

1

Stem Cells

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1.1

Introduction

Stem cells are the founder cells for every organ, tissue and cell in the body. They are undifferentiated cells that can give rise to several lineages of differentiated cell types. In addition, stem cells are able to self-renew and thus to produce undifferentiated descendents, some of which are stem cells again (Fig. 1.1). These features allow stem cells to fulfill their multiple functions, namely to provide enough cells during organogenesis, to control tissue homeostasis and, in addition, to ensure regeneration and repair, at least of certain tissues. It is because of these characteristics that stem cells are a prime target of applied research that seeks to treat degenerative diseases by cell replacement therapies. So far, researchers have used embryonic, fetal, and adult stem cells as a source from which to generate various specialized cell types. Any disease caused by tissue degeneration can be a potential candidate for stem cell therapies, including Parkinson's and Alzheimer's disease, stroke, spinal cord injury, heart diseases, burns, and many more. However, to realize the clinical potential of stem cells, it is crucial to have a deeper insight into the mechanisms regulating stem cell self-renewal and their ability to produce the correct cell type at the appropriate time and location in correct numbers. In this chapter, we review how extracellular signals influence stem cell behavior. This overview can by no means provide an exhaustive list of all signal transduction pathways reported to act on stem cells. Rather, we try to illustrate aspects of stem cell development by discussing some specific signals affecting stem cell proliferation, fate decision, and differentiation.

The stem cells with the broadest range of potential are cells isolated from the inner cell mass (ICM) of the blastocyst. These embryonic stem cells (ESCs) are pluripotent and able to respond to morphogenic signals and to differentiate into any desired cell type of the three germ layers. In culture, ESCs can be propagated almost indefinitely, demonstrating their unlimited potential with respect to growth and differentiation. Additionally, the developmental and therapeutic potential of adult stem cells isolated from various tissues is also being investigated. The bone marrow (BM) is composed of the non-adherent hematopoietic and adherent stromal cell compartment. The adherent BM stromal cell fraction contains pluripotent mesenchymal

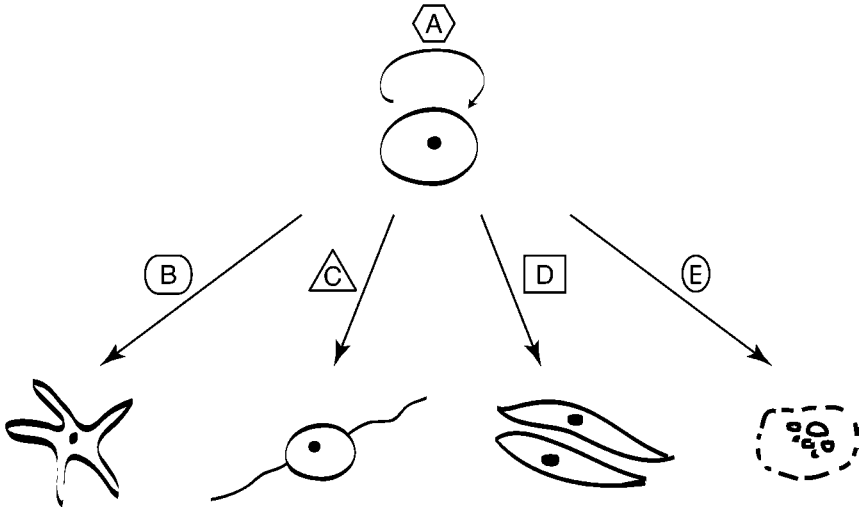


Fig. 1.1

Stem cell fates are regulated in a signal-dependent manner. Stem cells are multipotent, that is, they are able to generate many different derivatives. In addition, stem cells have the capacity to self-renew. At any time-point, neighboring cells, growth factors, and extracellular matrix components that adjust the balance between self-renewal, differentiation, or apoptosis influence the fate of stem cells. This decision is regulated by numerous cell-intrinsic and cell-extrinsic factors (identified here by A-E), some of which maintain the cells as stem cells whereas others induce cell death, or differentiation into various lineages. Usually, a combination of factors involving distinct signaling cascades is linked to a cell-specific output.

stem cells (MSCs) that can be induced to differentiate into various mesenchymal lineages as well as into most somatic lineages including derivatives of the brain [1–3]. Apart from bone marrow-derived stem cells, multipotent adult stem cells from the adult dermis [4], muscle, and brain [5] have been described to generate cells representing derivatives of multiple germ layers. These results have been explained by the capability of the cells to trans-differentiate. The term trans-differentiation describes the conversion of a cell type of a specific tissue lineage into a cell type of another lineage, involving reprogramming of gene expression due to altered microenvironmental cues. It has been hypothesized that tissue injury increases the rate at which bone marrow-derived stem cells trans-differentiate [3, 6]. These results have been debated, however, and it has been suggested that trans-differentiation events – if they occur at all – are rare and that the appearance of donor cell markers in host tissues might arise by other mechanisms. First, transplanted cells might undergo fusion with endogenous differentiated cells [7, 8]. In fact, the ability to fuse is characteristic of many cell types, such as myoblasts, hepatocytes, and others ([9] and references therein). Alternatively, cells from a given lineage might de-differentiate into a more naive state that allows the cell to re-differentiate along new lineages. Finally, a very rare pluripotent stem cell might persist until adulthood, and upon

transplantation would be able to generate a broad variety of cells representing derivatives of all three germ layers, depending on its environment. Thus, when elucidating the potential of stem cells in culture or *in vivo*, it is not sufficient to analyze the expression of appropriate lineage markers; rather, possible fusion events have to be excluded, and the purity of the stem cells has to be considered in order to rule out their contamination by additional cells with other potentials. This can be achieved by clonal analysis of prospectively identified cells that, if possible, have been minimally manipulated (for example without culturing) before use. The ultimate proof that a given stem cell can adopt a certain fate lies in the demonstration of its functional integration into the tissue.

1.2

Maintenance of Stemness in Balance with Stem Cell Differentiation

Many stem cells reside in a spatially restricted compartment called a niche. This niche provides an environment that supports the survival of the multipotent stem cell without induction of differentiation. Neighboring differentiated cell types secrete factors and provide a milieu of extracellular matrix that allows stem cells to self-renew and to maintain the capacity to respond to differentiation programs (Fig. 1.2). Physical contact between stem cells and their non-stem cell neighbors in the niche is critical in keeping the stem cells within this compartment and in maintaining stem cell character. Often, stem cells within the niche are quiescent or slow-cycling, but proliferation might be induced by injury. Niches have been described, for example, for germ cells, in the bulge of the hair follicle, the bone marrow, the crypt of an intestinal villus, and the subventricular zone of the brain (reviewed in [10]). It is still a matter of investigation which factors control stemness, that is, the maintenance of stem cell properties. It is likely that various signaling pathways are involved, including Notch, bone morphogenetic proteins (BMPs), transforming growth factor β (TGF β), and Wnt signaling (see below). Several groups have applied microarray technology with the goal of identifying genes that control stemness. The transcriptional profiles of ESCs, hematopoietic stem cells (HSCs), neural stem cells (NSCs), and neural crest stem cells (NCSCs) have been compared and analyzed [11–13]. However, only very few genes were found to be commonly expressed in all stem cells, and it appears to be difficult to define a valid genetic fingerprint that determines stemness of all stem cells or even of a specific stem cell subtype. This could be explained by the usage of different microarray chips, technical difficulties, or the purity of the analyzed cells. Furthermore, the data might reflect substantial intrinsic differences between different types of stem cells.

Cell-intrinsic properties determine how a stem cell interprets the signals present in its environment. At each cell division, stem cells have to choose between self-renewal and differentiation. The mechanisms determining how quiescent or slow-cycling stem cells are induced to start proliferation or differentiation are still largely unknown. One possibility might be that the stem cells, which are slowly cycling “fill“

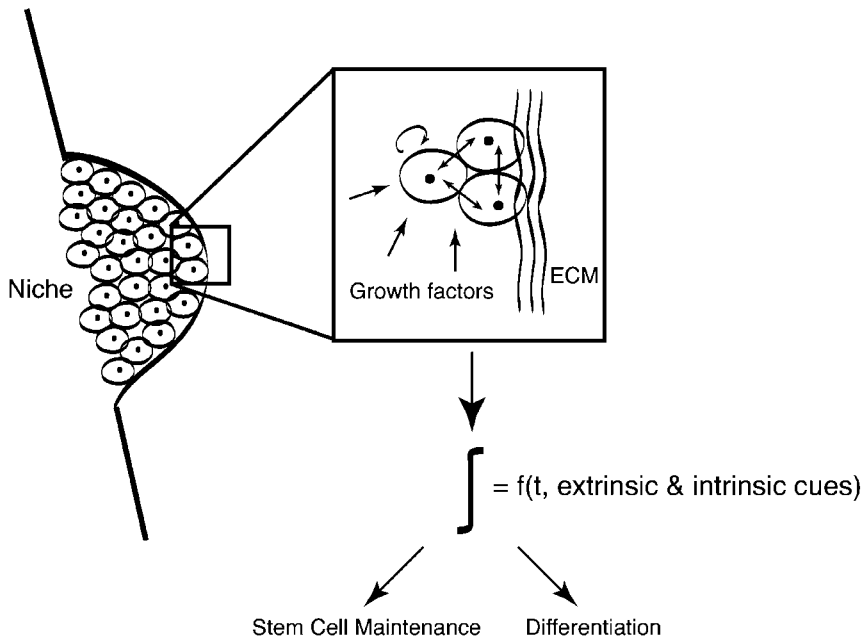


Fig. 1.2

Signaling in the niche. The niche provides an environment that attracts stem cells and keeps them in an undifferentiated state by supporting self-renewing cell divisions. Accordingly, differentiation may be initiated when the stem cell leaves the niche. The balance of quiescence, self-renewal, and cell commitment is influenced by secreted growth factors that initiate intracellular signaling cascades and activate distinct sets of transcription factors. Further, the extracellular matrix (ECM) plays an important role in retaining the stem cells in the niche. Thus, self-renewal versus lineage specification and differentiation are the result of the capacity of a stem cell to integrate multiple signals that vary with location and time.

the niche and subsequently leave it. Outside the niche the cells are exposed to an environment that is permissive or even inductive for differentiation. Alternatively, stress or injury might change the extrinsic signaling in a way that induces differentiation. Stem cells may undergo symmetrical divisions to generate identical twins to self-renew or to differentiate, or they may undergo asymmetric cell divisions, yielding one differentiated progeny and one stem cell daughter [14, 15]. Therefore, the total number of stem cells represents a dynamic balance between symmetric and asymmetric cell divisions in the niche. In addition, the stem cell number is controlled via programmed cell death. Due to the exponential expansion of a single progenitor cell, elimination of stem cells or precursors by programmed cell death at early stages will have a marked effect on the final number of terminally-differentiated cells. Again, the balance between maintenance and depletion of the progenitor pool size has to be tightly controlled by the extracellular environment. Extrinsic

factors could actively promote cell death, or the withdrawal of trophic support by growth factors that act as survival factors might induce cell death [16].

In principle, all stem cells and precursors respond to multiple growth factors, and their effects can be modulated by extracellular matrix components (reviewed in [17]). Several different integrins that bind to the extracellular matrix seem to be differentially involved in the regulation of proliferation, cell migration and differentiation. Binding to extracellular matrix proteins such as laminin activates an intracellular signaling pathway via phosphatidylinositol3 (PI3) kinase and Akt kinase [18]. In sum, stem cell development is controlled by the combinatorial activity of multiple factors, acting in signaling networks (Fig. 1.2, [10, 19]). The composition of such networks is dynamic, changing with time and location. To unravel the players involved, researchers have to elucidate the contribution of individual signal transduction pathways, knowing that this contribution is likely to be modulated by the cross-talk with other pathways.

1.2.1

Wnt Signaling

Wnt proteins are important regulators of cell proliferation and differentiation [20]. The Wnt signaling pathway involves proteins that directly participate in both gene transcription and cell adhesion. Nineteen Wnt genes with diverse functions exist in mammalian genomes. Wnt molecules are secreted lipid-modified signaling proteins [21] that bind to Frizzled receptors on the cell surface. Several cytoplasmic components transduce the signal to β -catenin (Armadillo in *Drosophila*), which enters the nucleus and forms a complex with a high mobility group (HMG) box-containing DNA binding protein such as TCF (T cell factor) and LEF (lymphoid enhancer factor). This complex activates many different target genes and is modulated by cross-talk of Wnt/ β -catenin signaling with various other signal transduction pathways including signaling by Notch, TGF β factors, FGFs, and Shh [22–24]. In addition, many proteins have been identified that interact with TCF and mediate repression. One such repressor is the Groucho protein in *Drosophila* (known as TLE in vertebrates). Groucho binds to TCF, repressing the expression of downstream target genes [25].

The central player of the canonical Wnt signaling pathway is β -catenin, which in the absence of Wnt is degraded in the cytoplasm. Excess β -catenin is phosphorylated by glycogen synthase kinase 3 β (GSK3 β) and then targeted for proteasome-mediated degradation. In the presence of Wnt signaling, Dishevelled (Dsh) becomes activated, which leads to the uncoupling of β -catenin from the degradation pathway by inhibition of GSK3 β activity. This results in the accumulation of β -catenin, which enters the nucleus and interacts with partners such as TCF/LEF. Therefore, stabilization of β -catenin and its accumulation in the cytoplasm is a crucial step in canonical Wnt-dependent target gene expression. Apart from GSK3 β , several proteins are instrumental in tightly regulating β -catenin levels in the cell, including adenomatous polyposis coli (APC) and Axin/Conductin. In addition to its function in the above-de-

scribed Wnt signaling pathway, β -catenin plays a role in the structural organization and function of cadherins. β -Catenin binds to the cytoplasmic domain of type I cadherins, linking cadherins through α -catenin to the actin cytoskeleton [24, 26, 27].

1.2.2

Wnt Signaling Regulates 'Stemness' in ESCs

The mechanisms controlling multipotency and differentiation of ESCs are of fundamental interest. So far, several factors have been described that affect cell-fate decisions and self-renewal of ESCs, including Oct4, Fgf4, Nanog and Sox2 [28–32]. The self-renewal capacity of mouse ESCs can be maintained by growth factors provided by feeder cells or exogenously [33]. In such cultures, ESCs from the inner cell mass of blastocysts remain multipotent and can be propagated nearly indefinitely. Various signaling pathways have been implicated in regulating the self-renewal capacity and multipotency of ESCs. One signal described in this process is the leukemia inhibitory factor (LIF) that is produced by feeder layers of inactivated mouse fibroblasts on which ESCs have been maintained in culture. LIF activates the Janus kinase (JAK) as well as signal transducer and activator of transcription-3 (Stat-3). However, while activation of Stat-3 is sufficient to maintain self-renewal of mouse ESC, LIF has no effect on human ESC self-renewal [34]. Large-scale gene expression profiling of undifferentiated human ESCs revealed that the main components of the canonical Wnt signaling pathway are expressed [34, 35]. Intriguingly, overexpression of Wnt1 or of stabilized β -catenin and lack of APC in ESCs results in the inhibition of neural differentiation and in activation of downstream targets of Wnt signaling such as cyclins, c-myc and BMP [35, 36]. Moreover, treatment of ESCs with a specific synthetic pharmacological inhibitor of GSK3 β activates the canonical Wnt pathway and allows both mouse and human ESCs to remain undifferentiated [37]. Such drug-treated cells display sustained expression of transcription factors including Nanog and Oct-3/4, which are important in controlling the pluripotent state of ESCs. Finally, mutations in APC associated with increased tumor incidence result in increased doses of β -catenin and interfere with the differentiation of ESCs into the three germ layers [38]. Taken together, canonical Wnt signaling has emerged as a crucial factor in regulating ESC maintenance.

1.2.3

Wnt Signaling in Hematopoietic Stem Cells

The hematopoietic stem cell is a multipotent cell in the bone marrow that has the capacity to provide for the life-long production of all blood lineages. The mechanisms regulating HSC lineage decisions and self-renewal *in vivo* have been difficult to define. However, it was possible to establish the importance of the hematopoietic microenvironment through the use of long-term bone marrow culture systems in which maintenance of HSCs at low frequencies is supported by culturing hemato-

poietic cells on stroma. Subsequently, candidate stem cell factors have been identified by direct addition of purified factors to *in vitro* cultures of HSC populations followed by transplantation of the cultured cells. Many early acting cytokines such as interleukin-3 (IL-3), IL-6, and Kit ligand stimulate proliferation of committed progenitor cells while allowing only limited expansion of HSCs capable of long-term multi-lineage repopulation [39]. In contrast, both conditioned media from cells expressing Wnt proteins and, more recently, addition of purified Wnt3a have been demonstrated to induce self-renewal of HSCs [21, 39]. Overexpression of β -catenin in long-term *in vitro* cultures leads to expansion of HSCs in an immature state, indicating an involvement of the canonical Wnt pathway in this process [40]. On the other hand, applying soluble inhibitors that prevent Wnt proteins from binding and activating the Frizzled receptors reduces HSC growth *in vitro*. Similarly, ectopic expression of Axin, which increases β -catenin degradation, has an inhibitory effect on growth of HSCs and on cell survival. Wnt signaling in HSCs might act through Notch (see below) and the transcription factor HoxB4, both of which have been shown to be involved in self-renewal of HSCs and are upregulated in response to Wnt in HSCs. Thus, Wnt signal activation and the nuclear functions of β -catenin

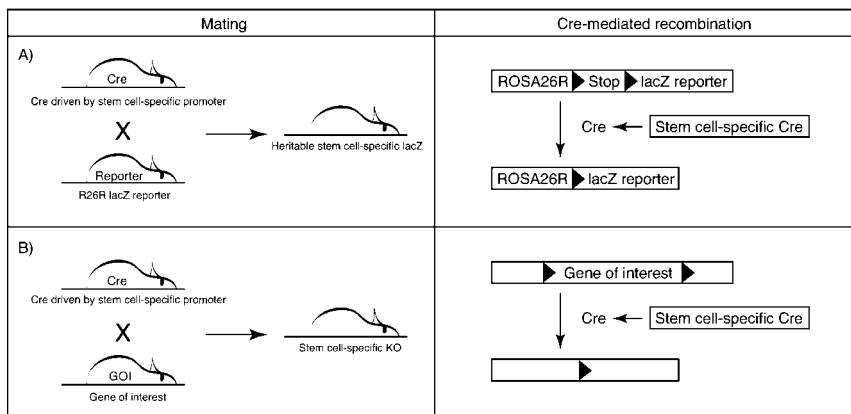


Fig. 1.3

In vivo fate mapping and conditional gene ablation in mice. Mice that express Cre recombinase from a stem cell-specific promoter mated with a reporter mouse line such as Rosa26R [203] produce mice that have heritable lacZ expression (A). Rosa26R reporter mice have the lacZ gene preceded by a transcriptional stop cassette that is flanked by loxP sites. All cells in which the Cre recombinase has been active, as well as their descendants, are lacZ-positive, allowing fate mapping. Conditional gene ablation is performed by using mice that carry alleles in which the gene of interest is flanked by loxP sites (B). Stem cell-specific expression of Cre eliminates the gene of interest. Additionally, inducible forms of Cre recombinase can be activated by injection or feeding of tamoxifen [204], allowing not only cell type-specific but also stage-specific gene manipulation.

enable HSCs to proliferate and to limit their differentiation potential, thereby sustaining self-renewal in long-term culture and functional reconstitution of hematopoietic lineages *in vivo*. However, conditional ablation of β -catenin using the cre/loxP technology (Fig. 1.3) in hematopoietic stem cells did not impair hematopoiesis and lymphopoiesis, suggesting that β -catenin is not required for self-renewal and development of hematopoietic stem cells under physiological conditions [41].

1.2.4

Wnt Signal Activation in the Skin

In the skin, cells in the basal layer proliferate, leave this layer, stop dividing and undergo terminal differentiation. Cells in the outermost layer of the skin are cornified and continually shed from the surface of the epidermis. Therefore, throughout the entire lifespan of the individual new differentiated cells must be produced. However, not all dividing cells within the basal layer are stem cells. As a stem-cell daughter is fated to undergo final differentiation, it first divides a small number of times as a transit-amplifying cell and thereby amplifies the number of terminally-differentiating cells generated by each stem cell. Skin stem cells reside in specific niches (bulge) of hair follicles and are bipotent, as they give rise to both keratinocytes of the hair follicle and the interfollicular epidermis [42, 43]. The niche is characterized by a variety of extracellular matrix proteins such as β 1 integrins that are expressed at higher levels in human interfollicular epidermal stem cells than in transit-amplifying cells. An association between Wnt signaling and skin stem cell development was suggested as a result of the finding that cultured human epidermal stem cells with high levels of β 1 integrins also displayed a higher level of β -catenin than transit-amplifying cells. Indeed, modulation of β -catenin activity affects the proportion of epidermal stem cells in culture [44], and mice expressing stable β -catenin under the control of an epidermal keratin promoter display excess skin epithelium and develop excess fur caused by postnatal hair follicle morphogenesis [45]. These data suggest that canonical Wnt signal activation might maintain the stem cell character of adult epidermal cells.

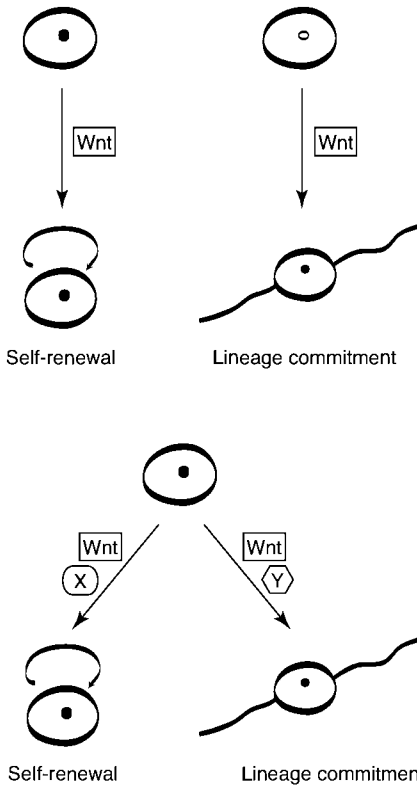
However, Wnt/ β -catenin might have further roles in the skin, promoting hair lineage proliferation and differentiation: Activation of c-myc, a possible downstream target of β -catenin, stimulates the exit from the stem cell compartment and cells turn into transit-amplifying cells [46, 47]. Moreover, *in vivo* manipulation of genes encoding Wnt signaling components indicates an essential role of Wnt signaling in fate decision processes of skin stem cells [48–50]. In particular, β -catenin-deficient stem cells fail to differentiate into follicular keratinocytes and instead adopt an epidermal fate [48]. Thus in the skin, canonical Wnt signaling can apparently elicit different cellular responses. How this is regulated remains to be determined, but it is conceivable that alterations in TCF/LEF transcription factors interacting with β -catenin are involved in controlling the fate of skin stem cells [49].

1.2.5

Multiple Roles of Canonical Wnt Signaling in Neural Stem Cells

Another tissue in which Wnt signaling has pleiotropic effects, presumably depending on location and developmental stage, is the nervous system (Fig. 1.4). In the central nervous system (CNS), gene deletion studies have demonstrated that Wnt signaling requires neural progenitor proliferation and hippocampal development [51] and the expansion of dorsal neural cells including the neural crest [52]. Conversely, Wnt signal activation by overexpression of Wnt or of constitutively activated β -catenin impairs neuronal differentiation and increases the progenitor pool, resulting in a massive enlargement of neural tissue in certain areas of the brain [53–55]. To address the question of whether the observed phenotypes are due to effects on multipotent, self-renewing neural stem cells or on transient amplifying progenitors, researchers rely on the availability of neural stem cell cultures. Using such systems, an increase in secondary neurosphere formation (indicating self-renewal activity of sphere-forming cells) has been reported from β -catenin-overexpressing cells derived from the ganglionic eminence [56]. In contrast, Wnt proteins were found to promote maturation and proliferation of neural progenitors from the cortex, apparently without affecting secondary or tertiary sphere formation [57]. These differences might be due to region-specific or context-dependent responses to Wnt signaling. Indeed, it has recently been reported that Wnts promote neuronal differentiation of neural stem cells at later stages of cortical development, while at early stages they control the expansion of neural stem cells [58].

The first example of Wnt promoting stem cell-fate decisions rather than proliferation in the nervous system was provided by studies carried out with neural crest stem cells (NCSCs). During vertebrate development, these cells delaminate from the closing dorsal neural tube and emigrate to various locations within the embryo to give rise to neuronal and glial cell types of most of the peripheral nervous system (PNS), and to several non-neural structures including pigmented cells, smooth muscle cells in the outflow tract of the heart, and craniofacial bones, cartilage, and connective tissues [59]. A variety of signals has been described that influence cell-fate decisions of NCSCs in culture [60, 61]. BMP signaling causes NCSCs to form autonomic neurons; TGF β promotes smooth muscle-like cells and, under certain conditions, autonomic neurogenesis; and neuregulin induces a glial phenotype. Several studies also reported that Wnt signaling plays a role at multiple stages of neural crest development. *In vivo*, Wnt signaling is involved in early neural crest induction and expansion [52, 62, 63]. Furthermore, both in avian cell cultures and in zebrafish *in vivo*, activation of the Wnt signaling pathway in neural crest cells promotes the formation of pigment cells [64, 65], while neural crest cells deficient in β -catenin fail to produce melanoblasts during development [66]. In addition, NCSCs lacking β -catenin fail to generate sensory neuronal precursors, and mutant neural crest cells are unable to aggregate in dorsal root ganglia (DRG) or to generate sensory neurons and satellite glia. Cell culture analysis revealed that NCSCs without β -catenin emigrate and proliferate normally but are unable to acquire a sensory neuronal fate. In a complementary set of gain-of-function experiments, Wnt/ β -

**Fig. 1.4**

Differences in Wnt signal interpretation. Different types of stem cells (depicted by different colored nuclei) respond differentially to Wnt signaling. In certain stem cell types Wnt signaling promotes stem cell self-renewal, while in other stem cells it regulates lineage commitment. Therefore, there are cell-intrinsic differences between stem cell types. Such differences are presumably due to distinct determinants that change spatially and over time. Furthermore, the microenvironment modulates Wnt activity by additional factors (indicated by X or Y). Thus, the biological activity of Wnt in a particular environment is influenced by the convergence of Wnt signaling with other signal transduction pathways.

catenin signal activation was shown to regulate sensory fate decisions in emigrating NCSCs while having little effect on the stem cell population size [67]. In particular, as shown by *in vivo* fate mapping of mutant cells (Fig. 1.3), NCSCs expressing a constitutively active form of β -catenin produce sensory neurons at the expense of other crest derivatives, some at ectopic cranial locations of the embryo that are usually devoid of neural derivatives of the neural crest. At locations of normal sympathetic ganglia formation, sensory rather than sympathetic neurons are generated in these mutant embryos. Clonal analysis of cultured cells further demonstrated that canonical Wnt signaling induces sensory neurogenesis by acting instructively on early NCSCs. Thus, in contrast to other types of stem cells, Wnt signaling does not control proliferation but rather promotes sensory fate decision in multipotent NCSCs (Fig. 1.4) [67].

1.2.6

Aberrant Wnt signal activation in carcinogenesis

Given that Wnt signaling is a crucial growth factor for many types of stem cells, its activity needs to be highly controlled to ensure proper organogenesis and tissue homeostasis. Indeed, deregulation of the Wnt signaling pathway affects cell-fate decision, adhesion, and migration, and results in induction and progression of several forms of cancer, indicating that cancers may be a consequence of dysregulation of stem cell programs. Accordingly, Wnt/ β -catenin signaling is not only essential for the homeostasis of the intestinal epithelium [68] but sustained β -catenin activity has also been directly implicated in the formation of colon carcinoma [69, 70]. Thus, mutations resulting in increased β -catenin levels have been found in genes encoding β -catenin itself, APC, or Axin, [71, 72], and the presence of constitutively active TCF/ β -catenin complexes in the nucleus is characteristic of some cancers [73]. The accompanying inappropriate activation of Wnt target genes is considered to be a critical, early event in the course of carcinogenesis. Thus, understanding how canonical Wnt signaling regulates cellular processes during normal development will likely yield important insights into the regulatory mechanisms involved in cancer progression in the adult.

1.3

The Notch Signaling Pathway

The Notch/Delta signaling pathway is highly conserved across species and is involved in cell-fate specification both in vertebrate and invertebrate development [74–76]. The Notch proteins are cell surface receptors that consist of a single transmembrane polypeptide with a ligand-binding extracellular domain containing several tandem epidermal growth factor (EGF)-like repeats. Mammals have four Notch receptors encoded by four different genes. Notch receptors are activated by Delta-like ligands (Dll-1, -3, and -4) and Serrate-like ligands (Jagged-1 and -2) presented by neighboring cells. The signaling pathway is initiated by ligand binding, which induces a proteolytic cleavage of the Notch intracellular domain (NICD). Once released from the plasma membrane, NICD translocates to the nucleus where it binds the transcriptional regulator CSL (CBF1/Suppressor of Hairless/Lag1), DNA-binding proteins [77], and the Mastermind (Mam)/Lag3 co-activator [78]. In an inactivated state, CSL associates with transcriptional co-repressors that inhibit target gene expression [79]. However, when the cleaved intracellular domain of Notch enters the nucleus, co-repressors are replaced, co-activators recruited, and expression of members of the Hairy enhancer of Split (HES) and HES-related (HERP) genes is initiated. HES proteins that act as transcriptional repressors belong to the basic helix-loop-helix (bHLH) family of transcription factors. These proteins are involved in several lineage-specification processes and mediate many of the primary effects of Notch activation. The HES proteins inhibit the expression of lineage-specifying

bHLH genes, such as Mash1 and Neurogenins (which regulate neurogenesis), MyoD (involved in myogenesis), and E2A (involved in B lymphopoiesis) [80]. Additionally, there exists CSL-independent signaling activities, although the mechanisms of these signaling pathways remain to be further elucidated (for review see [81]).

Notch signaling pathways are used in a variety of developmental contexts. The different output of Notch signaling strongly depends on the cellular context. Therefore, different target genes are expressed in different cells upon ligand stimulation [82]. Further, Notch signaling is modulated at several levels by extracellular, cytoplasmic, and nuclear proteins. At the extracellular level, Notch receptors and perhaps Notch ligands undergo posttranslational modification, as for example glycosylation by Fringe proteins [83, 84]. Fringes selectively alter the sensitivity of the Notch receptor to activation by different ligands [85], while several proteins, such as Numb inhibit Notch signaling by targeting cytoplasmic or nuclear NICD for ubiquitination and proteosomal degradation [86, 87].

Although most of our initial understanding of the Notch signaling pathway came from studies in worms and flies, Notch signaling has by now been shown to play several roles in vertebrates, ranging from controlling cell lineage decisions to pattern formation [76]. Notch receptors and ligands are widely expressed in the developing vertebrate embryo, and the generation of mutants of Notch ligands or receptors demonstrated important functions in cell-fate decisions in tissue derived from all three primary germ layers.

1.3.1

Notch Signaling During Hematopoiesis

Notch signaling is involved in many aspects of hematopoiesis. Both Notch receptors and ligands are widely expressed in the hematopoietic system, corroborating the important role of Notch signaling in hematopoiesis. For example, forced expression of Notch1 in HSCs can promote their ability to self-renew and suppress their differentiation into myeloid, erythroid, or lymphoid lineages [88]. In addition to Notch signaling, the Sonic hedgehog and Wnt signaling pathways have been implicated in adult HSC expansion and self-renewal [40, 89]. In the future it will be important to understand how these pathways interact and regulate the size of the HSC pool *in vivo*. However, although many gain-of-function experiments supported the idea that Notch signaling is involved in HSC maintenance, conditional loss-of-function approaches for Notch1 [90] and Notch2 [91] failed to demonstrate the role of Notch signaling in adult HSCs.

Notch1 function is best characterized in T/B lymphoid cell-fate specification [90, 92]. Loss-of-function and gain-of-function experiments revealed that Notch1 signaling is required for the determination of T cells from a progenitor that is capable of forming both T and B cells. Ablation of Notch1 function results in a hypotrophic thymus and bone marrow progenitors are instructed to develop into B cells at the expense of T cells after entering the thymus [93]. In addition, gain-of-function ex-

periments in which NICD or Delta are expressed in bone marrow precursors leads to ectopic T cell development in the bone marrow and blocks B cell development [94, 95]. Thus, Notch signaling must be absent or negatively regulated during B-cell development in the bone marrow. In sum, Notch signaling is critical for T cell-versus B cell-fate specification.

1.3.2

Notch1 Functions as a Tumor Suppressor in Mouse Skin

In the skin, the role of Notch signaling has been assessed by tissue-specific conditional gene ablation. Disruption of *RBP-J*, encoding a Notch signaling component, resulted in increased epidermal cell formation from hair follicle stem cells at the expense of hair cells [96]. A keratinocyte-specific conditional ablation of Notch1 results in deregulation of the normal balance between growth and differentiation [97]. Withdrawal from the cell cycle is a prerequisite of terminal keratinocyte differentiation [98]. It has been shown that activated Notch1 causes the arrest of keratinocyte growth via increased expression of the cyclin-dependent kinase inhibitor p21. Therefore, inactivation of Notch1 in young mice induces hyperproliferation of the basal epidermal layer and deregulates expression of multiple differentiation markers. This suggests a role for Notch1 as a critical integrator of signals which controls the induction of keratinocyte growth arrest and early versus late stages of differentiation in the epidermis [97]. Furthermore, the role of Notch1 in adult mice has been investigated by applying the same loss-of-function approach using tissue-specific inducible gene ablation [99]. Surprisingly, these studies indicated that long-term Notch1 deficiency leads to epidermal and corneal hyperplasia followed by the development of skin tumors in various parts of the body. These results are unexpected as Notch signaling has previously been associated with maintaining proliferative cell populations and with cancer progression [100, 101]. It appears that loss of Notch1 signaling in the epidermis of mouse skin de-represses Wnt signaling and leads to increased levels of free, signaling competent β -catenin [99]. In contrast, forced Notch1 signaling in the epidermis and primary keratinocytes represses β -catenin signaling. This supports the hypothesis that Notch1 signaling inhibits β -catenin-mediated signaling in keratinocytes, and acts as a tumor suppressor in the skin.

1.3.3

Notch Signaling in the Nervous System and its Role in Neural Differentiation and Stem Cell Maintenance

There is substantial evidence to show that in the CNS Notch signaling regulates neural differentiation and stem cell maintenance. Activation of Notch signaling has been associated with the inhibition of neuronal differentiation, whereas repression of Notch activity promotes neurogenesis (reviewed in [74, 102]). Several studies have suggested that interference with Notch signaling leads to premature neurogenesis

and a depletion of the neural stem cell pool [103–108]. For instance, mice deficient in Hes1, one of the downstream signaling effectors, display a decrease in the number of embryonic neural progenitor cells and commitment to the neuronal lineage is accelerated [104]. Moreover, conditional deletion of Notch1 in the cerebellar primordium *in vivo* results in upregulation of neuronal markers concomitant with reduced expression of the progenitor marker nestin [108].

Similarly, Notch activation inhibits oligodendrocyte differentiation in culture [109] while conditional ablation of Notch1 in oligodendrocyte precursors leads to their precocious maturation [110]. In turn, constitutive activation of Notch either renders multipotent progenitor cells permissive for cues inducing gliogenesis or instructs such cells to adopt a glial fate. In the retina, Notch1 and Hes1 are expressed in retinal progenitor cells and downregulated in differentiating and mature neurons [111, 112]. Forced expression of a constitutively activated Notch1 gene in rat retinal progenitor cells blocks the normal differentiation of the neuronal cell types and promotes formation of an unidentified cell type [112]. More recently these results have been complemented by forced expression of Hes1 or activated Notch1 in progenitor cells, which promotes formation of cells expressing Müller glia markers [113]. A possible mechanism by which Notch1–HES signaling exerts its function might be the repression of Mash1, a bHLH transcription factor required for neurogenesis [114]. Another study has shown that Notch activation in telencephalic progenitors promotes radial glia development [115]. In general, it remains to be elucidated whether activation of the Notch signaling pathway simply inhibits one fate (e.g. neurogenesis) leading to the promotion of a default pathway, or whether it directly promotes a specific fate.

Morrison and colleagues found that expression of activated Notch *in vivo* inhibits neuronal differentiation in the PNS. In addition, NCSCs in which the Notch signaling pathway is activated by soluble Delta are driven into the glial lineage *in vitro* [116]. Thereby, Notch instructs NCSCs to adopt a glial fate, even if exposure to Delta is only transient. It has been hypothesized that Notch signal activity is highly context-dependent and that the influence of Notch signaling is modified by additional signals. Addition of soluble Delta together with BMP2 revealed that neural crest cells become progressively more gliogenic and less neurogenic during development [117]. The decrease in sensitivity to the instructive neurogenic signal BMP2 as well as the increase in sensitivity to the anti-neurogenic and gliogenic signal Delta correlate with an increase in the ratio of expression of Notch1 to that of the Notch antagonist Numb [117]. Therefore, cells from distinct origins or isolated at different time-points display individual intrinsic properties that facilitate cell type-specific interpretation of Notch signaling, ranging from inhibition of differentiation and maintenance of the cells as progenitors to active instruction of progenitor cells to generate a particular cell lineage.

To further elucidate the role of Notch signaling in CNS stem cells, various research groups have made use of neurosphere cell culture systems. The self-renewing capacity of neural stem cells can be assessed in such neurosphere cultures [118]. In this assay, neural cells are cultured clonally and examined for their ability to form cell clusters (spheres). The differentiation potential of progenitors within spheres can be

demonstrated by dissociation and subsequent differentiation in appropriate culture media. Self-renewal capacity can be addressed by serial subcloning experiments in which the generation of secondary spheres from dissociated primary spheres is monitored (Fig. 1.5). Upon ablation of Notch1, cells derived from the forebrain are unable to generate neurospheres, indicating a depletion of the neural stem cell pool [119]. In particular, homozygous disruption of Notch1 or CSL in mice disturbs the self-renewal capacity of the stem cells while promoting neuronal and glial differentiation. Likewise, the sphere-forming capacity is reduced in Hes1- and Hes5-deficient cells from the embryonal telencephalon, confirming the reduced self-renewal capacity of mutant NSCs [107]. It has been proposed that Notch signaling is primarily involved in symmetric divisions of neural stem cells within the CNS. Therefore, as a consequence of attenuated Notch signaling, fewer symmetrical and self-renewing divisions of mutant neural stem cells take place, concomitant with an increase in neuronal and astroglial differentiation of the neural progenitor cells.

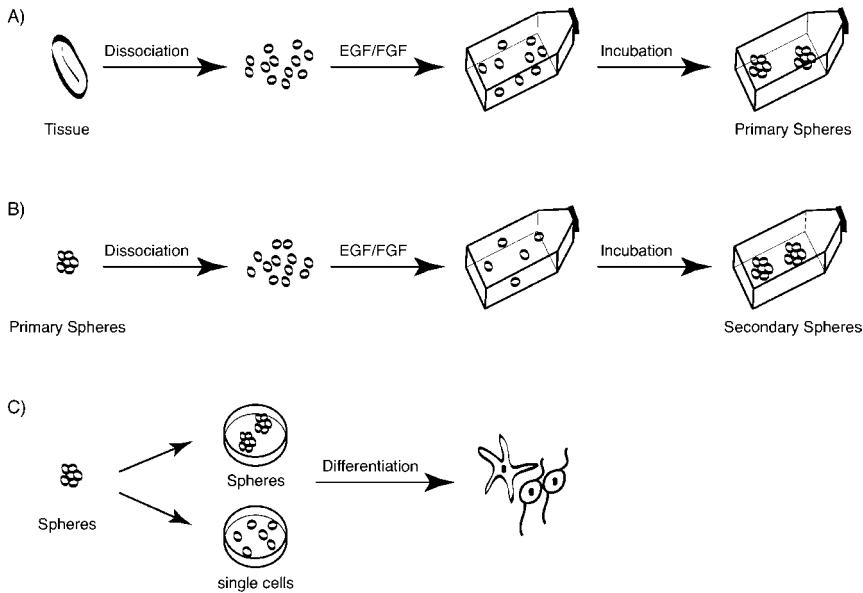


Fig. 1.5

The neurosphere assay demonstrates the self-renewal capacity and differentiation potential of stem cells. Stem cells from several different locations have been isolated and cultured as single floating cells in the presence of the growth factors EGF and FGF (A). Under these conditions the stem cells start to form floating cell aggregates termed neurospheres (first described in [118]). These primary spheres can be dissociated into single cells and again cultured clonally. The formation of secondary spheres proves the existence of stem cells that display the capacity for self-renewal (B). The differentiation potential of sphere-derived cells can be shown in adhesive cultures (C). The spheres are plated as entire spheres or as single cells, and after culturing in differentiation medium the cellular composition can be assessed.

This finding can be seen as an alternative to the idea that Notch signaling is directly and instructively involved in cell-fate decisions of neuronal and glial differentiation in the mammalian CNS [115]. Notch-dependent self-renewal is presumably mediated by endothelial cells that in co-culture promote the expansion of neuroepithelial cells and *in vivo* are thought to provide a vascular stem cell niche [120].

1.3.4

Aberrant Notch Signaling

Given its complex functions in normal tissue development and homeostasis, it is not surprising that aberrant Notch signaling gives rise to some human diseases. These include cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL; [121]), together with several human cancers. CADASIL is an autosomal dominant disorder mainly affecting the arteries of the brain. The cause of the disease is a systemic arteriopathy that is associated with mutations of Notch3 resulting in destruction of arteriolar vascular smooth muscle cells. Another known disease is the Alagille syndrome (AGS), which is caused by Jagged-1 mutations. It is an autosomal dominant disease characterized by defects in liver, heart, skeleton and eye [122–124]. Most of the patients suffering from AGS carry a mutation in the Jagged-1 gene or the entire gene is deleted. The molecular mechanism underlying the disease is largely unknown. It is hypothesized that in addition to Notch other environmental or genetic factors are involved, such as upstream and downstream modulators of Notch signaling.

Aberrant activation of Notch signaling promotes neoplastic transformation of many cell types, which might be explained by Notch inhibiting other signaling pathways [125]. So far, many human and murine cancers including certain neuroblastomas, and mammary, skin, cervical and prostate cancers, are correlated with alterations in expression of Notch proteins and/or ligands. Often, the causal relationships still remain to be proven. Nevertheless, the elucidation of the Notch signaling pathway might allow the manipulation of Notch signaling via delivery of soluble Notch ligands or other strategies, in order to establish possible therapeutic anti-cancer treatments in the future.

1.4

Signaling Pathway of the TGF β Family Members

Members of the TGF β superfamily play a role in many aspects of embryonic development and adult homeostasis by affecting cell proliferation, differentiation and migration. The family includes TGF β isoforms, BMPs, activins, and growth and differentiation factors (GDFs). Originally, they were identified as proteins capable of inducing ectopic cartilage and bone in mammals [126]. TGF β family members are secreted dimeric cytokines that bind to type II single transmembrane receptors with

intrinsic serine/threonine kinase activity, binding is followed by ligand-induced heterodimerization of type I and type II receptors [127]. Subsequently, the type I receptor is phosphorylated by the type II receptor and intracellular signaling is propagated by phosphorylation of specific Smad proteins that translocate to the nucleus where they control the transcription of target genes. There are eight vertebrate Smads that can be separated into three functional groups: common partner Smads (Co-Smads), receptor-regulated Smads (R-Smads) and inhibitory Smads (I-Smads). Smad2 and Smad3 are R-Smads that become phosphorylated and activated by TGF β and activin receptors, whereas Smad1, Smad5 and Smad8 are activated in response to BMP or other ligands [128, 129]. Once activated, R-Smads are released from the receptor complex and form a heterotrimeric complex with the Co-Smad Smad4. Finally, activated R-Smad/Co-Smad complexes efficiently translocate into the nucleus, and in conjunction with other nuclear factors, regulate transcription of target genes. I-Smads (i.e. Smad6 and Smad7) can negatively regulate TGF β signaling on several levels by binding to type I receptors and thereby preventing R-Smads from being activated by type I receptors. Additionally, I-Smads inhibit signaling by competing with Co-Smad interaction and by targeting the receptors for degradation. Smad proteins mediate transcriptional activation or repression depending on their associated partners. R-Smads and Smad4 are expressed in most cell types whereas the expression of the inhibitory Smad6 and Smad7 is highly regulated by extracellular signals. The level of the Smad pool is mainly regulated in a signaling-independent manner. Smad ubiquitination-related factor 1 (Smurf1) is an E3 ubiquitin ligase that catalyzes the transfer of the ubiquitin moiety to its target substrates. Smurfs appear to regulate BMP signaling by targeting non-activated Smad1 and -5 for protein degradation, thereby preventing spurious activation of the pathway. Additionally, the ubiquitin-proteasome pathway not only regulates the steady-state levels of R-Smads, but is also involved in the degradation of activated R-Smads. Smads also function as adaptors that recruit Smurfs to target proteins and thereby control the level of Smad-associating proteins. Two highly conserved negative regulators of Smad transcriptional function are c-Ski and SnoN, which are members of the Ski family of proto-oncogenes. Both antagonize TGF β signaling through direct interaction with Smad4 and R-Smads [130, 131]. Smad signaling is terminated by either dephosphorylation or by ubiquitination and proteasome-mediated degradation of activated R-Smads.

Although there are only few receptors and Smads, a great versatility of signaling is possible by combinatorial interactions of type I and II receptors, oligomeric interaction complexes formed with Smads, and specific transcription factors whose levels change temporally and spatially depending on the cellular context. Differences in stability of signaling components and their subcellular localization may also affect the cellular response. In addition to Smad-mediated transcription, TGF β also activates other signaling cascades such as the mitogen-activated protein kinase (MAPK) pathway. Some of these pathways regulate Smad activation, but others might induce Smad-independent responses [132, 133]. Additionally, other signaling pathways help to define the responses to TGF β factors in a context-dependent manner.

1.4.1

BMP Signaling in ESCs

It is still matter of investigation how many growth factors and signaling pathways are involved in ESC self-renewal. Recently, gene expression profiling suggested that in addition to Wnt signaling as previously mentioned, BMP4 might support ESC self-renewal [134]. Qi and coworkers showed that BMP4 inhibits MAPK pathways in ESCs. MAPK pathways are crucial for signal transduction of many mitogens including LIF, BMPs, and FGFs [135, 136]. Changes in the balance of MAPK activity might determine whether the cells remain undifferentiated or whether differentiation is induced. Furthermore, BMP4 acts synergistically with LIF to promote self-renewal of ESCs [134, 137]. Introduction of the inhibitory Smad family members Smad6 and Smad7 into ESCs in order to antagonize BMP signaling reduced the self-renewal capacity of ESCs and induced differentiation [137]. This is accomplished by the induction of Id proteins through BMP/Smad signaling. Id proteins are negative regulatory helix-loop-helix factors that prevent the transcriptional activity of bHLH factors such as MyoD and Mash1 [138, 139]. Therefore, the suppression of ESC differentiation by BMP4 is likely achieved via induction of Id genes. In summary, in ESCs the two signaling pathways initiated by LIF and BMP act in combination and are highly controlled in order to sustain self-renewal.

1.4.2

The Influence of TGF β Family Members on MSC Differentiation

MSCs isolated from bone marrow have the capacity to differentiate into a variety of cell types such as bone, cartilage, muscle and fat tissue [2, 3, 140]. MSCs can be isolated from the adult, and therefore it is likely that these stem cells participate in regeneration and repair. Members of the TGF β superfamily have important roles in regulating the differentiation of mesenchymal cells. BMPs can induce differentiation of mesenchymal cells into cells with chondroblastic and osteoblastic phenotypes. Furthermore, mesenchymal cell lines respond to multiple isoforms of BMP, including BMP2 and BMP7 [141–144]. BMPs induce specific transcription factors, such as Sox9, Dlx5, and c-fos that are known to determine the commitment of mesenchymal cells into chondrogenic or osteogenic lineages. This process, in which chondrogenic differentiation precedes osteogenesis, occurs in several steps that are dose- and time-dependent [145]. Furthermore, TGF β and activin provide competence for the early stages of chondroblastic differentiation, but at late stages in the osteoblastic differentiation pathway TGF β acts as an inhibitor. BMPs and TGF β also block differentiation into the myogenic lineage. TGF β inhibits muscle formation via direct interaction of Smad3 with MyoD [146], a bHLH transcription factor that plays an important role in myogenesis. Similarly, TGF β is also an inhibitor of adipogenesis mainly via Smad3. In sum, the TGF β s and the BMPs exert several functions demonstrating positive and negative effects on bone development. The cross-talk between TGF β and BMP signaling has not yet been fully elucidated but temporal expression and the dosage of the individual factors are important.

1.4.3

Tgf β Factors Act Instructively on NCSC Differentiation

Members of the TGF β superfamily have multiple functions during neural development, including lineage commitment, proliferation, survival, apoptosis, differentiation, and morphogenesis [147, 148]. In the CNS, BMP signaling is involved in the patterning of the neural tube, regulation of apoptosis, survival and maturation. In the PNS, factors of the BMP subclass together with other factors play a role in neural crest induction [149–152]. At later stages of PNS development, BMP2/4 promote autonomic neurogenesis *in vitro* and *in vivo* [153–155]. TGF β family members have been shown to act instructively on NCSCs. BMP2 promotes a neuronal and, to a lesser extent, a smooth muscle-like fate in clonal cultures of multipotent progenitors derived from neural crest, sciatic nerve, dissociated DRG, and enteric nervous system [156]. Likewise, single progenitor cells are instructed by TGF β to exclusively adopt a non-neural fate. Cardiac neural crest gives rise to smooth muscle cells in the outflow tract of the heart [157, 158] where TGF β isoforms are expressed [159, 160]. TGF β 2 null mice exhibit developmental cardiac defects but it is not clear whether the deficiency is in lineage determination, migration, or maturation of crest cells [161]. BMP2 and BMP4 are expressed in the dorsal aorta close to areas of autonomic neurogenesis [153, 155]. BMP2 induces and maintains the basic helix-loop-helix transcription factor Mash1 that is crucial for autonomic neuronal differentiation [162–164]. Thus, only if BMP2 expression persists in the environment is a neural crest-derived cell able to adopt an autonomic fate. The *in vivo* expression pattern of BMP and TGF β are consistent with the role of these factors in regulating cell-fate decisions in the developing PNS. In chicken embryos, a requirement for BMP signaling in autonomic neurogenesis has been demonstrated using the BMP agonist Noggin [154].

In vivo, however, progenitor cells of the PNS are exposed to multiple signals during migration and at sites of differentiation. Thus, it is conceivable that distinct signaling pathways act on a multipotent progenitor by modulating each other, thereby producing biological effects that are different from those elicited by the individual signals alone. In neural crest cultures, BMP2 and TGF β act co-dominantly, while these TGF β family members are dominant over other signals such as NRG1 [165]. In contrast, the gliogenic activity of Notch signaling suppresses induction of neurogenesis by BMP2 [116]. Additionally, cell-cell interactions termed community effects, influence lineage decisions [166]. Cell clusters of neural crest-derived progenitors, in contrast to single cells, display a reduced non-neural potential when exposed to TGF β factors (Fig. 1.6). Although individual progenitor cells have the potential to give rise to non-neural smooth muscle-like cells in response to TGF β factors, neurogenesis or, at slightly higher doses of TGF β , apoptosis is promoted at the expense of the non-neural fate in progenitor communities in the presence of these instructive signals [167, 168]. Thus, the community effect reveals a synergy between TGF β signaling and signal transduction pathways provided by short-range cell-cell interactions. Conceivably, this allows the fine tuning of the cell-fate decision and programmed cell death, which is an important process in development to con-

trol cell numbers and patterning [169–171]. The molecular basis of these effects has yet to be identified, but cellular interactions via cell-cell contact, local accumulation of secreted signals, or gap junctions are presumably involved [166]. In general, members of the TGF β superfamily can undertake a variety of different, context-dependent functions in developmental systems. TGF β signaling in early NCSC development represents an example of how TGF β signal transduction pathways are able to operate as part of a signaling network which integrates multiple environmental cues that a cell is exposed to.

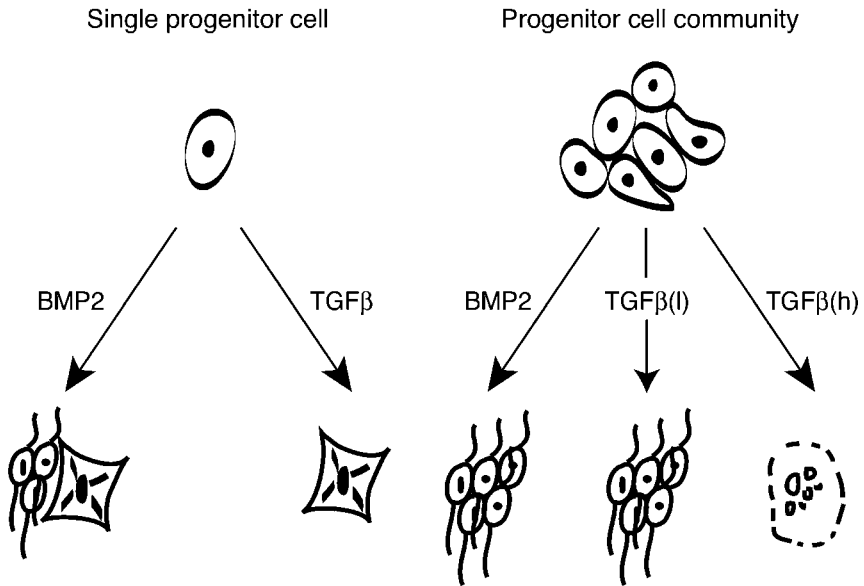


Fig. 1.6

Context-dependent TGF β signaling in neural crest-derived progenitor cells. In response to BMP2, single progenitor cells can produce neuronal as well as a non-neural, smooth muscle-like progeny. TGF β promotes only a non-neural fate in neural crest-derived single progenitors. In contrast, the non-neural cells are completely suppressed by short-range cell-cell interactions provided by progenitor cell communities. Instead, members of the TGF β factor family induce neurogenesis in such communities. Additionally, higher doses of TGF β promote cell death as an alternative fate. Thus, the fate of stem and progenitor cells is influenced by multiple signals that act in combination and at changing concentrations.

1.4.4

Aberrant Growth Regulation by Mutations in the Tgf β Signaling Pathway

The disruption of the TGF β signaling pathway has been implicated in the progression of several human diseases. For instance, TGF β signaling has been shown to be

involved in various forms of cancer such as breast, pancreatic, colon and lung cancer. TGF β signaling displays tumor suppressor activities, as it acts as an inhibitor of cell growth and an inducer of apoptosis that regulates the homeostasis of rapidly proliferating tissues, such as renewing epithelia and blood cells. On the other hand, TGF β also has pro-oncogenic activities that can lead to enhanced epithelial to mesenchymal transition (EMT), growth stimulation, increased motility, and invasiveness. The TGF β -mediated growth arrest in many cells can be attributed to the down-regulation of *c-myc*. This repression is achieved by the binding of a Smad complex to a TGF β -inhibitory element in the *c-myc* promoter [172]. A second important event that leads to TGF β -induced growth arrest is the induction of two major cell cycle inhibitors, the cyclin-dependent kinase (CDK) inhibitors p15 and p21, directly via Smad-dependent transcriptional activation [173, 174]. The components of the TGF β signaling pathway that are most commonly mutated in human cancers are Smad4 (originally termed "deleted in pancreatic carcinoma, locus4" or DPC4) and Smad2 [132]. Furthermore, the TGF β system and the Ras/MAPK pathways interact in tumorigenesis. TGF β is able to activate the MAPK pathways directly, and interacts with these pathways when they are activated by other cues. Many pro-oncogenic responses to TGF β seem to be either Smad-independent, or require cooperation of Smad with alternative pathways. Smad7 is upregulated in human pancreatic cancer, and its overexpression leads to a loss of TGF β -induced growth inhibition [175]. Thus, proteins that interact with Smads and modulate their activity might be direct targets of oncogenic change.

1.5 Shh Signaling

Sonic hedgehog (Shh) is a member of the Hedgehog (Hh) family of secreted signaling proteins carrying out diverse functions during vertebrate development. Originally, Shh was identified as a regulator of cell-fate determination and body segment polarity. In some contexts, Hh signals act as morphogens in a dose-dependent manner, in others as mitogens regulating cell proliferation. In many contexts, the Shh network functions as a "cell-fate switch" where the cell state is changed at a critical threshold level. For example, Shh is secreted from the notochord and organizes the developing neural tube by forming a concentration gradient. The distinct levels of Shh establish distinct regions of homeodomain transcription factor domains along the dorso-ventral axis, thereby specify neuronal identity [176–178].

A key component of the Shh signaling pathway is the 12-transmembrane domain receptor Ptc (patched in *Drosophila*), which acts as a key inhibitory regulator of the constitutively active G-protein coupled receptor component Smoothened (Smo). Binding of Shh inactivates Ptc and allows Smo to become active, which leads to transcription of downstream target genes of the Gli family and Ptc itself [179, 180]. There are three Gli proteins that interpret the Shh signal in a combinatorial fashion by having both activator and repressor activities [181–183]. Further, Ptc also regu-

lates the movement of Hh through tissue, as binding of Hh limits the spread of Hh from its source. The ability of Shh to exert its function is regulated by a series of posttranslational processes. The approximately 45-kDa Hh precursor molecule undergoes an autoproteolytic cleavage that removes the C-terminal end. During this cleavage a cholesterol moiety is covalently attached to the remaining active N-terminal fragment [184]. Additionally, the protein is palmitoylated at the N-terminal end [185]. These lipid modifications of Hh may play a role in targeting it to rafts, and may affect the ability of Shh to activate reporter constructs in cultured cells and target genes *in vivo* [186, 187].

1.5.1

Hematopoiesis and T-cell Maturation

As already mentioned in previous sections, factors regulating the pool of HSCs are still a matter of active research. Bhardwaj et al. showed in 2001 that Hh and its putative receptors, Ptc and Smo, along with the downstream transcription factors Gli1, Gli2, and Gli3, are expressed in primitive human blood cells and stromal cells of the hematopoietic microenvironment. Blocking of endogenously produced Hh or addition of exogenous soluble Hh can control the proliferation of uncommitted human hematopoietic cells [89]. Furthermore, Shh signaling influences T-cell differentiation, which depends on interactions between the thymic epithelium and developing thymocytes in the thymus [188]. It has been shown that Hh signaling is already active during early thymocyte development. Shh is produced by the thymic epithelium, and its receptors Ptc and Smo are expressed by thymocytes. Inhibition of Shh increases the differentiation of thymocytes and treatment with Shh inhibits their differentiation [189].

1.5.2

The Role of Shh in the Nervous System

During embryonic development Gli genes are expressed in proliferative zones of the brain. BrdU incorporation experiments demonstrated a mitogenic effect of Shh on nestin-positive progenitors [190]. Furthermore, neurosphere assays using embryonic neocortical progenitors showed that Shh signaling is required for normal proliferation and self-renewal [191]. In particular, cells isolated from the cortex of Shh-deficient animals produced neurospheres at a much lower frequency as compared to control cells. Therefore, Shh signaling provides a mechanism regulating the number of stem cells in the developing mouse neocortex.

It has been reported that until adulthood localized zones of active neurogenesis persist in the brain. Neurogenesis in the adult mammalian brain takes place in the SVZ of the lateral ventricular walls of the forebrain and in the subgranular layer of the dentate gyrus of the hippocampus [192]. Stem cells in these zones are periventricular astrocytes [193, 194] that are induced by inductive signals to produce new

neurons. Two recent reports by Lai et al. [195] and Machold et al. [196] showed that Shh signaling is involved in cell proliferation in adult neurogenic niches. Lai and colleagues report that Shh signaling regulates the proliferation of progenitor cells in the adult rat hippocampus, which can be blocked by applying an inhibitor of Shh signaling in the subgranular zone [195]. Moreover, the removal of Shh signaling results in a reduced number of neural progenitors in both the postnatal subventricular zone and the hippocampus [196]. Shh may directly regulate the cell cycle, as it upregulates the expression of type D and E cyclins [197]. Therefore, Shh appears to act on adult multipotent hippocampal progenitor cells by inducing proliferation. Consistent with this idea, an Hh agonist increases the proliferation and Gli1 expression in the SVZ and dentate gyrus [196]. Finally, the requirement for Shh in the maintenance of telencephalic stem cells has been assessed by the neurosphere assay, revealing that progenitors from the SVZ with impaired Shh signaling have a reduced potential to generate neurospheres. The combined data suggest that Shh is required for the maintenance of telencephalic stem cell niches in the adult brain. Possibly, Shh signaling acts at a certain concentration range together with other growth factors to establish an environment in which the stem cells are able to persist and to proliferate.

1.5.3

Shh Signaling in Tumorigenesis

Aberrant Shh signaling is thought to contribute to the neoplastic transformation of cells arising from two different cell types of ectodermal origin in the embryo: the epithelial cell of the skin (Gorlin syndrome; basal cell carcinomas, BCCs) and the neural precursors in the brain (gliomas, medullablastoma; [197]). Consistent with this idea, overexpression of Gli1 in the CNS of tadpoles as well as in the tadpole skin leads to tumor formation [190, 198]. Further, cyclopamine, a plant-derived drug that selectively inhibits the Hh-Gli pathway by suppressing the activity of Smo, is able to inhibit brain tumor growth [190]. Additionally, mice that carry a mutation in the patched gene are susceptible to medullablastoma formation [199, 200]. In humans, analysis of many sporadic brain tumors showed expression of three Gli genes [190]. In particular, inappropriate activation of the Shh-Gli pathway has been associated with familial brain tumors such as primitive neuroectodermal tumors (PNETs) of the cerebellum or medullablastoma. Medullablastomas represent the most common malignant brain tumors of childhood [201]. They form a heterogeneous group of tumors believed to arise from immature precursor cells of the cerebellar granule cells. Normally, Shh, which is produced by the Purkinje neurons, controls the growth of the cerebellum and promotes proliferation of granule neuron precursors in the external germinal layer (EGL) of the cerebellum. It is assumed that medullablastomas arise when granule neuron precursors inappropriately maintain Shh-Gli signal activation [190, 202]. More generally, it has been proposed that stem cells displaying sustained Shh signal activity might be responsible for the development of some tumors. Not surprisingly, the role of stem cells in tumorigenesis and of the signaling pathways involved has become a major focus of cancer research.

1.6

Conclusions

In recent years, stem cell research has made considerable progress and several of the signaling pathways that influence stem cell development have been brought to light. We have to be aware, however, that a complex orchestra of signaling cascades rather than individual signaling pathways controls stem cell specification, expansion, and differentiation. Distinct signaling pathways might activate, inhibit or modulate each other, thereby eliciting different biological responses. Moreover, the combination of signals involved likely changes in a spatiotemporal manner. Therefore, it will be a challenge for the future to identify the crucial key points in the signaling network that determines the fate of a particular stem cell type at a specific time-point and location. The use of functional genomics and proteomics should provide several candidate molecules. Cell culture experiments are helpful in the elucidation of the function of such candidate factors (and factor combinations), because they allow one to study the influence of multiple factors on cell-fate decisions in defined but changeable contexts. Furthermore, generating animal models carrying multiple mutations, possibly stem cell-specific and inducible, will be necessary to better understand signal integration by stem cells *in vivo*.

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