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Hedgehog Signaling in Hematopoiesis

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Abstract

The Hedgehog signaling pathway is highly conserved and plays an essential role in the embryonic development of a wide variety of organs. In adult tissues, such as the central nervous system, it may also be required for homeostasis and repair following injury. The role of Hedgehog signaling in regulating hematopoiesis is not entirely clear. Evidence exists that Hedgehog signaling is required for both primitive hematopoiesis in the developing embryo as well as definitive hematopoiesis in the adult. However, several studies also suggest that Hedgehog pathway activity is completely dispensable in post-natal hematopoiesis. In this review, we will discuss the current understanding of Hh signaling in vertebrate hematopoiesis as well as the contradictory findings that have been reported.

Keywords

Hedgehog signaling; hematopoiesis; hematopoietic stem cells

I. Hedgehog (Hh) signaling

A. Hh in development

The Hedgehog (Hh) signaling pathway was first identified in *Drosophila* almost 30 years ago as genetic loci required for proper anterior-posterior segmental patterning.[1] By examining mutations that disrupt the *Drosophila* larval body plan, Nusslein-Volhard and Weischaus identified several genes that resulted in the duplication of denticles, the spiked cuticular processes located on the anterior half of each body segment, as well as the loss of naked cuticles on the posterior segment. The appearance of a continuous lawn of denticles was reminiscent of the spines of a hedgehog, which thus gave rise to the characteristic name of the soluble factor and signaling pathway responsible for this phenotype. The disruption of antero-posterior positional information within the thoracic and abdominal segments suggested that Hh acts in a wide variety of developmental processes and serves as a classical morphogen to specify cell fates during embryogenesis.

Subsequent cloning and study of the *Hh* gene identified it as a unique secreted signaling factor that regulates cell proliferation, migration and differentiation during tissue and organ formation.[2] As exemplified by *Drosophila* wing development, Hh-mediated morphogenesis occurs in a spatial, temporal and dose-dependent fashion.[3] Here, Hh ligand is expressed and secreted by cells within the posterior compartment of the imaginal disc to establish a spatially defined concentration gradient across the anterior segment. Anterior segment cells respond to the local concentration of Hh ligand by modulating the expression of target genes and differentiating into distinct cell types to produce the mature wing.[4] Hh signaling is

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widely active during *Drosophila* development, and it is also required for proper formation of the leg and eye.[5, 6] Detailed studies in mice have demonstrated that the Hh pathway is highly conserved as it is required for the development of many organs, especially the skeletal and central nervous systems.[7] Furthermore, in some post-natal organs, the Hh pathway plays an important role in maintaining tissue homeostasis as well as repair and regeneration following injury.[8]

B. The Hh signaling cascade

In *Drosophila*, the Hh ligand is initially synthesized as a 45 kDa precursor that undergoes several post-translational modifications to form the active signaling molecule.[9, 10] A 19 kDa amino-terminal fragment is initially produced by an intramolecular cleavage reaction catalyzed by the carboxy-terminal portion of the precursor. It is then covalently coupled to two lipid molecules, cholesterol and palmitic acid, that further enhance its biologic activity and limit its diffusion within the extracellular space.[11, 12] These modifications are conserved within all Hh ligands from *Drosophila* to humans.[13] Cells responding to Hh ligand require two essential proteins, Patched (Ptch), a 12-pass transmembrane protein that serves as the Hh ligand receptor,[14] and Smoothened (Smo), a 7-pass transmembrane signal transducer.[15] Unlike many cellular pathways in which receptors function as signal transducers and directly induce pathway activity following ligand binding, Ptch represses Smo and inhibits pathway signaling in the absence of Hh ligand.[16] Upon ligand binding, the inhibitory effect of Ptch on Smo is relieved and signaling is activated. The nature of the interaction between Ptch and Smo remains poorly understood, but studies have suggested that they do not physically interact within the plasma membrane. Instead, Ptch is thought to catalytically regulate Smo by modulating the production or transport of a small molecule,[17] and recent studies in vertebrates have suggested that oxysterols, including vitamin D3, may serve as this intermediary.[18, 19] Smo activation ultimately results in activation of Cubitus interruptus (Ci), a zinc finger transcription factor that acts as either an activator or repressor of gene expression depending on its post-translational processing.[20] Cytoplasmic Ci is normally bound to the kinesin-like protein Costal2 (Cos2) allowing interaction with a number of other cellular factors including the serine/threonine protein kinase Fused (Fu), Suppressor of Fused (SuFu), Protein Kinase A, Glycogen Synthase Kinase 3, and Casein Kinase 1.[21, 22] In the absence of Hh ligand, full-length Ci is phosphorylated and undergoes limited proteolysis that removes the N-terminal transcriptional activation domain.[23] As a result, truncated Ci enters the nucleus and acts as a transcriptional repressor. Following the activation of Smo, Ci phosphorylation is altered and full-length Ci induces the expression of Hh target genes. The interpretation of different local concentrations of Hh ligand and specification of Ci transcriptional activity are beginning to be understood. Intermediate levels of Hh ligand result in the binding of Ci to SuFu in the cytoplasm, restricting nuclear import.[24] Stimulation by high levels of Hh ligand results in the dephosphorylation of Ci, allowing its dissociation from SuFu, and entry into the nucleus.

Divergence in Hedgehog signaling—The functional role of the Hh pathway and many of its components are conserved, but several differences exist between *Drosophila* and vertebrates. While only one *Hh* gene exists in *Drosophila*, three ligands have been identified in vertebrates, Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh).[2, 25] Dhh is most closely related to *Drosophila* Hh, while Ihh and Shh are more related to one another. Shh is widely expressed in vertebrates including three key signaling centers within the embryo, the notochord, the floor plate, and the zone of polarizing activity.[26] Hence Shh deficiency is embryonically lethal due to multiple defects in early to mid gestation.[2] Ihh is found within hematopoietic cells, bone, cartilage, and the eye, whereas Dhh is primarily expressed in the gonads, external genitalia, eyes and peripheral nerves. The activity of the single Ci transcription factor in Drosophila has been expanded to include

three homologues, Gli1–3, in mammals.[27, 28] Gli1 serves as a positive effector of Hh signaling, whereas Gli3 acts as a transcriptional repressor. Gli2 can function as both a positive and negative transcriptional regulator that is specified by both post-transcriptional and post-translational modifications.[29] The overall output of transcriptional activity is dictated by the balance between activator and repressor forms of these three transcription factors.[30]

The cellular localization of pathway components also plays an important role in Hh pathway activation. In *Drosophila*, Smo is localized within intracellular vesicles in the absence of ligand, then translocated to the plasma membrane upon ligand binding.[31] In vertebrates, the localization of pathway components occurs within the context of primary cilia.[32] In the absence of ligand, Ptch is located within the primary cilia whereas Smo is diffusely found within the plasma membrane.[33] Following ligand binding, Ptch moves out and Smo moves into the primary cilia where it interacts with Glis that subsequently enter the nucleus.[34, 35]

C. Developmental defects associated with aberrant Hh signaling

Given the conserved role of the Hh signaling pathway in development, it is not surprising that defects in pathway activity lead to congenital abnormalities in humans.[7, 36] Holoprosencephaly (HPE) is a cephalic disorder of varying severity characterized by the incomplete cleavage of the forebrain during embryogenesis.[37] The precise causes of HPE are unknown, but *SHH* haplosufficiency is clearly associated with this disorder in humans. In mice, the loss of a single copy of the *Shh* gene does not lead to HPE, but deletion of both alleles results in cyclopia and fusion of the cerebral hemispheres suggesting that forebrain development in humans is more sensitive to SHH loss than mice.[38] Mutations in *Ihh* result in brachydactyly that is characterized by shortened phalanges or metacarpals.[39, 40] Additionally, inactivation of Dhh in mice results in male sterility and defects in the peripheral nervous system.[41, 42] Mutations that inactivate *PTCH1* are among the best-recognized congenital defects arising from mutations within the Hh signaling pathway. Loss-of-function genetic lesions result in aberrant pathway activity and Gorlin Syndrome characterized by congenital defects of the brain and skeletal system.[43] Patients with Gorlin Syndrome are also predisposed to developing advanced basal cell carcinomas of the skin, medulloblastomas, and rhabdomyosarcomas suggesting that *Ptch1* functions as a tumor suppressor.[44–46] Finally, *GLI3* mutations have been identified in several congenital malformation syndromes that display characteristic limb abnormalities, including Greig Cephalopolysyndactyly and Pallister-Hall Syndrome.[47, 48]

II. Hedgehog signaling in Hematopoiesis

The hematopoietic system is required throughout embryonic, fetal and adult life to provide a continuous supply of functional blood cells. Hematopoiesis has been broadly divided into two major phases in vertebrates, primitive and definitive, based on the stage of development, location and cell types generated.[49] The Hh signaling pathway has been implicated in hematopoiesis, but the requirement for pathway activity and its precise role may differ depending on the specific developmental stage (primitive vs. definitive), cell type (hematopoietic stem cells (HSCs) vs. mature blood cells), and physiologic state (homeostatic vs. stress hematopoiesis) examined.

A. Role of Hh in primitive hematopoiesis

Primitive hematopoiesis takes place predominantly in the yolk sac and is characterized by the commitment of embryonic mesoderm to hematopoietic precursors including embryonic erythrocytes and macrophages.[50, 51] The formation of blood islands can be detected beginning at E7.5 in the mouse and is the first observable sign of hematopoietic activity.[52]

Ihh is expressed during this period by the visceral endoderm surrounding the epiblast as well as within the endodermal layer of the mature yolk sac.[53] The secretion of Ihh induces the expression of Hh target genes, such as *Ptch1* and *Gli1* within the epiblasts and results in both hematopoiesis and vasculogenesis.[54] Approximately half of all Ihh^{null} mice die at midgestation with yolk sac abnormalities indicating a role for *Ihh* in primitive hematopoiesis,[54, 55] but the lack of complete lethality also suggests that the requirement for *Ihh* is not absolute and may be compensated by other mechanisms. Additional evidence that Hh signaling is required for primative hematopoiesis has come from the examination of embryonic stem (ES) cell derived embryoid bodies (EB) that can form blood island-like structures *in vitro* and mimic the differentiation events that occur in the yolk sac.[56] *Ihh* deficient ES cell lines fail to form blood islands and exhibit reduced and disorganized vascular morphology.[57] Moreover, EB lacking *Smo* display similar defects suggesting that intact Hh signaling is required for normal yolk sac angiogenesis and blood island formation.

B. Role of Hh in definitive Hematopoiesis

Definitive hematopoiesis is characterized by the formation of multipotent hematopoietic stem cells (HSC). The aorta-gonad-mesonephros (AGM) region is thought to be the primary location for early definitive hematopoiesis,[58] but HSCs may also be found in the yolk sac and intraembryonically.[59, 60] By E9.5 hematopoiesis shifts to the fetal liver where it continues until birth.[61] Near term, the bone marrow becomes the major site of hematopoiesis and remains so for the remainder of life.[62] HSCs maintain blood production in the adult by maturing into multipotent progenitors (MPP) that subsequently differentiate into lineage-committed common myeloid (CMP) and lymphoid (CLP) progenitors that eventually produce mature blood cells.[63, 64] Furthermore, HSCs undergo self-renewal that allows the maintenance of blood production over the lifetime of the organism.

Similar to primitive hematopoiesis, the Hh pathway has been found to play a role in definitive hematopoiesis. Bhardwaj *et al* initially studied Hh signaling in definitive hematopoiesis and reported that primitive CD34+CD38-Lin- human cord blood HSCs express *PTCH1* and *SMO*, as well as the downstream transcription factors *GLI1*, *GLI2* and *GLI3*.[65] In addition, Hh pathway activation using exogenous Shh ligand stimulated the proliferation of HSCs while retaining their capacity to engraftment immunodeficient NOD/SCID mice in a multi-lineage fashion. Hh signaling may also be important in hematopoietic differentiation as treatment with cyclopamine, a naturally occurring inhibitor of SMO, can inhibit the production of human erythroid progenitors.[66] The Hh pathway may also play a role in angiogenesis as Shh can induce blood vessel formation that augments blood-flow recovery and limb salvage in a mouse limb ischemia model.[67] In this model, Shh has been reported to induce the expression of angiogenic cytokines such as vascular endothelial growth factor and angiopoietins from interstitial mesenchymal cells, suggesting that it may act as an indirect angiogenic factor.

These reports suggest that the Hh signaling pathway regulates several aspects of definitive hematopoiesis, and studies genetically targeting specific pathway components have provided additional insight into its specific roles. Given the large number of these studies, we will review experimental findings by each specific component.

Hh Ligand—Genetic manipulation of Hh ligand expression in zebrafish embryos has further demonstrated that pathway activity is essential for definitive hematopoiesis. Three Hh ligands are found in Zebrafish, *Shh*, *Tiggy-winkle hedgehog (Twhh)* and *Echidna hedgehog (Ehh)*.[68–70] Using loss of function *Shh* mutants or the pharmacologic pathway inhibitor cyclopamine, Gering and Patient reported that $Runx1^{+}$ cells that give rise to the first identifiable definitive blood cells were dramatically reduced.[71] In contrast, Hh-depleted

embryos had normal numbers of $\beta E1^+$ primitive erythrocytes, hence primitive hematopoiesis was not affected. Similarly, *Ihh* knockout mice exhibit a severe impairment in definitive erythropoiesis that manifests as a mid-gestation anemia that causes partial lethality.[72] Subsequent studies revealed that *Ihh* and *Gli1* expression were highest in the fetal liver stromal compartment. Thus, Ihh may act in a non-cell autonomous manner, perhaps within the fetal liver niche, to influence mature red blood cell production.

Patched—Mice heterozygous for *Ptch* ($Ptch1^{+/-}$) demonstrate increased Hh pathway activity and expansion of primitive HSCs in the bone marrow.[73] Following treatment with 5-fluorouracil (5-FU), these mice display improved recovery of peripheral blood counts compared to wild type mice, suggesting that HSCs and progenitors have increased proliferative capacity during the hematopoietic recovery. However, the proliferation of HSCs eventually leads to their exhaustion. In a separate report using the same mouse model, Dierks *et al* reported enhanced engraftment of $Ptch1^{+/-}$ fetal liver cells compared to wild type cells.[74] Mice engrafted with $Ptch1^{+/-}$ fetal liver cells also displayed enhanced count recovery following treatment with 5-FU, similar to the findings by Trowbridge *et al*.[73] Therefore, activation of the Hh signaling pathway enhances the regeneration potential of short-term repopulating HSCs. However, these results are not entirely consistent as Dierks *et al* failed to observe the exhaustion of long-term repopulating cells in mice transplanted with the $Ptch1^{+/-}$ fetal liver HSCs.

Smoothened—Both pharmacological and genetic manipulations of the Hh pathway suggest that active Hh signaling increases the proliferation of HSCs. Smo is a critical component of Hh signaling, but the embryonic lethality of somatic *Smo* knockouts pose a challenge to studying its role in hematopoiesis. Dierks *et al* examined the role of Smo in definitive hematopoiesis by studying fetal liver HSCs isolated from Smo^{null} mice and found no difference in engraftment compared to wild-type HSCs.[74] Two additional groups have used conditional models to inactivate *Smo* in HSCs of adult mice using the inducible *Mx1-cre* allele.[75, 76] Similar to the findings of Dierks *et al*, the loss of *Smo* had no effect on normal adult HSC function as peripheral blood counts, *in vitro* colony formation and HSC and progenitor subset frequencies were all unaffected. Furthermore, Smo^{null} HSCs exhibited no defects in homing, engraftment or long-term self-renewal during competitive and serial bone marrow transplantation. Hofmann *et al* further demonstrated that the pharmacologic inhibition of Hh signaling using a small molecule Smo antagonist also had no effect on normal hematopoiesis.[75] Moreover, *Smo* deletion in HSCs did not affect their ability to respond to 5-FU treatment. Gao *et al* also examined the expression of a constitutively active *Smo* allele in HSCs and found normal blood counts.[76] Together, these studies convincingly demonstrate that Hh signaling is dispensable for definitive hematopoiesis. However, another study in which *Smo* was conditionally deleted in hematopoietic cells using the *Vav-cre* allele found a profound defect in HSC function during primary and secondary transplantation.[77] The loss of *Smo* in zebrafish embryos also reduced the number of $Runx1^+$ definitive blood cells.[71]

Gli—All of the studies reviewed above have focused on up-stream modulators of Hh pathway activity (i.e., Hh ligand, Ptch1 and Smo). However, the main effectors of the Hh signaling pathway are the Gli transcription factors, and a recent report has characterized hematopoiesis in a $Gli1^{null}$ mouse model.[78] *Gli1* deficient mice are viable and show no gross developmental defects, and peripheral blood counts appear to be unaffected.[78, 79] Nonetheless, a detailed analysis of HSCs and progenitor subsets demonstrated decreased proliferation within the long term-HSC (LT-HSC), CMP and GMP compartments that correlated with lower levels of *Cyclin D1* expression. Increased HSC quiescence actually improved both short and long-term HSC engraftment as well as delayed peripheral blood

count recovery following 5-FU treatment. Furthermore, the differentiation of myeloid progenitors was also defective. These results suggest that Gli1 plays a role in definitive hematopoiesis and that Gli1 and Smo may not be functionally redundant.

III. Potential explanations for conflicting data

These data have generated conflicting views regarding the role of Hh signaling in hematopoiesis. In primitive hematopoiesis, Hh signaling, in particular the Ihh ligand, clearly plays a role.[53, 54, 56, 57] Pharmacologic and genetic manipulations of *SHh* ligand,[65, 71, 72] *Ptch1* or *Gli1* also suggest a role in definitive hematopoiesis.[73, 78] At least some of the conflicting reports in the literature can be directly attributed to differences in the experimental systems (human, mouse, zebrafish) and approaches (transgenic models, ES cells, *in vitro* cultures) that have been used to study Hh in hematopoiesis. Although the Hh pathway may be evolutionarily well conserved, it is likely that species-specific differences contribute to some of the controversies. Moreover, the spatial complexity of Hh signaling during embryonic development suggests that some of the differences may arise from the specific experimental approaches utilized. Although these factors may explain discrepancies between different species or approaches, they fail to explain stark inconsistencies in which the same organism is studied using the same approach. For example, it is difficult to reconcile the differences among the four separate reports examining complete disruption of *Smo* in mice. Three of these studies found that the conditional loss of *Smo* had no effect on hematopoiesis and HSC transplantation,[74–76] but a fourth report demonstrated that the conditional loss of *Smo* had a profound impact on engraftment.[77] Similarly, HSCs from $Ptch^{+/-}$ mice displaying increased Hh pathway activity were found to be more proliferative, and this enhanced proliferation eventually led to their exhaustion and decreased long-term repopulating capacity.[73] However, another report using the same $Ptch^{+/-}$ mice found enhanced long-term HSC engraftment.[74]

Both hematopoietic development and the activities of developmental signaling pathways are highly regulated in a spatial and temporal manner. Therefore, it is possible that conflicting data arise from differences in experimental design that impact these parameters. In studies examining conditional *Smo* loss of function, different promoters were used to express the cre recombinase. Zhao *et al* used the *Vav* promoter that is first expressed in the blood forming islands and megakaryocytes of the fetal liver and induces recombination starting at day E11.5.[80] In contrast, the *Mx1* promoter used by Gao *et al* and Hofmann *et al* is induced following injection of poly I:C that allows recombination to be carried out in adulthood.[81] Moreover, the *Vav* promoter induces recombination within both the hematopoietic and endothelial compartments,[82, 83] whereas *Mx1* is primarily activated within HSCs, lymphocytes and liver cells.[84] Thus, differences between these studies may be due to the distribution and timing of *Smo* inactivation, and it is possible that Smo plays a more critical function during early definitive hematopoiesis. It is also possible that some of these discrepancies may be a consequence of Hh signaling exerting its effects in a non-cell autonomous manner to influence hematopoiesis, such as through bone marrow endothelial cells which are in close proximity to primitive HSCs in the *Vav-cre* model.[85] Mandel *et al* reported another example, where *Drosophila* hematopoietic precursors are maintained in a quiescent precursor state by a Hh dependent niche.[86] Ihh overexpression in murine bone marrow stromal cells have also been shown to promote hematopoietic regeneration after bone marrow transplantation, suggesting a role for these stromally-derived Hh signals in HSC homeostasis.[87] In the case of the $Ptch^{+/-}$ heterozygotes, differences may be similarly accounted for by experimental conditions. In the Trowbridge *et al* study, HSCs were isolated from adult mice,[73] whereas Dierks *et al* studied fetal liver HSCs.[74] Thus, both the timing and source of HSCs may have influenced the observed results.

Discrepancies between the effects observed following the inhibition or activation of Hh signaling may also be explained by functional redundancy. During early embryonic development, Dhh may compensate for the loss of Ihh in hematopoiesis and vasculogenesis.[88] Similarly, Gli1 may compensate for the loss of Gli2.[89] It is also possible that other developmental signaling pathways, such as Wnt and Notch play overlapping roles in hematopoiesis and may partially or totally compensate for any deficiencies in Hh signaling.[90, 91]

Conclusions and future directions

Uncertainties surround the exact role of the Hh signaling pathway in hematopoiesis, but many of these controversies can be attributed to methodological differences or spatio-temporal relationships required for proper blood formation. A better understanding of the precise role of Hh signaling in primitive hematopoiesis may improve ongoing efforts to generate normal hematopoiesis and peripheral blood cells from ES or induced pluripotent stem cells. Resolution of these controversies surrounding the role of Smo in definitive hematopoiesis, especially within humans, may come from examining the incidence of cytopenias and studying HSCs from patients treated with highly specific SMO antagonists that have begun clinical testing as novel anti-cancer agents.[92] In addition, a better understanding of how the Hh pathway regulates normal hematopoiesis may provide important insights into the use of Hh inhibitors in hematopoietic cancers such as myeloid leukemias and myelodysplastic syndrome.

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