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Novel roles for Notch, Wnt and Hedgehog in hematopoesis derived from human pluripotent stem cells

CHANTAL CERDAN and MICKIE BHATIA*

McMaster Stem Cell and Cancer Research Institute (SCC-RI), McMaster University, Hamilton, Ontario, Canada

ABSTRACT Human pluripotent stem cells (PSCs) derived from a number of different sources, including reprogrammed adult somatic cells, provide a powerful cellular system to study signaling pathways implicated in cell fate decisions, and generate new sources of cells for regenerative medicine. To realize this potential, it is essential to control the direction and efficiency of human PSC differentiation. Although Notch, Wnt and Hedgehog (HH) signaling pathways have been implicated in the self-renewal/proliferation of hematopoietic stem/progenitor cells, both *in vitro* and *in vivo*, their roles in differentiation processes remain poorly explored. This review describes the role(s) of these pathways in the adult and embryonic hematopoietic system of mice and humans, with a particular emphasis on our recent studies on the hematopoietic development of human embryonic stem cells (hESCs). Understanding the individual and collective contributions of Notch, Wnt and HH signaling to the initial development of hematopoiesis is critical for achieving successful *ex vivo* expansion and differentiation of hematopoietic stem cells (HSCs) from human PSCs that will retain *bona fide* function comparable to adult-derived HSCs.

KEY WORDS: *human pluripotent stem cell, hematopoiesis, Wnt, Notch, Hedgehog*

Introduction

Hematopoietic stem cells (HSCs) are responsible for life-long production of the blood system. To this end, HSCs must establish a balance between the two opposing cell fates of self-renewal - for maintenance of the HSC pool - and differentiation into terminally mature progenies (reviewed in Giebel and Bruns, 2008). In clinical therapy, HSCs are typically derived from adult tissues [bone marrow (BM), umbilical cord blood (UCB), or mobilized peripheral blood (MPB)] and have been successfully used to treat a variety of acquired/genetic disorders and malignancies. However, the limited availability of adult-derived HSCs and their compromised *in vivo* potential after *ex vivo* culture (Attar and Scadden, 2004) have hampered their use in large-scale clinical applications; therefore, calling for a more readily available and renewable source of transplantable cells. In this regard, human embryonic stem cells (hESCs) (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000) and the more recently derived induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006; Park *et al.*, 2008; Yu *et al.*, 2007) provide two alternative sources of pluripotent stem cells (PSCs) that demonstrate both indefinite proliferative capacity *in vitro* (Avery *et al.*, 2006) and pluripotent differentiation potential (Yu and Thomson, 2008), including the potential to form blood.

HSCs are functionally defined by their ability to reconstitute the hematopoietic system of immunodeficient animals, *e.g.* NOD/SCID mice (Dick *et al.*, 1997; Mazurier *et al.*, 2003) and equally contribute to functional reconstitution in human transplant settings (Grewal *et al.*, 2003). While ectopic transcription factors of the *Cdx/Hox* pathway can modulate the functional behavior of adult HSCs (reviewed in Klump *et al.*, 2005) and confer HSC properties to mouse ESCs (Kyba *et al.*, 2002; Wang *et al.*, 2005a), this is not the case with hESCs where the generation of "putative" HSCs, using various methodologies, animal recipients and injection routes has yielded significant lower levels of reconstitution, as shown by our laboratory (Wang *et al.*, 2005b; Ji *et al.*, 2008) and later others (Lu *et al.*, 2007a; Lee *et al.*, 2008; Narayan *et al.*,

Abbreviations used in this paper: EB, embryoid body; hESC, human embryonic stem cell; HH, hedgehog; HSC, hematopoietic stem cell; PSC, pluripotent stem cell.

***Address correspondence to:** Mickie Bhatia. McMaster Stem Cell and Cancer Research Institute (SCC-RI), Michael G. DeGroote School of Medicine, McMaster University, 1200 Main Street West, MDCL 5029, Hamilton, Ontario, Canada, L8N 3Z5.
e-mail: mbhatia@mcmaster.ca - web: www.fhs.mcmaster.ca/SCCRI

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2006; Tian *et al.*, 2006; Ledran *et al.*, 2008).

In vitro, the hematopoietic potential of hESCs is routinely assessed by two methodologies that are largely adapted from the murine system: 1) coculture with stromal cells and 2) aggregation into three-dimensional structures known as embryoid bodies (EBs) (both reviewed in Tian and Kaufman, 2008). Both methodologies allow for the spontaneous differentiation of hESCs into blood lineages, albeit at low efficiency. This efficiency can be enhanced through manipulation of extracellular or intracellular regulators (Wang *et al.*, 2005c; Menendez *et al.*, 2005). However, the true contribution of extrinsic pathways that regulate this complex and dynamic process needs to be adequately defined for achieving controlled and efficient hematopoietic differentiation. By providing access to isolated cell populations at different developmental stages, human PSC cultures allow us to more precisely dissect the signaling and timing requirements for early hematopoiesis.

Based on the differentiation procedures outlined above, a series of studies (Chadwick *et al.*, 2003; Cerdan *et al.*, 2004; Chadwick *et al.*, 2003; Wang *et al.*, 2005d; Wang *et al.*, 2004; Ji *et al.*, 2008) from our laboratory have provided a detailed phenotypic and temporal roadmap of hESC-derived hematopoietic development towards myeloid/erythroid lineages. This work demonstrated that cells developing within EBs treated with hematopoietic cytokines (mainly SCF and FLT3L) and the ventral mesoderm inducer BMP-4 remain uncommitted to the hematopoietic cell fate up to 10 days of development, as defined by the lack of CD45 expression and colony forming unit (CFU) activity (Chadwick *et al.*, 2003). During this developmental window, a subset of endothelial-like cells expressing PECAM-1, Flk-1/KDR and VE-cadherin, but not CD45 (termed $CD45^{neg}$PFV) develops, which following clonal isolation and differentiation in culture, gives rise to both endothelial and hematopoietic progenies (Wang *et al.*, 2004); therefore, representing the equivalent of the "hemangioblast" or "hemogenic endothelial" precursor identified across species and hematopoietic sites (Huber *et al.*, 2004; Choi *et al.*, 1998; Nishikawa *et al.*, 1998; Jaffredo *et al.*, 2000; Zambidis *et al.*, 2005; Kennedy *et al.*, 2007; Lu *et al.*, 2007b).

The hematopoietic system is believed to arise from common progenitor cells of mesodermal origin and to proceed through an initial "primitive/yolk sac" stage comprising mostly erythrocytes and macrophages, followed by "definitive" hematopoiesis encompassing the full range of blood cells encountered in the adult organism, including HSCs. Signaling from critical growth/morphogenetic factors that are shared between vertebrates influences both primitive and definitive hematopoiesis. Contrary to hematopoietic cytokines such as FLT3L, SCF and TPO that can promote HSC survival but not expansion (Murdoch *et al.*, 2002), bone morphogenetic proteins (BMP) (Sadlon *et al.*, 2004), Notch (Radtke *et al.*, 2005), Wnt (Wodarz and Nusse, 1998) and Hedgehog (HH) (Baron, 2001) have been established as key stem cell signaling pathways involved in fate specification, self-renewal, and differentiation. With the exception of BMP signaling similarly required for hematopoietic differentiation of mouse and human ESCs (reviewed in McKinney-Freeman and Daley, 2007), none of the Notch, Wnt and HH pathways have been explored with respect to their ability to regulate early human hematopoiesis.

Accordingly, this review summarizes our current knowledge about the roles of Notch, Wnt and HH pathways in the regulation of adult and embryonic hematopoiesis with a particular emphasis on recent progress of our laboratory on the hematopoietic development of hESCs in the context of these pathways and comparison with the mouse system.

The Notch pathway

The Notch signaling pathway regulates a broad spectrum of stem cell fate decisions such as neurogenesis, myogenesis and hematopoiesis (Chiba, 2006). Notch signaling is activated through four receptors (Notch1-4) that can interact in a redundant manner with five ligands of the Delta/Jagged family (Bray, 2006). Ligand binding triggers a γ-secretase-dependent proteolytic cleavage of Notch receptor and the release of Notch intracellular domain (NICD) to the nucleus (De Strooper *et al.*, 1999), which in turn displaces the co-receptors associated with CSL transcription factors (CBF1 in humans; RBPJ in mice). Activating transcription factors are then recruited and expression of target genes such as Hairy and Enhancer of Split HES1, HES5 and Deltex1 is induced (Bray, 2006; Davis and Turner, 2001).

Although recently extended to the regulation of megakaryopoiesis (Mercher *et al.*, 2008), the role of Notch signaling has been best characterized in T cell lineage specification (reviewed in Radtke *et al.*, 2004) and its deregulation found associated with leukemia (mainly T-ALL) in humans (Grabher *et al.*, 2006). In adult hematopoiesis, activation of Notch signaling has been reported to promote HSC expansion/self-renewal in both mice and humans (Karanu *et al.*, 2000; Karanu *et al.*, 2001; Karanu *et al.*, 2003; Varnum-Finney *et al.*, 1998; Ohishi *et al.*, 2002; Carlesso *et al.*, 1999; Dando *et al.*, 2005); however, loss-of-function studies in mice have not clearly supported this conclusion (Radtke *et al.*, 1999; Saito *et al.*, 2003; Mancini *et al.*, 2005; Maillard *et al.*, 2008). In particular, inactivation of Notch receptors (Notch1, Notch2), ligands (Jagged-1) or downstream effectors (CSL/RBPJ, Mastermind-like1) does not impair HSC function. In mouse embryonic hematopoiesis, gene ablation strategies have revealed non overlapping roles for Notch1 and Notch2 in definitive hematopoiesis and B cell development, respectively (Hadland *et al.*, 2004; Robert-Moreno *et al.*, 2008; Huppert *et al.*, 2000; Krebs *et al.*, 2004; Kumano *et al.*, 2003; Xue *et al.*, 1999). However, while Notch seems essential for the establishment of definitive HSCs in the embryo, it inhibits the generation of mesodermal and subsequent hematopoietic and endothelial lineages from mouse ESCs (Schroeder *et al.*, 2006), leaving the role(s) of Notch signaling unclear during the earliest stages of hematopoietic development.

Corroborating the lack of convincing evidence that Notch pathway is involved in the maintenance of undifferentiated mouse ESCs (Nemir *et al.*, 2006), several laboratories (Noggle *et al.*, 2006; Fox *et al.*, 2008; Yu *et al.*, 2008), including ours (Lee, 2008), have confirmed the lack or minimal contribution of Notch signaling to the undifferentiated state of hESCs. Activation of Notch signaling by exogenous Jagged-1 promoted hESC hematopoiesis at greater levels than induced by any previously cytokine- or morphogen-mediated stimulation, thereby providing ready access to larger numbers of hematopoietic cells. Interestingly, knockdown of HES1 within the $CD45^{neg}$PFV subset resulted in reduced hematopoietic but enhanced endothelial output, suggesting that the commitment of bipotent precursors can be controlled by

regulation of HES1 (Lee, 2008)). This preliminary finding suggests that Notch signaling may function in an analogous manner to the "lateral inhibition" model of cellular control first observed in *Drosophila* (reviewed in Radtke *et al.*, 2005). This role of Notch may be of great value for directing human PSC specification into hematopoietic or endothelial fate exclusively. In this regard, our observation of a similar Notch-dependent regulation of hematopoietic development of human iPSCs indicates that the two cell types are similar with respect to their ability to respond to Notch signals. Since the biological effects of Notch activation are highly context-dependent, it is crucial to ascertain whether Notch-dependent promotion of hematopoiesis in human PSCs relies upon the presence of BMP-4 used in our differentiation system, which will likely provide a significant control over the biological functions of Notch and BMP within the endothelial/hematopoietic compartments. Although the role of Notch in the generation of *bona fide* HSCs from human PSCs remains to be assessed, our findings (Lee, 2008)) may have important implications as they suggest the possibility that activation of the Notch pathway may aid in stimulating the production of hematopoietic stem/progenitor or endothelial cells for both experimental and clinical applications.

The Hedgehog pathway

Conserved from *Drosophila* to humans, the Hedgehog (HH) pathway has a central role in embryonic development and adult tissue homeostasis by controlling cell fate specification and pattern formation (reviewed in McMahon *et al.*, 2003). The functional importance of this pathway is illustrated by the multiple birth defects and malignancies (notably leukemia) (Bai *et al.*, 2008) associated with mutations and/or aberrant activation of the pathway (Villavicencio *et al.*, 2000). Three HH ligands Sonic (SHH), Indian (IHH) and Desert (DHH) have been identified in mammals that can bind interchangeably to two related twelve-pass membrane Patched (Ptc) receptors (reviewed in Ingham and McMahon, 2001). In the absence of ligand, Ptc antagonizes the pathway by preventing the activity of another transmembrane protein Smoothened (Smo) (Lum and Beachy, 2004; Alcedo and Noll, 1997; Taipale *et al.*, 2002). Binding of HH ligands to Ptc relieves this inhibition, which activates target gene transcription through the regulation of Glioblastoma (Gli) family of transcription factors (Gli-1, Gli-2, Gli-3) (reviewed in Koebernick and Pieler, 2002; Aza-Blanc and Kornberg, 1999). The different Gli proteins exhibit activating or repressing transcriptional activities depending on proteolytic processing of the full-length proteins. Gli-1 and Gli-2 mainly act as transcriptional activators, while Gli-3 generates a repressor form (Gli3R) in the absence or inhibition of HH signaling (Dai *et al.*, 1999; Wang *et al.*, 2000; Ingham and McMahon, 2001). Although functional significance of Gli-3 has been demonstrated by genetic inactivation (Litingtung *et al.*, 2002), the molecular mechanisms that control Gli-3 interactions and targets are largely undefined, whereas the dynamic interplay between Gli-1 and Gli-2 signaling is well documented. Activation and repression of HH pathway, through interference with Smo activity, can be achieved with synthetic agonists (purmorphamine) (Sinha and Chen, 2006) and antagonists (cyclopamine) (Taipale *et al.*, 2000), respectively.

Studies using mouse embryos and ESCs have implicated the HH pathway (IHH) in early hemato-vascular development (Dyer *et al.*, 2001; Byrd *et al.*, 2002; Baron, 2003). However, *in vivo* genetic studies have been of limited utility in dissecting the role of the pathway due to either embryonic lethality induced by the targeted components (Chiang *et al.*, 1996) or the implication of other pathways (for example, Wnt or BMP).

In humans, our laboratory has been the first to reveal a role for HH pathway (SHH) in the regulation of adult HSCs (Bhardwaj *et al.*, 2001). Upon investigation of the role of this pathway in hESC-derived hematopoiesis, we found that hESCs activated by exogenous SHH or purmorphamine responded by a reduction in the size of the committed hematopoietic but not the hemogenic endothelial cell compartment. Conversely, antagonism of HH signaling with cyclopamine or siRNA against Smo increased blood development and was associated with the processing of Gli-3 into its repressor form (Ramos-Mejia, 2008). This effect was found to be BMP-4-dependent, in agreement with the established connection between HH and BMP pathways in adult HSC biology (reviewed in Baron, 2003). More importantly, cyclopamine favored the development of definitive erythropoiesis from hESCs as judged by adult β globin expression, providing circumstantial evidence of the potential to generate definitive hematopoietic lineages. Although the role of HH signaling in hESCs may seem contradictory to prior evidence in other species where HH has been established as a positive regulator of primitive or definitive hematopoiesis (Maye *et al.*, 2000; Dyer *et al.*, 2001; Byrd *et al.*, 2002; Gering and Patient, 2005), it is consistent with our previous report implicating activation of HH signaling in the exhaustion of mouse adult HSCs (Trowbridge *et al.*, 2006a).

Taken together, our findings indicate that inhibition of HH signaling in hESCs may be important for inducing definitive hematopoiesis, thereby raising the possibility to generate *bona fide* HSCs from hESCs. Assessing this possibility *in vivo* is key as it has been hypothesized that the compromised reconstituting capacity of HSCs derived from ESCs may be accounted for by their resemblance with cells derived from the yolk sac (reviewed in Palis and Yoder, 2001).

The Wnt pathway

The evolutionary conserved Wnt pathway diversifies into two main branches, *e.g.* canonical (β-catenin-dependent) and non-canonical (β-catenin-independent) that play critical roles in specifying cellular fates and movements, respectively, during both embryonic development and adult tissue regeneration (reviewed in Logan and Nusse, 2004; Reya and Clevers, 2005; Uusitalo *et al.*, 1999). Wnt ligands signal through binding to seven transmembrane Frizzled (Fzd) receptors and single transmembrane lipoprotein receptor-related protein (LRP) 5 or 6 co-receptors (Wu and Nusse, 2002). Canonical signaling mediated by ligands such as Wnt3a inhibits a multiprotein degradation complex consisting minimally of axin, adenomatous polyposis coli (APC) and glycogen synthase kinase 3 beta (GSK3β). This inhibition culminates in nuclear translocation of β-catenin, enabling it to interact with T-cell factor (TCF)/lymphoid enhancer factor (LEF) transciption factors to regulate gene expression (Khon and Moon, 2005). Non-canonical signaling, which is much less defined, is mediated by ligands such as Wnt11 that use the same Fzd receptors but ROR2/RYK as co-receptors (Nusse, 2008; Lu *et al.*, 2004). This pathway stimulates the Jun NH2-terminal kinase (JNK), Ca^{2+}/

CaMKII and PKC pathways (reviewed in Kohn and Moon, 2005). Both pathways interact with each other, and in some cases, non-canonical signaling antagonizes the canonical pathway (Kuhl, 2002).

Even though the role of canonical signaling on the regulation of adult hematopoiesis has been studied in great detail, controversy remains, possibly explained by differences in strength and duration of Wnt signaling or redundancy with other pathways (Reya *et al.*, 2003; Willert *et al.*, 2003; Trowbridge *et al.*, 2006b; Kirstetter *et al.*, 2006; Scheller *et al.*, 2006; Qian *et al.*, 2008; Koch *et al.*, 2008). In the context of development, genetic studies have demonstrated the requirement for canonical signaling in the formation of mesoderm (Liu *et al.*, 1999; Lako *et al.*, 2001; Kelly *et al.*, 2004; Huelsken *et al.*, 2000; Gadue *et al.*, 2006). However, studies using mouse ESCs have failed to assign specific roles for this pathway during commitment of mesoderm to the hematopoietic lineage (Naito *et al.*, 2006; Wang *et al.*, 2007; Nostro *et al.*, 2008), thus precluding tangible extrapolations from mouse to human. The role of non-canonical signaling on human hematopoiesis has been far less characterized as only one study by our laboratory has implicated the pathway in the regulation of adult HSCs *in vivo* (Murdoch *et al.*, 2003).

In hESCs, the role of Wnt pathways was thought to be irrelevant to hematopoiesis until the demonstration that canonical but not non-canonical signaling could support bipotent hemogenic cell development (Woll *et al.*, 2008). Since this study, recent advances from our laboratory have provided insights into the uniqueness of the biological functions of the two pathways. We found that non-canonical (Wnt11) and canonical (Wnt3A) Wnts affected different target populations and stages of hematopoietic development (Vijayaragavan *et al.*, 2009). Consistent with its previously defined role in human adult cells (Van Den Berg *et al.*, 1998) and mouse ESCs (Lako *et al.*, 2001; Lengerke *et al.*, 2008), canonical signaling increased proliferation of blood committed progenitors when administered during the proper window of time during EB development (Vijayaragavan *et al.*, 2009). However, we did not observe any positive influence of canonical Wnt signaling on mesoderm specification of hESCs as the work with mouse ESCs indicates (Lako *et al.*, 2001; Wang *et al.*, 2007). A short pulse of non-canonical signaling was necessary and sufficient to control exit of hESCs from the pluripotent state and subsequent entry into the mesendoderm/mesoderm lineages as mapped by the expression of representative markers and the induction of a unique cell population co-expressing Brachyury, Fzd7 and E-cadherin (Brachyury$^+$/Fzd7$^+$/E-cadherin$^+$). SiRNA-mediated knockdown of Fzd7 decreased the size of Brachyury$^+$/Fzd7$^+$/E-cadherin$^+$ population and subsequent hematopoietic compartments (Vijayaragavan *et al.*, 2009). In addition, the generation of this population was dependent upon EB formation and lost in monolayer cultures of hESCs, illustrating the importance of three-dimensional structures that more closely mimic gastrulation organization.

Taken together, our findings (Vijayaragavan *et al.*, 2009) provide the first evidence of a unique role for non-canonical signaling in early specification of hematopoiesis from hESCs, whereas canonical signaling affects the proliferation of cells already fated to blood. These studies provide a valuable model system for examining the possibility of chronological activation and interaction between non-canonical and canonical signaling in the cellular progression from mesoderm to blood. Furthermore, the non-canonical induced Brachyury$^+$/Fzd7$^+$/E-cadherin$^+$ population also provides the opportunity for addressing the relationship between this subset and the putative "mesendodermal" precursor described in zebrafish and *Xenopus* (Kimelman and Griffin, 2000; Rodaway and Patient, 2001), for which evidence is lacking in humans. As recently outlined in mouse ESCs (Lengerke *et al.*, 2008), the specific functions of both Wnt pathways will have to be revisited in the context of their cross talk with known hematopoietic regulators such as BMP and *Cdx/Hox*. The controversial function of canonical signaling on the reconstituting capacity of adult HSCs (Reya *et al.*, 2003; Kirstetter *et al.*, 2006; Scheller *et al.*, 2006; Koch *et al.*, 2008), combined with our present findings in hESCs, underscores the importance of fine tuning the strength and duration of Wnt signaling towards therapeutically exploiting the balance between self-renewal and lineage commitment of HSCs.

Towards an integrated view of HH, Wnt and Notch signaling in hematopoiesis

Multiple studies have suggested that Notch, Wnt and HH pathways can network together and with other signaling pathways to establish or regulate biological processes during embryogenesis and throughout adulthood (Hing *et al.*, 1994; Maloof *et al.*, 1999; Hooper, 1994). Despite the established connection between BMP and Notch, Wnt and HH pathways (Dahlqvist *et al.*, 2003; Itoh *et al.*, 2004; Nobta *et al.*, 2005; Takizawa *et al.*, 2003; Sumi *et al.*, 2008; Lengerke *et al.*, 2008; Baron, 2003), it remains unclear whether these pathways operate in direct network with one another in the context of the hematopoietic system. However, the importance of concerted regulation of hematopoiesis by distinct signaling pathways has been largely illustrated in the mouse (Pearson *et al.*, 2008). For example, interplay between Notch and cytokine-activated pathways such as FGF, PDGF, TGFβ, VEGF, G-CSF or GM-CSF has been found to modulate expression of Notch components (Bigas *et al.*, 1998; Reya *et al.*, 2003). Notch and Wnt signaling synergize to maintain the HSC/multipotent progenitor pool, which is likely accomplished through the regulation of expression of Notch target genes, and Notch-dependent inhibition of HSC differentiation by Wnt signaling (Duncan *et al.*, 2005; Trowbridge *et al.*, 2006b). Canonical Wnt and Notch signaling, which independently promote primitive erythropoiesis and cardiogenesis in mouse ESCs, respectively, set connections through reciprocal regulation of Notch (Numb) and Wnt (Sfrp1, Sfrp5, Dkk1, Wnt5a) pathway inhibitors to drive cellular differentiation specifically towards erythropoiesis (Cheng *et al.*, 2008). In addition to cooperating with Notch, Wnt signaling has been shown to work in concert with other pathways at different stages of mouse or human hematopoiesis. For instance, the induction of mesendodermal precursors seems to require cooperative interactions between canonical Wnt and TGFβ signaling in both mouse and human ESCs (Gadue *et al.*, 2006; Sumi *et al.*, 2008; Nostro *et al.*, 2008), although the balance between these pathways plays crucial roles in the ultimate decision of lineage specification. In addition, the connection of HH pathway with Notch and Wnt, as well as with proliferative/anti-apoptotic signaling pathways (FGF, IGF) regulates hematopoiesis. However, these connections have been best described in malignant he-

matopoiesis (Sengupta *et al.*, 2007) and poorly explored in normal development.

In addition to signaling cues, intrinsic determinants known to be involved in hematopoiesis engage with Notch and HH signaling. Of interest, hematopoietic transcription factors (SCL/TAL-1, RUNX1, HOXB4) (Orkin and Zon, 2002; Hochman *et al.*, 2006) and chromatin regulators (Palaparti *et al.*, 1997) suggest the participation of these pathways in the regulation of complex regulatory networks. Undoubtedly, further research is required to elucidate the integration of major extrinsic pathways into transcriptional and epigenetic regulatory networks. As recently explored during the undifferentiated state of mouse ESCs with respect to LIF and BMP pathways (Chen *et al.*, 2008), elucidation of the integration of HH, Notch and Wnt signals with core genetic and epigenetic networks during hematopoietic development of human PSCs should provide a strong foundation for more controlled manipulation of these pathways.

Concluding remarks

Capitalizing on insights gained from studies using vertebrate embryos, mouse ESCs, and most importantly adult-derived human HSCs, our findings have begun to unravel novel and distinct roles for Notch, Wnt and HH pathways in the regulation of hESC-derived hematopoiesis (see Fig. 1). Further work is needed to ascertain whether the increased "quantity" of hematopoietic cells derived from hESCs that we observed in response to the manipulation of Notch, Wnt and HH pathways can amount to a better "quality" of cells that possess *bona fide* HSC function.

These studies illustrate the value of (i) cell culture systems (EBs) and soluble growth/morphogenetic factors as a means to regulate the sequence of events and cell populations developing from the exit of pluripotent state to blood commitment and differentiation, (ii) Notch, Wnt and HH as important candidate pathways for regulating distinct and critical aspects of this development, (iii) temporal and stage specific manipulation of these pathways. This latter notion, which speaks to the invaluable contribution of concepts learned from developmental biology, is only beginning to be exploited in the hESC field. Although it is tempting to speculate that hESCs will behave more like mESCs as both systems share critical transcriptional pathways and pose a challenge for the generation of *bona fide* HSCs, our work underscores the existence of differences between the species through their disparate responsiveness to HH pathway or single-gene (HOXB4) reconstitution strategies. In light of these observations, it seems important to examine the situation in other model systems such as non-human primates, as divergent views remain on the origin of human definitive hematopoiesis between the epiblast-derived splanchnopleural mesoderm (Tavian *et al.*, 2001) and hypoblast (Bianchi *et al.*, 1993).

While the individual contributions of Notch, Wnt and HH pathways to the hematopoietic development of hESCs are only beginning to be investigated as outlined by our studies, the impact of their collective contribution should help determine whether combinatorial manipulation of these pathways may prove more powerful than single manipulation strategies towards influencing the development of hematopoietic lineages. However, the mechanistic complexity by which these pathways transmit intercellular and/or intracellular signals complicates the situation and raises a critical issue of selectivity. This is exemplified by (i) the growing

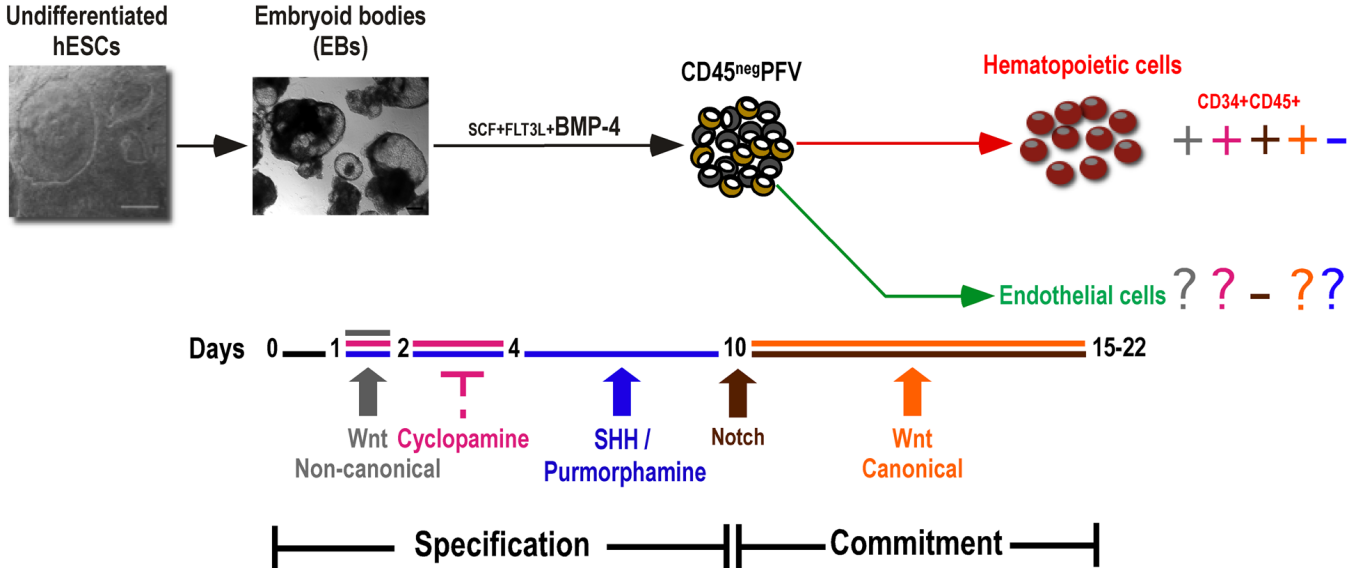

Fig. 1. Cellular and temporal roadmap of hematopoietic development of human embryonic stem cells (hESCs). *Undifferentiated hESC colonies were differentiated into embryoid bodies (EBs) in differentiation medium supplemented with SCF, FLT3L and BMP-4 for the duration (days 1 - 15-22) of EB development. Based on our previous studies (Wang* et al.*, 2004), this developmental scheme can be divided in two phases. Days 0-10 delineate the specification phase that is characterized by the emergence of the CD45^{neg}PFV hemogenic endothelial precursor and the lack of committed hematopoietic (CD34^{+}CD45^{+}) and endothelial cells. These latter populations emerge during the commitment phase from day 10 onwards. Different colors refer to activation of the different pathways as well as inhibition of the hedgehog (HH) pathway (cyclopamine). + and - signs indicate enhanced and decreased hematopoietic (or endothelial) output, respectively, as determined at days 15-22 of EB differentiation in response to manipulation of each pathway for the indicated time window. Question marks refer to unknown data.*

number of negative or positive regulatory loops within these pathways, (ii) the interrelationships with hematopoietic "niches", and (iii) the promiscuous receptor/ligand interactions, along with post-translational modifications of functional significance. In addition, the heterogeneous nature of ESC cultures, as demonstrated for human (Stewart *et al.*, 2006; Bendall *et al.*, 2007) and mouse (Hayashi *et al.*, 2008) ESC self-renewal, will need to be accounted for as the functional consequences of such heterogeneity extend to our ability to understand and control lineage specification of human PSCs. Notwithstanding the complexity of PSC cultures and Notch, Wnt and HH signaling, these issues are essential to better address in the context of hematopoietic development towards achieving signal specificity and unlocking the potential of these pathways.

Stem cell manipulation strategies using synthetic regulators may be of great value for specifically targeting the activation or inhibition of these pathways. However, this raises concerns towards therapeutic applications given the implication of these pathways in hematopoietic malignancies (Grabher *et al.*, 2006; Sengupta *et al.*, 2007; Aster *et al.*, 2008; Deshpande and Buske, 2007; Petropoulos *et al.*, 2008), and the exhaustion of HSC potential upon sustained activation (Trowbridge *et al.*, 2006b). Although the use of synthetic regulators of HH signaling has been encouraging in many systems (Stecca and Ruiz i Altaba, 2002), this will be more challenging for Wnt due to the intricate interplay of both pathways and the involvement of GSK3 in multiple pathways such as HH (Jia *et al.*, 2002). While our more advanced knowledge of the hESC biology sets the stage for characterizing the cell populations and developmental stages targeted by Notch, Wnt and HH signaling, the iPSC technology offers the attractive possibility of initiating a stepwise "dedifferentiation" of mature blood cells from somatic sources to multipotent HSCs without first passing through a pluripotent cell state. If true, manipulation of specific sets of extrinsic and intrinsic hematopoietic regulators may be facilitated by a more permissive epigenetic state of adult blood cells compared to PSCs, which may lead to *in vitro* generation of blood cells representing the multiples branches of the hematopoietic hierarchy. Moreover, the experimental model system of iPSCs provides a more relevant testing platform for *in vitro* models of hematological abnormalities, which is likely to contribute to a better understanding of how deregulation of these pathways could result in malignant hematopoiesis.

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