responses, they can do so without Smad4 (Dai et al., 1999; Sirard et al., 2000). A series of reports indicate that several MAP kinases (JNK, p38, and Erk) can be rapidly activated by TGFβ in a manner that is highly dependent on the cell type and conditions. The biochemical link between the TGFβ receptors and MAP kinase pathways has been elusive, although evidence suggests that the MAPKKK family member TAK1 (Takatsu et al., 2000) and Rho proteins (Engel et al., 1999) could be involved in this link. At least one TGFβ response, *fibronectin* induction, has been partly ascribed to JNK activation (Hocevar et al., 1999). Smads can interact in vitro with the JNK and p38 substrates c-Jun and ATF2, respectively, raising the possibility that TGFβ may simultaneously activate Smad and MAP kinase pathways that then physically converge on target genes (Zhang et al., 1998; Hanafusa et al., 1999; Sano et al., 1999; Wong et al., 1999). However, the physiological role of MAP kinases in TGFβ signaling remains uncertain due to a paucity of supportive genetic evidence.

Decisions in the Nucleus

By placing Smads in the nucleus, TGFβ conveys a signal but does not provide precise instructions. The cell's genetic makeup and responses to other signal inputs, more than the Smad complex itself, determine what genes will be recognized by this complex and the outcome of this target gene selection. This cellular context consists of at least two classes of Smad-interacting molecules: DNA binding cofactors and transcriptional coactivators and corepressors.

Why are DNA binding cofactors needed if Smads can bind DNA on their own? As it turns out, the affinity of Smads for their cognate sequence is too low to achieve unassisted binding to DNA (Shi et al., 1998). Cooperation between R-Smad and Smad4 might suffice for binding to certain genes that have two or more CACAG sequences appropriately spaced. However, many Smad gene responses are dependent on the cell type and conditions, implying that cell-specific factors dictate the choice of Smad target genes. Indeed, a group of structurally diverse proteins is emerging that plays such a role (Whitman, 1998, and references therein; Massagué and Wotton, 2000) (Figure 2). These molecules cooperate with activated Smads in binding only to those promoters that fulfill the combined sequence specificity requirements of a given Smad-cofactor combination. The expression of a Smad-DNA binding cofactor may be cell-type specific, conferring cell type specificity to a Smad response. Furthermore, each R-Smad subgroup is competent to associate with a different subset of DNA binding cofactors, thus achieving pathway specificity (Figure 2). Some Smad DNA binding cofactors, such as the winged-helix/forkhead family member FAST, the homeodomain protein Mixer, and the 30 zinc finger protein OAZ, have no detectable transcriptional activity on their own, whereas others, including c-Jun, TFE3, and Lef1/TCF, do (Derynck et al., 1998; Germain et al., 2000; Labbe et al., 2000; Massagué and Wotton, 2000; Nishita et al., 2000). Several of these are responsive to their own set of extracellular signals. For instance, c-Jun responds to diverse cytokines and cellular stress, and Lef1/TCF responds to Wnt/β-catenin signals.

On DNA, the Smad complex can recruit either transcriptional coactivators or corepressors (Luo et al., 1999; Sun et al., 1999; Wotton et al., 1999). Smad corepressors need not be viewed solely as negative regulators of Smad function. In theory at least, Smad corepressors might also serve as mediators of gene downregulation by TGFβ signals. Histone acetyl transferase activity associated with p300 and CBP and histone deacetylases (HDACs) recruited by TGIF, Ski, and SnoN give rise to Smad complexes of opposite chromatin remodeling activity. At a minimum, the choice between coactivators and corepressors depends on the relative abundance of these proteins. TGFβ can regulate

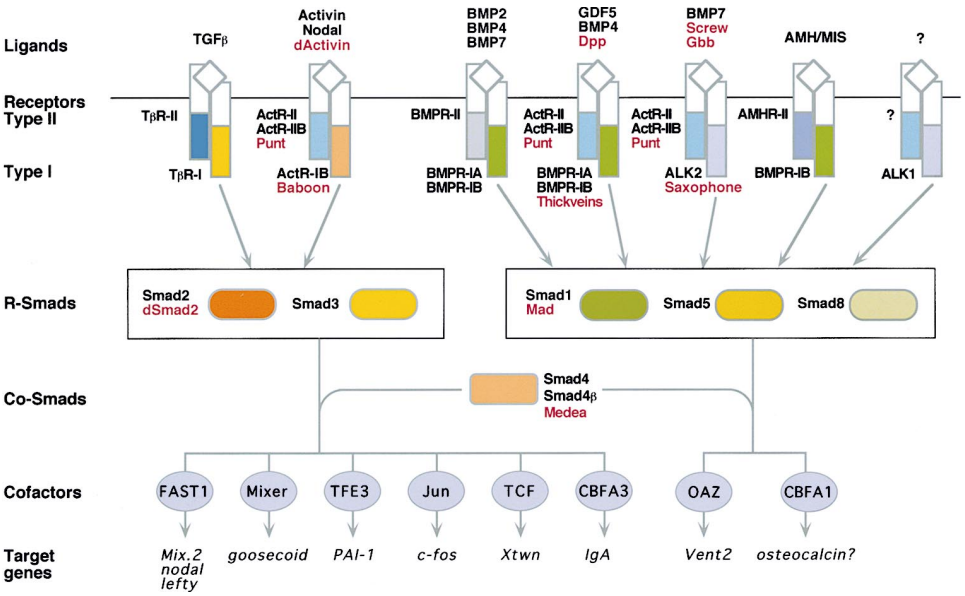

Figure 2. Ligand, Receptor, and Smad Relationships in the TGFβ System

Two branches of the Smad pathway mediate signaling by the two main groups of TGFβ family agonists. The TGFβs, Activins, and Nodals (and the Nodal-related Xnr factors from *Xenopus*) engage receptors that phosphorylate Smads 2 and 3. The BMPs and related GDFs, as well as AMH/MIS, engage receptors that signal through Smads 1, 5, and 8. Orthologs from *Drosophila* are listed in red color. Alternative type I receptor names are: ALK3 (BMPR-IA), ALK4 (ActR-IB), ALK5 (TβR-I) and ALK6 (BMPR-IB). Activins and BMPs share some of their type II receptors, as indicated. Activated R-Smads share co-Smads but not DNA binding cofactors. Smad4β has been reported only in *Xenopus*. The DNA binding cofactors belong to structurally different protein families (see text for details). BMP, bone morphogenetic protein; GDF, growth and differentiation factor; DPP, decapentaplegic; and AMH/MIS, anti-Müllerian hormone/Müllerian inhibiting substance.

both positively and negatively the levels of Ski and SnoN, but little else is known about the regulation of Smad corepressors and how Smad subunit composition might influence corepressor recruitment.

Networking

Ligand access to TGFβ receptors is so highly controlled that the ligand-receptor interaction may be viewed as the midpoint rather than the start of a TGFβ signaling pathway (reviewed in Massagué and Chen, 2000). Several structurally diverse soluble proteins have been identified that bind TGFβ factors, preventing their access to membrane receptors (Figure 4). The pro-peptide from the TGFβ precursor (referred to as "latency-associated protein", LAP) binds TGFβ; noggin, chordin, caronte, cerberus, and others bind BMPs; cerberus also binds Nodal; and follistatin binds activin (Massagué and Chen, 2000, and references therein). The expression or activity of these proteins is controlled by various signals such as Sonic Hedgehog in the case of Caronte, thrombospondin in the case of LAP, and BMP itself in the case of Noggin. In contrast, a group of membrane-anchored proteins function as enhancers of ligand binding to the receptors. Via its protein moiety, the proteoglycan betaglycan (also known as the TGFβ type III receptor) enhances TGFβ binding to its signaling receptors (López-Casillas et al., 1993) and enables the activin antagonist, inhibin, to bind to activin receptors (Lewis et al., 2000). A structurally related protein, endoglin, may have a similar role for TGFβ1 or a hitherto unknown family member (Arthur et al., 2000). Cripto in mouse and its ortholog in zebrafish are putative accessory receptors for Nodal-

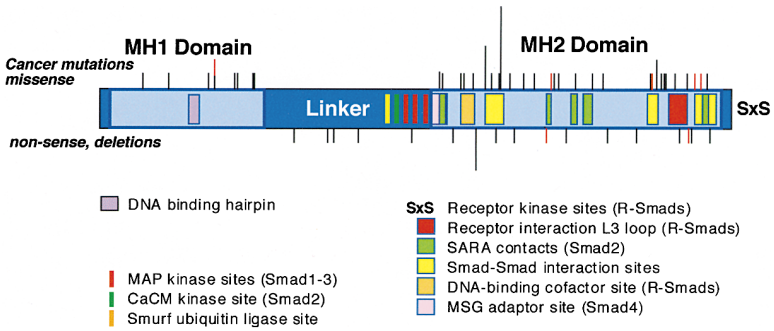

Figure 3. Smad Functional Domains and Cancer Mutations

The MH1 and MH2 domains are conserved in all R-Smads and co-Smads and form globular structures. They are linked by a more divergent region. The functions of these three regions are listed. Identification of the DNA binding site (hairpin) is based on the crystal structure of the Smad3 MH1 domain bound to its cognate sequence (Shi et. al, 1998). The Smad interacting regions in the MH2 domain are based on the crystal structure of this domain in Smad4 (Shi et. al, 1997). The multiple contact sites with SARA are based on the crystal structure of a SARA–Smad2 complex (Wu et. al, 2000). Other protein interaction sites have been defined by site-directed mutagenesis. Tumor-derived mutations are indicated by black bars for *Smad4* and red bars for *Smad2*.

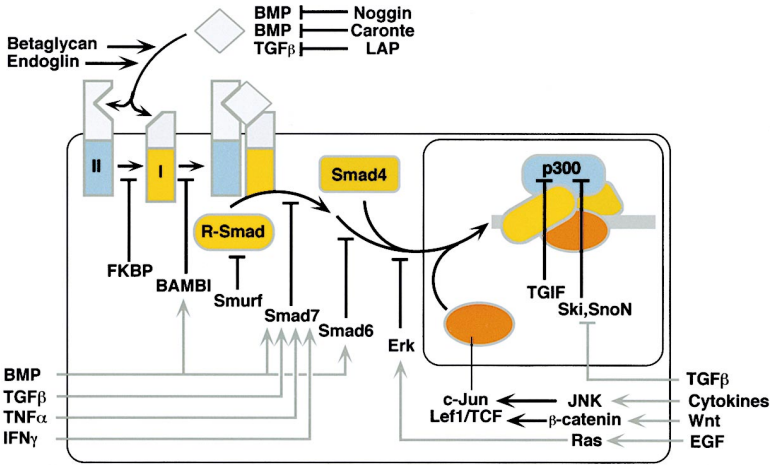

Figure 4. TGFβ Pathway Integration into a Signaling Network

A signaling network controls the activity of the TGFβ/Smad pathway at multiple levels. Only a few representative examples are shown. Noggin, Caronte, and LAP are inhibitors of ligand binding to the signaling receptors. Betaglycan and endoglin are enhancers of ligand-access to the signaling receptors. FKBP12 keeps the type I receptors in the basal state. BAMBI is a truncated receptor-like protein that inhibits type I receptor activation. Smurf is an E3 ubiquitin ligase that mediates Smad degradation. Smad7 and Smad6 are decoy Smads that interfere with receptor interaction with R-Smads or R-Smad interaction with Smad4. Erk MAP kinase phosphorylation attenuates nuclear accumulation of the Smads. TGIF, Ski, and SnoN are Smad transcriptional corepressors. TGIF competes with the coactivatior p300 for binding to the Smad complex. The level or activity of several of these components is controlled by diverse signals as indicated.

related factors (Schier and Shen, 2000). In humans, *noggin* mutations cause skeletal defects, and *endoglin* mutations cause vasculature malformations (see below), underscoring the physiological importance of these extracellular regulators.

The Smad signal transduction process itself may be simple but it is under the control of a complex web of regulators (Figure 4). Several of these molecules, including the truncated receptor-like molecule BAMBI, the ubiquitin ligase Smurf1, and the antagonistic Smads, Smad6 and Smad7, specialize in regulating this pathway. The levels of many of these molecules are controlled by diverse signals, providing feedback and cross-talk links. Additional control and integration are provided by signals that regulate the levels or activity of Smad DNA binding cofactors, including the aforementioned Wnt and diverse cytokine signals (reviewed in Massagué and Chen, 2000). The Smad pathway is therefore well integrated into the signaling networks of the cell at large.

Growth Control

Inhibition of cell proliferation is central to the TGFβ response in epithelial, endothelial, hematopoietic, neural, and certain types of mesenchymal cells, and escape from this response is a hallmark of many cancer cells. TGFβ can induce antiproliferative gene responses at any point during the division cycle. However, these responses are effective at inhibiting cell cycle progression only during G1. Once a cell becomes committed to executing DNA replication in late G1, the division cycle will proceed undeterred by TGFβ until the cell enters G1 again following mitosis, at which point the cell cycle will arrest. In most cases this arrest is reversible, but in some cases, it is associated with terminal differentiation or programmed cell death.

Two classes of antiproliferative gene responses are involved in TGFβ-mediated growth arrest: gene responses that inhibit cyclin-dependent kinases (cdks), and downregulation of c-*myc* (Figure 5A). In mammalian cells, cyclin D-cdk4, cyclin D-cdk6, cyclin E-cdk2, and cyclin A-cdk2 act sequentially during the G1/S transition and are required for cell-cycle progression through this period. Cdk activity is tightly regulated by diverse mechanisms, including changes in the levels of cyclins or cdks, phosphorylation of positive and negative regulatory sites, and interaction with stoichiometric inhibitors (Sherr and Roberts, 1999). The early observation that TGFβ inhibits phosphorylation of the retinoblastoma protein pRb (a cdk substrate) during G1, pointed at G1 cdks as targets of TGFβ action and eventually unveiled various gene responses that may vary among different cell types but in all cases suppress the activity of G1 cdks.

Cdk Inhibitory Gene Responses: Many Ways to the Same End

As first demonstrated in keratinocytes (Hannon and Beach, 1994), TGFβ rapidly induces the expression of p15Ink4b (henceforth p15) in a variety of different cell types, including lung, thyroid, and mammary epithelial cells, and astrocytes (Figure 5A). p15, a member of the Ink4 family of cdk inhibitors, specifically inhibits the cyclin D-dependent kinases, cdk4 and cdk6, by binding to the cdk subunit, inactivating the catalytic activity and preventing the assembly of new cyclin D-cdk complexes from latent pools. Cyclin D-cdk4/6 complexes function early in G1 and act as mitogen sensors. Their inhibition by TGFβ via p15 thus deprives the cell of this class of G1 cdk activities.

Cyclin D-cdk4/6 complexes also support cell cycle progression with a noncatalytic function, namely, the sequestration of the cdk inhibitor p27Kip1 (henceforth p27). This function, too, is targeted by p15 (Reynisdóttir et al., 1995; Sandhu et al., 1997). Cip/Kip cdk inhibitors, including p21Cip1, p27Kip1, and p57Kip2, can interact with cyclin D-cdk4/6, cyclin E-cdk2, and cyclin A-cdk2. This interaction is mediated by two conserved subdomains, one docking on the cyclin subunit and the other

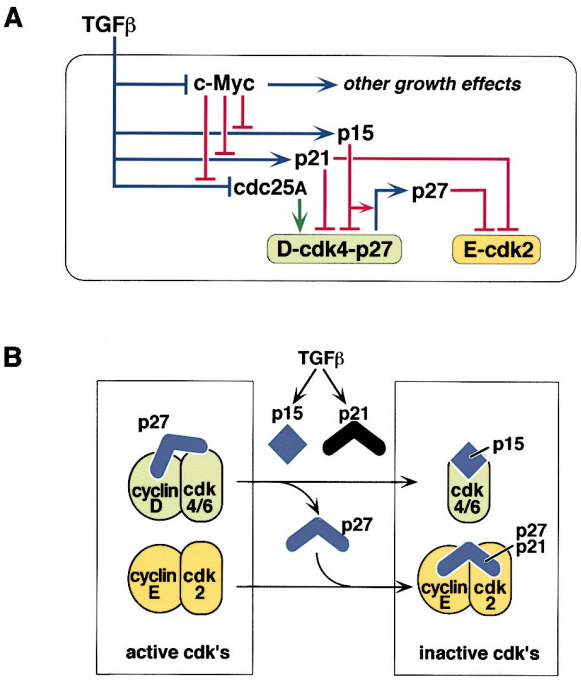

Figure 5. The Cell Cycle Arrest Response to TGFβ

(A) Two classes of antiproliferative gene responses are known to be induced by TGFβ. The first is c-Myc downregulation, observed in most cell types that are growth inhibited by TGFβ. The second are cdk-inhibitory responses, including the induction of p15 and p21 and the downregulation of cdc25A. Most cells that are growth inhibited by TGFβ have different combinations of cdk-inhibitory responses. c-Myc antagonizes TGFβ signaling by acting as a repressor of cdk-inhibitory responses. Downregulation of c-Myc is thus necessary for TGFβ-induced cell cycle arrest. Loss of cdc25A and the induction of p21 or p15 lead to the direct inhibition of cyclin D-cdk4.

(B) p15 binding to cyclin D-cdk4 leads to the shuttling of p27 from active cyclin D-cdk4-p27 complexes to cyclin E-cdk2 complexes, resulting in their ultimate inhibition as well.

contacting the associated cdk subunit (Pavletich, 1999). Through these contacts, p27 acts as an inhibitor of cyclin E/A-cdk2 complexes but it is not an obligate inhibitor of cyclin D-cdk4/6, In fact, p27 and p21 have been proposed to facilitate the assembly of cyclin D-cdk complexes. Most of the p27 protein in a proliferating cell is found in association with cyclin D-cdk4/6, thus sparing cdk2 from inhibition. The association of p27 with cdk4/6 in proliferating cells ends once TGFβ induces a rise in p15 levels (Figure 5B). Owing to partially overlapping binding sites for the two inhibitors and to an equilibrium of cdk4/6 between a cyclin-bound state in the nucleus and a cyclin-free state in the cytoplasm (Reynisdóttir and Massagué, 1997), p27 is displaced by p15 from cyclin D-cdk4/6 and shuttled to cyclin E-cdk2, inhibiting this kinase (Reynisdóttir et al., 1995; Reynisdóttir and Massagué, 1997; Sandhu et al., 1997). Thus, by increasing the level of one single cdk inhibitor, TGFβ can inhibit both classes of G1 cdks.

In addition to blocking catalytic activity, binding of p27 can occlude a cdk2 complex from phosphorylation by cdk-activating kinase (CAK), and this may explain the accumulation of cdk2 lacking this phosphorylation seen in TGFβ-treated cells. However, in HepG2 hepatocellular carcinoma cells, TGFβ inhibits this phosphorylation without a detectable change in the levels or association of known cdk inhibitors (Nagahara et al., 1999). Thus, these mechanisms have backups, as illustrated also by the fact that *p15*$^{-/-}$ or *p27*$^{-/-}$ mouse embryo fibroblasts remain at least partly growth inhibited by TGFβ (Nakayama et al., 1996; Latres et al., 2000). Furthermore, TGFβ induces the expression of p21 in keratinocytes, colon, and ovarian epithelial cells (Figure 5A). TGFβ addition can also prevent the increase in cdk4 levels that occurs under the particular conditions of mitogen-deprived cell cultures replenished with serum; this effect occurs at the translational level, requires the 5′UTR of cdk4, and is p53 dependent (Ewen et al., 1995). Another cdk inhibitory response to TGFβ is the downregulation of *cdc25A* (Iavarone and Massagué, 1997) (Figure 5A). The cdc25 family of tyrosine phosphatases removes inhibitory tyrosine phosphorylation from cdks. Cdc25A downregulation by TGFβ in MCF-10A mammary epithelial cells leads to accumulation of tyrosine phosphorylation on cdk4 and cdk6 and subsequent inhibition of these kinases. A mutant form of cdk6 lacking the phosphorylatable tyrosine residue is resistant to inhibition by TGFβ in these cells. This multiplicity of antiproliferative TGFβ gene responses assures that growth inhibition will generally be achieved in nontransformed cells.

TGFβ also downregulates cdc25A in keratinocytes (Iavarone and Massagué, 1999). In these cells, however, cdc25A downregulation is a secondary event following the initial drop in cdk kinase activity caused by the cdk inhibitors, p15 and p21. TGFβ-induced cdc25A downregulation involves formation of a transcriptional repressor complex containing E2F, the pRb-related protein p130, and the histone deacetylase HDAC1. Binding of this complex to an E2F site in the *cdc25A* promoter represses expression of this gene. This type of response may be representative of the large number of adaptive changes in gene expression that follow the entry of a cell into a quiescent state. Other TGFβ-induced changes in cell cycle components that occur with slow kinetics (i.e., several hours after TGFβ addition), such as the downregulation of various cyclins and cdk's (Geng and Weinberg, 1993; Reynisdóttir et al., 1995), may fall in this category. Transcript profiling of mammary epithelial cells using DNA microarrays indicates that the levels of nearly 1% of all transcripts in the cell change several fold after four hours of TGFβ addition (J. M., unpublished data).

Upstream of cdk Inhibition: Downregulation of c-Myc

c-Myc, a member of the basic helix-loop-helix leucine zipper (bHLH-LZ) family of transcription factors, is a ubiquitous promoter of cell growth and proliferation (Facchini and Penn, 1998). c-Myc has both transcriptional activation and repression effects depending on the nature of its associated factors (Dang, 1999). As an activator, c-Myc, in association with another bHLH-LZ protein, Max, interacts with a consensus sequence termed the E-box in enhancer elements. In various TATA-less promoters, c-Myc represses transcription through an interaction with the transcriptional initiator (Inr) region, a DNA sequence distinct from the E-box.

In contrast to the cell type–dependent diversity of cdk inhibitory gene responses induced by TGFβ, transcriptional downregulation of c-*myc* is a rapid and general effect observed in most cells with an antiproliferative response to TGFβ (Alexandrow and Moses, 1995) (Figure 5A). As c-Myc has a short half-life, this downregulation results in a rapid loss of protein. The exact mechanism of downregulation remains unknown but its importance seems clear: artificially preventing c-Myc downregulation renders cells resistant to the antiproliferative action of TGFβ.

c-Myc downregulation by TGFβ is required for the TGFβ-mediated inactivation of G1 cdks (Warner et al., 1999; Claassen and Hann, 2000). A drop in c-Myc protein levels in a TGFβ-induced response may deprive a cell of various functions to which c-Myc contributes in support of cell proliferation. However, in lung epithelial cells conditionally expressing a human *c-myc* allele, expression of low levels of exogenous c-Myc blocks the rapid transcriptional activation of *p15* by TGFβ (Warner et al., 1999). In keratinocytes, expression of c-*myc* blocks the TGFβ induction of *p21* (Claassen and Hann, 2000). These findings suggest that TGFβ must downregulate c-Myc in order to activate the p15 and p21 G1 arrest pathways (Figure 5A).

How could c-Myc prevent the induction of cdk inhibitory immediate gene responses by TGFβ? c-Myc could maintain *p15* in a basal inhibited state by acting as a repressor. In this model, TGFβ would have to remove this repression in order to proceed with activation of the *p15* (and *p21*) promoter. However, *p15* is not induced in epithelial cells whenever the levels of c-Myc decline, such as following serum deprivation. In addition to c-Myc downregulation, other TGFβ-dependent signaling events, perhaps involving Smad proteins, must be involved in p15 induction by TGFβ. Also of note, c-Myc has been implicated as a positive regulator of cdc25A expression (Galaktionov et al., 1996), a mechanism that would also antagonize the effect of TGFβ on cdc25A expression (Figure 5A).

Resistance to TGFβ-mediated growth arrest has been ascribed to many other proteins, such as the ras and MDM-2 oncoproteins. However, a distinction must be made between bona fide members of the TGFβ antiproliferative pathway described above and factors which secondarily circumvent TGFβ cell cycle arrest signals. For example, hyperactive (oncogenic) Ras, which can overcome TGFβ-mediated arrest in vivo, has been shown to increase cyclin D levels and increase p27 degradation as well as attenuate Smad2/3 nuclear accumulation (Marshall, 1999; Kretzschmar, et al., 1999). While these effects would confer TGFβ resistance, they do not place Ras directly in the TGFβ cell-cycle arrest pathway. Likewise, while chronic MDM2 overexpression may eventually select for cells resistant to TGFβ (Sun et al., 1998), transient overexpression of MDM2 does not alter a cell's sensitivity to TGFβ-mediated growth arrest (Blain and Massague, 2000), suggesting that, unlike c-Myc, MDM-2 is not a direct participant in the TGFβ cell cycle arrest pathway.

Terminal Arrest

In addition to causing reversible cell cycle arrest in some cell types, TGFβ can induce programmed cell death in others. In fact, apoptosis induced by TGFβ family members is an essential component of the proper development of various tissues and organs, including the rhombencephalic neural crest (Graham et al., 1996), the interdigital fields of the limb (Zou et al., 1997), and the mammary gland ductal system (Nguyen and Pollard, 2000). After lactation, a rise in TGFβ3 levels mediates the induction of programmed cell death of epithelial cells that precedes mammary gland involution (Nguyen and Pollard, 2000). TGFβ-induced apoptosis and the selective elimination of preneoplastic cells may also be involved in the tumor suppression mediated by TGFβ, as a body of largely circumstantial evidence suggests (reviewed in Gold, 1999). This is especially relevant in the case of colon cancer, as colonic epithelial homeostasis is dependent on the rates of both cell proliferation and apoptosis near the tips of villi. Just as loss of TGFβ-mediated growth arrest might predispose a cell to cancer, loss of TGFβ-mediated apoptosis may permit selective accumulation of premalignant cells. The mechanisms that trigger apoptosis in response to TGFβ are largely unknown, although Bcl family members and caspases that participate in the apoptotic effector system are activated in cells undergoing TGFβ-induced apoptosis (Chen and Chang, 1997; Saltzman et al., 1998).

TGFβ and Cancer

Although TGFβ is a potent growth inhibitor in epithelial tissues, it is both a suppressor and a promoter of tumorigenesis. On the one hand, TGFβ has a tumor suppression function that is lost in many tumor-derived cell lines (Reiss, 1997; reviewed in Gold, 1999). It has been estimated that nearly all pancreatic cancers (Goggins et al., 1998; Villanueva et al., 1998) and colon cancers (Grady et al., 1999) have mutations disabling a component of the TGFβ signaling pathway. Some of these mutations occur in the TGFβ receptors, Smad4 or Smad2 (see below); others may occur in hitherto untested or unknown components of the signaling pathway. Experiments in mice have provided additional evidence for a role of TGFβ in protection against tumor progression in the early stages. TGFβ1 heterozygous null mice show increased hepatocyte proliferation, decreased apoptosis in the lung and liver (Tang et al., 1998), and accelerated mammary epithelial proliferation and ductal outgrowth in response to hormone (Barcellos-Hoff and Ewan, 2000). When challenged with carcinogens, these mice develop liver and lung tumors of greater size, number, and malignant potential than the controls, suggesting a role for TGFβ1 in tumor suppression (Tang et al., 1998). These tumors retain the remaining TGFβ1 allele, suggesting haploinsufficiency in the tumor suppressor function of TGFβ (Tang et al., 1998). Transgenic expression of a dominant-negative TβRII construct in the mammary gland or the epidermis diminishes epithelial responsiveness to TGFβ and increases the tumor incidence in these tissues when the mice are challenged with a carcinogen (Bottinger et al., 1997; Go et al., 2000).

On the other hand, TGFβ exacerbates the malignant phenotype of transformed and tumor-derived cells in experimental systems, and there is some evidence that it may be doing the same in human cancer. High levels of

TGFβ expression are correlated with advanced clinical stage of the tumor (Gold, 1999). Tumor-derived TGFβ could contribute to tumor growth indirectly by suppressing immune surveillance or stimulating production of angiogenic factors. However, TGFβ can also act directly on cancer cells to foster tumorigenesis. Tumor cells that have selectively lost their growth-inhibitory responsiveness to TGFβ but retain an otherwise functional TGFβ signaling pathway may exhibit enhanced migration and invasive behavior in response to TGFβ stimulation (Cui et al., 1996; Oft et al., 1998; Yin et al., 1999). Expression of dominant-negative TβRII in human mammary adenocarcinoma cells reduces the size and number of bone metastases they generate in athymic mice (Yin et al., 1999). TGFβ signaling could promote tumor cell metastasis in many different ways. Of interest is the ability of TGFβ to induce an epithelial to mesenchymal transition (EMT) in these cells (Oft et al., 1998). EMT is characterized by the downregulation of proteins involved in cell–cell adhesion and upregulation of molecules important for cell-extracellular matrix associations, ultimately leading to enhanced migratory and invasive properties of the cell. A switch from an epithelial to fibroblastoid phenotype occurs frequently during late stages of carcinoma progression and correlates with the metastatic potential of tumor cells. Provocative as these observations are, an important limitation is that the majority of this evidence is derived from experimental metastasis assays that utilize engineered carcinoma cell lines.

TGFβ Receptor Mutations in Cancer

Inactivating mutations in *TβRII* occur in most human colorectal and gastric carcinomas with microsatellite instability (MSI) (Markowitz et al., 1995). Stable transfection of wild-type TβRII into a human MSI colon cancer cell line (Wang et al., 1995) and a human gastric cancer cell line (Chang et al., 1997) restored TGFβ-mediated growth arrest and reduced tumorigenicity in athymic mice, providing further evidence that mutational inactivation of TGFβ receptors is a pathogenic event. MSI is common to many sporadic cancers and results from DNA mismatch repair (MMR) defects causing nucleotide additions or deletions in simple repeated sequences, or microsatellites, throughout the genome. MMR in one such microsatellite, a 10 bp polyadenine repeat within the *TβRII* sequence encoding a part of the extracellular domain (referred to as the *BAT-RII* track), results in a frameshift and a truncated, inactive TβRII product (Markowitz et al., 1995). *BAT-RII* inactivating mutations are also found in colorectal and gastric tumors from patients with hereditary nonpolyposis colon cancer (HNPCC), a familial cancer syndrome in which affected individuals inherit defects in genes encoding components of the DNA MMR pathway (Lu et al., 1996; Akiyama et al., 1997). Although *BAT-RII* mutations are found in subsets of colon cancers, gastric cancers and gliomas with MSI (Markowitz et al., 1995; Myeroff et al., 1995; Parsons et al., 1995; Izumoto et al., 1997), these mutations are uncommon in MSI tumors from the endometrium, pancreas, liver, and breast. Thus, the loss of TβRII is selected for in only cancers of specific tissue origins.

Most commonly, *BAT-RII* mutations are biallelic, but mutation in one allele may also be accompanied by a non-*BAT-RII* mutation that inactivates the kinase domain in the other allele (Markowitz et al., 1995; Parsons et al., 1995; Takenoshita et al., 1997). Recently, missense mutations of *TβRII,* most of which target the kinase domain, have been reported in 15% of microsatellite stable colon cancers examined (Grady et al., 1999). Thus, inactivating mutations of *TβRII* may be present in as many as one quarter of all colon cancers. Mutational inactivation of the TGFβ type I receptor, or TβRI, has also been detected in human cancers. An inactivating mutation in *TβRI* occurs in one third of ovarian cancers examined; notably, in the same tumor cohort, no inactivating mutations were identified in *TβRII* (Wang et al., 2000). A missense mutation in the kinase domain of TβRI, resulting in a hypomorphic allele, has been identified in one cohort of metastatic breast cancers (Chen et al., 1998b) but not in another (Anbazhagan et al., 1999). In addition, deletions of *TβRI* occur at a low frequency in pancreatic and biliary carcinomas (Goggins et al., 1998) as well as cutaneous T cell lymphoma (Schiemann et al., 1999). Notably, homozygosity of a common germline polymorphism, *TβRI(6A)*, is associated with an increased incidence of colon cancer (Pasche et al., 1999).

Smad Mutations in Cancer

The TGFβ signaling network is also disrupted in cancer by mutations in *Smad4* and *Smad2*. *Smad4*, initially identified as *DPC4* (*deleted in pancreatic carcinoma locus 4*) located on 18q21, suffers biallelic loss in one half of all of pancreatic cancers (Hahn et al., 1996), one third of metastatic colon tumors (Miyaki et al., 1999), and smaller subsets of other carcinomas. In addition, germline mutations in *Smad4* cosegregate with a subgroup of patients with juvenile polyposis syndromes (JPSs), an autosomal dominant disorder characterized by hamartomatous intestinal polyps and an increased risk of gastrointestinal cancers (Howe et al., 1998). Occasionally, *Smad4* mutations have been found in conjunction with *TβRI* mutations in biliary cancer (Goggins et al., 1998) and with *TβRII* mutations in colon cancer (Grady et al., 1999). Smad4 and the TGFβ receptors may therefore have certain nonoverlapping tumor suppressive activities. *Smad2*, also located on 18q21, is the target of inactivating mutations in a small proportion of colorectal cancers (Eppert et al., 1996; Uchida et al., 1996).

Inactivation of *Smad2* and *Smad4* occurs by loss of the entire chromosome region, small deletions, frameshift, nonsense mutations, or missense mutations. Missense mutations mostly target the MH2 domain, resulting in loss of stability or disruption of homo- and hetero-oligomerization of the Smads (Figure 3). Among the missense mutations in the MH1 domain, one targeting the same conserved residue in both Smad2 and Smad4 results in an enhanced autoinhibitory interaction between the MH1 and MH2 domains and additionally decreases protein stability (Hata et al., 1998; Xu and Attisano, 2000). Smad-deficient mice display phenotypes in support of a tumor suppressor role for the Smads. Although mice with homozygous loss of *Smad2* and *Smad4* die in utero, their heterozygous counterparts are viable. In fact, mice heterozygously null for *Smad4* develop gastric polyps that can develop into tumors at

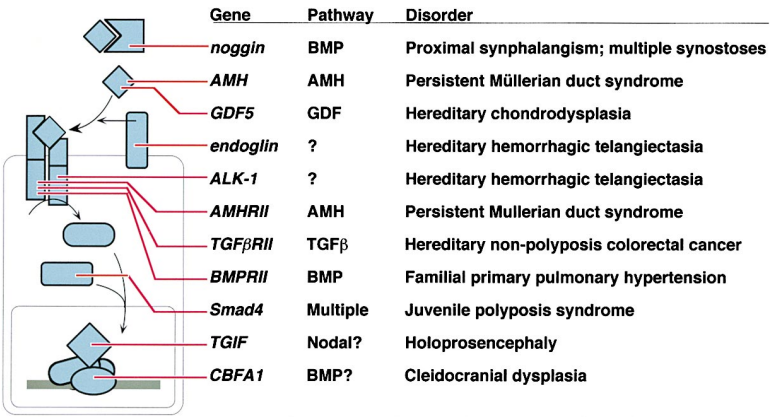

Figure 6. Heritable Mutations in the TGFβ Pathway

Mutations that target components of the TGFβ signaling pathway contribute to diverse human disorders. The basic signaling components are indicated with similar icons as those depicted in Figure 1. The modes of inheritance and the mechanisms of disease are discussed in the text.

a late age (Xu et al., 2000b). Furthermore, mice defective in *APC* (*adenomatous polyposis coli*) develop numerous indolent intestinal polyps. When mice with one mutated APC allele are crossed with heterozygous null *Smad4* mice, the compound heterozygotes develop larger polyps that can progress into malignant adenocarcinomas with loss of the remaining copies of both *APC* and *Smad4* (Takaku et al., 1998). Moreover, although no *Smad3* mutation has been found in human cancer, mice with a homozygous deletion of *Smad3* develop aggressive metastatic colorectal cancer at an early age in a manner that seems to be highly dependent on the genetic background of the mice (Zhu et al., 1998).

TGFβ Signaling Defects in Developmental Disorders

TGFβ signaling orchestrates critical roles in mammalian embryogenesis and organogenesis (Hogan, 1996; Whitman, 1998; Goumans and Mummery, 2000; Schier and Shen, 2000; Tremblay et al., 2000). BMP4 acts at distinct stages of development, beginning with epiblast proliferation and survival preceding gastrulation and later with instructive interactions among many different cell types, especially neural, cartilage, bone, and dermal cells. Nodal is required for primitive streak formation, anterior patterning, and the generation of left-right asymmetry formation. Several other members of the TGFβ family, such as Activin, Vg1, BMPs, and Lefty, have also been shown to be important for left-right axis formation. Various TGFβs are involved in the morphogenesis of many organs and tissues: TGFβ1 in vascular development; TGFβ2 in cardiac, lung, craniofacial, and urogenital development, and TGFβ3 in proper palate closure. Smad functions are also important during mammalian development. Smad2-dependent signals generated from the extraembryonic tissues are essential for anterior-posterior identity within the underlying epiblast; later, during gastrulation, Smad2 signaling directs epiblast derivatives toward formation of the definitive endoderm, which gives rise to the gut tube. Smad4 function is required for both epiblast proliferation and primitive or extraembryonic endoderm formation.

Given this, it should come as no surprise that various heritable developmental disorders in humans turn out to be caused by mutations in the TGFβ system (Figure 6). In addition, abnormal TGFβ signaling has also been implicated in widespread human disorders including fibrosis, hypertension, and osteoporosis. Although genetic alterations in the TGFβ system are not known to be a direct cause of these disorders, polymorphisms in *TGFβ1* have been associated with ischemic heart disease and hypertension, osteoporosis, and fibrosis (Blobe et al., 2000, and references therein).

noggin Mutations in Hereditary Synostosis

Endochondral bone development and formation of articulations between skeletal elements occur simultaneously from the same initial mesenchymal condensation. The BMP-related factor GDF5 (growth and differentiation factor 5) regulates both the size of the early cartilage condensation and formation of the joints (Francis-West et al., 1999; Storm and Kingsley, 1999). Noggin has been proposed as an upstream modulator of GDF5 signaling, which is consistent with the similar pattern of joints affected occurring in humans and mice with either *noggin* or *GDF5* mutations.

Two autosomal dominant disorders, proximal symphalangism and multiple synostoses syndrome, trace to heterozygous, missense mutations in *noggin*, the gene product of which antagonizes BMP/GDF receptor binding (Gong et al., 1999). In proximal symphalangism, synostosis (osseous union between the bones resulting in fusion of joints) affects mainly the proximal interphalangial and carpal joints of the hands and tarsal joints of the feet. In multiple synostoses syndrome, additional sites are involved including the hip and cervical spine (Gong et al., 1999). In both disorders, the precise mechanisms of the missense mutations remain unknown, although case reports of chromosomal deletions suggest functional haploinsufficiency (Gong et al., 1999). *noggin*$^{-/-}$ mice die at birth from multiple defects including excessive cartilage and bony fusions of the appendicular skeleton (Brunet et al., 1998; McMahon et al., 1998). Thus, noggin suppresses chondrogensis and joint restriction in the limbs of humans and mice.

GDF5/*CDMP1* Mutations in Hereditary Chondrodysplasias

In the developing limb, early expression of GDF5 is both necessary and sufficient to stimulate cartilage development and inhibit joint marker expression, including GDF5 itself and Gli3, thus restricting joint formation later to the appropriate locations. Subsequently, a narrower

expression of GDF5 in the joint region contributes to joint morphogenesis (Storm and Kingsley, 1999). In addition, GDF5 accelerates the initial stages of chondrogenesis such as mesenchymal condensation by increasing cell adhesion and later can increase chondrocyte proliferation (Francis-West et al., 1999).

Mutations in the human ortholog of *GDF5*, *CDMP1* (cartilage-derived morphogenetic protein 1), are associated with several human hereditary chondrodysplasias including Hunter-Thompson type acromesomelic chondrodysplasia (Thomas et al., 1996), autosomal dominant brachydactyly type C (Polinkovsky et al., 1997), and Grebe type chondrodysplasia (Thomas et al., 1997). These are all characterized by pronounced shortening of the skeletal elements in the limbs, with more severe effects distally and the loss of one or more joints. The *brachypodism* (*bp*) phenotype in mice is caused by inactivating mutations in *GDF5* (Storm et al., 1994; Storm and Kingsley, 1996). Mouse *bp* sydrome and human Hunter-Thompson type chondrodysplasia are both caused by missense mutations in both alleles of *GDF5*, resulting in a total loss of function. Thus, these syndromes are inherited in an autosomal recessive manner. In contrast, Grebe type chondrodysplasia and brachydactyly type C follow an autosomal-dominant mode of inheritance. In the former, mutation in a conserved cysteine (C400Y) of GDF5 yields a dominant negative partner in the production of dimeric ligand (Thomas et al., 1997), which may cause more severe phenotypes than those seen in Hunter-Thomas type chondrodysplasia. Similarly, brachydactyly type C, which is characterized by the shortening and the occasional loss of some phalanges, is due to haploinsufficiency of GDF5 (Polinkovsky et al., 1997).

CBFA1 Mutations in Cleidocranial Dysplasia

The core binding factor (CBF) family of transcription factors, which consists of a DNA binding α subunit (CBFA1, CBFA2, and CBFA3) in association with a common β subunit, plays critical roles in tissue growth and differentiation. CBFA1 (also known as AML3 and PEBP2αA) functions in bone formation, CBFA2 in hematopoiesis, and CBFA3 in B lymphocyte IgA class switching (Westendorf and Hiebert, 1999). Provocative but still tentative evidence suggests that CBFA members may associate with Smads and collaborate in transcriptional activation of certain TGFβ target genes. CBFA1 appears to show a preference for BMP-activated Smad1, and CBFA2 and 3 form a functional complex with receptor-activated Smad3 and 4 to transactivate the germline Igα constant region (IgCα) (Hanai et al., 1999; Pardali et al., 2000). Both the N-terminal Runt homology DNA binding domain and the C-terminal transactivation domains of CBFA proteins have been implicated in direct CBFA-Smad interaction.

Cleidocranial dysplasia (CCD) is an autosomal-dominant disease characterized by abnormal clavicles, patent sutures and fontanelles, supernumerary teeth, short stature, and a variety of other skeletal changes (Mundlos, 1999). Heterozygous mutations in the *CBFA1* gene have been identified in CCD patients (Lee et al., 1997; Mundlos et al., 1997). Most CCD-associated mutations are missense and cluster in the Runt domain, not excluding the carboxy-terminal region. *CBFA1* homozygous null mice lack both endochondral and intramembranous bones, display defects in chondrocyte maturation, and die minutes after birth due to the inability to breathe. *CBFA1* heterozygous null mice show specific bone defects that phenocopy CCD patients, consistent with CBFA1 haploinsufficiency in the pathogenesis of CCD (Komori et al., 1997; Otto et al., 1997). Thus, the phenotypes of CCD individuals with mutations in *CBFA1* and of mice deficient in *CBFA1* support an early and critical role of CBFA1 in osteoblast differentiation and chondrocyte maturation. It will be important to determine whether the CCD phenotype of inherited *CBFA1* mutations specifically results from a Smad signaling loss.

ALK1 and *endoglin* Mutations in Hereditary Hemorrhagic Telangiectasia

Hereditary hemorrhagic telangiectasia (HTT), or Rendu-Osler-Weber syndrome, is inherited as an autosomal dominant trait (1 in 10,000) and exhibits age-related penetrance with variable expressivity (Guttmacher et al., 1995). The earliest and most common clinical manifestations include nosebleeds and mucocutaneous telangiectasia; gastrointestinal bleeding usually occurs later in life. Some patients also develop life-threatening complications involving arteriovenous malformations (AVMs) in the pulmonary, cerebral, and hepatic circulations. This clinical heterogeneity has been explained in part by the identification of two distinct loci, *endoglin* in HHT1 and *ALK-1* in HHT2. HHT1 is associated with a higher incidence of AVMs than HHT2, which is considered a milder form with a delayed onset. Moreover, at least one other gene, still unknown, is involved in the pathogenesis of HHT (Piantanida et al., 1996).

Both endoglin and ALK-1 are highly expressed on endothelial cells and are involved in TGFβ superfamily signaling (Massagué, 1998). ALK-1 is a member of the TGFβ type I receptor family, and its physiologic ligand is unknown. HHT2-associated mutations in ALK-1 are found in the extracellular, transmembrane, and intracellular kinase domain and include frameshift, nonsense, and missense mutations. HHT2 thus appears to result from a loss-of-function of the mutant (Abdalla et al., 2000). Endoglin was originally shown to be a nonsignaling ancillary receptor component homologous to betaglycan, which enhances TGFβ access to the type I and II receptor complex. However, endoglin shows cross-reactivity with multiple members of the TGFβ superfamily in vitro (Massagué, 1998). The majority of the HHT1-associated mutations in *endoglin* causes frameshifts and premature stop codons, and all the missense mutations identified so far occur in the extracellular domain. Based on biochemical analyses of these mutants, it appears that HHT may result from either dominant-negative protein interactions or haploinsufficiency (Pece-Barbara et al., 1999; Lux et al., 2000). Mice heterozygous for a null *endoglin* allele phenocopy human HHT; those homozygous for a null *endoglin* allele die in utero at day 10.5 due to angiogenesis defects (Bourdeau et al., 1999). While *ALK-1*$^{-/+}$ mice are normal and fertile, the *ALK-1*$^{-/-}$ conceptuses also die in utero at day 10.5 due to defects in angiogenesis (Oh et al., 2000).

The identity of the physiologic ligand for endothelial ALK-1 and endoglin has remained controversial. Although overexpressed ALK-1 can bind TGFβ or activin when coexpressed with the corresponding type II receptors, this binding is much weaker than the binding of the TGFβ type I receptor TβR-I/ALK-5. A constitutively active form of ALK-1 has been shown to phosphorylate and activate Smad1 and 5 but not Smad2 (Macias-Silva et al., 1998; Chen and Massagué, 1999), suggesting that ALK-1 mediates BMP-like signaling. Given that the expression pattern of *Smad5* overlaps with those of *TGFβ1* and *TβR-II*, and that *TGFβ1*$^{-/-}$ mice also display vascular defects, it has been proposed that TGFβ1 may be a natural ligand for ALK-1 (Oh et al., 2000). However, the vascular defects observed in *TGFβ1*$^{-/-}$ conceptuses result from alterations in vasculogenesis, not angiogenesis, due to inadequate endothelial terminal differentiation (Dickson et al., 1995). Furthermore, *TGFβ1*$^{-/-}$ conceptuses die either at 10.5 dpc due to defects in vasculogenesis (as do *TβRII*$^{-/-}$ conceptuses) or later at 3 weeks post-partum due to severe inflammatory disease (Dickson et al., 1995). The distribution of these lethal phenotypes varies with the genetic background such that a modifier allele on chromosome 5 of the NIH mouse strain can resue *TGFβ1*$^{-/-}$ conceptuses from lethal vasculogenesis defects. This background, however, cannot rescue the endoglin null phenotype (Arthur et al., 2000), suggesting that TGFβ1 may not lie in the same pathway as endoglin in regulating vascular development.

BMPRII Mutations in Familial Primary Pulmonary Hypertension

Familial primary pulmonary hypertension (PPH) is a rare autosomal dominant disorder that has reduced penetrance: inheriting one of at least two genes confers a 10%–20% likelihood of developing the disease (Peacock, 1999). This disorder usually affects the arterial side of the pulmonary circulation; left untreated, it usually progresses to severe pulmonary hypertension and right-sided heart failure. With the mean age at onset in the fourth decade, familial PPH allows a median survival of only two years following diagnosis. Recently, familial PPH has been shown to be caused by mutations in *BMPRII* (Deng et al., 2000; The International PPH Consortium et al., 2000). Nonsense or frameshift mutations predicting premature termination of the receptor in the extracellular domain, the transmembrane domain, the serine/threonine kinase domain, or a carboxy-terminal domain of unknown biochemical function have been found in familial PPH *BMPRII* alleles. Monoclonality of the hyperproliferating endothelial cells found in the plexiform lesions of familial PPH suggests a need for loss of the remaining wild-type BMPRII allele or a cooperative mutation in a different gene, which may help explain the low penetrance of familial PPH (Peacock, 1999).

The histopathologic changes in PPH, including endothelial and smooth muscle cell proliferation and in situ thrombosis, reflect tissue remodeling in response to endothelial injury, which may result in an imbalance between vasoconstriction and vasodilation (Peacock, 1999). Homozygous disruption of *BMPRII* in mice results in embryonic lethality, whereas the heterozygotes are overtly normal (Beppu et al., 2000). However, mice homozygously null for *Smad6*, an antagonist of the BMP pathway (Figure 4), display imbalances in cardiovascular homeostasis such as hypertension and a defective nitric oxide response (Galvin et al., 2000). Thus, BMP signaling likely plays critical roles in maintaining cardiovascular homeostasis.

AMH and *AMHRII* Mutations in Persistent Müllerian Duct Syndrome

In the male fetus, Anti-Müllerian hormone (AMH) (also known as Müllerian inhibiting substance, MIS), a relatively distant member of the TGFβ family, causes the regression of the Müllerian duct, the anlagen of the uterus, oviducts, and the upper portion of the vagina. AMH is produced by the Sertoli cells of the fetal testis and acts on the mesenchymal cells adjacent to the ductal epithelium (Belville et al., 1999). Thus, AMH induces ductal epithelial regression through a paracrine mechanism originating from the periductal mesenchyme, and both apoptosis and epithelio-mesenchymal transformation are involved in AMH-mediated Müllerian duct regression (Allard et al., 2000).

The critical role of AMH and its type II receptor, AMHR-II, in mediating sexual dimorphism is demonstrated in humans by the persistent Müllerian duct syndrome (PMDS), a rare autosomal recessive disorder characterized by the presence of Müllerian duct derivatives, such as the uterus and the fallopian tubes, in genetic males who are otherwise normally virilized (Belville et al., 1999). About eighty percent of cases are due to inactivating mutations in either *AMH* or *AMHRII*. These include missense and nonsense mutations throughout the length of the coding regions, insertions, and a common (45% of probands) 27 bp deletion in the intracellular domain of AMHR-II, which is either homozygous or coupled with a missense mutation in the other allele. The etiology of the remaining twenty percent of PMDS cases remains unknown, although sex-linked inheritance has been reported (Belville et al., 1999). Recent biochemical evidence points to BMPR-IB and Smad1 as mediators of AMH and AMHRII (Figure 2), suggesting that AMH gains access to a shared type I receptor and Smad system through a type II receptor (i.e., AMHR-II) whose tissue expression pattern is highly restricted (Gouedard et al., 2000). PMDS-associated mutations in BMPR-IB seem unlikely, as PMDS patients do not exhibit any bone and joint abnormality in the appendicular skeleton. The clinical phenotypes of mutations in either *AMH* or *AMHRII* are the same and are specifically phenocopied in mice with mutations in the corresponding genes. Introduction of homozygous *AMHRII* null mutations into female *AMH* transgenic mice rescues all the reproductive abnormalities (Mishina et al., 1999), suggesting a high level of specificity between AMH and AMHR-II serving a temporally and spatially restricted role during development.

TGIF Mutations in Holoprosencephaly

Signaling defects in ventral forebrain induction underlie the developmental anomalies characterizing the heritable human disease holoprosencephaly (HPE; 1 in 250 conceptuses and 1 in 10,000 live births), in which the

forebrain (prosencephalon) fails to cleave into left and right hemispheres, telencephalon and diencephalon, and olfactory and optic bulb tracks (Muenke and Beachy, 2000). In the severest forms, a single brain ventricle is present without evidence of an interhemispheric fissure, and, in the absence of ventral forebrain structures, the optic primordia develops as a single evagination from the floor of the forebrain, resulting in facial anomalies such as cyclopia (single eye) and displacement of the nasal structures superiorly. Among at least twelve chromosomal loci associated with HPE, four HPE genes have been identified: *Sonic Hedgehog (SHH)*, *ZIC2*, *SIX3*, and *TGIF* (Muenke and Beachy, 2000). HPE-associated mutations in the Smad transcriptional corepressor *TGIF* generally involve loss of a single copy of the *TGIF* gene or hypomorphic point mutations within one copy, resulting in only a partial loss of function (Gripp et al., 2000). Thus, a slight reduction in TGIF levels can have severe developmental consequences.

Signals from the prechordal plate mesoderm and/or anterior definitive endoderm formed early during gastrulation help pattern the ventral forebrain. In the zebrafish, mutations in the *Nodal*-related genes *cyclops* and *squint* and the putative *Nodal* accessory receptor *one-eyed pinhead (oep)* disrupt the formation of the prechordal plate mesoderm, resulting in floorplate and ventral forebrain defects and cyclopia. The phenotypic effects of the *cyclops* and *oep* mutations can be rescued by the expression of *Smad2*. Furthermore, in mice doubly heterozygous for null alleles in both *Nodal* and *Smad2*, HPE phenotypes were observed in half of the embryos; this is likely due to defects in the formation of the prechordal mesoderm and/or anterior endoderm that provide signals patterning the ventral forebrain (Gripp et al., 2000, and references therein). That haploinsufficiency in human *TGIF* also results in similar defects raises the possibility that TGIF may normally mediate Nodal-induced downregulation of genes involved in specifying normal forebrain structures. Interestingly, the low penetrance of the HPE phenotype caused by *TGIF* mutations in humans suggests the existence of modifiers that may bring the levels of TGIF over the threshold needed for proper ventral forebrain development. How TGIF levels are regulated remains largely unknown.

The currently available data, however, do not exclude a role for TGIF in modulating signals other than Nodal and related factors. In fact, BMP can induce a Smad1-TGIF interaction when overexpressed (Wotton et al., 1999). In the chick, late exposure to high doses of BMP4 and 5 leads to HPE-like phenotypes by inducing apoptosis in the ventral forebrain. In addition, TGIF has been shown in vitro to compete with RXR for binding to the RXR response element. Thus, it is possible that hypomorphic TGIF mutants may result in hyperactive BMP or retinoic acid signaling (Muenke and Beachy, 2000, and references therein).

Prospects

A better understanding of the TGFβ signaling pathway has allowed a deeper appreciation for its integration into the signaling networks at large as well as its disruption in human disorders. The identification of heritable disorders of the TGFβ system may provide insights into the etiology of related but more common disorders arising from somatic mutations in the TGFβ pathway. For example, the role of BMP signaling in vascular wall homeostasis revealed by the phenotype of *BMPRII* mutations in PPH points to the possibility that other primary forms of hypertension may arise from somatic alterations of BMP signaling in the vasculature. Conversely, the identification of somatic mutations in TGFβ signaling components may facilitate the discovery of heritable forms of these mutations, as has happened with the identification of mutations in *TBR-II* and *Smad4* in colon cancer. The TGFβ system may also be perturbed by alterations in the embedding network. Think, for example, of the many forms of cancer in which loss of TGFβ responsiveness or its degeneration into an instigator of metastasis cannot be ascribed to a mutation in TGFβ receptors or Smad proteins. Identifying the defect in these cases is essential and will require a better knowledge of the links between TGFβ and other signaling pathways.

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