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Review Article

Cell signaling pathways involved in hematopoietic stem cell specification

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Introduction

Generation of HSCs for regenerative medicine

Hematopoietic stem cells (HSCs) are self-renewing, tissue-specific stem cells that give rise to all mature blood cell types. The capacity of HSCs to reconstitute the entire adult hematopoietic system after transplantation makes them invaluable for the treatment of various blood disorders. A significant limitation of this treatment is the need for immune compatibility between donor and host, thus there has always been an acute need for reliable cultivation or generation of HSCs. The recently demonstrated ability to generate induced pluripotent stem (iPS) cells that resemble embryonic stem cells (ESCs) now make generation of HSCs from stem cells a realistic goal. To date, pluripotent stem cells have been instructed by a variety of experimental approaches to recapitulate waves of hematopoiesis such as primitive and transient definitive cells [1], myelomonocytic cells [2], and multilineage progenitors with lymphoid potential [3] (Fig. 1A). Surprisingly, concerted efforts to generate functional HSCs in vitro from pluripotent stem cells have thus far proven unsuccessful, indicating that our understanding of de novo generation of HSCs is insufficient [4] (Fig. 1B). Therefore, it is crucial to precisely characterize the mechanisms of cell signaling events that occur in vivo to form functional HSCs. Importantly, recent studies mapping the process of HSC generation in vertebrate embryos demonstrated that HSCs emerge from hemogenic endothelium present in the floor of the dorsal aorta (DA) [5–9]. For this reason, the generation of hemogenic endothelium likely represents a critical prerequisite for successfully generating HSCs in vitro. While many major cell-signaling pathways conserved throughout the animal kingdom have been demonstrated as requirements for DA and/or HSC formation, the molecular mechanisms that each required effector molecule exerts in this context is unclear. In this paper we summarize the roles of select cell-signaling pathways in HSC generation in the embryo and provide perspective on the in vitro instruction of HSCs fate for use in regenerative medicine.

Hematopoietic stem cell emergence in the vertebrate embryo

The HSCs that maintain homeostasis of the adult hematopoietic system are generated during embryogenesis, but are not the first blood cells to be formed in the embryo. HSC emergence is preceded by primitive and definitive waves that are defined by limited differentiation potentials. Primitive myeloid and erythroid cells are the first hematopoietic cells to emerge, but unlike adult blood progenitors, do not possess multilineage potential or the capacity to self-renew [10,11] (Fig. 1C). Following these primitive waves, the first transient definitive progenitors arise that possess multipotent erythromyeloid potential (EMPs) [10,12,13]. EMPs are similar to HSCs in that they have multilineage potential, but are separated by the fact that they do not possess lymphoid potential or the capacity to appreciably self-renew (Fig. 1D). The anatomical sites of emergence from which these waves arise vary according to species as shown by transplantation, imaging, and lineage tracing studies. The first three hematopoietic waves are found in the yolk sac in mammals and birds, in anterior/posterior ventral blood islands in frogs, and anterior/posterior lateral mesoderm in fish, the details of which are reviewed elsewhere [14,15]. In contrast, HSCs emerge from hemogenic endothelium within the floor of the dorsal aorta in a process termed endothelial to hematopoietic transition (EHT) in all vertebrate species analyzed [6,7,9,16] (Fig. 1E). Nascent HSCs have been defined by their

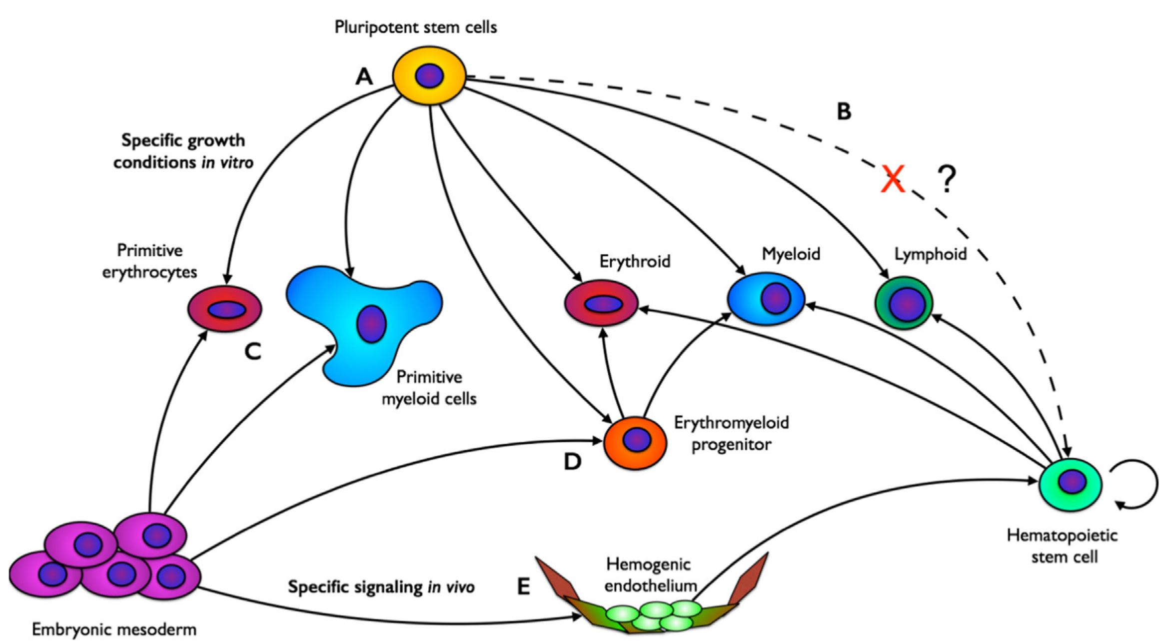

Fig. 1 – Pathways to hematopoietic differentiation in vitro and in vivo. (A) Pluripotent cells from embryonic or induced pluripotent sources have not been successfully instructed to hematopoetic stem cell fate (B), but have been successful in generating primitive (C) and transient definitive blood (D) cell fates. Embryonic hematopoiesis proceeds in four ordered waves with primitive erythroid and myeloid waves preceding a definitive EMP wave, and culminates with the establishment of adult definitive hematopoiesis through specification of hematopoietic stem cells via ventral aortic endothelium (E).

capacity in mammals to long-term reconstitute immune-deficient adult recipients and colonize adult hematopoietic organs [17,18]. Developmental hematopoiesis thus progresses through four ordered waves, the last of which generates HSCs.

A major question regarding the ontogeny of the hematopoetic system is how different regions of mesodermal derivatives are specified into each of these related but distinct fates. Transplantation experiments performed in frogs demonstrate that mesodermal precursors to blood cells experience bipotency for either primitive or definitive fate potential until the neurula stage [19], indicating that environmental signals are important during key stages of programming to different fate outcomes. Many of the signaling molecules involved in HSC specification are dispensable for other hematopoietic waves, indicating that the combined inputs that bestow HSC potential may be specific. Here we highlight the major signaling pathways involved in HSC emergence that are conserved across vertebrates.

Bmp signaling

Bmp signaling is part of the transforming growth factor-β (TGFβ) [2] superfamily that regulates many cellular processes and fate decisions during early embryonic development. Bmps signal through Type I and II receptors that heterodimerize in response to ligand binding. As activated complexes, these proteins phosphorylate Smad proteins that regulate the expression of a multitude of genes [20]. Bmp is required for embryogenesis during gastrulation and for the specification of mesoderm [21,22], but has also been implicated in a later role in HSC emergence. Bmp4 is expressed in the mesenchyme surrounding the developing dorsal aorta, and antisense knockdown leads to a loss of HSCs in zebrafish [23], suggesting it is a key determinant of HSC fate. In mammals, chemical inhibition of Bmp signaling reduced HSC numbers contained within the aorta–gonads–mesonephros (AGM) region, indicating that this requirement for Bmp4 is conserved in vertebrates [24]. In addition, mouse AGM explants that contain nascent HSCs were enhanced for repopulating potential following the addition of Bmp4 [24]. These results suggest that Bmp promotes the generation, homing efficiency, and/or survival of HSCs. Downstream effectors of Bmp signaling, Smad1 and Smad5, are expressed in tissues around the sites of HSC emergence [25]. Endothelium-specific inactivation of Smad1/5 results in embryonic lethality in mice before HSC specification [26], but recent studies in zebrafish demonstrate that low-dose knockdown of Smad1/5 that bypass early embryonic requirements have specific defects in HSC formation [27]. Specific genetic excision of Smad1 and Smad5 in specified blood had no effect on hematopoiesis [28], suggesting that Bmp signaling is dispensable after HSC commitment. These findings position the requirement for Bmp signaling during two distinct time windows during differentiation; first during mesoderm commitment, and later just prior to HSC specification in the local HSC microenvironment (Fig. 2A).

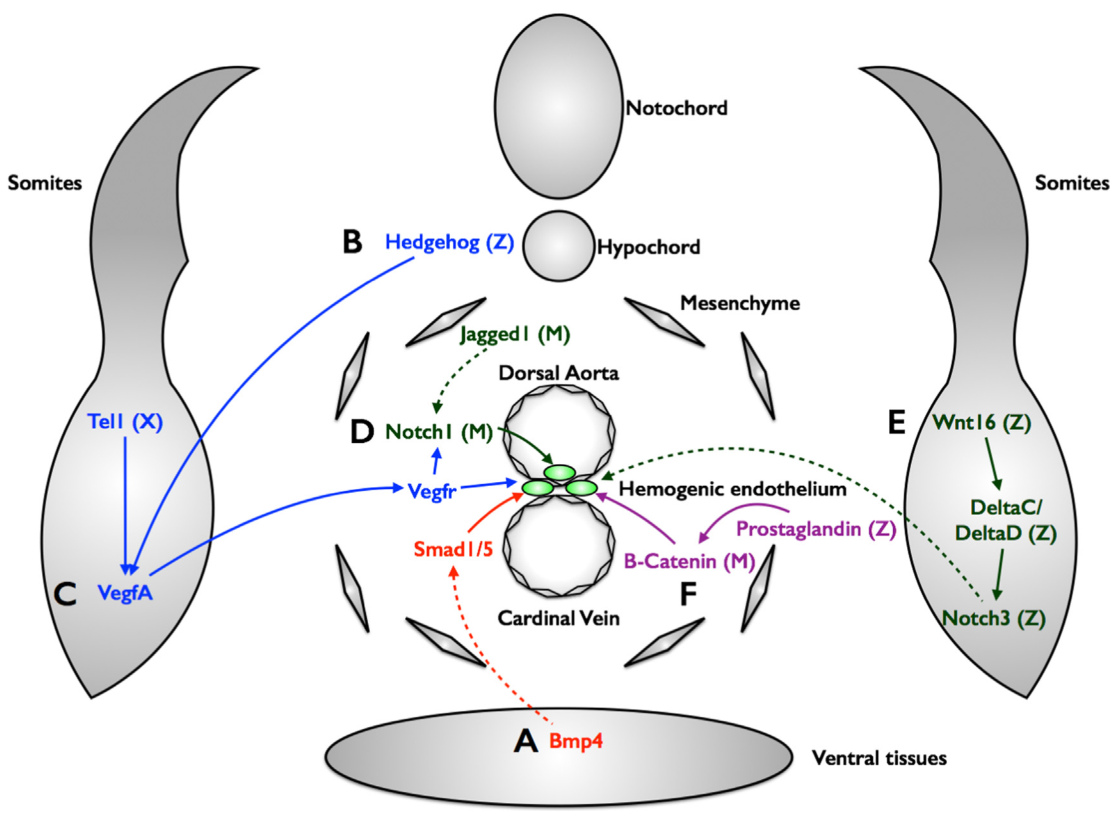

Fig. 2 – Cell signaling pathways involved in HSC specification, at a glance. A virtual cross section through the vertebrate embryo (zebrafish) with Bmp (A), Hedgehog (B), Vegf (C), Notch (D), non-canonical Wnt/Notch (E), and canonical Wnt signaling requirements depicted (F). Genes required for HSC emergence are annotated, including their anatomical site of expression and known epistasis within each pathway. Species is indicated in instances where gene function has only been identified in mouse (M), Xenopus (X), or zebrafish (Z). Solid lines indicate that there is evidence for a genetic relationship between proteins, while dotted lines indicate uninvestigated but plausible relationships.

Hedgehog signaling

Hedgehog (Hh) signaling is involved in a wide range of activities during development that are conserved throughout the animal kingdom, including axis and segment patterning of the vertebrate body plan. Hh binds to the Patched transmembrane receptor [29], that in the absence of ligand-binding, inhibits a required transmembrane signal transducer Smoothened [29]. Uninhibited smoothened is then free to activate the zinc-finger transcription factor Cubitus interruptis (Ci) that becomes phosphorylated and available to interact with and activate a wide range of kinase pathways, ultimately translocating to the nucleus to activate expression of Hh target genes [30]. In mammals, the loss of Sonic Hedgehog (Shh) or Smoothened results in embryonic lethality. However, murine AGM explants cultured with exogenous Hh generated HSCs with increased transplantation efficiency [31]. In zebrafish, the notochord and floor plate are the main sources of Hh ligand during the time window of HSC specification, suggesting that these tissues play important signaling roles during HSC formation. Importantly, genetic loss of function or chemical inhibition of Hh signaling by cyclopamine results in a specific loss of HSC specification in the DA [23,32]. As demonstrated by epistasis experiments, Hh signaling is genetically upstream of vascular endothelial growth factor (Vegf) signaling, which controls Notch activation in the endothelium [33] (Fig. 2B). Collectively, these studies indicate that Hh signaling is a key regulator of an essential signaling cascade responsible for vascular patterning and the subsequent generation of hemogenic endothelium.

Vegf signaling

Unlike the previous pathways mentioned, Vegf signaling is required after gastrulation and axis formation and exclusively by endothelial cells. There are four Vegf ligands (VegfA, B, C, and D), and multiple known isoforms of VegfA are produced by alternative splicing, which play distinct roles in regulating proliferation, migration, survival, and/or permeability [34,35]. Vegf ligands bind with variable affinity to the Vegf receptor tyrosine kinases, Vegfr-1, 2, or 3, leading to the hetero- or homodimerization of receptors and subsequent activation by autophosphorylation [36]. In mice, the loss of a single Vegf allele results in severe vascular defects, resulting in embryonic lethality before or during HSC specification [37,38]. In zebrafish, the Vegf receptor Kdrl is expressed throughout the vasculature and genetic or pharmacological inhibition results in the combined loss of DA and HSC specification [32,33]. Studies in Xenopus elucidated that VegfA is required for HSC formation through multiple inputs; longer $VegfA_{170}$ and $VegfA_{190}$ isoforms are required for HSC specification but dispensable for DA specification, whereas the shorter diffusible $VegfA_{122}$ isoforms lacking extracellular matrix-binding domains are required for both processes, as shown by analysis of isoform-specific mutants [39]. In addition, VegfA production in lateral plate mesoderm and somitic tissues is dependent upon the transcriptional activity of Tel1. Dorsal lateral plate (DLP) mesoderm that normally give rise to HSCs in vivo are capable of hematopoiesis in vitro when co-cultured with wild-type somites, but Tel1-deficient somitic cells that do not secrete VegfA are deficient in promoting hematopoiesis from wild-type DLP [40]. This data is in agreement with the observation that VegfA is produced in the somites of zebrafish [33]. Thus, Vegf signaling is important for the formation of the DA and HSCs from endothelial precursors (Fig. 2C).

Notch signaling

Notch signaling is a cell-to-cell signaling pathway involved in a wide range of cellular fate decisions including lineage commitment, lateral inhibition between neighboring cells, and maintenance of homeostasis [41]. Key proteins involved in Notch signaling include Notch receptors (Notch1–4 in mammals), their cognate Jagged/Delta ligands that vary in number across species, enzymes that modify Notch ligands during activation (Mindbomb), proteases that cleave activated receptors (gamma secretase/ADAM TACE) to release a transcriptionally active Notch intracellular domain (NICD), as well as an array of intracellular proteins that facilitate transcriptional repressive (RBPj/CSL) and/or activating complexes (Mastermind and Mastermind-like) [reviewed in depth in [41,42]]. Many Notch signaling pathway proteins are required for HSC specification. Loss of Mindbomb and RBPj, both of which are essential for Notch signaling, leads to loss of HSCs in developing embryos [43–45]. Additionally, the Notch1 receptor is required in a cell-autonomous manner to specify HSCs as shown by blastula chimera experiments in [46,47]; mouse mutants also display vascular and aortic defects [48]. The necessity for Notch1 in both of these processes may reflect a dual requirement for Notch, since many studies have implicated, but not directly shown, that DA specification is a functional prerequisite for HSC specification. Unlike Notch1 mutants, mutants for the Notch ligand Jagged1 are not defective in DA formation but similarly fail to specify HSCs, suggesting that there are likely multiple requirements for Notch signaling in HSC specification [49] (Fig. 2D). Recently our laboratory has uncovered, through loss of function and spatiotemporally-controlled NICD rescue experiments, that Notch3 is required in the somites to specify HSCs [50]. This non-cell-autonomous requirement is genetically downstream of a previously indentified Wnt16 regulated somitic signaling cascade [51] (Fig. 2E). Collectively these findings indicate that Notch signaling orchestrates intrinsic as well as environmental programs to instruct HSC fate.

Wnt signaling

Canonical Wnt signaling is involved in the specification and homeostasis of many tissues. In mammals, the Wnt pathway is comprised of 19 secreted ligands that directly associate with Frizzled receptors and co-receptors expressed on the surface of many diverse cell types [52,53]. In the absence of ligand binding, β-catenin is normally targeted for degradation by a 'destruction complex' of proteins [54,55]. However, upon the ligand-induced activation of Wnt receptors, this protein complex is inactivated and β-catenin translocates to the nucleus to bind the TCF/LEF transcription factors that activate target gene transcription [56]. In mice, genetic deletion of β-catenin in VE-Cadherin^{+} endothelium results in hematopoietic defects, but has no effect when genetically deleted in Vav1^{+} committed blood cell precursors, suggesting that this requirement for Wnt signaling is in cells during or just before they become hemogenic endothelium [57]. Interestingly, in this context arterial specification is unaffected in the endothelium of mutant embryos, suggesting that Wnt signaling is dispensable for the aortic program. In contrast, gain-of-function studies demonstrate that over-activation of β-catenin results in an upregulation of arterial markers. These data indicate that Wnt signaling plays discrete roles in HSC and arterial fates (Fig. 2F).

Interaction between signaling pathways

The fact that specific molecules from Bmp, Hedgehog, Vegf, Notch and Wnt signaling pathways are required for HSC formation during development raises important questions about when and where each is required, and regarding interactions between pathways. The expression pattern of most of these required molecules is dynamic, suggesting that location and timing of these signals is tightly regulated and related to their functional roles. The fact that diffusible Hh and Vegf are secreted from tissues physically separated from the DA, while direct cell-to-cell contact through Notch and Jagged1 occurs proximal to the DA, suggest that these diverse signals must be initiated and received at specific spatial locations during ontogeny (compare Fig. 2C–D). Additionally, timing is an important consideration, as evidenced by the requirement for non-canonical Wnt16 function in the regulation of somitic expression of DeltaC/DeltaD in zebrafish [58]. The failure to specify HSCs in Wnt16-deficient embryos can be rescued by ectopic activation of Notch signaling during mid-somitogenesis, despite the fact that HSCs are not specified for many hours afterwards (Fig. 2E). This surprising inflexibility in the timing of this molecular requirement may be attributed to coordination between multiple signaling pathways. Canonical Wnt signaling also interacts with other signaling pathways to specify HSCs, as demonstrated by the requirement for prostaglandin via a β-catenin-dependent mechanism [59] (Fig. 2F). It is likely that other signaling pathways are utilized iteratively for HSC specification at multiple sites and/or times during ontogeny that trigger different genetic, cellular, and morphogenetic outcomes.

Conclusion

While only a subset of the known signaling requirements in HSC specification have been discussed in this review, many have been utilized in attempts to recapitulate hemogenesis in vitro. Wnt, Bmp, Vegf, and Notch have been utilized in combination with other factors in well-defined supportive conditions in vitro to generate multilineage blood cell precursors from pluripotent stem cells. These approaches have not yet, however, led to generation of engraftable HSCs [1,3,60]. One possible explanation for these difficulties is that the growth conditions utilized are missing key signals and/or that some of these signals are provided in an inappropriate context for HSC emergence. Investigation of the mechanisms involved in HSC specification in the embryo is a rapidly advancing field (Fig. 3A), therefore strategies for the generation of HSCs must continue to evolve accordingly.

Alternative strategies involving reprogramming also hold great promise for generating HSCs. Recently, a study demonstrated that terminally differentiated myeloid and lymphoid cells could be dedifferentiated back to HSCs by brief induction of transcription factors (Run1t1, Hlf, Lmo2, Prdm5, Pbx1, and Zfp37) and subsequent transplantation [61], indicating that HSC identity can be reacquired in blood cells by molecular reprogramming and may thus represent a source of HSCs in the foreseeable future (Fig. 3B). Surprisingly, even non-hematopoietic differentiated fibroblasts

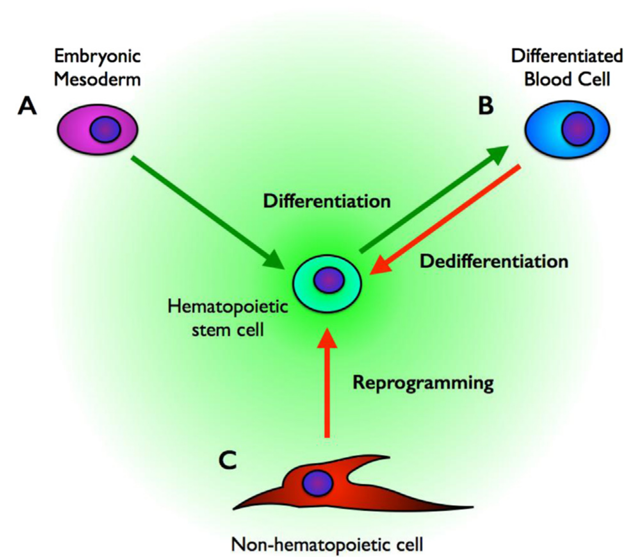

Fig. 3 – Multiple sources for HSC generation. Normal embryonic developmental processes specify HSCs from mesoderm, and differentiated blood from committed progenitors with instrinsic and extrinsic signals denoted by green arrows (A). Recent studies have established conditions using ectopic induction of transcription factors and supportive signaling conditions denoted by red arrows to dedifferentiate hematopoietic cells (B) or reprogram non-hematopoietic lineages to HSC-like fates (C).

have been transformed to hematopoietic progenitor fates (Fig. 3C), as shown by ectopic expression of transcription factors Oct4 or Gata2, Gfi1b, cFos, and Etv6 in fibroblasts [62,63]. Combining reprogramming along with supportive microenvironmental cells is also a promising strategy; recently, human umbilical vein cells induced with transcription factors (Fosb, Gfi1, Runx1, and Spi1) plated on supportive vascular monolayers designed to mimic the hemogenic endothelial niche acquired hematopoietic fate and engrafted into immune-deficient mice [64]. In summary, the major goals now in the fields of regenerative medicine and HSC biology are to understand how certain tissues can be reprogrammed to an HSC-like fate, how reprogramming and normal embryonic programming of HSCs compare to one another, and how normal HSC development can be recapitulated in vitro without the use of potentially oncogenic gene transduction approaches (Fig. 3). Key advancements in investigating and replicating HSC induction will involve the generation of reagents and protocols that allow unprecedented precision in the observation, induction, and manipulation of essential, cooperative molecular inputs.

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