Regulation of dendritic cell differentiation in bone marrow during emergency myelopoiesis[1]:

Dendritic cells, Notch, and emergency myelopoiesis

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Abstract

Although accumulation of dendritic cell (DC) precursors occurs in bone marrow (BM), the terminal differentiation of these cells takes place outside BM. The signaling, regulating this process, remains poorly understood. We demonstrated that this process could be differentially regulated by Notch ligands: Jagged-1 (Jag1) and Delta-like ligand 1 (Dll1). In contrast to Dll1, Jag1, *in vitro* and during induced myelopoiesis *in vivo,* prevented DC differentiation by promoting the accumulation of their precursors. Although both ligands activated Notch in hematopoietic progenitor cells (HPC), they had an opposite effect on Wnt signaling. Dll1 activated Wnt pathways; whereas, Jag1 inhibited it via down-regulation of the expression of the Wnt receptors Frizzled (Fzd). Jag1 suppressed *fzd* expression by retaining histone deacetylase 1 in the complex with the transcription factor CSL/CBF-1 on the *fzd* promoter. Our results suggest that DC differentiation, during induced myelopoiesis, can be regulated by the nature of the Notch ligand expressed on adjacent stroma cells.

Introduction

Dendritic cells (DCs) are professional antigen presenting cells critically important for the induction of immune responses (1, 2). The regulation of DC differentiation is a complex, spatially controlled process. Although it is initiated in bone marrow (BM), most DCs became terminally differentiated cells in the peripheral lymphoid organs or tissues. There are only a few differentiated, functionally competent DCs in BM (3). This plays an important biological role by limiting the possible inflammatory reaction in BM. The molecular mechanisms regulating this process remained largely unclear.

DC differentiation in BM is controlled by a complex network of soluble factors and cell surface bound molecules. Among the latter, the Notch family of transcriptional regulators plays a major role. Notch signaling is initiated by the binding of the Notch receptor to specific ligands that result in the proteolytic cleavage of the intracellular domain (ICN); followed by the ICN translocation to the nucleus, where it interacts with the transcriptional repressor CSL/CBF-1 (4, 5). One of the most puzzling questions is the role played by different Notch ligands in defining the biological effects of Notch. At present, two major

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Notch ligand families, Delta (Dll1, Dll3, and Dll4) and Jagged (Jag1 and Jag2), have been described in mammals (6). Although these ligands activate Notch signaling, in recent years, evidence has emerged of the contrasting effects of Delta and Jagged on different cells (7–13). However, the mechanisms these effects remain largely unclear.

It is known that the Notch pathway is involved in the differentiation and function of DCs. However, its role remains controversial. Most of the studies with "gain-of-function" experiments have demonstrated an up-regulation of DC differentiation; whereas, many studies with "loss of function," using knockout mice, showed either a lack of the effect or an inhibition of DC differentiation (rev in (14)). Dll1 was shown to potently promote DC differentiation (11, 15, 16): whereas, Jag1 prevented the terminal DC differentiation *in vitro* by inducing an accumulation of immature myeloid cells (IMCs) and DC precursors (11). The mechanism of such an opposite effect remained unclear. The goal of this study was to understand the mechanisms that prevent DC differentiation in BM and possible role of Jag1 in this process.

Material and Methods

Mice

All mouse experiments were approved by the University of South Florida Institutional Animal Care and Use Committee. Female C57BL/6 mice (aged 6–8 weeks) were obtained from the National Cancer Institute. Mx1-Cre mice (mouse strain #003556), -catenin^{flox+} mice (mouse strain #004152 on C57BL/6 background) and CD45.1$^+$ congenic mice (B6.SJL-PtrcaPep3b/BoyJ) were purchased from Jackson Laboratories. Jagged-1^{flox+} mice were provided by Dr. Lewis (10). The conditional -cat or Jag1 knock-out mice were generated by crossing homozygous floxed -cat or Jag1 mice with Mx1-Cre mice and the offspring carrying a floxed -cat or Jag1 allele and Mx1-Cre were back crossed to the homozygous floxed -cat or Jag1 mice. Homozygous floxed -cat or Jag1 mice carrying Mx1-Cre transgene were selected and were referred to as -cat$^{fl/fl}$Cre$^{+/-}$ or Jag1$^{fl/fl}$Cre$^{+/-}$ mice. Jag1$^{fl/fl}$Cre$^{/-}$ or -cat$^{fl/fl}$Cre$^{-/-}$ mice were used as controls. To induce the -cat or Jag1 deletion, 250 μg poly (I:C) were injected *i.p.* every other day, for three or five times. Mice were used within 3 weeks after their last poly (I:C) injection. For adoptive transfer experiments, Jag1$^{fl/fl}$ mice were further back-crossed for 8 generations with C57BL/6 mice.

Reagents

Antibodies against mouse I-A/E, CD11b, CD86 (B7-2), CD45, CD45.1, CD11c, Gr1, B220, and isotype control antibodies were obtained from BD PharMingen (San Diego, CA). Siglec H, DEC-205, and DCIR-2 (33D1) antibodies were from ebioscience (San Diego, CA). F4/80 antibody was purchased from Serotec (Raleigh, NC). Recombinant murine GM-CSF and IL-4 were obtained from Research Diagnostics (Flanders, NJ), IL-3, FLT-3, M-CSF, recombinant human cytokines GM-CSF, SCF, TNF-, mouse Wnt3a were obtained from R&D systems (Minneapolis, MN). Jagged-IgG was from Alexis Biochemical. SB216763, human IgG, and anti-human IgG were from Sigma (St Louis, MO). -catenin, Jagged-1, HDAC1,2,5,7 antibodies were from Cell Signaling Technology, Inc. (Beverly, MA). Antibodies against Frizzled 6, 7, 10, -actin, Delta-1, CBF1 and siRNA for Jagged-1, HDAC1, Delta and CBF-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). CBF1 reporter, shRNA for mouse -catenin were from SABiosciences (Frederick, MD), dual-luciferase assay kit from Invitrogen (Carlsbad, CA), and primers for Real-time RT-PCR were synthesized by Integrated DNA technologies (Coralville, IA). The monomeric form of the Notch ligand Delta-1 was made by fusing the extracellular domain of Delta-1 to a series of myc epitopes Delta-1$^{ext-myc28}$ and was a gift from Dr I. Bernstein. -cat-GFP

plasmid was provided by Dr. Ira Mellman (Genentech, Inc). HDAC activity assay kit was from Cayman Chemical (Ann Arbor, MI, USA).

Cell lines, cell culture, and flow cytometric analysis

Bone marrow and spleen stroma cells were generated as described previously (11). The NIH 3T3 cell lines transfected with Delta-1, Jagged-1 or control plasmids were described previously (11). Mouse HPCs from mouse bone marrow were enriched with a lineage cell depletion kit from Miltenyi Biotec (Auburn, CA, USA). Enriched HPC cells, from naive bone marrow, were placed onto 3T3 cells or primary stromal cells the following day and cultured in RPMI medium supplemented with 10% FBS and 20 ng/ml GM-CSF for various time. HPC progenies were transferred to new wells and coated with corresponding cell lines every 2–3 days. CD45$^+$ cells were gated so that hematopoietic cells could be distinguished from contaminated stroma. For the analysis of gene and protein expression, hematopoietic cells were isolated with biotin-conjugated CD45 antibody and magnetic beads (Miltenyi Biotec) (17). 32D cells (murine hematopoietic cell line) were cultured in RPMI medium with 10% FBS and 20% conditioned medium containing IL-3 (WEHI-3B cell line). The incubation of 32D cells with different 3T3 cell lines was similar to HPCs. Cells was analyzed by flow cytometry on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA, USA) or LSR II (BD Biosciences, Mountain View, CA, USA), and data were analyzed with the CellQuest program (Becton Dickinson, Mountain View, CA, USA) or FlowJo program (Treestar Inc., Ashland, OR).

The immobilization of Notch ligands on plastic was performed, as described previously (18). In brief, 20 μg/ml of antibody against human IgG was placed into each well of a 24-well plate and incubated for 30 min at 37°C. Wells were blocked with complete culture medium (CCM) for 30 min and then incubated for 3 hr at 37°C with 7.5 μg/ml Dll1, Jag1 or IgG in CCM.

Plasmid construction and transfection

The 5 -regulatory sequence (−1233 bp to +235 bp) of the mouse *fzd10* gene (NM_175284) was cloned into pGL3-enhancer vector (Promega), using the following pair of oligonucleotides: forward primer, 5 -CGACGCGTGATTAGCCCAAAACCAACCA-3 ; reverse primer, 5 -CCGCTCGAGGTCCTTGCACATGGGAATCT -3 . Transient transfections were performed on 32D cells using the GenePORTER2 transfection agent (Genlantis, San Diego, CA, USA) and following the manufacturer's guideline. For the reporter activity assay, Renilla plasmid was co-transfected with luciferase plasmids and Relative light units (RLUs) of luminescence were measured 48 hr after transfection with a dual-luciferase reporter assay system (Promega, Madison, WI, USA). HPCs were transfected with mouse macrophage buffer and Y01 program at AMAXA nucleofector (AMAXA, Gaithersburg, MD, USA).

Hydrodynamic gene transfer (HGT)

HGT was performed as described (19). Briefly, GM-CSF plasmid (20) was purified with an Endofree plasmid mega kit (Qiagen). 80μg of plasmid, resuspended in 1.6 ml saline, was injected *i.v.* within 5 seconds.

Quantitative real-time polymerase chain reaction, immunoprecipitation and immunoblotting

PCR was performed by usingSYBR® Green RT-PCR reagent kit (Applied Biosystems, Foster City, CA, USA) and target gene-specific primers. Amplification of endogenous *cyclophilin* or *hprt* was used as an internal control. Primers sequence was reported

previously (18). The analysis of gene expression by immunoblotting was performed as previously described (11). For immunoprecipitation, 500 μg pre-cleared cell lysate was incubated with specific antibody for overnight and the antigen-antibody complex was precipitated by using ProteinA/G agarose beads (Santa Cruz Inc., Dallas, TX) and then subjected to electrophoresis in 8% sodium dodecyl sulfate-polyacrylamide gels. The specific bands were visualized by an enhanced chemiluminescence (ECL) detection kit (Amersham Life Sciences, Arlington Heights, IL, USA).

Chromatin Immunoprecipitation Assay

ChIP assay was performed with the reagents and protocol from Millipore (Billerica, MA, USA). In brief, we fixed 2×10^6 32D cells by adding formaldehyde into culture media to a final concentration of 1%. The cells were then lysed and sonicated to shear the chromatin DNA. Cell lysate was precleared with protein G-agarose, before immunoprecipitation overnight at 4°C with 2 μg of specific antibody or normal mouse IgG. The precipitated antibody-chromatin complex was collected by incubation with protein G-agarose, for 1 hr at 4°C, and subsequently washed and eluted in elution buffer. DNA, in the precipitated samples, was recovered by reverse crosslinking at 65°C for 4 hr. Ten percent of lysate, before antibody precipitation, was used as the input. Real-time PCR was carried out with *Fzd10*-specific primers. Amplification of cyclophilin from the input was used as the loading control.

Primers used in ChIP of *Fzd10*

CHIP primer set 1—ACCTATGCAGTTGGGGGAGT

ACCGCAGTCCTTAGATGTCC

CHIP primer 2—GGTTGAAGCTCTGTGTCCTTATC

GGCTGGTGGTTTCTCCAA

CHIP primer 3—CCCTAAACTCCGGCTTCC

GGGATTTGCGTTTTATGACC

CHIP primer 4—GCAGGTGATGATAGGCTCGT

AACACAGGAAGAAGCGAAGG

Gapdh—TACTAGCGGTTTTACGGGCG

TCGAACAGGAGGAGCAGAGAGCGA.

Putative CBF-1 binding sites

SITE 1   GCTCTA**TTCCCA**GGCCAG
SITE 2   GGTAGT**TTCCCA**GACACT
SITE 3   GGCTGG**TTCCCA**CGCAGA
SITE 4   TCCGGC**TTCCCA**AGTCCC
SITE 5   TGGAGA**TTCCCA**TGTGCA

Allogeneic Mixed Leukocyte Reaction

The C57BL/6 BM HPCs were cultured on immobilized Jag-1, on plate, in the presence of SB216763 plus GM-CSF or GM-CSF only for 5 days. The HPC progenies were co-cultured with T cells (10^5/well) from allogeneic BALB/c mice for 4 days, in triplicate, in U-bottom 96-well plates at different ratios. [^{3}H]-thymidine (1 μCi) was added to each well 18 hr prior to cell harvesting. T cell proliferation was measured by [^{3}H]-thymidine incorporation in a liquid scintillation counter (Packard Instrument, Meriden, CT, USA).

Statistical methods

The data were analyzed with a two-tailed Student t-test using GraphPad Software. P values < 0.05 were considered to be statistically significant.

Results

Jag1 negatively regulates DC differentiation in bone marrow

To evaluate the role of Jag1 in DC differentiation, we used mice with conditional knockout of this protein. Jag1$^{fl/fl}$ mice (10) were crossed with Mx1-Cre mice and the Jag1 deletion was induced by repeated injections of poly (I:C). As a control, we used Jag1$^{fl/fl}$Cre$^{-/-}$ littermates treated with poly(I:C). To simplify abbreviation, in this manuscript, Jag1$^{fl/fl}$Cre$^{-/+}$ with inducible deletion of Jag1 are referred as Jag1 knockout (KO) and control mice as wild-type (WT). We generated stroma from bone marrow (BMS) and spleen (SPS) with inducible deletion of Jag1 (Fig. S1) and evaluated their effect on DC differentiation from enriched hematopoietic progenitor cells (HPC) in the presence of GM-CSF. SPS supported DC differentiation substantially better than BMS (control in Fig. 1A vs. B). The absence of Jag1 in SPS did not affect DC differentiation (Fig. 1A). In contrast, lack of Jag1 on BMS significantly (p<0.05) promoted DC differentiation (Fig. 1B). Absence of Jag1 on BMS had no effect on differentiation of DCs generated in the presence of FLT3L (Fig. 1C).

Next, we evaluated the DC differentiation in Jag1 deficient mice *in vivo*. In steady state, no differences in the total number or the proportions of different DC populations in BM, spleen (SPL), or lymph nodes (LN) were found (Fig. S2A). Since our *in vitro* experiments showed that Jag1$^{-/-}$ BMS had effect on DC differentiation only in the presence of GM-CSF, which is associated with emergency hemtopoiesis, we evaluated the role of Jag1 in a model of inflammation-induced myelopoiesis caused by injection of complete Freund's adjuvant (CFA). In both, wild-type (WT) and Jag1 KO mice, CFA induced rapid and equal increase of IMCs in peripheral blood (PB) (Fig. 1D). In contrast, Jag1 KO mice had significantly higher number of CD11c$^+$ cells than WT mice (Fig. 1E). CD11c$^+$ cells (21) are comprised of DCs (CD11c$^+$MHC II$^+$) (3), DC progenitors (mainly CD11c$^+$MHCII$^-$) (22) and few activated leukocytes (reviewed in (23)). Analysis of the population of CD11c$^+$MHC II$^+$ DCs in PB confirmed significant increase in the presence of these cells in Jag1 KO mice (Fig. 1F). These results might reflect differences in the mobilization of DCs. To test this possibility, we evaluated DCs in BM 8 days after CFA injection (at the peak of DC presence in PB). The Jag1 KO mice had a significantly higher proportion and absolute number of plasmacytoid DCs (pDCs) and CD11c$^+$MHC II$^+$DCs, but not MΦ, than their control littermates (Fig. 1G). An increased presence of DCs in BM of Jag1 KO mice was confirmed in allogeneic MLR, a hallmark of DC activity (Fig. 1H). Jag1 KO mice also had a significantly higher presence of DCs in LNs (Fig. 1I). The total number of cells in WT and Jag1 KO mice was the same (Fig. S2B) and, as a result, the total number of DCs showed the same changes as the proportion of the cells.

Similar effect of Jag1 was observed in the different model of induced myelopoiesis caused by hydrodynamic gene transfer (HGT) of GM-CSF plasmid *in vivo*. An equal increase in IMCs in the PB of both WT and Jag1KO mice was seen (Fig. 2A). In Jag1KO mice, GM-CSF effect on DC presence in PB (Fig. 2B), BM (Fig. 2C) and LNs (Fig. 2D) was similar to the effect of CFA. There were no difference in the absolute cell number between WT and Jag1 KO BM.

To clarify the role of Jag1 in DC differentiation, we used an adoptive transfer of BM cells from congenic CD45.1[+]WT mice to lethally irradiated Jag1 KO or WT CD45.2[+] mice. Jag1 deletion was induced by the poly(I:C) injections. Three weeks later mice were irradiated and congenic BM cells were transferred. Six weeks after the transfer, recipient mice were injected with CFA and evaluated 8 days later. Jag1 KO and WT mice were equally reconstituted with donor's BM (Fig. S3). However, Jag1 KO mice had a significantly higher presence of donor's DCs in BM (Fig. 2E, Fig. S3) than WT mice. Thus, taken together these results indicated that Jag1 in BM inhibited DC differentiation.

Dll1 and Jag1 have opposite effect on wingless (Wnt) signaling in HPCs

What could be the mechanism of Jag1 negatively regulating the DC differentiation? Activation of the canonical Wnt pathway in HPC positively regulates DC differentiation; and the Dll1 effect on DC differentiation could be mediated via activation of Wnt signaling (17). Canonical Wnt pathway is activated by Wnt proteins binding to Frizzled (Fzd) receptors and co-receptors, which leads to stabilization and translocation of -catenin to the nuclei to form co-activators with T cell factor (TCF)/lymphoid enhancer factor (LEF) to regulate the target genes (24–26). We asked whether Wnt signaling is involved in Jag1-mediated effects on DC differentiation. Enriched BM HPCs were incubated for 18 h on a monolayer of NIH3T3 fibroblasts overexpressing Dll1, Jag1, or the corresponding control vectors (11). Jag1-cells did not significantly increase expression of any Fzd receptors but inhibited the expression of number of them (Fig. 3A). This was in contrast to the effect of Dll1, which dramatically up-regulated members of Fzd family (17). The down-regulation of several Fzd receptors was confirmed by Western blotting (Fig. 3B). The inhibition of Wnt signaling was determined by down-regulation of -catenin (Fig. 3C), Wnt-target genes (Fig. 3D) and TCF/LEF reporter activity (Fig. 3E). To avoid a possible confounding effect of fibroblasts, we used an experimental system where Notch signaling was activated by Notch ligands directly immobilized on plastic in the presence of recombinant Wnt3a. Dll1 and Jag1 activated the Notch pathway as was measured by the activity of CBF-1 reporter and expression of *hes5* (Fig. 3F, G), suggesting that both ligands are able to activate the conventional Notch signaling. Jag1 inhibited the expression of *fzd10* and Wnt-target genes (Fig. 3G), decreased the amount of Fzd6 and Fzd10 protein (Fig. 3H), and down-regulated the activity of TCF/LEF reporter (Fig. 3I). In contrast, Dll1 induced up-regulation of Wnt signaling (Fig. 3G–I).

We asked whether Jag1 can neutralize activating effect of Dll1 on Wnt signaling. Dll1-IgG and Jag1-IgG were mixed at a 1:1 ratio and immobilized on plastic. As a control, Dll1-IgG were mixed at a 1:1 ratio with control IgG. Jag1 did not inhibit Dll1-inducible up-regulation of *hes5* expression indicating that mix of these two ligands did not affect Notch signaling (Fig. 4A). In contrast, the presence of Jag1 dramatically reduced the expression of *fzd6* and *fzd10,* as well as *wisp1* and *wisp2* induced by Dll1 (Fig. 4A). Jag1 also decreased the activity of the TCF/LEF reporter induced by Dll1 (Fig. 4B). Thus, Jag1 was able to neutralize up-regulation of Wnt pathway caused by Dll1.

Incubation of HPC on BMS generated from Jag1 KO mice resulted in increased expression of Wnt–targeted genes (Fig. 4C). HPCs isolated from BM of CFA-treated Jag1 KO mice (described in Fig. 1) had a significantly higher expression of Wnt target genes and *fzd10* as

well as -cat and Fzd10 proteins than the HPCs from control mice (Fig. 4D). These results were confirmed in experiments *in vitro* using BMS with Jag1 down-regulated with specific siRNA (Fig. 4E). Thus, Jag1 in contrast to Dll1 substantially reduced Wnt signaling in HPC via down-regulation of Fzd receptors.

Jag1 negatively regulate DC differentiation via down-regulation of Wnt signaling

Next, we investigated the mechanism of Jag1 mediated regulation of DC differentiation. Consistent with previous observations (11, 15, 16, 27), Dll1 promoted DC differentiation from HPCs (Fig. 5A). Addition of Jag1 to Dll1 completely abrogated this effect (Fig. 5A). We evaluated the effect of activation of Wnt pathway downstream of Fzd receptors on the DC differentiation. Wnt pathway was activated by overexpression of a constitutively active -cat mutant (28) (Fig. 5B) or by inhibition of the GSK-3 kinase, which is critically important for activation of Wnt signaling, using specific inhibitor SB216763(29) (Fig. 5C). In both cases activation of Wnt pathway abrogated the inhibitory effect of Jag1 on DC differentiation (Fig. 5B, C) and restored the proportion of DCs to the control level. It also restored the ability of cells differentiated in the presence of Jag1 to stimulate allogeneic T cells, the function attributed to DCs (Fig. 5D).

Next, we investigated the consequences of down-regulation of Wnt signaling for DC differentiation, using mice with a conditional knockout of the -cat gene. -cat$^{fl/fl}$ mice were crossed with Mx1-Cre mice and -cat deletion was achieved by repeated injections of poly(I:C) (Fig. 5E). These mice were further referred as -cat KO. BM cells from -cat KO mice and Cre$^-$ WT littermates were adoptively transferred into lethally irradiated congeneic CD45.1 mice. Four weeks after the transfer, the mice were treated with GM-CSF HGT or with a control vector, as described in Fig. 2C, and analyzed on day 4. No differences in DC differentiation between recipients of WT and -cat KO BM cells were seen in mice treated with the control vector. GM-CSF HGT caused significant increase in the presence of WT donors' DCs in BM, which was consistent with the recent report (30). In contrast, no increase was observed in recipients of -cat KO BM (Fig. 5F). M differentiation was not impaired in the -cat KO BM (Fig. 5F).

To evaluate DC differentiation from their precursors, BM Gr-1$^+$CD11b$^+$ IMC were isolated from WT and -cat KO mice (CD45.2$^+$) and transferred to sub-lethally irradiated CD45.1$^+$ recipients. Donor's cells were evaluated 4 days later. The proportion and absolute number of splenic cDCs (MHCII$^+$CD11c^{hi}) but not M s, generated from -cat KO IMCs was significantly lower than that from WT IMCs (Fig. 5G). The differentiation of CD8 $^-$ cDC subset was also significantly impaired, which was consistent with previous observations that the development of splenic CD8 $^-$ cDCs was dramatically blocked in CBF-1 KO mice (31). The culture of enriched HPCs from -cat KO mice with GM-CSF and IL-4 produced smaller proportion of CD11c$^+$CD11b$^+$F4/80$^-$ DCs than that of WT HPC (Fig. 5H). Treatment of mice with selective Wnt inhibitor ICG-001 (32) reduced the proportion of DCs in BM of Jag1 KO mice treated with CFA but not in control mice (Fig. S4). Thus, these data support the hypothesis that Jag1 regulates DC differentiation in emergency hematopoiesis via inhibition of Wnt signaling.

Molecular mechanism of opposing effects of Dll1 and Jag1 on Wnt signaling

Histone deacetylase (HDAC) is a major component of the constitutive CSL/CBF-1 repressor complex. We explored the possible role of HDAC in the regulation of *fzd* expression by Jag1. HDAC inhibitor trichostatin-A (TSA) completely abrogated the Jag1-inducible inhibition of *fzd6* and *fzd10* expression (Fig. 6A) and restored the expression of the Wnt-target genes (Fig. 6B). We made a luciferase reporter construct by cloning the *fzd10* promoter region into a PGL3 enhancer vector. 32D myeloid progenitor cells were

transfected with this vector and placed on fibroblasts, overexpressing different Notch ligands. Dll1 significantly up-regulated the reporter (Fig. 6C), whereas Jag1 inhibited it (Fig. 6D). The TSA slightly up-regulated the *fzd10* reporter in the cells cultured on Dll1, but completely restored its activity in 32D cells cultured on Jag1 (Fig. 6C,D). To clarify these findings, ChIP assays were performed in 32D cells using antibody specific for acetylated histone H3 and primers specific for *fzd10* promoter. Dll1 up-regulated the association of acetylated H3 with the promoter, but it was significantly inhibited by Jag1 (Fig. 6E). The opposite effect was observed when chromatin was precipitated with HDAC1 antibody (Fig. 6F). Thus, in contrast to Dll1, Jag1 did not cause the displacement of HDAC1 from the *fzd10* promoter, which could result on silencing of the gene expression.

Since both Jag1 and Dll1 induced CBF-1 activation, we asked whether these ligands differently regulated the physical interaction between HDAC1 and CBF-1. HPCs were cultured on immobilized Dll1 or Jag1 in the presence of GM-CSF for 4 h. Cell lysates were precipitated with CBF-1 antibody and then probed with HDAC1 antibody. The amount of HDAC1 co-precipitated with the CBF-1 in HPC incubated on Dll1 was substantially lower than in HPC cultured on Jag1 (Fig. 6G). To test if HDAC1 association with the *fzd10* promoter depends on the presence of CBF-1, HPCs were transfected with CBF-1 siRNA (Fig. 6H, inset). The down-regulation of CBF-1 completely abrogated the Jag1-inducible HDAC1 association with the *fzd10* promoter (Fig. 6H), indicating that HDAC1 interacts with the *fzd10* promoter in complex with CSL. HDAC1 was knocked down in 32D cells by using siRNA (Fig.6I, inset). This did not affect the Dll1-inducible up-regulation of the expression of *fzd10* and Wnt-target genes. However, the Jag1-inducible down-regulation of these genes was abrogated (Fig. 6I).

Discussion

This study suggests the novel mechanism of regulation of DC differentiation in BM, which depends on the expression of the Notch ligand, Jag1. Under steady state conditions Jag1 was dispensable for DC differentiation. However, it was important in the conditions of forced myelopoiesis. Among several models of emergency myelopoiesis, we used two: inflammation-related - caused by the injection of CFA; and induced by the administration of GM-CSF. Although both treatments caused an equal increase of IMCs in WT and Jag1KO mice, the presence of DCs in blood and BM was significantly higher in Jag1KO than in WT mice. These data were consistent with previous observations that Notch signaling had no effect on long-term, repopulating HSC self-renewal when assessed during homeostasis (33); but had a strong effect on the differentiation and enhanced generation of myeloid progenitors during stress hematopoiesis (34). The fact that the effect of Jag1 deletion on DCs was confined largely to BM could be explained by the fact that Jag1 expression is predominant in BMS but not in SPS (11). Jag1 was also previously shown to be the main Notch ligand that is expressed in the HSC niche in BM (35). It appears that Jag1 may play an important role in the inhibition of DC differentiation from the precursors inside BM. When DC precursors leave BM, they undergo a terminal differentiation in tissues where the expression of Jag1 is substantially reduced.

The main focus of this study was on the mechanism of the opposite effect of Jag1 and Dll1 on DC differentiation. Both are ligands for the same receptors and both are able to activate Notch signaling. One of the possible explanations is the different strength of the Notch signaling, mediated by Jag1 and Dll1. It was shown that Jag1 had the weakest ability to activate Notch1; whereas, Dll4 was the strongest Notch1 activator, followed by Dll1 and Jag2 (12). The different effect of Jag1 and Dll1 was also associated with the Lunatic-Fringe–mediated glycosylation of Notch1. Glycosylation of Notch1 potentiated Notch signaling through Dll1 ligands and Jag2, in contrast to Jag1 (12, 36). Upon glycosylation of Notch,

Dll4-Notch signaling was enhanced; whereas, Jag1 had a weak signaling capacity (7). Individual Notch receptors have different transcriptional activity (37). Jagged family members, in the presence of Fringe, can activate Notch2 (38–40). It was recently shown that Notch2 enhanced the rate of formation of HSCs; while delaying myeloid differentiation in BM, during non-homeostatic conditions, including after chemotherapy or during marrow regeneration after stem cell transplantation. However, both Jag1 and Dll1 activated Notch2 in the HSC-enriched population that inhibited myeloid differentiation and enhanced the generation of myeloid progenitors during stress hematopoiesis (34). In our study, lack of Jag1, during emergency hematopoiesis, did not affect the total number of myeloid cells. No decrease in the presence of IMCs or macrophages was seen. This suggested that deletion of Jag1 did not result in a modified expansion of progenitor cells, but rather influenced the specific differentiation of DCs in the compartment with prevalent Jag1 expression (BM). Jag1 was able to actively neutralize the effect of Dll1 on DC differentiation. These data argue that the inhibitory effect of Jag1 on DC differentiation was unlikely, due to its inability to reach the required Notch signal strength threshold to induce DC differentiation.

Our data suggest a novel mechanism of a negative regulation of DC differentiation by Jag1 that involves Wnt signaling. Previous studies have implicated the Wnt pathway in DC differentiation and activation (17)(28, 41). Dll1 promoted DC differentiation via up-regulation of the expression of the Fzd family of Wnt receptors and activation of Wnt signaling (17). This effect was different from the recently reported post-translational inhibition of -catenin in stem and colon cancer cells by membrane-bound Notch (42). To our surprise, Jag1 directly inhibited Wnt signaling in HPCs via the transcriptional down-regulation of the expression of several Wnt receptors Fzd. Jag1 was able to neutralize the Dll1-inducible up-regulation of Fzd, despite the fact that Dll1 has a much stronger effect on Notch signaling.

These data suggest that Wnt signaling could affect the differentiation of DCs. However, recipients of -cat KO BM cells, in steady state, had no defects in DC differentiation. This was consistent with a previous report (43). However, when recipients of -cat KO BM were treated with GM-CSF by HGT, they failed to up-regulate DC production in BM; which suggested that -cat may play an important role in DC differentiation during emergency hematopoiesis.

In the absence of Notch activation, CBF-1 exists in nuclei as a repressor complex, in association with the transcriptional co-repressors SMRT, NCoR, CIR, SHARP, KyoT2, and Skip. Some of these co-repressors (SMRT, CIR, SHARP) are shown to recruit HDACs to Notch target genes (44, 45). After activation, cleaved ICN displaces these co-repressors and HDACs, from CBF-1, to form a transcription-activating complex (46). In addition, ICN, binding to CBF-1, recruits mastermind (MAML) to the complex; which can recruit HDACs to the activation complex (47, 48). It appears that the interaction of Notch with Dll1 in HPCs follows this scheme. As a result, expression of *fzd* and Wnt signaling is dramatically increased. However, Jag1 fails to displace HDAC1 from co-repressor complexes on *fzd* promoters that shut-down *fzd* expression and inhibited Wnt signaling and terminal DC differentiation. This effect was not global, since Jag1 activated transcription of *hes1* and *hes5* target genes. The detailed mechanism of this process needs to be further elucidated. However, it is possible that the number of CBF-1 binding sited in different genes can be important. Our data may help to explain the diversity and context-dependent nature of Notch-mediated effects and provide a novel model of regulation of the DC differentiation in various tissue compartments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Shortman K, Heath WR. The CD8+ dendritic cell subset. Immunol Rev. 2010; 234:18–31. [PubMed: 20193009]

2. Dominguez PM, Ardavin C. Differentiation and function of mouse monocyte-derived dendritic cells in steady state and inflammation. Immunol Rev. 2010; 234:90–104. [PubMed: 20193014]

3. Sapoznikov A, Pewzner-Jung Y, Kalchenko V, Krauthgamer R, Shachar I, Jung S. Perivascular clusters of dendritic cells provide critical survival signals to B cells in bone marrow niches. Nat Immunol. 2008; 9:388–395. [PubMed: 18311142]

4. Artavanis-Tsakonas SRM, Lake RJ. Notch signaling: cell fate control and signal integration in development. Science. 1999; 284:770–776. [PubMed: 10221902]

5. Allman D, Punt J, Izon DJ, Aster JC, Pear WS. An invitation to T and more: Notch signaling in lymphopoiesis. Cell. 2002; 109:S1–S11. [PubMed: 11983148]

6. Osborne B, Miele L. Notch and the immune system. Immunity. 1999; 11:653–663. [PubMed: 10626888]

7. Benedito R, Roca C, Sorensen I, Adams S, Gossler A, Fruttiger M, Adams RH. The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. Cell. 2009; 137:1124–1135. [PubMed: 19524514]

8. Bugeon L, Gardner LM, Rose A, Gentle M, Dallman MJ. Cutting edge: Notch signaling induces a distinct cytokine profile in dendritic cells that supports T cell-mediated regulation and IL-2-dependent IL-17 production. J Immunol. 2008; 181:8189–8193. [PubMed: 19050233]

9. Kwon SM, Eguchi M, Wada M, Iwami Y, Hozumi K, Iwaguro H, Masuda H, Kawamoto A, Asahara T. Specific Jagged-1 signal from bone marrow microenvironment is required for endothelial progenitor cell development for neovascularization. Circulation. 2008; 118:157–165. [PubMed: 18591437]

10. Brooker R, Hozumi K, Lewis J. Notch ligands with contrasting functions: Jagged1 and Delta1 in the mouse inner ear. Development. 2006; 133:1277–1286. [PubMed: 16495313]

11. Cheng P, Nefedova Y, Corzo CA, Gabrilovich DI. Regulation of dendritic cell differentiation by bone marrow stroma via different Notch ligands. Blood. 2007; 109:507–515. [PubMed: 16973960]

12. Van de Walle I, De Smet G, Gartner M, De Smedt M, Waegemans E, Vandekerckhove B, Leclercq G, Plum J, Aster JC, Bernstein ID, Guidos CJ, Kyewski B, Taghon T. Jagged2 acts as a Delta-like Notch ligand during early hematopoietic cell fate decisions. Blood. 2011; 117:4449–4459. [PubMed: 21372153]

13. Ramasamy SK, Lenka N. Notch exhibits ligand bias and maneuvers stage-specific steering of neural differentiation in embryonic stem cells. Mol Cell Biol. 2010; 30:1946–1957. [PubMed: 20154142]

14. Cheng P, Gabrilovich D. Notch signaling in differentiation and function of dendritic cells. Immunol Res. 2008; 41:1–14. [PubMed: 18008056]

15. Hoshino N, Katayama N, Shibasaki T, Ohishi K, Nishioka J, Masuya M, Miyahara Y, Hayashida M, Shimomura D, Kato T, Nakatani K, Nishii K, Kuribayashi K, Nobori T, Shiku H. A novel role for Notch ligand Delta-1 as a regulator of human Langerhans cell development from blood monocytes. J Leukoc Biol. 2005; 78:921–929. [PubMed: 16037408]

16. Olivier A, Lauret E, Gonin P, Galy A. The Notch ligand delta-1 is a hematopoietic development cofactor for plasmacytoid dendritic cells. Blood. 2006; 107:2694–2701. [PubMed: 16357328]

17. Zhou J, Cheng P, Youn JI, Cotter MJ, Gabrilovich DI. Notch and wingless signaling cooperate in regulation of dendritic cell differentiation. Immunity. 2009; 30:845–859. [PubMed: 19523851]

18. Ohishi K, Varnum-Finney B, Flowers D, Anasetti C, Myerson D, Bernstein ID. Monocytes express high amounts of Notch and undergo cytokine specific apoptosis following interaction with the Notch ligand, Delta-1. Blood. 2000; 95:2847–2854. [PubMed: 10779430]

19. Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Ther. 1999; 6:1258–1266. [PubMed: 10455434]

20. Takebe Y, Seiki M, Fujisawa J, Hoy P, Yokota K, Arai K, Yoshida M, Arai N. SR alpha promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. Mol Cell Biol. 1988; 8:466–472. [PubMed: 2827008]

21. Metlay JP, Witmer-Pack MD, Agger R, Crowley MT, Lawless D, Steinman RM. The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. J Exp Med. 1990; 171:1753–1771. [PubMed: 2185332]

22. del Hoyo GM, Martin P, Vargas HH, Ruiz S, Arias CF, Ardavin C. Characterization of a common precursor population for dendritic cells. Nature. 2002; 415:1043–1047. [PubMed: 11875574]

23. Hashimoto D, Miller J, Merad M. Dendritic cell and macrophage heterogeneity in vivo. Immunity. 2011; 35:323–335. [PubMed: 21943488]

24. Reya T, Clevers H. Wnt signalling in stem cells and cancer. Nature. 2005; 434:843–850. [PubMed: 15829953]

25. Katoh M, Katoh M. WNT Signaling Pathway and Stem Cell Signaling Network. Clin Cancer Res. 2007; 13:4042–4045. [PubMed: 17634527]

26. Nusse AR. Wnts as ligands: processing, secretion and reception. Oncogene. 2006; 25:7461–7468. [PubMed: 17143290]

27. Ohishi K, Varnum-Finney B, Serda RE, Anasetti C, Bernstein ID. The Notch ligand, Delta-1, inhibits the differentiation of monocytes into macrophages but permits their differentiation into dendritic cells. Blood. 2001; 98:1402–1407. [PubMed: 11520788]

28. Jiang A, Bloom O, Ono S, Cui W, Unternaehrer J, Jiang S, Whitney JA, Connolly J, Banchereau J, Mellman I. Disruption of E-cadherin-mediated adhesion induces a functionally distinct pathway of dendritic cell maturation. Immunity. 2007; 27:610–624. [PubMed: 17936032]

29. Owen R, Gordon-Weeks PR. Inhibition of glycogen synthase kinase 3beta in sensory neurons in culture alters filopodia dynamics and microtubule distribution in growth cones. Mol Cell Neurosci. 2003; 23:626–637. [PubMed: 12932442]

30. Li HS, Yang CY, Nallaparaju KC, Zhang H, Liu YJ, Goldrath AW, Watowich SS. The signal transducers STAT5 and STAT3 control expression of Id2 and E2–2 during dendritic cell development. Blood. 2012; 120:4363–4373. [PubMed: 23033267]

31. Caton ML, Smith-Raska MR, Reizis B. Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. J Exp Med. 2007; 204:1653–1664. [PubMed: 17591855]

32. Emami KH, Nguyen C, Ma H, Kim DH, Jeong KW, Eguchi M, Moon RT, Teo JL, Kim HY, Moon SH, Ha JR, Kahn M. A small molecule inhibitor of beta-catenin/CREB-binding protein transcription [corrected]. Proc Natl Acad Sci U S A. 2004; 101:12682–12687. [PubMed: 15314234]

33. Maillard I, Koch U, Dumortier A, Shestova O, Xu L, Sai H, Pross SE, Aster JC, Bhandoola A, Radtke F, Pear WS. Canonical notch signaling is dispensable for the maintenance of adult hematopoietic stem cells. Cell stem cell. 2008; 2:356–366. [PubMed: 18397755]

34. Varnum-Finney B, Halasz LM, Sun M, Gridley T, Radtke F, Bernstein ID. Notch2 governs the rate of generation of mouse long- and short-term repopulating stem cells. J Clin Invest. 2011; 121:1207–1216. [PubMed: 21285514]

35. Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, Martin RP, Schipani E, Divieti P, Bringhurst FR, Milner LA, Kronenberg HM, Scadden DT. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature. 2003; 425:841–846. [PubMed: 14574413]

36. Kato TM, Kawaguchi A, Kosodo Y, Niwa H, Matsuzaki F. Lunatic fringe potentiates Notch signaling in the developing brain. Mol Cell Neurosci. 2010; 45:12–25. [PubMed: 20510365]

37. Ong CT, Cheng HT, Chang LW, Ohtsuka T, Kageyama R, Stormo GD, Kopan R. Target selectivity of vertebrate notch proteins. Collaboration between discrete domains and CSL-binding site architecture determines activation probability. J Biol Chem. 2006; 281:5106–5119. [PubMed: 16365048]

38. Visan I, Tan JB, Yuan JS, Harper JA, Koch U, Guidos CJ. Regulation of T lymphopoiesis by Notch1 and Lunatic fringe-mediated competition for intrathymic niches. Nat Immunol. 2006; 7:634–643. [PubMed: 16699526]

39. Yang LT, Nichols JT, Yao C, Manilay JO, Robey EA, Weinmaster G. Fringe glycosyltransferases differentially modulate Notch1 proteolysis induced by Delta1 and Jagged1. Mol Biol Cell. 2005; 16:927–942. [PubMed: 15574878]

40. Hicks C, Johnston SH, diSibio G, Collazo A, Vogt TF, Weinmaster G. Fringe differentially modulates Jagged1 and Delta1 signalling through Notch1 and Notch2. Nat Cell Biol. 2000; 2:515–520. [PubMed: 10934472]

41. Staal FJ, Luis TC, Tiemessen MM. WNT signalling in the immune system: WNT is spreading its wings. Nat Rev Immunol. 2008; 8:581–593. [PubMed: 18617885]

42. Kwon C, Cheng P, King IN, Andersen P, Shenje L, Nigam V, Srivastava D. Notch post-translationally regulates beta-catenin protein in stem and progenitor cells. Nat Cell Biol. 2011; 13:1244–1251. [PubMed: 21841793]

43. Cobas M, Wilson A, Ernst B, Mancini SJ, MacDonald HR, Kemler R, Radtke F. Beta-catenin is dispensable for hematopoiesis and lymphopoiesis. J Exp Med. 2004; 199:221–229. [PubMed: 14718516]

44. Oswald F, Winkler M, Cao Y, Astrahantseff K, Bourteele S, Knöchel W, Borggrefe T. RBP-J/SHARP Recruits CtIP/CtBP Corepressors To Silence Notch Target Genes. Mol Cell Biol. 2005; 25:10379–10390. [PubMed: 16287852]

45. Hsieh JD, Zhou S, Chen L, Young D, Hayward S. CIR, a corepressor linking the DNA binding factor CBF1 to the histone deacetylase complex. Proc Natl Acad Sci USA. 1991; 96:23–28. [PubMed: 9874765]

46. Miele L. Notch Signaling. Clin Cancer Res. 2006; 12:1074–1079. [PubMed: 16489059]

47. Nam Y, Weng A, Aster J, Blacklow S. Structural requirements for assembly of the CSL. intracellular Notch1. Mastermind-like 1 transcriptional activation complex. J Biol Chem. 2003; 278:21232–21239. [PubMed: 12644465]

48. Fryer C, Lamar E, Turbachova I, Kintner C, Jones K. Mastermind mediates chromatin-specific transcription and turnover of the Notch enhancer complex. Genes Dev. 2002; 16:1397–1411. [PubMed: 12050117]
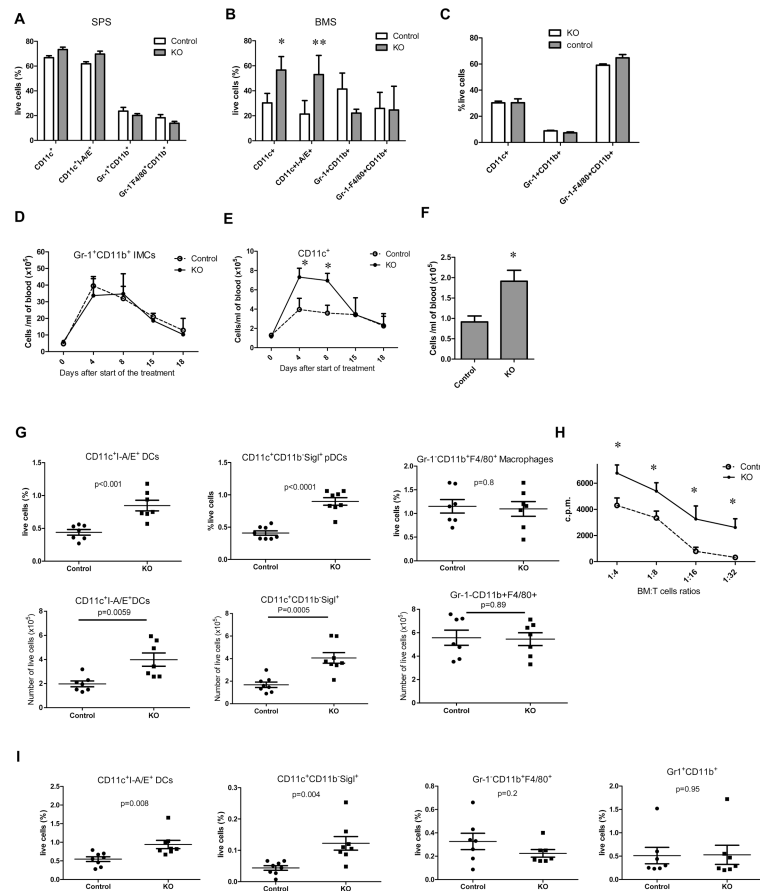
Figure 1. Jag1 negatively regulates DC differentiation on bone marrow stroma and CFA treated mice

A, B. CD45.1[+] HPCs were cultured in the presence of 20ng/ml GM-CSF for 5 days on the monolayer of CD45.2[+] SPS **(A)** or BMS **(B)** generated from Jag1 KO (KO) or WT mice. Cells were labeled with cocktail of indicated antibodies and examined by flow cytometry on day 5. Only CD45.1^{+} cells were evaluated. Data are presented as Mean ± SEM from 3 independent experiments. *-statistically significant (p<0.05) and ** (p<0.01) differences between groups. **C.** CD45.1[+] HPCs were cultured in the presence of 100 ng/ml FLT3L for 10 days on the BM stromal from Jag1 KO or control mice. CD45.1^{+}cells were evaluated by FACS. Data are presented as Mean ± SEM from 3 independent experiments. **D,E.** The kinetic of Gr-1^{+} CD11b^{+} IMCs (**D**) and CD11c^{+} (**E**) in PB of WT and Jag1KO mice at different time points after CFA injection. Mean±SEM from 5 mice per group are shown. **F.** The total cell numbers of CD11c^{+}I-A/E^{+} DCs, in 1 ml PB of WT and Jag1KO mice on day 8 after CFA injection Mean±SEM from 5 mice per group are shown. *-statistically significant (p<0.05) differences between groups. **G.** Jag1 KO and control mice were injected with CFA. The proportions and absolute number of indicated populations of cells in BM of mice on day 8 after CFA injection are shown. Individual data from mice (7 mice per group) are shown. **H.** Allogeneic MLR. BM cells from Jag1 KO or WT mice were stimulated with 100 ng/ml LPS for 16 h and cultured at different ratio with allogeneic (BALB/c) T cells for 5 days. Cell proliferation was measured in triplicate by [3H]-thymidine uptake in the last 16 h. Data are presented as Mean ± SEM (n=3 mice per group). **I**. The proportions of indicated cell populations in LNs of mice on day 8 after CFA injection. Numbers in each figure indicated p-value. Individual data from mice (7 mice per group) are shown.
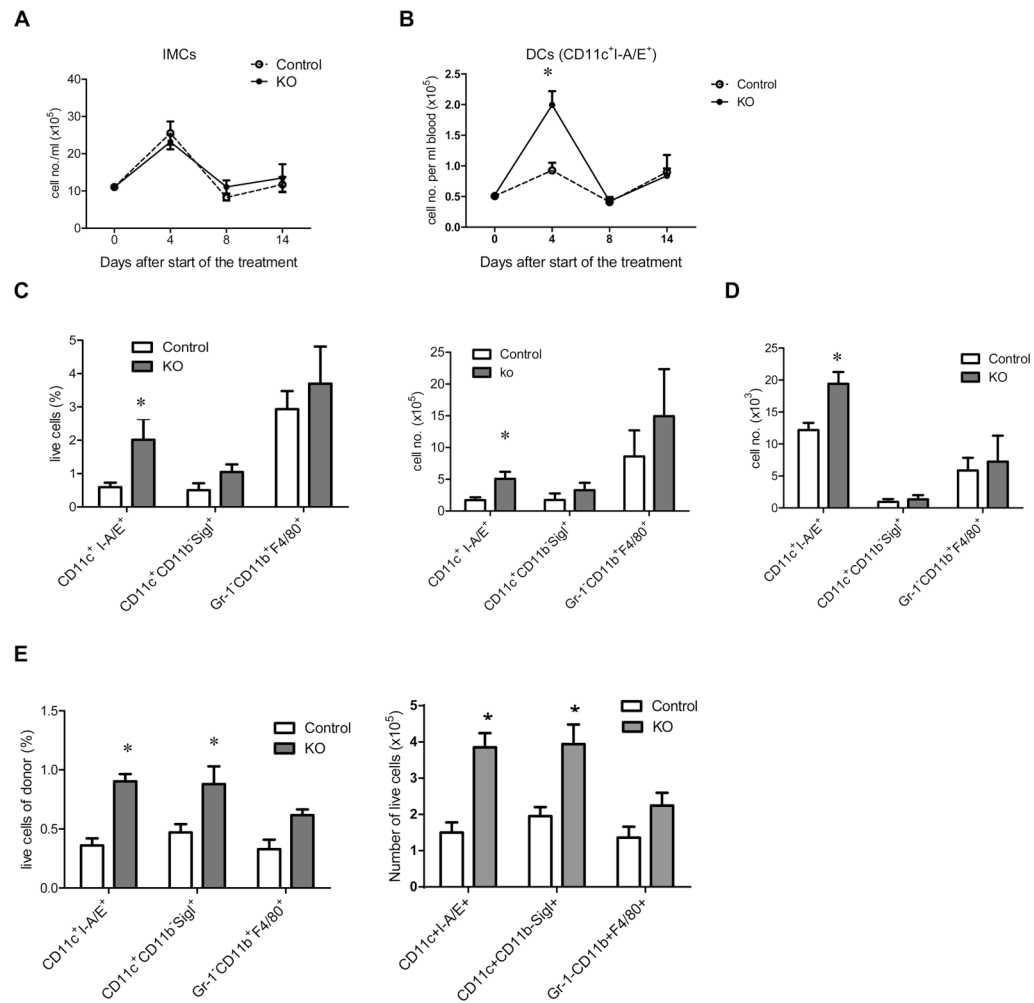
Figure 2. Jag1 negatively regulates DC differentiation in bone marrow after GM-CSF stimulation

A, B. The kinetic of IMCs **(A)** and CD11c^{+} I-A/E^{+} DCs **(B)** in PB of Jag-1 KO and WT mice after treatment with GM-CSF HGT. Each group included 3–4 mice. *-statistically significant (p<0.05) differences between the groups. **C.** The proportion and absolute number of indicated populations of cells in BM of mice obtained on day 4 after HGT. Mean±SEM from 5 mice per group are shown. * - statistically significant differences between groups (p<0.05). **D.** Absolute number of indicated cell populations in LNs of the same mice as in Fig. 2C. E. Lethally irradiated CD45.2[+] Jag1 KO or WT mice were reconstituted with 10[6] CD45.1[+] donor's HPCs from WT mice. After 6 weeks, mice were treated with CFA as described in legend to Fig.1G and the presence in BM of indicated donor's cells was evaluated 8 days after CFA injection. Data are presented as Mean ± SEM (n=5 mice per group). *-statistically significant (p<0.05) differences between the groups.
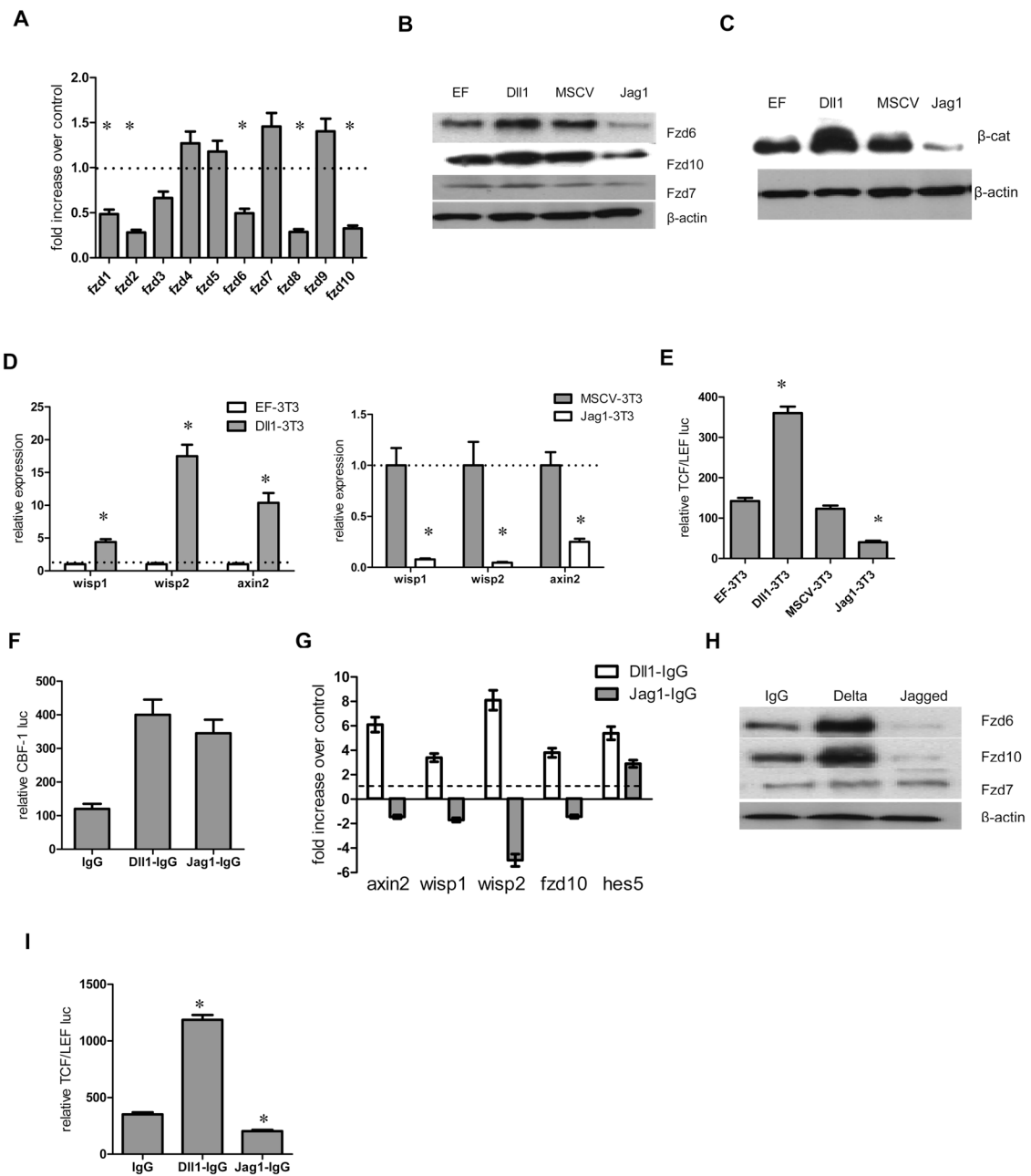
Figure 3. Notch ligands differentially regulate Wnt signaling in HPCs
A. Enriched HPCs were incubated on a monolayer of NIH3T3 fibroblasts overexpressing Jag1, or control MSCV-vectors in medium containing 20 ng/mL GM-CSF. CD45^{+} hematopoietic cells were isolated using magnetic beads after 18 hrs culture and expression of *fzd* gene family were evaluated using qRT-PCR. Relative gene expression was calculated by normalizing to internal control (cyclophilin). Mean ± SEM of three experiments performed in triplicates are shown. **B,C.** The amounts of selected Fzd proteins (**B**) and -catenin protein (**C**) evaluated by Western blot after 3 day culture. Typical examples of three performed experiments are shown. **D.** mRNA level of Wnt-target gene were by measured by qRT-PCR after 18 h culture. Mean ± SEM of three experiments performed in triplicates are shown. Please note differences in the scale. **E.** HPCs were transfected with TCF/LEF

reporter plasmid and control Renilla plasmid and then cultured on fibroblast cells for 48 h. Reporter activity was measured using dual-luciferase reporter assay. Three experiments were performed in duplicates. * - statistically significant differences from corresponding controls (p<0.05). **F–I.** The effect of immobilized Notch ligands on Notch signaling in HPCs. **F**. Enriched HPCs were transfected with luciferase CBF1 reporter plasmid and control renilla plasmid. Cells were cultured for 24 hr on plates coated with 7.5 μg/ml control IgG, Dll1, or Jag1. Luciferase activity was measured in triplicates in dual-luciferase assay. Results of two experiments are shown. **G.** Enriched HPCs were cultured on a 24-well plate coated with 7.5 μg/ml Delta-IgG, Jag-IgG or control IgG protein in the presence of 100 ng/ml recombinant Wnt3A. Expression of *fzd* and Wnt-targeted genes evaluated using qRT-PCR after 24 hrs culture. Fold changes in relative gene expression over the control IgG levels was calculated. The negative values indicate down-regulation of genes expression. Mean ± SEM of three experiments performed in triplicates are shown. **H.** The amount of selected Fzd proteins was evaluated in Western blotting using indicated antibodies. **I.** HPCs were transfected with TCF/LEF reporter plasmid and control Renilla plasmid before cultured on ligands coated plate. Reporter activity was measured 48 hr later. Mean ± SEM of three experiments performed in duplicates are shown.
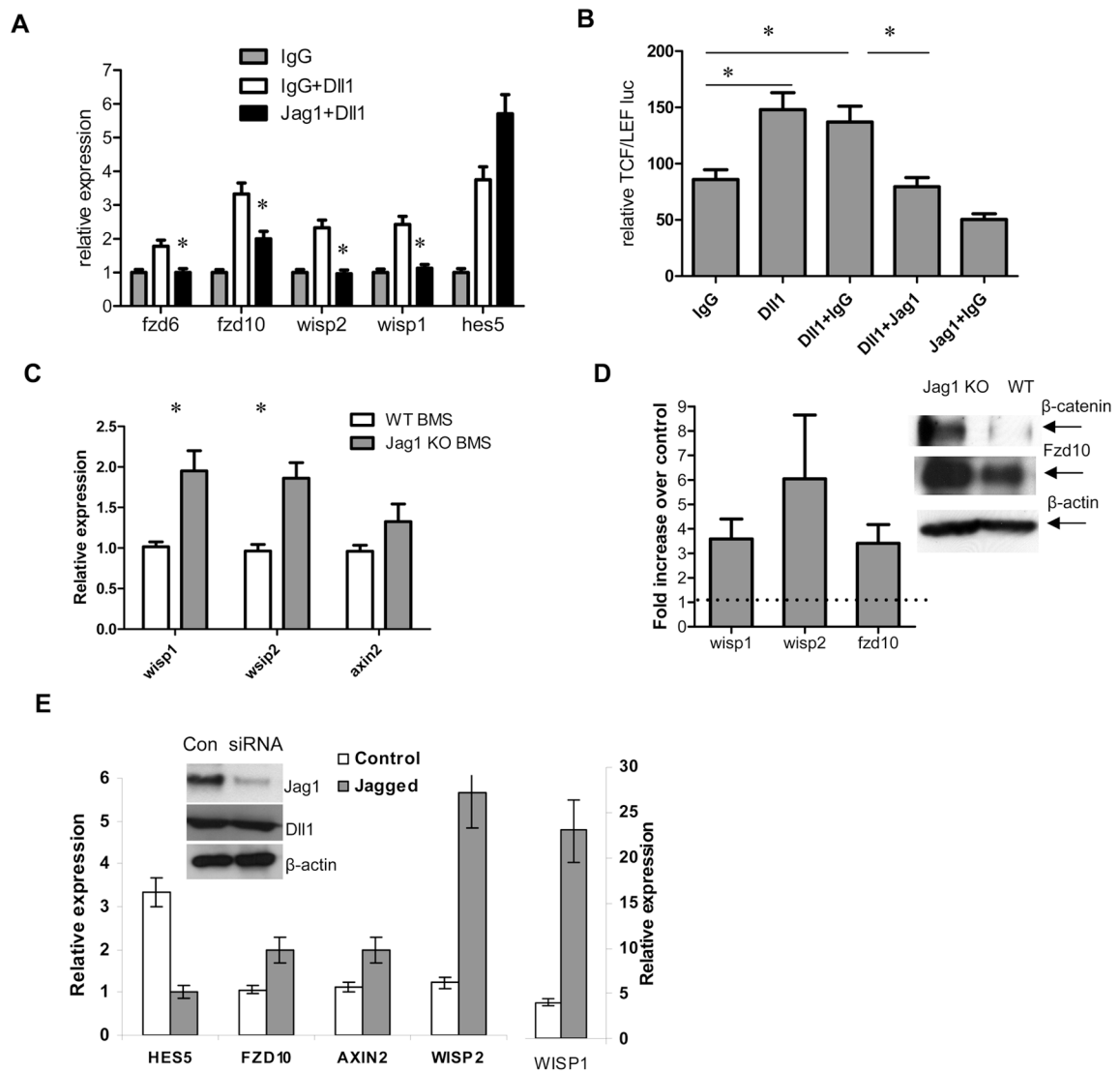
Figure 4. Jag1 neutralized the effect of Dll1 on Wnt signaling

A. HPCs were cultured for 24 hr on 24-well plates coated with Dll1 or 1:1 mixture of Dll1 with control IgG or Jag1 in the presence of 100 ng/ml Wnt3a. Expression of *fzd* and Wnt-targeted genes was measured by qRT-PCR. Mean ± SEM of three experiments performed in triplicates are shown. * - statistically significant differences between IgG+Dll1 and Jag1+Dll1 groups (p<0.05). **B.** Enriched HPCs were transfected with luciferase TCF/LEF reporter plasmid and control renilla plasmid. Cells then were cultured for 24 hr on plates coated with control IgG, Dll1 or 1:1 mix of Dll1 with IgG or Dll1 with Jag1 protein. Luciferase activity was measured in HPCs in triplicates in dual-luciferase assay. Mean ± SEM of three experiments are shown. * p<0.05. **C.** CD45.1[+] HPC were cultured for 24 h on the monolayer of Jag1^{+/+} or Jag1^{−/−} BMS cells in the presence of 20 ng/ml GM-CSF. Total mRNA was extracted from isolated CD45.1^{+} cells and expression of Wnt target genes was evaluated in qPCR. Each experiment was performed in triplicates. Cumulative results from three experiments are shown as Mean ± SEM. * - p<0.05 between groups. **D**. mRNA and whole cell lysate **(inset)** were isolated from BM HPCs of Jag1KO or control mice treated as described in legend to Fig. 1G. Expression of *fzd* and Wnt-targeted genes was evaluated by qRT-PCR in triplicates. Each group included three mice. Fold increase of gene expression in

Jag1KO over control mice is shown. All differences were significant (p<0.05). Indicated proteins were detected by Western blotting (**inset**). **E**. BMS prepared from CD45.1[+] congenic mice were transfected with control or Jag1 siRNA for 24 h. Enriched HPCs from CD45.2[+] mice were cultured on BMS for 48 h. Expression of *fzd* and Wnt-targeted genes in CD45.2[+] HPCs was measured by qRT-PCR. Inset – protein level in BMS by Western blotting demonstrating specificity of Jagged-1 knock-down.
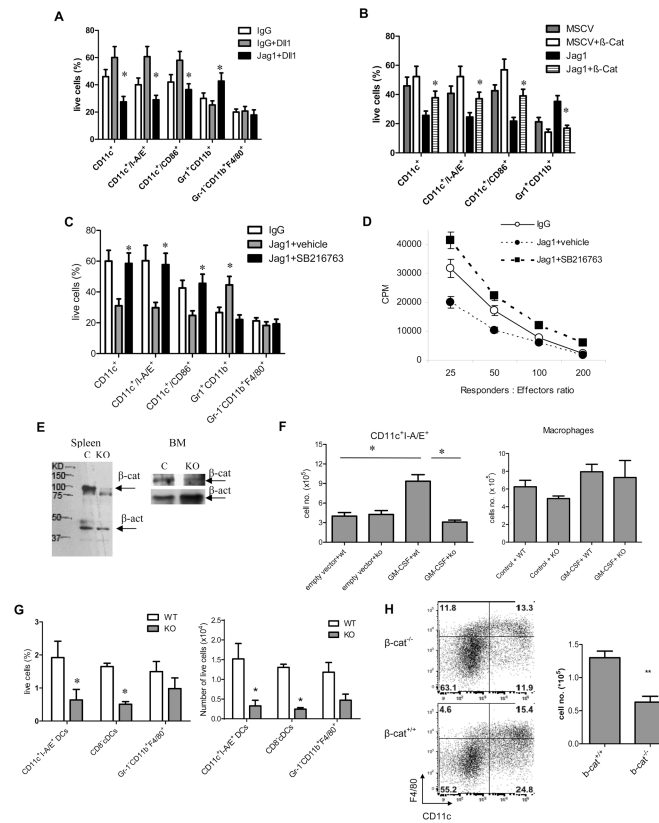
Figure 5. Jag1 negatively regulate DC differentiation via down-regulation of Wnt signaling
A. Enriched HPCs were cultured on 24-well plates coated with a 1:1 mixture of Dll1 and
Jag1 or IgG proteins in the presence of Wnt3a (100 ng/ml) for 5 days in medium with 20 ng/
ml GM-CSF. Mean ± SEM of three experiments are shown. * - statistically significant
differences between IgG + Dll1 and Jag1 + Dll1 groups (p<0.05). **B.** Enriched HPCs were
transfected with -catenin-GFP or GFP vector, and then cultured on monolayer of
fibroblasts for 5 days. The percentage of the indicated population of cells was calculated
within gated CD45^{+}GFP^{+} cells. Mean ± SEM of three experiments are shown. *, p<0.05
between Jag1 and Jag1+ -cat groups. **C.** Enriched HPCs were cultured on plates coated with
IgG or Jag1-IgG for 4 days. SB216763 (10 nM) was added on day 0. The percentage of the
indicated population of cells were evaluated on day 5. Cumulative results of two performed
experiments. * - statistically significant differences between Jag1 and Jag1+SB216763
groups (p<0.05). **D.** Allogeneic MLR. Cells from experiments described in panel C were
cultured at different ratios with T cells isolated from allogeneic BALB/c mice for 4 days.
Cell proliferation was measured in triplicate by [^{3}H]-thymidine uptake. Values are the mean
± SEM. **E.** -catenin expression in splenocytes from -cat^{fl/fl}Mx1-Cre^{+/−} knockout (KO) or
control -cat^{fl/fl} Mx1-Cre[−] (control) mice. **F.** Lethally irradiated CD45.1[+] recipients were
reconstituted with 1.5x10^{6} cells BM from CD45.2[+] -cat KO or WT mice. Four weeks after
the adoptive transfer mice were treated with GM-CSF HGT or control vector and evaluated
4 days later. Data show the number of indicated populations of donors cells presented as
Mean ± SEM (n=5 mice per group). *-statistically significant differences (p<0.05) between
WT and -cat KO mice; **G.** 8x10^{6} CD45.2[+] Gr-1^{+} cells were isolated from BM of -cat KO
or WT mice and transferred *i.v.* into sub-lethally irradiated CD45.1[+] mice. The presence of
indicated population of cells among donors cells were evaluated in spleens 4 days after the
transfer. Mean ± SEM from 4 mice per group are shown. * - statistically significant (p<0.05)
differences from control. **H**. Differentiation of DCs in the presence of GM-CSF and IL-4 in

serum free medium for 6 days from 1.5×10^5 enriched BM HPCs from -cat KO or WT mice. Representative FACS profiles from four individual experiments and cumulative results of the total number of F4/80$^-$ CD11c$^+$CD11b$^+$ DCs are shown. **. p<0.01 between groups.
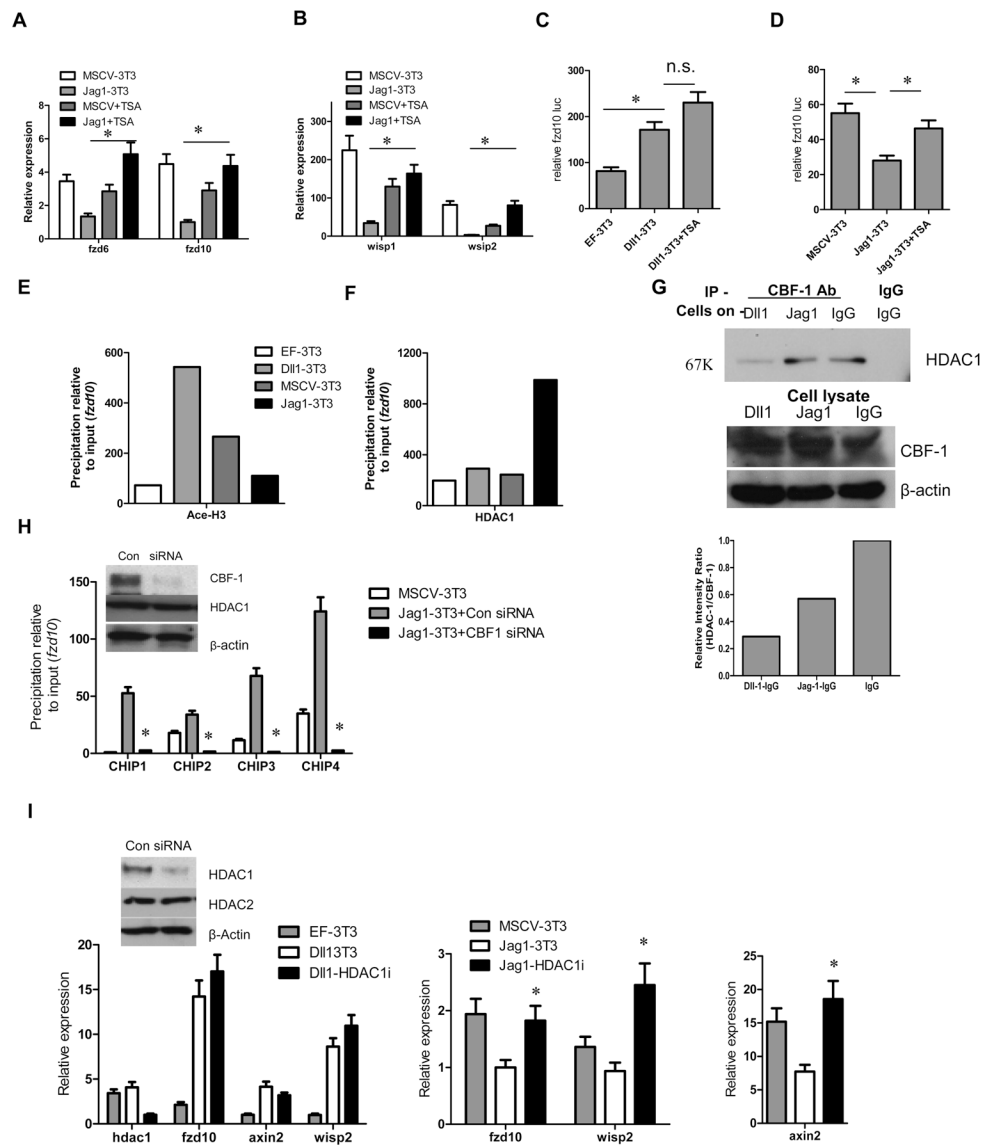
Figure 6. Jag1 regulates the expression of *fzd10* via HDAC

A,B. HPCs were incubated on monolayer of NIH3T3 fibroblasts expressing either Jag-1 or control vectors for 18 hr, followed by treatment with 10 nM TSA for 12 hr. Expression of *fzd* (**A**) or Wnt-target (**B**) genes was measured in HPC by qRT-PCR. Mean ± SEM of three experiments performed in triplicates are shown. *, p<0.05. **C, D.** 32D cells were transfected with *fzd10*-Luc reporter plasmid, followed by incubation with fibroblasts expressing Dll1, Jag1 or their control vectors for 18 h and then treated with 10 nM TSA for another 12 h. Renilla plasmid was used as internal control. Luciferase activity was measure using Dual-reporter assay system. Mean ± SEM of three experiments are shown. *, p<0.05; n.s., not significant. **E, F.** ChIP assay in 32D cells cultured on different fibroblasts with antibodies against acetylated H3 histone (**E**) or HDAC1 (**F**). Association of *Fzd10* promoter with acetylated histone 3 or HDAC1 was evaluated by qPCR and presented as the fold increase over input DNA. Two experiments in triplicates were performed. **G.** HPCs were cultured on plates with immobilized Dll1, Jag1, or control IgG for 4 h. Cell lysates were pulled down with CBF-1 antibody followed by immunoblotting with HDAC1 antibody. The level of CBF-1 and -actin was measured in cell lysates. Intensity of HDAC-1 band normalized to

CBF-1 is shown in the bottom panel. Three experiments with similar results were performed. **H.** Enriched HPCs were transfected with control or CBF-1 siRNA and then were placed on MSCV or Jag1 fibroblasts for 36 h. ChIP assay was performed with HDAC1 antibody. Association of *Fzd10* promoter with HDAC1 was evaluated by qPCR and presented as the fold increase over input DNA. Two experiments in triplicate with the same results were performed. Different primers sets were used. Their location in promoter region of *Fzd10* is shown in supplementary data. Inset - protein level by western blot demonstrating down-regulation of CBF-1 in HPCs. **I.** 32D cells were transfected with control siRNA or HDAC1 siRNA. 24 h post-transfection cells were incubated with different fibroblasts for another 18 h. Expression of *fzd* and Wnt-targeted genes was determined by qRT-PCR. Mean ± SEM of three experiments performed in triplicates are shown. Inset – protein level demonstrating specificity of down-regulation of HDAC1. *-statistically significant differences from control siRNA (p<0.05).