Roles of Notch in Hematopoietic Stem Cell Proliferation After Injury

The stem cells found in many tissues of adult organisms can regenerate differentiated cells to maintain tissue homeostasis. The stem cell niche is the microenvironment that provides the signals required for maintenance of self-renewing adult stem cells, thus ensuring a constant supply of progenitors throughout the entire life of an organism (Morrison and Spradling *Cell* 2008). In mammals, hematopoietic stem cells (HSCs) are a small population of progenitor cells that differentiate into diverse types of blood cells including lymphocytes, dendritic cells, erythrocytes, megakaryocytes, and myeloid cells (Tsiftsoglou et al. *Pharm and Therap* 2009). During development HSCs are generated mainly in the mammalian liver, but after birth they migrate to the bone marrow where they are maintained in a perisinusoidal niche and facilitate adult hematopoiesis (Ding et al. *Nature* 2012). HSCs and lineage-restricted progenitor populations must undergo massive expansion during differentiation to repopulate rapidly turned-over blood cells. As a result, loss or mis-regulation of HSCs quickly exhibits major affects on the derived cell populations (Kiel and Morrison *Nat Rev Immunol* 2008). Therefore, it is not surprising that there are a wide variety of leukemias associated with the hematopoietic lineages. Signaling pathways originating from the niche are often mis-regulated in cancer and in degenerative diseases. Thus, understanding their molecular nature and their cells of origin is critical to human health.

Although several signaling pathways are mis-regulated in the initiation of leukemia, improper regulation of the well-characterized Notch pathway appears to be essential for most of these cancers (Klinakis et al. *Nature* 2011). Notch signaling is known to be involved in embryonic HSC generation and self-renewal, but its role in the adult niche in HSC maintenance and self-renewal is unclear (Bigas and Espinosa *Blood* 2012). Notch1 in adult HSC fate determination has been extensively analyzed, especially in the context of myeloid and lymphoid differentiation (Radke et al. *Immunity* 1999). The role for Notch at this step in differentiation has implications for cancers such as chronic myelomonocytic leukemia (CMML) and T-cell acute lymphoblastic leukemia (T-ALL) (Klinakis et al. *Nature* 2011).

Recent reports, however, indicate that HSC identity does not depend on Notch signaling. In these studies, HSCs were not lost *in vivo* under basal, unstressed conditions when Notch or members of the Notch pathway were mutated or inhibited. The major phenotype was an alteration in the proportion of myeloid to lymphoblastic cells (Klinakis et al. *Nature* 2011; Maillard et al. *Cell Stem Cell* 2008). It has been suggested, however, that expansion of both long and short-term HSC populations after injury is dependent on Notch2. In cell culture, exposing HSCs to the Notch ligand Jagged leads to proliferation without differentiation (Bigas and Espinosa *Blood* 2012). In mouse studies *in vivo*, deletion of Notch2 from the bone marrow inhibits the expansion of the HSC population after injury to the bone marrow and hematopoietic lineages (Varnum-Finney et al. *JCI* 2011). Because of background expression of the Cre-driver in other cell types, however, conclusive interpretation of these results is impossible.

Notch2 does appear to be constitutively expressed, at least at low levels, in adult HSCs, but it is unclear whether activation of the Notch ligand Jagged is required in specific cell types to activate Notch2 in HSCs during the injury response. Sufficiency studies have shown that Jagged1 up-regulation in osteoblast cells triggers the expansion of the HSC population, but it is unknown whether bone marrow stress induces expression of Jagged1 in the HSC niche cells (Calvi et al. *Nature* 2003). Furthermore it is also unknown which cell types are required to express Jagged1 and stimulate the proliferation of HSCs, as necessity studies have not yet been performed in the HSC niche (Ding et al. *Nature* 2012).

Finally, the RNA-binding protein Musashi2 (Msi2), which is expressed at high levels in adult HSCs, has been implicated in the regulation of Notch receptor expression in neural stem cells (Sakakibara et al. *J Neuro* 2001). The presence of Msi2 suggests that expression of Notch may be required for some role in HSC activity prior to differentiation. In HSCs, down-regulation of Msi2 is associated with down-regulation of the Notch receptor in

culture and up-regulation of the Notch inhibitor Numb (Hope et al. *Cell Stem Cell* 2010). It is thought that Msi2 regulates Notch signaling through binding *numb* mRNA and inhibiting its translation, but this mechanism has not yet been tested. Msi2 has several targets in stem cells and could be regulating Numb and Notch expression through one of these targets. **I propose that Notch2 is activated in HSCs during times of injury or hematopoietic stress to expand the HSC population. This is achieved by up-regulation of Jagged1 in sinusoidal endothelial cells rather than through change in expression of Notch itself, which I propose is controlled by Msi2 through direct inhibition of Numb (See Figure 1).** The following aims are designed to test this hypothesis in a murine model system:

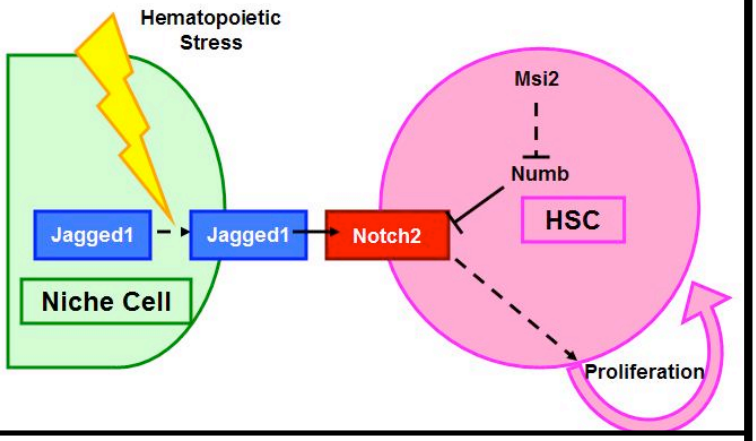

Figure 1: A model for the activation and role of Notch in adult hematopoietic stem cells. Steps being tested in this proposal are indicated by dashed lines.

Aim 1: Determine if activation of the Notch pathway through Notch2 signaling in HSCs is required cell-autonomously for HSC proliferation after injury to the bone marrow. The controversy over the effect of Notch on HSC proliferation stems in part from a lack of cell-type specificity of current studies. I will use cell-type specific tamoxifen-inducible Cre drivers to delete Notch2 specifically from adult HSCs (Vav1-CreERT) (Ding et al. *Nature* 2012) or from lineage-specific hematopoietic progenitors (CD48-CreERT) (Kiel et al. *Cell* 2005). Using the chemotherapeutic agent 5-FU to induce injury (Varnum-Finney et al. *JCI* 2011), I will test if Notch2 is required cell-autonomously in HSCs for expansion and tissue regeneration by quantifying HSC number with immunohistochemistry and fluorescence activated cell sorting (FACS). I will also use serial bone marrow transplantation and competitive reconstitution assays as standard tests to analyze whether there is a selective advantage of Notch2 expression in HSCs. A constitutive transgene of the primary Notch target Hes1 (Klinakis et al. *Nature* 2011) will be used for rescue.

Aim 2: Analyze whether Jagged-1 up-regulation in vascular endothelial cells after injury activates the Notch2 receptor in HSCs to promote proliferation, and if exogenous expression of Jagged-1 is sufficient to expand the niche beyond the perisinusoidal region. I will use immunohistochemistry to compare the expression of Jagged-1 in the bone marrow between control and 5-FU-treated mice to determine if and in which cell type(s) expression of Jagged-1 is up-regulated in response to injury. I will then use cell-type specific Cre-drivers as described in Ding et al. *Nature* 2012 to discern in which of these cell types Jagged-1 expression is required for Notch2 activation in HSCs both during homeostasis and in response to injury. I will also determine sufficiency of Jagged-1 to ectopically expand the niche using cell-type specific over-expression.

Aim 3: Test whether Msi2 binds and directly inhibits the Notch inhibitor Numb *in vivo* to activate Notch2. Up to this point the connection between Msi2 and Notch activation in HSCs has been correlative and the mechanism unclear. I will use a previously described *in vitro* binding assay to test whether Msi2 binds to the predicted Msi1 binding site in the Numb 3'UTR (Imai et al. *Mol Cell Bio* 2001). I will then use a GFP-tagged Numb transgene containing a wild type or mutated Msi2 binding site to determine whether direct Msi2 interaction is necessary for reduction in Numb protein expression in HSCs in whole mice. Finally, using the Numb with a mutated binding site in either a wild type or Msi2 null background will show whether Msi2-mediated activation of Notch2 and associated HSC proliferation in the context of bone marrow injury requires the interaction of Msi2 and Numb.

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