

REVIEW

Roles of TGF- β family signaling in stem cell renewal and differentiation

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Transforming growth factor (TGF)- β s and their family members, including bone morphogenetic proteins (BMPs), Nodal and activins, have been implicated in the development and maintenance of various organs, in which stem cells play important roles. Stem cells are characterized by their ability to self-renew and to generate differentiated cells of a particular tissue, and are classified into embryonic and somatic stem cells. Embryonic stem (ES) cells self-renew indefinitely and contribute to derivatives of all three primary germ layers. In contrast, somatic stem cells, which can be identified in various adult organs, exhibit limited abilities for self-renewal and differentiation in most cases. The multi-lineage differentiation capacity of ES cells and somatic stem cells has opened possibilities for cell replacement therapies for genetic, malignant and degenerative diseases. In order to utilize stem cells for therapeutic applications, it is essential to understand the extrinsic and intrinsic factors regulating self-renewal and differentiation of stem cells. More recently, induced pluripotent stem (iPS) cells have been generated from mouse and human fibroblasts that resemble ES cells via ectopic expression of four transcription factors. iPS cells may have an advantage in regenerative medicine, since they overcome the immunogenicity and ethical controversy of ES cells. Moreover, recent studies have highlighted the involvement of cancer stem cells during the formation and progression of various types of cancers, including leukemia, glioma, and breast cancer. Here, we illustrate the roles of TGF- β family members in the maintenance and differentiation of ES cells, somatic stem cells, and cancer stem cells.

Keywords: embryonic stem cells, somatic stem cells, cancer stem cells, BMP, Wnt

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TGF- β family signaling in the maintenance of pluripotency and self-renewal of embryonic stem (ES) cells

ES cells are derived from the inner cell mass of blastocysts, multicellular structures originating from several cleavages of fertilized eggs. One of the defining features of mouse ES cells is their potential to undergo symmetric cell divisions without differentiation in order to produce identical progeny, which is known as self-renewal. Mouse ES cells are also capable of differentiating into all three germ layers, i.e. ectoderm, mesoderm and endoderm, which is known as pluripotency [1]. Pluripotency of ES cells has been demonstrated *in vivo* by the complete integration of mouse ES cells into a developing

embryo after introduction into the blastocyst [2]. Mouse ES cells can undergo multi-lineage differentiation *in vitro* and generate cells with well-differentiated phenotypes [3]; thus, ES cells are useful as *in vitro* models to study mammalian development. Moreover, these observations raise the possibility that ES cells can be used as sources of differentiated cells for regenerative medicine, and generation of human ES cells has opened a new gate for using ES cells for regenerative medicine [4]. More recently, mouse and human fibroblasts were transformed into a pluripotent state that resembles ES cells by ectopic expression of four transcription factors, Oct4, Sox2, c-Myc and KLF4 [5–7]. These ES-like pluripotent cells, termed induced pluripotent stem (iPS) cells, have moved the dream of cell transplantation-based regenerative medicine one step closer to reality by overcoming the immunogenicity and ethical controversy of ES cells.

Undifferentiated states of mouse and human ES cells are maintained by co-culture with appropriate feeder cell

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layers, such as mouse embryonic fibroblasts (MEFs). Subsequently, leukemia inhibitory factor (LIF), provided by feeder cells or as a recombinant protein, was shown to be sufficient for maintenance of mouse ES cell identity, although it was not sufficient for maintenance of human ES cell identity [8]. While maintenance of human ES identity is less understood than mouse ES cells, activation of the Wnt pathway by a GSK-3 β inhibitor, known as 6-bromoindirubin-3'-oxime (BIO), was shown to be able to replace the requirement of MEF-conditioned medium for the maintenance of the undifferentiated state of human ES cells [9]. In addition to the signaling pathways mediated by LIF and Wnts, certain transcription factors such as Oct-3/4 [10] and Nanog [11, 12] play critical roles in the maintenance of identity of both human and mouse ES cells.

Transforming growth factor (TGF)- β family signaling has been reported to be involved in the maintenance of ES cell identity. Mouse embryos deficient in *Smad4* display defective epiblast proliferation and delayed outgrowth of the inner cell mass [13], and mice lacking ALK-3 bone morphogenetic protein (BMP) type IA receptor exhibit reduced cell proliferation in the epiblast [14], demonstrating that BMP signaling plays important roles in the maintenance of mouse ES cell identity. Qi *et al.* [15] reported that BMP-4 provided by feeder cells is necessary for the maintenance of ES cell self-renewal, and that effect of BMP-4 is accomplished by means of inhibition of both extracellular receptor kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways. Inhibition of the p38 MAPK pathway by SB203580 was shown to overcome the block in deriving ES cells from blastocysts lacking ALK-3. Ying *et al.* [16] also reported that BMP-4 sustains self-renewal of mouse ES cells in concert with LIF, and that the critical contribution of BMP-4 to self-renewal is mediated by the induction of the helix-loop-helix protein Id (inhibitor of differentiation). Since BMPs are potent inhibitors of neural differentiation in vertebrate embryos [17], the growth-stimulatory activities of BMPs may be mediated by their inhibitory effects on neural differentiation of mouse ES cells.

Nodal signals are also implicated in the maintenance of ES cell properties by the finding that Nodal-deficient mouse embryos exhibit an epiblast that is substantially reduced in size with very low levels of Oct-3/4 expression [18, 19]. Large-scale gene expression profiling of ES cells and somatic stem cells has revealed that TGF- β family signaling networks, especially Nodal signals, are likely to play important roles in the maintenance of the characteristics of ES cells [20]. Moreover, phosphorylation and nuclear localization of Smad2 induced by

TGF- β , activin, or Nodal signaling were observed in undifferentiated human ES cells, and decreased upon early differentiation [21]. Inhibition of TGF- β /activin/Nodal signaling by SB-431542, a chemical inhibitor of the kinases of type I receptors for TGF- β /activin/Nodal [22, 23], resulted in decreased expression of the markers of undifferentiated states [21, 24]. Xiao *et al.* [25] also found that activin is able to support long-term feeder-free culture and maintenance of pluripotency of human ES cells possibly by inducing the expression of Oct-4 and Nanog, which was shown by microarray analysis. Recently, TAZ (also known as WWTR1), a transcriptional regulator, was shown to play important roles in the maintenance of pluripotency of human ES cells by controlling nucleocytoplasmic shuttling of Smad2/3-Smad4 complexes in response to TGF- β [26].

We also found that SB-431542 dramatically decreases the proliferation of mouse ES cells without decreasing their pluripotency [27], suggesting that activin, Nodal, and/or TGF- β signaling is indispensable for proliferation of mouse ES cells. Furthermore, supplementation of the culture medium with recombinant activin or Nodal enhances the proliferation of serum-free cultured mouse ES cells without affecting their pluripotency, suggesting that endogenously activated activin and/or Nodal signaling promotes mouse ES cell self-renewal.

Thus, TGF- β family signals play important roles in the maintenance of self-renewal and pluripotency of both human and mouse ES cells [28], while the mechanisms of their action seem to diverge between these two species (Figure 1).

TGF- β family signaling during *in vitro* differentiation of ES cells

Germ-layer specification

During early stages of embryogenesis, three germ layers, ectoderm, mesoderm, and endoderm, are generated during gastrulation. At the beginning of gastrulation of mouse embryo, a transient structure known as primitive streak is formed. During this process, uncommitted epiblast cells migrate through the primitive streak, and leave either as mesoderm or definitive endoderm. Lineage mapping analyses have defined that posterior, mid, and anterior regions of the primitive streak are different in gene expression profiles and developmental potentials. Brachyury is expressed throughout the primitive streak, while Foxa2 and Goosecoid are expressed preferentially in anterior regions. As gastrulation proceeds, cells transit more anterior parts of the primitive streak, generate cranial and cardiac mesoderm, and subsequently form paraxial and axial mesoderm. Definitive endoderm de-

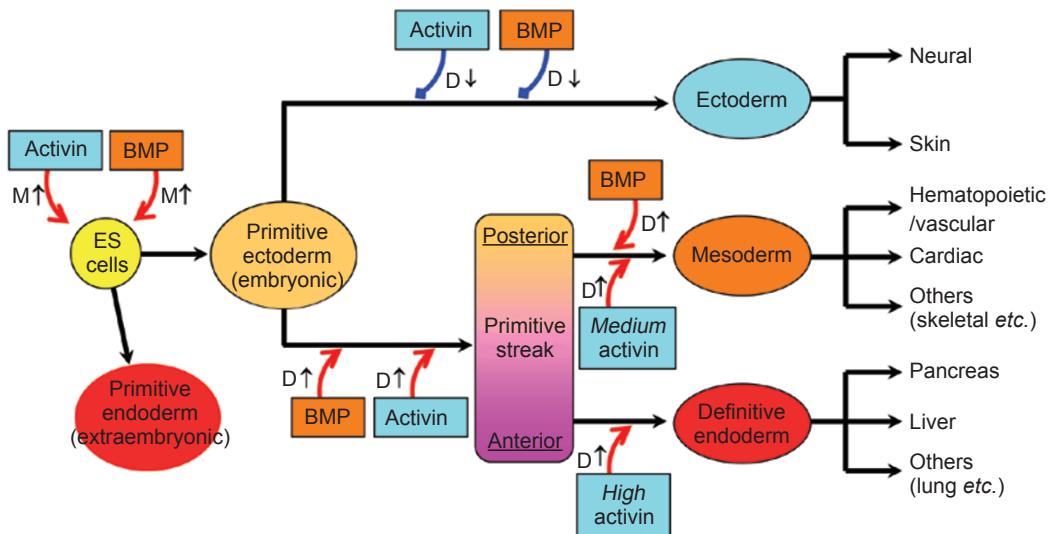


Figure 1 Roles of TGF- β family members in the maintenance and differentiation of ES cells. Red and blue arrows indicate the inductive and inhibitory effects of TGF- β family members on the maintenance (M) and differentiation (D) of ES cells and their derivatives.

velops from epiblast cells that migrate through the most anterior region of the primitive streak. Furthermore, existence of bi-potent mesendoderm cells that can develop into both mesoderm and endoderm has been reported from *Caenorhabditis elegans* to zebrafish [29]. In contrast to mesoderm and definitive endoderm, ectoderm is originated from the anterior region of the epiblast that does not enter the primitive streak. Expression analyses and genetic studies have shown that members of the TGF- β family including BMP-4 and Nodal as well as members of the Wnt family, are essential for these developmental steps. Moreover, different levels of expression of agonists of these pathways, together with regionalized expression of inhibitors of these pathways, form a signaling network that regulates germ-layer induction and specification. Thus, specification of germ layers is a dynamic process controlled by the coordinated activation and regional inhibition of the Wnt, Nodal, and BMP signaling pathways.

When the factors that maintain the undifferentiated state of ES cells are removed, ES cells start to differentiate and generate progeny that consists of derivatives of all three germ layers under appropriate culture conditions [1]. By recapitulating these developmentally regulated patterns of gene expression, developmental processes can be analyzed on a cellular level *in vitro*. ES cells have, therefore, been used as an experimental model to analyze early differentiation events complementary to *in vivo* studies. In ES cell differentiation studies, expres-

sion of Brachyury is useful for monitoring the formation of a primitive streak-like population and the onset of mesoderm formation, whereas expression of Foxa2 and Goosecoid can be used to monitor the anterior region of the primitive streak. Recently, reporter mouse ES cell lines in which reporter cDNAs (*e.g.* GFP) are targeted to specific marker genes have been engineered to model the regional specification of primitive streak *in vitro* [30].

When BMP was added to ES cell differentiation cultures in the absence of serum, Brachyury-positive mesoderm was formed, followed by the development of Flk1 (vascular endothelial growth factor receptor 2, VEGFR2)-positive mesoderm [31]. Activation of Nodal pathways by addition of activin induced a primitive streak population that expresses Foxa2 and Goosecoid, and subsequent formation of definitive endoderm or mesoderm depending on the concentrations of activin. Clonal analysis revealed that individual Goosecoid-positive cells have the potential to develop into both mesoderm and endoderm, suggesting that they may represent mesendoderm progenitors [30]. In contrast to Nodal/activin signals, BMP signals alone induce the formation of posterior primitive streak population. Interestingly, when added together with activin, BMP dominantly posteriorizes the anterior primitive streak population. Progression of the anterior primitive streak population to definitive endoderm requires sustained activation of Nodal/activin signals, consistent with the observation that Nodal signals are required for development of definitive endoderm

in embryos [29]. Furthermore, the induction of ectoderm in ES cells is often referred to as the default pathway, since neuroectoderm readily develops in the culture without serum or inducers of primitive streak.

These findings suggest that there are correlations of TGF- β family signals in the specification of germ layers between *in vivo* systems and *in vitro* ES cell differentiation systems. Differentiation of the three germ layers from ES cells is regulated by a combination of TGF- β family signals [28] (Figure 1). Ectoderm is differentiated from mouse and human ES cells in the absence of TGF- β family signals, while primitive streak differentiation is induced by BMP and activin/Nodal. Mesoderm is differentiated from the primitive streak region in the presence of BMP and a medium level of activin/Nodal signals. Differentiation into endoderm is induced by a high level of activin/Nodal signals in the absence of serum. Further differentiation from each germ layer will be discussed below.

Mesoderm derivatives

The hematopoietic, vascular, cardiac, myogenic, adipogenic, and chondrogenic cell lineages develop from mesoderm in embryos, which is recapitulated by *in vitro* ES cell differentiation systems [32]. An ES cell system for *in vitro* hematopoietic-vascular differentiation showed striking similarities to differentiation of hematopoietic and vascular cells in early embryos. Initial differentiation of hematopoietic cells and vascular endothelium during mouse embryogenesis occurs in the mesodermal layer of the yolk sac, yielding structures named blood

islands, which consist of common progenitors for embryonic blood and blood vessels. These progenitors express Flk1 and are called hemangioblasts [33, 34] (Figure 2). Importantly, differentiated ES cells contain a significant proportion of Flk1-positive (Flk1 $^+$) hematopoietic-endothelial progenitor cells when cultured under optimized conditions [33-36]. Park *et al.* [31] demonstrated that in the presence of VEGF, BMP-4 is able to induce hematopoietic differentiation of mouse ES cells in serum-free culture. Recently, Lee *et al.* [37] showed that ER71, a member of the Ets transcription factor family, functions downstream of BMPs and regulates the generation of Flk1 $^+$ blood and vessel progenitors.

These Flk1 $^+$ cells are able to differentiate into all types of myeloid and lymphoid progeny [38]. In addition, several groups have shown differentiation of human ES cells into hematopoietic cells [39-41]. Lengerke *et al.* [42] showed that BMP signals play two distinct and sequential roles in hematopoietic differentiation from ES cells; they initially induce mesoderm and later specify blood lineages via activation of Wnt signals and the Cdx-Hox pathway. However, differentiation of ES cells into hematopoietic stem cells that are capable of long-term engraftment in host animals still remains a challenge in the field.

Vascular development from ES cells has also been well documented [43, 44]. When Flk1 $^+$ hematopoietic-endothelial progenitors isolated from differentiation cultures are re-differentiated in the presence of VEGF, lineages of both endothelial cells and mural cells (pericytes and vascular smooth muscle) develop from a common

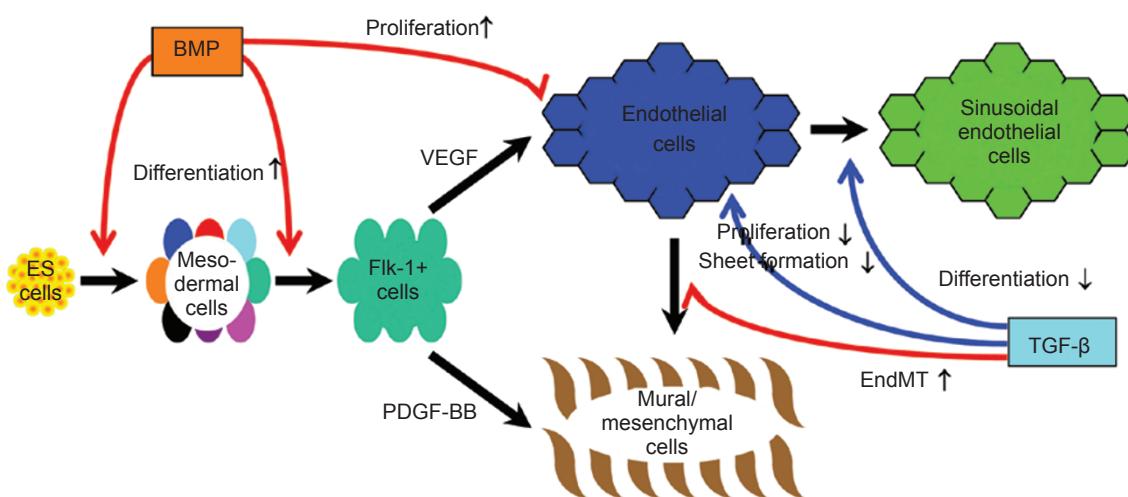


Figure 2 Roles of TGF- β family members in vascular differentiation of ES cells. Red and blue arrows indicate the inductive and inhibitory effects of TGF- β family members on the various biological processes of ES-cell-derived vascular cells. EndMT: endothelial-mesenchymal transition.

ES-cell-derived Flk1⁺ progenitor cell type [45] (Figure 2). Furthermore, arterial, venous and even lymphatic endothelial cells have been obtained from ES-cell-derived Flk1⁺ cells [46, 47]. Endothelial differentiation has also been demonstrated in human ES cell differentiation cultures [48].

We have been studying the roles of TGF- β family signals during vascular development from mouse ES cells. Suzuki *et al.* [49] found that BMP signals promote the proliferation of mouse ES-cell-derived endothelial cells (MESECs) by inducing the expression of Flk1 and Tie2, both of which promote endothelial-cell proliferation. TGF- β and activin inhibit the proliferation and formation of a sheet-like structure by endothelial cells by inducing the expression of p21 and inhibiting the expression of claudin-5, a component of tight junctions, respectively [50]. Inhibition of endogenous TGF- β and activin signaling by kinase inhibitors of the type I receptors for TGF- β /activin/Nodal not only facilitates proliferation and sheet formation of MESECs but also induces the expression of stabilin and LYVE-1, both of which are markers for sinusoidal endothelial cells [51]. Furthermore, long-term culture of MESECs with TGF- β 2 results in the endothelial-mesenchymal transition (EndMT) [52], an event that is also observed during cardiac development and the formation of cancer-associated fibroblasts [53, 54]. These results suggest that TGF- β family members play important roles in the proliferation, sheet formation, and subsequent differentiation of MESECs.

Generation of cardiomyocytes from ES cells has been of interest to many investigators, as it will be clinically useful for the treatment of cardiac diseases, such as myocardial infarction. Activin and BMP-4 were used to induce human ES cells to differentiate into cardiomyocytes in chemically defined medium [55]. The primary function of activin and BMP-4 in cardiomyocyte differentiation may be the induction of mesoderm, which differentiates into the cardiac lineage, since the effects of activin and BMP-4 are most dramatic when they are added early in differentiation cultures.

Recently, Narazaki *et al.* [56] succeeded in inducing the differentiation of mouse iPS cells into Flk1⁺ cardiovascular progenitor cells, which generated arterial, venous and lymphatic endothelial cells, and self-beating cardiomyocytes. Utilization of various TGF- β family members will benefit more efficient differentiation of cardiovascular cells from iPS cells established from patients suffering from cardiovascular diseases.

Endoderm derivatives

Generation of endoderm derivatives from ES cells, particularly pancreatic and hepatic cells, remains one of

the greatest challenges in the field of ES cell biology, as generation of these types of cells is clinically useful for the treatment of type I diabetes and liver diseases, respectively. However, progress in inducing the differentiation of these endoderm-derived cell types had been relatively slow until the establishment of an efficient protocol for obtaining definitive endodermal cells by treating differentiating ES cells with activin in the absence of serum [57].

Gouon-Evans *et al.* [58] generated an endoderm progenitor population by culturing ES cells in the presence of activin in serum-free conditions, and specified these progenitors with BMP-4, activin, and fibroblast growth factor (FGF) in the development of hepatic populations highly enriched (45–70%) for cells expressing albumins. During embryogenesis, expression of Pdx1 (pancreatic and duodenal homeobox gene 1) marks the dorsal and ventral pancreatic buds, which generate all pancreatic lineages, i.e. endocrine, exocrine, and duct cells [59]. Shiraki *et al.* [60] established a culture condition that yields Pdx1-positive cells at a high efficacy (30%) using activin and BMP-4 at various steps. These findings suggest that it is critical to understand the developmental mechanisms by which hepatic and pancreatic cells differentiate from definitive endoderm, as it is now possible to efficiently generate endoderm populations from ES cells.

Ectoderm derivatives

Neural lineages and skin are derived from ectoderm. ES-cell-derived ectoderm can give rise to both neural and epidermal lineage cells. Especially, it is well established that ES cells can generate tri-lineage neural progenitors that can differentiate into neurons, oligodendrocytes, and astrocytes. Derivation of neurons and glial cells from ES cells has been an important goal in cell replacement therapy for Parkinson's disease and spinal-cord injury.

In amphibian systems, BMPs inhibit neural differentiation of animal cap ectoderm and induce its epidermal differentiation [17, 61]. Consistently, BMP signaling inhibits neural induction and stimulates the formation of the epidermis from mouse ES cells [62–64]. Moreover, activation of Nodal signals suppresses neural differentiation from mouse ES cells [65]. Together, these findings indicate that TGF- β family signaling inhibits the commitment of ES cells to neuroectoderm.

Neural crest cells originate from dorsal tube, migrate throughout the embryo and differentiate into diverse derivatives, such as peripheral (sensory and autonomic) neurons and cranial mesenchymal cells [66]. While BMP signals inhibit neural differentiation at early gastrula stages, the same signals induce differentiation of neural crest cells at later stages in embryos. In accordance with

in vivo status, late exposure of mouse and primate ES cells to BMP-4 results in the generation of neural crest cells [67]. Furthermore, low and high concentrations of BMP-4 induced sensory and autonomic neurons from ES cells, respectively, which is consistent with the results of *ex vivo* experiments using neural tube explants of rats [68]. These findings show that BMP signals induce differentiation of multiple types of ectodermal cells in stage- and concentration-dependent fashions.

TGF- β family signaling in somatic stem cells

While studies of ES cells and iPS cells have demonstrated their potential for generating tissues of therapeutic value, they have also highlighted problems associated with inefficient differentiation and tumorigenicity [69]. Therefore, identification of somatic stem cells in a variety of adult tissues has raised hope that they may be harnessed for a broad range of therapeutic usages. As somatic stem cells can be obtained from a patient's own tissues, we can avoid both ethical and immunological problems. Furthermore, somatic stem cells are non-tumorigenic when transplanted.

The stemness of somatic stem cells is regulated by their intrinsic properties and non-autonomous features that are maintained by the niche (a limited microenvironment that supports stem cells). Somatic stem cells receive multiple external cues from soluble factors as well as membrane-bound molecules and extracellular matrix proteins, and intrinsic cues to select a specific cell fate. To determine the key components that govern the maintenance and differentiation of somatic stem cells, it is critical to elucidate the signals released from the surrounding niche. Signals mediated by TGF- β family members have been implicated in the maintenance and differentiation of various types of somatic stem cells [28]. In this section, we focus on their roles in intestinal, hair follicle, mesenchymal, and neural stem cells.

Intestinal stem cells

Many tissues in the adult retain extensive regenerative potential throughout life. The mammalian intestine is covered by a single layer of epithelial cells that is replaced every 4-5 days in order to serve as protective barriers against the external environment. The adult gastrointestinal epithelium has a well-defined and organized structure, consisting of a functional region, i.e. the villi covered with differentiated cells, and a proliferative region, i.e. crypts of Lieberkühn. The proliferative region contains stem cells, which differentiate into four lineages: enterocytes, goblet cells, enteroendocrine cells, and Paneth cells. These cells (excluding Paneth cells) migrate

up and out of the crypts onto the adjacent villus as they differentiate. When they reach the tips of the villi, they undergo apoptosis. The continuous and rapid turnover of intestinal epithelia requires a steady supply of newly formed cells maintained by the intestinal stem cells.

Before Lgr5 was identified as a reliable intestinal stem cell marker [70], their location was identified using the label-retaining cell approach, which takes advantage of their slow-cycling, quiescent property [71]. After pulse-chase labeling of DNA-synthesizing cells with ^3H -thymidine or bromodeoxyuridine (BrdU), rapidly dividing cells dilute out the label, while terminally differentiated cells are lost on tissue turnover. Therefore, only stem cells can be observed as label-retaining cells in the self-renewing epithelia. Stem cells in kidney [72] and mammary gland [73], which are scattered among epithelial cells and not confined to an organized niche, have been identified using the BrdU labeling technique.

The intestinal stem cell niche provides a three-tiered hierarchical system [74]. During the development of crypt, an ancestral stem cell establishes the adult crypt niche, constituting the first tier of cells. This ancestral stem cell gives rise to four to six active stem cells, which constitute the second tier of cells. These second tier of cells undergo asymmetric division, and produce a daughter stem cell that remains anchored to the niche and a second daughter cell that further divides to produce partially differentiated cells. These partially differentiated cells, termed "transiently amplifying" cells, constitute the third tier, and are located just above the active stem cells. These 20-30 transiently amplifying cells commit to terminally differentiated cell fates, or de-differentiate into stem cells to ensure the maintenance of stem cell populations in the crypts. Upon exhaustion of their proliferative potential, transiently amplifying cells execute their terminal differentiation program.

Proliferation and differentiation of intestinal stem cells are regulated by factors secreted from the underlying mesenchymal layer, which include fibroblasts, enteric neurons, blood vessels, and the extracellular matrix. The Wnt signaling pathway positively regulates the proliferation of stem cells and transiently amplifying cells in crypts [75]. Activation of Wnt signals, as detected by the nuclear localization of β -catenin and expression of target genes, including *EphB2* and *c-Myc*, is observed in a graded fashion, with the highest level of activation at the bottom of the crypts [76]. Aberrant Wnt signaling caused by mutations in the *APC* gene has been shown in hereditary and sporadic cases of colorectal cancers [77].

BMP signaling acts as a negative regulator of stem cell proliferation in crypts. BMP-4 protein is highly expressed in the intravillus mesenchyme [78], and phos-

phorylation and nuclear localization of the BMP-specific R-Smads are observed in differentiated villus epithelial cells and intestinal stem cells [79], suggesting that BMP signaling from the mesenchyme acts on the adjacent non-proliferating epithelial cells and stem cells in a paracrine fashion. A functional role of BMP signaling in the intestinal stem cell niche has also been suggested by the association of *SMAD4* [80] and *BMPRIA/ALK-3* [81] mutations with juvenile polyposis in humans. Consistently, exogenous expression of noggin in the mouse intestine leads to *de novo* ectopic formation of normal crypt-villus units, and at later stages, formation of a complex architecture of branching villi with dilated cysts, similar to human juvenile polyposis [78]. Furthermore, conditional deletion of the *Bmpr1a* gene in crypts in mice disturbs intestinal epithelial regeneration with expansion of the stem and transiently amplifying cells, eventually leading to a type of intestinal polyposis, reminiscent of human juvenile polyposis [79].

BMP signaling inhibits Wnt signaling and ensures a balanced control of stem cell self-renewal. Inhibition of Wnt signaling by BMPs has been shown to be mediated by their activation of PTEN, leading to inactivation of the PI3 kinase-Akt signaling pathway, which in turn decreases the amount of nuclear β-catenin [79]. BMP signaling thus plays important roles in the maintenance of quiescent stem cells and terminal differentiation of intestinal epithelial cells in villi through competing with Wnt signaling, which positively regulates the proliferation of active stem cells and transiently amplifying cells in crypts.

Hair follicle epidermal stem cells

The hair coat protects most mammals from various injurious agents. It requires a constant supply of new hairs throughout the lifetime, which makes hair follicle an excellent model for studying stem cells. To produce new hairs, follicles undergo cycles of growth (anagen), regression (catagen), and rest (telogen) [82]. A follicle produces an entire hair shaft during each anagen phase. Then, follicles reset and prepare their stem cells during the catagen and telogen phases so that they could receive the signal to begin the next anagen phase. The structure of hair follicle is multilayered and complex. Dermal cells surround and underlie the epidermal cells and are believed to secrete external cues towards hair follicle stem cells. Hair follicle stem cells give rise to cells of the dermal papillae and sebaceous glands. ^3H -thymidine labeling studies revealed that most label-retaining cells in the skin are located in the bulge region of the hair follicle. Transplantation of β-galactosidase-marked bulge cells into host animals indicated that stem cells that are

originated from the bulge differentiate into dermal papilla cells [83].

Similar to that in intestinal epithelium, BMP signals are involved in the maintenance of quiescent hair follicle stem cells. When the *Bmpr1a* gene was ablated in postnatal skin epithelium using an inducible conditional targeting strategy, quiescent hair follicle stem cells were activated to proliferate, leading to the loss of slow-cycling cells [84]. However, hair follicle stem cells were not lost, and they generated long-lived and tumor-like cells that failed to terminally differentiate to make hairs. Conversely, expression of a constitutively active *Bmpr1a* in skin promoted premature hair follicle differentiation, suggesting that balancing the BMP signals is important to maintain the properties of quiescent stem cells.

The actions of BMPs in the hair follicle stem cell niche are mediated by two mechanisms, inhibition of Wnt signaling and induction of nuclear factor of activated T cells c1 (NFATc1). Activation of Wnt signaling is observed in the developing and adult hair follicles [85]. Expression of a stabilized β-catenin molecule in the skin, which activates Wnt signaling, results in *de novo* hair follicle generation, followed by the formation of tumors, revealing that Wnt signaling induces proliferation of hair follicle stem cells [86]. Ablation of the *Bmpr1a* gene in skin in mice enhanced β-catenin stabilization in the hair follicle stem cell niche through activation of the PI3 kinase-Akt pathway [84]. Furthermore, when skin from a *noggin*-deficient mouse was engrafted onto a wild-type mouse, expression of β-catenin was decreased and secondary follicles were not formed [87]. These findings illustrate that the crosstalk between Wnt and BMP signaling pathways plays important roles in the maintenance and differentiation of various types of stem cells.

Recently, Horsley *et al.* [88] showed that NFATc1, which belongs to the NFAT family of transcription factors, is a downstream component of BMP signaling, and balances quiescence and proliferation of hair follicle stem cells. Expression of NFATc1 is restricted to quiescent hair follicle stem cells, and dependent on BMP signals. Loss-of-function study showed that NFATc1 maintains stem cell quiescence via repression of cyclin-dependent kinase 4 (CDK4). These results suggest that BMP signals control the quiescent state of hair follicle stem cells through multiple mechanisms.

Mesenchymal stem cells (MSCs)

MSCs are a heterogeneous population of cells that can be isolated from various adult tissues. While MSCs are abundant in bone marrow, similar somatic stem cells have also been isolated from other tissues, including muscle, fat, dermis, and peripheral and cord blood. They

can differentiate into various mesoderm-derived cells. Bone marrow-derived MSCs are capable of differentiating into bone, cartilage, muscle, and hematopoiesis-supporting stromal cells, but not into hematopoietic cells [89]. Muscle-derived cells are able to give rise to myogenic, osteogenic, chondrogenic, adipogenic, and hematopoietic cells [90], while adipose-derived cells can differentiate into adipogenic, myogenic, osteogenic, and chondrogenic cells [91]. Owing to their potential for use in clinical applications, such as tissue engineering, MSCs have attracted much attention [92]. Human MSCs derived from bone marrow can be expanded more than a billion-fold in culture without losing their stem cell capacity. However, since the molecular mechanisms governing proliferation and differentiation of MSCs are not fully understood, their practical use is currently limited.

Similar to epithelial stem cells, Wnt signaling stimulates the proliferation of human MSCs [93]. TGF- β 1 also induces the proliferation of human MSCs [94]. TGF- β 1 induces Smad3-dependent nuclear accumulation of β -catenin in MSCs, which is required for the stimulation of MSC proliferation. In contrast, BMP-2 antagonizes Wnt3a signaling and inhibits the proliferation of mouse bone marrow-derived MSCs through the interaction of a BMP-specific R-Smad with Dishevelled-1, a component of the Wnt signaling pathway [95]. TGF- β family signaling may thus differentially regulate MSC proliferation through crosstalk with Wnt signaling, as demonstrated also in epithelial stem cells. Recently, using transcriptome analyses, Ng *et al.* [96] predicted that signaling pathways mediated by TGF- β , platelet-derived growth factor (PDGF), and FGF play important roles in the proliferation of MSCs. Inhibition of any of these signals decreased the growth of MSCs, whereas a combination of these three factors enabled multiple passages of MSCs, suggesting that these signaling pathways are necessary and sufficient for MSC growth.

Members of the TGF- β family have also been implicated in directing decisions regarding the fate of MSCs [97]. BMPs induce differentiation of mesenchymal cells into cells with chondroblast or osteoblast phenotypes *in vitro*. TGF- β and activin also provide competence for chondroblast differentiation at early stages, while TGF- β inhibits osteoblast maturation at late stages in the differentiation pathway. Inhibition of endogenous TGF- β and activin signaling in human MSCs by SB-431542 strongly induces osteoblastic maturation [98]. The inhibitory effects of TGF- β /activin signaling on osteoblast maturation are mediated by the induction of inhibitory Smads, which in turn represses BMP signaling. These findings identify TGF- β receptor inhibitors as a novel therapeutic effector for certain bone diseases.

Adipose tissue consists of two functionally distinct types of fat: white adipose tissue, which is the primary site of triglyceride storage, and brown adipose tissue, which is specialized in energy expenditure and can counteract obesity. Recently, BMP-7 was shown to induce the generation of brown, but not white, adipose tissue from MSCs in the absence of the normally required hormonal induction cocktail [99]. Implantation of these differentiated cells into nude mice resulted in a significant increase in brown fat mass, leading to an increase in energy expenditure and a reduction in weight gain. These results suggest a potential new therapeutic strategy of using BMP-7 for the treatment of obesity.

Neural stem cells

Inhibition of TGF- β family signaling directs commitment of ES cells to neuroectoderm lineages, resulting in the formation of embryonic neural stem cells [100]. Embryonic neural stem cells differentiate into complex arrays of neurons and glia (astrocytes and oligodendrocytes) of the central nervous system. Recent progress in neural stem cell research has been facilitated by the development of culture methods that allow the maintenance of neural stem cell populations *in vitro* [101]. Neural stem cells can be easily propagated as monolayer cultures or as floating aggregates, i.e. neurospheres, in the presence of FGF-2 (also known as basic FGF) and epidermal growth factor (EGF). The enriched neural stem cells give rise to both neural and glial lineages *in vitro* depending on culture conditions. These studies have revealed the presence of somatic neural stem cells in adult tissues [100].

TGF- β signals play important roles in the maintenance and growth of neural stem cells [102]. Ablation of *Tgfb2* gene (encoding the TGF- β type II receptor) in the mid/hind brain enhanced the self-renewal, but not multipotency, of neural stem cells, resulting in enlargement of the midbrain. Ectopic expression of FGF and Wnt signaling components was observed in mutant brains, suggesting that TGF- β signaling controls the size of a specific area of the brain by negatively regulating the self-renewal of neuroepithelial stem cells through antagonizing FGF and Wnt signaling pathways.

BMP signals are also implicated in the maintenance of neural stem cells in adult brain. Somatic neural stem cells reside at two neurogenic regions, the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) in the dentate gyrus of the hippocampus [103]. The germinal region of the SVZ, which continually generates new neurons, consists of multiple cell types, such as migrating neuroblasts, immature precursors, astrocytes, and ependymal cells. SVZ astrocytes, but not

ependymal cells, act as neural stem cells in both normal and regenerating brain [104]. Signals mediated by factors such as BMPs, noggin, sonic hedgehog, and Notch play important roles in maintaining the somatic neural stem cell niche. BMP signaling during development induces differentiation of neural stem cells into astrocytes at the expense of oligodendroglogenesis and neurogenesis. Adult SVZ cells produce both BMPs and their receptors, suggesting that BMP signals are activated in the SVZ cells. The BMP signals may confer the self-renewal and multi-lineage differentiation potential of neural stem cells to SVZ cells by maintaining them as partially differentiated astrocytes. In contrast, strong expression of noggin is observed in ependymal cells [105]. This local expression of noggin may contribute to forming a neurogenic niche for SVZ stem cells, as it promotes neurogenesis. These findings suggest that BMPs play a role in the maintenance of neural stem cells, i.e. the SVZ astrocytes, by inhibiting their commitment to neurons and oligodendrocytes.

TGF- β family signaling in cancer stem cells

Recently, accumulating evidence has demonstrated that certain types of tumors consist of a heterogeneous population of cells, and contain a subset of cells that initiate and propagate tumors with high efficiency. These cells often exhibit stem cell properties such as self-renewal, multipotency and the expression of stem cell markers. These findings suggest a hierarchical model in which cancer arises from transformed stem cells and progresses through mechanisms similar to a developmental process. Such cancer stem cells have been identified in tumors of various organs and tissues, including blood [106], breast [107], and brain [108]. Initially, cancer stem cells were retrospectively identified in the hematopoietic system as a phenotypically and functionally distinct population of cells within acute myeloid leukemia [106]. Such leukemic stem cells exhibited the characteristics of normal hematopoietic stem cells and exclusively possessed the capacity to initiate acute myeloid leukemia after transplantation into immunodeficient mice.

In many cases, cancers are notorious for their ability to survive treatment, and recur. Recent studies have revealed that certain cancer stem cells are resistant to chemotherapy [109] and radiotherapy [110]. If cancer stem cells are the major culprits in tumor development and recurrence, conventional therapeutic approaches targeting the overall population of cancer cells may spare cancer stem cells and allow recurrence of more aggressive tumors [111]. Therefore, it is critical to understand the aberrant control of signaling pathways responsible for

the propagation and maintenance of cancer stem cells in order to identify appropriate regulators of these pathways that may have novel therapeutic roles in cancer treatment.

Brain cancer stem cells constitute a small fraction of human glioblastoma cells and display striking similarities to normal neural stem cells, namely self-renewal and multipotency. They express specific markers that are exhibited by fetal and adult neural stem cells, including CD133 and nestin. Self-renewal ability was demonstrated *in vitro* by limiting dilution assays showing their potential to form primary, secondary, and tertiary neurospheres, and *in vivo* by serial transplantation and tumor formation in immunodeficient mice [108]. Upon exposure to differentiation conditions, brain cancer stem cells generate a large number of progeny and differentiate into neural and glial cells *in vitro*. BMPs induce differentiation of embryonic neural progenitor cells into astrocytes, and play an instructive role in the adult brain stem cell niche through maintaining neural stem cells as immature astrocytes [105]. Under the conditions that support the growth of cancer stem cells derived from human glioblastoma *in vitro*, BMP-4 inhibits cell proliferation and induces differentiation predominantly into cells resembling mature astrocytes [112]. Furthermore, *in vivo* delivery of BMP-4 reduces the tumor growth and associated mortality that occur in mice after intracerebral inoculation of human glioblastoma cells. These observations suggest that BMP-4 may serve as a promising therapeutic agent to prevent the growth and recurrence of human glioblastomas by inducing terminal differentiation of cancer stem cells.

However, Lee *et al.* [113] recently demonstrated that BMP-mediated astroglial differentiation is impaired in a subpopulation of brain cancer stem cells due to epigenetic silencing of the BMP type IB receptor (BMPRIB/ALK-6), which causes a differentiation block contributing to the pathogenesis of glioblastoma. These findings suggest that cancer stem cells, despite their striking similarities to normal stem cells, are different in their differentiation potential, and identify BMPRIB as a promising molecular target for inducing terminal differentiation of brain cancer stem cells.

During the progression of cancer, epithelial-mesenchymal transition (EMT) confers malignant properties, including motility and invasiveness to tumor cells. TGF- β signaling plays important roles in inducing EMT by upregulating the expression of Snail transcription factor family members. Mani *et al.* [114] found that stem-like cells isolated from normal and cancerous mammary glands exhibit EMT properties with decreased expression of E-cadherin and increased expression of mesenchymal

markers, including N-cadherin and vimentin. Furthermore, normal and transformed mammary epithelial cells that have undergone EMT acquire stem-cell properties, including expression of CD44 and CD24, both of which are stem-cell markers, and are capable of forming mammospheres *in vitro* and in the case of transformed cells, tumors *in vivo*. The connection between EMT and epithelial stem-cell properties illustrates how cancer can generate more malignant cancer stem cells, and also suggests a possibility that pharmacological means regulating the process of EMT may help generate normal epithelial stem cells that are useful for regenerative medicine.

Perspectives

Recently, stem cell biology has been advancing at an incredibly rapid pace. ES cells will be useful sources for cell replacement therapy, although there still remain technical problems to be addressed [69]. Advancement of iPS cell technology may solve the problems of immunogenicity and ethic issues associated with the use of ES cells. The ability to isolate somatic stem cells suggests that they may also be useful for treating human genetic disorders after genetic correction of the abnormal stem cells. Since cancer stem cells have been implicated in the development and recurrence of various types of cancers, the molecular mechanisms that govern the self-renewal and differentiation of embryonic and somatic stem cells need to be understood, not only for the purpose of efficient propagation of stem cells and directing their differentiation towards desired cell types, but also for the development of new strategies for the treatment of cancer. As members of the TGF- β family play important roles in embryogenesis and adulthood, a better understanding of the functional roles of TGF- β family signaling in embryonic and somatic stem cells will aid both basic research of stem cells and their potential applications.

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