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Signaling in adult stem cells

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1. ABSTRACT

Adult stem cells are set aside during development in order to provide a source for replenishment of tissue over time in response to damage or simply wear and tear. The literature suggests that stem cells can be found in most major organ systems, and that they possess defining characteristics, namely the ability to both self-renew and differentiate down one or more specific lineages. Many groups have sought to define stem cell specific physiology in a molecular fashion by identifying those genes specifically expressed in stem cells. Although these data suggest that there are genes frequently found to be upregulated in stem cells from various tissues, they do not definitively demonstrate that these cells all function similarly. There is also considerable data showing how various signaling pathways influence stem cell growth and differentiation. A review of this literature suggests that many of the well-described pathways affect adult mammalian stem cells from different tissues similarly, and that these effects are sometimes unique to stem cells as opposed to their progeny. In this review we summarize the effects of well-known signaling pathways on several of the most well defined stem cells and argue that the similarity with which unique stem cells from different tissues respond to external stimuli suggests that they share functional mechanisms.

2. INTRODUCTION

For the most part, adult SCs are more quiescent than their progeny. How is this maintained? Most stem cells seem to have intrinsic mechanisms to maintain quiescence, and the growth and differentiation in these cells seems to be at the discretion of various signaling pathways. This review will focus on how various signaling pathways impinge on SCs in their niche. Specifically we try to address: Do different stem cells respond similarly to the

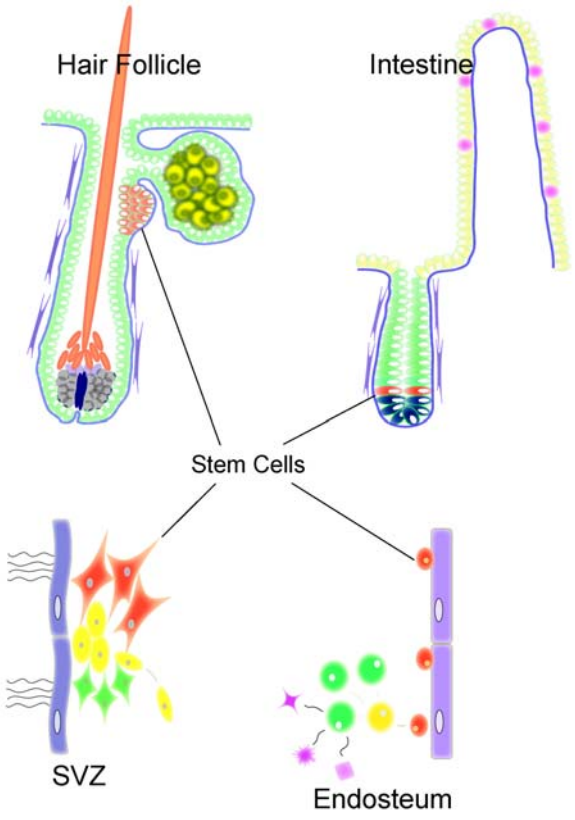

Figure 1. Stem cells reside within most organs in adult mammals. Here, we highlight four of the most well described adult stem cell niches. The niches shown are found in the skin, intestine, brain (sub-ventricular zone), and the endosteal lining of the end of bone. These niches are made up of several different types of cells that together allow for the proper regulation of stem cell fate decisions. The effects of gain or loss of function of various signaling pathways are known and have been described in this review. Less well understood is how multiple signaling pathways converge on stem cells in these niches to influence more subtle effects and maintain homeostasis. These cartoons are meant to highlight the idea that stem cells are not solitary entities, but more likely send and receive signals from neighboring cells to function properly.

same signal? Is this response shared by the progeny of the SCs? Is the different response a result of different target genes being turned on, or different amounts of the same targets?

This survey of the current literature suggests that several signaling pathways exert the same effect on stem cells from different tissues. We chose to look at four well described signaling pathways implicated in the growth and differentiation of not only stem cells, but also their progeny: Wnt, TGF-beta (including BMP), Notch, and Shh. These pathways are reviewed in the context of their effects on four different tissues: Intestine, Brain, Epidermis, and the Hematopoietic system. These tissues all set aside and maintain a population of stem cells and the four signaling pathways have been implicated in the maintenance, self-renewal, or differentiation of each population. Furthermore, these pathways also seem to influence cell fate decisions of lineage restricted cells within these same tissues. Overall we find that each pathway seems to play a conserved role in stem cells from at least three of the four different tissues. In addition, the effects of these pathways are frequently not conserved between stem cells and their progeny. In essence, the literature suggests that stem cells from different tissues share common mechanisms for receiving and interpreting extrinsic signals.

2.1. Intestinal Stem Cells (ISCs)

The intestine is one of the most proliferative epithelia in mammals, repopulating itself every 5 days (1,2). The basic architecture of this tissue includes an invaginated crypt from which cells grow upwards toward the villi, which takes nutrients from the interstitial surface epithelium (Figure 1). Intestinal stem cells were originally identified as slowly cycling cells near the base of the crypt. These cells are thought to give rise to all of the cells of the crypt and villi, including enterocyte, goblet, enteroendocrine, and paneth (3,4). Unfortunately, no system has been developed to study the ability of purified ISCs to reconstitute the crypt and villi as of yet. However, the stem cells were shown to be able to repopulate in the crypt and villi through experiments involving varying doses of irradiation in order to ablate enough cells so that single cells were then tracked for their ability to undergo a clonogenic reconstitution of the tissue (5,2). These fascinating experiments suggested that the stem cells were more sensitive to irradiation, and more prone to undergoing apoptosis. This finding becomes relevant for consideration of stem cells serving as a trigger for tumorigenesis, i.e. the cancer stem cell hypothesis.

A number of proteins have been proposed to be faithful markers of the ISCs (6,7,8), but as of yet, there are no consensus candidates. This makes drawing conclusions about the effects of various signaling cascades on these cells challenging. The accumulated evidence currently suggests that the stem cells reside four to five cells up from the bottom of the crypt (9,5), though some groups have evidence for stem cell characteristics in other positions as well (10). In order to uncover specific markers of ISCs, new approaches will have to be undertaken, such as laser capture microdissection. This technique allows for the purification of tissue at single cell resolution. This technique, when employed in conjunction with microarray expression profiling, has already begun to uncover the molecular identities of specific cells in many different tissues, including intestinal stem cells (11), and may be necessary for the isolation of others, such as Neural Stem Cells (NSCs).

2.2. Neural Stem Cells (NSCs)

Cell proliferation in the nervous system was thought for years to end pre-natally. Seminal findings from Altman in the 1960s showed that in fact neurogenesis proceeds into adulthood (12,13,14). More recently, two niches have been found that support adult neurogenesis and NSCs. Both the subventricular zone of the lateral ventrical and the subgranular zone within the dentate gyrus are now

established sites for NSCs (Figure 1) (15,16,17,18). It was proposed that new neurons formed at these sites are then thought to migrate in a rostral migratory stream to provide new neurons as needed to olfactory and hippocampal regions (19). It is now thought that the newly generated neuroblasts migrate in parallel to the cerebrospinal fluid flowing through the ventricle (20). Adult neurogenesis is thought to be influenced by many factors, including exercise, stress, and learning, and recently some of the signaling pathways influencing this process have been defined (21,22,23,24).

As in the intestine, there are no well established molecular markers of the NSCs, but elegant work has defined their origin, location, and their identity (15). The radial glia were once thought to simply support NSCs, but the current consensus is that these cells are in fact the source of NSCs (25), as NSCs retain remnant radial glia attributes. Another group argues that the ependymal cells lining the niche actually give rise to the stem cells, further complicating the issue (26). More recently, the same group that identified radial glial cells as NSCs has proposed that the PDGF receptor represents a specific marker for NSCs (27). If verified, this could represent an enormous leap forward in the NSC field and allow for the same kinds of experiments so readily exploited in other stem cell systems. In addition, the existence of fascinating interactions between NSCs and endothelial cells suggest that additional plasticity can be found in these niches. Beginning with the finding that cell proliferation in the brain is accompanied by increased numbers of endothelial cells, one group showed that NSCs can give rise to endothelial cells, while another showed that endothelial cells provide critical support to NSC maintenance (28,29).

2.3. Hematopoietic Stem Cells (HSCs)

The stem cells responsible for populating the entire repertoire of the blood are the most well functionally characterized adult stem cells in the least well characterized niche. Early work fractionating the hematopoietic system established the basis for experimental manipulation of stem cells and the concepts of self-renewal and differentiation (30,31,32,33). More recently, single HSCs have been shown to be able to reconstitute the entire hematopoietic repertoire, firmly establishing them as stem cells (34,35). In addition, these reconstitution assays have been exploited to identify these cells on the basis of their cell surface markers. The work of many labs has shown that these cells can now be purified by various combinations markers until the point that 1 in 3 cells transplanted into a depleted niche can repopulate the entire hematopoietic system (36,37). This suggests that various combinations of cells surface markers either can be used to purify cells to the point where only two or three different kinds of cells remain, or that technical limitations remain in the grafting protocol.

These cells were originally thought to reside in the endosteal lining of the bone marrow cavity (38). Recently, a HSC niche was described more specifically by demonstrating the presence of label-retaining cells (LRCs) in the endosteal lining of the trabeculum at the end of the bone. It was also shown that the LRCs were in intimate contact with osteoblastic cells (Figure 1). Interestingly, the number of osteoblasts was directly related to the numbers of LRCs in this niche (39,40). Another group compared the transcriptional profiles of purified long term (LT) and short term (ST) HSCs. A new family of cell surface proteins emerged, which, when used in combination, demarcated LT-HSCs from their progeny. Antibodies against these new markers were then used to illuminate the location of HSCs in their niche (41). It is thought that HSCs mobilize in response to injury and repopulate any of the hematopoietic lineages as necessary (42). Recent work has begun to elucidate the mechanisms behind this mobilization and the signaling pathways that control their growth and differentiation (43,44). HSCs were also shown to give rise to non-hematopoietic lineages in a process suggested to be transdifferentiation. These controversial findings were found to be most likely be the result of cell fusion between different cell types (45,46). Whether cell fusion occurs naturally and what role it might play in tissue repair is as of yet unclear.

2.4. Hair Follicle Stem Cells (HFSCs)

The epidermis and its hair follicle appendage is one of the best characterized models in stem cell biology. The follicle itself goes through cycles of growth, degeneration, and rest throughout the life of the animal, reaffirming each time the presence and capabilities of the stem cells therein (47,48). Originally identified as label-retaining cells, a group of cells residing in the hair follicle demonstrated the ability to regenerate the epidermis, hair follicle and sebaceous gland (49,50,51). These stem cells reside in a niche called the bulge, and along with the sebaceous gland, represent the only permanent portions of the hair follicle (Figure 1). More recently, several groups have shown that these cells can be isolated on the basis of either their slow-cycling nature, or cell surface markers. As with stem cells from other tissues, epidermal stem cells display a unique transcriptional profile, which partially overlaps with that of other stem cells (49,50,52). Within this niche, it has also been shown that there are at least two populations of cells with stem cell characteristics. One of these is associated with the basal lamina surrounding the follicle, while the other is not associated (49). The two populations share many characteristics, but have somewhat different transcriptional and cell cycle profiles. Despite this, the purpose of having two populations is still unclear. Furthermore, we know that epidermal stem cells go through periods of quiescence and activation, but the signaling pathways involved in these transitions are only just beginning to be elucidated.

2.5. Contribution of Stroma to stem cell biology

There is ample evidence that the various cell types surrounding a stem cell niche make significant contributions to the maintenance, quiescence and activation of stem cells. The niche cannot be thought of as simply the stem cells and their progeny. Niches are frequently composed of many different cell types that can play roles in signaling to or preventing the signaling to stem cells (Figure 1). Recent work from our group and others have begun to identify stromal factors with significant roles in the stem cell niche (39,90,28,40, 173). These findings are

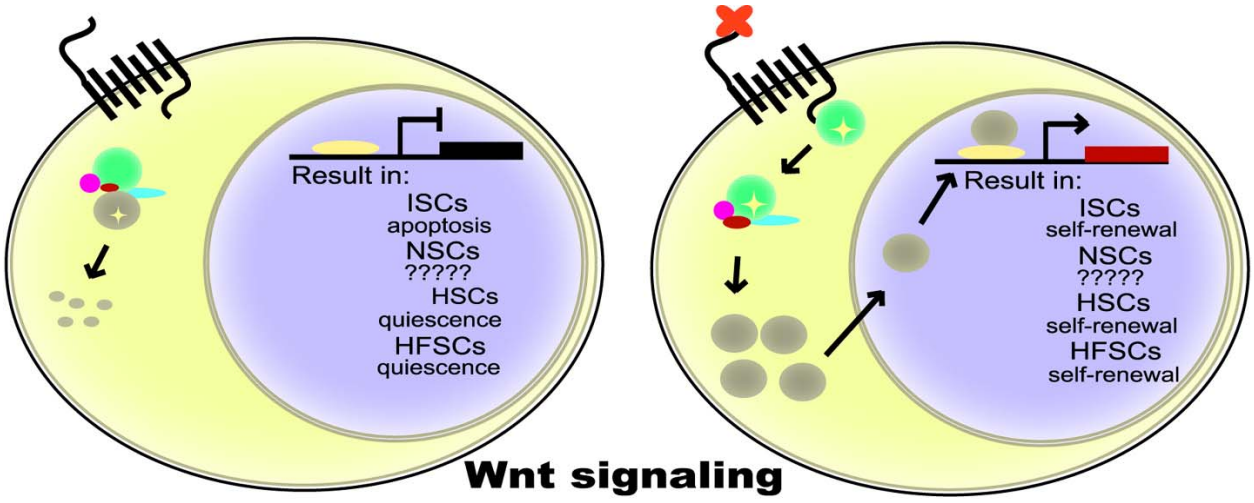

Figure 2. A schematic depicting the inactive state of the Wnt signaling pathway. Left: in the absence of the Wnt ligand, a degradation machinery phosphorylates and targets beta-catenin for destruction. In most cases, an absence of Wnt signaling leads to apoptosis or quiescence. Right: when Wnt signaling is activated, beta-catenin is stabilized and accumulates in the cytoplasm, where a portion may then enter the nucleus and associate with a transcription factor, such as a member of the Lef/Tcf family. Nuclear beta-catenin and activation of Lef/Tcf is normally associated with self-renewal in stem cells, whereas lineage restricted cells also utilitze Wnt activation during differentiation.

fascinating and critical to stem cell biology, but unfortunately, due to space constraints cannot be summarized in this review.

3. The Wnt signaling pathway

The term Wnt originally derives from a fusion of two well described biological phenomena. The Int locus was identified as a MMTV viral integration site in murine breast tumors, which led to activation of the Int gene product (54). The Drosophila Int gene (Dint) was then shown to be identical to the locus mutated in the Wingless phenotype (55). The Dint/Wingless gene was then renamed Wnt, and the literature exploded with data relating to the subsequently described Wnt signaling cascade which was shown to be critically involved in both development and tumorigenesis. The Wnt gene product serves as a ligand for a serpentine receptor with seven transmembrane domains first identified in drosophila called Frizzled (56). While it remains unclear as to whether this receptor directly couples to heterotrimeric G-proteins (57,58), it is certain that activation of this receptor leads to cytosolic stabilization of its critical intracellular mediator, beta-catenin. Beta-catenin is most often associated with cadherins in the cell adhesion machinery, but when stabilized, it accumulates in the cytoplasm, translocates to the nucleus, associates with transcription factors and activates or suppresses target gene expression (Figure 2). Cytoplasmic beta-catenin is normally degraded by a complex machinery involving Dsh, Gsk3beta, Axin, and APC, which collaborate to phosphorylate beta-catenin, targeting it for ubiquination and subsequent degradation. Upon receipt of the Wnt signal, Dsh is activated, beta-catenin is displaced from the degradation machinery, accumulates, and translocates to the nucleus to influence gene expression through either Lef/Tcf or Sox transcription factors (Figure 2)(59,60). These transcriptional complexes have been shown to either stimulate or inhibit expression of a great many target genes (http://www.stanford.edu/~rnusse/pathways/targets.html). Recently, a large number of other players in this signaling cascade have been identified and shown to play critical modulatory roles in this pathway, however this work will not be described here (61,62,63,64,65).

The Wingless pathway has been implicated in a myriad of developmental paradigms. The role of Wnt signaling in adult stem cells has only more recently been described, owing to the development of novel techniques for specifically characterizing stem cells and the impact of this pathway. Furthermore, the wingless genes were first described as oncogenes, and this pathway has been shown to play specific roles in tumor formation. A connection between SC biology, the wingless pathway, and tumor formation lies at the heart of many theories for “cancer stem cells”. While these theories will not be discussed in this review, it is worth noting that many of the gain of function paradigms generated for the Wnt pathway in stem cells lead to tumor formation. It remains to be determined if tumor formation is caused by aberrant activation of this pathway in stem cells, or in their progeny, or both.

3.1. Wnt in the intestine

The Wnt signaling cascade has been proposed to be the dominant force in growth and differentiation in the intestinal crypt (66,67,68). The first evidence for this idea came from the understanding of a human disorder Familial Adenomatous Polyopsis (FAP). Patients with this disease present with aberrant growths which seem to be linked to inactivating mutations in the APC gene. A fraction of these

polyps are known to transform into tumors with metastatic potential. As APC is required for cytoplasmic degradation of beta-catenin, this mutation mimics a hyperactive Wnt signal, resulting in aberrant nuclear beta-catenin and hyperplasia (69,70).

More recently, diverse roles for Wnt in the intestine have been clarified. In the ISCs, which normally display nuclear beta-catenin, expression of Wnt ligand and canonical pathway inhibitor Dkk1 blocks proliferation in the crypt (67). In addition, in mice lacking Tcf4, a critical downstream effector of the Wnt signal, proliferation in the base of the crypt is blocked (71,72). On the other hand, expression of a beta-catenin-Lef fusion protein induced apoptosis in ISCs (73). However this fusion protein was expressed mosaically during development of the intestine and those cells expressing the transgene were eliminated by apoptosis, so the functional result of expression of beta-catenin-Lef in adult ISCs could not be ascertained. Hyperactivation of beta-catenin, due to the inactivation of APC, leads to premature differentiation of ISCs and enhanced proliferation of cells not normally receiving a Wnt signal such as mid-crypt progenitors (74). Essentially, in ISCs which are normally receiving a Wnt signal and cycling (though more slowly than their immediate progeny), a loss of function for the Wnt cascade seems to lead towards a block in proliferation or even apoptosis, whereas stimulating the Wnt cascade in these cells leads to terminal differentiation. Activation of beta-catenin in the more differentiated cells of the villi which are normally not receiving the Wnt signal instead leads to massive proliferation and eventually tumor formation (70, 74).

3.2. Wnt in the nervous system

In the nervous system the Wnt pathway has been implicated in numerous developmental contexts. In the adult brain, Wnt was first shown to drive proliferation in neural precursors by expression of truncated, and therefore non-degradable beta-catenin. In these gain of function mice, neural progenitors were expanded at the expense of other cell types (75,76). Recently, Lie *et al* nicely showed that elevated expression of Wnt3a in regions of the brain where NSCs are thought to reside led to increased neurogenesis (77). This was a result of proliferation of the neuroblast pool which eventually generated differentiated neurons. The authors also used a dominant negative molecule to block the Wnt cascade and showed that neurogenesis was blocked. These data provided the first clue about a role for Wnt in driving cell fate in the adult brain, but the effect on the stem cells which give rise to the neuroblasts was not clearly elaborated. Either the NSCs are not responsive to the Wnt signal or the transgene was not expressed in those cells. On the other hand, in more lineage restricted neural cells there is a great deal of evidence that Wnt plays a role in promoting the differentiation down various lineages both *in vivo* and *in vitro* (78,79,80). While the role for Wnt in quiescent NSCs remains unclear because of the difficulty of describing these cells *in vivo*, this pathway certainly can drive either neurogenesis or terminal differentiation of more lineage restricted cell types depending on the context.

3.3. Wnt in the Hematopoietic System

The role for Wnt in hematopoietic stem cells has been described both *in vivo* and *in vitro*, but is still somewhat controversial. While gain of function experiments clearly show that Wnt can promote proliferation of HSCs, it is unclear whether this proliferation represents self-renewal and whether this effect is physiological. It has been estimated that 75% of HSCs are quiescent (81). These LT-HSCs are thought to be responsible for replenishing the supply of ST-HSCs which can quickly reconstitute the entire hematopoietic system. It was first shown *in vitro* that HSCs can be expanded in culture only in the presence of Wnts (82,83). A loss of function experiment with ectopic expression of Axin showed that HSC growth is impaired when stabilization of beta-catenin is blocked. In addition, a reporter mouse for Tcf/Lef activation demonstrated that Wnt signaling was active in LT-HSCs *in vivo*. Furthermore, another group used *in vivo* administration of a GSK3beta inhibitor to augment the reconstitution capability of human HSCs in a mouse recipient, suggesting a role for active beta-catenin in HSC self-renewal (84). Two studies also employed expression of constitutively active beta-catenin as a model for Wnt activation in the hematopoietic system (85,86). These groups showed that constitutive beta-catenin signaling led to aberrant proliferation of HSCs at the expense of multilineage differentiation. Eventually, the HSC pool was depleted, suggesting that this proliferation was not self-renewal, but probably generation of transit-amplifying cells.

The fly in the ointment seems to be data derived from a conditional knockout mouse for beta-catenin which has a completely normal hematopoietic system (87). Perhaps the discrepancy can be explained by a complementation in these mice by plakoglobin, an isoform of beta-catenin, or perhaps the Wnt cascade plays an insignificant role in HSCs, unless present at high doses. It is also possible that stressing the system, such as in a wounding model may reveal a role for the canonical Wnt pathway, as the authors only looked for a phenotype during normal homeostasis. Conversely, gain of function experiments highlight a role for Wnt in HSCs. The issue of why gain and loss of function experiments for the Wnt pathway in HSCs do not agree will persist until more is learned about how beta-catenin acts through Lef/Tcfs to mediate responses.

In lineage restricted cells of the hematopoietic system, it seems as though the Wnt cascade can drive cell fate. Wnt has been shown to be able to drive terminal differentiation down several lineages (88,89,90). In one study, expression of constitutively active beta-catenin in lymphoid and myeloid progenitors led to an expansion of these cells and reduced lineage restriction (91). In essence, ectopic activation of beta-catenin drove the cells to become more stem-like, perhaps analogous to the effect of beta-catenin gain of function on lineage restricted intestinal cells

as mentioned previously and also with interfollicular epidermis as will be discussed below.

3.4. Wnt in the Epidermis

A role for Wnt in the epidermis became clear years ago with the development of transgenic animals expressing Lef or Tcf, critical mediators of the canonical pathway (92,93). A role in proliferation and morphogenesis of the epidermis was highlighted by expressing constitutively active beta-catenin (94). These mice developed normally but during adulthood formed de novo hair follicles and eventually developed tumors (94). It was suggested that the interfollicular de novo follicles were the result of activation of an existing stem cell, or dedifferentiation of a lineage restricted cell. At least a part of this phenotype corresponded to a precocious activation of stem cells, but without methods for isolating these cells, definitive claims were elusive. The role of Wnt in the epidermis was first demonstrated by the identification of nuclear beta-catenin in the terminally differentiating cells of the hair cortex (92,95). Our group generated a Tcf/Lef reporter mouse and showed that canonical Wnt signaling is indeed found in the hair cortex, but also to a lesser degree in the stem cell niche during the transition period of the hair cycle where the niche goes from a quiescent to a proliferative state (96). More recently, our lab and others have developed new techniques with which to characterize the HFSCs (49,50,97,52). Armed with these new methods we re-visited the gain of function model for beta-catenin signaling and found that, indeed, elevation of this pathway leads to activation of the stem cell compartment as witnessed by nuclear beta-catenin, proliferation (BrdU, Ki67) and an altered cell cycle profile (47). In the face of the elevated beta-catenin signal, the stem cells retained all of their signature markers and homeostasis of the niche was maintained i.e. the niche did not become swollen with extra SCs (47).

These data demonstrated that the Wnt cascade can promote proliferation, leading to one daughter cell remaining in the niche while the other exits. The exiting cells remain proliferative and form the hair germ, which then goes on to remake the entire hair. The gain of function experiment was informative, but in order to demonstrate whether this result was physiologically relevant, we asked whether canonical Wnt signaling was required for SC activation in the epidermis. A conditional knockout of beta-catenin during development suggested that beta-catenin was required for maintenance of hair follicles (99). To characterize the role for beta-catenin in the mature HFSCs, we created an inducible knockout to monitor the effect of the loss of beta-catenin during adulthood. We found that beta-catenin is required for not only SC activation, but maintenance of the follicular nature of the SCs (47). Without beta-catenin, the hair follicle stem cells quickly adopted a more epidermal nature and eventually the entire follicle converted into epidermis. While it seems clear that the Wnt pathway plays a role in activation of ESCs, data from the TOPGAL reporter mouse suggests that the highest Wnt activity in the epidermis is found in the terminally differentiating cells of the hair follicle, suggesting that either Wnt plays opposing roles in different cell types in the epidermis depending on how primitive the cell is, or that simply the dose of the Wnt signal determines the outcome.

A role for Tcf3 in stem cells of the epidermis and hair follicle has been assumed for years based on its specific expression in the most primitive epidermis during development and in the bulge of the adult follicle. A new study employing an inducible Tcf3 transgenic has illuminated the role of the transcription factor in stem cell biology. In relatively mature stratified epidermis, induction of Tcf3 forces all of the cells adopt a more primitive fate reminiscent of more rudimentary single layer epidermis (100). In the follicle, induction of Tcf3 also drove a reversion to a more primitive, undifferentiated state (100). These data argue that, in the adult bulge, Tcf3 expression acts to maintain a somewhat primitive state to avoid premature differentiation.

4. The TGF-beta signaling pathway

Another pathway known to play various roles in many developmental contexts is the TGF-beta signaling pathway. This superfamily of signaling molecules is divided into two major categories: TGF-beta and Bone Morphogenic Protein (BMP). Both families of ligand bind to a receptor tyrosine kinase to stimulate downstream effectors. The TFbeta ligand and their receptors signal through SMADs 2 and 3, whereas BMP ligands signal through SMAD 1,5,7. These two different SMAD pathways lead to activation of distinct transcriptional target genes, thus distinguishing the effects of TGF-beta from BMP (Figure 3). These two signaling cascades are modulated by many different intracellular interactions, but the most robust modulation comes from a large number of proteins that bind TGF-beta superfamily ligands to prevent their interaction with the receptor. Both TGF-beta and BMP ligands have specific inhibitors such as Noggin, Follistatin, Gremlin, Chordin. Interestingly, Noggin was identified as the gene responsible for a null mutation leading to the absence of neuroectoderm (101). It was later shown that BMP inhibition was required for development of the central nervous system (102).

4.1. TGF-beta in the Intestine

In the intestine, BMP signaling has been shown to be strongest in the more differentiated cells of the colon (103). In mice lacking the BMPR1a receptor, ISC self-renewal is induced (6). The authors argue that BMP actually inhibits Wnt signaling in order to maintain a proper balance of self-renewal versus differentiation. These mice had five times the normal number of ISCs, and eventually developed polyps and supernumerary crypts. Mice overexpressing Noggin under control of a villi-specific promoter, also formed ectopic crypts with excessive branching and budding (104). In addition, ablation of SMAD4, a co-SMAD active in transducing both TGF-beta and BMP signals, also led to aberrant crypt formation and lack of control of ISC proliferation. Finally, inactivating mutations for some of the key molecules in the BMP pathway have been found in human patients with Juvenile Polyopsis (JP), suggesting that BMP plays similar roles in ISCs in both murine and human models (105). All

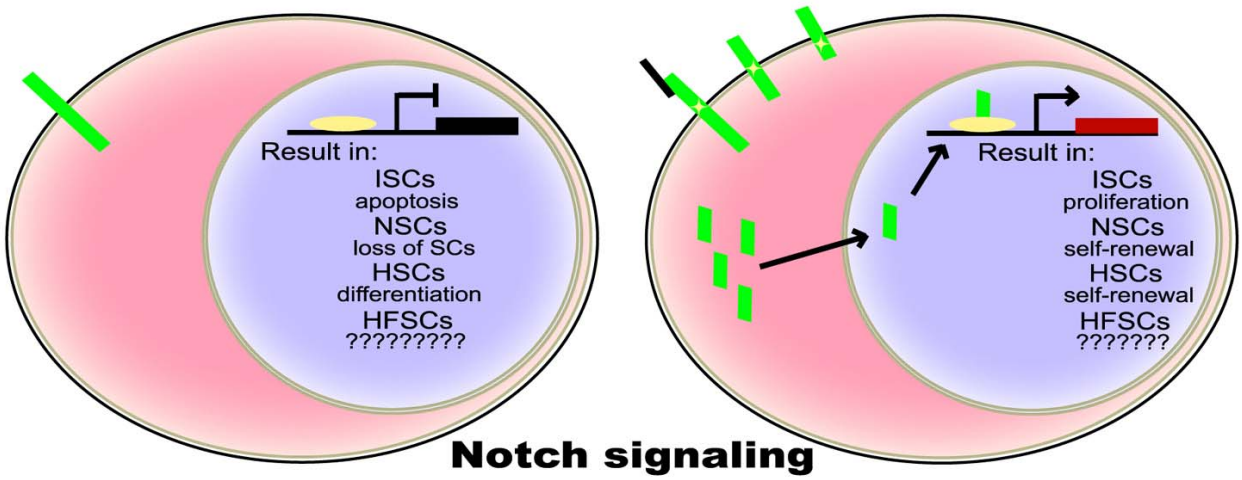

Figure 3. In the absence of ligand engagement, the Notch receptor remains at the membrane. Upon binding of a ligand such as Delta, the Notch receptor undergoes a series of cleavage events leading to an accumulation of soluble, cytoplasmic protein. Truncated Notch can then enter the nucleus where is binds to RBP-J (Csl) to drive transcription of target genes. In most stem cells, at least some activity of this pathway is required for the maintenance of self-renewal, whereas this pathway is also exploited to drive differentiation in stem cell progeny.

these data are consistent with the notion that BMP signaling is important in maintaining proper self-renewal of ISCs. TGF-beta, on the other hand, seems to be active in all cell types of the villi (106). SMAD2 takes advantage of ELF, an adaptor protein to transduce the TGF-beta signal. Animals lacking ELF display a distinctive pattern of flattened gut epithelia and a loss of entire villi, demonstrating that this pathway is critical to all the cells of the intestine (107,108,109).

4.2. TGF-beta in the Brain

In the neural progenitors of the olfactory epithelium, GDF11, a TGF-beta family member, drives cell cycle arrest, whereas Follistatin an inhibitor of GDF11 was shown to drive neurogenesis (110). This pathway was shown to function in an autocrine manner, as the neurons seem to be regulating their own numbers by secreting GDF11. The TGF-beta pathway seems to be involved in neurogenesis in the olfactory bulb as it is required for the FGF2 pathway (111). Conversely, in the ELF mutant mice, proliferation is unabated and differentiation impaired (108). As SMAD is thought to act as a mediator for both TGFb and BMP pathways, ablation of this gene should mimic a block of both arms of the TGF-beta superfamily pathway. Mice lacking SMAD4 show increased numbers of neurons at the expense of other fates (112Zhou 2003).

The BMP pathway has also been show to act in the brain to drive NSCs to a glial fate (113,114Hsieh 2004), and can also drive glial progenitors down astrocytic lineages (115,116). Consistent with these findings, is the idea that inhibition of this pathway drives neurogenic development, as expression of Noggin can drive neurogenin expression and neural fate in the spinal cord (117). In neurons, the BMP pathway can promote astrocytic fate and inhibit oligodendrocyte fate (118). *In vitro*, BMP can direct Neural Crest Stem Cells (NCSCs) to undergo neurogenesis by inducing MASH1 eventually creating neurons with characteristics of the autonomic nervous system (119Shah 1999). As SMAD is thought to act as a mediator for both TGFb and BMP pathways, ablation of this gene should mimic a block of both arms of the TGFb superfamily pathway. Mice lacking SMAD4 show increased numbers of neurons at the expense of other fates (112.).

4.3. TGF-beta in Blood

A role for BMP signaling in HSCs was first uncovered by Bhatia *et al* 1999 (120). The authors showed that BMP4 can promote the ability of the HSCs to reconstitute the hematopoietic system. Several years later, it was shown that BMP signaling is essential to maintain the size of the HSC niche. In fact, the defects seen in the BMPR1A knockout mouse led to the discovery of the niche itself. In the absensce of BMP signaling, the niche was small and poorly maintained, leading to fewer numbers of LRCs (39,40). These data showed that BMP seems to have an indirect role in HSC maintenance. In fact, BMP determined the number of osteoblastic cells making up the HSC niche which, in turn, affected the number of LRCs. Similarly, quantitative trait analysis in TgfB2 knockout mice demonstrated a positive role for TGFB2 in regulating the number of HSCs both *in vitro* and *in vivo* (121). Unlike the other adult SCs, both arms of the TGF-beta pathway seem to be required for HSC maintenance, and stimulation of this pathway *in vitro* even seems to promote self-renewal.

4.4. TGF-beta in the Epidermis

Our own lab has studied BMP signaling during hair follicle development and found that this signaling cascade is required to maintain the proper balance of growth and proliferation. Without the BMPR1A receptor, hair follicles become cysts and terminal differentiation is disrupted (122,123,124). In addition, it was shown that

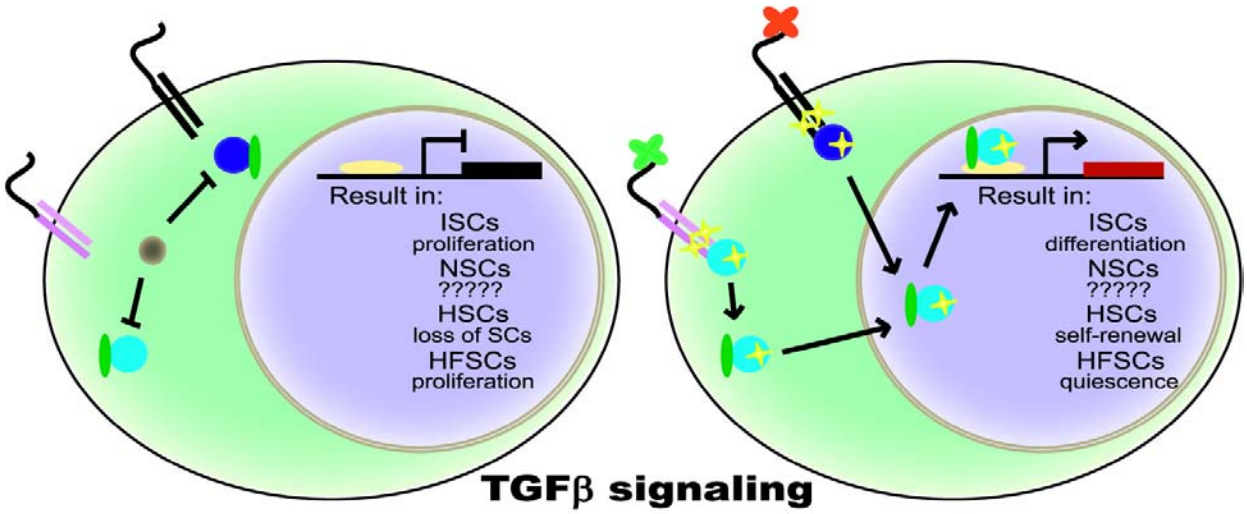

Figure 4. Tgf-beta family receptors are tyrosine kinases that form dimers and cross-phosphroylate each other to activate downstream cascades. Without this phosphorylation, inhibitory factors (SMAD 6/7) block downstream signaling. Upon ligand engagement and receptor phosphorylation, activating factors (SMAD 1/5/8 for BMP, SMAD 2/3 for Tgf-beta) can enter the nucleus and drive transcription of target genes. The majority of stem cells studied to date are thought to be driven by Tgf-beta to enter quiescence or differentiate.

both Wnt signaling and inhibition of BMP are required for proper downgrowth of follicles during development because of a collaborative effort through Lef to downregulate E-cadherin (125). Neither of these studies, however, demonstrated a role for BMP signaling in the maintenance or growth of adult follicles. The first evidence for a role for BMP signaling in adult HFSCs came from microarray profiling that suggested the presence of a gradient of BMP signaling in the quiescent cells of the epidermal stem cell niche. The most quiescent cells in this niche expressed BMP6, and when HFSCs were treated *in vitro* with purified BMP6, their growth was impaired without inducing differentiation (49). This suggested that BMP6 is exploited by the HFSCs to maintain their quiescence until the next hair cycle when they could be re-activated, perhaps in a similar manner to GDF11 in neurogenesis.

The most definitive evidence to date for a role of the BMP pathway in the HFSCs comes from recent work employing an inducible ablation of the BMPR1a receptor in fully mature hair follicles. In this model, the normally quiescent stem cells immediately became activated and proliferated (126). These findings, coupled with the data on BMP6, convincingly argue that the BMP pathway is required to maintain quiescence in this niche, similar to its role in other SC models.

TGF-beta signaling components were also shown to be upregulated in the quiescent bulge by gene expression profiling (52). In addition, the TGF-beta pathway was shown to be active in this compartment by an activity dependant antibody for SMAD2/3 of the TGF pathway. Recently, another group created mice lacking SMAD4, and although an analysis of a specific role for SMAD4 in the HFSCs was not performed, these mice showed a defect in the hair cycle and increased proliferation throughout the epidermis and hair follicle (127). The TGF-beta pathway has also been implicated in epidermal maintenance because of its clear role in carcinogenesis in this tissue (128).

5. The Notch signaling pathway

The Notch receptor and its ligand Delta were first described in C. Elegans and Drosophila Melanogaster as signaling molecules important for lateral inhibition of cell fate. That is, a cell whose fate has been determined signals to surrounding cells thereby inhibiting them from adopting the same fate (129,130). The mechanism for this pathway has been worked out in great detail first in Drosophila and later in mice, but briefly, upon receipt of the ligand (delta or jagged), the Notch receptor undergoes a series of proteolytic cleavages to produce a soluble cytoplasmic domain (NICD). The final cleavage is performed by gamma-secretase. Gamma-secretase inhibitors were developed for potential therapeutic application in Alzheimer's disease, but have proved to be quite useful for blocking the Notch pathway in many different cell types (131). Truncated Notch protein can enter the nucleus and bind to a protein, which in Drosophila is called suppressor of hairless (RBPsuh, RBPkj, Csl or Cbf in mice). This protein is normally a suppressor of the hairy enhancer of split genes (Hes, Hey), but upon binding to the NICD molecule, transforms into a potent stimulator of an ever expanding list of target genes (Figure 4). Most of these target genes, such as Hairy enhancer of split, are actually transcriptional repressors, thereby suppressing cell fate choice in lateral inhibition. The Notch pathway has been shown to be active and important in almost every developmental context, and in stem cells in particular. The canonical pathway is dependent on the RBP-J, a transcription factor. Notch has been shown in lower organisms to have unique effects during development that

are independent of RBP-J, but similar findings in mammalian systems are more controversial. For now, it is assumed in mammals that the majority of Notch signaling observed is dependent on the RBP-J target genes of the Hairy Enhancer of Split (Hes) family. While gain and loss of function studies have begun to uncover roles for Notch in SCs, it is less clear how Hes family members mediate the effects of Notch signaling in SCs.

5.1. Notch in the Intestine

In the intestine, Notch signaling seems to play a role in every cell type. Expression of a constitutively active NICD molecule leads to an increased number of progenitors in the crypt and a suppression of differentiation (132). This phenotype was ascribed to increased expression of Notch target gene Hes1, which is known to suppress Math1, a critical transcription factor in intestinal differentiation. In a mouse model lacking Math1, goblet, paneth, and enterocyte lineages are all depleted (133). In mice with reduced Hes activity, Math1 is induced and cells are driven down goblet and enteroendocrine lineages (134). In Hes1 knockout mice, the intestine and gut displayed increased differentiation of endocrine lineages (135). Mice treated with gamma-secretase inhibitors show a lack of proliferation in the ISCs and marked increase in the production of Goblet cells (134). Mice can only survive for a few days of Notch pathway ablation with this inhibitor, suggesting that replenishment of the crypts and villi by ISCs is vital to the ability of this organ to function. It has been postulated that gamma-secretase inhibitors, on the basis of their ability to suppress ISC proliferation, might be useful as therapeutics in cancers of the gastrointestinal tract (131).

5.2. Notch in the Brain

The Notch pathway was originally discovered to be critical for lateral inhibition and cell fate decisions in the Drosophila nervous system. While not nearly as well defined, multiple roles for Notch in the murine nervous system have been elucidated. Notch seems to play a critical role in NSCs by inhibiting premature differentiation (136). Several groups have shown Notch signaling to be essential for proper self-renewal of NSCs by both gain and loss of function studies (137,138). More recently, another group showed that Notch is required for maintenance of NSCs not only in the adult, but also during neuronal development by using the neurosphere formation to assay for stemness (139). The gain of function Notch led to an accumulation of NSCs in the subventricular zone at the expense of neurogenesis (140). *In vitro* it was suggested that endothelial cells can promote self-renewal of NSCs by secreting some soluble factor. Two groups suggest that this phenomenon is due to a signal emanating from endothelial cells that activates the Notch pathway (28,29). In neural crest stem cells (NCSCs) during development, a transient gain of function of Notch led to glial specification. This was also suggested to be the case for neuroblast cell fate decisions (141). Essentially, it seems clear that Notch activity is required for general maintenance and self-renewal of adult NSCs, however, this pathway plays distinct roles in the more specified lineages. In glial progenitors, Notch has been shown to promote astrocytic fate, while also blocking the final steps of oligodendrocyte differentiation in lower mammals, which is also thought to hold true in mouse models. (142).

5.3. Notch in the Blood

Using a Notch reporter mouse, it was shown that the Notch pathway is active in HSCs *in vivo* and is downregulated as cells differentiate (143). Then, using either gamma-secretase inhibitors or expression of a dominant negative Csl construct, it was demonstrated that suppression of Notch led to differentiation. This effect could perhaps be ascribed to the role of Hes1, a Notch target gene that was recently described to be upregulated *in vivo* in HSCs relative to their progeny (144). These data were consistent with previously published work which showed that induction of Notch in hematopoietic progenitors can promote multipotency while inhibiting differentiation down granulocyte lineages (145). On the other hand, the most well-defined role for Notch in the hematopoietic system is in lineage determination in immune cell precursors. Notch is required for the B versus T cell lineage as shown by both gain and loss of function in the hematopoietic system (146,147,148,149).

5.4. Notch in the Epidermis

The role of the notch pathway in HFSCs remains unclear. An epidermal knockout model for Notch1 displayed a marked defect in differentiation of the epidermis. In addition, it was argued that Notch can actually act as tumor suppressor in this system – a unique role for notch specific to the epidermis (150). The idea that Notch functions as a tumor suppressor in the epidermis while acting as a proliferative agent in most other systems, was called into question by recent findings describing the loss of function of RBP-J and gain of function of Notch1 in the embryonic epidermis (151). These more recent data argued that Notch's role in the epidermis is probably consistent with that in other developmental paradigms. Gain or loss of function of the Notch pathway in the specified cells of hair follicles led to dramatic defects in follicular differentiation, but as of now, no one has identified a role for Notch in adult HFSCs (152,153). Another loss of function study for RBP-J was done specifically in the hair follicle, but the dramatic loss of follicular integrity did not allow for a detailed examination of the SC niche (154). The RBP-J knockout animal eventually lost all its hair and formed cysts with epidermal markers, suggesting that the Notch pathway is required for maintenance of follicular fate. *In vitro*, the gain of function NICD protein induced differentiation into a spinous layer fate (155), and proliferation was thought to be impaired by upregulation of cell cycle inhibitor p21. Now that stem cell specific gain and loss of function methods for Notch have been developed, perhaps we will soon know what role, if any, Notch plays in the SC niche.

6. The Sonic hedgehog pathway

Hedgehog was first described in drosophila as a mutant in segment polarity (156). A murine homolog was soon cloned and renamed sonic hedgehog (157). Soon after, it was shown that sonic hedgehog is a ligand for a receptor called patched. This receptor acts as an inhibitor

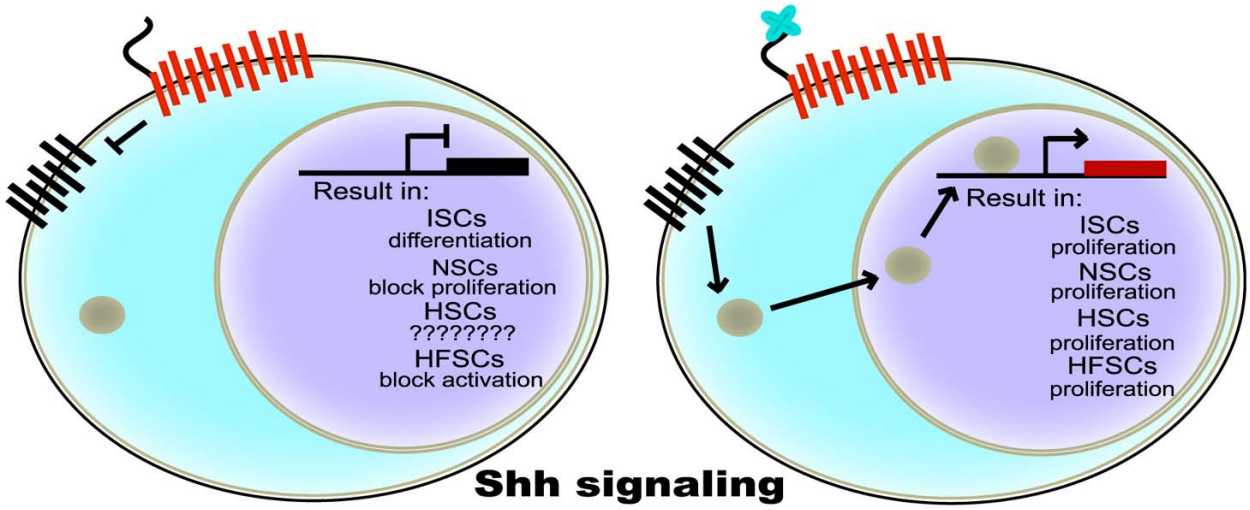

Figure 5. Activation of the Shh pathway in adult contexts most frequently leads to proliferation, as opposed to its role in development as a morphogenic determinant. Blockade of this pathway usually results in impaired growth, frequently leading to differentiation.

of another transmembrane protein called smoothened. The Shh signal actually inhibits patched which then ceases to inhibit smoothened, leaving smoothened free to stimulate a distinct set of transcription factors called Gli. Activated Glis enter the nucleus and drive expression of many newly identified genes (Figure 5). The sonic hedgehog pathway has been implicated in most developmental contexts, most frequently as a potent stimulator of proliferation. This pathway was also identified to be blocked in the phenomenon of cyclopia. This occurs frequently in bovine animals who happen to eat plants with high Cyclopamine content. This naturally made poison blocks the Shh pathway, leading to abnormal neural tube defects, where in the most severe cases only a single eye is formed during development. Many groups now take advantage of this toxin to specifically block the pathway in the study of tumorigenesis and development. In addition, many groups argue that the Shh pathway is subordinate to the Wnt cascade (94,158).

6.1. Shh in the Intestine

A definitive role for the hedgehog pathway in the intestine was first defined in Indian Hedgehog null mice. These mice had impaired ISC proliferation and smaller that normal villi (159). Another group used a blocking antibody for Shh to show that this pathway is important to maintain integrity of the tissue and in the organization of the villi. More recently, it was shown that intake of cyclopamine can block terminal differentiation of some lineages but drive almost all cells to a goblet cell fate (160). As evidence for crosstalk between the Wnt and Shh pathways, this group also showed that some well established Wnt target genes were upregulated upon cyclopamine intake.

6.2. Shh in the Brain

Shh was originally implicated in NSCs by the apparent expression of Gli factors in the NSC niche (162,163). Several groups have shown that Shh signaling is required for maintenance of NSCs in both the SGZ and the SVZ (164,165,162). Using a reporter sensitive to Shh target Gli1, another group recently demonstrated the *in vivo* role of Shh in maintenance of NSCs and their role in neurogeneis (166). In addition, a gain of function for Shh expressed by adenoviral transduction demonstrated that this pathway promotes proliferation in this niche. Conversely, cyclopamine, the Shh inhibitor, blocks proliferation (164,165). *In vitro*, the Shh pathway seems to play a critical role in neurosphere formation arguing either that proliferation is blocked or that no neurosphere forming cells are found in the absence of the Shh pathway (167). Interestingly, the Shh pathway, which is normally implicated in rapid proliferation also seems to be capable of more measured response such as that seen in NSCs. In addition, the Shh pathway acts as a morphogen during spinal cord development, and we could therefore speculate that Shh acts as more than simply a proliferation factor in adult stem cells (168).

6.3. Shh in the Blood

There is scant evidence for a role of Shh in HSC function. One report, however, showed that Shh can promote the repopulating efficiency of these cells in a reconstitution assay that apparently depended on a downstream BMP signal (169). This was presumably a result of increased proliferation of LT-HSCs, driving the production of progenitors for each of the various lineages.

6.4. Shh in the Epidermis

While much is understood about the role Shh plays in the epidermis, very little is actually known about whether this pathway affects adult HFSCs. Gain of function analysis has shown that Shh can drive the formation of basal cell carcinomas (170 This pathway is also known to be very active during initial hair follicle formation and in driving proliferation in the hair matrix (171). A recent study concluded that Shh does play a role in the adult HFSC niche (172). This group used a gain of

function model to show that Shh can drive proliferation in the niche, as well as throughout the epidermis. These mice displayed an expansion of all basal layer epidermis to such an extent that some mice actually had extra skin. Interestingly, other mice with the same transgene had a very different phenotype, where the skin was taught and translucent. The authors suggested that the same transgene could either induce p63 leading to a wrinkled phenotype or suppress p63 in the translucent phenotype. In the end, it was unclear whether either of these phenotypes were suggestive of a role for Shh in the follicular HFSCs. More likely, Shh simply acted as a general proliferative signal as it has in most other tissues. Years ago, our lab showed the Shh pathway was activated in the epidermis downstream of Wnt activation in the hair matrix (94). More recently, our study on elevated Wnt signaling in the HFSC niche demonstrated that in fact Shh is not induced in the niche in response to Wnt, but is induced later in the nascent hair germ (47). These data suggested the Shh might not normally play a role in the quiescent stem cell niche, but certainly is important in proliferation of the hair germ, a progenitor of the hair matrix, and certainly in the hair matrix itself.

7. PROSPECTIVE

The accumulated literature suggests that many of the known signaling pathways affect most adult stem cells in a similar manner. For instance, active Wnt signaling drives growth in ISCs, Neuroblasts, HFSCs, and ESCs. Interestingly, in intestine and epidermis, the cell division is asymmetric, as each division driven by nuclear beta-catenin creates both a self and a more differentiated daughter cell. On the other hand, in more lineage restricted/differentiated progeny, elevation of the Wnt signal drives terminal differentiation in intestine, brain, blood, and epidermis. This suggests that the response to the Wnt signal, although shared amongst stem cells, is different in their progeny. This conservation of response also holds true to some extent with regards to BMP signaling. In 3 out of 4 stem cells studied, inhibition of BMP drives proliferation, whereas stimulation of BMP promotes differentiation of both stem cells and more differentiated progeny. For the Notch pathway, clear data on adult stem cells only exists for ISCs, NSCs, and HSCs, but in each case Notch signaling is required for proliferation of progenitors. In the absence of Notch, these adult stem cells are thought to undergo apoptosis or differentiate. Lineage restricted cells, on the other hand, differentiate in the presence of the Notch pathway. Not surprisingly, the Shh pathway seems to drive proliferation in almost all cell types without regard to whether the cell is primitive or not.

The similarity with which adult stem cells from different tissues respond to various signals suggests that these cells share common physiological mechanisms for responding to these pathways. If that is the case, then perhaps these cells share other characteristics that make them uniquely stem cells. For instance, all stem cells can undergo self-renewal to create an exact duplicate daughter cell, but do they all use the same physiological mechanisms to achieve that end? Some groups have argued that overlapping gene expression profiles demonstrate that stem cells from different tissues share a “stemness” quality, but until the functions of these genes are rigorously tested, the answer will remain elusive. Given that the niche is composed of many different cell types, perhaps identifying the genes commonly found upregulated in the niche, as opposed to just stem cells, would shed more light on the possibility of a common physiology amongst stem cells. On the other hand, a look at the existing data in the literature on signaling pathways suggests that different stem cells do share a common interpretation of an extracellular signal, and that they do so differently from their more differentiated progeny.

Finally, the data discussed in this review describes the role of extracellular signals received in different contexts leading to different outcomes between stem cells and their progeny. In fact, this interpretation of the data ignores the idea that different doses of these signals might lead to different outcomes. None of the experiments outlined here described systems where each different cell type receives the same amount of signal. The possibility exists that the outcome of signaling is more a matter of signaling dose rather than the identity of the receiving cell. For instance, in the epidermis both the differentiated cells of the hair follicle and the undifferentiated cells of the bulge receive and respond to a Wnt signal. In the follicle, the Wnt signal is required for differentiation, while in the bulge the same signal drives proliferation. Is this discrepancy due to the context of the signal, or to the fact that the follicle cells sense a much higher dose of the signal? The next frontier in signaling in stem cells should include a more detailed examination of all signaling pathways with respect to any relation between dose, context and physiological response. Even more intriguing is the probability that we currently only understand the roles individual signaling pathways have on these cells, when, in fact, these cells are constantly sending and receiving signals to and from surrounding cells. Presumably, stem cells must integrate all these signals into a coherent response. This suggests that we will not be able to accurately predict or manage stem cell behavior until we understand how all the known and unknown signaling pathways act in concert in stem cells.

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