Haematopoietic differentiation is inhibited when Notch activity is enhanced in FLK1+ mesoderm progenitors

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Abstract Notch signalling has been implicated during haematopoietic development in vivo and in the differentiation of haematopoietic cells from pluripotent cells in vitro. However, interpretation of data from many of these studies has been complicated by the heterogeneous nature of cell populations under study and by the fact that the Notch pathway is active during embryogenesis prior to the development of the haematopoietic system. To define the role of Notch signalling in more precise cell populations during the early stages of haematopoietic development within the aorta–gonad–mesonephros (AGM) microenvironment we co-cultured differentiating ESCs on a stromal cell line derived from this region of the embryo. Our co-culture system had no effect on the production of FLK1+ mesoderm progenitor cells but promoted their subsequent haematopoietic differentiation. We assessed the role of Notch signalling on haematopoietic differentiation of isolated FLK1+ cells. Notch activity is dynamic and drops to basal levels as FLK1+ cells commit to a haematopoietic fate. Further reduction of Notch activity by the inducible expression of dominant negative MAML had no functional consequences. In contrast, induction of Notch activity using an inducible NotchIC expression system had an inhibitory effect on haematopoietic differentiation. We used a Cre-mediated recombination strategy whereby NotchIC-expressing cells were marked with the hCD2 receptor and observed a reduction in the number of multi-lineage and myeloid colonies derived from NotchIC+ compared to NotchIC− FLK1+ cells isolated from the same culture. We believe that our culture system represents a good model for haematopoietic development within the AGM microenvironment and our data suggest that haematopoietic commitment of FLK1+ cells in this setting occurs when Notch activity is below a specific threshold.

Introduction Directed differentiation of pluripotent cells into haematopoietic lineages has been widely used as a tool to study the molecular mechanisms involved in haematopoietic development as well as a potential source of cells for regenerative therapies (Kolios and Moodley, 2013; Okano et al., 2013). However, there are significant challenges facing the production of therapeutic cell populations from these sources. It has not been possible to produce haematopoietic stem cells (HSCs) capable of long-term reconstitution from human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) and differentiated cell types that have been generated tend to display immature phenotypes. ESC-derived erythrocytes, for
example, are incapable of undertaking the final enucleation step in their maturation and do not switch on the appropriate adult globin genes (Dias et al., 2011; Lapillonne et al., 2010; Mountford et al., 2010). Some of these problems have been partly resolved by co-culturing differentiating ESCs on stromal cell lines derived from various sites of haematopoietic activity in vivo such as bone marrow, foetal liver or the aorta–gonad–mesonephros (AGM) region. This highlights the importance of appropriate signals from the haematopoietic niche. A fuller understanding of the molecular mechanisms associated with haematopoietic niche in vivo and in co-culture systems in vitro is an essential step towards the development of robust differentiation protocols that can be translated into the clinic.

Murine haematopoiesis first occurs in the extra-embryonic yolk sac around embryonic day 7.5 with the formation of blood islands containing primitive erythrocytes surrounded by developing endothelial cells (Ferkowicz and Yoder, 2005; Moore and Metcalf, 1970; Palis et al., 1999; Palis and Yoder, 2001). This transient wave of haematopoiesis is followed by the permanent "definitive" process where multi-potential HSCs and haematopoietic progenitor cells (HPCs) appear in several anatomical sites (Cumano et al., 1996; Godin et al., 1995; Muller et al., 1994; Ottersbach and Dzierzak, 2005; Palis et al., 1999; Yoder et al., 1997). HSCs first arise de novo in the AGM region at E10.5 indicating that this region is a potent source of haematopoietic inducing signals (Kumaravelu et al., 2002; Medvinsky and Dzierzak, 1996; Medvinsky et al., 1993, 1996,). Indeed a number of studies, including our own, have demonstrated that signals from primary AGM tissue and/or stromal cells derived thereof can promote the haematopoietic differentiation of both mouse and human pluripotent cells (Krassowska et al., 2006; Ledran et al., 2008; Matsuoka et al., 2001; Ohneda et al., 1998; Oostendorp et al., 2002a,b; Weisel et al., 2006; Xu et al., 1998). One of these cell lines derived from the aorta and surrounding mesenchyme (AM) also promoted the production of cells capable of limited engraftment in the peripheral blood from human ESCs in vivo (Ledran et al., 2008). We demonstrated previously that one of the haematopoietic-promoting cell lines derived from the AM region exerted its effects after the formation of T/Bry mesoderm. Our finding that cell–cell contact was required for the promoting activity and that this was partially inhibited by the gamma secretase inhibitor (GSI) is consistent with the idea that the Notch pathway could be involved in this process (Gordon-Keylock et al., 2010). In this study we assessed the effects of the AM microenvironment on the production of FLK1+ mesoderm progenitor cells and the role of the Notch pathway on the further differentiation of FLK1+ cells into haematopoietic lineages.

The Notch signalling pathway has been implicated as an important regulator of haematopoietic development (Bigas and Espinosa, 2012). Four Notch receptors (Notch1–4) and five ligands (Delta-like 1, 3, & 4 and Jagged 1 & 2) have been identified in vertebrates. The transduction of Notch signalling requires a series of processes including receptor proteolytic cleavage producing the intracellular domain, NotchIC, recruitment of mastermind-like protein (MAML), p300 to form RBP-Jκ activator complex and ultimately the activation of downstream genes including members of the Hes/Hey families and Gata2 (Lai, 2004; Robert-Moreno et al., 2005; Weng et al., 2003).

Both gain and loss of function studies have demonstrated a critical role for Notch signalling during haematopoietic development in vivo and during ESC differentiation in vitro. Several studies have suggested a differential effect of Notch signalling on the primitive and definitive wave of haematopoietic development (Bigas and Espinosa, 2012). For example ectopic Notch1 has been reported to have an inhibitory effect on the haematopoietic differentiation of ESCs and on the expansion or survival of HPCs cultured on OP9 stromal cells (Schroeder et al., 2006) (Ganapati et al., 2007) and loss of function studies using Notch1 deficient ESCs indicated that Notch signalling was dispensable for primitive but not definitive haematopoiesis (Cheng et al., 2008; Hadland, 2004). More recently activation of Notch signalling in differentiating human ESCs enhanced the generation of haematopoietic cells from committed progenitors (Yu et al., 2008). However, most of these experiments have been performed using heterogeneous cell populations so it is not clear precisely when during the differentiation that Notch signals are active and it is difficult to define the precise cell–cell interactions that are responsible for any phenotypic effects. In ESC systems the fact that ectopic NotchIC inhibited the differentiation of mesoderm and the inherent problems associated with clonal variation between ESC lines have further complicated the interpretation on haematopoietic commitment (Schroeder et al., 2003a, 2006).

To assess the involvement of Notch signalling on defined cell populations within the AGM microenvironment we established a novel system whereby we co-cultured isolated FLK1+ cells on AGM-derived stromal cell lines. We used inducible expression of either NotchIC or dominant negative MAML as gain- and loss-of-function strategies respectively to manipulate Notch activity within this defined cell population at specific time points during differentiation. Potential problems associated with transgene silencing during differentiation have been avoided by targeting our inducible constructs into the permissive Rosa26 and Hprt loci. The Cre-mediated recombination strategy that we have used to express NotchIC allows us to compare directly NotchIC+ and NotchIC− cells from the same culture, avoiding clonal variability between ESC subclones (Schroeder et al., 2003a). Using this elegant system we have been able to dissect precisely the role of Notch signalling on the specification of haematopoietic cells from FLK1+ precursors within the AGM microenvironment, uncovering an inhibitory effect of Notch signalling on haematopoietic commitment.

**Materials and methods**

**Cell maintenance**

ESC lines that were used in this study included control wild type E14IV; control wild type, constitutively expressing, 7a-GFP (Gilchrist et al., 2003); the parental A2lox.Cre (Iacovino et al., 2011); a tamoxifen-inducible NotchIC, NIC-C5 (Lowell et al., 2006); doxycycline (dox)-inducible dominant negative MAML, iDML.1 (see below) and a dox-inducible EGFp, iEGFP.1. All ESC lines were maintained in their undifferentiated state as described previously (Gordon-Keylock et al., 2010; Krassowska et al., 2006). The AM stromal cell line (AM14/AM14.1C4) was derived from the aorta and surrounding mesenchyme of E11 AGM tissue (Oostendorp et al., 2002a,b) and maintained on gelatinized flasks in medium as described (Gordon-Keylock et al., 2010; Krassowska et al., 2006).
Co-culture on AM14 stromal cells does not affect the production of FLK1+ cells but promotes their subsequent haematopoietic differentiation. A. Schematic of experimental strategy: Day 1 EBs were co-cultured on AM14 stromal cells or gelatin control. Cells were harvested and sorted from stromal cells at days 3, 4, and 5 and FLK1 expression kinetics was assessed by flow cytometry and qRT-PCR.

B. Kinetics of Flk1 mRNA assessed by qRT-PCR in EBs sorted from co-cultures on gelatin or AM14 stromal cells. 18s was used as the internal control and the calibrator (day 1EB RNA) was assigned a value of 1. C. Kinetics of production of FLK1+ cells assessed by flow cytometry in EBs cultured on gelatin or AM14 stromal cells. D. Schematic of experimental strategy: FLK1+ cells were magnetically sorted from differentiating day 4 EBs, co-cultured on AM14 stromal cells or gelatin control for a further 2 days (day 6) then analysed by flow cytometry and in vitro colony forming assays. E. The number of CFU-C (per 10⁵ cells) was approximately 3-fold higher in FLK1+ cells derived from two independent ESC lines (E14IV and NIC-C5) that were cultured on AM14 stromal cells compared to gelatin controls. F. Percentage of haematopoietic progenitors (defined by co-expression of CD41 and cKit) produced from isolated FLK1+ cells derived from E14IV and NIC-C5 day 4EBs was significantly higher in AM14 co-cultures compared to gelatin control. This was assessed as both % and total number of CD41+cKit+ cells. Data in B and C represent the mean of 3 independent experiments; error bars represent SEM; P-values calculated using Mann–Whitney test or Wilcoxon signed rank test (*p < 0.05).
Figure 2  AM14 and OP9 stromal cell lines have a distinct profile of Notch ligand expression. A. q-RT-PCR of Jag1, Jag2, Dll1 and Dll4 in AM14 and OP9 stromal cells. AM14 stromal cells expressed significantly lower Jag1 but higher Jag2 compared to OP9. No significant difference was observed in the expression of Dll1 or Dll4 between the two cell lines. B. Flow cytometry analysis of Jag1 and Dll1 in AM14 and OP9 stromal cells. A significant higher proportion of OP9 cells expressed Jag1 compared to AM14, whereas a similar proportion of Dll1 positive cells were observed in the two cell lines. C. Immunohistochemistry analysis of Dll4 expression (green) in AM14 and OP9 cell lines. Nuclei are stained with DAPI (blue). Data in A represent the mean of 2 or 3 independent experiments; error bars represent SEM. Data in B represent the mean of 4 independent experiments; error bars represent SEM. P-values calculated using Mann–Whitney test or Wilcoxon signed rank test (*p < 0.05, *** p < 0.001).
Construction of iDML-EGFP/iEGFP ESC lines

A MAML peptide fused to GFP (DML-GFP) has been shown previously to act in a dominant negative manner (Weng et al., 2003). This DML-EGFP cDNA was excised (BglII (blunted)/NotI) from pEGFP-DML-N3 plasmid (Weng et al., 2003) and cloned into the p2lox (XhoI (blunted)/NotI) (Iacovino et al., 2011). A2lox.Cre ESCs (Iacovino et al., 2011) were passaged twice then treated with 1 μg/ml dox for 24 h to induce CRE recombinase expression before electroporation. ESC (0.77 ml of 10^7/ml) were electroporated with 30 μg (1 μg/μl) of the p2lox.DML-EGFP or p2lox.EGFP plasmid using a BIORAD gene pulser electroporator then selected in 270 ng/ml G418 (Geneticin) for 9 days. Single ESC clones were picked and expanded for further validation.

Haematopoietic differentiation

Embryoid bodies (EBs) were formed in hanging drops in the presence of leukaemia inhibitory factor (LIF) for 2 days, placed in suspension culture for 1 day in the absence of LIF then co-cultured on confluent γ-irradiated (40 Gy) stromal cell layers or gelatinized flasks for a further 5 days (Gordon-Keylock et al., 2010; Krassowska et al., 2006). To isolate FLK1^+ cells, EBs were maintained in suspension cultures (3 × 10^4 cells/ml in ES medium without LIF) in sterile bacteriological grade Petri dishes for 4 days. EBs were then dissociated and incubated with biotin-conjugated anti-mouse-FLK1 antibody (eBioscience) then fractionated using anti-biotin MicroBeads (Miltenyi Biotec) according to the manufacturer’s protocol. Isolated FLK-1^+ cells (4 × 10^4/cm^2) were plated directly onto irradiated stromal cells in EBD medium (IMDM supplemented with 15% FCS, 200 μg/mL iron-saturated transferrin (Roche), 4.5 mM monothioglycerol (Sigma), 50 μg/mL ascorbic acid (Sigma), penicillin/streptomycin (Gibco), and 2 mM glutamine) for further differentiation.

Cells were assayed for haematopoietic colony (CFU-Cs (Colony Forming Unit-culture)) formation after harvesting and dissociating with trypsin solution to generate a single cell suspension. 5 × 10^4 or 1 × 10^5 cells were then seeded into methylcellulose culture medium (MethoCult® M3434) and all the total number of colonies counted were normalised and expressed as CFU-C per 10^5 cells in all figures. The
Colonies were classified based on the morphology by light microscopy and scored between day 7 to day 12. Defined concentrations of 4-hydroxy tamoxifen (4-OHT) (H6278, http://www.sigmaaldrich.com) dissolved in ethanol were added to co-cultures or methylcellulose cultures when required. ESC were distinguished from stromal cells either by GFP expression in ESCs or by staining stromal cells with Vybrant DiD (Invitrogen) and the percentage of ESC-derived cells in co-cultures was determined using the BD™ FACS Calibur or BD™ LSRFortessa cell analyzer. The number of colonies derived from...
ESC was then calculated by dividing the total number of colonies scored by the percentage of ESC-derived cells in the sample.

**Flow cytometry analysis and fluorescent-activated cell sorting (FACS)**

Flow cytometry analysis was carried out on BD™ FACS Calibur or BD™ LSRFortessa cell analyzer and data analysed using FACSDiva or Flowjo. Cell sorting was performed on BD™ FACSAria II cell sorter or BD™ FACS Jazz. Cells were incubated with optimal concentrations of antibodies as described (Gordon-Keylock et al., 2010). Directly conjugated antibodies against cKit, CD45 and CD41 were purchased from eBioscience. Human-CD2+ (hCD2) cells were stained with anti-hCD2-PE (BD Bioscience). Stromal cells were stained with anti-Jagged 1 (R&D) and anti-Delta-like 1 (Abcam) followed by secondary conjugated antibody staining.

**Quantitative reverse transcription (RT)-polymerase chain reaction (PCR)**

Total RNA was extracted using RNaseasy Mini Kit (Qiagen) and cDNA prepared by reverse transcription using SuperScript™ III First-strand Synthesis SuperMix or SuperScript® Vilo™ Master (Invitrogen). Quantitative real-time RT-PCR was performed on an ABI 7500FAST qPCR machine using TaqMan PCR chemistry or Sybr green under universal conditions (Applied Biosystems). Samples were analysed in triplicate, using Hprt (hypoxanthine-guanine phosphoribosyl transferase) or 18s (18S ribosomal RNA) as the endogenous control. Relative quantitation was calculated using the \( \Delta \Delta C_T \) method in ABI SDS1.4 software and data are presented as fold change in gene expression relative to an internal calibrator. Primers and probes information are listed in Supplemental Table 1.

**Luciferase assay**

2 x 10^5 cells were plated into 24 well plate in ESC culture medium. 24 h later cells were co-transfected with 0.75 μg 12xRBPJκ–luciferase reporter plasmid plus 15 ng of internal control plasmid (pEFBos/renilla luciferase) in the presence or absence of 4-OHT (1 μM). As a positive control, 0.25 μg of pCAG-NotchIC plasmid was co-transfected. As a negative control, 0.75 μg pGL3 control plasmid plus 15 ng of internal control plasmid were transfected. Cells were collected 48 h later and analysed with Promega Dual Luciferase Kit (Promega) according to manual instruction. Ratios of firefly luciferase reporter/renilla luciferase control were compared to the reference sample to give a fold change.

**Western blotting**

Cells were harvested and lysed with cold RIPA buffer (ThermoScientific) in the presence of 1% protease inhibitor (Sigma). Protein samples were separated using NuPAGE® SDS-PAGE Gel System (Invitrogen) with NuPAGE® 4–12% Bis Tris Gels then semidy electro-transferred onto nitrocellulose membranes. Membranes were probed with anti-GFP or control anti-STAT3 antibodies (Cell signalling technology) then incubated with Horseradish-conjugated anti-rabbit antibody (Santa Cruz) before adding ECL substrate and autoradiography.

**Immunocytochemistry**

Stromal cells were grown on glass coverslips in 24 well plates coated with 0.1% gelatin and irradiated when getting around 100% confluent then further cultured for another 24 h. Cells were fixed with 4% PFA (Sigma) and blocked in 5% donkey serum (Sigma) in PBST (0.001% Triton X-100 in PBS) then incubated with rat anti-Delta-like 4 (R&D) at 4 °C overnight followed by an incubation of FITC conjugated anti-rat antibody together with 1 μg/ml DAPI (Invitrogen). Coverslips were mounted with Prolong Gold mounting medium (Invitrogen).

**Results**

**Co-culture on AM14 stromal cells does not affect the production of FLK1+ cells but promotes their subsequent haematopoietic differentiation**

Our previous work demonstrated that AM stromal cell lines derived from the AGM region provide a haematopoietic promoting microenvironment during the differentiation of ESCs and, using the Bry-GFP reporter cell line and one of AM stromal cell lines-AM14.1C4, we showed that the promoting effect acted after the formation of mesoderm (Gordon-Keylock et al., 2010; Krassowska et al., 2006). To further define the haematopoietic promoting activity of this co-culture system we assessed the expression of FLK1, a marker for a mesodermal
progenitors that have the potential to form cardiac, endothelial and haematopoietic lineages (Fehling et al., 2003). We co-cultured differentiating EBs on AM14 (AM14.1C4) stromal cells, sorted ESC-derived cells from contaminating stromal cells then analysed the proportion of FLK1+ cells by flow cytometry and the level of expression of Flk1 mRNA by qRT-PCR analysis (Fig. 1A). The kinetics of FLK1 expression was not significantly altered by co-culture on stromal cells suggesting that they must exert their haematopoietic promoting effects after the emergence of cells that express this receptor (Figs. 1B, C). We noted that the production of FLK1+ cells and CFU-Cs after co-culture with the AM14 stromal cell line was slightly higher (although not statistically significant) to the commonly used bone marrow-derived OP9 cell line (Supplementary Figs. 1A–C). In contrast co-culture of differentiating ESC on stromal cell lines (UG26) from the urogenital ridge (UG) region of the AGM appeared to have an inhibitory effect on the production of FLK1+ cells (Supplementary Figs. 1A, B).

We next tested whether FLK1+ cells, produced in the absence of stromal cells, could respond directly to the AM14 stromal cells. We isolated FLK1+ cells that were generated in differentiating EBs at day 4 which was the peak time point of their production (data not shown). Sorted FLK1+ cells were co-cultured on irradiated AM14 stromal cells for a further two days then their haematopoietic colony forming ability was assessed (Fig. 1D). Co-culture with AM14 stromal cells significantly increased the number of CFUs derived from FLK1+ cells by approximately threefold compared to the gelatin control in two independent ESC lines (Fig. 1E). The increase in haematopoietic progenitor number after AM14 co-culture was confirmed by the percentage and absolute numbers of cells co-expressing CD41 and cKit which have been reported to mark definitive ESC-derived haematopoietic progenitors (Mikkola et al., 2003) (Fig. 1F). Taken together these data demonstrate that the AM14 stromal cells did not affect the emergence of FLK1+ cells but could further enhance their haematopoietic development and/or survival. We believe that this model system provides a simple in vitro model system to dissect the role of specific signalling pathways on the haematopoietic development within the AGM microenvironment. Our previous work had demonstrated that cell–cell contact was required (Gordon-Keylock et al., 2010) and this led us to hypothesise that the Notch pathway could be playing a role at this stage in haematopoietic differentiation as previously suggested by others (Kumano et al., 2003; Robert-Moreno et al., 2005, 2008). To test this hypothesis we first assessed the expression of Notch ligands in AGM stromal cell lines then used inducible gain-and loss-of-function strategies to manipulate of Notch activity within FLK1+ mesoderm progenitor cells.

Profile of Notch ligand expression in AM14 stromal cell line is distinct from OP9

We analysed the expression of the Notch ligands, Jagged 1, Jagged 2, delta-like 1(Dll1) and delta-like 4 (Dll4) in the AM14 stromal cell line compared to the commonly used bone marrow derived OP9 cell line (Fig. 2). OP9 cells expressed significantly higher levels of Jagged 1 but lower levels of Jagged 2 compared to the AM14 stromal cell line as assessed by q-RT-PCR analysis and flow cytometry (Figs. 2A, B). There was no significant difference in the expression of Dll1 and Dll4 between the two cell lines (Figs. 2A–C). These data suggest that stromal cell lines derived from different haematopoietic sites have distinct Notch ligand profiles and are thus likely to exert their enhancing effects by distinct mechanisms.

Ectopic NotchIC abrogates haematopoietic differentiation of FLK1+ cells

We tested whether enhancing Notch activity using a gain-of-function strategy would subsequently affect the differentiation potential of FLK1+ cells (Fig. 3A). We used a 4-OHT inducible ES cell line (NIC-C5) which expresses NotchIC-IREs-hCD2 upon the addition of 4-OHT (Lowell et al., 2006). 4OHT induces the translocation of Cre-ERT2 to the nucleus where it mediates recombination between loxP sites in the Rosa26 locus resulting in the expression of NotchIC and hCD2 (Supplementary Fig. 2A). hCD2 therefore marks all cells that express NotchIC providing a convenient marker for recombination efficiency and for the isolation of NotchIC+ and NotchIC− cell populations (Lowell et al., 2006). We confirmed an enhancement of Notch activity upon 4-OHT induction using a luciferase assay for transcription activity and qRT-PCR for Notch target gene expression (Supplementary Figs. 2B, C). We differentiated ESCs in EBs for four days, fractionated FLK1+ cells by MACS and cultured these on AM14 stromal cells for an additional two days in the presence or absence of 4-OHT. There was no significant difference in total cell numbers in the presence and absence of tamoxifen (Supplementary Fig. 2D). Cells were harvested and analysed by qRT-PCR, flow cytometry and CFU-C assays (Fig. 3A). The frequency of CD41+cKit+ cells was slightly lower when NIC-C5 ESCs, but not control E14IV ESCs, were treated with 4-OHT (Fig. 3B) suggesting that high levels of Notch had a slight inhibitory effect on the differentiation of definitive haematopoietic progenitors from FLK1+ cells. Induction of NotchIC by addition of 4-OHT also had an inhibitory effect on the formation of multi-potent CFU-GEMM, CFU-M and CFU-GM colonies (Figs. 3C & D). In contrast, induction of NotchIC had no effect on the production of erythroid colonies (BFU-E, CFU-E) (Fig. 3E). This suggests that there is a differential effect of NotchIC on the commitment of FLK1+ cells to specific haematopoietic lineages in this system (Figs. 3C–E).

Interpretation of these experiments could be limited by incomplete Cre-mediated excision that had been calculated to be approximately 50% (Supplementary Fig. 2E). Therefore, to further define the effect of NotchIC, we fractionated NotchIC+ and NotchIC− populations from the same co-culture based on hCD2 expression (Fig. 4A) (Lowell et al., 2006). Consistent with our analysis of unfractionated cultures, the frequency of CD41+cKit+ haematopoietic progenitors was slightly lower in the hCD2+ (NotchIC−) compared to the hCD2− (NotchIC+) fraction suggesting that activation of Notch inhibited the production of definitive progenitors (Fig. 4B). When co-cultures were treated with 4-OHT for a longer period of time (to day 9) the frequency of cells expressing the pan-haematopoietic marker CD45 was more profoundly reduced in the hCD2− compared to hCD2+ fraction (Fig. 4B).

The hCD2+ cell population formed significantly less CFU-GM and CFU-M colonies in methylcellulose compared
to the hCD2– population supporting the notion that Notch activity was inhibitory to production of myeloid progenitors (Fig. 4C). In contrast, and again consistent with the results from unfractionated cultures, there was no significant difference in the number of erythroid colonies produced from CD2+ and CD2– cell populations (Fig. 4D). No reduction in the number of myeloid colonies was observed when 4-OHT was added to the methylcellulose culture of CD2– cells indicating that NotchIC abrogated the formation of myeloid progenitors during co-culture but had no effect on their subsequent proliferation and differentiation in the colony assay (Fig. 4E). There was no effect of 4-OHT on the proliferation of E14IV-derived myeloid cells in methylcellulose assay excluding a toxic effects of 4-OHT (Fig. 4F) (Shi and Petrie, 2012).

The effect of NotchIC was further assessed by qRT-PCR of target genes. The Notch downstream gene, Hey1 was increased 8-fold in the hCD2+ fraction compared to hCD2– fraction. However there was no significant effect on the expression of Gata2 and only a modest effect on the expression of Runx1 that are reported to be downstream of the NotchIC signalling pathway and to regulate definitive haematopoiesis in the AGM region (Fig. 5A). A significantly lower level of expression of haematopoietic marker genes including Pu1, Gata1, Scl/Lmo2, β-H1 and β-major was observed in cells with elevated Notch activity (Fig. 5B).

NotchIC had no dramatic effect on the expression levels of Flk1 or VE-cad indicating that ectopic Notch signalling did not maintain cells at the haemangioblast cell state (Fig. 5C). However we could speculate that the modest, but significant increase in both VE-cad and Runx1 might indicate that the Notch activation favours the transition to haemogenic endothelium but further experiments are necessary to prove this hypothesis. There was no significant difference in the expression of Sox17 that is reported to be a critical marker to distinguish the AGM-derived from yolk sac derived haematopoiesis as well as for proliferation of haemogenic endothelium cells (Irion et al., 2010; Nakajima-Takagi et al., 2013). We observed a modest increase in the expression of Tbx6 but notDll3 that are reported as markers of paraxial mesoderm. Taken together, our data suggest that inhibition of haematopoietic differentiation by ectopic NotchIC occurs after haemogenic endothelium formation and proliferation.

![Figure 5](image_url)

**Figure 5**  
A. The level of expression of Hey1 was significantly higher in hCD2+ (i.e. NotchIC-expressing cells) compared to hCD2– cells. There was a modest, but significantly higher level of expression of Runx1 but there was no significant difference in the expression of Gata2.  
B. The level of expression of the haematopoietic-related genes (Scl, Lmo2, Pu.1, Gata1, β-H1 and β-major) was significantly lower in CD2+ (NotchIC+) compared to CD2– cells.  
C. There was no significant difference in the level of expression of Flk1 and a modest but significantly higher level of expression of VE-Cad in CD2+ (NotchIC+) compared to CD2– cells. Of genes associated with non-haematopoietic lineages, we noted a modest increase in Tbx6 but no difference in expression levels of Dll3 or Sox17 between CD2+ (NotchIC+) and CD2– cells. Expression levels in hCD2+ (NotchIC+) fraction were expressed as fold increase over the related to hCD2– fraction (assigned as 1). HPRT was used as the endogenous control. Data represented 9 reactions from 3 independent experiments; error bars, SEM. P values were calculated using the Mann–Whitney test (**p < 0.05**).
iDML can inhibit exogenous and endogenous Notch activity but has no effect on haematopoietic differentiation of FLK1⁺ cells. We also used a dominant negative approach to regulate Notch activity in our FLK1⁺/AM14 stromal co-culture system. Mastermind-like (MAML) proteins are critical transcriptional co-activators for Notch signalling and the truncated, dominant-negative form (DNMAML) can act as a potent inhibitor of the pathway (Weng et al., 2003). DNMAML encodes amino acids 13 to 74 of MAML providing the binding site for NotchIC but this truncated protein cannot recruit other co-activators such as p300 to form the fully functional
Haematopoietic differentiation when Notch activity is enhanced

There was a significant reduction in endogenous Notch activity (as assessed by Hey1 expression) in FLK1+ cells at day 6 compared to day 4 in three independent ESC lines (Fig. 6G). This suggests that Notch activity drops to a basal level at this stage of the differentiation process. Taken together, these results indicate that Notch activity in FLK1+ derived cells in this system is dynamic, reducing to basal levels as they differentiate and as a result there are no functional consequences of our dominant negative strategy nor pharmacological Notch inhibition. However we cannot exclude the possibility that the reduction in Hey expression is due to a lack of endothelial cells that our culture may not support.

To our knowledge this is the first time that an inducible DNAMAML has been used to modulate Notch activity in differentiating ESCs and represents a powerful approach to assess the role of the Notch pathway in a wide range of developmental pathways.

**Discussion**

We describe the co-culture of ESC-derived FLK1+ cells on AGM-stroma that provides a simple model to study the molecular pathways involved in haematopoietic development in the AGM microenvironment. We demonstrate that the AGM derived stromal cell line promotes the differentiation of FLK1+ mesoderm progenitor cells into haematopoietic lineages and we have used our system to assess the role of the Notch pathway at this point in the differentiation of a defined subpopulation of cells. AM14 stromal cells have a distinct Notch ligand profile compared to OP9 with the expression of Jagged 1 being significantly lower and the expression of Jagged 2 being higher than OP9. This indicates that the different microenvironments have distinct Notch ligand activity. Our goal in this study was to study the role of Notch signalling within the AGM-type microenvironment. We noted that Notch signalling is dynamic during the co-culture with the lowest level being associated with the time of haematopoietic progenitor production. Further reduction of this low level had no functional consequences with the haematopoietic differentiation of FLK1+ cells being unaffected by the overexpression of dominant negative MAML. In contrast, activation of the Notch pathway by NotchIC inhibited the subsequent production of haematopoietic progenitors from FLK1+ cells as assessed by CFU-C formation and the expression of haematopoietic cell surface markers and transcription factors. Our Cre-mediated recombination strategy, whereby NotchIC-expressing cells were marked with the hCD2 receptor,

**Figure 6**  
IDML can inhibit exogenous and endogenous Notch activity but has no effect on haematopoietic differentiation of FLK1+ cells.  
A. Scheme of the dox inducible iDML-EGFP ESC line where the TRE-DML-EGFP is targeted to the HPRT locus and the rtTA to the ROSA26 locus.  
B. Expression of DNAMAML-EGFP induced by 0.5 μg/ml dox was confirmed by western blot using an EGFP antibody.  
C. Expression of DML-EGFP was demonstrated on our system to assess the role of the Notch pathway at this point in the differentiation of a defined subpopulation of cells. AM14 stromal cells have a distinct Notch ligand profile compared to OP9 with the expression of Jagged 1 being significantly lower and the expression of Jagged 2 being higher than OP9. This indicates that the different microenvironments have distinct Notch ligand activity. Our goal in this study was to study the role of Notch signalling within the AGM-type microenvironment. We noted that Notch signalling is dynamic during the co-culture with the lowest level being associated with the time of haematopoietic progenitor production. Further reduction of this low level had no functional consequences with the haematopoietic differentiation of FLK1+ cells being unaffected by the overexpression of dominant negative MAML. In contrast, activation of the Notch pathway by NotchIC inhibited the subsequent production of haematopoietic progenitors from FLK1+ cells as assessed by CFU-C formation and the expression of haematopoietic cell surface markers and transcription factors. Our Cre-mediated recombination strategy, whereby NotchIC-expressing cells were marked with the hCD2 receptor,
has been a powerful approach because it has enabled us to directly compare FLK1<sup>+</sup> cells that express exogenous NotchIC with control, FLK1<sup>+</sup> NotchIC<sup>−</sup> cells from the same culture. Thus we overcome some of the problems that have been associated with the interpretation of data derived from complex heterogeneous cell populations that has resulted in confusing and sometimes contradictory reports in the literature (Bigas and Espinosa, 2012). At this stage during haematopoietic differentiation the FLK1<sup>+</sup> cell population includes progenitors for a number of mesodermal lineages including endothelial and cardiac cells and NotchIC could be acting via interactions between these cell types.

**Inhibitory effects of ectopic NotchIC on FLK1<sup>+</sup> derived haematopoiesis**

Ectopic Notch-IC expression inhibited the production of haematopoietic progenitors from FLK1<sup>+</sup> cells and this is in keeping with our hypothesis that the level of Notch activity must be below a specific threshold for haematopoietic commitment. We noted that the severity of inhibition negatively correlated with the level of endogenous Notch activity: myeloid colony production was inhibited to a greater extent by Notch IC compared to the modest inhibitory effect on activity: myeloid colony production was inhibited to a greater extent by Notch signalling in our experiments is also supported by the fact that moderate overexpression level of NotchIC in our system is not sufficient and/or requires co-factors to activate Gata2. The fact that NotchIC inhibited the expression of other haematopoietic-related genes such as *Scl*, *Lmo2*, *Gata1*, *PU.1* & *β-globin* and the production of CFU-Cs and definitive progenitors (defined as CD41<sup>−</sup>cKit<sup>+</sup>) suggests that both primitive and definitive haematopoietic programmes are affected in this culture system.

**Effect of DNMAAML in regulating Notch signalling pathway**

Notch activity decreased significantly during the co-culture of FLK1<sup>+</sup> cells on AM stromal cells and the introduction of DNMAAML1 at this point in the differentiation system had no functional consequence. This result confirms other findings where DNMAAML1 has been shown to have minimal effects in systems where Notch activity is low. For example, one study demonstrated that bone marrow derived LSK progenitors were exposed to a lower level of Notch signalling compared to more committed progenitors and that induction of DNMAAML1 did not abrogate the expression of *Hes1*, *Dtx1* or *Runx1* in this context. However after exposure to the exogenous Delta-like 1 ligand, the expression of *Hes1* and *Dtx1* expression was enhanced and this enhanced expression could then be inhibited by DNMAAML1 (Maillard et al., 2008). Similarly, DNMAAML1 prevented megakaryocyte differentiation from megakaryocyte-erythroid progenitors possessing higher Notch activity compared to multi-lineage progenitors (Mercher et al., 2008). Thus, DNMAAML1 can only have functional consequences in systems where there is a high level of Notch activity. Our data suggest that haematopoietic
commitment of FLK1\(^+\) cells in the AM microenvironment requires Notch activity to be at a basal level and is therefore consistent with several studies that have demonstrated an inhibitory effect of Notch signalling on haematopoietic differentiation and lineage commitment from progenitors (Bigas et al., 1998; Li et al., 1998; Milner et al., 1996).

Haematopoietic enhancing effect of AM derived stromal cells

The AM14 stromal cell line used in our study was derived from the dorsal aorta and surrounding mesenchymal area of the AGM region. During haematopoietic ontogeny the underlying mesenchyme has been suggested as a potential niche for the further differentiation of the haemangioblast into pre-HSCs, the formation of haemogenic endothelium and/or the maturation of pre-HSCs (Costa et al., 2012). Our co-culture system might therefore provide an *in vitro* model of this developmental process. The AM14 stromal cell line expresses SCF, G-CSF as well as chordin-like protein that are known to be expressed in mesenchymal cells (Charbord et al., 2002; Oostendorp et al., 2002) and therefore have the potential to promote the survival and self-renewal ability of haematopoietic progenitors (Durand et al., 2007; Jackson et al., 2012).

Conclusion

In summary, using an inducible genetic system which has allowed us to compare NotchIC\(^+\) and NotchIC\(^−\) cells from the same culture we have resolved some of the discrepancies in the literature about the role of this signalling pathway during haematopoietic differentiation. We conclude that within the AGM microenvironment the production of haematopoietic cells from FLK1\(^+\) progenitors is optimal when Notch signalling is below a specific threshold. The opportunity to modulate Notch signalling via its interaction with other signalling pathways such as Wnt and Hedgehog could lead to new strategies for regenerative medicine and the production of haematopoietic cells from pluripotent cells for therapy.

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Appendix A. Supplementary data

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