

## Fucose-Dependent Differentiation and Gene Expression of Common Myeloid Progenitor Cells through Notch Signaling Pathways

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### Abstract

The Notch pathway is an extensively utilized, evolutionarily maintained regulatory system which mediates a wide range of fate decisions among multipotent precursor cells by inhibiting differentiation along one pathway while promoting self-renewal or differentiation along an alternative pathway. Notch signaling has been shown to affect haematopoietic stem cell (HSC) self-renewal and differentiation, T cell versus B cell fate specification, and myeloid cell differentiation. The diverse functions of Notch in vertebrates are facilitated by complex interactions between four Notch receptors and five Notch ligands, all of which are expressed by hematopoietic cells and stromal cells. Moreover, Notch signaling is modulated by genes such as *fringe* as well as two unusual types of *O*-linked glycosylation; the addition of *O*-linked glucose (*O*-glucose) and *O*-linked fucose (*O*-fucose). Our goal is to determine whether *in vitro* myeloid differentiation is regulated by Notch activation, and whether this is a fucose-dependent process. Specifically, we focused our research on common myeloid progenitor (CMP) cell differentiation and the dynamic change of Notch-targeted genes during Notch regulated myeloid differentiation that is modified by fucosylation.

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### Introduction

The Notch signaling pathway is a broad regulatory system initially described in one strain of *Drosophila melanogaster* with notches at the wing margins due to the partial loss of the function of the gene (Kidd *et al.*, 1986). Mutations in some of the Notch components result in a number of human genetic disorders including T-cell leukaemia, congenital diseases associated with bone, vasculature, and internal organ defects, and the pathogenesis of multiple sclerosis (Haines and Irvine, 2003). Previous studies of Notch function have shown that Notch signaling affects haematopoietic stem cell (HSC) self-renewal and differentiation, T cell versus B cell fate specification, and myeloid cell differentiation (Ohishi *et al.*, 2003). In addition, analyses of Notch signaling in both invertebrates and vertebrates have shown that Notch plays an important role in neurogenesis, myogenesis, wing formation, eye development, and oogenesis. In general, Notch is able to direct the fate choices of precursor cells by inhibiting differentiation along one pathway while promoting self-renewal or differentiation along an alternative pathway.

Notch is comprised of a set of central constituents which allow for its diverse capabilities and functions. Notch receptors are transmembrane proteins whose extracellular domain consists of 29 to 36 tandem epidermal growth factor (EGF-like) repeats, which bind DSL ligands (Delta-Serrate-Lag2) (Maillard *et al.*, 2005). In vertebrates, there are

four Notch receptors (Notch 1-Notch 4) and five Notch ligands: Jagged1 and 2 which are Serrate homologs, and Delta-like-1 (DLL-1), Delta-like-3 (DLL-3), and Delta-like-4 (DLL-4) which are homologs of Delta. All Notch receptors and ligands are expressed by hematopoietic cells and stromal cells (Radtke *et al.*, 2004). Upon ligand interaction, the intracellular domain of Notch is released through two proteolytic cleavage events and translocated into the nucleus. The intracellular domain of Notch (NotchIC) includes the RAM domain and ankyrin repeats that interact with transcription factor CBF1/RBPJ $\kappa$  and regulate gene expression. NotchIC also binds to several proteins such as the cytoplasmic zinc finger-domain protein, Deltex, through CBF1-independent pathway (Weinmaster *et al.*, 1997).

However, recent studies have shown that activation of Notch by its ligands is much more complex. For example, Notch signaling has been found to be positively or negatively modulated by genes such as *fringe* as well as two unusual types of *O*-linked glycosylation; the addition of *O*-linked glucose (*O*-glucose) and *O*-linked fucose (*O*-fucose) (Haines and Irvine, 2003). Fucose is present on some of the EGF domains of the mammalian Notch receptors and Notch ligands, where it is in direct linkage to hydroxyl groups of serine and threonine residues, known as *O*-fucosylation (Moloney *et al.*, 2000). *O*-fucose, attached to EGF domains by *O*-fucosyltransferase (OFUT1), is a rare form of

glycosylation that has been identified on several proteins in addition to Notch and Notch ligands (Haltiwanger *et al*, 2002). Fucosylation is important for ligand-induced Notch signaling in cell lines and *Drosophila* (Chen *et al*, 2001; Moloney *et al*, 2000), but its role in controlling Notch-dependent signaling in post-developmental events, including in the immune and hematopoietic systems, is not yet understood.

*Fringe* encoding a fucose specific  $\beta$ 1,3 N-acetylglucosaminyltransferase can further modulate Notch by elongation of O-fucose present on its extracellular EGF repeats (Moloney *et al*, 2000b; Bruckner *et al*, 2000). Fringe-mediated extension of O-fucose differentially influences Notch ligand binding and the ligand specificity of Notch pathway (Shao *et al*, 2002; Shi *et al*, 2003). Research done in Dr. Lan Zhou and Dr. John Lowe's laboratories furthered the understanding of the Notch signaling pathway in myelopoiesis which also appears to be fucose dependent. Briefly, severe peripheral leukocytosis was observed in  $FX^{-/-}$  mice that are genetically deficient in FX protein (GDP-4-keto-6-D-deoxymannose epimerase/NADPH-dependent reductase), a key enzyme complex directing *de novo* synthesis of GDP-fucose from GDP-mannose (Collins *et al*, 1987). Leukocytosis in  $FX^{-/-}$  mice is also associated with an enhanced granulocytopenia in the marrow as a result of an increased number of CFU-GM (Smith *et al*, 2002). When fucosylation is restored through the salvage pathway in  $FX^{-/-}$  mice by feeding exogenous fucose, the elevated number of CFU-GM is quickly normalized. Fucose depletion had an opposite effect. Dr. Zhou has also observed that LSK cells ( $lin^{-}Sca-1^{+}c-kit^{+}$  cells; encompassing HSCs with myeloid differentiation potential) from  $FX^{-/-}$  mice have aberrant granulocytic differentiation when co-cultured with S17 stromal cells transduced to express Notch ligand Delta-like-1 (Dll1) (unpublished data). Such aberrant granulocytic differentiation from  $FX^{-/-}$  LSK cells were not seen when fucose is supplemented in the culture medium.

Although it is well established that Notch1 is essential for T cell fate specification and development, the exact role of Notch in myelopoiesis remains controversial. In addition, many studies focused on the effect of Notch on myelopoiesis by using cell lines rather than bone marrow HSC cells or myeloid progenitor cells. In this paper, our goal is to determine whether *in vitro* myeloid differentiation is regulated by Notch activation, and whether this is a fucose-dependent process. Specifically, we focused our research on common myeloid progenitor (CMP) cell differentiation. CMPs are myeloid-committed progenitors ( $Lin^{-}c-kit^{+}Sca1^{-}IL7R^{+}CD34^{+}Fc\gamma RII^{low}$ ) that are capable of differentiating into either

megakaryocyte/erythrocyte progenitors (MEPs) or into granulocyte/macrophage progenitors (GMPs). In addition, we examined the dynamic change of Notch-targeted genes, *Hes1* and *Deltex*, and genes implicated in myeloid differentiation, PU.1 and C/EBP $\alpha$ , during Notch regulated myeloid differentiation that is modified by fucosylation.

## Materials and Methods

### Mice

For our experiment, three different mouse strains all at 12 weeks of age were used: wild type mice C57Bl/6J (CD45.2) were purchased from Jackson Laboratories;  $FX^{-/-}$  (CD45.2) mice (Smith *et al*, 2002) were reared with fucose-supplemented chow until 8 weeks old and then kept on regular chow;  $FX^{-/-}$  (CD45.1) mice were fed on a fucose-supplemented diet after weaning ~ 4 weeks old and kept on fucose-supplemented chow until use. All mice are reared in the Animal Facility at Wolstein Research Building.

### Lineage Depletion of Bone Marrow Cells

Mice were euthanized by cervical dislocation and femurs and tibias were removed into Hank's buffered saline solution (HBSS) (without calcium and phenol blue). Bone marrow was then flushed into 50 mL centrifuge tubes containing 45 mL working media, HBSS with 5% bovine serum albumin (BSA), at room temperature. After spinning down in a centrifuge, the solutions were aspirated with a fine needle and taken into 500  $\mu$ L working media at room temperature. Fifteen mL of red blood cell lysis buffer (Sigma) was then added to each centrifuge tube and allowed to lyse the red blood cells for 5 minutes at room temperature. Each cell suspension was then passed through a 70 micron cell strainer, spun down in a centrifuge, and washed with ice-cold working media. Using a hemacytometer, the remnant leukocytes from each mouse strain were subsequently counted.

After being resuspended in ice-cold working media at 2 mL/100 x 10<sup>6</sup> cells, a cocktail of Biotin-rat IgG-anti mouse antibodies was added for lineage depletion at 20  $\mu$ L/100 x 10<sup>6</sup> cells: Gr-1 for granulocytes, CD11b for monocytes, B220 for B cells, CD3e, CD4, CD8a for T cells, Ter-119 for erythrocytes, Sca-1 as a phenotypic marker for mouse haematopoietic stem cells, and CD127 for IL-7 receptor  $\alpha$  chain. The subsequent cell solutions were then incubated for 20 minutes at 4°C, spun down in a centrifuge, and then washed twice and resuspended in ice-cold working media at 87  $\mu$ L/10 x 10<sup>6</sup> cells.

Next, Miltenyi goat-anti-rat IgG microbeads were added at 13  $\mu$ L/10 x 10<sup>6</sup> cells and incubated for

20 minutes at 4°C. Following the incubation period, Miltenyi Midimacs columns were used to remove lineage-positive cells and collect the lineage depleted fraction of cells. One Midimacs column is used to deplete up to 100 x 10<sup>6</sup> cells. After passing cells on to the column, 3 mL cold working media was used to wash through the column, and the flow-through was collected into a 15 mL centrifuge tube. The cells were then counted and subsequently stained with FITC CD34, APC cKit, SA-APC-Cy7, and PE-FcγRII for detection of common myeloid progenitor cells (CMPs) (Lin<sup>-</sup> c-kit<sup>+</sup> Sca1<sup>-</sup> IL7R<sup>-</sup> CD34<sup>+</sup> FcγRII<sup>low</sup>). After incubation for 20 minutes at 4°C, the cells were washed twice and then placed in 500 μL working media before sorting.

#### *Flow Cytometry and Immunophenotyping*

Lineage-depleted bone marrow cells were analyzed and CMP cells were sorted and collected on a BD FACSAria. After sorting, CMP cells were cultured on OP9 stromal cells transfected with either vector only (Ret10), Jagged1, Jagged2, Delta-like 1, Delta-like 3, or Delta-like 4 Notch ligands in a 24-well cell-culture plate. CMPs were grown in RPMI medium (10% Fetal Calf Serum) with cytokines that support cell growth and myeloid differentiation (SCF 50 ng/ml, FLT3 ligand 30 ng/ml, IL-3 10 ng/ml, IL7 10 ng/ml, and GM-CSF 10 ng/ml). In plates where CMPs were obtained from FX(-/-) mice reared with fucose-supplemented chow, 1 mM of fucose was also added in the culture medium. For immunophenotyping, 500 CMPs were placed in each well. For RNA extraction, 5000-8000 cells were placed in each well. Cells were allowed to grow for different periods of time (ranging from 3 hours to 6 days) before they were collected for RNA preparation or immunophenotyping (ranging from 10-14 days). Immunophenotyping was then carried out using antibodies Sca-1, Gr-1, CD11b, and CD45, a leukocyte antigen for exclusion of OP9 cells. Granulocytes were then sorted on the BD FACSAria based on the expression of Gr-1 and CD11b.

#### *Isolation of RNA and cDNA*

Isolation of RNA was done according to the manufacturer's recommendation by using Qiagen's RNeasy Plus Mini kit. Following attainment of RNA, cDNA was acquired for use in Real-Time PCR. Ten μL-13 μL of RNA sample was added to a 0.2 mL PCR tube along with 2 μL random primers to reach a final volume of 15 μL. Up to 3 μL of Nuclease-free water was added when necessary. This solution was then incubated at 65°C for 5 minutes and snap-chilled on ice. Next 4 μL of 5x *iScript* select reaction mix and 1 μL of *iScript* reverse transcriptase was added to the tube. Together these components were incubated

in a thermocycler at the following conditions: 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes.

#### *Real-Time PCR*

Following acquisition of cDNA, real-time PCR was set up in a 96 well plate using 1 μL cDNA sample and 24 μL reaction mixture composed of 1 μL of forward and reverse primers (400 nM final concentration), 12.5 μL BioRad SYBR Green Supermix, and 10.5 μL nuclease free water. Real-time PCR was then carried out in a BioRad thermal iCycler.

#### *Relative Quantification of RT PCR*

Quantification of the studied gene expression by real-time PCR was done using a mathematical model which compares a target gene transcript to a reference gene transcript (GAPDH). This method, the "Delta-delta method" is outlined by the formula

$$\text{Ratio} = 2^{-[\Delta\text{CP sample} - \Delta\text{CP control}]}$$

where CP, crossing point, is defined as the point at which the fluorescence signal rises significantly above the background fluorescence during real-time PCR. GAPDH was used as the internal reference gene.

#### *Statistical analysis*

Student's t-test was performed to compare the average of Gr-1 positive and CD11b positive cells differentiated from CMPs of different sources.

## **Results**

#### *Fucosylation-dependent inhibition of CMP differentiation by Notch ligands*

To evaluate the effects of Notch ligands on myeloid progenitor cell differentiation, we used a co-culture system in which OP9 mouse stromal cells transduced to express either Notch ligands (Jagged 1 and 2, Delta-like 1, 3 and 4) or none (Ret10) were incubated with isolated CMP cells (Schmitt and Zuniga-Pflucker, 2002). CMPs were obtained from wild type (WT) mice, Fx (-/-) mice that had been reared for 4 weeks on a diet without fucose supplementation (FX-/- without fucose), and Fx (-/-) mice that have been reared on a fucose-supplemented diet (Fx-/- with fucose). CMPs from WT and FX(-/-) mice with fucose, but not from FX(-/-) mice without fucose, express selectin ligand activity that is fucosylation-dependent (Zhou and Lowe, data not shown). After 10-14 days of incubation, cells were collected and scored for the expression of Gr-1 and CD11b, markers for mature granulocytes. Upon co-

culture with OP9 cells expressing DLL-1 or DLL-4, the percentage of granulocytes differentiated from wild type CMPs are only 27% ( $p < 0.001$ ) and 23% ( $p < 0.001$ ), respectively, of those obtained from cultures with stromal cells expressing no Notch ligand (Fig.1). Incubation with OP9 cells expressing Jagged 1 and Jagged 2 also resulted in production of decreased number of granulocytes from CMPs (33%,  $p < 0.005$ , and 47%,  $p = 0.055$ , respectively). DLL-3 had no effect on CMP differentiation. Interestingly, the inhibitory effect by Notch ligands on CMP differentiation was not seen in CMP cultures when cells were obtained from FX (-/-) mice without fucose and grown in the medium without fucose. This is in contrast with CMP cultures when cells were obtained from FX (-/-) mice with fucose and grown in the presence of 1 mM fucose. All Notch ligands except DLL-3 inhibited differentiation of CMPs obtained from FX(-/-) mice with fucose. The suppressed percentages of granulocytes were comparable to those obtained from WT mice.

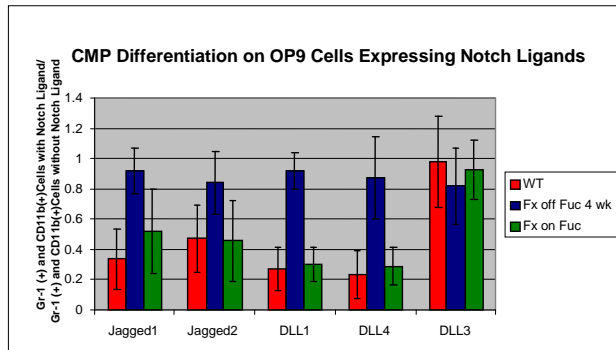


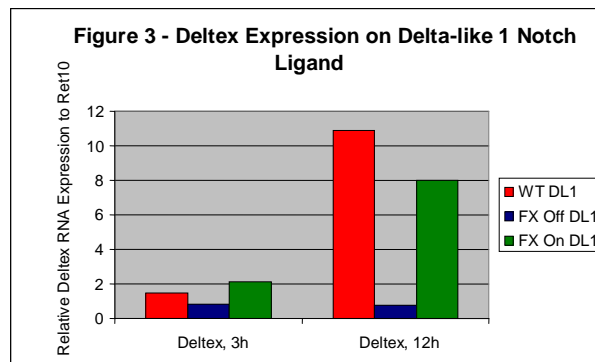
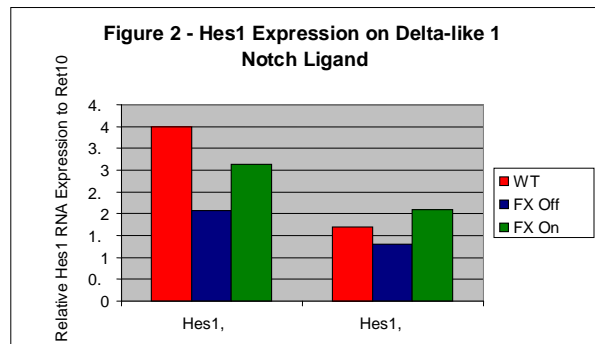
Figure 1 - CMP differentiation on OP9 cells expressing Notch ligands.

*Expression of Notch pathway target genes is affected by fucosylation*

We further examined the expression of two genes in CMPs, Hes1 and Deltex, reported to be directly modulated by Notch pathway. Since DLL-1 and DLL-4 showed much more potent inhibition of CMP differentiation, we focused our gene expression analysis on CMPs that are activated by DLL-1. Three hours after CMPs from WT mice were plated onto OP9 stromal cells expressing DLL-1, the expression of Hes1 was increased 4 fold when compared with Hes1 level from CMPs plated on the OP9 expressing no Notch ligand. The level of Hes1 in CMPs from FX(-/-) mice without fucose supplementation activated by DLL-1, however, was only 50% of that from WT CMPs (2.1 fold increase). Hes1 level from CMPs from FX(-/-) mice with fucose supplementation was increased by 3.1 fold. The expression of Hes1 went down by 12 hours after co-

culturing with DLL-1 expressing OP9 (Fig.2). In addition, the difference in Hes1 expression between WT and FX(-/-) without fucose was not obvious by 12 hrs post culture, indicating an early and transient up regulation of Hes1 in CMPs upon DLL-1 activation.

In comparison, there is no significant change of Deltex expression in CMPs when activated by DLL-1 after 3 hrs of plating on OP9, regardless of sources of CMPs (Fig. 3). However, by 12 hrs, Deltex expression was increased by 10.9 fold in WT CMPs activated by DLL-1 compared with CMPs cultured on OP9 without Notch ligand. But Deltex expression in CMPs from FX(-/-) without fucose showed no change under the same culture condition. In comparison, Deltex in CMPs from FX(-/-) mice with additional fucose increased by 8 fold upon activation by DLL-1.

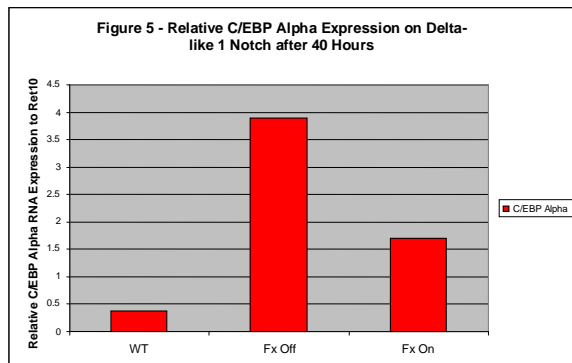
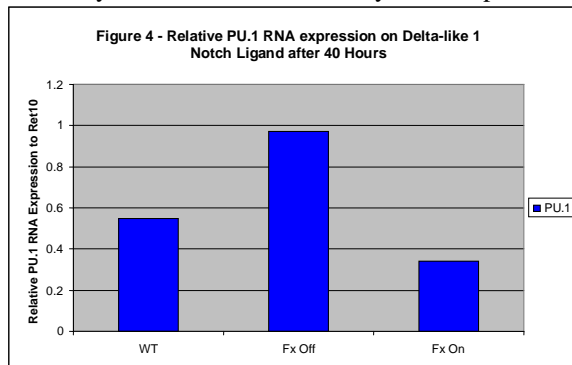


*Expression of myeloid differentiation regulatory genes is affected by fucosylation*

To confirm the altered differentiation of CMPs stimulated by Notch activation is through modulation of myeloid differentiation transcription factors, we examined the expression of PU.1 and C/EBP $\alpha$  (Tenen, 1997) in CMPs to see if such a correlation is present. The results were obtained from CMP cells collected 40 hours after being cultured on OP9 stromal cells transfected with DLL-1 or no Notch ligand. The expression of PU.1 in WT CMPs stimulated by DLL-1 was only 55% of that in CMPs

not seeing any Notch ligand. PU.1 expression was 97% in CMPs from FX(-/-) mice without fucose, indicating sustained myeloid differentiation under PU.1 regulation. The level of PU.1 was also significantly lowered in CMPs from FX(-/-) mice with fucose, suggesting a similar suppression of PU.1 by DLL-1 when fucosyl-modification is present (Fig.4).

Similarly, while CMPs from WT mice had only 37% level of C/EBP $\alpha$  expression upon DLL-1 activation, CMPs from FX(-/-) mice without fucose had a 3.9 fold increase of expression instead, and CMPs from FX(-/-) with fucose was mildly increased after DLL-1 activation (1.7 fold) (Fig. 5). The difference in C/EBP $\alpha$  expression in CMPs between WT and FX(-/-) mice with or without fucose strongly suggests that DLL-1 inhibits C/EBP $\alpha$  function in directing granulocytic differentiation, and the inhibitory effect of DLL-1 is fucosylation-dependent.



## Discussion

This study is the first to show that Notch activation inhibits the development of granulocytes/monocytes from bone marrow myeloid-committed progenitor cells, ie, CMPs. Although several studies have indicated that Notch affects myelopoiesis, the exact role of Notch signaling remains controversial. It is generally accepted that Notch1 inhibits differentiation of immature myeloid

precursors rather than promoting differentiation (Milner *et al*, 1996; Bigas *et al*, 1998). The discrepancy of these results mainly stems from differences in the myeloid cell lines used, or to differences in the Notch constructs used. In our study, we isolated common myeloid progenitors from mouse bone marrow and co-cultured them with stromal cells expressing different Notch ligands. Our results indicate that most Notch ligands (except DLL-3) are capable of suppressing myeloid differentiation into mature granulocytes *in vitro*. Such inhibitory effect on granulocyte differentiation was accompanied by activation of Notch target genes, Hes1 and Deltex, and down regulation of myeloid transcription factors PU.1 and C/EBP $\alpha$ .

As Notch receptors are modified on their extracellular EGF repeats by O-fucose, which can be further modified by *fringe*, it remains unclear whether O-fucosylation itself or its further extension by *fringe* actually modulates Notch signals in its function in hematopoiesis. To answer this question, we used hematopoietic progenitor cells from a genetically-modified mouse that is deficient in fucosylation due to the deletion of an enzyme essential for *de novo* synthesis of fucose. By providing exogenous fucose in the diet and in the culture medium, we have shown in previous studies that cellular fucosylation can be restored via a salvage pathway. Therefore, we are able to change the status of O-fucosyl modification of Notch by adding or depleting fucose. Our results provide clear evidence that the inhibitory effect of Notch on granulocyte differentiation is significantly attenuated in CMPs from FX(-/-) mice cultured without fucose but unaffected when cells are cultured with addition of fucose. The aberrant granulocyte differentiation associated with fucosylation deficiency is also accompanied by suppressed activation of Notch target genes (Hes1 and Deltex) and sustained activation of myeloid transcription factors (PU.1 and C/EBP $\alpha$ ). Addition of fucose to cell culture restores suppressed expression of Notch regulated genes and down regulates myeloid transcription factors as observed in wild type CMPs.

We conclude from this study that Notch activation inhibits myeloid differentiation. Absence of fucosylation results in escape from Notch-dependent suppression of granulocyte differentiation. Together with observations that FX(-/-) mice have neutrophilia and expanded granulocytic/monocytic progenitor cells in the marrow, our *in vitro* studies indicate a key role for O-fucose, and possibly its modification by *fringe*, in modulating Notch-dependent regulation of granulocytogenesis.

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