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Notch Signaling and the Bone Marrow Hematopoietic Stem Cell Niche

Jonathan M. Weber and **Laura M. Calvi**
Endocrine Division, Wilmot Cancer Center, Department of Medicine and Center for Musculoskeletal Research, University of Rochester School of Medicine, Rochester NY

Abstract

Recently there has been increased interest in the regulatory interactions between osteoblasts and cells in the surrounding bone marrow microenvironment. The proximity of hematopoietic stem cells (HSCs) with osteoblastic cells first suggested regulatory interactions and recent data have highlighted the role of osteoblastic cells in providing a HSC niche. Reports have indicated that direct contact is necessary to mediate the osteoblastic effects and that these effects could be mediated through Notch activation. Notch signaling is important throughout development and also appears to play a critical role in cellular maturation and differentiation of osteoblastic cells and hematopoietic cells as disregulation can lead to bone loss and leukemias respectively. In this review we discuss the current understanding of Notch signaling and how it functions in hematopoiesis, osteoblastic cells, and the interactions between HSC and their osteoblastic niche.

Introduction

In the late 70's Schofield used the term *niche* to define custom microenvironments composed of subset of cells and extracellular substrates required to support localization, survival, and self-renewal of stem cells *in vivo* [1]. Conceptually the niche can be thought of as a region that can harbor stem cells and maintain balance between self renewal and differentiation. Over the years, a body of evidence has accumulated in a number of animal models which supports the concept of microenvironmental regulation of stem cells [2]. Notch signaling is in many ways ideal for instructive communication between the niche and stem cells as it is highly conserved, requires direct contact of adjacent cells, and Notch signaling can influence cell fate decisions [3]. While HSCs are fairly well understood, only recently data have indicated cellular and molecular components of the bone marrow HSC niche [4], with some of the first evidence suggesting that osteoblastic cells are supportive of HSCs coming from *in vitro* work [5]. The important role of Notch signaling in hematopoiesis was highlighted by its association with hematological malignancies [6,7], which initiated a great deal of interest in the role of Notch signaling in HSC regulation and in hematopoietic lineage allocation and differentiation, as we will review. Most recently, *in vivo* evidence has strongly implicated Notch signaling in regulation of osteoblastic differentiation [8] [9]. Together, these lines of investigation would suggest that Notch signaling in the bone marrow microenvironment could be essential in HSC-osteoblastic

Address correspondence to: Laura M. Calvi, Endocrine Division, Department of Medicine, University of Rochester School of Medicine, 601 Elmwood Avenue Box 693 Rochester 14642 NY USA, Tel (585) 275-5011; Fax (585) 273-1288; laura_calvi@urmc.rochester.edu.

regulatory interactions, and in fact, data defining the role of osteoblastic cells in HSC regulation point to Notch signaling as a potential intermediary [10]. In this review we will discuss the Notch signaling pathway and how it functions in the hematopoietic system, osteoblastic cells, and the interaction between HSC and their microenvironment which have emerged so far.

Notch Signaling

The canonical Notch Signaling pathway is highly conserved and plays roles in many cellular functions including cellular growth, differentiation, and fate choices [3]. In mammals four Notch receptors (Notch 1–4) and five ligands (Jagged1-2 and Delta like 1, 3, and 4) have been identified, all of which are single span transmembrane proteins that require cell-cell contact for activation [11]. When a Notch ligand comes in contact with Notch, the external portion of Notch is cleaved by tumor necrosis factor (TNF) -α-converting enzyme, a member of the ADAM metalloprotease family. A second intracellular cleavage event occurs through the γ-secretase complex, which releases the activated Notch intracellular domain (NICD) which initiates the signal. Presenilin (PS) 1 and 2, nicastrin, APH1 and PEN2 form the complex which has γ-secretase activity[12]. After the NICD has been cleaved it can translocate to the nucleus and bind to CSL (**C**BF1 in humans, RBPJ in mice, **S**uppressor of hairless in Drosophila, **L**ag1 in C. elegans), which turns it from a repressor to activator by displacing corepressor complexes [13–15], and recruiting coactivators such as Mastermind-like (MAML) proteins [16,17]. Canonical downstream signals of Notch include HES1 and HES5 and Hey1.

Notch and Hematopoiesis

1. Notch, Leukemias and Hematopoietic Lineage Allocation

Strong evidence for the role of Notch signaling in hematopoiesis is provided by the finding that aberrations in Notch can lead to leukemias. Notch was first linked to T-cell neoplasias in the late 80's and early 90's with the identification of the t (7;9) chromosomal translocation which was cloned from a subset of T-cell acute lymphoblastic leukemias (T-ALL) [6,7]. This translocation resulted in a truncated form of Notch1 termed translocation associated Notch homolog (TAN1), which correlates to the activated NICD. When bone marrow was retrovirally transduced with TAN1 50% of the mice developed T-ALL with similar presentation to the human disease [18]. Additional studies with the dominant active forms of Notch receptors in hematopoietic progenitor cells or immature thymocytes also induced development of T-cell leukemias [18–20]. In a study conducted on pediatric cases of T-ALL over 50% of the subjects had a Notch mutation [21]. Further studies have suggested that that Notch signaling in the thymic microenvironment also plays a role in T-cell/B-cell lineage commitment [22].

Notch has been implicated in many lymphoid leukemias but there have been only a few reports of Notch mutations in myeologenous leukemias, where it is unclear whether the Notch abnormalities caused the leukemia [23]. However, some studies have demonstrated Notch effects on myelopoiesis, with Notch ligands suppressing myeloid differentiation of progenitor cells [24]. Together, these data of effects of Notch signaling in both lymphoid and myeloid lineages suggest that Notch signaling may be involved in hematopoietic lineage allocation and differentiation. These Notch effects have been extensively reviewed elsewhere [25,26].

2. Notch and HSC

In an effort to test Notch signaling's effect on HSC, Varnum-Finney et al. transduced lineage negative (lin-), c-kit positive, sca-1 positive cells (LSK), which represent a

population phenotypically enriched for HSC, with the active Notch1 intracellular domain (N1ICD) [27]. Compared with untransduced cells, which were no longer viable by 25 days, the cells transduced with N1ICD continued to maintain an undifferentiated appearance and proliferate for over 8 months, suggesting that Notch activation increases HSC self-renewal. *In vivo* transplant studies with these cells showed that repopulation could occur in all blood lineages although B-cell reconstitution was reduced, consistent with a Notch effect also on lineage allocation. Interestingly, if the transduced LSK were transplanted without other cells, the animals would die after two weeks, indicating that Notch1 activation kept HSC in either a more immature state and prohibited them from differentiating into mature cells or that they could not enter into the periphery [27]. In a similar study, Stier et al transduced lin-sca-1+ cells with activated Notch1. They found that Notch1 activation inhibited differentiation of HSC both *in vitro* and *in vivo* by impeding the differentiation of stem cells from the progenitor pool resulting in an increase of HSC which was confirmed by secondary transplantation experiments [28]. Taken together, these results suggest that Notch signaling maintains and may expand HSCs.

To identify and quantify Notch activation *in vivo* transgenic Notch reporter (TNR) mice were generated, which express GFP when Notch signaling is activated [29]. The LSK compartment from the TNR mice had a higher percentage of GFP expression than their progeny, which indicates that Notch signaling is active in more undifferentiated populations. LSK that were either GFP+ or GFP− formed the same number of colonies (CFU-C), but the GFP+ LSK had significantly more multiple cell type colonies, suggesting that these cells were multipotent and more undifferentiated. These results were also confirmed *in vivo* when GFP+ LSK were transplanted into irradiated recipients and had a greater long term reconstitution as demonstrated by secondary transplantation experiments [30]. Additionally transplanted Notch-inhibited HSC were found to deplete the HSC pool as they preferentially differentiated instead of undergoing self renewal [29]. Using GFP+ LSK from TNR mice this group was also able to demonstrate that the precursors divide symmetrically in a prorenewal environment and asymmetrically in prodifferentiation environment [30]. Again, these data suggest that Notch signaling maintains and expands the most primitive HSC.

Thus, Notch-related data generated so far in general support the concept that Notch signaling affects stem cell regulation by favoring self renewal over differentiation [28].

Notch Signaling in Osteoblastic Cells

1. *In vitro* Studies

Many independent studies have established that Notch1 and the Notch ligands Jagged1 and Delta-like1 are expressed in osteoblasts [10,31–36]. *In vitro* studies attempting to elucidate the role of Notch signaling in bone have led to contradictory conclusions that Notch activation either impairs [32,33,37–39] or facilitates [34,40,41] osteoblastic differentiation. The disparity in these studies could be due to different cellular models, different stages of differentiation when cells were used for experimentation, as well as the stable or transient transfection of Notch signaling elements. Although the *in vitro* studies do not offer conclusive evidence about the role of Notch signaling in osteoblastic cells, it is it apparent that Notch signaling has important effects on osteoblastic cells. Some of the contradiction in the *in vitro* results may stem from the likely possibility that Notch may play different roles depending on the maturation stage of the osteoblastic cell, as it does in other biological systems.

2. *In vivo* Studies

Recent *in vivo* studies gave us more insight into the role of Notch signaling in bone using genetic models with Notch signaling elements altered at different stages of differentiation.

Engin et al. overexpressed the N1ICD under the control of the type I collagen (Col1α 1) promoter and found that genetically altered animals had an osteosclerotic phenotype with thickened bone mass and a marrow cavity filled with trabecular bone [8]. Additionally, all Notch signaling was removed *in vivo* from osteoblastic cells by targeted deletion of the required components of the γ-secretase complex. Presenilin1/2 (PS1/2) are components of the γ-secretase complex that cleaves and activates the NICD. A mouse model was generated in which Presenilin2 is globally deleted and Presenilin 1 is selectively removed from osteoblastic cells only ($Col1\alpha1^{cre/+}$ $PS1^{fl/fl}$ $PS2^{-/-}$ mice) [8]. Previous studies had demonstrated that removal of both Presenilins completely abolishes NICD production [42], however, since γ-secretase activity also plays a major role in cleaving and regulation the amyloid precursor protein to form β-amyloid proteins as well as Cadherins and other proteins [42], the results from these animals must be interpreted in the context of other Notch genetic models. $Col1\alpha1^{cre/+}$ $PS1^{fl/fl}$ $PS2^{-/-}$ mice appeared normal at 3 months compared to littermate controls ($PS1^{fl/fl}$ $PS2^{-/-}$), but developed an osteoporotic phenotype at 6 months. These results suggest that loss of canonical Notch signaling led to osteoporosis through activation of osteoclastogenesis (see below) and increased bone resorption compared to formation rates with age[8].

Hilton et al. also used the $PS1^{fl/fl}$ and $PS2^{-/-}$ mice, but crossed them with a Prx1 cre, which is expressed earlier, at the stage of mesenchymal stem cells (MSCs) [9]. The $Prx1^{cre/+}$ $PS1^{fl/fl}$ $PS2^{-/-}$ mice had shorter, denser long bones than controls ($PS1^{fl/fl}$ $PS2^{-/-}$) at eight weeks but died unexpectedly at 9–10 weeks of age from unknown causes. The $Prx1^{cre/+}$ mice were also mated with $Notch1^{-/fl}$ $Notch2^{fl/fl}$ mice (PNN) with the hope that these animals would survive through adulthood. These animals had a similar phenotype at 8 weeks to the $Prx1^{cre/+}$ $PS1^{fl/fl}$ $PS2^{-/-}$ with increased bone mass and trabecular bone in the marrow cavity. Interestingly by 15 and 26 weeks the PNN mice lost the increased bone mass which was reduced to a level far below the control animals, likely also due to effects on osteoclasts [9].

Similar to some of the *in vitro* results, these two studies report contrasting data which is very likely due to Notch's divergent roles at different stages of differentiation. An example of opposite effects of Notch signaling depending on differentiation stage is provided by neuronal differentiation. In neuronal development Notch signaling has been well characterized and is either required to be up or down regulated in order for certain cells types to form during differentiation [43]. It is likely that Notch signaling works in a similar fashion in osteoblastic differentiation. It appears that Notch signaling is important for maintaining osteoblastic progenitors because removal from MSCs results in a short term dramatic increase in bone. Therefore Notch signaling could be used to maintain a progenitor population and also be involved in terminal differentiation. We can also speculate that Notch signaling in mature or terminally differentiating osteoblastic cells might ultimately feedback on the MSC population and call for further self renewal. Additionally, both these studies suggest that Notch signaling may play a further role in bone homeostasis by having direct effects on osteoclasts. Additional data have suggested an important role of Notch signaling in osteoclastic differentiation and activation [44,45]. Further investigation into these models and direct comparison of developmental activation and inactivation of Notch signaling is necessary to determine the mechanisms by which Notch signaling alters osteoblastic maturation and bone maintenance.

Notch and Stem Cell Niches

As a system providing rapid environmental cell-initiated signals to stem cells, the Notch signaling pathway would be predicted to play an important role in stem cell niches. In fact, much data support the role of Notch signaling as a key mediator of niche-stem cell

interactions in a number of organisms and in numerous organ systems. For example, Notch is reported to be important in gonadal niches in both worms [46] and flies [47,48], where Notch signaling may also determine niche size. In the murine heart, cardiac progenitor cells express Notch1, while supporting cells express Jagged1, and Notch activation regulates early fate choices in cardiac progenitor cells [49]. Thus, a strong precedent exists for Notch ligands in the microenvironment to initiate regulatory Notch activation in stem cells.

Notch and the Microenvironmental control of Hematopoiesis

Examples exist of the importance of Notch signaling in relaying instruction from the microenvironment to stem cells in numerous systems, but what about mammalian HSCs? Mammalian HSCs are some of the best characterized stem cells. One of the difficulties in determining the signals involved in HSC regulation by the microenvironment has been that the cellular regulatory components of the bone marrow microenvironment have only recently come to light. Currently the HSC niche is thought to be comprised of either osteoblastic cells [10,50,51], endothelial cells (reviewed in Colmone et al. [52]), or potentially both. As we have already discussed, Notch signaling has been shown to play roles in both HSC regulation and in osteoblastic cells. Multiple *in vitro* studies have demonstrated that cells expressing Notch ligands or plates with immobilized Notch ligands can maintain or enhance HSC self renewal in culture [53–59]. The *in vitro* studies all show a Notch-dependent consistent increase in HSCs, but as we review below the growing body of evidence studying *in vivo* Notch signaling between HSCs and the bone marrow microenvironment remains contradictory.

The Notch ligand Jagged1 is expressed in bone marrow stromal cells [53,60] and murine osteoblastic cells [32,35], and increased Jagged1 in human stroma is sufficient to expand HSC [60]. Additionally Jagged1 is expressed by human derived mesenchymal-like CD146+ cells which can form a hematopoietic supportive niche [61]. Although the functionality of Jagged1 in these cells was not examined this provides further evidence of the involvement of Notch signaling in the niche. Our own work also implicated Notch signaling in the parathyroid hormone (PTH) mediated increase of HSC by osteoblastic cells [10]. Mice with a constitutively active PTH receptor expressed in osteoblastic cells [62] had an increase in osteoblastic Jagged1, increased HSC NICD levels, and increased numbers of HSCs [10]. The results could also be replicated with PTH injections [10]. We and others have also found that PTH administration leads to an increase in Jagged1 both *in vivo* and *in vitro*[35,63]. Further *in vitro* studies suggested that the PTH-dependent HSC increase requires direct contact of stroma and HSC [10]. Moreover, this increase could be abrogated by administration of a γ-secretase inhibitor [10]. Together, these *in vitro* data are consistent with an important role of Notch in niche regulation of HSC behavior. As constitutively active PTH receptor mice only have an altered receptor in osteoblastic cells, and HSC do not express the PTH receptor [64] the response of HSC to PTH must be initiated by osteoblastic cells, however the differentiation stage of these HSC-supportive osteoblastic cells is currently unknown.

In contrast to these studies, others have reported that Jagged1 and Notch signaling are not important for maintaining HSC populations. Mancini et al. generated a Jagged1 floxed ($^{fl/fl}$) mouse and bred it to the interferon inducibile Mx1 cre mice [65,66]. After cre induction, the basal levels of HSC were not significantly different from the control animals. The same was true when using the induced Mx1 cre in combination with the Notch1 $^{fl/fl}$ animal or the combination of Jagged1 $^{fl/fl}$/Notch1$^{fl/fl}$. Mx1 cre Notch1 $^{fl/fl}$ HSC were transplanted into Mx1 cre Jagged1 $^{fl/fl}$ mice and had similar basal levels to control HSC after induction of the cre. This study suggests that Notch signaling is not necessary in maintaining HSC in basal conditions. Additionally, only Jagged1 and Notch1 were removed in the experimental

conditions and other Notch signaling elements could be compensating for their loss. Following this publication, Maillard et al. developed a dominant negative Mastermind-like1 (dnMAML) which binds to the activated NICD and inhibits Notch 1–4 from signaling [67]. Whether dnMAML was transfected into LSK or if the LSK came from genetically altered animals, these cells reconstituted the bone marrow equally to cells from controls, except for changes in T-cells which would be expected with models affecting Notch signaling. They also found no difference after secondary transplantation suggesting that the long term repopulating capabilities of HSC and the LT-HSC population do not require Notch signaling. These results were confirmed with Mx1 cre RBPJ $^{fl/fl}$, where RBPJ encodes for a DNA-binding factor that is necessary for signaling of all Notch receptors. The models used in this study rule out the possibility of compensation by alternative Notch receptors as all Notch signaling is removed and they still do not see a change in basal HSC or reconstitution ability. Both of these studies indicate that Notch signaling is not necessary for the maintenance of HSC at steady state and in some situations of myeloablative injury.

In contrast to the latter studies, evidence that Notch in the microenvironment is important for normal hematopoiesis was recently provided by Kim et al. [68]. In these experiments, inactivation of Mind bomb-1 (Mib1), which is essential for Notch ligand endocytosis and Notch activation, resulted in the development of myeloproliferative disease (MPD). The surprising finding was that transplantation of wild-type bone marrow cells into the Mib-1 null microenvironment resulted in *de novo* MPD. Activated N1ICD cells were also transplanted into the Mib1 null microenvironment which significantly slowed progression of the MPD [68]. These results suggest that the MPD resulted from the non-hematopoietic microenvironmental cells with defective Notch signaling. These experiments demonstrate that Notch signaling in the microenvironment does play a role in maintaing normal hematopoiesis.

Concluding thoughts

Although the verdict is still out on the role of Notch in HSC maintenance, it is evident from *in vitro* studies that Notch ligands can increase HSC and maintain progenitor populations. It is also appears that normal Notch signaling in the bone marrow microenvironment is necessary to maintain normal hematopoiesis. Based on the data available so far, it is unlikely that Notch signaling is the only pathway to regulate HSC within the niche. Many other pathways, such as N-cadherin [50], Angiopoietin1/Tie2 [69], Osteopontin [70,71], Annexin II [72] and Wnt [73] have been implicated in HSC regulation by the osteoblastic niche. Removing only one of these and looking at basal levels may not be enough to definitively determine the role of the pathway and may explain some of the varied results that have been presented to date.

Further studies are necessary to determine the roles of Notch signaling throughout osteoblastic maturation and the effects of Notch on bone maintenance. This will require removing or activating Notch signaling at maturation stages to determine how Notch is acting as it can cause self renewal, binary fate decisions, or terminal differentiation. However, given the roles identified for Notch in both osteoblastic and hematopoietic cells, it is likely that Notch is an important regulator of hematopoietic osteoblastic regulatory interactions. Notch can therefore potentially be regarded as a target for pharmacological expansion of HSC in situations of clinical need, such as in the recovery from environmental or iatrogenic myeloablation.

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