Chapter 12
Notch Ligands in Hematopoietic Stem Cell Production

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Abstract Hematopoietic transplantation has been a therapeutic option for leukemia patients for more than 50 years. Its possible applications have expanded in recent years with the success of gene therapy and gene-editing approaches that can now offer promising treatments for monogenic incurable diseases. Nowadays, the main limitation to apply this therapy is the availability of compatible donor stem cells and the complications of hematopoietic recovery, which could be attenuated by the recent breakthrough discoveries on the field of reprogramming. However, our knowledge how to produce hematopoietic stem cells is still limited to safely use this technology. In this review, we covered the key elements that should be considered for a better understanding of hematopoietic cell production in the embryo proper or from in vitro protocols and how Notch participates in this process.

Keywords Embryonic hematopoiesis · HSC · AGM · Fetal liver · Bone marrow · ES cells

12.1 Hematopoietic Stem Cells

12.1.1 Overview of Hematopoietic Stem Cell Production

Hematopoiesis is the process that generates the different types of blood cells and takes place during the whole life of an organism. Blood cells derive from a common ancestor or hematopoietic stem cell (HSC). HSCs are somatic, tissue-specific stem cells with multipotency and self-renewal capacity [4]. A single HSC is able to reconstitute the whole hematopoietic system of an immunodeficient receptor, and

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this unique trait is used to define a bona fide functional stem cell [27]. Thus, HSCs are the most robust source of blood cells, being the base of the hematopoietic transplantation therapies, which are common practice for leukemia treatments and other blood-related diseases. The main limitation for these treatments is the source of HLA-matched immune cells, which usually relies on family members or rare unrelated altruistic donors. Obtaining an unlimited source of compatible blood cells in vitro that could be used for transplantation would provide an alternative option for many patients. The understanding of the process that regulates HSC maintenance and differentiation is crucial for this purpose.

In the recent years, the stem cell scientific community has turned the focus of the investigations onto reprogramming and generation of newly formed HSC from embryonic cells. This is a promising and exciting field not only for hematopoietic production but also for other somatic stem cell types that could provide different sources of cell replacement. The demonstration that a combination of transcription factors can reprogram differentiated cells into pluripotent stem (PS) cells [82] led to a change of the paradigm and laid high expectations about the possibility of generating HSCs from autologous induced PS (iPS) or to directly reprogram cells into HSCs (iHSC). Investigations on this area have revealed striking parallelisms between the molecular mechanisms for HSC generation in vitro and the developmental processes that lead to HSC specification in the embryo. Thus, there is a clear need for a better understanding of this embryonic process.

Studies focused on the regulation of de novo formation or the expansion of HSCs in the embryo have identified candidate signals that will be relevant for this process.

For example, and after many years of discussion, Notch signaling is slowly settling in the field as a promising tool to amplify or generate HSC in vitro. However, many questions still remain related to the physiological Notch function in the hematopoietic system, likely associated with its complex and context-dependent effects. In this review, we will discuss the physiological involvement of Notch in the formation and maintenance of the hematopoietic system and how this signal can be manipulated to our benefit.

12.1.1.1 Overview of Embryonic and Adult Hematopoietic Development

Hematopoietic development in the vertebrate embryo occurs in waves (reviewed in [22]), adapted to fulfill the embryo necessities likely as a result of an evolutionary process. In the mammalian embryo, the first wave of hematopoietic cells takes place in the yolk sac, and it is known as primitive hematopoiesis. In the yolk sac, endothelial-like cells organize in vessel structures called blood islands where the first erythrocytes and macrophages are found. Next, the yolk sac will produce progenitors with definitive characteristics and erythroid/myeloid potential (EMPs) but without or with limited capacity to self-renew [17]. After the organization of the embryonic vascular tubes and alongside with artery specification, the next wave of hematopoiesis takes place closely associated with the endothelium of the aorta, the

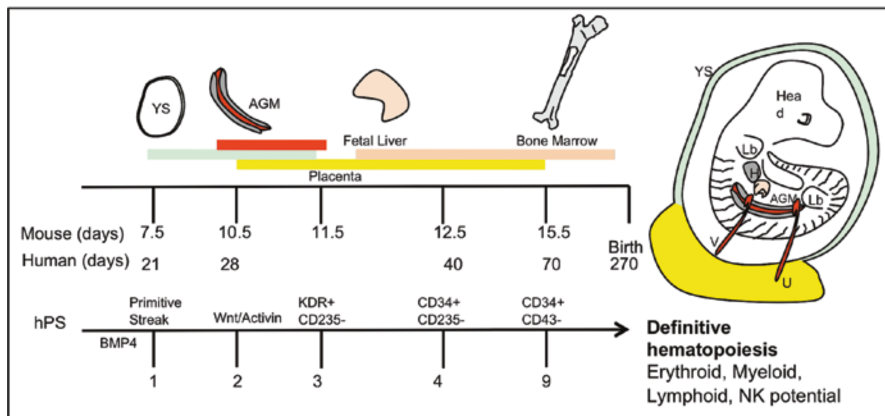

Fig. 12.1 Timing of hematopoietic development waves in the mouse and human embryo (upper) and in vitro human pluripotent (hPS) differentiation protocol for definitive hematopoiesis (lower) adapted from [80] (YS, yolk sac; Lb, limb buds; NK, natural killer; V, vitellin; U, umbilical)

umbilical and vitelline arteries, and the placenta [22] restricted to a very limited temporal window (E10–E12 in the mouse and week 4–5 in human). The aorta surrounded by the gonad and mesonephros (AGM) structures is the best characterized hemogenic tissue, both in mouse and human, with the capacity for HSC production [39, 57]. Cells with endothelial characteristics transit into a hematopoietic (EHT) phenotype and form cell clusters that emerge into the lumen of the aorta. It is within these clusters that a few cells are specified as definitive HSCs. Hematopoietic cells formed in the different embryonic sites (including the HSCs) and migrate then to the fetal liver, which becomes the main hematopoietic organ after E12 and until birth. Close to birth, hematopoiesis moves to the bone marrow of the long and flat bones, where it will reside through life (see scheme in Fig. 12.1).

12.1.1.2 Development of the Hematopoietic System In Vitro

Following the establishment of murine and human embryonic stem cell (ESC) lines [25, 83], important progress has been made to induce the differentiation into hematopoietic products and, ideally, use them as a source of HSCs [60]. Unfortunately, engraftment of ESC-derived hematopoietic cells has reproducibly failed. More recently, the implementation of induced pluripotent stem (iPS) cell techniques and the generation of personalized HSCs for clinical applications are viewed as realistic goals. Since in vitro HSC production is still a big limitation, a diversity of cytokine cocktails and differentiation protocols have been developed to mimic the normal differentiation of embryonic cells into hemogenic endothelium or hematopoietic stem cells. Most of these protocols can successfully reproduce the EHT process that occurs in embryonic sites, but cannot reproducibly generate HSCs. Thus, in vitro differentiation of PS cells is likely reproducing the first embryonic hematopoietic

waves that do not produce HSCs. With these results, scientists have turned the attention to the stromal signals and the AGM niche and obtained a successful long-term repopulating HSC activity using different stromal-incubation strategies [37]. Within this scenario, the role of Notch ligands and Notch signals will be further developed in this review.

12.1.2 The Niche for HSC Specification and Generation: AGM

As mentioned, HSC generation is spatially and temporarily restricted during embryonic development. The AGM region, which corresponds to the caudal part of the dorsal aorta, shelters the endothelial-like precursors that will undergo endothelial to hematopoietic transition. Only few of these cells, which constitute the also called hemogenic endothelium, will become HSCs. However, to date there are no specific markers that single out HSC precursors from the rest of the hemogenic endothelium. This fact has greatly postponed defining the signals that affect HSC and HSC precursors directly from those affecting other cells in the AGM niche that can also influence HSC formation. Some of the identified cellular elements that are important in this process are endothelial/arterial cells, macrophages, mesenchyme, and neuronal/adrenal gland cells.

12.1.2.1 Endothelial/Arterial Cells in the AGM

Endothelial cells are important elements in the process of HSC formation for several reasons. First of all, there is a common ancestor of both lineages that, based on recent investigations, could be previous to the commitment of hemogenic endothelium and arterial specification [21, 30]. Secondly, hemogenic endothelium is immersed in the endothelial layer of the dorsal aorta at the time of HSC commitment. Third, the endothelial cell layers deliver signals that can activate Notch and Wnt pathways, which are essential for HSC commitment but also arterial specification [72, 76]. Moreover, arterial and HSC specification in the embryonic dorsal aorta occurs simultaneously or at least in the same time frame (for more details, see Sect. 12.2.1).

12.1.2.2 Subaortic Cells

The aorta is surrounded by mesenchymal cells originated from the splanchnic lateral plate and is later replaced by the sclerotomal-derived mesenchyme [68, 69, 92, 96]. Detection of cells expressing Gata2 and Gata3 mRNA or CD41 within the mesenchymal layer supported the idea that HSC precursors are located in the subaortic patches [8]. However, other structures also stain for these genes being difficult to interpret this result. For example, the adrenal gland precursors

also express and depend on Gata3. Alternative attempts to demonstrate the putative mesenchymal origin of HSCs have also failed [68, 104]. It is likely that mesenchymal cells may serve as signal-sending cells required for hematopoietic development as was recently shown for BMP regulatory molecules [55]. In fact, stromal cell lines that have been obtained from AGM and support HSC development display mesenchymal features [16, 62]. However, the composition of this mesenchymal layer is not resolved, and cells with hemogenic characteristics with capability to become HSCs have been described (Rybtsov, Medvinsky). It is then conceivable that pre-HSC cells are intermingled in the mesenchyme in the subluminal layer of the aorta or that cells from the committed pre-HSC cells from the endothelial layer migrate to the subaortic layers.

12.1.2.3 Sympathetic Nervous System

A connection between the AGM niche and the nervous system was found by analyzing the Gata3 mutant embryos, which lack functional HSCs. Neuronal precursors for the adrenal gland are Gata3-expressing cells, reside in the subaortic mesenchyme, and are not found in the absence of Gata3. Thus, defective HSC formation in the GATA3-null mice was due to the absence of a catecholamine, which is normally synthesized by these neuronal precursors and required for the survival of nascent HSC. As a proof of concept, addition of exogenous catecholamines partially rescued the production of transplantable Gata3-deficient HSCs [28]. Interestingly, the production of adrenal gland derivatives and components of the nervous system are also critical in the migration and homing of adult HSCs to the bone marrow niche [40].

12.1.2.4 Macrophages

Macrophages are key cellular elements for the remodeling of developing tissues and during adulthood (rev. [97]). Studies with CSF$^{op/op}$ mice lacking macrophages due to a mutation in the colony-stimulating factor-1 gene (CSF-1 or M-CSF) were crucial to understand the role in osteogenesis, remodeling of bone marrow cavities, and the hematopoietic system [5]. More recently, macrophages were found to play an essential function in HSC emergence in zebrafish [85]. In this study, it was elegantly demonstrated that primitive macrophages are responsible for extracellular matrix degradation that permits the migration of HSPC through the AGM stroma to the posterior cardinal vein. Although one could argue that migration of the newly formed HSPC through the AGM stroma is a unique trait of the zebrafish system, the fact that CD68+ macrophages are also detected among the CD34+ human aortic clusters supports the possibility that this is a more general HSC regulatory mechanism.

12.1.3 The Niche for HSC Expansion: Fetal Liver and Placenta

HSCs are first detected in the AGM, but soon after, the placenta contains much higher HSC activity (about 15-fold compared to the AGM) [31, 63]. This observation revealed that the placenta was an important site for HSC amplification, before or simultaneous to the fetal liver [31]. Since circulation from the placenta to the embryo occurs through the umbilical artery, just upstream of the fetal liver, and HSC number in the placenta decreases concomitant to the increase in the fetal liver HSC potential, it was suggested that placenta-derived HSCs (and not the AGM-produced HSCs) colonize the fetal liver. Moreover, mutant embryos with heartbeat and circulation defects (ncx mutants) can produce lymphoid and myeloid cells, thus suggesting that HSCs may not only be generated in the AGM [71]. Moreover, some pre-HSCs present in the AGM require a maturation step in the fetal liver to become functional HSCs [10], but it is possible that the placenta can serve as a maturation niche similar to the fetal liver. In any case, the fetal liver is unequivocally a HSC expansion organ, as shown by detailed limiting dilution transplantation assays [11]. In these assays, fetal liver HSCs displayed a higher transplantation capacity than adult bone marrow cells that was directly linked to differences in their expression profile [41].

In the human embryo, the site of origin of the HSCs is uniquely associated with the AGM region as early as 32–33 days postfertilization [38], but it is not until week 9 of gestation that HSCs are robustly detected [75].

12.1.4 Bone Marrow Niche: Maintenance of HSC

Close to birth, HSCs migrate to the bone marrow where they reside all through adulthood. A tightly controlled balance between self-renewal and differentiation is required to avoid life-threatening hematopoietic malignancies. For this reason, the detailed characterization of the elements that compose the bone marrow niche and the signals that control hematopoietic homeostasis is still an important topic of investigation.

Inside the long and flat bones, there is soft tissue formed by an endosteal layer that recovers the osteoblast tissue. This endosteal tissue and the endothelium of the small vessels contain the cells that more closely associate with the HSCs and regulate their functions [15, 42, 100]. Discussion on the nature of the bona fide HSC niche is still ongoing although imaging technology is rapidly developing and its application to bone marrow imaging is already happening [52]. For example, it was recently shown that different types of capillary structures affect HSC activity [1]. Current and future studies using this technology should be able to uncover most of these questions.

Other elements of the bone marrow niche include the arterioles, which are surrounded by sympathetic nerves, smooth muscle cells, and matrix components and differ from other venule sinusoids. They express specific markers such as sca1 and NG2+ and localize adjacent to the HSCs. Other stromal cell types that are also important in the maintenance of HSCs are mesenchymal stem cells characterized by nestin expression [58].

The bone marrow contains not only the HSC population but also most of the differentiating hematopoietic cell types that originate from them. Importantly, HSC, progenitors, and differentiated cells were shown to send signals to the HSCs, thus regulating its self-renewal capacity and differentiation [12, 103]. Recently, the identification of dormant HSC (in comparison to the active HSCs) has provided valuable data in relation with stromal specific cells that may regulate particular HSC stages. Dormant HSCs divide about five to six times in the whole life of a mouse in homeostatic conditions; however, they become crucial in stress conditions such as bone marrow transplantation or hematopoietic recovery after insults (infections or chemotherapy). Arterioles have been shown to be essential to maintain HSC quiescence, in the dormant population [47].

12.1.5 Ex Vivo Production of HSC

Since the discovery of ES cells (mouse and human), many efforts have concentrated on using them as a source of HSC. Although ES cells can produce many types of hematopoietic cells and progenitors, no reliable protocols are currently available to generate cells with engraftment capacity or HSC activity. In fact, hematopoietic production from ES cells closely resembles the primitive hematopoietic wave that occurs in the yolk sac. This observation strongly suggests that signals required for HSC specification are different from that required for hematopoietic production and highlights the need for their identification. Recently, different reprogramming strategies have been more successful in obtaining cells with hematopoietic transplantation capacity. For example, some HSPC activity has been obtained from fibroblasts reprogrammed with the transcription factors Gata2, Gfi1b, cFos, and Etv1 [67] or Erg, Lmo2, Runx1, Gata2, and SCL [3]. However, only in one study in which murine B cells were reprogrammed using Runt1t1, Hlf, Lmo2, Pbx1, and Zfp37 has reported long-term repopulation capacity [91]. Another recent report has been able to induce ESC differentiation into HSC by using a combination of another seven transcription factors (erg, HoxA5, HoxA9, HoxA10, LCOR, Runx1, and Spi1) [81]. Lastly, a very promising strategy has come from direct conversion of human adult endothelial cells into long-term engrafting HSCs, which was achieved by Rafii's group. By exploring combinations of transcription factors, they found that Fosb, Gfi1, Runx1, and Spi1 (FGRS) can reprogram endothelial cells, generating HSCs with engraftment potential [77] and T-cell immunity potential [49].

Together these studies show that de novo generation of HSCs in vitro is feasible, but the translation into clinical application resides in activating and controlling the

expression of endogenous transcription factors without genetic manipulation. In this sense, the control of developmental pathways (Notch, Wnt, HH, or FGF) that respond to extracellular cues should be crucial to achieve this regulation.

12.2 Notch and Notch Ligands in Hematopoietic Stem Cell Niches

From development to adulthood, each hematopoietic wave and process has a different requirement for Notch activity. Since Notch activation is governed by cell-cell interaction and ligand-receptor availability, the specific Notch activity is delivered and controlled by the niche.

12.2.1 Notch Ligands in HSC Specification

Work from different groups unequivocally shows that Notch is required for the proper development of the hematopoietic system. Although this is still a matter of investigation, data in mouse and zebrafish embryos demonstrated that Notch is essential to specify the hemogenic endothelium and/or HSCs [14, 46, 74]. Notch is however dispensable for the generation of primitive and definitive blood cells in the yolk sac [7, 36, 73]. The pioneer study from Hirai's lab in 2003 analyzed Notch1- and Notch2-deficient embryos and observed a profound hematopoietic defect with lack of HSC activity specifically in the Notch1-deficient embryos. Other studies in mouse and zebrafish models confirmed that definitive hematopoiesis was impaired in Notch mutants [14]. The fact that Notch loss-of-function embryos also lacked arterial identity, which disturbs the niche for nascent HSCs, was not appropriately considered by most of these studies. However, in the zebrafish embryo, the ectopic activation of Notch in the vein resulted in hematopoietic production, which suggests that the preexistence of arterial identity was not required [14]. In 2008, we found that Jag1 mutant embryos preserved the arterial identity but lacked definitive hematopoiesis in the AGM [74]. This observation was crucial in the understanding of Notch signaling in HSC determination, allowed uncoupling the role of Notch in arterial and HSC specification, and demonstrated that a productive Notch signal was required for both processes. The latter was confirmed by analyzing Notch target genes that are important for the correct specification of HSC [34, 35]. However, Notch signals are not just on and off but rather defined by levels of activity. Using Notch activity reporters with different sensitivities, we have recently confirmed that HSC precursors in the AGM do not experience high Notch activity as opposed to the arterial cells, but they descend from cells that experienced low Notch activity [30]. The right Notch activity level is achieved by the interplay of Jag1- and Dll4-delivered signals, being Jag1 required to maintain low Notch active levels and

prevent arterial specification in the hemogenic precursors. Recently, other mechanisms involved in maintaining lower levels of Notch activity have been described in EHT. For example, inhibition of sox17 is required to downregulate Notch1 transcriptionally [51], while G-protein-coupled receptor (gpr) 183 contributes to Notch degradation [101]. These results can be reconciled in a model in which hemogenic endothelial precursors refrain from turning on the arterial program by partially inhibiting the Notch pathway. Afterward, they will require Notch activation (at a lower level) to activate HSC-specific Notch-dependent target genes such as Gata2 [35]. Finally, hematopoietic development will become Notch independent at the end of HSC maturation [79].

12.2.2 Notch Signaling in the Fetal Liver

As mentioned, the fetal liver is the main site for HSC amplification during embryonic development. The understanding of this process is crucial for improving protocols for HSC expansion. Until recently, the role of Notch in this process has remained unknown. Evidence that Notch is not only involved in HSC determination but also in HSC amplification comes from the analysis of a Notch1 hypomorphic mutant, carrying a deletion of the transcriptional activation domain (TAD) [32]. In this model, the amount of Notch1 signal is enough to allow the generation of the HSC in the AGM, but it is not enough for proper expansion of the HSC pool in the liver. This activity has not been associated with any specific ligand yet although early studies did show the presence of Jag1 and Delta1 expression in the E12–E17 murine fetal liver by in situ hybridization [90], while Delta4 and Jag2 were not determined. Different ligands seem to signal similarly downstream of Notch receptor; however, they exhibit different efficiencies in activating Notch, which results in different Notch signal strengths [86]. Taking into account results from other cell types, the strength of Notch activity delivered by Dll4 is higher than that delivered by Dll1, which is also higher than that induced by Jagged ligands. We speculate that Dll1 ligand is a good candidate for regulating Notch-mediated fetal liver HSC amplification. In fact, most of the in vitro amplification studies have been performed with Dll1 (see Sect. 12.3).

Furthermore, several tissues simultaneously express two or more Notch ligands, and it is the activity of fringe glycosyltransferases that modulate the ability of the Notch receptor to interact with each individual ligand [98]. For example, it was first described in the Drosophila wing margin formation that glycosylated Notch has higher affinity to bind to Delta but lower affinity to bind to Serrate (orthologue for Jagged) [29]. This observation has been reproduced in vertebrates, not only involving trans-activation but also cis-inhibition of Notch signals, which result in several Notch-sending and Notch-receiving cellular states [48]. In mammals, four Notch receptors, five Notch ligands, and three different fringes can produce a whole range of Notch states that can explain several contradictory observations for Notch

phenotypes. Further research in this direction should help to elucidate the appropriate Notch state that is compatible with HSC formation and HSC expansion.

12.2.3 Notch Ligands in the Bone Marrow

The bone marrow niche is involved in maintaining the HSC fitness during the lifespan of an organism. As described above, different cellular components residing in the bone marrow are involved in this function through both cell-cell interactions and secreted molecules. As example, the Notch ligand Jag1 that is present in the surface of the endosteal cells has indirectly been associated with the maintenance of the quiescent state of LT-HSC [15]. However, other studies using loss-of-function mouse models did not confirm the requirement for Notch in the adult HSC compartment. These studies include the Notch1- [70] and Jagged1 [54]-deficient mice, the dominant-negative of mastermind transgenic (blocks Notch signaling), and the RBPj-deficient mice [53]. Interestingly, detailed analysis of Notch2 mutant mice revealed a role for Notch2 in the proliferation and myeloid differentiation of short-term HSC that affected regeneration of the bone marrow after 5-FU treatment [88]. In contrast, the best characterized effect of Notch1 activity disruption is the impairment of T-cell development, which is also observed after specific Notch pathway abrogation in the hematopoietic system [70].

Analysis of other Notch pathway mutants uncovers the presence of myeloid proliferative defects, which are both cell autonomous and non-cell autonomous. This is the case of hematopoietic deletion of Nicastrin; compound Notch1, Notch2, and Notch3; RBPj [44]; presenilin [78]; and pofut [99]. Thus, although interpretation of all these data remains controversial, it highlights the importance of Notch in the regulation of hematopoietic homeostasis.

12.2.4 Notch in Hematopoietic Differentiation and Leukemia

Notch1 and Notch3 are required to control T-cell development, and their constitutive activation in hematopoietic progenitors unequivocally results in T-cell leukemia [6, 65]. During normal T-cell differentiation, cells need to activate Notch at the double-negative (DN) 1 stage and turn it off after the DN3 stage. This is a tightly regulated process that involves essential transcriptional Notch targets such as hes1, il7r, and deltex.

Following the initial cloning of Notch1 as a rare translocation present in T-ALL patients [23], its pathological relevance was confirmed by the identification of

Notch1 mutations in more than 60% of all T-ALL patient samples [94]. This finding has been extensively confirmed, and it is now evidenced that most T-cell transformation requires Notch activity to occur. However, which are the mechanisms and the downstream effectors of Notch in this disease is not totally understood. Many Notch target genes have been identified in T-ALL models including Myc [95], CCR7 [13], IL7 receptor alpha [33], Hes1 [93], and Notch3 itself [66], among others. The relevance of these targets in T-ALL has been confirmed in different experimental models, and each one has a specific impact on the leukemic process. For example, CCR7 is responsible for the infiltration of tumor cells in the central nervous system (CNS). Hes1 is involved in repressing important tumor suppressor genes such as PTEN [64] and CYLD [24], which directly impinge on the PI3K/Akt and NF-κB pathways, respectively. Surprisingly, several years after the identification of Notch as a tumor driver in T-ALL, it is now clear that Notch mutations confer good prognosis in response to current chemotherapy treatment protocols [26].

In addition to its role in T-ALL, Notch has also some effect on myeloid differentiation and can affect early erythropoiesis and/or megakaryopoiesis [59, 61], as it has been mentioned. Also, recent data suggests that Notch is essential for hematopoietic regeneration after immunodepletion [43, 88].

12.3 Manipulation of Notch Signal Ex Vivo

12.3.1 Notch Ligands in the Expansion of HSC and Progenitors

Notch activity associates with stem cell self-renewal and inhibition of cell differentiation in many different tissues (reviewed in [9]. More recently, endosomal segregation of Notch components has been found to regulate stem cell fate in the drosophila CNS and the gut but also in the neural precursors of the spinal cord in zebrafish [18, 45]. These observations have led to nominate Notch as a good candidate to promote HSC self-renewal and amplification, although the experimental evidences for this assumption are still weak. Among them, early studies showed that constitutive activation of Notch in the murine hematopoietic precursors (lin-sca1 + kit+ cells) resulted in a self-renewal multipotent cell line capable of lymphoid and myeloid reconstitution [89]. This result was a proof of concept that Notch could be used to amplify HSPC. In this line, the Bernstein group has pioneered studies demonstrating the possibility to expand hematopoietic progenitors, and to improve their engraftment capacity, by culturing them on recombinant ligands such as Delta1 (the most used in these studies) [87]. Of special interest is the expansion of cord blood (CB) progenitors on Delta1 ligand as described below [20].

12.3.2 Notch Ligands in Hematopoietic Transplantation

The number of putative clinical application for recombinant Notch ligands is rapidly increasing, and in fact, there is an ongoing clinical trial for CB expansion and transplantation. Among the advantages of CB as a potential source of hematopoietic progenitor cells for transplantation are the availability and the easy access to HLA-compatible units worldwide. However, the limited number of transplantable cells that are provided from each cord blood unit is still a problem that can lead to engraftment failure. One approach to circumvent this problem has been the transplantation of double CB (dCB) units, which has greatly improved the donor engraftment but not neutrophil recovery. Thus, dCB transplantation is becoming a fantastic model to functionally test expansion protocols. Based on promising preclinical studies, Bernstein and colleagues have now designed a phase 1 clinical trial for patients undergoing CB transplantation. In this trial, one non-manipulated CB unit is transplanted along with a second CB unit (CD34+ cells) that has undergone ex vivo expansion on Delta1. This trial is still ongoing, but some early conclusions have already been reported from the first ten patients such as the short-term engraftment and the faster recovery of absolute neutrophil count [19].

In addition to HSC expansion, Notch signaling could be modified to prevent graft-versus-host disease (GVHD) in transplantation procedures. In this sense, different biochemical and genetic approaches in several models showed that Notch depletion in the T cells efficiently protects from acute GVHD in allo-HCT recipients [102]. Mechanistically, Notch inhibition blocked the production of multiple inflammatory cytokines by alloreactive T cells. In addition, Notch-depleted T cells retained potent cytotoxic effects against allogeneic targets and were able to eliminate host-type leukemic cells, leading to long-term disease-free survival while preventing GVHD. More recently, Maillard and colleagues have identified Dll4 as the ligand responsible for alloreactivity in T cells. This is therapeutically relevant since blockage of Dll4 with antibodies at early time points after transplantation of allo-HCT in murine models decreased GVHD incidence and severity without causing global immunosuppression [84].

12.3.3 Notch Ligands in HSC Generation

Notch is essential for the first steps of HSC development in vivo, although the mechanistic base is not completely understood (see Sect. 12.1.2.1). The lack of knowledge and the complexity of HSC specification have delayed the success of reproducing this process in vitro from ESC or iPS. To gain light into the HSC specification process, Daley and colleagues have compared the transcriptome of different embryo-derived subpopulations of cells with HSC potential and ES-derived cells with HSC phenotype (CD41+, CD45+, CD34+) [56]. One of the conclusions of this study was that ESC-derived HSC lacked the activation of the Notch pathway when

compared with their embryo-derived counterparts. This pioneer study was performed before the general implementation of RNA-Seq, and the authors needed more than 2,500 embryos to perform the analysis. Thus, although the reported observation highlighted the importance of Notch signaling in the generation of actual HSCs, improvement of current genomic techniques should provide more accurate information on this process, especially at a single-cell level. In a more

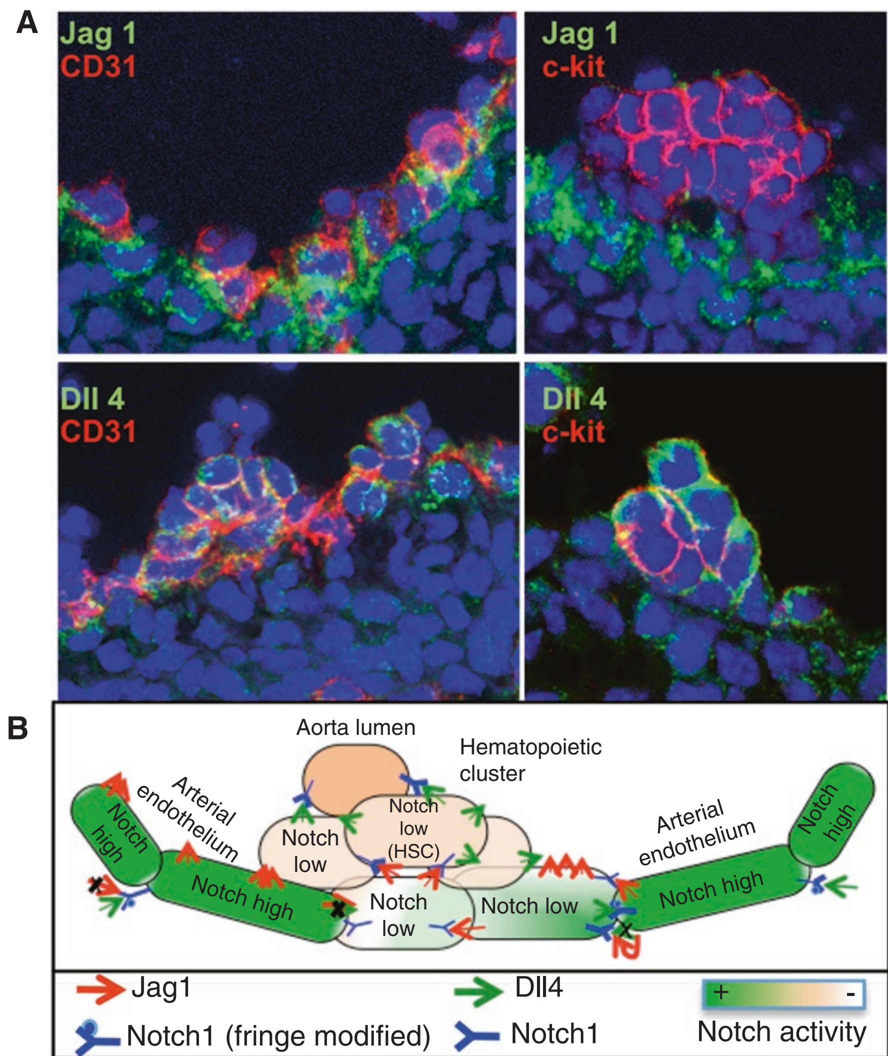

Fig. 12.2 Model for Notch signaling in HSC specification. (**a**) Notch ligands (Jag1 and Dll4) distribution in embryonic aortic endothelium (expressing CD31) and cluster cells (expressing c-kit and CD31). (**b**) Model for HSC and arterial endothelium specification in the embryonic aorta (AGM)

comprehensive characterization of cells arising in the ESC-derived cultures, Menendez and colleagues observed that embryo body-derived cells with endothelial or hematopoietic potential can be separated by the levels of the Dll4 protein [2].

On the other hand, studies from the Keller lab have been essential to improve the current protocols for ESC-HSC development. By using activin/nodal/TGFb and Wnt-b-catenin activation, they distinguished between primitive and definitive hematopoieses [80] and found that definitive hematopoiesis with lymphoid capacity was Wnt-dependent specifically at the hemogenic endothelium specification stage. More recently, this precursor was shown to be different from the one undergoing arterial/venous fate decision. Moreover, the definitive hemogenic precursor was shown to be Notch dependent [21]. These observations are in agreement with our results obtained with the Notch activation reporters N1::CRE [50], which can differentially report Notch activity levels. Our conclusions from the analysis of these embryos were that arteries require high Notch activity levels, whereas in hematopoietic precursors, Notch levels are kept low (only reported with the more sensitive mouse, N1::Cre^{HI}) to induce/preserve the hematopoietic commitment while preventing arterial specification [30] (see model in Fig. 12.2).

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