ES cell-derived hemangioblasts remain epigenetically plastic and require PRC1 to prevent neural gene expression

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Running Title: Epigenetic plasticity of hemangioblast-committed cells

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Abstract

Many lineage-specific developmental regulator genes are transcriptionally primed in ES cells; RNA Pol II is bound at their promoters but is prevented from productive elongation by the activity of Polycomb Repressive Complexes (PRC) 1 and 2. This epigenetically poised state is thought to enable ES cells to rapidly execute multiple differentiation programs and is recognised by a simultaneous enrichment for H3K4me3 and H3K27me3 modifications (bivalent chromatin) across promoter regions. Here we show that the chromatin profile of this important cohort of genes is progressively modified as ES cells differentiate towards blood-forming precursors. Surprisingly however, neural specifying genes such as Nkx2-2, Nkx2-9 and Sox1 remain bivalent and primed even in committed hemangioblasts, as conditional deletion of PRC1 results in overt and inappropriate expression of neural genes in hemangioblasts. These data reinforce the importance of PRC1 for normal hematopoietic differentiation and reveal an unexpected epigenetic plasticity of mesoderm-committed hemangioblasts.

Introduction

Tractable mouse ES cells in which differentiation pathways can be accurately modelled in vitro and in vivo has revolutionised our understanding of lineage specification\(^1\) and provided critical information on the linear relationships between precursor cells and their progeny\(^2\-4\). Using a well-characterised model of hematopoietic differentiation\(^3\,5\) we have investigated stage-specific changes in chromatin at specific genes in ES cells upon mesoderm induction. Undifferentiated ES cells contain hyperdynamic chromatin\(^6\) where much of the genome is transcribed at a low level\(^7\) and many developmental regulator genes are transcriptionally primed and ‘bivalently marked’ with histone modifications associated with both gene activation (such as tri-methylation of lysine 4 of histone 3, H3K4me3) and Polycomb-mediated repression (such as H3K27me3)\(^8\-10\). This marking is thought to reflect the lineage plasticity of ES cells since it is resolved upon differentiation (for example in neural precursors\(^10\,11\)), and regained upon reprogramming of differentiated cells to induced pluripotent stem (iPS) cells\(^12\). Initial comparisons between ES, hematopoietic stem cells, and lymphocytes showed reduced chromatin accessibility and loss of bivalent marking in hematopoietic cells\(^8\) but did not evaluate whether such changes occurred before, coincident with, or after cells become functionally restricted. By
examining the chromatin status of a panel of genes in intermediate stages of differentiation we show here that several lineage-inappropriate (neural-specifying) genes remain bivalent and transcriptionally poised in committed hemangioblasts, since the removal of Polycomb Repressive Complex 1 (PRC1) activity results in overt gene de-repression.

Materials and methods

Cell culture and differentiation
Bry-GFP \(^5\) and Ring1a \(^-\)/Ring1b \(^{fl/fl}\)/Cre-ERT \(^13\) ES cells were cultured and differentiated as described \(^5\). Embryoid bodies were dissociated, stained using a PE-conjugated anti-Flk1 antibody (BD Biosciences) and cells FACS-sorted on GFP (Bra/T) and Flk1 expression. Hematopoietic potential was tested using colony assays as described in supplementary methods.

Epigenetic analysis
Replication timing and gene expression was analysed using previously published methods \(^14\). Embryoid bodies were BrdU pulse-labelled before dissociation and sorting. Chromatin from Bra/T-GFP+/Flk1+ cells was immunoprecipitated as described in supplementary methods.

Results and Discussion
Bry-GFP ES cells that carry a GFP reporter under the control of the mesoderm-associated Brachyury (Bra/T) gene \(^3,5\) were differentiated into embryoid bodies and cells representing epiblast (GFP-/Flk1-, day 2.5), early mesoderm (GFP+/Flk1-, day 3.75) and hemangioblast stages (GFP+/Flk1+, day 3.75) of differentiation were isolated by FACS, as previously described \(^5\) (Figures 1A-B) and verified by expression analysis. Expression of pluripotency-associated factors declined (Oct4/Pou5f1) or was lost (Rex1/Zfp42, Sox2) upon differentiation, while mesoderm-associated genes (such as Bra/T, Flk1/Kdr and Ikaros/Ikzf5) were induced, and genes important for neuroectoderm specification were detected only at low levels (Math1/Atoh1, Nkx2-2, Nkx2-9, Sox1) \(^15-18\) similar to undifferentiated ES cells (Figure 1B and S1A). Importantly, epiblast-stage cells readily formed colonies when re-plated in ES media (170/1000 cells), but this capacity was not retained by cells expressing Bra/T-GFP (Figure 1C), consistent with a loss of pluripotency and commitment to mesoderm at this stage.
As accessible chromatin domains tend to replicate earlier in S-phase than heterochromatic condensed domains\textsuperscript{19}, we used replication timing analysis to assess dynamic chromatin changes accompanying differentiation\textsuperscript{8,20-22}. Temporal replication of ES-associated (\textit{Rex1, Sox2}), mesoderm- (\textit{Bra/T, Flk1, Ikaros, Myf5}) and neuroectoderm-associated (\textit{Math1, Sox1, Nkx2-2, Nkx2-9}) genes was evaluated at different stages of hemangioblast differentiation (Figure 1D, summarised in S1B) using established methods and controls\textsuperscript{14}. As anticipated, \textit{Rex1} and \textit{Sox2} replication was earlier in ES cells (black lines) than hemangioblasts (red lines), consistent with reduced chromatin accessibility and gene expression upon differentiation, while replication of constitutively accessible (\textit{\alpha-Globin}) or heterochromatic (X141) controls remained unaffected. Three mesoderm-associated genes showed slight (\textit{Bra/T, Ikaros}) or significantly (\textit{Flk1}) advanced replication in response to differentiation, while neural-specifying genes either retained early replication profiles throughout (\textit{Nkx2-2, Nkx2-9} and \textit{Sox1}) or, in the case of \textit{Math1}, were delayed progressively upon differentiation (Figures 1D, S1B). These data showed that while mesoderm commitment results in changed epigenetic profiles, several genes encoding neural specifiers remained early replicating in hemangioblasts in contrast to lymphocytes and hematopoietic stem cell lines (FDCPmix A4 and \textit{Pax5-/-} pro-B) characterised previously\textsuperscript{8} (summary Figure S1B).

To directly determine whether these promoters were bivalently marked in hemangioblasts, we performed chromatin immunoprecipitation (ChIP) on purified Bra/T-GFP+/Flk1+ samples, using antibodies specific for H3K4me3, H3K27me3 and total H3. Previous reports had shown that the promoters of \textit{Bra/T, Flk1, Ikaros, Math1, Sox1, Nkx2-2, Nkx2-9} (but not \textit{Myf5}) are bivalent in mouse ES cells\textsuperscript{10}. In hemangioblasts, we detected H3K4me3 at the promoters of expressed genes (\textit{Bra/T, Flk1}, green, Figure 2A), while H3K4me3/H3K27me3 was co-detected at \textit{Ikaros, Sox2, Math1, Sox1, Nkx2-2} and \textit{Nkx2-9}. Similar bivalent marking of these genes was observed in CD41+-enriched hematopoietic progenitor populations (data not shown). Verification that the neural-associated genes \textit{Sox2, Math1, Sox1, Nkx2-2} and \textit{Nkx2-9} contained bivalently marked chromatin was shown by sequential ChIP\textsuperscript{9} in which H3K4me3-containing fragments were enriched by a second round of precipitation with antibodies to H3K27me3 (Figure 2B). Furthermore, locus-wide profiling of \textit{Nkx2-2}, a gene-poor region on mouse chromosome 2 that has been extensively characterised as a prototype ‘bivalent domain’ in ES cells\textsuperscript{9,23}, showed that the distributions of H3K4me3 and of H3K27me3 were largely preserved in Bra/T-GFP+/Flk1+hemangioblasts (Figure 2C).
Bivalent marking of developmental regulator genes in ES cells is associated with a primed transcriptional state in which RNA PolII is bound, phosphorylated at Serine5, but prevented from elongating by polycomb repressive complexes; removal of PRC1 or PRC2 results in inappropriate gene up-regulation (de-repression). Abundant PRC1 transcripts (Ring1a, Ring1b, Mel18, Bmi1) and Ring1b protein was detected in both undifferentiated ES cells and cells at successive stages of hemangioblast differentiation (Figures S3A-B) and ChIP analysis showed Ring1b binding at Math1, Sox1, Nkx2-2, and Nkx2-9 promoters in Bra/T-GFP+Flk1+ cells (Figure 2D). To ask whether these genes were indeed functionally primed in mesoderm-committed hemangioblasts we used Ring1a-deficient ES cells where PRC1 activity can be withdrawn upon tamoxifen-induced Ring1b removal (CreER$_{T2}$ cells) (Figure 2E). Removal of Ring1b in Flk1+ hemangioblasts (Figures S3C-D) resulted in overt expression of Nkx2-2, Nkx2-9 and Sox1 at day 3 (Figure 2F). In contrast to ES cells, where PRC1 loss also caused an up-regulation of downstream haematopoietic regulators (such as Scl, Runx1 and Fli, upper panel Figure S3E), expression of these genes by hemangioblasts was unaffected by PRC1 withdrawal (Figure S3E, lower panel). Taken together, these data show that there is extensive remodelling of lineage-appropriate genes upon mesoderm commitment, yet several lineage-inappropriate genes capable of executing an alternative fate, remain bivalent and primed for expression. Ring1b-depleted Flk1+ hemangioblasts were unable to generate hematopoietic colonies (Figure 2G), a defect that was evident after a single day of tamoxifen treatment (Figure S3F) confirming the importance of PRC1 in the proliferation and differentiation of stem cells.

In summary, our data demonstrate that many neural-specifying genes remain bivalently marked and functionally primed for expression in ES-derived committed hemangioblasts. These include Sox1, Nkx2-2, Nkx2-9 (Figure 2A-B), Ngn1, Ngn2, Msx1 (data not shown) and Math1, a gene that although bivalent showed a delayed timing of replication and insensitivity to PRC1-withdrawal upon mesoderm induction. Collectively, these data suggest that hemangioblast precursors may be much more plastic than has previously been appreciated and argue that lineage restriction is probably not the result of an abrupt stage-specific loss of potential (as has been suggested), but rather reflects a more gradual and progressive decline in accessibility of multiple lineage-inappropriate genes over time.
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Authorship
Contribution: LM, HJF, JSR and SP did the experiments, the authors collectively designed the study and LM, HFJ, MM and AGF wrote the paper.
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References


**Figure legends**

**Figure 1. Progressive changes in the replication timing of developmental regulator genes upon mesodermal differentiation of ES cells.** (A) Isolation of cells at sequential stages of ES cell induction to hematopoietic progenitors. Bry-GFP ES cells were differentiated in embryoid bodies (EB), harvested at different times and samples isolated by FACS-sorting on the basis of GFP and Flk1 expression. (B) Upper panel shows GFP (Bra/T) and Flk1 expression by ES cells (Day 0) and in dissociated embryoid bodies (2.5, 3.75 days of differentiation), lower panel: Western blot analysis of Oct4 and Sox2 levels in sequential 3-fold dilutions of whole cell extracts from ES and FACS-sorted cell populations a, b, c and d. Detection with tubulin antibody is shown as a loading control. (C) Equal numbers of undifferentiated ES cells and FACS-sorted differentiated cell populations were replated in ES cell medium containing LIF for 10 days on feeder layers and colonies scored after staining with methylene blue. Values are the average and standard deviation from two experiments carried out in triplicate. Asterisks indicate significantly different numbers of colonies compared to ES cells (p<0.05, student’s t-test). (D) Replication timing of control loci (α-Globin, X141), ES- (Rex1, Sox2), mesodermal- (Bra/T, Flk1, Ikaros, Myf5) and neural-specific genes (Math1, Sox1, Nkx2-2, Nkx2-9) in undifferentiated ES cells (black), and ES-derived GFP-/Flk1- (epiblast, grey), GFP+/Flk1- (mesoderm, green) and GFP+/Flk1+ (hemangioblast, red) samples. The non-expressed non-bivalent Myf5 locus replicates late throughout, whereas the replication profile of Ikaros, a bivalent locus, is variable (according to developmental stage) but remains early. For each locus, the abundance of newly replicated DNA was measured by quantitative real-time PCR in six cell-cycle fractions; G1, S1-S4 and G2/M as described in supplementary Figure S2. Values shown are the amount of newly synthesized DNA, calculated as a percentage of total (sum of all fractions). Average and standard deviation from two-three experiments are shown.

**Figure 2. Bivalent chromatin and PRC1-mediated repression of neural genes is retained in hemangioblast-committed cells.** (A) Analysis of modified histones at the promoters of ES- (Sox2, Oct4, Rex1; blue bars), neural- (Math1, Nkx2-2, Nkx2-9, Sox1; red bars), mesodermal- (Ikaros, Bra/T, Flk1, Myf5; green bars) and control genes (Gapdh, black bar) by immunoprecipitation of chromatin from FACS-sorted GFP+/Flk1+ (hemangioblast) cells using antibodies specific for H3K4me3 (upper panel) or H3K27me3 (lower panel). Enrichment was measured by quantitative real-time PCR and is presented.
relative to total H3. As a control, IgG (open bars) was used for ChIP in parallel (values for most genes were low and barely visible). The average and standard deviation of four experiments are shown. Threshold levels, based on the enrichment at negative control loci (Myf5 for H3K4me3, Flk1 for H3K27me3), are indicated in grey. (B) Sequential ChIP analysis of GFP+/Flk1+ (hemangioblast) cells using first anti-H3K4me3 followed by a second round of IP using anti-H3K27me3 antibody (filled bars) or non-specific IgG control (open bars). Genes are colour-coded as in panel A, average and standard deviation of three experiments are shown, and threshold levels, based on the enrichment at a negative control locus (Flk1), is indicated in grey. (C) Domain-wide profiling of histone methylation along the Nkx2-2 locus; the positions of a conserved upstream control element (grey), the transcription start site (arrow), coding regions (black), UTR (light grey), an adjacent gene (Rik) and PCR amplicons are indicated in the upper panel, where scale = 1 kb. Enrichment of H3K4me3 (upper) and H3K27me3 (lower) across the Nkx2-2 locus was assessed by ChIP using IgG as controls (white bars, barely visible) in ES and GFP+/Flk1+ (hemangioblast) cells. Average and standard deviation from 3 experiments are shown. (D) ChIP analysis of GFP+/Flk1+ (hemangioblast) samples using anti-Ring1b antibody (filled bars) or IgG (open bars). Enrichment levels were measured by real time-PCR and are expressed relative to 10% input. Genes are colour-coded as in panel A. The average and standard deviation from three experiments are shown. Threshold levels, based on the enrichment at the expressed (Ring1b negative) Flk1 locus is indicated in grey. (E) Strategy for withdrawal of Ring1a/Ring1b activity from CreERT2 ES cells, or from differentiated hemangioblasts, based on tamoxifen treatment (3 days). (F) Quantitative RT-PCR analysis of candidate gene expression (colour-coded as in panel A), in Ring1a<sup>-/-</sup>/Ring1b<sup>fl/fl</sup>/Cre-ER<sub>T2</sub> undifferentiated ES cells (upper panel) or embryoid body-derived Flk1+ hemangioblast cells (lower panel) three days after addition of 800 nM Tamoxifen (Tam) to delete Ring1b (coloured bars) versus untreated controls (white bars). Values were normalized to a housekeeping gene (Hmbs) and expressed as fold change relative to untreated. The average and standard deviation from three experiments are shown. Asterisks indicate significantly upregulated expression in tamoxifen treated samples (p<0.05, student’s t-test). (G) Hematopoietic colony assays were performed in triplicate for untreated (-) and tamoxifen treated (+) samples; FACS-sorted Flk1+ Ring1a<sup>-/-</sup>/Ring1b<sup>fl/fl</sup>/CreER<sub>T2</sub> cells were replated in differentiation medium (Exp1 and Exp2) or blast medium (Exp2*) with or without tamoxifen for 3 days before dissociation and scored 6 days after replating in
semisolid hematopoietic medium. Asterisks indicate significantly different numbers of colonies in treated compared to untreated samples (p<0.05, student’s t-test).
Figure 1
Figure 2

Enrichment relative to input

Enrichment relative to H3

mRNA level (fold change relative to -Tam)
ES cell-derived hemangioblasts remain epigenetically plastic and require PRC1 to prevent neural gene expression

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