The NOTCH pathway contributes to cell fate decision in myelopoiesis

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ABSTRACT

Background

Controversy persists regarding the role of Notch signaling in myelopoiesis. We have used genetic approaches, employing two Notch zebrafish mutants *deadly seven* (DES) and *beamter* (BEA) with disrupted function of *notch1a* and *deltaC*, respectively, and Notch1a morphants to analyze the development of leukocyte populations in embryonic and mature fish.

Design and Methods

Myelomonocytes were quantified in early embryos by *in situ* hybridization using a myeloperoxidase (*mpx*) probe. Morpholinos were used to knock down expression of Notch1a or DeltaC. Wound healing assays and/or flow cytometry were used to quantify myelomonocytes in 5-day post-fertilization (dpf) Notch mutants (BEA and DES), morphants or *pu.1*:GFP, *mpx*:GFP and *fms*:RFP transgenic embryos. Flow cytometry was performed on 2-3 month old mutant fish.

Results

The number of mpx^+ cells in embryos was reduced at 48 hpf (but not at 26 hpf) in DES compared to WT. At 5 dpf this was reflected by a reduction in the number of myelomonocytic cells found at the wound site in mutants and in Notch1a morphants. This was due to a reduced number of myelomonocytes developing rather than a deficit in the migratory ability since transient inhibition of Notch signaling using DAPT had no effect. The early deficit in myelopoiesis was maintained into later life, 2-3 month old BEA and DES fish having a decreased proportion of myelomonocytes in both the hematopoietic organ (kidney marrow) and the periphery (coelomic cavity).

Conclusions

Our results indicate that defects in Notch signaling affect definitive hematopoiesis, altering myelopoiesis from the early stages of development into the adult.

Key words: NOTCH signaling, hematopoiesis, myelopoiesis, cell fate.

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Introduction

Discovered in *Drosophila*, Notch signaling is well known for its role in cell fate decisions and the development of multiple tissues. Its importance in hematopoiesis in vertebrates has become a recent focus of interest. Notch receptors and their ligands are expressed on hematopoietic cells and play a role in hematopoietic stem cell (HSC) emergence, self-renewal, differentiation and lineage choices.[1,2] The canonical Notch signaling pathway is activated by binding of Notch receptors to transmembrane ligands of the DSL (Delta, Serrate and Lag-2) family expressed on neighboring cells. Following ligand binding, two proteolytic cleavage events mediated by ADAM-family metalloproteases and gamma-secretase take place, releasing the Notch intracellular domain (NiC) into the cytoplasm. NiC translocates to the nucleus where it binds to the DNA-binding protein RBPjK and its co-activator Mastermind (MamL) turning a co-repressor complex into a transcriptional activating complex. A number of genes have been identified as targets of the canonical Notch pathway with the most well characterized being the Hes and Hes-related family of basic helix-loop-helix transcription factors.[3]

The role of Notch signaling in early T-cell development in the thymus is well established[1] and Notch signaling is widely considered to be a positive component of normal T-cell development while a mutated gain-of-function Notch allele is responsible for T-cell acute lymphoblastic leukemia (T-ALL).[4] The precise function of Notch genes in myeloid cell differentiation, however, remains controversial with a number of studies using different approaches arriving at contradictory conclusions. Constitutive expression of Notch1 through retroviral transfection of NiC in 32D myeloid progenitors has been reported to inhibit granulocytic differentiation.[5] However, Schroeder *et al.*[6] showed that transient activation of Notch1 signaling reduced self-renewal of multipotent progenitor cells and increased differentiation of granulocyte, macrophage and dendritic cell (DC) lineages through a direct increase in the expression of the PU.1 transcription factor. Retroviral transduction of NiC in recombination activating gene (RAG)$^{-/-}$ mouse derived bone marrow (BM) cells resulted in enhanced self-renewal of HSC and favored lymphoid over myeloid lineage differentiation.[7] In contrast, Burns *et al.*[8] reported that transient Notch activation in irradiated adult zebrafish accelerated repopulation of all lineages without any skewing to lymphoid or myeloid lineages. Finally, Notch deficiency in hematopoietic progenitor cells and embryonic stem cells was reported to result in impaired development of myeloid and lymphoid DCs in the mouse.[9]

Recent evidence derived from the analysis of human mammary epithelial cells has shown that the dichotomous functional activities displayed by Notch signaling is dose dependent[10] with high activation levels leading to downregulation of matrix-adhesion genes and inhibition of proliferation, whereas low activity induced sustained matrix adhesion and promotion of hyper-proliferation. This observation might provide some explanation for the contradictory reports in the literature using different models of over and underexpression of Notch pathway components.

In a different approach, conditional inactivation of Mindbomb, a ubiquitin ligase essential for signaling to Notch through its ligands, in the microenvironment in adult mice using a CRE-Lox system induced increased numbers of granulocyte progenitors leading to myeloproliferative disease.[11] In zebrafish, Mindbomb mutants analyzed at 28 h post fertilization (hpf) showed no differences in the number of cells expressing the myeloid markers *pu.1*, *mpx* and *l-plastin* as compared to wild-type fish[8] (WT).

Several zebrafish lines exist with mutations in genes encoding components of the Notch pathway. These mutants were identified from a large-scale ENU screen for zygotic mutations that affected the embryonic development in zebrafish.[12] Mutations in four Notch pathway related genes all resulted in abnormal somatogenesis.[13] Two such mutants are *beamter* (BEA) and *deadly seven* (DES). These carry mutations in *delta C* and *notch1a* genes, respectively.[13,14] These mutant zebrafish have developmental defects in somite patterning[13,14] but are nevertheless viable, probably due to a historical gene duplication of the zebrafish genome. Defects caused by mutation of the *notch1a* gene include perturbed motor axon outgrowth, neurogenesis, and somitogenesis.[15] Genetic manipulation of the Notch pathway in the mouse is hampered by the fact that, in most cases, inactivation of genes involved in this pathway causes embryo lethality restricting this approach to conditional or cell specific targeting of mutations. The availability of viable mutant zebrafish lines with defects in the Notch pathway provides a novel tool to investigate the function of this pathway in inflammatory responses and hematopoiesis. To investigate the role of Notch in myelopoiesis in a whole organism model, we made use of the BEA and DES mutant zebrafish. *bea*^{tw212b} mutant carries a mutation in the 7th EGF repeat of DeltaC while *des*^{tp37} carries a mutation within the hydrophobic domain of the signal peptide of Notch1a.

Like other vertebrates, zebrafish have a primitive and definitive wave of hematopoiesis, self renewal of HSCs taking place only during the definitive wave which occurs after the first 24 hpf.[8] Signaling pathways and transcription factors regulating HSC formation and differentiation are conserved between zebrafish and mammals. Zebrafish embryos are optically transparent allowing direct visualization of all hematopoietic cells at different stages of early development. These features, together with the availability of Notch mutants, make zebrafish an attractive model to study the role of Notch in hematopoiesis.

In this report, we studied immune cell populations in Notch mutant zebrafish embryos and found decreased numbers in the myeloid compartment at 48 hpf. By using Notch1a knockdown via morpholinos in *pu.1:GFP*[16], *mpx:GFP*[17] and *fms:*RFP[18] transgenic fish we found a reduced proportion of myelomonocytes. In embryonic zebrafish this was reflected functionally by less cells being recruited and/or retained by a wound injury in morphants and Notch mutants. Finally, analysis in older fish revealed that the defect in the myeloid compartment was maintained in Notch1a mutants and in Notch ligand DeltaC mutants. Altogether, our results indicate that Notch signaling affects cell fate decision in myelopoiesis at the definitive but not primitive stage of hematopoiesis.

Design and Methods

Zebrafish care and breeding

Fish were maintained according to standard practices and all

procedures conformed to UK Home Office requirements (ASPA 1986). WT, *bea*^{tw212b} and *des*^{tp37} were obtained from Tubingen. *zpu.1:eGFP* zebrafish were from Thomas Look.[16] Tg(*mpx:GFP*)*i114*[17] (called mpx:GFP in this paper) zebrafish were produced and provided by Stephen A. Renshaw (University of Sheffield, UK). Tg(fms:Gal4.VP16)i186;Tg(UAs:nfsB.mCherry) i149[18] (called *fms:*RFP in this paper for clarity) was produced and provided by Timothy Chico (University of Sheffield, UK). The genotype of Notch mutants was confirmed by PCR and SNP custom assays using real time PCR (Applied Biosystems) for the mutation in *bea*^{tw212b} and *des*^{tp37}, respectively.

In situ *hybridization*

Whole-mount *in situ* hybridization (ISH) was performed as previously described.[19] Briefly, paraformaldehyde (4%) fixed embryos were treated with proteinase K prior to incubation with digoxigenin-labeled antisense RNA probes for *mpx* at 70°C overnight. After 2 x SSC and 1 x PBS/ 0.1% Tween20 washes, embryos were incubated with anti-digoxigenin antibody followed by Nitro blue tetrazolium/5-Bromo 4-chloro 3-indolyl phosphate (BCIP; Sigma) color development.

Morpholino injection

The following morpholino oligonucleotides (MO) were purchased from GeneTools, LLC (Philomath, OR, USA): 5' TTCACCAAGAAACGGTTCATAACTC 3' (zebrafish Notch1a translational blocking morpholino),[14] 5' AGCACGTTAATAAAACACGAGCCAT 3' (zebrafish DeltaC translational blocking morpholino), 5' GCCTCGGCGTTACAACTTCTTTAAA 3' (zebrafish Notch1a second non-overlapping translational blocking morpholino) and 5' CCTCTTACCTCAGTTACAATTTATA 3' (standard control morpholino). Between 4-10 ng of MOs were microinjected into the yolk of 1-4 cell stage embryos. Embryos injected with MOs against Notch ligand or receptor genes were screened at 48 hpf by selecting those exhibiting somite disorganization.

Tail transection and MPO staining of embryos

5 dpf WT or transgenic embryos were anesthetized by immersion in 0.6 mM MS-222 (Sigma) in system water and transection of the tail performed with a sterile scalpel. After 4 h embryos were fixed in 4% paraformaldehyde overnight at 4°C, washed in 0.1% Tween 20 in PBS and stained for MPO with 0.075 mg/ml diaminobenzidine (Sigma), 0.03% H_2O_2 in PBS. Embryos were then imaged for MPO positive cells using a Leica DMIL inverted microscope.

In some experiments embryos were exposed to the inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine-t-butyl-ester, Calbiochem) resuspended at 50 μM in DMSO. Fish were treated 30 min prior to the tail transection and during the 4 h after the injury.

Whole embryo, whole kidney marrow (WKM) and coelomic cavity cell analysis

Pu.1:GFP, *mpx*:GFP and *fms*:RFP (5 dpf) whole embryo morphants were dissociated using PBS containing 0.25% trypsin and 1 mM EDTA and incubated for 60 min at 37°C with mechanical disruption by pipetting every 15 min. The dissociated cell suspension was strained using a 70 μm strainer (BD Falcon) into PBS supplemented with 5% fetal bovine serum (FBS), washed once and resuspended in PBS. Hematopoietic cells from adult zebrafish WKM and coelomic cavity were isolated as described.[20,21] Cells were washed, and resuspended in ice-cold Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, passed through a 70 μM strainer. All cells were then analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences). The WKM and coelomic cavity cell populations were identified as previously described.[20,21]

RNA, cDNA and quantitative real-time PCR (qRT-PCR)

Embryos were processed following a lethal overdose of MS-222, homogenized using a pestle in lysis buffer and RNA extracted using MagMAX™-96 Total RNA Isolation Kit (Applied Biosystems) according to the manufacturer's instructions. The quantity and quality of RNA was assessed using a Thermo Scientific NanoDrop™ 1000 spectrophotometer and 125 ng of total RNA was used for reverse transcription using High-Capacity cDNA Archive Kit (Applied Biosystems). qRT-PCR was performed with Taqman assays (Applied Biosystems) using a 7500 Fast instrument (Applied Biosystems). Results were normalized to 18S and calibrated to untreated samples for relative quantitation.

Statistical analysis of FACS and leukocyte recruitment data

In datasets statistically analyzed in which the data in both groups passed the Kolmogorov-Smirnov test for normal distributions, an unpaired two-tailed t-test was performed. In datasets statistically analyzed in which the data failed the Kolmogorov-Smirnov test for normal distributions, a Mann Whitney test was performed.

Results

DES mutant embryos have reduced numbers of myeloid cells from 48 hpf

While the adaptive immune system of the zebrafish is only functional after 4-6 weeks post fertilization, myelomonocytic cells of the innate immune system develop rapidly in the embryo.[22] This offers the opportunity to study the consequences of a Notch defect on myelopoiesis and the function of myelomonocytic cells in the absence of adaptive immunity in the early embryo.

DES mutants were analyzed by whole mount ISH for *mpx* in 26 hpf and 48 hpf embryos. The primitive wave of hematopoiesis occurs during the first day post fertilization while the definitive waves follow after that. Total numbers of *mpx*$^{+}$ cells were assessed following whole mount ISH with an *mpx* probe on siblings of heterozygous DES matings. While the cell counts were comparable between WT and DES siblings at 26 hpf (Figure 1A), we found a significant reduction in *mpx* cell number at 48 hpf in DES mutants (Figure 1B). This result indicates that Notch1a is not required for primitive hematopoiesis but that a Notch1a defect could affect myelopoiesis during definitive hematopoiesis.

Reduced number of myeloid cells in embryos at 5 dpf

To assess the effect of Notch on myelomonocytes later on in development, embryos at 5 dpf were analyzed. We made use of three transgenic zebrafish lines in which myeloid cells are fluorescently labeled, *pu.1*:GFP in which GFP is expressed in all myeloid cells, *mpx:*GFP which marks neutrophils and *fms:*RFP (otherwise called CSF1R) in which RFP is expressed in macrophages but not neutrophils.[14] We used morpholino injection to knock down Notch1a. Zebrafish embryos were injected just after fertilization with a translation blocking morpholino.[14] Somite disorganization was observed at day 2 in the Notch1a morphants (Figure 2A) confirming the efficiency of the interruption of Notch1a translation mediated by the morpholino and as previously reported.[14] Transgenic GFP/RFP

morphant embryos were analyzed by flow cytometry of trypsin treated and dispersed whole embryos. Results show that the percentage of GFP/RFP positive cells was significantly lower in *pu.1*:GFP, *mpx*:GFP and *fms*:RFP morphant fish when compared to controls (Figure 2B-D). A second non-overlapping translation blocking Notch1a morpholino was also used as control and showed similar results (Figure 3). These results indicate that the myeloid compartment (neutrophils and macrophages) is affected during the definitive wave of hematopoiesis by a Notch1a defect.

DES mutant embryos exhibit a reduced number of myeloid cells recruited to a wound

Several studies have demonstrated that tail fin wounding in zebrafish embryos, including tail transections, medial fin incisions and laser-inflicted wounds induce the migration of leukocytes to the site of tissue damage.[23-25] In particular, neutrophil mediated responses to acute wounding have been demonstrated using a combination of histochemical staining of fixed embryo tissue for the neutrophil specific marker MPO and transgenic zebrafish line *mpx*:GFP. To compare the number of functionally intact myelomonocytic cells in Notch mutants and WT fish we performed a tail transection wound assay on embryos at 5 dpf. Four hours post wounding (hpw), the number of MPO positive cells present at the site of injury (Figure 4A) was compared in WT and Notch1a mutant fish (Figure 4B). The result showed that a significantly lower number of MPO positive cells were present at the wound site in DES mutants than in WT fish ($P<0.001$).

Similar experiments were performed in WT zebrafish embryos injected with the Notch1a morpholino. Figure 4C shows that fewer MPO positive cells accumulated at the wound of zebrafish embryos injected with Notch1a morpholinos compared to injected controls ($P<0.001$) confirming the results obtained in the DES mutants. Collectively, our results with mutant and Notch1a morphant embryos suggest that zebrafish with defects in Notch signaling have reduced numbers of myeloid cells recruited to and retained at the site of injury.

In order to assess whether the lower myeloid cell number present at the wound in embryos with Notch defects could also be due to an impaired migratory function of these cells rather than a deficit in cell numbers, embryos were treated temporally with DAPT, a γ-secretase inhibitor. DAPT inhibits Notch signaling by blocking the cleavage of NiC necessary for activation of transcription of downstream target genes. DAPT has previously been used efficiently in zebrafish embryos to inhibit the Notch pathway[26] and has also been shown to inhibit Notch signaling in myeloid cells.[27] Figure 4D shows that DAPT did not affect the number of cells recorded at the wound site in WT fish when added 30 min prior to the time of the tail transection and for 4 h after injury and before the cells at the wound site were counted. Confirmation that DAPT treatment had transiently inhibited Notch signaling was obtained by demonstrating that Notch target hes1 mRNA was significantly reduced in DAPT treated embryos when compared to controls (Figure 4E). Therefore, the reduced number of cells observed is likely to be due to a defect in total number of myelomonocytes generated in Notch mutants rather than a defect in their ability to migrate.

Collectively our results show that the myeloid compartment is affected in embryos from 48 hpf with a reduced number of myelomonocytes developing in fish with a defective Notch pathway.

Notch1a mutant zebrafish have altered myelomonocyte:lymphocyte ratios in both the primary hematopoietic organ and the periphery

Next we analyzed whether the defect observed at early stages of development was retained in more mature fish.

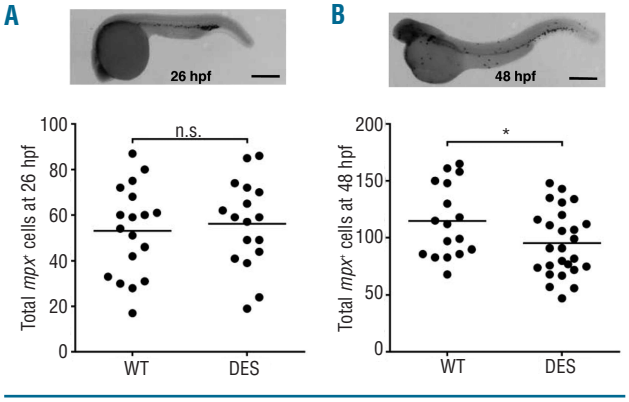

Figure 1. Reduced number of neutrophils in DES mutant embryos at 48 hpf. Heterozygote DES adult fish were crossed and embryos were harvested and fixed at (A) 26 hpf or (B) 48 hpf. Following whole mount ISH with an *mpx* probe, the total number of stained cells was counted, genotyping by SNP analysis and real time PCR was performed on individual embryos and each dot shows the total number of *mpx* cells in each embryo according to their genotype for WT and DES mutants. A representative image of an *mpx* probed embryo is shown for each stage of development above the corresponding graph. Scale bars: (A) 250 μm, (B) 400 μm. (*$P<0.05$).

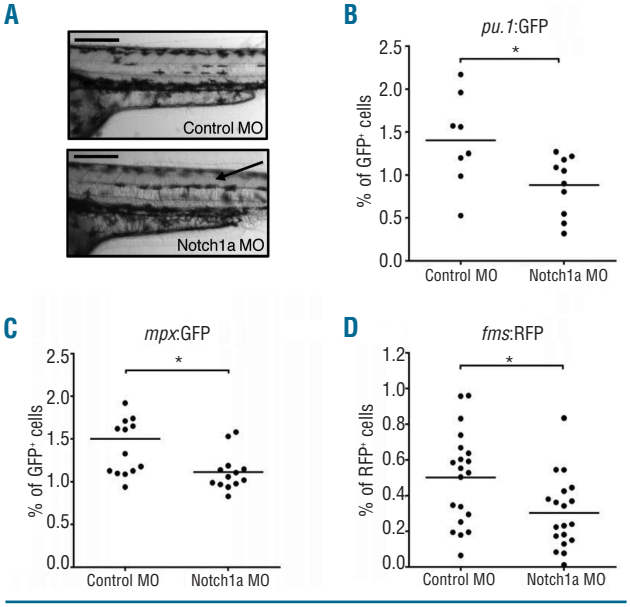

Figure 2. Total number of myelomonocytes is reduced in 5 dpf Notch1a morphants. WT (A), *pu.1*:GFP (B), *mpx*:GFP (C) and *fms*:RFP (D) fertilized embryos were injected with Notch1a or control MO. (A) Representative images of somite organization in control and Notch1a morphants at 2 dpf (arrow shows somite disorganization), scale bars: 100 μm. (B-D) Percentage of GFP+ or RFP+ cells obtained from flow cytometry analysis of single cell suspensions prepared from individual 5 dpf whole morphant embryos (each dot represents one embryo) (*$P<0.05$).

In zebrafish, different leukocyte populations can be distinguished by flow cytometry based on their forward and side scatter profile.[20] Single cell suspensions prepared from WKM and cells gently flushed from the coelomic cavity were obtained from 2-3 month old Notch1a mutants and WT fish. Cell suspensions were then analyzed by flow cytometry. Figure 5A and B show representative scatter plots of WKM and coelomic cavity derived cells, respectively. The gates demonstrate the various cell populations that were analyzed and are as follows: R2 for myelomonocytes, R3 for precursors and R4 for lymphocytes from WKM, R5 for myelomonocytes, R6 for lymphocytes and, finally, R7 for non-specific cytotoxic cells (NCC)[21] from the coelomic cavity. Analysis showed that in WKM the percentage of myelomonocytes was significantly lower in DES mutants (P<0.01) when compared to WT fish while percentages of lymphocytic cells were higher in DES mutants *versus* WT (P<0.01) (Figure 5C and D). The percentage of precursor cells was similar in both groups (Figure 5E). The total numbers of cells collected from WKM were similar in all fish indicating that overall, DES mutants have lower numbers of myelomonocytes than WT fish. Notch receptors and Notch ligands are expressed on leukocytes[1,2] and we found that Notch1a transcripts were indeed readily detectable in all sorted WT cell populations (R2, R3 and R4, *data not shown*). The trend observed in WKM derived cells was reflected in the periphery with a significantly lower percentage of myelomonocytic cells in the coelomic cavity of DES mutants when compared to WT (P<0.01) (Figure 5F). Again the lymphocyte containing population was increased in DES compared to WT (P<0.05) (Figure 5G). The NCC population was also increased in DES compared to WT (P<0.05) (Figure 5H).

BEA mutants showed altered numbers of myelomonocytes in embryos and 2-3 month old zebrafish

To confirm the role of the Notch pathway in leukocyte differentiation we analyzed the effect of a defect in one of the Notch ligands, DeltaC, on myeloid cell numbers and function. *mpx:*GFP transgenic fish were injected with a DeltaC morpholino (Figure 6A, P<0.05) and the proportion of GFP positive cells analyzed at 5 dpf. A lower number of myelomonocytes was observed in DeltaC morphants compared with injected controls. We also performed tail transections in 5 dpf BEA mutants which carry a defect in *deltaC* gene. Again a decreased number of MPO$^+$ cells were found at the wound site compared to WT fish (Figure 6B, P<0.01).

Analysis of 2-3 month old fish showed that, as in the DES mutants, the percentage of myelomonocytes in the WKM was significantly lower in BEA (P<0.001) when compared to WT fish, while percentages of lymphocytic cells were higher in the mutants (BEA (P<0.01) *vs*. WT) (Figure 6C and D). The percentage of precursors was not affected by the mutation (Figure 6E). In the coelomic cavity of BEA mutants there was a significantly lower percentage of myelomonocytic cells when compared to WT (BEA P<0.05; Figure 6F). The lymphocyte containing pop-

Figure 3. Decreased myelomonocyte numbers in Notch1a morphants. *mpx:*GFP fertilized embryos were injected with a second non-overlapping translation blocking Notch1a morpholino or control MO. Percentage of GFP$^+$ cells obtained from flow cytometry analysis of single cell suspension prepared from individual 5 dpf whole morphant embryo were plotted (each dot represents one embryo) (*** P<0.001).

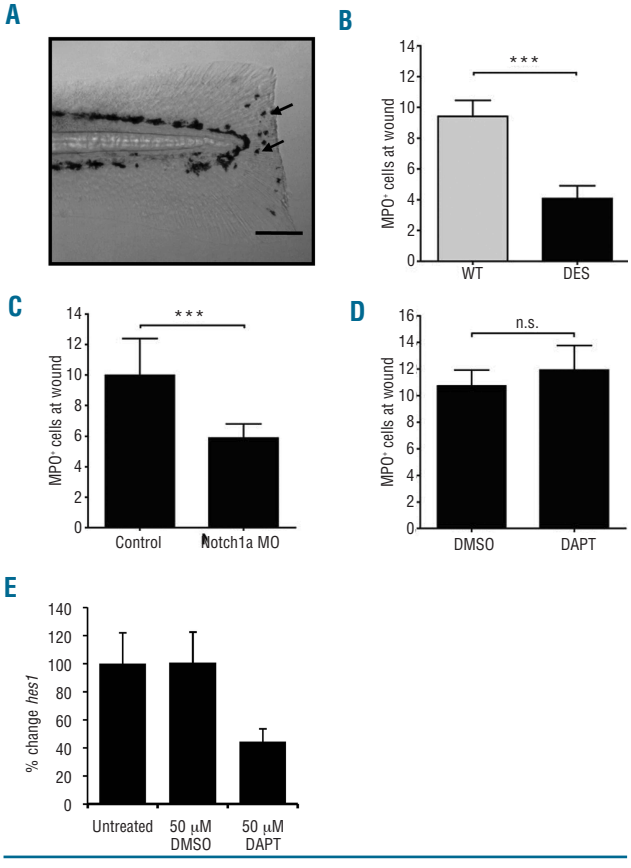

Figure 4. Reduced numbers of neutrophils at a tail wound in 5 dpf embryos with Notch1a defect. (A) Representative image of MPO cells recruited to tail injury. Embryos at 5 dpf were submitted to tail transection and were fixed and stained for MPO 4 h post injury. Scale bar: 50 μm, arrows indicate examples of MPO positive cells. (B) MPO positive cells present at the wound site were counted and groups compared as follows: WT (light gray bar) and DES (black bar). (C) WT embryos were injected with Notch1a or control MO, at 5 dpf tail transection was performed on morphants and stained for MPO after 4 h. Graph shows average cell counts obtained. (D) 5 dpf WT embryos were treated with DAPT (50 μM) or with equivalent levels of DMSO control 30 min prior to tail transection and for 4 h post injury. Fish were then stained for MPO and MPO positive cells present at the wound site were counted. (E) Embryos were collected following DAPT and DMSO treatment and RNA extracted for analysis of *hes1* mRNA levels by quantitative (TaqMan) RT-PCR. Results were normalized to 18S and relative expression compared to untreated controls. Graphs display mean values and error bars represent the 95% confidence intervals of the data sets. Graphs B (n>150 in each group), C (n>60 in each group) and D (n>125 in each group). (*** P<0.001).

ulation was increased in mutant fish compared to WT (BEA $P<0.05$; Figure 6G) while no significant differences were observed in the NCC population (Figure 6H). Altogether the results observed using BEA mutants were consistent with those obtained in DES mutants and Notch1a morphants.

Discussion

Numerous approaches have been used to study the role of Notch in hematopoiesis, but controversy remains regarding its contribution to a number of key processes including myelopoiesis.[1] In the whole organism, gain of function via retroviral or transgenic expression, although valuable, tends to provide an excessive level of Notch signaling. This may lead to misleading conclusions since it has been shown that the effects of Notch activation can depend on the level of signaling.[10] Loss of function studies using knockout mice have been limited since embryonic lethality results from silencing most rate limiting genes of this pathway. Mice heterozygous for null mutations have nevertheless provided useful models as they may show functional haploinsufficiency. Studies in heterozygous mice have focused on thymus development and to our knowledge there is no description of the development of the myeloid compartment in these mice.[28] However, Notch1$^{-/+}$ mice exhibit a decreased number of macrophages at wound sites, a result that is consistent with the present data. A similar observation was made when the authors used mice with conditional deletion of Notch1 in macrophages (LysMCre;Notch1flox/flox), although Outtz *et al.*[29] have suggested that this may be due to a functional defect in macrophages rather than a deficit in cell numbers.

Zebrafish have experienced a genome wide duplication event and as a consequence often have two co-orthologs in contrast to the single copy gene in humans and other mam-

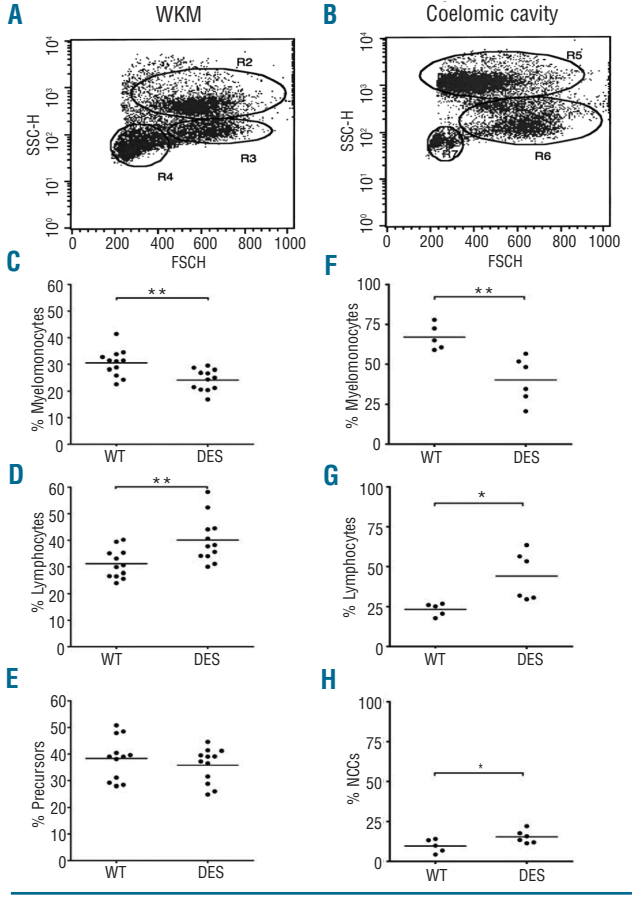

Figure 5. Leukocyte proportions in whole kidney marrow and coelomic cavity are affected in Notch1a mutants. Cells isolated from WKM and coelomic cavity of WT and DES mutants were obtained from 2-3 month old zebrafish and analyzed using flow cytometry based on their forward and side scatter characteristics. (A-B) Typical profiles of live cells obtained for WKM and coelomic cavity of WT fish. The gates used for the analysis of various cell populations were as follows: R2=myelomonocytes, R3=precursors and R4=lymphocytes for WKM, R5=myelomonocytes, R6=lymphocytes, R7=NCC for coelomic cavity. Percentage of gated cells were plotted for individual fish (C-E) in WKM and (F-H) in coelomic cavity. (*$P<0.05$, **$P<0.01$).

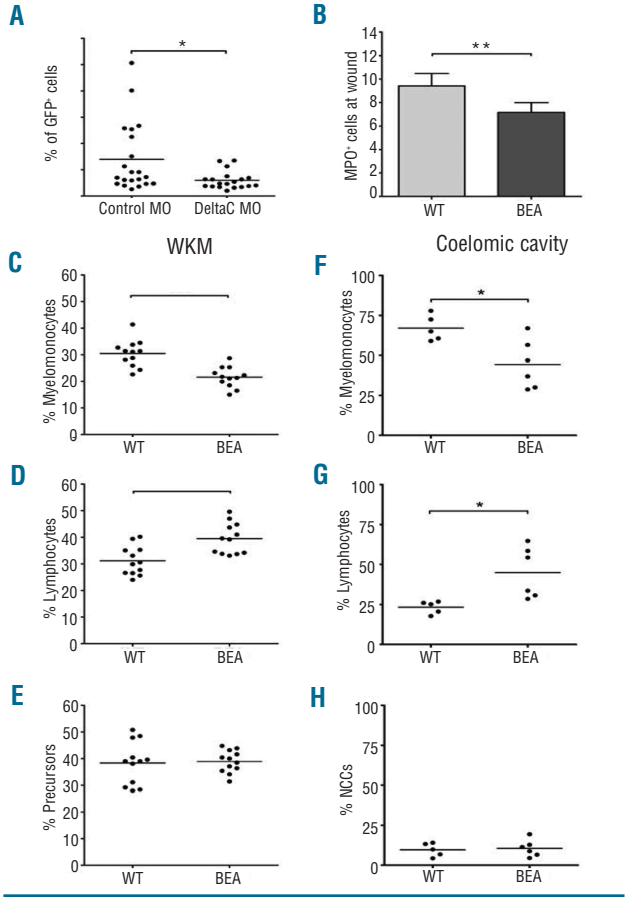

Figure 6. Decreased myelomonocyte numbers in DeltaC morphants and BEA mutants. (A) *mpx*:GFP fertilized embryos were injected with DeltaC or control MO. The percentage of GFP+ cells obtained from flow cytometry analysis of single cell suspensions prepared from individual 5 dpf whole morphant embryos (each dot represents one embryo). (B) 5 dpf BEA zebrafish embryos were subjected to a tail transection and stained for MPO after 4 h. MPO positive cells present at the wound site were counted and groups compared as follows: WT (light gray bar, n>150), BEA (dark gray bar, n>150). (C-H) Cells isolated from WKM and coelomic cavity of WT and BEA mutants were obtained from 2-3 month old zebrafish and analyzed using flow cytometry based on their forward and side scatter characteristics. Percentage of gated cells were plotted for individual fish (C-E) in WKM and (F-H) in coelomic cavity. Data were produced in the same experiment as shown in Figures 4 for (A) and 5 for (C-H). (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

mals.[30] This is the case for some of the Notch receptors and ligands, and, therefore, a null mutation of single genes in this pathway, otherwise embryo lethal in mammals, offers the potential for novel analyses. DES and BEA carry null mutations in *notch1a* and *deltaC*, respectively, but are able to develop to adulthood. Although duplicated, the copies are not fully redundant since a phenotype can be observed, particularly in DES mutants which show markedly disrupted somites from the early stages of development. Use of these mutants have been informative in the context of hematopoiesis since we were able to detect significant differences between mutants and WT in myeloid lineages. A quantitative analysis in BEA and DES mutants showed that defects in the Notch pathway affect myelopoiesis from an early stage of development. We further confirmed our findings by morpholino-mediated Notch1a knockdown in the embryo but since Notch signaling is dose dependent and involved in many aspects of the organism development we did not try to rescue the phenotype observed in the mutant by injection of WT transcript of the gene. The results are consistent with the results of Cheng *et al.*[9] who showed that Notch1 deficient ES cells or HPCs had a reduced ability to differentiate into myeloid cells, as well as with those of Schroeder *et al.*[6] who found an increased and accelerated differentiation along the myeloid lineage following co-culture of hematopoietic progenitors with Notch ligand expressing cells through direct upregulation of PU.1. Moreover in a microarray analysis performed on murine hematopoietic progenitor cell lines with conditional activation of Notch signaling, Schwanbeck *et al.*[31] found that a number of genes related to myeloid differentiation were regulated. For example, the Irf1 transcription factor shown to be involved in granulocyte[32] and macrophage[33] differentiation was directly up-regulated by Notch signaling. In the same study, HMGA1 which interacts with PU.1 complex, was also regulated by Notch signaling. Finally, they found that c-Myb was down-regulated. c-Myb downregulation has been shown to be necessary for myeloid differentiation to progress.[34] A defect in Notch signaling in our model might affect directly the expression of these genes and as a consequence alter the development of a normal myeloid compartment. Notch signaling can take place in hematopoietic cells as well as in the microenvironment as both express Notch and Notch ligands. In conditional Mindbomb mutants,[11] myeloproliferative disease developed when defective Notch ligand regulation occured in the microenvironment itself. In our mutants, a single Notch receptor in DES or Notch ligand in BEA is defective in the whole organism and the phenotype observed could be due to a defect in Notch signaling in either or both the microenvironment and the HSC.

The first myeloid cells emerge from the primitive wave of hematopoiesis that occurs during the first day post fertilization in zebrafish. In Mindbomb zebrafish mutants, which lack functional Notch ligands, primitive hematopoiesis occurs normally and *mpx* cells are detected at levels similar to those of WT at 28 hpf[8] which is consistent with our findings using Notch1a (DES) mutants. The definitive wave takes place immediately after and gives rise to erythromyeloid progenitor (EMP) cells and HSCs. EMPs are non-self renewing progenitors and have been shown to be specified normally in the absence of Notch signaling while HSCs are able to self-renew and are Notch dependent.[35] We found that at 48 hpf a significant decrease in *mpx* cells was observed in DES compared to WT siblings. Bertrand *et al.*[35] found that *mpx* expressing cells were present at similar levels at 36 and 48 hpf in Mindbomb zebrafish mutants to that in WT. However, in the latter studies a qualitative only assessment was performed and our results indicate that at this stage a detailed, quantitative analysis is necessary to reveal the effects observed. By day 5 we found that *pu.1*:GFP, *mpx:*GFP and *fms:*RFP marked cells showed reduced proportions in Notch1a morphants indicating that all compartments of myelomonocytes were affected.

The role of Notch signaling in self-renewal of HSCs is still controversial. Whilst transient overexpression of NiC in *hsp70:gal4;uas:NICD* adult fish resulted in expansion of multi-lineage precursor cells in BM,[8] Maillard *et al.*[36] found that Notch signaling was dispensable in maintaining adult HSCs in a transgenic mouse expressing a dominant negative form of MAML (which inhibits canonical Notch signaling). We found that *notch1a* or *deltaC* null mutants had a normal proportion and numbers of precursors but an altered ratio of the myeloid and lymphoid cell compartments. This is consistent with the possibility that in zebrafish, a single Notch family member gene defect does not significantly affect HSC specification and HSC self-renewal as suggested by Maillard *et al.*,[36] although in our system we cannot rule out the possibility that gene redundancy results in this observation.

Notch plays an important role in the differentiation and maturation of T cells.[37] In fact, Notch signaling must be tightly regulated and suppressed within the BM in order to prevent ectopic differentiation of T cells therein. Several groups have found that interrupted canonical Notch1 signaling results in inhibition of many cell fates including B-cell and myeloid cell types.[38] Here we report that mutations in Notch-related genes result in higher proportions of lymphocytes in the WKM. Since the lymphocyte population comprises T cells and B cells, it is possible that the impaired signaling in the Notch pathway results in inhibited T-cell differentiation accompanied by enhanced B-cell differentiation, which overall results in greater numbers of lymphocytes.

The role played by Notch signaling in cell fate commitment in hematopoiesis is complex and remains controversial. A recent report by Poirault-Chassac *et al.*[39] demonstrated conflicting data in human and mouse megakaryopoiesis of Notch signaling mediated by DeltaL ligands. This finding raises the possibility that some of the controversy observed regarding the role of Notch signaling in myelopoiesis could be explained by species-specific phenomena. However, it could also be argued that differences between the types of stem cell used for studies in mouse and human may account for the difference observed.

The data presented here contribute to the debate regarding the role of Notch signaling in hematopoeisis, supporting its role in promoting myeloid cell differentiation at the definitive, but not primitive, stage of hematopoiesis.

Authorship and Disclosures

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