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Transforming Growth Factor β Superfamily Signaling in Development of Colorectal Cancer

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Abstract

Transforming growth factor (TGF)-β cytokines signal via a complex network of pathways to regulate proliferation, differentiation, adhesion, migration, and other functions in many cell types. A high percentage of colorectal tumors contain mutations that disrupt TGF-β family member signaling. We review how TGF-β family member signaling is altered during development of colorectal cancer, models of study, interaction of pathways, and potential therapeutic strategies.

Keywords

Transforming Growth Factor β; Activin; Colon Cancer

Colorectal cancer (CRC) is the third leading cancer by incidence and the second leading cause of cancer mortality in the United States.[1] CRC initiation and progression involve loss of tumor suppressor proteins, including transforming growth factor (TGF)-β. In colon epithelial cells, TGF-β signaling reduces proliferation and promotes apoptosis and differentiation.[2,3] Loss of TGF-β signaling and its antiproliferative effects is a feature of CRC cells[4–7] and is observed in transformed intestinal epithelial cells.[8,9] TGF-β superfamily proteins are found in vertebrates and invertebrates. The family includes 30 proteins that signal via a common mechanism, through serine/threonine kinase transmembrane receptors to SMAD proteins, which regulate transcription.[10] We review the activities of the TGF-β superfamily members TGF-β, activin, and bone morphogenetic proteins (BMPs) in colon carcinogenesis. Members of these signaling pathways are frequently mutated in sporadic CRCs, and germline mutations are causative for hereditary CRC syndromes.

Canonical and Noncanonical Signaling Pathways

TGF-β superfamily ligands bind and signal through type II and type I serine/threonine kinase receptors (also called activin receptor–like kinases). These receptors include TGFBR2, TGFBR1, BMPR2, BMPR1A/1B, ACVR2A/2B, and ACVR1A/1B (see Figure 1

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Conflicts of interest

The authors disclose no conflicts.

and Table 1). There are varying degrees of specificity and cross-reactivity between ligands and receptors, which adds complexity to the study of TGF-β family signaling and should factor into interpretation of study results.

Ligand access to receptors is regulated by ligand-trap proteins that selectively bind to specific ligands.[3,11] In response to ligand binding, receptors interact at the cell surface and the constitutively active type II receptor trans-phosphorylates type I receptor, leading to downstream activation of pathway-specific receptor-associated Smad proteins (R-SMAD proteins).[3] These R-SMAD effectors complex with the common Smad for all TGF-β superfamily members, SMAD4, and translocate to the nucleus to regulate transcription of target genes[3,11,12] (Figure 1). There are 8 vertebrate SMAD proteins (SMAD1–SMAD9). After interaction between TGF-β or activin ligands and their receptors, the R-SMAD proteins 2 and 3 are phosphorylated and activated by type I receptors (Figure 1). R-SMAD proteins 1, 5, and 9 (historically also called SMAD8) are activated in response to BMP ligand association with type II/I BMP receptors (Figure 1). Recent studies have questioned the strict distinction between ligands activating R-SMAD proteins 2/3 and 1/5/9, possibly pointing toward a complex and underappreciated interaction of different superfamily members on a SMAD level.[13] SMAD6 and SMAD7 can inhibit BMP, TGF-β, and activin signals by interfering with R-SMAD phosphorylation by type I receptors.[14,15] Other proteins also contribute to the inhibition of SMAD signaling at the level of R-SMAD phosphorylation and include DPR2, PP2A, STRAP, EIF2A, and EIF3/TRIP1.[16] Multiple proteins, including SARA, endofin, axin, DAB2, and DOK1, contribute to the recruitment of R-SMAD proteins to the type I receptors and enhance Smad activation.[16]

Steady-state Smad protein levels are regulated through the ubiquitin-proteasome degradation pathway.[17] The best studied mechanism of ubiquitin-dependent SMAD degradation is through Smad ubiquitination regulatory factors (SMURF) 1 and 2, which target R-SMAD proteins for degradation. SMURF1 has specificity for the BMP-associated SMADs 1 and 9,[18] whereas SMURF2 seems to be less specific to the BMP pathway and interacts with all R-SMAD proteins.[19] A number of other ubiquitin ligases have been implicated in SMAD protein degradation and modulation of TGF-β superfamily signaling, but the interactions between and the biological significance of most factors are not completely understood.[20]

As many as 80% of CRC cell lines, depending on their genomic subtype, have a defect in the TGF-β signaling pathway and escape TGF-β–induced growth arrest. Studies of mutational frequencies have shown that TGF-β pathway mutations occur in approximately one-third of tumors,[21] which is somewhat lower than that observed in studies of cell lines. Tumors can escape the growth-suppressing effects of TGF-β signaling via many mechanisms, including mutations in receptors, R-SMAD proteins, or SMAD4; overexpression of inhibitory SMAD6 or SMAD7 proteins; blocking phosphorylation of R-SMAD proteins; or increased ubiquitin-mediated proteolysis (see Kang et al[16] for review). It is plausible that most CRCs have alterations in TGF-β superfamily signaling, although the exact frequency of inactivation is hard to determine due to the many mechanisms of colorectal carcinogenesis.

Ligand binding to the TGF-β receptors also activates several non-Smad signaling pathways, known as noncanonical signaling. These pathways involve activation of several kinase

cascades, including Rho, Rac, and Cdc42 guanosine triphosphatases; mitogen-activated protein kinase pathways that include MEK1/2 and ERK1/2, as well as TRAF4/6, TAK1, MKK3/6 and p38 kinases; and the phosphoinositide 3-kinase–AKT–mTOR pathway.[22] Activation of each of these pathways may contribute to the ability of TGF-β to promote the epithelial-to-mesenchymal transition (EMT), probably cooperating with canonical SMAD-mediated signaling. However, noncanonical signaling can occur in the absence of functional SMAD proteins (Figure 1).

SMAD-mediated signaling pathways also interact with other pathways that are important for intestinal stem cell maintenance and differentiation. For example, glycogen synthase kinase 3β (GSK3B) phosphorylates SMAD1 and SMAD3, targeting them for degradation; Wnt signaling inactivates GSK3B to increase BMP and TGF-β signaling through SMAD1 and SMAD3, respectively.[22] Notch signaling and TGF-β/BMP signaling pathways regulate components of each other's signal transduction pathways to cooperatively suppress epithelial cell proliferation.[23] TGF-β–and Notch-mediated transcriptional activation integrate to cooperatively induce expression of HEY1, which contributes to EMT.[24] Conversely, Notch signaling with HEY1 induction represses BMP2 expression and signaling.[25] TGF-β and BMP signaling integrate with Notch-activated signaling to regulate expression of subsets of target genes in several experimental systems,[22] although little is known about how these signaling pathways integrate in the colon cells.

Transcriptional coactivator proteins TAZ and YAP regulate cell proliferation and differentiation downstream of the Hippo pathway. TAZ and YAP signaling integrate with Wnt and TGF-β signaling at several intracellular levels and are likely to have significant effects on intestinal cell proliferation, differentiation, and function. For example, YAP associates with SMAD7 at the type I receptor and increases the binding affinity of SMAD7 for this receptor, thereby inhibiting TGF-β and BMP signaling.[26] SMAD7 and SMAD3 are also regulated to some degree by GSK3B, again connecting the SMAD signaling pathway with the β-catenin signaling pathway. TAZ and YAP, along with β-catenin and TCF and Smad proteins, have complex cooperative and antagonistic roles on gene transcription that are highly dependent on cellular and tissue context and have not been clearly defined in intestinal epithelial or stromal tissues.

Disruption of TGF-β Signaling in Human CRC

Expression and activity of TGF-β receptors and the SMAD protein signal transducers determine whether cell proliferation is inhibited by TGF-β.[3] For example, in CRC cells, mutation of the type II TGF-β receptor can prevent signaling to SMAD proteins; loss of TGF-β–mediated transcriptional activity prevents cells from responding to TGF-β signals that inhibit proliferation.[27,28] Similar findings have been shown for the activin type II receptor *ACVR2A*.[29] Mutations in *SMAD4*, *SMAD2*, and *SMAD3* have been identified in CRC, supporting the concept that SMAD proteins function as tumor suppressors in the colorectal epithelium.[30] Similarly, loss of BMP signaling through mutations of BMP receptors can also contribute to the initiation and progression of CRCs.[31] Furthermore, germline mutations in genes in the TGF-β family signaling pathway strongly increase the risk of colonic neoplasia.

Germline Mutations

Juvenile polyposis syndrome (JPS) is an autosomal-dominant condition that was first described in 1964.[32] Patients with JPS develop juvenile polyps of the stomach as well as the small and large intestine. Juvenile polyps are characterized by overgrowth of the lamina propria with inflammatory cells and cystic glands in the stroma and a spherical appearance. They are not younger polyps, as the name may suggest, but rather are hamartomas. Interestingly, these polyps are at increased risk for developing into tumors.[33,34] The lifetime risk of gastrointestinal (GI) cancers in patients with JPS is as high as 50%.[35,36]

Clinical criteria for the diagnosis of JPS are at least 5 juvenile colorectal polyps in the absence of a family history of JPS, any juvenile polyp in other parts of the GI tract, or any number of juvenile polyps with a family history of JPS.[37] Germline mutations in TGF-β superfamily members have been detected in approximately one-half of JPS cases. Approximately 20% to 30% of patients carry a mutation in the *BMPR1A* gene,[38] with another 20% to 30% of patients carrying a mutation in *SMAD4*.[39] Interestingly, no mutations were found in other BMP receptors (*BMPR1B*, *BMPR2*, or *ACVR1A*)[40] or R-SMAD genes (*SMAD1*, *SMAD2*, *SMAD3*, or *SMAD5*).[41] Although these findings were in small cohorts, they indicate a specific role for *BMPR1A* in GI physiology or an important role of other BMP receptors in prenatal development.[42] An infrequent but more severe form of JPS, called JPS of infancy, is caused by microdeletion of chromosome 10q22-23, which contains the *BMPR1A* and *PTEN* genes. JPS of infancy has onset in the first 2 years of life. Patients most often present with profound rectal bleeding. The disorder is associated with macrocephaly and has high mortality.[43]

In patients with JPS, *SMAD4* mutations are associated with a more severe gastric phenotype than *BMPR1A* mutations and are associated with a worse prognosis.[44] A subset of patients with *SMAD4* mutations have hereditary hemorrhagic telangiectasia (HHT), an autosomal-dominant disease characterized by multifocal telangiectasias and arteriovenous malformations. HHT can be caused by mutations in endoglin (a coreceptor to TGF-β receptors) or the type I TGF-β superfamily receptor activin receptor–like kinase 1 (*ACVRL1*)[45,46] (Table 1). People with germline mutations in *SMAD4* can present with HHT, JPS, or a combination of these, indicating the involvement of environmental factors. It is not clear whether people with HHT who carry a *SMAD4* mutation are at higher risk for intestinal neoplasms, but screening for GI polyps in patients with HHT who are positive for this mutation should be considered. Approximately 50% of patients with JPS do not carry a germline mutation in either *BMPR1A* or *SMAD4*, illustrating the high variance of this elusive GI syndrome.

Studies of JPS have shown that SMAD and BMP signaling prevents carcinogenesis in the GI tract. Disruption of these pathways can lead to the formation of malignant tumors. Given the different histopathologic features of hamartomas and colorectal adenocarcinomas and the low frequency of BMP receptor mutations in sporadic CRCs, it is not clear whether JPS and CRC have similar mechanisms of pathogenesis.

There have been many studies of the effects of germline mutations in genes encoding TGF-β receptors in CRCs because of the antiproliferative effects of TGF-β signaling. Interestingly,

*TGFBR1*6A*, one of the first susceptibility alleles identified, is found in a large proportion of the general population (13.7%) and has been associated with a 24% increase in risk of CRC.[47] The variant *TGFBR1*6A* encodes the deletion of 3 alanines within a 9-alanine (*9A) repeat at the 3°-end of the exon 1 coding sequence.[48] The *TGFBR1*6A* and *TGFBR1*9A* polymorphisms encode a normal mature TGFBR1 after cleavage of the signal sequence.[49] However, Pasche et al observed that the receptor encoded by *TGFBR1*6A* was a less effective mediator of TGF-β antiproliferative signals[50] compared with the protein encoded by *TGFBR1*9A*. Notably, a recent meta-analysis of 13,662 cases and 14,147 controls identified a modest increase in the risk of breast and ovarian cancer associated with the TGFBR1*6A allele but no significant increase in CRC, bladder cancer, prostate cancer, or lung cancer.[51] The impact of germline mutations in TGF-β receptors for CRC might therefore be modest, even though they do occur and appear to be causative for other conditions such as Marfan syndrome (OMIM #154700).[46]

Somatic Mutations in TGF-β Receptors and SMADs

Proliferation of a subset of CRC cell lines is no longer inhibited by TGF-β,[52] and TGF-β signaling is frequently disrupted in CRC tissues from patients. *TGFBR2* mutations are frequently detected in colon cancer cells with microsatellite instability (MSI). Colon cancer cells with MSI have mutations in mismatch repair genes that lead to accumulations of mutations in microsatellite DNA sequences. Most colon cancers with MSI have been found to have frameshift mutations in the *TGFBR2* gene, in the polyadenine micro-satellite in exon 3.[27] More than 80% of primary colon cancer cells with MSI contain biallelic mutations in *TGFBR2* that encode a truncated protein.[53] Loss of TGFBR2 expression via mutation in late adenomas is associated with their progression to colon carcinomas with MSI[54] and then microsatellite-stable colon cancers.[28] However, a subset of biallelic mutations in TGFBR2 that encode a truncated product do not completely disrupt TGF-β signaling,[55] and there have been reports of active TGF-β signaling in cells with *TGFBR2* mutations.[56,57] TGF-β might therefore signal directly through mutant forms of TGFBR2.

Genome-wide screening studies led to the identification of *ACVR2A* mutations in CRC cell lines.[58] *ACVR2A* mutations have been found to be the second most common mutations in MSI CRC cells. The identification of biallelic mutations and associated loss of protein in primary colon cancers indicates that these mutations might contribute to development of colon cancer.[29] More than one-half of colon cancers with MSI contain mutations in *ACVR2A* and *TGFBR2.*

The correlation of *ACVR2A* mutations with grade and larger size of primary CRCs is consistent with a loss of growth-suppressive properties.[59] However, lack of correlation with stage, although difficult to interpret at the time, is now consistent with our knowledge of a dual role for TGF-β, suppressing growth of early-stage tumors but promoting dissemination of later-stage tumors. This model is supported by the observation that in patients with MSI cancers, loss of TGF-β signaling due to loss of TGFBR2 is associated with longer survival times.[60] In microsatellite-stable tumors, development of CRC appears to involve inactivation of the TGF-β receptor kinase domain via point mutations and reduced expression of ACVR2 via promoter hypermethylation.[61]

TGF-β receptor and activin signaling are therefore commonly disrupted during development of colon cancer. Studies are needed to determine at what specific time points in tumor progression signaling is lost, possibly to develop targeted treatments. It will also be important to study the effects of epithelial loss on stromal ligand regulation and cross-regulation of pathways in the presence or absence of mutations.

SMAD4 is the Smad family gene most commonly found to be disrupted in cancers. *SMAD4* is located on chromosome 18q21.[62,63] Mutations of *SMAD4* have been identified in 50% of pancreatic cancers,[62] 20% to 30% of CRCs,[64–66] and up to 20% of small-bowel carcinomas.[67] Loss of SMAD4 is correlated with loss of E-cadherin,[68] metastasis to liver,[69] and poor prognosis of patients with Dukes' stage C CRC.[70] SMAD4 loss and chromosome 18q deletions have been associated with incidence of lymph node metastasis in CRC.[71] These findings indicate that loss of SMAD4 expression contributes to colorectal carcinogenesis.

Somatic mutations in other *SMAD* genes have been less frequently identified. In a study of more than 700 sporadic CRCs, somatic inactivating mutations were identified in *SMAD4* (8.6% of samples), *SMAD2* (3.4% of samples), and *SMAD3* (4.3% of samples) for a combined prevalence of 14.8%. This frequency of mutation is smaller than previously reported in other cohorts but consistent with *SMAD* genes having important roles as tumor suppressors.[30] It is important to remember that SMAD4 activity can be disrupted by other mechanisms, such as those that alter its posttranslational modification or localization.

TGF-β Signaling Maintains Homeostasis of the Small Intestine and Colon

Different TGF-β family members have overlapping functions, which are poorly understood. We will refer to the groups of ligands and not differentiate specific isoforms, although we acknowledge potential differences that need to be addressed in further studies.

Wnt and TGF-β superfamily members interact during embryonic development and in homeostasis of the adult intestinal epithelium.[72,73] Barnard et al first showed an increasing gradient of TGF-β in colonic epithelium, from the crypt to the surface.[74] Kosinski et al noted an inverse gradient of BMP or Smad and Wnt pathway activation in intestinal epithelium, indicating the interactions between these antagonistic pathways[75] (Figure 2).

Studies of tissues from patients with JPS have provided evidence for the interaction between SMAD4 and Wnt pathways.[39,40] Transgenic overexpression of the BMP antagonist noggin and mutation of the *Bmpr1a* gene (which encodes a BMP receptor) results in development of polyps in mice that resemble those observed in patients with JPS. Cells from these polyps have increases in nuclear and cytoplasmic β-catenin[76] and increased transcriptional activity of Wnt[77] (see the preceding text). Intestine-specific disruption of *BMPR1* leads to expansion of intestinal stem and progenitor cell populations that precede polyp formation. Incubation of cultured intestinal segments with noggin increases levels of phosphorylated PTEN (the inactive form), levels of phosphorylated AKT, and nuclear levels of β-catenin; this increases expression of a β-catenin and TCF-responsive reporter gene.[77]

These studies show that BMP signaling promotes PTEN activity, leading to decreased levels of PIP3, counteracting phosphoinositide 3-kinase, and reducing Akt activity in the regulation of Wnt signaling.[77] Consistent with these observations, the BMP antagonist gremlin 1 activates Wnt signaling in cultured rat intestinal epithelial cells.[75] Taken together, these findings show the importance of the interaction between TGF-β and BMP signaling, via SMAD4 and the Wnt pathway, in intestinal homeostasis and carcinogenesis (Figure 3). However, the exact mechanisms of this interaction have not been fully determined.

Tumor Suppression

Nonsteroidal anti-inflammatory drugs block prostaglandin synthesis and reduce the risk of development of colon adenomas.[78] Cyclooxygenase-2 is the most abundant, inducible form of prostaglandin synthase; it is upregulated in one-half of colorectal adenomas and in 85% of colorectal adenocarcinomas.[78,79] 15-Hydroxyprostaglandin dehydrogenase (15-PGDH) metabolizes prostaglandins.[80] In a gene expression profile study, Yan et al[81] identified 15-PGDH as one of the most downregulated genes in colon cancer cells. They also found that cultured colon epithelial cells continuously exposed to TGF-β upregulate 15-PGDH. This role of TGF-β is consistent with its function as an anti-inflammatory cytokine. 15-PGDH is highly expressed by normal colonic epithelia but is nearly undetectable in colon cancer samples. Not surprisingly, levels of 15-PGDH are reduced in CRCs because TGF-β signaling is disrupted in more than 80% of CRCs.[28] CRC cells that overexpress 15-PGDH form fewer xenograft tumors in mice than control cells that express an inactive mutant.[73]

Calon et al[82] associated gene expression patterns of the tumor stroma with tumor phenotype. Specifically, TGF-β signaling in the stroma was associated with more aggressive CRCs. It is therefore important to look beyond the tumor epithelial cells and investigate stromal signaling, and stromal TGF-β signaling, in development of CRC.

Antitumor Immunity

Chronic inflammation promotes carcinogenesis and tumor progression. One observation to support the link between inflammation and CRC is that patients with inflammatory bowel diseases are at increased risk for CRC. Furthermore, there is increasing evidence that changes in the colonic microbiota can create an inflammatory environment in the colon that contributes to carcinogenesis.[83] Cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, and TGF-β contribute to the inflammatory microenvironment to promote colon cancer development and progression.[84] The role of TGF-β in CRC and other cancers is particularly complex and often paradoxical to its role in normal tissues as a tumor suppressor and inhibitor of inflammation. TGF-β regulates proliferation, differentiation, and functions of immune cells that include macrophages, dendritic cells, natural killer cells, B cells, and T cells. TGF-β also modulates innate and adaptive immunity (reviewed by Caja and Vannucci[85]). The role of TGF-β in the regulation of T-cell maturation, proliferation, and expansion is particularly relevant to cancer progression.

TGF-β inhibits IL-2–induced proliferation of T cells[86] and TNF production.[87] Highly immunogenic tumor cells engineered to overexpress TGF-β failed to stimulate primary

cytotoxic T-cell responses and were able to evade eradication by the immune system in mice.[88] Mice with homozygous disruption of the *Tgfb1* gene are viable at birth but invariably develop an acute wasting syndrome within 20 days. This is characterized by multifocal areas of mixed inflammatory cell infiltration into numerous organs, including the heart and lungs, and the GI tract as well as tissue necrosis, organ failure, and death by 4 weeks of life. These findings indicate the prominent role for TGF-β1 in homeostatic regulation of immune cell function[89,90] (Table 2).

Data on the role of activin in the normal gut epithelium are scarce, but this protein could have a role in intestinal wound repair.[91] The low baseline expression of activin in the bowel, plus the lack of strong GI phenotype in *Acvr2a*$^{-/-}$ mice, indicates that activin does not have an important role in gut homeostasis. Studies indicate an important immune-modulatory function of activin that resembles but does not copy TGF-β function. Activin expression peaks early in the inflammatory response and has been associated with inflammatory diseases including inflammatory bowel disease,[92] asthma,[93,94] and viral infections.[95] Activin increases secretion of inflammatory factors such as IL-1β, TNF, and IL-6[96–98] in vitro and in vivo, reduces secretion of the anti-inflammatory cytokine IL-10,[99] and inhibits secretion of inflammatory cytokines such as TNF, IL-18, and IL-6.[100] Inhibition of activin by its binding partner, follistatin, reduces the severity of inflammation and even mortality in animal models of inflammatory bowel disease[101] and lipopolysaccharide-induced endotoxemia.[102] Activin is involved in development of cachexia in patients with CRC.[103,104] Overexpression of activin in mouse models[105,106] leads to a cachexia phenotype, most probably through interaction with the myostatin pathway.[107] Furthermore, levels of activin correlate with cachexia in patients with cancer.[108]

Preclinical Studies of CRC and TGF-B Family Signaling

SMAD-Knockout Mice

SMAD family members are the point of convergence for canonical TGF-β signaling pathways. Investigators have disrupted many of the Smad genes in mice (Table 2). Most Smad-knockout mice die in utero (overview in Goumans and Mummery[109]). Downregulation of SMAD7 by injection of antinucleotides leads to reduced tumor formation in *APC*$^{+/min}$ mice,[110] indicating that SMAD7 signaling inhibits colorectal tumorigenesis. Genome-wide association studies identified single nucleotide polymorphisms in *SMAD7* that correlate to risk of CRC, but their effects on expression or function of the product are unknown.[111]

SMAD2-knockout embryonic mice do not undergo mesoderm induction.[112] The combination of heterozygous loss of *Smad2* and heterozygous loss of *Apc* increases the size and invasiveness, but not the number, of bowel tumors,[113] indicating an anticarcinogenic effect of activating Smad proteins downstream of activin and TGF-β signaling.

In initial studies, knockout of exon2 of *Smad3* in a 129/Sv background led not only to minor growth suppression and skeletal deformities but most prominently to metastasizing large-bowel cancers in 100% of mice.[114] However, the frequency of colorectal neoplasms was lower in mice when exon2 of *Smad3* was knocked out in a 129/Sv C57BL/6 hybrid

background; only 30% of mice developed bowel cancers and with no evidence of metastases.[115] Thus, even though SMAD3 appears to prevent against development of colorectal tumors, its effects on metastasis might depend on the mouse strain studied.

Mice with knockout of exon 8 of *Smad3* develop an autoimmune phenotype with abnormally activated T cells, colon inflammation, and infrequent adenocarcinomas.[116] Crossing $Apc^{+/Min}$ mice with $Smad3^{-/-}$ mice increases the frequency of intestinal tumor formation compared with $Apc^{+/Min}$ or $Smad3^{-/-}$ alone; despite the invasive character of adenocarcinomas, again no metastases are observed.[115] In summary, loss of SMAD3 appears to promote colorectal tumorigenesis, but the pro-oncogenic effects observed in some mouse models seem to be much stronger compared with human correlates.

Knockout of SMAD4 is embryonic lethal due to defects in gastrulation.[117] Heterozygous mice reach adulthood and develop inflammatory gastric and duodenal polyps with histopathologic features comparable to JPS.[118] Hohenstein et al reported that a spontaneous single nucleotide mutation in *Smad4*, which reduced messenger RNA, produced a similar phenotype with duodenal polyps.[119] Mice heterozygous for disruption of *Smad4* and *Apc* develop more GI tumors than mice with heterozygous deletion of only *Smad4* or *Apc*, indicating that tumor formation due to loss of *Apc* is augmented by loss of *Smad4*. Depletion of SMAD4 from T cells in mice leads to de novo epithelial carcinomas in the gut, but epithelial-specific knockdown of SMAD4 did not promote development of GI cancers.[120] Notably, the histological phenotype of the tumors observed in these studies was closer to hamartomas than to adenocarcinomas.

Freeman et al reported that conditional knockout of SMAD4 in $Apc^{\Delta 1638/+}$ mice resulted in a 10-fold increase in small intestinal and colonic adenomas, whereas $Apc^{\Delta 1638/+}$ mice developed fewer than 5 adenomas in the ileum and only occasional adenomas in the colon.[121] The high frequency of somatic *SMAD4* mutations observed in human CRCs and a growing amount of in vivo data[122] indicate that loss of *SMAD4* from the epithelium contributes to tumorigenesis.

Although the role of Smad proteins in colorectal carcinogenesis has been studied in a number of animal models, most investigate either knockout of all SMAD signaling (SMAD4) or SMAD signaling downstream of TGF-β and activin (SMAD2/3) but not of BMPs (SMAD1/5/9). Different TGF-β superfamily members signal through the same canonical SMAD-dependent pathways, so it is impossible to discern the respective contributions of specific TGF-β superfamily members to observed phenotypes. It is not yet possible to evaluate the effects of canonical BMP signaling in development of sporadic CRC, although studies of conditional knockouts will likely provide further insights into the roles of canonical epithelial BMP signaling. Even though canonical TGF-β superfamily signaling is important in a number of animal models, its multifunctional and context-specific nature should not be underestimated. As for β-catenin signaling.[123] rather than being an analog on-off switch, SMAD signaling should be seen as a finely tuned system in which the extent and timing of activation and inhibition affect cell homeostasis. Lastly, the role of noncanonical signaling pathway activation, which might be differentially regulated when

canonical signaling is disrupted, has not been explored in mouse models. This is an important area of research for future studies.

TGF-β Knockout Mice

When the *Tgfb1* gene is disrupted in *Rag2*$^{-/-}$ mice, which are immune deficient, the mice develop proximal colon tumors with a mucinous phenotype in the absence of APC or P53 pathway disruptions. Interestingly, this phenotype is seen in mice of a 129 genetic background but not in mice of a C3H genetic background (reviewed by Doetschman[124]).

Disruption of other components of TGF-β signaling, including inactivating mutations in the TGF-β receptor or Smad proteins, in mice promotes development or progression of colon tumors, either in the presence of initiating mutations in APC signaling via Wnt and β-catenin or in conjunction with chronic inflammation.[124] One study compared transcriptomes of colon tumors from mice with azoxymethane-induced cancer, *Apc*$^{Min/+}$ mice, *Tgfb1*$^{-/-}$; *Rag2*$^{-/-}$ mice, and *Smad3*$^{-/-}$ mice. The azoxymethane-induced tumors and the tumors from *Apc*$^{Min/+}$ mice activated transcription of genes in the canonical Wnt signaling pathway, genes encoding stem cell markers, and genes that regulate cell proliferation. The tumors from *Tgfb1*$^{-/-}$; *Rag2*$^{-/-}$ mice and *Smad3*$^{-/-}$ mice altered expression of genes that regulate the immune and inflammatory responses.[125] Interestingly, the tumors that formed in the *Tgfb1*$^{-/-}$; *Rag2*$^{-/-}$ mice and *Smad3*$^{-/-}$ mice altered expression of genes linked to inflammation associated with *Helicobacter* infection.[124]

Apc$^{Min/+}$ mice mostly develop benign intestinal adenomas and only rare invasive cancers. However, disruption of the *Tgfbr2*,[126] *Smad4*,[121,127,128] or *Smad3*[115] genes in *Apc*$^{Min/+}$ mice increases the numbers of tumors that form and the number of invasive adenocarcinomas. Tumors that form in cis-*Apc*$^{+/\Delta 716}$; *Smad4*$^{+/-}$ mice increase production of the chemokine CCL9. CCL9 production has been associated with loss of SMAD4 from CRC epithelial cells and recruitment of myeloid cells that express the CCL9 receptor, CCR1, to the tumor stroma. CCL9 thereby promotes tumor invasion and metastasis.[128] In human CRC cells, loss of SMAD4 results in upregulation of CCL15 (the human orthologue of mouse CCL9).[129] In human tumor samples, there is an inverse correlation between levels of CCL15 and SMAD4, and liver metastases that express CCL15 contained 3-fold more CCR1+ cells than those without CCL15 expression. Furthermore, patients with CCL15-expressing metastases have significantly shorter times of disease-free survival after resection intended for cure than patients with metastases that do not express CCL15.[129]

Further evidence for the role of TGF-β signaling in maintaining homeostasis and regulating inflammatory responses in the colon was recently provided in a study of intestine-specific and inducible knockout of the type II TGF-β receptor, using *Tgfbr2flox/flox* mice crossed with *Villin-CreER* mice, which express a tamoxifen-activated form of Cre recombinase specifically in the intestinal epithelial cells.[130] In these mice, *Tgfbr2* can be selectively disrupted in the intestinal epithelial cells of adult mice by administration of tamoxifen. Crossing the *Tgfbr2flox/flox*; *Villin-CreER* mice with *Apc*$^{+/\Delta 716}$ mice resulted in the expected intestinal adenocarcinomas with submucosal invasion after tamoxifen exposure; surprisingly, the *Tgfbr2flox/flox*; *Villin-CreER* mice developed invasive colon carcinomas several weeks after tamoxifen exposure, followed by induction of colitis with dextran

sodium sulfate, and without the need for *Apc* mutation or exposure to azoxymethane. Furthermore, in the *Tgfbr2flox/flox*;*Villin-CreER* mice, mucosal regeneration after radiation-induced injury was impaired, and in the absence of TGF-β signaling there was an expansion of proliferating and undifferentiated intestinal epithelial cells. Interestingly, colon surface epithelial cells from patients with ulcerative colitis have nuclear staining for phospho-SMAD2, but this is lost from colon tumors that develop in patients with ulcerative colitis. These findings indicate that loss of TGF-β signaling is an event in ulcerative colitis–associated carcinogenesis.[130]

The association between germline mutations in *BMPR1A* and GI cancer in patients with JPS sparked a strong interest in BMP signaling in CRC.[131] The disruption of BMP signaling in animal models revealed the main effector cells in bowel cancers to be nonepithelial. This has broadened our horizon to reevaluate involvement of nonepithelial compartments in all TGF-β superfamily signaling.

For instance, loss of *Bmpr1a* from the gut epithelium, via inactivation by *Villin-Cre*, surprisingly did not lead to tumor or polyp formation but instead impaired differentiation of secretory cells and increased crypt proliferation.[132] In contrast, nonspecific disruption of BMP signaling in the stromal and epithelial compartment, either through mx1-cre-mediated disruption of *Bmpr1a*[76] or epithelial overexpression of the BMP antagonist noggin via *Villin-Cre*[75] or *Fabp1-Cre*,[133] led to the formation of hamartomatous polyps in the small intestine. A similar phenotype was observed following knockout of SMAD4.[134] BMPR2-knockout mice (via *Nestin-Cre*)[135] develop hamartomatous polyps and epithelial hyperplasia. Inhibition of TGF-β superfamily canonical signaling in T cells through disruption of *Smad4* by *Lck-Cre* or *Cd4-Cre*[120] also leads to hamartomatous polyps in the small and large intestine, adding further to the complexity of BMP signaling in the colonic microenvironment.

Taken together, these studies provide evidence for the role of BMP signaling in formation of juvenile polyposis and hamartomas but not in development of sporadic CRC. Loss of BMP activation in sporadic human CRC, as measured by loss of nuclear SMAD1, 5, and 9, is believed to be important in the development of sporadic CRC.[31] However, its true effects may be modest because BMP receptor mutations are infrequently detected in CRCs. The presence of *SMAD4* mutations in sporadic CRC and a subset of hamartomatous syndromes could indicate the importance of upstream BMP family member signaling in CRC. However, SMAD4 is a shared downstream pathway member of all canonical TGF-β superfamily members (see Figure 1) and net effects may be due to loss of other pathway members, compensatory noncanonical signaling, or both. There have been few studies of BMP signaling in a model of sporadic CRC.

Activin knockout mice have severe developmental defects (see Table 2). No studies investigating ligand deficiency have reported formation of neoplasms, although these studies focused on reproductive tissues and intrauterine development and did not analyze phenotypes of older animals. However, overexpression of activin in the skin of mice from a keratin promoter promoted development of chemical-induced skin cancers.[136] Knockout of

the activin antagonist inhibin led to spontaneous gonadal sex cord tumors and later to adrenocortical tumors, accompanied by cachexia if animals had their gonads removed.[137]

Activin receptor knockout mice do not have the same phenotypes as activin knockout mice, indicating the complex, multifunctional, and promiscuous nature of activin receptor interactions. Knockout of ACVR1B in mice that express an activated form of Kras in pancreatic cells reduced formation of pancreatic tumors.[138] The applicability of this study to human disease is unclear because levels of activin were increased in serum and pancreas tissue, indicating increased activin signaling in stroma and the hematopoietic compartment, whereas there was only partially repressed activin function in the epithelial compartment. To date, there are no studies investigating activin signaling, either on the ligand or receptor level, in models of sporadic CRC, despite the availability of conditional models.[139,140]

Overall, despite the correlations between activin and BMP pathway members and more aggressive CRC,[141,142] and their substantial effects on CRC cells in vitro,[143,144] few animal studies have investigated the roles of TGF-β superfamily members in the pathogenesis of sporadic CRC.

Future Directions

Gene expression analyses of human CRCs provide further evidence for the roles of TGF-β signaling in colorectal tumor development. Combined molecular profiles from 6 independent primary CRC gene expression analyses led to a classification into 4 distinct consensus molecular subtypes.[145] One subtype (subtype 4) was characterized by upregulation of genes associated with activation of TGF-β, stromal invasion, and angiogenesis. This subtype had the shortest times of overall and recurrence-free survival.

In an interesting follow-up study,[146] cells in tubular adenomas that progressed to classic CRCs underwent apoptosis in response to TGF-β, consistent with its role in suppressing tumor growth, whereas a subgroup of sessile serrated adenomas progressed to subtype 4–like CRCs, with activation of genes in the TGF-β pathway. Expressing the $BRAF^{v600E}$ mutant in tubular adenoma organoids through CRISPR/Cas9 gene editing, mimicking the sessile serrated adenoma pathway in CRC, changed the response to TGF-β from apoptosis to the EMT. These findings indicate that TGF-β mediates the change of sessile serrated adenomas into the mesenchymal subtype 4 CRC.[146]

Agents that inhibit TGF-β signaling have been tested in clinical trials of patients with fibrosis in lung and kidney disease as well as patients with glioblastomas, melanomas, pancreatic cancer, or metastatic colon cancers.[147,148] These trials were based on preclinical studies showing that TGF-β inhibition substantially reduced the frequency of metastases that formed in animal models of breast cancer,[149] pancreatic cancer,[150] and CRC.[151]

Inhibitors of TGF-β ligands and receptors were found to be safe in humans,[152–154] but no compound has completed a stage 3 trial or shown clinical efficacy. Current studies are under way for patients with glioblastoma or pancreatic cancer[155] but not CRC. One key challenge of TGF-β–directed therapy for CRC is the identification of markers to classify patients as responders, because inhibiting TGF-β in the wrong subpopulation could be detrimental. A

study found that systemic and epithelial-specific inhibition of TGF-β signaling in mice with *Apc* mutations led to wasting, autoimmunity, and significantly shorter survival times.[156]

Targeting Activin Signaling

Despite growing evidence of the substantial role of activin in the development of CRC, few studies have focused on tumor-specific rather than overall effects of activin inhibition. A soluble activin receptor 2A–IgG fusion protein reduces osteolytic lesions in models of multiple myeloma and breast cancer metastasis[157] and increases hematocrit in humans with cancer-associated anemia.[158] Perhaps even more exciting, administration of an activin receptor 2B decoy to mice with CRC xenograft-induced cachexia, to sequester activin and myostatin, leads to markedly increased muscle mass and reduced mortality.[159] Even though two stage 2 studies were recently terminated early due to poor enrollment,[160] activin inhibition in patients with solid cancer appears to be safe and feasible. Besides the possible, but as yet uninvestigated, positive direct actions on tumor cells, activin inhibitors could have effects beyond the tumor epithelium.

Targeting BMP Signaling

BMP signaling could be an attractive therapeutic target because of its roles in induction of a metastatic phenotype and the promotion of tumor stem cells. Given the oncogenic function of BMP signaling inhibition in the colonic stroma plus the multifunctional protumorigenic and anti-tumorigenic effects in epithelial cells, and the altogether less understood role of BMP signaling in sporadic CRC, BMP inhibition is unlikely to enter clinical studies soon, especially with few findings from animal studies. Furthermore, currently available type 1 BMP receptor inhibitors inhibit other TGF-β superfamily type 1 receptors as well, hampering their translation to the clinic.[161]

Targeting SMAD Proteins

SMAD proteins are downstream of several TGF-β super-families, so SMAD inhibitors might be used to inhibit several interacting TGF-β signaling pathways. Little is understood about the interactions between canonical and noncanonical TGF-β superfamily signaling pathways, which inhibit proliferation but promote metastasis. These uncertainties remain a challenge for SMAD-directed therapy in CRC. Nevertheless, oligonucleotides against *Smad7* have entered clinical trials for Crohn's disease and have met safety end points,[162] so they might be tested in patients with CRC.

There has been exciting progress in our understanding of TGF-β family member signaling in colon cancer. To develop treatments, however, we need to increase our understanding of the interactions between TGF-β signaling pathways and epithelial and mesenchymal compartments. Better preclinical models are required to identify biomarkers for subpopulations of patients most likely to benefit from targeted and timed inhibition of TGF-β superfamily proteins.

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Abbreviations used in this paper

BMP	bone morphogenetic protein
CRC	colorectal cancer
EMT	epithelial-to-mesenchymal transition
GI	gastrointestinal
GSK3B	glycogen synthase kinase 3β
HHT	hereditary hemorrhagic telangiectasia
IL	interleukin
JPS	juvenile polyposis syndrome
MSI	microsatellite instability
15-PDGH	15-hydroxyprostaglandin dehydrogenase
TGF	transforming growth factor
TNF	tumor necrosis factor

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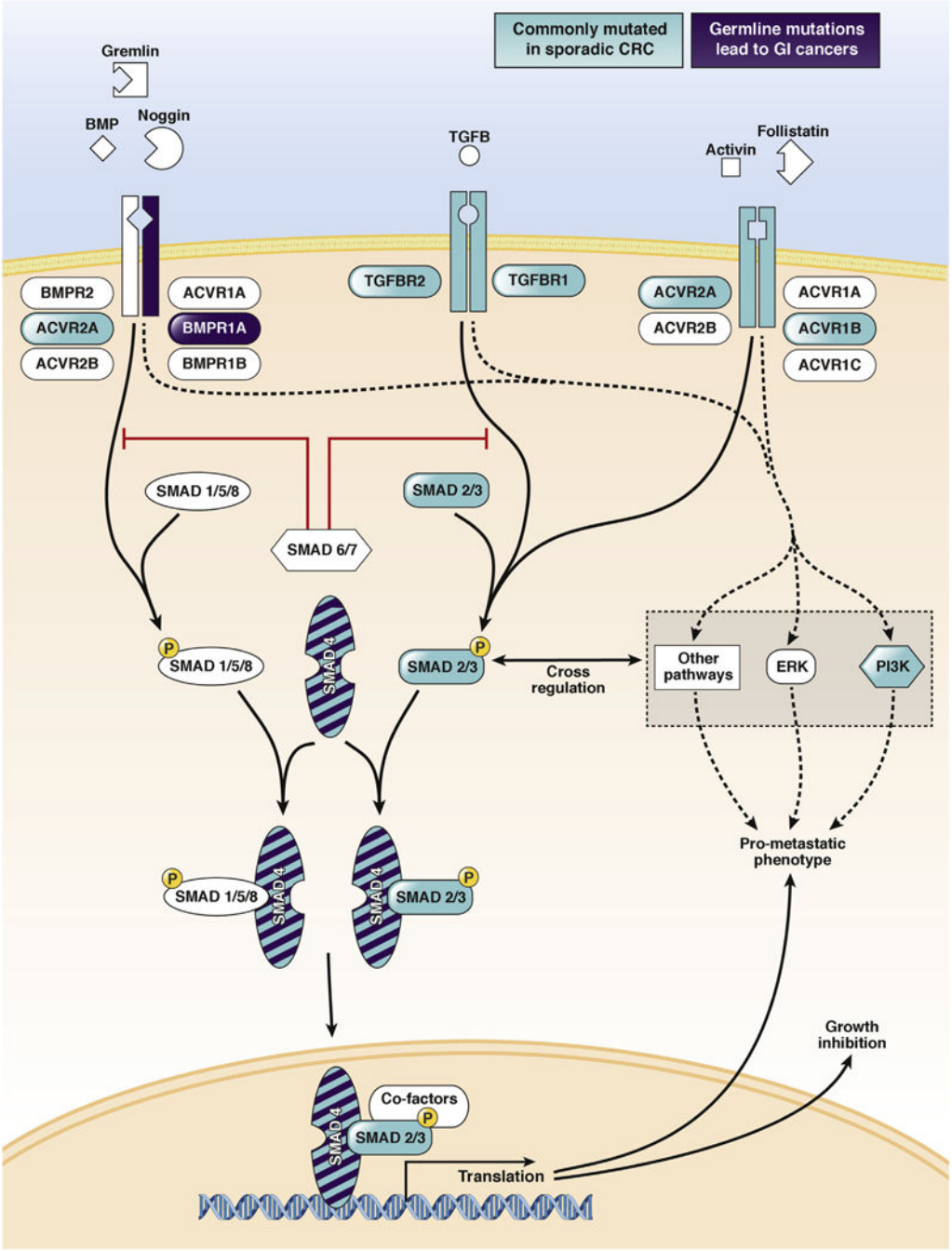

Figure 1.

TGF-β family member signaling and its target in CRC. Members of the TGF-β family are commonly mutated in CRCs. Various ligands bind to specific cell surface receptor systems to affect downstream SMAD and non-SMAD signaling. Pathway members commonly mutated in CRC are in *green*, members affected in other GI cancers are in *purple*, and members that have been found altered in both are *striped*. As depicted in this simplified cartoon, there is frequent cross-regulation among upstream and downstream pathway members that are context dependent.

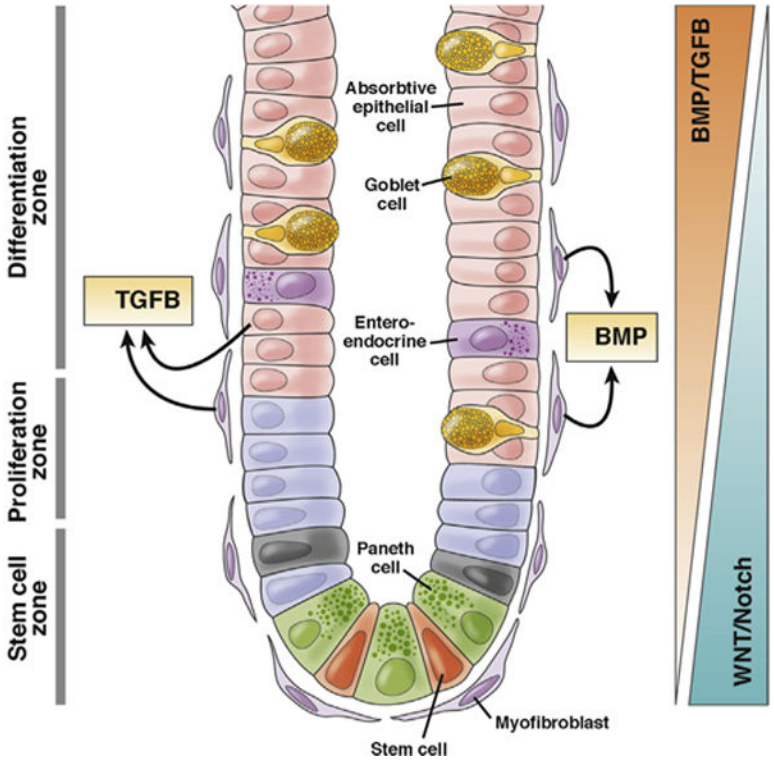

Figure 2.

Epithelial-stromal signaling of TGF-β family members in normal colonic mucosa. In the differentiated normal intestinal cell crypt, various gradients of TGF-β family members maintain homeostasis. Importantly, while BMP appears to be secreted mostly by epithelial cells, fibroblasts are a significant source of TGF-β secretion.

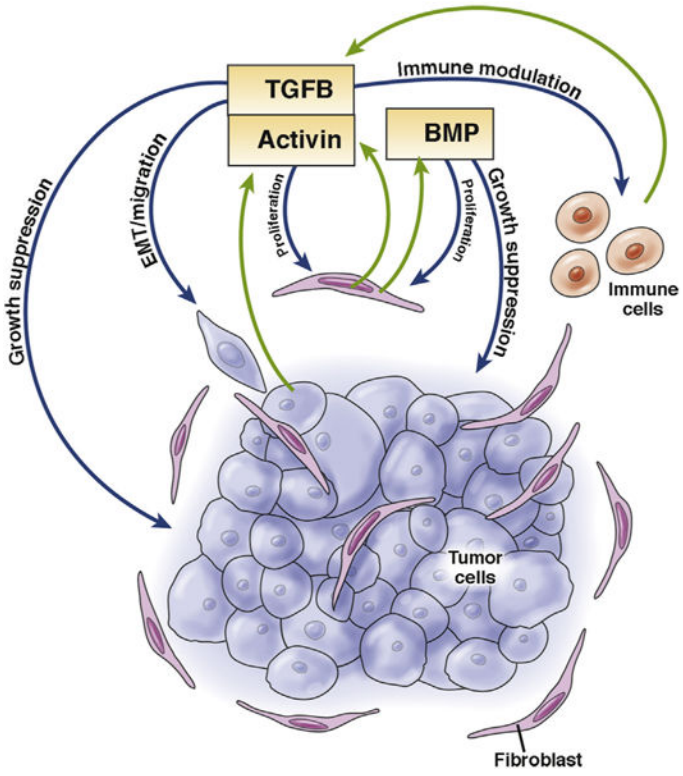

Figure 3.

Epithelial-stromal signaling of TGF-β family members in CRC. In CRC, there is enhanced secretion of TGF-β family ligands by both stroma and epithelial cells leading to autocrine enhanced secretion, immune modulation, and EMT as well as fibroblast proliferation and tumor cell growth suppression.

Table 1

Synonymous Nomenclature for TGF-β Family Receptors

Alk1	ACVRL1
Alk2	ACVR1A
Alk3	BMPR1A
Alk4	ACVR1B
Alk5	TGFBR1
Alk6	BMPR1B
Alk7	ACVR1C

Table 2

Phenotypes in Murine Models and Human Disease Correlates

Gene	Phenotype of global loss	Conditional models of CRC	Human disease correlate
TGFB1	Autoimmune phenotype and wasting, short survival[89,90] or intrauterine death due to defective angiogenesis,[163] depending on background	In combination with *Rag*$^{-/-}$ tumor suppressive mice when compared with *Rag*$^{-/-}$ mice alone[164]	Camurati-Engelmann syndrome[165]
TGFB2	Wide range of developmental defects, perinatal lethal[166]	N/A	Balanced chromosomal translocation t(1;7)(q41;p21) (TGFb2 and HDAC9) → Peters' anomaly[167]
TGFB3	Palatal shelves do not fuse, reduced survival[168]	N/A	De novo mutation → clinical features overlapping with Marfan and Loeys-Dietz syndrome[169]
TGFBR1	Intrauterine lethal[170]	Haploinsufficiency increases the number of adenomas compared with *APC*$^{Min+/-}$ alone[171]	Loeys-Dietz syndrome[172]
TGFBR2	Intrauterine lethal[173]	Epithelial knockout leads to increased number of tumors after challenge by azoxymethane[130] and invasive carcinomas after dextran sulfate sodium challenge[174] Epithelial knockout combined with APC mutations increases invasiveness of lesions compared with APC alone[125,130]	Loeys-Dietz syndrome[172] Marfan syndrome (subset)[175] (pT315M) Hereditary nonpolyposis colorectal cancer, type 6[176]
Activin ligands	Activin A (INHBA): severe whisker and palate defects; mortal within 24 hours of birth[177] Activin B (INHBB): eyelid deformation in males; female infertility[178]	N/A	N/A
ACVR1A	Intrauterine lethal[179]	N/A	Gain of function → fibrodysplasia ossificans progressiva[180]
ACVR1B	Intrauterine lethal[181]	N/A	N/A
ACVR2A	Craniofacial deformation in a subset/female infertility[182]	N/A	N/A
ACVR2B	Postnatally lethal heart defects Left-right and anterior-posterior axis malformation[183]	N/A	Heterotaxy syndrome[184]
BMP ligands	More than 15 isoforms – intrauterine lethality and affected bone formation common – reviewed in Wang et al[42]	N/A	N/A
BMPR1A	Intrauterine lethal/failure to form mesoderm[185]	Stromal knockout via Mx1-Cre leads to hamartomatous polyps in the small intestine[76] Epithelial knockout via Villin-Cre leads to increased crypt proliferation but no polyps[132]	JPS[40]
BMPR1B	Skeletal deformities[186]	N/A	p.Cys53Arg Acromesomelic chondrodysplasia type Grebe[187] p.Arg31Cys du Pan Acromesomelic dysplasia[188]

Gene	Phenotype of global loss	Conditional models of CRC	Human disease correlate
			Idiopathic pulmonary arterial hypertension[189]
BMPR2	Intrauterine lethal/failure to form mesoderm[190]	Stromal knockout via Nestin-Cre leads to intestinal hamartomas and epithelial hyperplasia[135]	Hereditary pulmonary arterial hypertension[191]
SMAD1	Intrauterine lethal/failure of allantois formation[192]	N/A	N/A
SMAD2	Intrauterine lethal due to failure of mesoderm induction[112]	Haploinsufficiency increases size and invasiveness of tumors in $APC^{+/-}$ animals[113]	N/A
SMAD3	Invasive CRC (30%– 100% of animals) and minor growth suppression[114,115] or colonic inflammation and infrequent adenocarcinomas[116]	Global knockout increases tumor frequency in the colon of $APC^{+/-}$ animals[115]	N/A
SMAD4	Intrauterine lethal[118]	Haploinsufficient animals develop gastric and duodenal inflammatory tumors[118] Knockout in T cells via Lck-Cre or Cd4-Cre leads to bowel cancers[120] Haploinsufficiency increases frequency of tumors in $APC^{+/-}$ animals[193] Epithelial knockout via K19-Cre leads to strongly increased number of adenomas in $APC^{\Delta 1638/+}$ mice[121]	JPS[39] HHT[45]
SMAD5	Intrauterine lethal due to embryonic deformities[194]	N/A	N/A
SMAD6	Severe cardiovascular deformation[195]	N/A	Congenital cardiovascular malformation[196]
SMAD7	Lethal[197] or minor growth suppression,[197] depending on background	Treatment with antinucleotides leads to less tumors in $APC^{+/-}$ animals[110]	N/A
SMAD8	Changes in pulmonary vasculature leading to pulmonary arterial hypertension[198]	N/A	Pulmonary arterial hypertension[199]

N/A, not applicable.