Hematopoietic Jagged1 is a fetal liver niche factor required for functional maturation and engraftment of fetal hematopoietic stem cells

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Abstract: Notch signaling is essential for the emergence of definitive hematopoietic stem cells (HSCs) in the embryo and their development in the fetal liver niche. However, how Notch signaling is activated and which fetal liver cell type provides the ligand for receptor activation in HSCs is unknown. Here we provide evidence that endothelial Jagged1 (Jag1) has a critical early role in fetal liver vascular development but is not required for hematopoietic function during fetal HSC expansion. We demonstrate that Jag1 is expressed in many hematopoietic cells in the fetal liver, including HSCs, and that its expression is lost in adult bone marrow HSCs. Deletion of hematopoietic Jag1 does not affect fetal liver development; however, Jag1-deficient fetal liver HSCs exhibit a significant transplantation defect. Bulk and single-cell transcriptomic analysis of HSCs during peak expansion in the fetal liver indicates that loss of hematopoietic Jag1 leads to the downregulation of critical hematopoietic factors such as GATA2, Mllt3, and HoxA7, but does not perturb Notch receptor expression. Ex vivo activation of Notch signaling in Jag1-deficient fetal HSCs partially rescues the functional defect in a transplant setting. These findings indicate a new fetal-specific niche that is based on juxtracrine hematopoietic Notch signaling and reveal Jag1 as a fetal-specific niche factor essential for HSC function.

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Hematopoietic Jagged1 is a fetal liver niche factor required for functional maturation and engraftment of fetal hematopoietic stem cells

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Notch signaling is essential for the emergence of definitive hematopoietic stem cells (HSCs) in the embryo and their development in the fetal liver niche. However, how Notch signaling is activated and which fetal liver cell type provides the ligand for receptor activation in HSCs is unknown. Here we provide evidence that endothelial Jagged1 (Jag1) has a critical early role in fetal liver vascular development but is not required for hematopoietic function during fetal HSC expansion. We demonstrate that Jag1 is expressed in many hematopoietic cells in the fetal liver, including HSCs, and that its expression is lost in adult bone marrow HSCs. Deletion of hematopoietic Jag1 does not affect fetal liver development; however, Jag1-deficient fetal liver HSCs exhibit a significant transplantation defect. Bulk and single-cell transcriptomic analysis of HSCs during peak expansion in the fetal liver indicates that loss of hematopoietic Jag1 leads to the downregulation of critical hematopoietic factors such as GATA2, Mllt3, and HoxA7, but does not perturb Notch receptor expression. Ex vivo activation of Notch signaling in Jag1-deficient fetal HSCs partially rescues the functional defect in a transplant setting. These findings indicate a new fetal-specific niche that is based on juxtracrine hematopoietic Notch signaling and reveal Jag1 as a fetal-specific niche factor essential for HSC function.

hematopoietic stem cells | fetal liver | Notch signaling | embryonic development

Significance

During embryonic development, newly emerged hematopoietic stem cells (HSCs) migrate to the fetal liver where they undergo expansion without exhaustion of hematopoietic potential. Why are fetal HSCs capable of robust functionality? We hypothesized that it due to the microenvironmental cues of the fetal liver niche, one of which is activation of Notch signaling. Through the use of single-cell analysis and transplantation assays, we provide evidence of Jagged1 as a novel niche factor for fetal liver HSCs. Multiple hematopoietic cells present Jagged1 to HSCs in the fetal liver. Notch activity in HSCs drives expression of critical hematopoietic factors such as GATA2. A better understanding of the fetal liver niche can be leveraged to new discoveries that enhance efficacy of hematopoietic transplants.

During hematopoietic development, the fetal liver (FL) functions as an intermediary niche for developing hematopoietic stem cells (HSCs) (1). Cell intrinsic characteristics of FL HSCs as well as the overall cellular organization of the FL niche have been studied in recent years (2–10). Yet there remains a significant gap in our understanding of the molecular cues and cell types in the FL niche that regulate HSC function. One of the unique signaling pathways in the FL niche is Notch signaling, which is highly conserved in multicellular organisms (11) and regulates the emergence of the first definitive HSCs from the hemogenic endothelium in the aorta-gonad-mesonephros (AGM) region in days 9.5 to 10.5 postconception (E9.5-10.5) in mice (12–14). Notch signaling is critical for survival and function of definitive HSCs in the FL microenvironment (3, 15). Importantly, while Notch signaling is active in the FL niche, it is dispensable for the maintenance and function of postnatal BM HSCs (16, 17). Thus, the Notch pathway represents a unique signaling mechanism that distinguishes proliferative and highly functional FL HSCs from the predominantly quiescent adult BM HSCs.

In mammals, there are four Notch receptors (Notch1-4) and five Notch ligands, Delta-like (Dll1, 3, and 4) and Jagged (Jag1 and Jag2) (18, 19). The direct interaction of a ligand and receptor on adjacent cells initiates a series of proteolytic cleavages causing the intracellular domain of Notch receptor (ICN) to release from the plasma membrane, translocate to the nucleus, and form a transcriptional activation complex along with the DNA-binding protein RBPJ and co-activator Mastermind-like homolog (20). Though the components and the activation mechanism are both highly conserved and widely utilized in many developing and adult tissues, the various combinations and permutations of ligands and receptors in different cell types ensure a highly effective and tightly regulated signaling mechanism (21–26). In the specific context of fetal liver hematopoiesis, we have shown that Notch1 receptor is expressed on the surface of E14.5 FL HSCs and its activation is essential for fetal HSC survival and function (3).

Here we used single-cell analysis to determine that FL HSCs express Jag1 and that loss of Jag1 expression in the hematopoietic lineage severely decreases expression of key stem cell genes, and limits engraftment and function of the FL HSCs without perturbing hepatic and endothelial development. A partial rescue of the reconstitution defect seen in Jag1-deficient HSCs can be achieved by presenting synthetic Jag1 ligand to fetal liver

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The authors declare no competing interest.

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HSCs that lack Jag1 but retain Notch receptor expression. Our findings reveal the existence of a hematopoietic-specific niche for Notch activation in the FL and suggest a distinctive mechanism for the generation of highly proliferative and functionally potent HSCs.

Results

Constitutive Deletion of Endothelial Jag1 Prevents Development of Vasculature and Hematopoietic Colonization of the FL. Previous studies have suggested that endothelial cells (ECs) may impact HSC development in the FL (9, 27). We analyzed ECs ($CD45^-CD31^+CD144^+$) by flow cytometry from wildtype (WT) E14.5 FL and detected high surface expression levels of the Notch ligands Dll4 and Jag1 in ECs (*SI Appendix*, Fig. S1 *A* and *B*). Constitutive Cdh5-Cre/VE-cadherin strain, which is active early in development ~E8.5, also referred to as VEC-Cre (28), was crossed with $Jag1^{f/f}$ mice (*SI Appendix*, Fig. S1*C*) and yielded efficient loss of Jag1 expression in FL ECs (*SI Appendix*, Fig. S1*D*). The deletion of Jag1 in FL ECs, also affected expression of canonical Notch target Hes1, indicating a perturbation of Notch target expression in FL ECs (*SI Appendix*, Fig. S1*D*).

$Jag1^{f/f}$VEC-Cre^+ E14.5 embryos were stunted and poorly vascularized and presented with a substantially smaller FL compared to the heterozygous $Jag1^{+/f}$ littermates (*SI Appendix*, Fig. S1*E*). A colony-forming assay using complete Methocult media showed a complete lack of hematopoietic potential from $Jag1^{f/f}$VEC-Cre^+ FL when compared to heterozygous $Jag1^{+/f}$ littermates (*SI Appendix*, Fig. S1 *F* and *G*). 3D confocal imaging was used to visualize the FL microenvironment of $Jag1^{f/f}$VEC-Cre^+ mutants. Complete Jag1 deletion under the VEC-Cre driver severely disrupted the formation of FL sinusoidal vessels and the limited colonization of the FL by hematopoietic cells. This is evident by a massive reduction of Lyve-1 expression in $Jag1^{f/f}$VEC-Cre^+ (*SI Appendix*, Fig. S1*H*) a profound loss of cellular organization and defective accumulation of c-kit^+ hematopoietic progenitors (*SI Appendix*, Fig. S1*I*). Heterozygous $Jag1^{+/f}$ littermates had normal FL morphology (*SI Appendix*, Fig. S1*H*) comparable to WT littermates (*SI Appendix*, Fig. S2*A*). In fact, loss of a single copy of Jag1 showed no significant downregulation of overall Jag1 expression levels (*SI Appendix*, Fig. S2*B*) or defect in hematopoietic potential or overall numbers of FL ECs and HSCs (*SI Appendix*, Fig. S2 *C* and *D*). These findings indicate that Jag1 plays a prominent role in early vascular and hematopoietic development and that its efficient deletion precluded further analysis of its role in hematopoietic development in the FL.

Inducible Deletion of Endothelial Jag1 during Mid-gestation Shows No Effect on Function of FL HSCs. To bypass the severe early development defects seen in constitutive endothelial deletion mutants of Jag1, we utilized an inducible VE-cadherin-Cre/Cdh5-Cre^{ERT2} (VE-cadCre^{ERT2}) strain (29). In timed matings, the $Jag1^{f/f}$VE-cadCre^{ERT2+} mice were treated with a regiment of 4-hydroxytamoxifen (4-OHT) injections started at E10.5 (0.5 mg) and incrementally increased to a higher dose of 2.0 mg by E12.5. The treatment is graphically shown in Fig. 1*A*. To test the timing and efficiency of Jag1 deletion, we first harvested FLs from E12.5 embryos and measured Jag1 expression in both FL ECs ($CD45^-CD31^+CD144^+$) and HSCs (*SI Appendix*, Fig. S3*C*). We observed that at E12.5 the tamoxifen treatment had no significant effect on the expression of Jag1. Comparable overall embryonic morphology was observed between $Jag1^{f/f}$VE-cadCre^{ERT2-} and $Jag1^{f/f}$VE-cadCre^{ERT2+} littermates (Fig. 1*B*), and by E14.5, mRNA expression of Jag1 was markedly reduced in FL

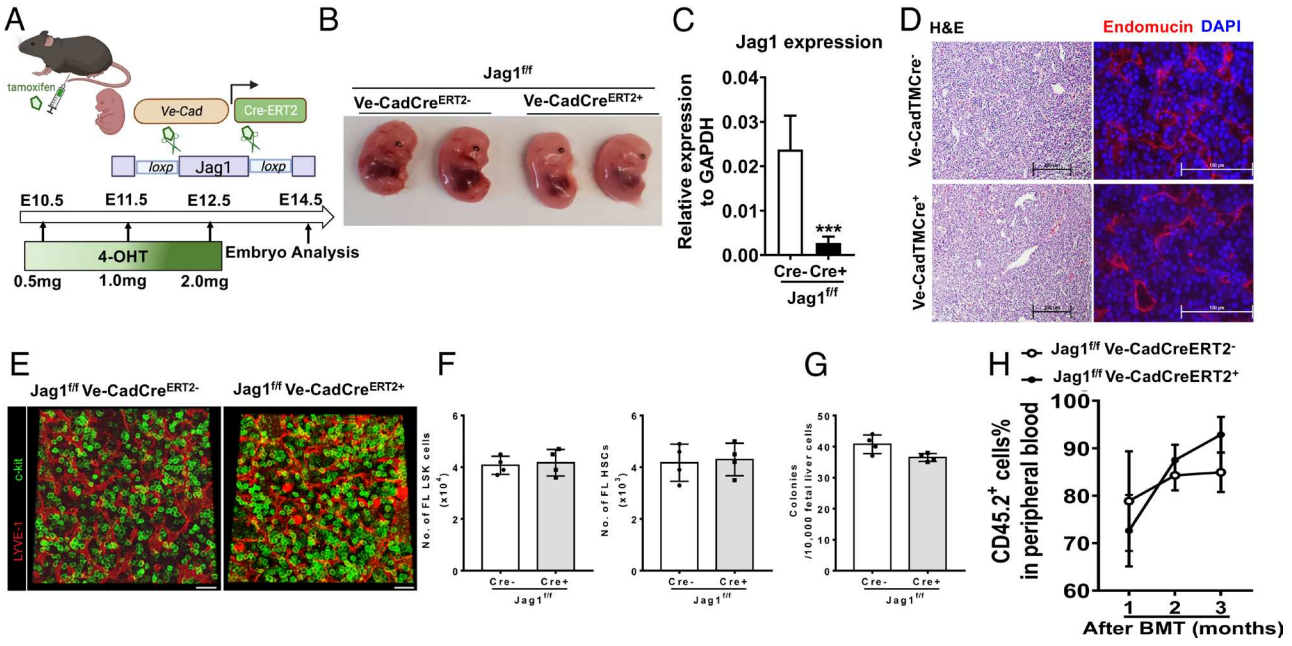

Fig. 1. Inducible loss of endothelial Jag1 does not affect function of HSCs in E14.5 fetal livers. (*A*) Schematic diagram of inducible loss of endothelial Jag1. Pregnant $Jag1^{f/f}$Ve-CadherinCre^{ERT2+} female was administrated treatment with 4-OHT at times shown. Embryos were analyzed at E14.5. (*B*) Gross comparative view of E14.5 embryos from $Jag1^{f/f}$Ve-CadherinCre^{ERT2-} and $Jag1^{f/f}$Ve-Cadherin Cre^{ERT2+} after tamoxifen treatment. (n = 3). (*C*) Jag1 expression by qRT-PCR in sorted FL ECs from $Jag1^{f/f}$Ve-Cadherin Cre^{ERT2-} and $Jag1^{f/f}$Ve-CadherinCre^{ERT2+} embryos. Expression data normalized to GAPDH. ***$P < 0.001$. (n = 3). (*D*) H&E staining of E14.5 fetal livers from each genotype (*Left*). Immunofluorescent staining for endomucin (red) in E14.5 FL (*Right*). DAPI (blue) nucleus. Bar = 100 μm. (n = 3). (*E*) High resolution images of FL sinusoids (red) and distribution of c-kit^+(green) progenitors in FL from E14.5 $Jag1^{f/f}$Ve-Cadherin Cre^{ERT2-} and $Jag1^{f/f}$Ve-Cadherin Cre^{ERT2+}. (Scale bars, 50 μm.) (n = 3). (*F*) Numbers of $Lin^-Sca^+cKit^+$ (LSK) progenitor cells and LSK $CD48^-CD150^+$ (SLAM-LSK) HSCs in E14.5 FL from $Jag1^{f/f}$Ve-Cadherin Cre^{ERT2-} and $Jag1^{f/f}$ Ve-Cadherin Cre^{ERT2+} embryos. (n = 4). (*G*) Numbers of hematopoietic colonies per 10,000 E14.5 fetal liver cells from E14.5 $Jag1^{f/f}$Ve-CadherinCre^{ERT2-} and $Jag1^{f/f}$ Ve-CadherinCre^{ERT2+} embryos. (n = 4). (*H*) Direct transplantation of 2×10^5 $CD45.2^+$ cells from $Jag1^{f/f}$Ve-CadherinCre^{ERT2-} and $Jag1^{f/f}$Ve-CadherinCre^{ERT2+} E14.5 FL donors into lethally irradiated recipient mice ($CD45.1^+$). Percentage of donor-derived cells in peripheral blood analyzed at 1, 2, 3 mo after transplantation (n = 5).

ECs (Fig. 1*C*). E14.5 FL sections from Jag1$^{f/f}$ VE-cadCre^{ERT2-} and Jag1$^{f/f}$VE-cadCre^{ERT2+} littermates were further analyzed by hematoxylin and eosin (H&E) staining and immunofluorescence for sinusoidal capillary marker endomucin. In both cases, no significant differences were observed between Jag1$^{f/f}$VE-cadCre^{ERT2-} and Jag1$^{f/f}$VE-cadCre^{ERT2+} littermates (Fig. 1*D*). Whole fetal lobe imaging (*SI Appendix*, Fig. S3 *A* and *B*) revealed no significant perturbation of the sinusoidal endothelial network or the cellularity of c-kit$^+$ hematopoietic progenitors (Fig. 1*E*). Flow cytometry analyses further showed that the numbers of LSK cells and HSCs in the FL of Jag1$^{f/f}$VE-cadCre^{ERT2+} embryos were comparable to VE-cadCre^{ERT2-} littermates (Fig. 1*F*). Colony forming of FL hematopoietic cells from Jag1$^{f/f}$VE-cadCre^{ERT2+} embryos showed similar numbers to littermate controls (Fig. 1*G*). Direct transplants of FL hematopoietic cells from Jag1$^{f/f}$VE-cadCre^{ERT2+} or Cre^{ERT2-} littermates (CD45.2+) into irradiated recipients (CD45.1$^+$) showed no significant difference in reconstitution potential over 3 mo (Fig. 1*H*) and no difference in multilineage differentiation potential (*SI Appendix*, Fig. S3*D*). Collectively, these findings show that deletion of endothelial Jag1 during mid-gestation in the FL does not perturb development and functionally of FL HSCs at E14.5.

FL HSCs Express Notch Ligands and Notch Receptors. To assess alternative niche cells that activate Notch signaling in FL HSCs, we turned our attention to hematopoietic cells, which are both numerous and diverse in the FL microenvironment (30, 31). We began by screening FL hematopoietic populations at E14.5 for surface expression of Jag1 by flow cytometry. CD71$^+$Ter119$^+$ erythroblasts did not express Jag1 on their surface (*SI Appendix*, Fig. S4*B*). Both CD41$^+$ megakaryocytes and CD11b$^+$ myeloid cells express Jag1 (*SI Appendix*, Fig. S4 *C* and *D*). Interestingly, our analysis also shows that LSK progenitors and HSCs in the FL express Jag1 on the surface (Fig. 2*C*). This raised the possibility that FL HSCs express both Notch ligands and receptors on their surface.

To comprehensively characterize Notch signaling in sorted FL HSCs (Fig. 2*A*), single-cell RT-PCR was performed for all the canonical Notch pathway components, such as receptors, ligands, and cofactors. We analyzed E14.5 FL HSCs and directly compared them to similarly isolated adult BM HSCs from 8-wk-old mice. A clustered heatmap, shown in Fig. 2*B*, indicates the percentage of single cells positive or negative for the expression of all the canonical Notch pathway components in FL and adult BM HSCs. While many of the cells shared expression of key hematopoietic factors such as Gata2 and Runx1, nearly all (~95%) of FL HSCs expressed Jag1 which was not detected in adult BM HSCs. Of the receptors, Notch1, Notch2, and Notch3 all were expressed in over ~70% of FL HSCs, with only Notch2 continuing to be expressed in adult BM HSCs (Fig. 2*B*). Specific RT-qPCR examples of representative genes from dataset of Fig. 2*B* in single FL HSC or adult BM HSCs are shown in *SI Appendix*, Fig. S4*A*.

We then performed flow cytometric analysis for surface expression of two Notch ligands, Jag1 and Dll4, in HSCs during three stages of hematopoietic development: AGM (CD41$^+$cKit$^+$) (32, 33), FL (SLAM-LSK), and adult BM (SLAM-LSK). Our data indicate that Dll4 is expressed more on nascent AGM HSCs, but in the FL, nearly 50% of all HSCs express Jag1 on their surface

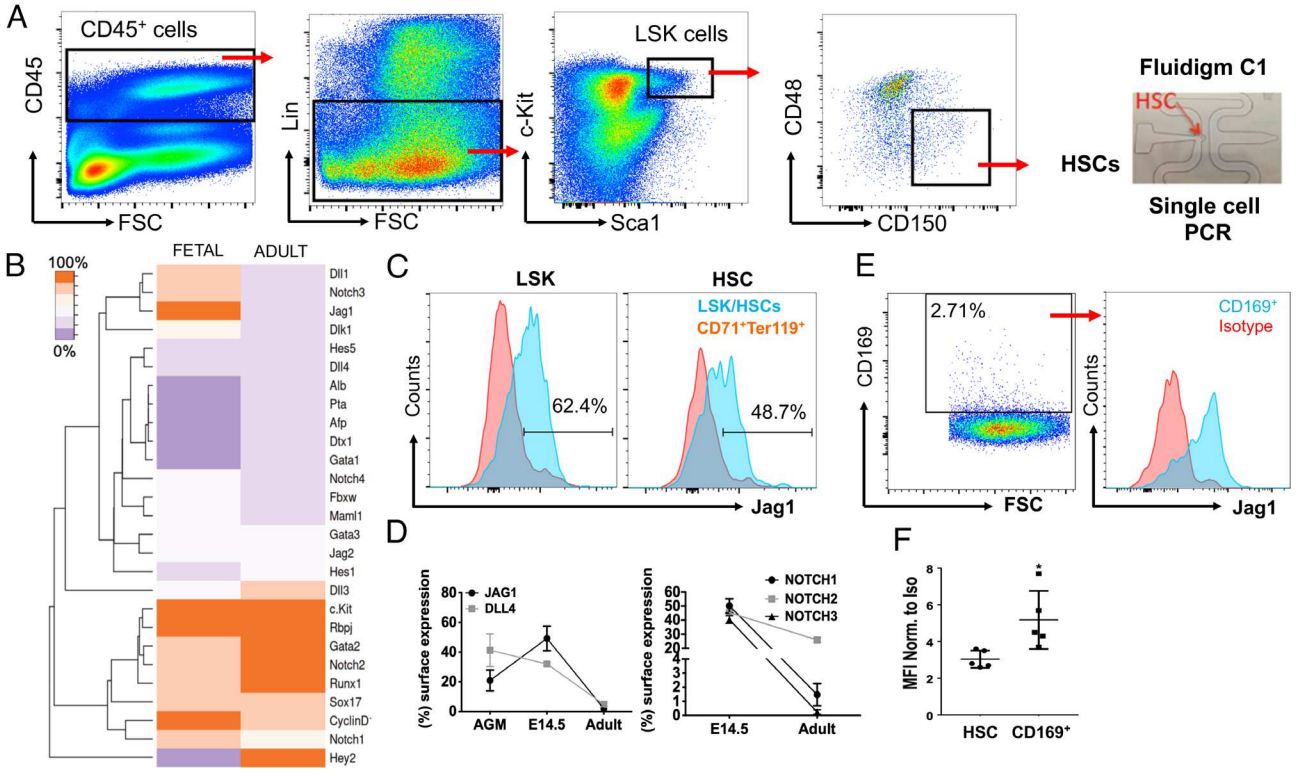

Fig. 2. Jag1 is highly expressed in HSCs from E14.5 fetal livers. (*A*) HSC gating strategy for isolation of SLAM-LSK cells from E14.5 fetal livers. Similar gating was used for adult BM. Individual cells were loaded and visually confirmed in the chamber of the Fluidigm C1 system. (*B*) Representative data analysis from single-cell RT-PCR shown in heatmap format with color intensity indicative of the percentages of single HSCs that expressed listed genes relative to two internal controls (GAPDH and EF1a). Complete gene list is shown in *SI Appendix*, Table S2. (*C*) Surface expression of Jag1 in LSKs and HSCs from E14.5 FL. CD71$^+$Ter119$^+$ cells used as a gating control. (*D*) Percentages of surface expression of listed Notch ligands and receptors by flow cytometry analysis on HSCs from AGM (CD41$^+$cKit$^+$), E14.5 FL and adult bone marrow (SLAM-LSK) (n = 3 to 5). (*E*) CD169$^+$ cell gating strategy. Wild type E14.5 fetal liver cells were stained as described in *Materials and Methods*. Jag1 or isotype IgG expression was analyzed in DAPI$^-$CD45$^+$CD169$^+$ cells. (*F*) Mean fluorescence intensity (MFI) of Jag1 expression in HSCs and CD169+ FL macrophages presented as mean ± SD normalized to isotype control. ****$P < 0.05$, n = 5 for each.

(Fig. 2*D*). In the adult BM, Jag1 and Dll4 are not detected on the surface of HSCs (Fig. 2*D*). This matched our initial screen of Jag1 surface expression in FL HSCs (Fig. 2*C*) and suggests that Jag1 surface expression in HSCs peaks during their time in the FL. We performed a similar analysis for the surface expression of Notch receptors (Notch1-3). This shows that half of FL HSCs express Notch1, Notch2, and Notch3, but that only Notch2 is detected on the surface of adult BM HSCs (Fig. 2*D*). Of all the hematopoietic cells thus tested, $CD169^+$ macrophages, which are not an abundant population in the FL (Fig. 2*E*), express the highest levels of surface Jag1 (*SI Appendix*, Fig. S4*I*), even when compared to FL HSCs (Fig. 2*F* and *SI Appendix*, Fig. S4*E*). We also compared the expression of Jag1 on the surface of FL $CD11b^+$ monocytes and $CD41^+$ megakaryocyte progenitors with their adult BM counterparts (*SI Appendix*, Fig. S4 *F* and *G*). Our results show that adult BM monocytes and megakaryocytes express lower levels of Jag1 on their surface when compared to their FL counterparts (*SI Appendix*, Fig. S4*H*). These observations demonstrate a dynamic expression pattern of Notch signaling components, particularly highlighted by elevated expression of the Jag1 ligand and several Notch receptors in highly proliferative and self-renewing FL HSCs.

Loss of Hematopoietic Jag1 Expression Does Not Affect HSC Numbers or FL Endothelial Development. To assess the consequences of Jag1 deletion in hematopoietic cells, we generated $Jag1^{f/f}$*Vav*-Cre transgenic mice (Fig. 3*A*) which cause pan-hematopoietic loss of Jag1 expression as early as E12.5 (34). Loss of Jag1 expression in E14.5 FL HSCs by RT-qPCR (Fig. 3*B*) and protein levels in $CD45^+$ cells (*SI Appendix*, Fig. S5*G*) was confirmed. The deletion of Jag1 in E14.5 FL HSCs did not affect expression of Notch1 (Fig. 3*B*). $Jag1^{f/f}VavCre^+$ embryos appeared morphologically similar to littermates at E14.5 and the percentages (*SI Appendix*, Fig. S5*A*) and absolute numbers (*SI Appendix*, Fig. S5*B*) of FL ECs from $Jag1^{f/f}VavCre^+$ embryos and Cre^- littermates showed no significant difference. There was no significant difference in the percentages (*SI Appendix*, Fig. S5*E*) and absolute numbers (Fig. 3*D*) of FL HSCs between $Jag1^{f/f}VavCre^+$ and wildtype littermates in the FL at E14.5.

To visualize the effect of Jag1 deletion in hematopoietic cells on the overall FL microenvironment, we performed microscopy

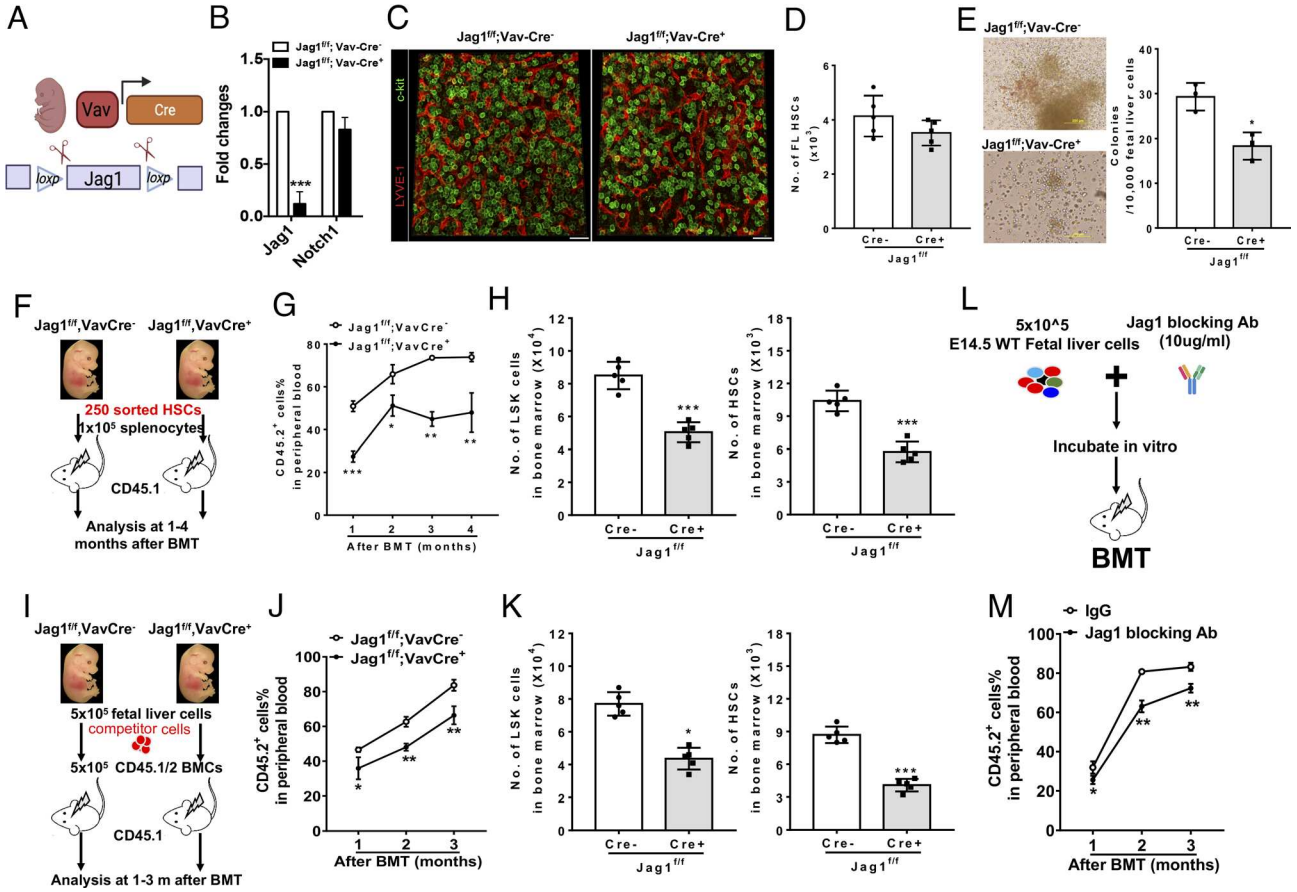

Fig. 3. Loss of hematopoietic Jag1 negatively affects HSC function. (*A*) Schematic showing conditional deletion of hematopoietic Jag1 in $Jag1^{f/f}$Vav-Cre^+ mice. Predicted Cre expression is at E12.5. (*B*) qRT-PCR results of Jag1 and Notch1 expression in sorted HSCs from $Jag1^{f/f}VavCre^-$ and $Jag1^{f/f}VavCre^+$ E14.5 FL. ***P < 0.001 normalized to $Jag1^{f/f}VavCre^-$ and GAPDH (n = 3). (*C*) High magnification images depicting details of vascular sinusoidal structures ($Lyve1^+$) and hematopoietic progenitors ($cKit^+$) of FL from E14.5 $Jag1^{f/f}$Vav-Cre^- and $Jag1^{f/f}$Vav-Cre^+ embryos. (Scale bars, 50 μm.) (n = 3). (*D*) Absolute numbers of HSCs from E14.5 FL for each genotype. (n = 5). (*E*) Representative images (*Left*) of day 12 hematopoietic colonies from $Jag1^{f/f}VavCre^-$ and $Jag1^{f/f}VavCre^+$ E14.5 FL in murine MethoCult™ medium. Quantification of CFU per 10,000 plated cells (*Right*). *P < 0.05. (n = 3). (*F*) Diagram of 250 sorted HSCs ($CD45.2^+$) transplantation from E14.5 $Jag1^{f/f}$Vav-Cre^- and $Jag1^{f/f}$Vav-Cre^+ fetal livers. (*G*) Percentages of donor-derived cells in peripheral blood after transplantation. *P < 0.05, **P < 0.01, **P < 0.01, ***P < 0.001. (n = 5). (*H*) Absolute numbers of donor-derived LSKs and HSCs in the bone marrow at 4 mo after transplantation. ***P < 0.001. (n = 5). (*I*) Diagram of competitive FL cell transplantation. 5×10^5 E14.5 FL donor cells ($CD45.2^+$) from each genotype were transplanted into lethally irradiated recipient mice ($CD45.1^+$) along with 5×10^5 adult bone marrow competitor cells ($CD45.1/.2^+$). (*J*) Percentages of donor-derived cells in peripheral blood after transplantation. *P < 0.05, **P < 0.01. (n = 5). (*K*) Absolute numbers of donor-derived LSK progenitors and HSC in the BM at 3 mo after transplantation. *P < 0.05, ***P < 0.001. (n = 5). (*L*) Diagram of experimental design of E14.5 FL donor cells ($CD45.2^+$) incubated with Jag1 neutralizing antibody. FL cells directly transplanted into lethally irradiated recipient mice (CD45.1). (*M*) Percentages of total $CD45.2^+$ cells in peripheral blood after transplantation of donor cells treated with anti-IgG or anti-Jag1 antibodies. *P < 0.05, **P < 0.01. (n = 5).

of whole-mount immunostained FL lobes at E14.5 from Jag1$^{f/f}$VavCre^{+} and wildtype littermates (*SI Appendix*, Fig. S5*C*). Deletion of Jag1 in the hematopoietic compartment had no significant impact on the endothelial network or the widespread distribution of c-kit^{+} hematopoietic progenitors in FL (Fig. 3*C*). H&E and endomucin staining, for detection of the sinusoidal capillary marker, showed no significant differences observed between WT and Jag1$^{f/f}$VavCre^{+} littermates (*SI Appendix*, Fig. S5*D*).

FL HSCs were also isolated and assayed by RT-qPCR for the expression of apoptotic-related genes such as BCL2, MCL1, PUMA, and BAX (35). Loss of Jag1 expression had no effect on pro-apoptotic factors in Jag1$^{f/f}$VavCre^{+} HSCs (*SI Appendix*, Fig. S5*F*). To study Notch receptor activation, we isolated FL HSCs from Jag1$^{f/f}$VavCre^{+} and wildtype littermates and assessed Notch1 activation using cleaved Notch1 ICN -Val1744 antibody (3). Our results indicate that loss of hematopoietic Jag1 decreases Notch1 activation in FL HSCs (*SI Appendix*, Fig. S5*H*). To begin to evaluate the functional potential of FL HSCs that lack Jag1 expression, we plated 10,000 FL cells in a colony-forming assay. Interestingly, despite comparable overall numbers and a similar FL microenvironment, the cells from Jag1$^{f/f}$VavCre^{+} embryos failed to generate robust colonies and produced a significantly lower numbers of colonies overall when compared to WT littermates (Fig. 3*E*). These results suggest a very specific role for Jag1 in the induction of functional potential of HSCs during their time in the FL niche.

HSC Reconstitution of FL HSCs Is Impaired by Loss of Jag1. Next, we characterized the frequency and function of other FL hematopoietic cellular populations in the Jag1$^{f/f}$VavCre^{+} mutants. No defect was observed in the abundance of monocytes and neutrophils in Jag1$^{f/f}$VavCre^{+} mutants (*SI Appendix*, Fig. S6 *A* and *B*). A very recent study has shown that macrophages are critical to determine the quality control of newly emerged HSCs during development in zebrafish (36). We compared the frequency and numbers of CD169^{+} and F4/80^{+} macrophages or CD41^{+} megakaryocytes in the E14.5 FL of Jag1$^{f/f}$VavCre^{+} mutants (*SI Appendix*, Fig. S6 *C* and *D*). In all cases, no significant differences were observed. CD11b^{+} cells from E14.5 FL of Jag1$^{f/f}$VavCre^{+} mutants and Cre- littermate controls were purified to test their differentiation and function (*SI Appendix*, Fig. S7*A*). Neither phagocytosis (*SI Appendix*, Fig. S7*B*) as observed by uptake of FITC labeled pellets, nor functional response to LPS treatment (*SI Appendix*, Fig. S7*C*) as observed by production of IL-1β, iNOS and IL-6, were affected by loss of Jag1, though a decrease in levels of IL-1β was observed in Jag1-null mutants (*SI Appendix*, Fig. S7*C*). We wanted to further characterize the effect of Jag1 deletion on fetal liver macrophages, so we sorted CD169^{+} from E14.5 FL from Jag1$^{f/f}$VavCre^{+} or Cre^{-} littermates. Bulk RNA sequencing was performed and though the analysis revealed no significant pathway disruption, there were several interesting differentially expressed genes (DEGs) between Jag1$^{f/f}$VavCre^{+} and Cre^{-} littermate controls (complete DEG list in *SI Appendix*). A representative group of these findings, shown in *SI Appendix*, Fig. S7*D*, highlights the role of several downregulated genes in macrophages activation and cell survival. For example, two DEGs negatively affected by loss of Jag1 are VegfB and CD52 which have been shown to have roles in preventing cell death (37, 38). Furthermore, there is a significant decrease in the expression of Rbp4, the transport protein for vitamin A (retinol) in circulation, which has been shown to induce expression of ICAM1 and VECAM1 (39), and may broadly affect the FL microenvironment by disrupting retinol levels and expression of adhesion proteins on ECs and other cell types. Overall, these findings indicate a generally unperturbed cellular hematopoietic environment at E14.5 in the FL of Jag1$^{f/f}$VavCre^{+} mutants, but suggest potential roles of Jag1^{+} macrophages in the later embryonic development of the FL niche.

To further test the functionality of Jag1-null FL hematopoietic cells, we directly transplanted E14.5 FL cells from Jag1$^{f/f}$VavCre^{+} or WT littermate embryos into lethally irradiated recipient mice (*SI Appendix*, Fig. S8*A*). Within the first 4 wk after transplantation, a significant defect in total peripheral blood (PB) was observed in the percentage of CD45.2^{+} Jag1$^{f/f}$VavCre^{+} donor cells (*SI Appendix*, Fig. S8*A*), which was persisted over the course of 3 mo after reconstitution (*SI Appendix*, Fig. S8 *B-C*). At 3 mo post-transplant, we analyzed the LSK and HSC engraftment to the BM or Jag1$^{f/f}$VavCre^{+} donors where we observed significantly lower numbers of LSK progenitors and HSCs by Jag1$^{f/f}$VavCre^{+} FL cells compared to WT controls (*SI Appendix*, Fig. S8*D*).

To test the cell-autonomous functionality of FL HSCs from Jag1$^{f/f}$VavCre^{+} and Cre- littermates, 250 FL HSCs were sorted from each genotype and transplanted along with Lin^{-} splenocyte helper cells into lethally irradiated recipients (Fig. 3*F*). Within the first 4 wk after transplantation, a significant defect in PB reconstitution was observed in the Jag1$^{f/f}$VavCre^{+} donor cells (*SI Appendix*, Fig. S9*A*) which continued over the course of 4 mo post-transplant (Fig. 3*G*) and was also observed for multi-lineage reconstitution (*SI Appendix*, Fig. S9*B*). Bone marrow analysis at 4 mo indicated a significant defect in donor-derived engraftment of LSK progenitors and HSCs (Fig. 3*H*) These findings demonstrate a defect in the function of Jag1-deficient FL HSCs during reconstitution of irradiated recipients.

To directly test the fitness of Jag1-deficient FL hematopoietic cells, WT marrow cells were transplanted in competition with either WT or Jag1$^{f/f}$VavCre^{+} FL hematopoietic cells were injected into CD45.1^{+} recipients (Fig. 3*I*). A decrease in the PB reconstitution by Jag1$^{f/f}$VavCre^{+} FL hematopoietic cells was observed after transplantation. This defect was first observed at the 1-mo timepoint and persisted over the course of 3 mo (Fig. 3*J*). Similarly, to the direct transplant, Jag1$^{f/f}$VavCre^{+} donors had lower BM engraftment of LSKs and HSCs (Fig. 3*K*). These findings confirm the defect in the functional potential of the Jag1-deficient fetal liver hematopoietic cells and specify that Jag1-null FL HSCs have a cell autonomous reconstitution defect. This indicates that while a Jag1 deficiency does not disturb the cell numbers and overall frequency of FL HSCs, it significantly impairs their functional potential in a transplant setting.

HSCs Function Is Not Affected by Loss of Myeloid Jag1. Since macrophages and myeloid cells express Jag1 on the surface in the FL (Fig. 2*E* and *SI Appendix*, Fig. S4 *D* and *E*), we leveraged the LysMCre+ driver to delete Jag1 in the myeloid hematopoietic lineage (40) *SI Appendix*, Fig. S10*A*. CD11b+ cells were sorted from E14.5 FL of LysMCre+ or LysMCre– Jag1$^{f/f}$ littermates and expression of Jag1 was assessed by RT-qPCR (*SI Appendix*, Fig. S10*B*) and western blot (*SI Appendix*, Fig. S10*C*). A significant loss of Jag1 expression and protein levels was detected, however no defect was observed in the absolute numbers of LSK progenitors and HSCs in the FL of Jag1$^{f/f}$ LysMCre+ embryos (*SI Appendix*, Fig. S10*D*). Furthermore, colony forming assays showed no significant difference when cells were harvested from the LF of Jag1$^{f/f}$LysMCre^{+} embryos (*SI Appendix*, Fig. S10*E*), suggesting no defect in hematopoietic potential of FL HSCs when myeloid Jag1 was deleted. In support of these findings, we also performed whole marrow transplants with Jag1$^{f/f}$LysMCre^{+} cells (CD45.2+) into lethally irradiated congenic (CD45.1+) recipients and observed no defect in peripheral blood reconstitution when donors were Jag1$^{f/f}$LysMCre^{+} cells (*SI Appendix*, Fig. S10*F*), and though there was a

trend decrease in the peripheral blood reconstitution of CD11b+ CD45.2+ myeloid cells when the donors originated from Jag1$^{f/f}$LysMCre^{+}, this trend was not significantly different from controls (*SI Appendix*, Fig. S10*G*). Finally, BM engraftment analysis showed no difference in the donor-derived LSK progenitor and HSC numbers in recipients at 3 mo post-transplant (*SI Appendix*, Fig. S10*H*).

Blocking Jag1 on FL Hematopoietic Cells Limits Reconstitution in a Transplant Setting. To directly test the hypothesis that surface expression of Jag1 is specifically required for early engraftment of FL hematopoietic cells, we treated WT E14.5 FL hematopoietic cells in vitro with a neutralizing anti-Jag1 antibody (10 μg/mL) or IgG isotype control (Fig. 3*L*). The colony forming potential of FL hematopoietic cells treated with anti-Jag1 neutralizing antibodies was decreased (*SI Appendix*, Fig. S11*A*). After transplantation, the reconstitution potential of WT FL hematopoietic cells with Jag1-blocking antibody treatment was impaired when compared to control cells (Fig. 3*M*). Multi-lineage differentiation of donor hematopoietic cells was significantly impaired in Jag1-blocking antibody-treated donor cells when compared to IgG-treated FL donor cells (*SI Appendix*, Fig. S11*B*). A similar experiment using anti-Dll4 antibody (10 μg/mL) or IgG isotype control (*SI Appendix*, Fig. S11*C*) on WT E14.5 FL hematopoietic cells had no effect in colony formation potential (*SI Appendix*, Fig. S11 *D* and *E*), indicating that the functional defect is unique to the role of hematopoietic Jag1. These findings are consistent with our previous loss-of-function experiments, and show that either genetic deletion of Jag1 or disruption of Jag1 protein function on the surface of FL hematopoietic cells limit their functional potential. Specifically, the reconstitution defect observed by what is admittedly a transient blocking of Jag1 surface protein prior to transplant suggests a narrow temporal role for hematopoietic Jag1 during engraftment of FL progenitors and HSCs in the bone marrow.

Downregulation of Key Hematopoietic Genes Identified by RNA-seq in Jag1-Null FL HSCs. To begin to investigate the underpinnings of how loss of Jag1 expression in the FL hematopoietic cells negatively impacts the function of HSCs, we sorted E14.5 FL HSCs from Jag1$^{f/f}$VavCre^{+} or Cre^{-} littermates. Bulk RNA sequencing was performed. The analysis revealed 132 DEGs between Jag1$^{f/f}$VavCre^{+} and Cre^{-} littermate controls. A representative group of these findings, shown in Fig. 4*A*, highlights the consistency of DEGs among FL HSCs of three different litters. While a number of upregulated genes are seen in the Jag1$^{f/f}$VavCre^{+}, LogFC column, most of these have low expression levels in the FL HSCs and are not known for roles in developmental hematopoiesis. In stark contrast, the majority of genes that are downregulated after loss of hematopoietic Jag1 are highly expressed in FL HSCs, and include critical hematopoietic factors such as GATA2, Mllt3, Hoxa7, and Angp1, each of which show at least a twofold decrease in expression (Fig. 4*A*, blue arrows). Among this group of DEGs, GATA2, and Mllt3 have been extensively studied and their roles have been shown to be intricately connected to HSC function (41–43).

To validate our findings, we isolated FL HSCs from Jag1$^{f/f}$VavCre^{+} or Cre- littermates at E14.5 and tested the expression

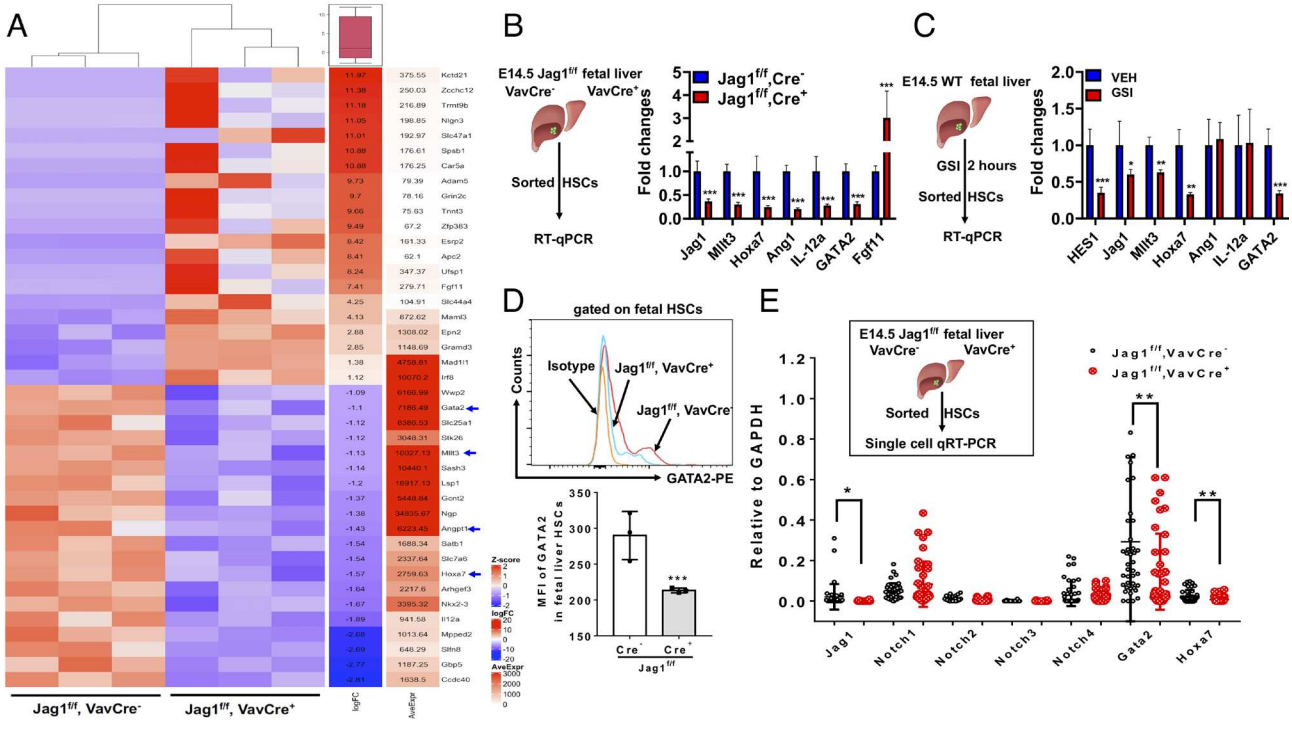

Fig. 4. Loss of hematopoietic Jag1 downregulates expression of key HSC genes. (*A*) Bulk RNA-Seq analysis of sorted HSCs from E14.5 FL of Jag1$^{f/f}$Vav-Cre^{-} and Jag1$^{f/f}$Vav-Cre^{+} embryos. Differential gene expression shown as clustered Z-score heat map of n = 3 for each genotype shown in clustered left columns. log^{FC} and average expression of representative genes shown in left columns, respectively. Genes known for hematopoietic roles indicated by blue arrows. (n = 3). (*B*) Validation by qRT-PCR of differential expression for selected genes from RNA-seq. HSC were freshly sorted from E14.5 and values were normalized to Jag1$^{f/f}$, Vav-Cre^{-} and GAPDH levels. ***P < 0.001. n = 3. (*C*) Gamma-secretase inhibitor (GSI) 2 h treatment of WT E14.5 FL cells followed by qRT-PCR of sorted HSCs to measure the expression of the indicated genes. *P < 0.05, **P < 0.01, ***P < 0.001. n = 3. (*D*) Expression of Gata2 in Jag1$^{f/f}$Vav-Cre^{-} and Jag1$^{f/f}$Vav-Cre^{+} HSCs. Fetal liver HSCs from Jag1$^{f/f}$Vav-Cre^{-} and Jag1$^{f/f}$Vav-Cre^{+} embryos were stained with Gata2 antibody. Expression levels of Gata2 presented as MFI. ***P < 0.01 vs Jag1$^{f/f}$Vav-Cre^{-} HSCs. (n = 3). (*E*) TaqMan single cell qRT-PCR. Single HSCs from E14.5 FL of Jag1$^{f/f}$Vav-Cre^{-} and Jag1$^{f/f}$Vav-Cre^{+} fetal livers were sorted into lysis buffer (*Inset*). Expression of Jag1, Notch1, Notch2, Notch3, Notch4, Gata2 and HoxA7 was examined and compared between Jag1$^{f/f}$Vav-Cre^{-} and Jag1$^{f/f}$Vav-Cre^{+} HSCs at a single cell level. *P < 0.05, **P < 0.01. (n = 40).

GATA2, Mllt3, Hoxa7, Angpl1 by RT-qPCR, all of which were shown to be downregulated in Cre$^+$ HSCs (Fig. 4*B*). To determine which of the genes that are dysregulated by hematopoietic loss of Jag1 are specifically dependent on Notch signaling for transcriptional activation, we harvested CD45$^+$ cells from the WT E14.5 FL and cultured them ex vivo in the presence of gamma-secretase inhibitor Compound E (GSI) (Fig. 4*C*). RT-qPCR analysis in HSCs for the same genes assessed in Fig. 4*B* as well as canonical Notch target Hes1 and Jag1 showed that only GATA2, Hoxa7 and Mllt3 are sensitive to GSI treatment, suggesting that they are dependent on Notch activation in FL HSCs. The expression levels of Angpl1 and IL21a were not affected by the GSI treatment, likely suggesting an indirect effect carried out by a downstream Notch target gene. We also tested the expression of these same genes in adult BM HSCs in Jag1$^{f/f}$VavCre$^+$ or Cre- 8-wk–old littermates. These results show that in the adult BM, despite a dramatic loss of Jag1, only Angp1 shows a significant decrease in expression (*SI Appendix*, Fig. S4*J*). To validate FL targets at the protein level, we performed intracellular flow cytometry for GATA2 levels in the FL and detected a significant decrease in expression in Cre$^+$ HSCs when compared to Cre$^-$ littermates and isotype controls (Fig. 4*D*), thus confirming the loss of GATA2 protein expression in Jag1$^{f/f}$VavCre$^+$ FL HSCs.

To further investigate the mechanistic interplay between loss of Jag1 expression and functional potential of individual FL HSCs, we performed single-cell RT-qPCR on Jag1$^{f/f}$VavCre$^+$ or Cre$^-$ littermate E14.5 HSCs (Fig. 4*E*). We specifically wanted to know whether loss of Jag1 expression had any impact on the ability of individual FL HSCs to express the Notch receptors or critical Notch target genes. Our results indicate that single HSCs that lost Jag1 expression still have comparable levels of the Notch receptors when compared to Cre$^-$ HSCs, suggesting they have the capacity for activation of the pathway. However, these same HSCs that are harvested from a Jag1$^{f/f}$VavCre$^+$ FL show a significant decrease in the expression of GATA2 and HoxA7 (Fig. 4*E*). Comprehensive analysis of all the single FL HSCs analyzed along with a catalogue of the genes and expression values can be found in *SI Appendix*, Table S4. These results imply a unique hematopoietic-driven mechanism of Notch receptor activation for HSCs during expansion and functional maturation in the FL.

Functional Rescue of Jag1-Deficient FL HSC by Immobilized Jag1 Protein Ex Vivo. The use of immobilized Jag1 to activate Notch signaling has been previously used in angiogenic studies (44) and in bone marrow mesenchymal stem cell expansion (45). Because Jag-deficient HSCs retain expression of Notch receptors, we cultured 1 × 10^6 Jag1$^{f/f}$VavCre$^+$ E14.5 FL cells for 24 h in plates coated with FC or FC-Jag1 protein (Fig. 5*A*). For a positive control and to account for defects in self-renewal potential due to ex vivo maintenance, Jag1$^{f/f}$VavCre$^-$ E14.5 FL cells were plated for 24 h in FC-coated plates. After the incubation period, a portion of the CD45$^+$ FL hematopoietic cells was used to assess activation of Notch singling. We observed that this treatment upregulated the expression of Mllt3 and HoxA7, but not GATA2 in the Jag1$^{f/f}$VavCre$^+$ cells that had been cultured in the FC-Jag1 coated plate (Fig. 5*B*), indicating partial activation of the Notch pathway. Colony forming assay showed a significant increase in hematopoietic potential evident by more CFUs in cells that had been cultured with immobilized Jag1 protein (Fig. 5 *C* and *D*). Improvement in function was seen as early as 1 mo after transplant of recipients (CD45.1$^+$) by Jag1$^{f/f}$VavCre$^+$ CD45.2$^+$ donor cells (Fig. 5*E*). This trend of a significant improvement in reconstitution persisted over the course of 4 mo after transplant (Fig. 5*F* and *SI Appendix*, Fig. S9*C*) and was also evident in the engraftment of the recipient BM at 4 mo (Fig. 5*G*). While this treatment does not fully rescue the defect when compared to WT littermate controls, which may depend on the means, the dose and length of ex vivo immobilized Jag1 treatment as well as on attaining robust GATA2 expression, it strongly suggests that activation of Notch signaling in Jag1-deficient FL hematopoietic cells is sufficient to improve functional hematopoietic potential in a transplantation setting.

Loss of Hematopoietic Jag1 Has No Effect on Adult BM HSCs Function. Lastly, to determine if the functional defect seen in the Jag1-deficinent FL HSCs is persists once they home and engraft to the BM, we isolated (CD45.2$^+$) HSCs from the BM of primary recipients (Fig. 3) and used 500 cells as donors for secondary transplants (Fig. 5*H*). In striking contrast to the FL transplants, no significant defect in PB reconstitution was observed in the Jag1$^{f/f}$VavCre$^+$ donor cells that originated from the BM (Fig. 5*I*). Bone marrow analysis at 3 mo showed no defect in donor-derived engraftment of LSK progenitors and HSCs (Fig. 5*J*). Overall the results show that hematopoietic Jag1 is dispensable for HSC function once the cells have engrafted in the BM. These findings further highlight the unique role for Jag1-mediated Notch activation in the FL niche and support previous findings that show no role of Notch signaling in the maintenance and self-renewal of BM HSCs (16, 17).

Discussion

Since the discovery of HSCs, the goal of regenerative medicine has been to harness and expand functional and self-renewing HSCs for the purpose of therapeutic application. Many strategies over the years have yielded scientifically interesting yet ultimately impractical results (46). In the FL niche, HSCs have an astonishing capacity for natural proliferation and self-renewal. Over the course of 4 d during murine development (E12-E16), a small number of definitive HSCs that have migrated from the AGM (47, 48) undergo expansion in the FL (6, 7, 49). The rapidly dividing FL stem cells are remarkably competent in both engraftment and reconstitution of adult hosts (50, 51). In this study, we set out to determine the molecular and cellular components of the FL niche that support and enhance the function of FL HSCs.

We initially investigated whether FL ECs serve as niche cells that activate Notch signaling in FL HSCs. This was based on multiple previous reports indicating an essential role for sinusoidal EC in the BM niche (52, 53). Furthermore, ECs express high levels of Notch ligands and receptors (54–60). More specifically, VE-cadherin$^+$ FL ECs have been shown to closely associate with colonizing HSCs in the FL (27) while Lyve1$^+$ FL sinusoidal ECs have been shown to support functional potential of HSCs ex vivo (9). Here, we focused on Jag1 due to the early and essential role that Dll4 plays in vascular development (55). Through the use of both conditional and inducible transgenic murine model systems, we established a temporal requirement for endothelial Jag1 during early embryonic development, but which had no effect on endothelial or hematopoietic development in the FL at E14.5. Though we cannot rule out the contribution of other EC-expressed Notch ligands, such as Dll4 or Jag2, our findings show that in the FL, during peak expansion of definitive HSCs, endothelial Jag1 is not required for expansion, survival or function.

Our attention next turned to the FL hematopoietic compartment (30, 31). We conducted a surface protein analysis of Notch ligands in the E14.5 FL where we found that developing erythroid cells lack expression of Jag1, while CD169$^+$(Siglec1) macrophages, other CD11b$^+$ myeloid cells as well as megakaryocytes all express

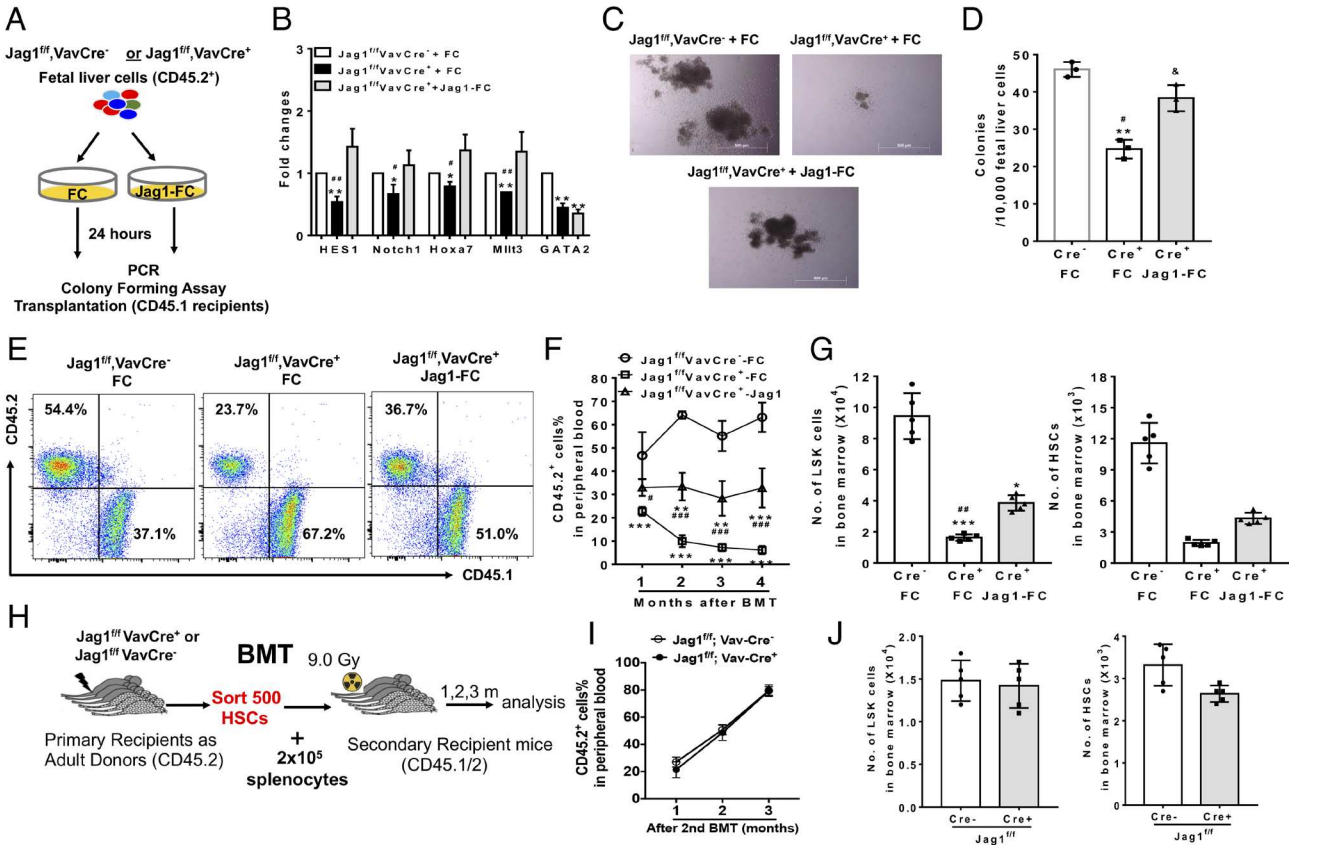

Fig. 5. Immobilized Jag1 Ligand Rescues the Functional Defect of Jag1-Null FL HSCs. (*A*) Diagram of experimental design with 5×10^5 FL cells from Jag1$^{f/f}$VavCre$^-$ or Jag1$^{f/f}$VavCre$^+$ embryos cultured onto FC- or Jag1-FC-coated plates for 24 h. (*B*) Expression of indicated genes in sorted HSCs by qRT-PCR after 24-h incubation with FC- or Jag1-FC. $^*P < 0.05$, $^{**}P < 0.01$ vs. Jag1$^{f/f}$VavCre$^-$ with FC, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. Jag1$^{f/f}$VavCre$^+$ with Jag1-FC. Expression is normalized to Jag1$^{f/f}$Vav-Cre$^-$ and GAPDH. (*C*) Representative images of day 12 hematopoietic colonies from colony forming assay with 10,000 cells from FC- or Jag1-FC treatment. (*D*) Quantification of colony-forming assay for each treatment. $^{**}P < 0.01$ vs. Jag1$^{f/f}$VavCre$^-$ with FC; $^{\#}P < 0.05$ vs. Jag1$^{f/f}$VavCre$^+$ with Jag1-FC; and $^{\&}P < 0.05$ vs Jag1$^{f/f}$VavCre$^-$ with FC. (*E*) Representative flow cytometric analysis indicating the engraftment of ex vivo treated donor-derived cells (CD45.2$^+$) in the peripheral blood of recipients (CD45.1$^+$) 1 mo after transplantation. (*F*) Quantification of donor-derived cells (%) in peripheral blood of recipients at 1,2,3 and 4 mo after transplantation. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.01$ vs. Jag1$^{f/f}$VavCre$^-$ with FC; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. Jag1$^{f/f}$VavCre$^+$ with FC. (n = 5). (*G*) Absolute numbers of donor-derived LSKs and HSCs in the bone marrow at 4 mo after transplantation. $^*P < 0.05$, $^{***}P < 0.01$ vs. Jag1$^{f/f}$VavCre$^-$ with FC; $^{\#\#}P < 0.01$ vs. Jag1$^{f/f}$VavCre$^+$ with FC. (n = 5). (*H*) Diagram of experimental design for secondary transplant with 500 HSCs from the bone marrow of Jag1$^{f/f}$VavCre$^+$ or Jag1$^{f/f}$VavCre$^-$ (CD45.2$^+$) transplanted into irradiated (CD45.1.2$^+$) recipients. (*I*) Quantification of donor-derived cells (%) in peripheral blood of recipients at 1,2 and 3 mo after transplantation. (n = 5). (*J*) Absolute numbers of donor-derived LSKs and HSCs in the bone marrow at 3 mo after transplantation. (n = 5)

Jag1 on their surface. These findings suggest that Jag1 expression is widely expressed on the surface of multiple hematopoietic cells in the FL. Even more striking was the fact that FL LSK progenitors and FL HSCs (~95%) both express Jag1 on their surface. FL HSCs also express multiple Notch receptors which, in combination with the prevalent presence of Jag1 in hematopoietic cells, suggests a robust and redundant mechanistic failsafe to sustain hematopoietic activation of Notch signaling in HSCs. We hypothesized that the reason for this activation is to enhance the functional potential of FL HSCs. Due to the wide array of Jag1-expressing hematopoietic cells in the FL, we made use of the pan-hematopoietic VavCre transgenic driver which effectively removed Jag1 expression in CD45$^+$ cells by E14.5. The loss of pan-hematopoietic, but not myeloid-specific, Jag1 had a profoundly negative effect on reconstitution potential of FL HSCs in vivo while having no discernable defect in their viability in the FL niche.

As a canonical Notch ligand, Jag1 has been studied in several contexts, yet its functional role has consistently been shown to be the activation of Notch receptors (18, 19). Our results indicate that Jag1 is specifically important for HSC function in the FL niche and we provide novel evidence of a hematopoietic-driven mechanism that promotes the robust potential of FL HSCs. Mechanistically we propose that Jag1 activates Notch signaling which in turn regulates the transcription of key HSC factors, among which are GATA2, Mllt3, and Hoxa7. GATA2 is recognized as a key transcriptional factor important in orchestrating many aspects of hematopoietic development, including proliferation and maturation of developing HSCs (43, 61, 62). We observed a twofold decrease in GATA2 expression after loss of hematopoietic Jag1, which can begin to explain some of the defects of Jag1-null FL HSCs in colony forming and transplantation assays where even a moderate decrease in GATA2 expression can have substantial effect on cell-autonomous function of HSCs (63). Mechanistically, GATA2 has also been previously shown to be a Notch target during the emergence of definitive HSCs in the AGM (13). Further support for Notch-dependent GATA2 expression has also been observed in vitro, where active Notch signaling sustained GATA2 expression levels and inhibited progenitor cell differentiation (64). While GATA2 and Notch have previously been associated in developmental hematopoiesis, here we present the first evidence of Notch regulation of Mllt3 and Hoxa7. Both transcription factors are highly expressed in FL HSCs, both were downregulated by loss of hematopoietic Jag1 and were confirmed as sensitive targets to Notch inhibition in WT FL HSCs. This finding is especially relevant in the context of recent work that describes a profound role for Mllt3 in reconstitution

and engraftment potential of HSCs (41). Hoxa7 has also been studied in the context of FL hematopoiesis and along with other Hox genes has been shown to regulate HSC self-renewal (65). Furthermore, *Hox* genes are positively regulated by *Mll* in hematopoietic development (66) suggesting interplay between key fetal hematopoietic factors.

Our work shows that hematopoietic activation of Notch signaling by Jag1 is required for the robust function of FL HSCs but does not significantly affect HSC numbers or survival. This would suggest that the expansion and survival of the HSCs in the FL is supported by other cell types. Recent work indicates endothelial and Pdgfra$^+$ hepatic stellate cells are sources of stem cell factor, an essential secreted factor for self-renewal and expansion of HSCs (6), is in line with our conclusions. Another report suggests that macrophages are enriched in the "expansion pockets" of CD93$^+$ HSCs and progenitors in the FL (67), which is supported by our data that show Jag1 expression is elevated in macrophages in the FL. Very recently, several studies have shown that definitive fetal HSCs do not substantially contribute to embryonic hematopoiesis, which is sustained and populated by primitive waves of yolk sac and erythro-myeloid progenitors (68, 69). Considering that the overall configuration of the hematopoietic compartment in our Jag1$^{f/f}$VavCre$^+$ FL is not perturbed; our findings agree with these reports and indicate with a unique role for Jag1 with regard to definitive FL HSCs and not primitive or progenitor hematopoiesis.

Taken together we propose a working model where the FL EC and stromal populations provide the signaling cues for expansion and survival of the proliferating HSCs while hematopoietic progenitors and macrophages present Jag1 to Notch receptor-expressing FL HSCs. This interaction between Jag1$^+$ hematopoietic cells and HSCs is responsible for a signaling event which is important for their functional maturation by transcriptional activation of key hematopoietic identity factors. These findings exemplify the complexity of the FL niche, which is likely as diverse and nuanced as the BM niche, where multiple signals and interactions combine to generate a specific behavioral outcome in HSCs illustrated by proliferation, self-renewal and superior function. Understanding of these signaling pathways is an important step toward using the fetal liver niche as a model for the ex vivo expansion and functional rejuvenation of adult HSCs.

Materials and Methods

Experimental details on the use of transgenic mouse animal models and cell extraction from embryonic tissues is described in depth in the extended supporting information section. All experiments using animal were conducted according to the rules and regulations of the University of Illinois Institutional Animal Care and Use Committees. Furthermore, the antibody staining procedure for flow cytometry and the sorting panels sorting are further described in the Materials and Methods section of the supporting information. Finally, the RNAseq and single-cell methodology as well as statistical analyses of the HSC and macrophage datasets for this study are described in detail in the *SI Appendix, Materials and Methods* section.

Data, Materials, and Software Availability. RNA sequencing data have been deposited in NCBI Gene Expression Omnibus (GSE186190) (70).

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