ENFORCED EXPRESSION OF HOXB4 IN HUMAN EMBRYONIC STEM CELLS ENHANCES THE PRODUCTION OF HAEMATOPOIETIC PROGENITORS BUT HAS NO EFFECT ON THE MATURATION OF RED BLOOD CELLS

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LF was the principal investigator and takes primary responsibility for the paper. MJ, RA, AHT, RA, JE, EO, LM and AE performed the lab work for this study. MJ and RA participated in the statistical analysis. LF, JM, AE and ES co-ordinated the research. LF and MJ wrote the paper. The authors report no potential conflicts of interest.

ABSTRACT (227 words)

We have developed a robust, GMP-compatible differentiation protocol that is capable of producing scalable quantities of red blood cells (RBCs) from human pluripotent stem cells (hPSCs). However, translation of this protocol to the clinic has been compromised by the fact that the RBCs produced are not fully mature; they express embryonic and foetal, rather than adult globins and they do not enucleate efficiently. Based on previous studies we predicted that activation of exogenous HOXB4 would increase the production haematopoietic progenitor cells (HPC) from hPSCs and hypothesised that it might also promote the production of more mature, definitive RBCs. Using a tamoxifen-inducible HOXB4-ER^{T2} expression system we first demonstrated that activation of HOXB4 did indeed increase the production of HPCs from hPSCs as determined by CFU-C activity and the presence of $CD43^{+}CD34^{+}$ progenitors. Activation of HOXB4 caused a modest but significant increase in the proportion of immature $CD235a^{+}/CD71^{+}$ erythroid cells but this did not result in a significant increase in more mature $CD235a^{+}/CD71^{-}$ cells. RBCs produced in the presence of enhanced HOXB4 activity expressed embryonic (ε) and foetal (γ) but not adult (β) globins and the proportion of enucleated cells was comparable to that of controls cultures. We conclude that programming with the transcription factor HOXB4 increases the production of haematopoietic progenitors and immature erythroid cells but does not resolve inherent challenges associated with the production of mature adult-like enucleated RBCs.

INTRODUCTION.

Cell-based therapies such as bone marrow transplantation and blood transfusion are used to treat diseases of the haematopoietic system but these procedures are completely reliant on a limited supply of donor tissue. Thus one goal has been to produce therapeutic haematopoietic cells from a bankable and limitless source of human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPSCs) [1-3]. There are many reports on the production of haematopoietic progenitors cells (HPCs) and mature haematopoietic cells from ESCs and iPSCs but it is widely acknowledged that significant challenges face this field. Differentiation protocols that have been used to date are relatively inefficient and it has proven particularly difficult to produce the most potent HSC capable of long-term reconstitution (LTR-HSCs) and fully mature red blood cells (RBCs) that express adult β globin and enucleate [4-6]. Most of the strategies employed to produce erythroid cells have required the use of stromal cell feeder layers and/or ill-defined serum that would present significant safety issues in clinical translation [7-11]. Feeder-free haematopoietic differentiation strategies have been developed but many of these required lengthy embryoid body stages that prove difficult to control and replicate effectively [8, 12, 13]. Production of fully mature adult-like erythroid cells that undergo appropriate globin switching has been particularly challenging with most studies detecting embryonic (ζ, ε) and fetal (γ) but not adult (β) globin [8, 11, 14]. Although some studies report the production of some enucleated cells none of these demonstrate a convincing and robust degree of enucleation in pluripotent stem cell-derived RBCs [9, 12].

The first wave of erythropoiesis *in vivo* occurs in the yolk sac and is responsible for the production of primitive nucleated erythrocytes. A second wave initially arises in the aorta-gonad-mesonephros region where the first definitive haematopoietic stem cells (HSC) arise that are capable of long term multi-lineage reconstitution (LTR) and production of adult nucleated erythrocytes [15]. We hypothesised that the limited production of nucleated erythrocytes that can be achieved from pluripotent stem cells *in vitro* might reflect the “primitive” state of the progenitors that are produced in this system. This is supported by the fact that the production of LTR-HSCs *in vitro* from pluripotent stem cells that are capable of multi-lineage long-term reconstitution has proven

particularly difficult to achieve. We reasoned that HOXB4 might be a transcription factor that could programme hPSC-derived haematopoietic cells to a more 'definitive' phenotype because it had been shown to confer long-term reconstitution ability on primitive yolk sac cells and mouse ESCs [16-18]. Furthermore our studies in the murine ESC system demonstrated that HOXB4 could promote a paracrine effect and thus its actions could mimic the action of stromal cell co-culture [19-21]. The effects of exogenous HOXB4 on human ESCs has been less clear with contradictory outcomes likely related to the range of differentiation protocols used and the different expression strategies used leading to variations in the level and timing of expression[19]. In this study we used a controllable HOXB4 expression system where we could activate HOXB4 at different time points in a well-defined differentiation protocol.

We have developed a step-wise, serum- and feeder-free clinical grade protocol for both the growth and maintenance of hESC and hiPSCs and for their subsequent expansion and differentiation into erythroid cells [22](Olivier, submitted for publication). We have tracked the emergence of haematopoietic progenitor cells (HPCs) by CFU-C and flow cytometry and have then tested the effects of a tamoxifen-inducible expression of HOXB4 in this defined and robust haematopoietic differentiation protocol. In keeping with murine studies we report that enforced expression of HOXB4 results in a specific increase in the production of multi-lineage HPCs. We also noted a modest increase in the production of immature erythroid cells but overall there had no significant effect on their subsequent maturation. Erythroid cells that were produced in the presence of HOXB4 failed to undergo efficient enucleation and expressed embryonic (ε) and foetal (γ) but not adult (β) globin. Thus enforced expression of HOXB4 does not overcome the major hurdle in the production of mature enucleated cells required for transfusion.

MATERIALS AND METHODS

Maintenance and differentiation of hESCs

Human pluripotent stem cell lines including H1 [23] and Runx1C-GFP [24] were routinely maintained in StemPro® hESC serum free medium on CELLstart™ matrix (Invitrogen) with the addition of bFGF 20 ng/ml (Life Technologies PHG0021), and passaged (1:4) when 70-80-%

confluent using StemPro® EZPassage™ tools (Invitrogen). Differentiation was carried out in a step-wise, serum and feeder free protocol as described (Olivier et al., submitted for publication). Briefly one confluent well of human ESCs was cut and transferred to 2 wells (6 well plates (cell repellant surface), Greiner Bio one, Cat No. 657970) then cultured in 3 ml per well of Stemline® II Hematopoietic Stem Cell Expansion Medium (Sigma, Cat No. S0192) with the addition of BMP4 10ng/ml (R+D systems, cat 314-BP-010), VEGF 10ng/ml (R+D systems, cat 293-VE010), Wnt3a 10ng/ml (R+D systems, cat 5036-WN010), Activin A 5ng/ml (R+D systems, cat 338-AC010 and GSK-3β Inhibitor VIII 2μM (Merk Millipore, cat A014418). EBs formed spontaneously and after 2 days the following cytokines were added in 500μl Stemline II per well: BMP4 20ng/ml, VEGF 30ng/ml, Wnt3a 10ng/ml, Activin A 5ng/ml, FGFa 10ng/ml (Life Technologies , cat PHG0014), SCF 20ng/ml (Life Technologies PHC 2111) and inhibitor VIII 2μM. EBs were disaggregated on day 3 with 0.5ml/well StemPro® Accutase® Cell Dissociation Reagent for 3 min at 37^0C, before gently pipetting 10 times and adding pre-warmed media. After centrifugation, cells were replated at 2 x 10^5 cells per well (6 well plates (Corning)) in 3 ml Stemline II with the addition of BMP4 20ng/ml, VEGF 30ng/ml, FGFa 10ng/ml, SCF 30ng/ml, IGF2 10ng/ml (R+D systems, cat 292-G2), TPO 10ng/ml (R+D systems, cat 288-TPN-25), Heparin 5μg/ml (Sigma, cat H3149), IBMX 100μM (Sigma, cat I5879) and β-estradiol 0.4ng/ml (Sigma, cat E2257). On day 7 the floating cells and medium were carefully removed and centrifuged before replating all cells back into the same dishes (containing any adherent cells) in 3 ml fresh Stemline II containing BMP4 20ng/ml, VEGF 30ng/ml, FGFa 10ng/ml, SCF 30ng/ml, IGF2 10ng/ml, TPO 10ng/ml, Heparin 2.5μg/ml and IBMX 100μM.. On day 9 the same day 7 cytokines were topped up in 500 μl Stemline II per well. On day 10 all cells were aspirated from the well, centrifuged and accutased in 500μ□ 37^0c for 3 min and 3 x 10^5 cells were replated per well in IBIT media (240ml Iscove Basal Medium, Biochrome FG-0465), 1%BSA (Invitrogen G10008-01), 10 μ g/ml insulin (Sigma I9278), 0.2mg/ml transferrin (Sigma, cat T0665) and 500μl β-mercaptoethanol (Invitrogen, cat 31550-010), with the addition of Hydrocortisone 10μM, SCF 50ng/ml, Flt3L 16.7ng/ml (Peprotech 300-19), BMP4 6.7ng/ml, IL-3 6.7ng/ml (Peprotech, cat 213-13), IL-11 6.7ng/ml (Peprotech 200-11) EPO 3U/ml (R+D 287-TC-500) and IBMX 100μM. Every 2 days the cytokines were topped up in a volume of 500 ul per well.

On day 17 cells were centrifuged, counted and 1 x 10^6 cells were plated per well in IBIT with the addition of Hydrocortisone 10 μ M, SCF 20ng/ml, IGF1 (20ng/ml, Peprotech 100-11), IL-3 6.7ng/ml, IL-11 6.7ng/ml and EPO 3U/ml, Cytokines were replenished at the same concentrations every 2 days in a volume of 500 μl per well.

Flow Cytometry

Cells were harvested and 2 x 10^5 cells were aliquoted in PBS containing 1% BSA (PBS/BSA) and centrifuged (200g) for 5 minutes. Antibodies were added directly to cell pellets that were then re-suspended to a final volume of 100□l, incubated for 30 minutes on ice, washed in 5 ml BSA/PBS then re-suspended in 300□l. Samples were analysed on a LSR Fortessa (BectonDickinson) using FACS Diva acquisition and FlowJo analysis software, or Sorted on a FACS ARIA (Becton Dickinson). Anti-human antibodies included CD71-FITC (eBioscience, cat 11-0719) CD43-APC (eBioscience, cat 17-0439-42), glycophorinA (M) (CD235a) efluor450 (eBioscience, cat 48-9884), CD34-PE (eBioscience, cat 12-0349) CD41a-FITC (BD Biosciences) CD45-V450 (BD Biosciences) and CD144-PE (Beckman Coulter). Mouse IgG1 APC isotype control (eBioscience 17-4714-81) and Mouse IgG1 PE Isotype Control (eBioscience 12-4714-81) were used and shown in Supplementary Figure 2).

CFU-C assay

Single cells (5x 10^3 or 10^4) were plated into1.5 ml Methocult media containing SCF, G-CSF, GM-CSF, IL-3, IL-6, Erythropoietin (Stem Cell Technology) in 35 mm dish low attachment plates (Greiner), incubated at 37^{o}C in a humid chamber then scored for haematopoietic colony formation after 12-15 days.

Magnetically activated cell sorting (MACS)

MACS was carried out according to manufacturers instructions (Miltenyi). Briefly, cells were resuspended in PBS containing 2mM EDTA, incubated in FC blocking agent for 10 minutes at room temperature, combined with microbead-labelled antibody (Miltenyi (CD43:130-091-333 or

CD34:130-046-702) for 20 minutes at 4℃ then washed and resuspended in PBS/EDTA. Labelled cells were then applied in 250μ□ aliquots to the MACS column, left to stand in the magnet for 5 minutes then washed three times with 0.5ml PBS/EDTA. Cells were released from the column according to manufacturers instruction and the purity of sorted cells was assessed by flow cytometry.

hESC transfection

H1 hESC were fed with fresh Stempro medium containing 20 ng/ml bFGF (R+D systems) and 10 μM Rock inhibitor (Y-27632 Calbiochem) at least 1 hour prior to electroporation as described [25]. Single cell suspensions were generated using accutase then cells were washed and resuspended in Stempro medium (10^7 cells per 0.5ml) with 30μg of linearised pCAG-HOXB4ER^{T2}-IRES-PURO vector [20]. Cells were electroporated (Bio Rad Gene pulser: 320V 250 μFd), plated on CellStart matrix in StemPro media containing 20 ng/ml bFGF (R+D systems) and 10 μM Rock inhibitor (Y-27632 Calbiochem). Resistant colonies were selected in 0.6μg/ml puromycin for 10 days then single colonies were picked, expanded and screened for expression of HOXB4 by Western blot (Supplementary Figure S1).

High-performance liquid chromatography (HPLC)

HPLC globin chain separation was performed using a protocol modified from Lapillonne et al [9]. Erythroid cells were washed 3 times in PBS, lysed in 50μl 0.1% trifluoroacetic acid (TFA) in water. The cells were then centrifuged at 13,000g at 4^{o}C for 10mins and the supernatant collected for HPLC analysis. Globin chain separation was performed by injecting 10μl of the supernatant onto a 1.0 x 250 mm C4 column (Phenomenex, UK) with a 42% to 56% linear gradient between mixtures of 0.1% TFA in water and 0.1% TFA in acetonitrile at flow rate of 0.05mL/min for 50min on a HPLC Ultimate 3000 system (Dionex, UK). The column temperature was fixed at 50^{o}C during analysis and the UV detector was set at 220nm. Lysates from adult peripheral blood and fetal liver were used as positive controls. Elution times of peaks generated were compared with that of the control

peaks for identification. The area under the curve was used to calculate the proportion of each globin peak from each sample.

RESULTS

Monitoring haematopoietic progenitor production.

We monitored the production of HPCs throughout the differentiation protocol by flow cytometry and CFU-C assays (Figure 1). The proportion of cells expressing CD34 and CD43 peaked between day 6 and 10 (Figure 1A, B) and the appearance of cells carrying these HPC markers coincided with the presence of CFU-C colonies (Figure 1E). The majority of CFU-C generated from day 7 cells were small primitive-like colonies (Figure 1Di-iv) with larger, more robust colonies being produced by cells in day 10 cultures (Figure 1Dv-viii). The absolute numbers of the $CD34^+$ and $CD43^+$ pupulations within the cultures at day 3 and day 10 were calculated. The most abundant population at day 10 was the $CD34^+/CD43^+$ double positive population (Figure 1C). To further characterise the HPCs generated in our differentiation protocol we used the $RUNX1C^{GFP/w}$ hESC line carrying a GFP reporter under the control of the endogenous Runx1C promoter [24] that is considered to be a marker of definitive haematopoietic stem cells [26]. The majority of double positive, $CD34^+CD43^+$ cells at day 10 also expressed Runx1C-GFP confirming their definitive progenitor-like phenotype (Figure 1F). To determine which cell population contained the haematopoietic activity, we MAC-sorted differentiating ESCs at day 7 based on expression of CD34 (Figure 2A) and at day 10 based on CD43 expression (Figure 2C) then analysed the enriched populations for haematopoietic colony activity (Figure 2B,D). The maximum number of CFU-Cs was found within the $CD34^{hi}$ population at day 7 and $CD43^+$ population at day 10. Further analyses of FAC-sorted day10 cells demonstrated that CFU-C activity was contained within the $CD43^+$ $CD34^+$ and the $CD43^+$ $CD34^-$ (Figure 2E,F).

Enforced expression of HOXB4

HOXB4 has been widely reported to enhance the production of haematopoietic cells from murine ESCs but its effect on the differentiation of hESCs is less clear. Variable results in hESC studies are likely due to the variety of differentiation strategies employed and the different systems used to

express HOXB4 resulting in differing levels of expression [19]. Based on our previous studies we hypothesised that activation of exogenous HOXB4 would increase the production of HPC during the differentiation process [20] and might also act to promote a more "definitive-like progenitor capable of differentiating into mature adult-like RBCs that could express adult globins and enucleate efficiently. To assess the effects of HOXB4 in our defined differentiation protocol we transfected H1 hESCs with a tamoxifen-inducible HOXB4-ER^{T2} transgene under the control of the CAG promoter as previously described [20]. We previously confirmed and published that the HOXB4-ER^{T2} fusion protein can localise to the nucleus upon tamoxifen addition and that it is functional in this context by demonstrating the induced expression of the HOXB4 target gene, *Frzb* upon addition of tamoxifen [20]. The expression of the HOXB4-ER^{T2} protein and the haematopoietic differentiation potential of hESCs carrying this transgene were confirmed by Western blotting and CFU-C assays (Supplementary Figure S1).

We first tested the effects of HOXB4 activation on the production of HPCs in differentiating hESCs carrying this transgene in the presence and absence of tamoxifen for 10 days by CFU-C assays and flow cytometry (Figure 3). We observed a significant increase in the number of CFU-Mix and CFU-GM colonies in the presence of tamoxifen in cells expressing the HOXB4-ER^{T2} transgene but not control H1 ESCs (Figure 3B). There was no significant difference in the number of CFU-M, or BFU-E in the presence and absence of tamoxifen in either cell line indicating that enforced expression of HOXB4 preferentially enhanced the production, proliferation or survival of multi-potent HPCs. Consistent with the CFU-C data, the proportion of $CD34^+CD43^+$ double positive cells was increased when HOXB4 was activated by tamoxifen (Figure 3C,D). The overall proportion of cells expressing CD43 did not change when HOXB4 was activated so the increase in double positive $CD34^+/CD43^+$ cells was due to an increase in the number of cells expressing CD34 (Figure 3C).

To assess the effects of enforced HOXB4 expression on RBC maturation we generated HPCs in the absence of tamoxifen and then added tamoxifen to the later stages of differentiation (day 10-24) (Figure 4A). We observed no effect of tamoxifen on the overall proportion of erythroid cells

that expressed CD235a at day 24 but we noted differences in the proportion of those $CD235a^+$ cells that co-expressed CD71 (Figure 4B-D). The CD71 marker is lost as erythroid cells mature and we noted that the proportion of the less mature $CD235a^+/CD71^+$ was significantly increased upon HOXB4 activation (Figure 4C). However there was no significant difference in the proportion of the more mature cells (Figure 4D). Haemoglobinised cells were observed in cultures both in the presence and absence of tamoxifen and the globin profile of these cell populations was analysed by HPLC at day 24 (Figure 5A,B). β globin was detected in control fetal and adult blood samples but hESC-derived RBCs generated predominantly embryonic (ε) and fetal ($\gamma\alpha$ and $\gamma\gamma$) globins. Activation of HOXB4 from either day 10 to 24 (shown here) or from day 0 to 24 did not alter this profile indicating that it had no effect on the maturation of erythroid cells. We predict that altering the activation time period for example, from days 10 to 17 or days 17 to 24 would have a similar negative effect but cannot exclude the possibility that different effects might be observed. We also noted that activation of HOXB4 did not alter the efficiency of enucleation of hESC-derived RBC as assessed by staining with the SYTO Red fluorescent nucleic acid stain (Figure 5C) and compared directly with the white and red cell fractions from peripheral blood (Figure 5D). Globin profiles and enucleation rates were also analysed in cultures that were activated with HOXB4 throughout the differentiation protocol (ie from day 0 to 24) with similar results (data not shown).

As one of the aims of this study was to determine whether HOXB4 activation could enhance the number of RBCs that could be generated, we also assessed the effects of HOXB4 on cell expansion throughout the culture period. There was a 10,000 fold expansion in cell numbers over the 24 day culture period in control cells and in hESCs carrying the HOXB4-ER^{T2} transgene in the absence of tamoxifen but a significant reduction in expansion was observed upon HOXB4 activation (Figure 6). Previous studies have indicated that HOXB4 enhanced the proliferation of HPCs *ex vivo* without significant effect on their differentiation potential [27, 28] but we demonstrate that this cannot be applied to more committed progenitors.

DISCUSSION

The development of strategies to produce transfusion-competent RBCs from a limitless supply of human PSCs would have an enormous impact on this widely used therapy. However, to date, the majority of RBCs produced *in vitro* from this source have an immature phenotype characterised by an embryonic and/or foetal globin profile and nuclear retention [1]. Although there is some evidence that iPSC-derived RBCs can undergo terminal differentiation when transplanted *in vivo,* the nucleated state would preclude the clinical use of these cells [29]. In contrast RBCs differentiated *in vitro* from adult, fetal liver or umbilical cord blood cells do enucleate efficiently demonstrating that it is possible to create a microenvironment *in vitro* that supports the enucleation process [30, 31]. Adult stem cell-derived enucleated RBCs have been used in a proof-of-principal clinical transfusion study [31]. Although co-culture with foetal liver stromal cells can improve the enucleation efficiency of PSC-derived cells the proportion of enucleated cells remains prohibitively low and this type of strategy could not be translated into the clinic. A recent study that analysed the molecular profile of encleating cells derived from hESCs identified a micro RNA (miR-30a) as a potential regulator of the enucleation process demonstrating that it is also possible to modulate the process by altering the intrinsic genetic environment [32].

Based on our previous findings that enhanced expression of HOXB4 could exert a paracrine effect in differentiating ESCs [20] and that combining co-culture with HOXB4 expression did not have an additive effect [21], we set out to test the hypothesis that enhanced expression of this transcription factor could create an microenvironment that would allow the production of haematopoietic progenitors capable of differentiating into mature erythroid cells. We used a defined, feeder-free culture system designed for the production and expansion of erythroid cells [22] (Olivier et al., 2015 (submitted for publication)).

Production of haematopoietic progenitors.

We first monitored the production and phenotype of haematopoietic progenitors produced in this defined step-wise protocol. Over 60% of cells expressed HPC markers between day 8 and 10 and the time course $CD43^{+}CD34^{+}$ cell production correlated with the production of CFU-C clonogenic progenitors. At this stage, $CD43^{+}CD34^{+}$ cells predominantly expressed the Runx1C-GFP reporter supporting their progenitor-like phenotype. The phenotype and potential of PSC-derived

haematopoietic progenitors is consistent with that described for the differentiating hiPSCs on OP9 co-culture system [33-35] and thus our robust step-wise protocol is an appropriate system to assess the effects of specific transcription factors on the differentiation process.

Enhanced expression of HOXB4

We hypothesised that the deficiency in erythroid maturation could be due to the primitive nature of haematopoietic progenitors that are generated *in vitro*. We considered that HOXB4 might be a transcription factor that could programme hPSC-derived haematopoietic cells to a more definitive phenotype because it had been shown to confer long-term reconstitution ability on primitive yolk sac cells and mouse ESCs [16, 17]. The results described in previous studies using human PSCs were contradictory and this was likely due to differences in expression strategies employed and in the differentiation protocols that were used [19]. To resolve discrepancies in the published studies, we used a defined differentiation protocol without feeder cells and an inducible expression system where we could control the timing of expression of HOXB4. We demonstrate that enforced expression of HOXB4 increases the production of multi-lineage haematopoietic progenitors but has no significant effect on the production and maturation of RBCs. Erythroid progenitors that were produced in the presence of HOXB4 failed to undergo efficient enucleation and expressed embryonic (ε) and foetal (γ) but not adult (β) globin. In addition we observed a detrimental effect of HOXB4 activity on cell expansion, thus negating any small increases in cell populations seen by inducing HOXB4.

In summary this defined differentiation system generates haematopoietic progenitors and is robust enough to test the effects of the numerous transcription factors that have been shown to programme haematopoietic development [36-38]. We demonstrate that HOXB4 increased the proportion of haematopoietic progenitor production, but enhanced activity of this transcription factor did not promote red blood cell maturation.

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FIGURE LEGENDS

Figure 1. Representative flow cytometry analysis of cells from day 0 to day 24 of the differentiation protocol using antibodies against CD34 and CD43 (A) and the quantification of % positive (B) was performed from at least three independent experiments. Absolute cell numbers of the differentiation represented in A is shown for day 3 and day10 (C). CFU-C activity was assessed by methylcellulose assays on days 7 and 10 (E) and representative colonies generated from day 7 (D i,- iv) and day 10 cells (D v-viii) are shown. These data represent 3 independent experiments and bars are standard error of the mean. Flow cytometry analysis to assess the expression of Runx1C-GFP in the of CD34/CD43 double positive cell population where Runx1C-GFP$^+$ cells are shown as green and Runx1C-GFP$^-$ cells as red (F).

Figure 2. CD34$^+$ cells from day 7 cultures (A), and CD43$^+$ cells from day 10 cultures (C) were sorted using MACS enrichment and re-analysed by flow cytometry to assess purity (unsorted (red), enriched +ve cells (blue) and flow-through low/negative cells (green). Haematopoietic activity was quantified by plating these cell populations in CFU-C assays (B, D). Day 10 cultures were stained for both CD34 and CD43 (E) and CD43$^+$/CD34$^-$, CD43$^+$/CD34$^+$ double positive and CD43$^-$ CD34$^-$ double negative were plated in CFU-C assays to quantify their haematopoietic activity (F).

Figure 3. Tamoxifen (4OHT, labelled + or -) was added to cultures to activate HOXB4 in H1 hESCs expressing HOXB4-ER^{T2} (labelled HOXB4) or parental H1 hESCs (labelled Control) between day 0 and day 10 (A) then assessed for CFU-C activity (B) and expression of haematopoietic progenitor markers, CD34 and CD43 by flow cytometry (C,D). Data were generated from four independent experiments with error bars representing standard error of the mean (*, p=0.03; **, p=0.04)

Figure 4. HOXB4 was activated with tamoxifen (4OHT) from day 10 to 24 (A). Production of erythroid cells was monitored by expression of CD235a and CD71 by flow cytometry at day 24 (B-

D). Data were generated from four independent experiments with error bars representing the standard error of the mean (* p=0.05).

Figure 5. High performance liquid chromatography (HPLC) analysis of cell lysates generated at day 24 of the differentiation protocol from Control (H1) and HOXB4 activated cells (in the presence (+T) and absence (-T) of tamoxifen)(A). The amount of the different globins detected as a proportion of the total globin content was calculated in six independent experiments (B). Adult peripheral blood and foetal blood were included for comparison. Enucleation of Control and HOXB4-expressing hESCs in the presence (+T) and absence (-T) of tamoxifen was assessed by flow cytometry using the nuclear Sytored stain and the erythroid marker, CD235a (C). A representative histogram of Sytored staining of control enucleated peripheral blood samples (blue), nucleated mononuclear cells (green) and hESCs after HOXB4 activation (orange) are shown together with unstained control hESCs (D).

Figure 6. Cell expansion data for H1 hESCs (Control) and HOXB4-ER^{T2}-expressing hESCs (HOXB4) in the presence (+) and absence (-) of tamoxifen for 24 days. Data are expressed as fold expansion compared to the number of cells at day 3 and represent the mean of six independent experiments (+/- standard error).

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Figure 1

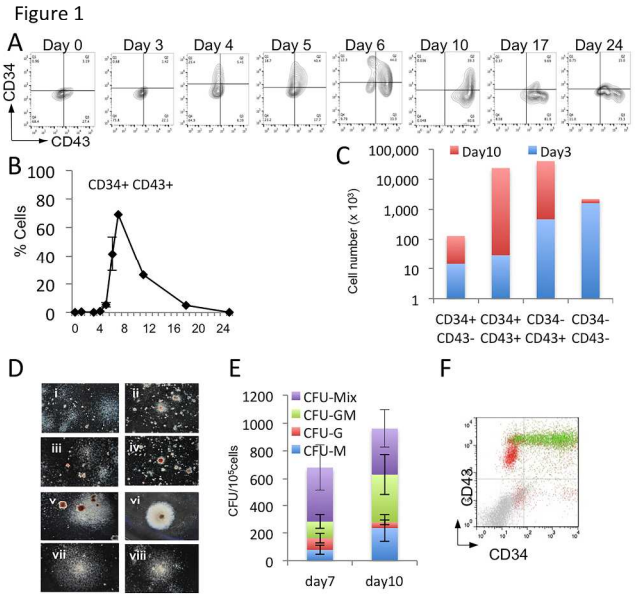

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Figure 2

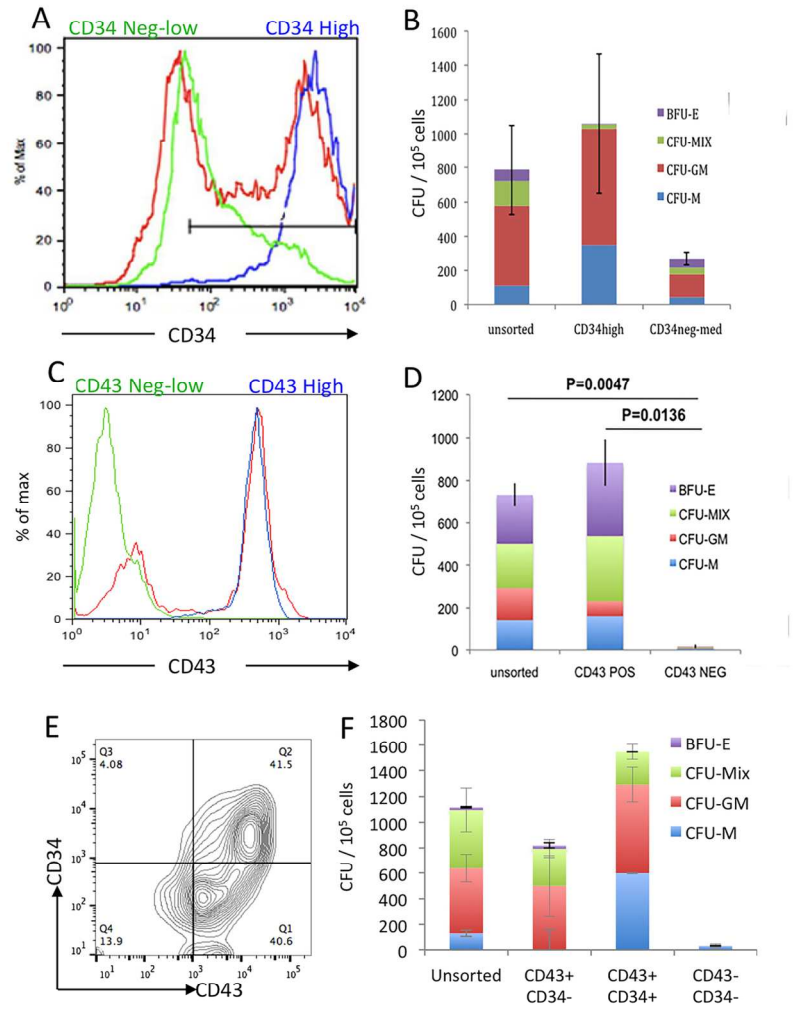

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Figure 3

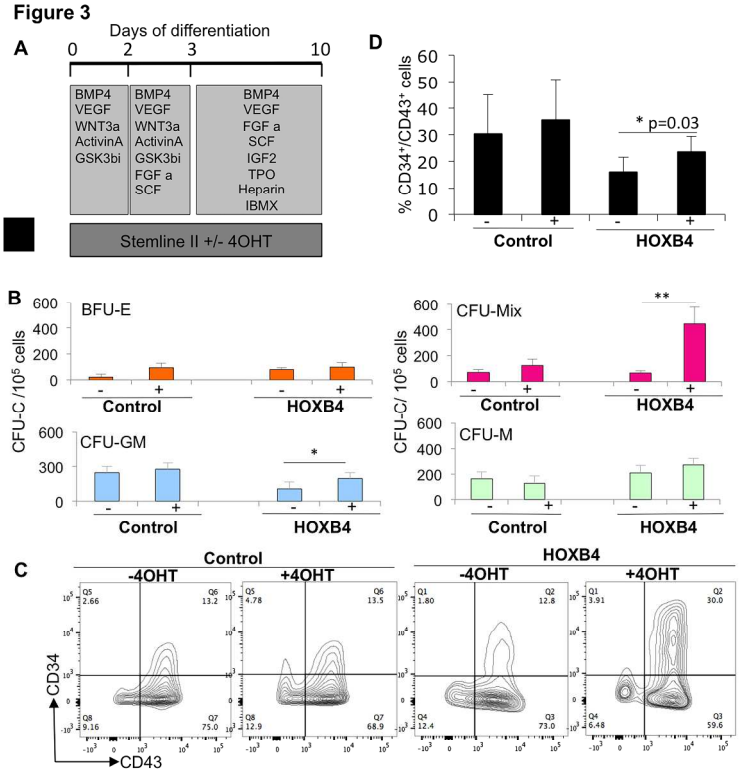

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Figure4

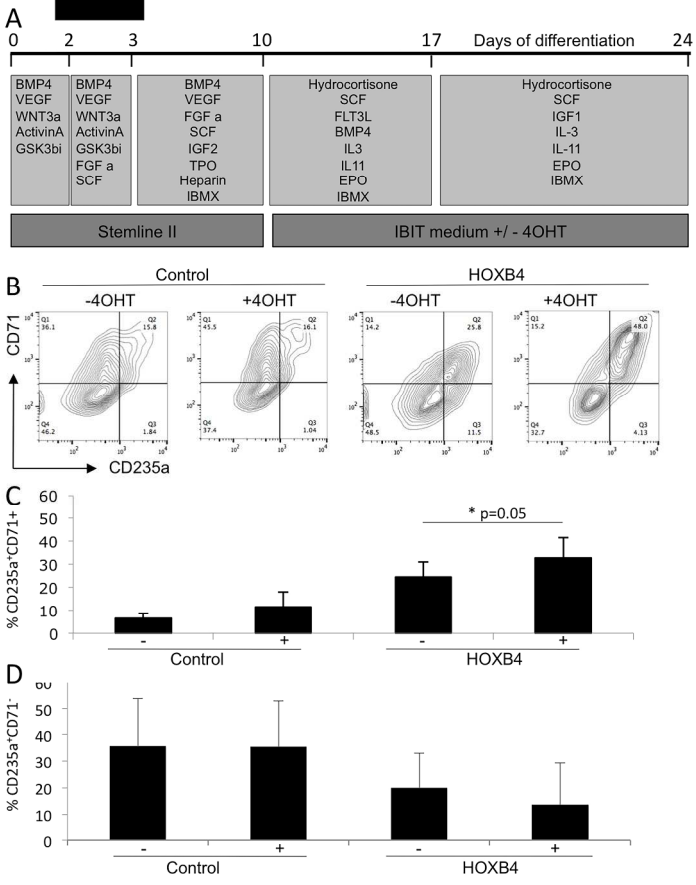

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Figure 5

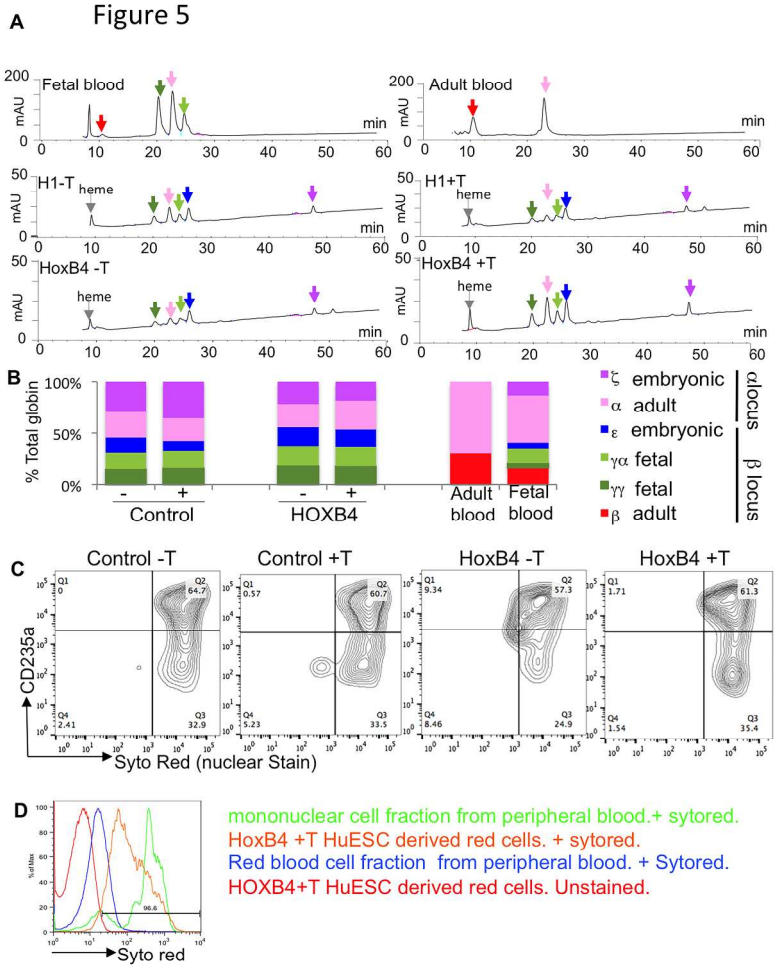

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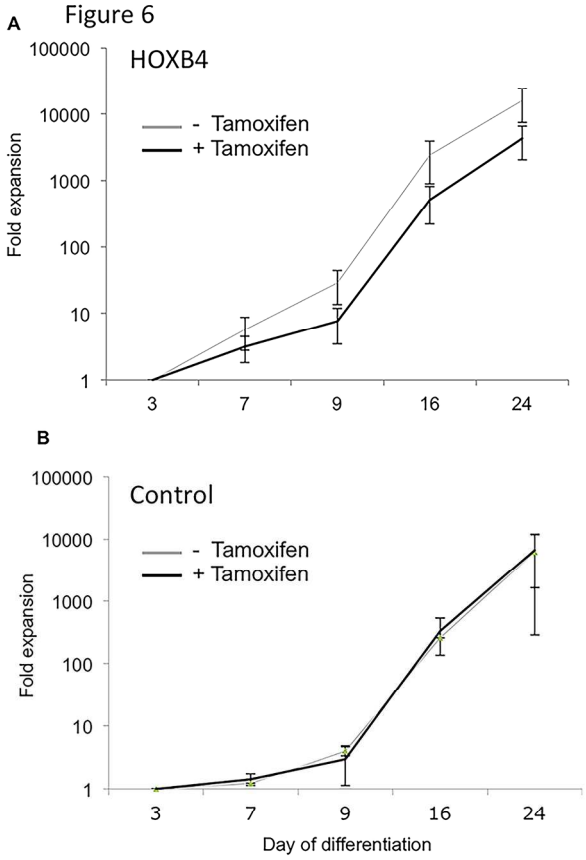
Figure 6



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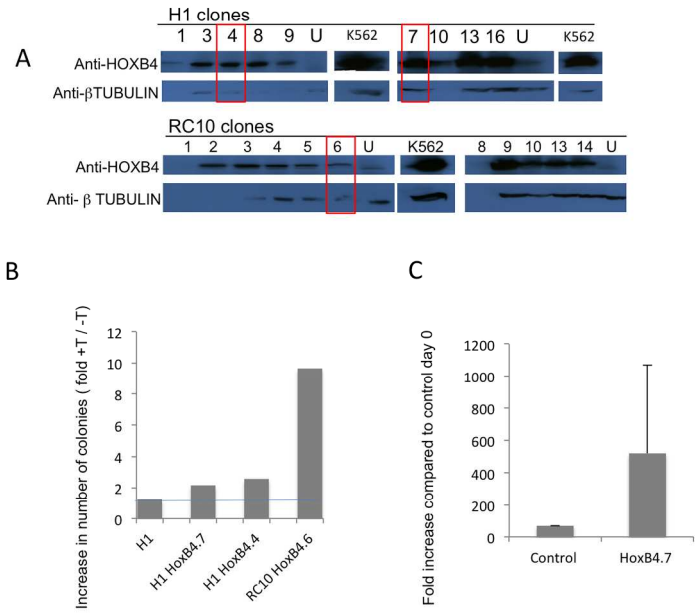
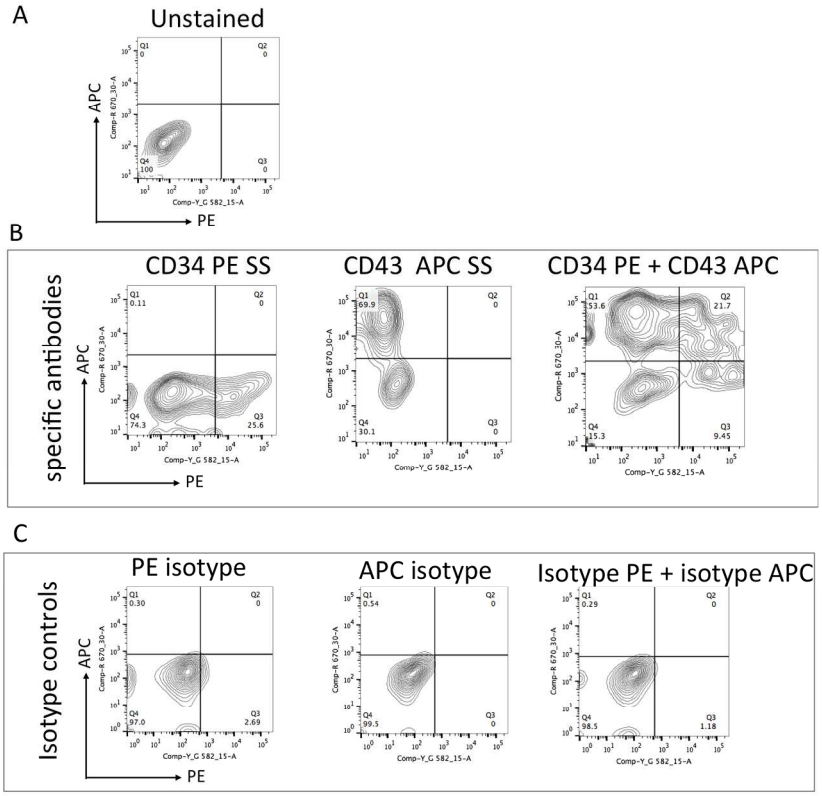
Supplementary Figure S1



Supplementary Figure S1. hESC lines, H1 and RC10 (Roslin cells http://roslincells.com) were transfected with the HOXB4-ER^{T2} expression plasmid and drug-resistant transfectant clones were screened by Western blotting using an anti-HOXB4 and the loading control (anti-β-tubulin) antibodies (A). H1HOXB4.7, H1 HOXB4.4 and RC10HOXB4.6 were further screened for their potential to differentiate into haematopoietic progenitors using CFU-C assays (B). Clone H1HOXB4.7 was selected for further study as clone H1HoxB4.4, proliferated less well and RC10HOXB4.6 proliferated faster than controls. The hESC line HoxB4.7 demonstrated enhanced expression of HOXB4 compared to control cells at day 0 and later in the differentiation protocol at day 17 (C) and was used for further study.

240x346mm (300 x 300 DPI)

Supplementary Figure S2. Isotype control antibody staining



Day 10 differentiated hESCs were either unstained (A), stained with specific antibodies (B) or isotype controls (C) before flow cytometry analysis. No staining was observed with the isotype control antibodies.

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