# SOX7 regulates the expression of VE-cadherin in the haemogenic endothelium at the onset of haematopoietic development

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## SUMMARY

At early stages of vertebrate ontogeny, blood and endothelial cells develop from a common mesodermal progenitor, the haemangioblast. Upon haematopoietic commitment, the haemangioblast generates blood precursors through populations of endothelial cells with haemogenic properties. Although several transcription factors have been implicated in haemangioblast differentiation, the precise mechanisms governing cell fate decisions towards the generation of haemogenic endothelium precursors remain largely unknown. Under defined conditions, embryonic stem (ES) cells can be differentiated into haemangioblast-like progenitors that faithfully recapitulate early embryonic haematopoiesis. Here, we made use of mouse ES cells as a model system to understand the role of SOX7, a member of a large family of transcription factors involved in a wide range of developmental processes. During haemangioblast differentiation, SOX7 is expressed in haemogenic endothelium cells and is downregulated in nascent blood precursors. Gain-of-function assays revealed that the enforced expression of *Sox7* in haemangioblast-derived blast colonies blocks further differentiation and sustains the expression of endothelial markers. Thus, to explore the transcriptional activity of SOX7, we focused on the endothelial-specific adhesion molecule VE-cadherin. Similar to SOX7, VE-cadherin is expressed in haemogenic endothelium and is downregulated during blood cell formation. We show that SOX7 binds and activates the promoter of VE-cadherin, demonstrating that this gene is a novel downstream transcriptional target of SOX7. Altogether, our findings suggest that SOX7 is involved in the transcriptional regulation of genes expressed in the haemogenic endothelium and provide new clues to decipher the molecular pathways that drive early embryonic haematopoiesis.

KEY WORDS: Haemangioblast, Haematopoiesis, Haemogenic endothelium, SOX7, VE-cadherin (cadherin 5), Mouse

## INTRODUCTION

The first haematopoietic and endothelial cells of vertebrates emerge extra-embryonically within the yolk sac. Long-standing observations early in the 20th century initially suggested that both lineages originated from a common precursor, the haemangioblast (Sabin, 1920). However, the existence of this progenitor was only demonstrated much later in vitro using the differentiation of mouse embryonic stem (ES) cells as a model system (Choi et al., 1998) and more recently through in vivo studies (Huber et al., 2004; Vogeli et al., 2006). Human and mouse ES-derived embryoid bodies (EBs) contain blast colony-forming cells (BL-CFCs) that emerge from mesodermal progenitors, with kinetics that closely mimic the generation of the haemangioblast in vivo (Choi et al., 1998; Fehling et al., 2003; Huber et al., 2004; Kennedy et al., 2007). BL-CFCs express VEGF receptor 2, FLK1 (KDR – Mouse Genome Informatics), and can generate blast colonies when cultured in the presence of VEGF. Upon differentiation, these colonies give rise to blood and endothelial precursors and represent a good developmental model with which to study the mechanisms that drive the specification of both lineages (Kennedy et al., 1997; Choi et al., 1998; Faloon et al., 2000). In a series of comprehensive experiments, it has been reported that, at this developmental stage, CD41^{+} (ITGA2B – Mouse Genome Informatics) blood precursors arise from an intermediate endothelial population of cells with haemogenic properties (Eilken et al., 2009; Lancrin et al., 2009). These studies have revealed that the haemangioblast has the potential to generate haemogenic endothelium cells and have contributed to a deeper understanding of the transition process that occurs during the generation of CD41^{+} blood precursors from the haemogenic endothelium.

The first observations that described the haemogenic potential of cells with endothelial properties led to the notion of specialised endothelial cell populations that generate blood cells (reviewed by Jaffredo et al., 2005). Histological analysis of chicken embryos demonstrated that intra-aortic clusters of CD45^{+} (PTPRC – Mouse Genome Informatics) blood cells originated from endothelial cells lining the floor of the dorsal aorta (Jaffredo et al., 1998). Moreover, Nishikawa and colleagues isolated populations of VE-cadherin^{+} CD45^{–} cells from murine embryos and mouse ES cells that co-expressed other endothelial markers and ultimately differentiated into haematopoietic cells (Nishikawa et al., 1998a; Nishikawa et al., 1998b; Fraser et al., 2002). Yet, it was not until recently that the existence of haemogenic endothelium precursors was demonstrated in a series of elegant studies. Cell fate-tracing experiments showed that activation of a reporter gene driven by the promoter of VE-cadherin (Cdh5 – Mouse Genome Informatics) during intra-embryonic haematopoiesis labelled blood precursors that generated descendants throughout ontogeny and during adult life (Zovein et

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al., 2008; Chen et al., 2009). In addition, live-imaging microscopy of transgenic mouse and zebrafish embryos monitored the emergence of haematopoietic progenitor cells from the dorsal aorta and their subsequent entry into circulation (Bertrand et al., 2010; Boisset et al., 2010; Kissa and Herbomel, 2010; Lam et al., 2010). Nevertheless, the precise molecular mechanisms that underlie the specification of haemogenic endothelium cells still remain to be determined. It is known that the transcription factor SCL (TAL1 – Mouse Genome Informatics) is crucial for the generation of haemogenic endothelium cells and that these cells require RUNX1 expression to generate definitive haematopoietic progenitors (Chen et al., 2009; Lancrin et al., 2009). Furthermore, HOXA3 has recently been implicated in haemogenic endothelium development. Its overexpression was shown to impair the haematopoietic differentiation of haemogenic endothelium cells and to activate an endothelial signature in early blood precursors that could only be antagonised by RUNX1 (Iacovino et al., 2010).

SOX7 belongs to the SRY-related HMG-box (SOX) family of transcription factors and shares similar protein structure with SOX17 and SOX18, two other members of the F subgroup (Bowles et al., 2000). The HMG-box domain of SOX proteins encompasses 79 residues that recognises preferentially hexameric $5'-^A/_T^A/_TCAA^A/_T-3'$ sequences (reviewed by Wegner, 1999). Early in murine development, *Sox7* transcripts can be detected in the parietal endoderm and in vascular tissues (Takash et al., 2001; Kanai-Azuma et al., 2002; Murakami et al., 2004; Young et al., 2006; Sakamoto et al., 2007) and compelling evidence has revealed that SOX7, together with SOX17 and SOX18, plays an important role during vertebrate cardiovascular development (reviewed by Francois et al., 2010). Interestingly, the combined but not single morpholino knockdown of SOX7 and SOX18 promotes severe vascular and cardiac defects in zebrafish and *Xenopus* embryos, respectively (Zhang et al., 2005; Cermenati et al., 2008; Herpers et al., 2008; Pendeville et al., 2008). The role of SOX F proteins in haematopoiesis is far less understood. Kim and colleagues have demonstrated that SOX17 regulates the proliferation of neonatal and foetal haematopoietic stem cells (Kim et al., 2007), whereas we have recently established the involvement of SOX7 and SOX18 at the earliest stages of blood development (Gandillet et al., 2009; Serrano et al., 2010). In our studies, a genome-wide expression analysis of differentiated murine ES cells revealed that *Sox7* was upregulated at the onset of haemangioblast development. Subsequently, using transgenic mouse and ES cell lines carrying a GFP reporter under the control of the regulatory sequences of *Sox7*, we showed that *Sox7* was transiently expressed during the generation of CD41$^+$ precursors from FLK1$^+$ haemangioblasts (Gandillet et al., 2009). Moreover, the enforced expression of *Sox7*, or even *Sox18*, in CD41$^+$ blood precursors resulted in the maintenance of the proliferative state of these cells and in a reversible arrest of the haematopoietic differentiation program (Gandillet et al., 2009; Serrano et al., 2010).

In the present study, we demonstrate that, within blast colonies, SOX7 is expressed in haemogenic endothelium cells and downregulated upon haematopoietic differentiation. When the expression of *Sox7* is maintained beyond its normal time frame, blast colonies retain cell populations with haemogenic endothelium properties at the expense of haematopoietic maturation. Moreover, we show that SOX7 binds and activates the promoter of VE-cadherin, suggesting that SOX7 is part of the molecular machinery conferring VE-cadherin$^+$ identity to haemogenic endothelium cells. In summary, our data reveal that SOX7 is specifically expressed in haemogenic endothelium cells at the onset of haematopoietic

development and that SOX7 controls the expression of VE-cadherin, a gene that is critically associated with the endothelial nature of the haemogenic precursors.

## MATERIALS AND METHODS

### Cell culture
ES cells were cultured and differentiated as previously described (Pearson et al., 2008; Sroczynska et al., 2009). The differentiation of FLK1$^+$, haemogenic endothelium and CD41$^+$ cells was carried out as previously described (Pearson et al., 2008; Lancrin et al., 2009). Where indicated, 1 µg/ml of doxycycline was added to the culture medium. Time-lapse microscopy was performed in an IncuCyte FLR device (Essen Instruments). For lymphocyte differentiation, c-KIT$^+$ cells were co-cultured with OP9 and OP9-DL1 stromal cells for 20 days in α-MEM (PAA) supplemented with 20% foetal bovine serum, 1% KIT ligand, IL7 (1 ng/ml) and FLT3 ligand (5 ng/ml). Brain endothelial (bEnd.3), human embryonic kidney 293 (HEK293) and Mile Sven 1 (MS1) cell lines were cultured in DMEM (Sigma) supplemented with 50 µg/ml penicillin/streptomycin and 10% FCS (Sigma). bEnd.3 cells were cultured in the presence of 10 ng/ml VEGF (R&D Systems). All cell lines were maintained in standard culture conditions, with 5% $CO_2$ at 37°C.

### Flow cytometry
For fluorescence-activated cell sorting (FACS), cells were disaggregated by trypsinisation, washed in IMDM 20% FCS and incubated with combinations of conjugated monoclonal antibodies on ice. The following antibodies were used: CD34-biotin, CD41-biotin, FITC or PE, CD45-Biotin, c-KIT-APC, TIE2-PE, VE-cadherin-AF647, FLK1-biotin and strep-PECy7 (all from eBioscience). Analyses were performed on a FACSCalibur and cell sorts on a FACSAria (BD Biosciences). Data were analysed with FlowJo (TreeStar), gating first on the forward scatter versus side scatter to exclude non-viable cells.

### Immunofluorescence
Day 3 EB-derived FLK1$^+$ cells were cultured for 48 hours onto gelatinised µ-Slides (Ibidi). Cells were permeabilised in PBS 0.2% Triton X-100 (Sigma), fixed with 2% paraformaldehyde (Sigma) then washed and stained with PBS containing 10% FCS and 0.2% Tween 20 (Sigma). Cells were pre-blocked for 15 minutes, incubated with primary antibodies for 1 hour and then secondary antibodies for 1 hour. Slides were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). Blast colony images were captured using an Axiovert 200M (Zeiss) and processed with Metamorph (Molecular Devices).

### Protein extraction and western blotting
Nuclear proteins were prepared with Nuclear Extract Kit (Active Motif), aliquoted and stored at –80°C. Protein preparations were incubated for 15 minutes at 70°C in SDS gel-loading buffer (NuPage LDS sample buffer and NuPage reducing agent; Invitrogen) then separated by SDS-PAGE with NuPAGE Bis-Tris gels in Xcell Surelock electrophoretic chambers containing MOPS buffer (Invitrogen).

### Polyclonal antibody against mouse SOX7
A 25 kDa fragment of SOX7 (amino acids 159 to 380) expressed in bacteria was used for a series of five immunizations in sheep (Scotland Diagnostics). The purification of anti-SOX7 antibodies from immunised sheep serum was performed on immobilisation of His-SOX7-fragment linked to an AminoLink Plus column (Thermo Scientific) according to the manufacturer's directions.

### Luciferase assays
VE-cadherin promoter regions were amplified by PCR from a bacterial artificial chromosome (CH29-557J22; Chori) (supplementary material Table S1) and cloned into pGL3b (Promega). Mutant versions of plasmids were generated by nucleotide substitution with the QuikChange II XL Site-Directed Mutagenesis kit (Stratagene). *Sox7* sequence was isolated from an expression plasmid previously described (Gandillet et al., 2009) and subcloned into pcDNA3 (Invitrogen). The day before transfection, HEK293 cells were transferred onto 24-well plates at 125,000 cells/cm$^2$.

Cells were co-transfected with 250 ng firefly and 1 ng Renilla luciferase plasmids (pRL-SV40, Promega) using GeneJuice (Novagen). For co-transactivation assays, cells were co-transfected with 125 ng firefly luciferase vectors, 125 ng of expression constructs and 1 ng Renilla luciferase plasmids. Luciferase activity levels were analysed after 48 hours with Dual-Luciferase Reporter System (Promega). Luciferase activity was measured in a Berthold Luminometer (Berthold Technologies) and firefly luciferase records were standardised to the respective Renilla luciferase.

### Biotin pull-down and chromatin immunoprecipitation
For biotin pull-down assays, complementary single-stranded DNA oligonucleotides biotinylated at the 5′ ends (Sigma) were annealed at a 1:1 molar ratio and bound to Dynabeads M-280 Streptavidin (Invitrogen). Binding reactions were carried out by incubating 25 µl DNA-labelled Dynabeads with 250 µg nuclear protein extracts, and 10 µg poly(dG-dC)•poly(dG-dC) (Sigma) in 12 mM HEPES (pH 7.9), 4 mM Tris (pH 7.9), 150 mM KCl, 1 mM EDTA, 1 mM DTT, 12% glycerol and 0.1% Tween 20 with rotation for 4 hours at 4°C. Dynabeads were then isolated and washed with binding buffer. Proteins were eluted from Dynabeads with SDS gel-loading buffer and subjected to western blotting analysis.

Chromatin IP (ChIP) were carried with Transcription Factor ChIP Kit (Diagenode), using $1 \times 10^6$ day 4 EB cells for each IP and input samples. IP mixtures contained 2 µg SOX7 antibody or 2 µg immunoglobulin (Ig) isotype (sheep IgG, I5131, Sigma). For data analysis, ChIP signals were first normalised to input levels, then to IgG reference levels and expressed as $2^{-\Delta\Delta ct}$.

### Gene expression assays
Total RNA was isolated with RNeasy Mini Kit (Qiagen). Quantitative RT-PCR (qPCR) assays were performed as previously described (Pearson et al., 2008). Gene-specific primers are detailed in supplementary material Table S1.

### Embryo generation
Timed matings were set up between ICR male and female mice, and the morning that a plug was observed in a female mouse was taken to be E0.5. Morphological landmarks were used to stage gastrulating embryos. All animal work was performed under regulations set out by the Home Office Legislation under the 1986 Animal Scientific Procedures Act.

### Lentivirus production
PCR amplified *VE-cadherin* (supplementary material Table S1) and *GFP* cDNAs separated by a 2A sequence (de Felipe et al., 1999) were cloned into a lentiviral vector containing the human EF1 promoter (Gilham et al., 2010). VSVg pseudotyped lentiviral particles were produced and titred as previously described (Dull et al., 1998; Zufferey et al., 1998; Gilham et al., 2010).

### Generation of *Sox7* knockdown ES cells
A tandem of three *Sox7* shRNAs (Gandillet et al., 2009) was designed using a strategy described by Sun et al. (Sun et al., 2006) and synthesised by GeneArt. This sequence was cloned downstream of *dTomato* cDNA into a plox plasmid and introduced in Ainv18 cells as previously described (Kyba et al., 2002) to generate the doxycycline-inducible *Sox7* knockdown ES cell line.

## RESULTS
### SOX7 is transiently expressed at the onset of haematopoietic development
The differentiation of ES cells into EBs containing blood precursors is a powerful method with which to explore the molecular and cellular mechanisms that regulate embryonic haematopoiesis. The emergence of the first blood precursors within EBs can be monitored around day 3 by the presence of FLK1$^+$ BL-CFCs (Faloon et al., 2000; Chung et al., 2002). In order to characterise the pattern of *Sox7* expression in early haematopoiesis, we studied its detailed kinetic of transcription during haemangioblast differentiation. Hence, FLK1$^+$ cells were isolated

from day 3 EBs and cultured in presence of VEGF to stimulate the generation of blast colonies. Blast colony cells cultured in such conditions gradually lost their endothelial identity as shown by the downregulating VE-cadherin and TIE2 over the 4-day time course of differentiation (supplementary material Fig. S1). Inversely, they gained a haematopoietic immunophenotype, with the rapid upregulation of CD41 followed by a slower acquisition of CD45. This transition was accompanied by a transient increase in the expression of the haematopoietic/endothelial marker c-KIT (supplementary material Fig. S1). Interestingly, and in agreement with results previously reported by our group (Gandillet et al., 2009), the transcription of *Sox7* was strongly enhanced on the first day of blast colony development, followed by a sharp downregulation to levels lower than those of freshly isolated FLK1$^+$ cells (Fig. 1A). Taking into account the crucial role of VEGF in the generation of blast colonies from FLK1$^+$ precursors (Choi et al., 1998; Faloon et al., 2000), we hypothesised that this striking initial upregulation of *Sox7* expression might be linked to the stimulation of the FLK1$^+$ cells by this growth factor. To test this prediction, *Sox7* transcript levels were compared in day 1 colonies grown in media supplemented or not with VEGF (Fig. 1B). Indeed, the absence of VEGF resulted in cultures that failed to upregulate *Sox7* suggesting that the expression of *Sox7* is downstream of the VEGF signalling pathway and associated with the initiation of the haematopoietic program.

The haemangioblast-derived haemogenic endothelium has been described as a population of CD41$^-$ TIE2$^+$ c-KIT$^+$ cells. Upon differentiation, this population first acquires the expression of CD41 and then loses the TIE2$^+$ c-KIT$^+$ immunophenotype (Lancrin et al., 2009). As the downregulation of *Sox7* at day 2 of blast colony development coincides with the dramatic increase in CD41$^+$ cells, we wanted to gain further insight into the dynamics of *Sox7* expression in the transition from haemogenic endothelium to CD41-expressing cells. Therefore, the relative levels of *Sox7* transcripts were compared between CD41$^-$ TIE2$^+$ c-KIT$^+$ cells and the three subsets of CD41$^+$ cells: TIE2$^+$ c-KIT$^+$, TIE2$^-$ c-KIT$^+$ and TIE2$^-$ c-KIT$^-$, found within day 2 blast colonies (Fig. 1C, left). Whereas virtually all CD41$^-$ TIE2$^+$ c-KIT$^+$ cells expressed VE-cadherin, CD41$^+$ subpopulations showed decreasing frequency in VE-cadherin$^+$ cells in correlation with TIE2 and c-KIT downregulation, confirming a loss of endothelial identity associated with this process (Fig. 1C, right). Interestingly, the expression of *Sox7* in any of the CD41$^+$ populations was much lower than in CD41$^-$ TIE2$^+$ c-KIT$^+$ cells, suggesting that the downregulation of *Sox7* is concurrent to the emergence of blood precursors from the haemogenic endothelium (Fig. 1D). Similar to blast colonies, CD41$^-$ TIE2$^+$ c-KIT$^+$ cells isolated from E7.5 embryos were enriched in *Sox7* transcripts when compared with CD41$^+$ cells (supplementary material Fig. S2). To further support these observations, the expression of SOX7 was analysed by immunofluorescence in day 2 blast colonies relative to endothelial and haematopoietic markers. At this stage, SOX7 was co-expressed with VE-cadherin in clusters of cells that formed compact aggregates. By contrast, SOX7 was not detected in CD41-expressing cells that marked blood progenitors emerging at the periphery of the colonies (Fig. 1E) and that were mostly VE-cadherin$^-$ (supplementary material Fig. S3). Altogether, these data show that the expression of SOX7 during the generation of blood precursors from mouse ES cells is transient and tightly linked to the signalling activity of VEGF in BL-CFCs. Importantly, within blast colonies, SOX7 appears to be expressed in haemogenic endothelium cells and downregulated in haematopoietic precursors.
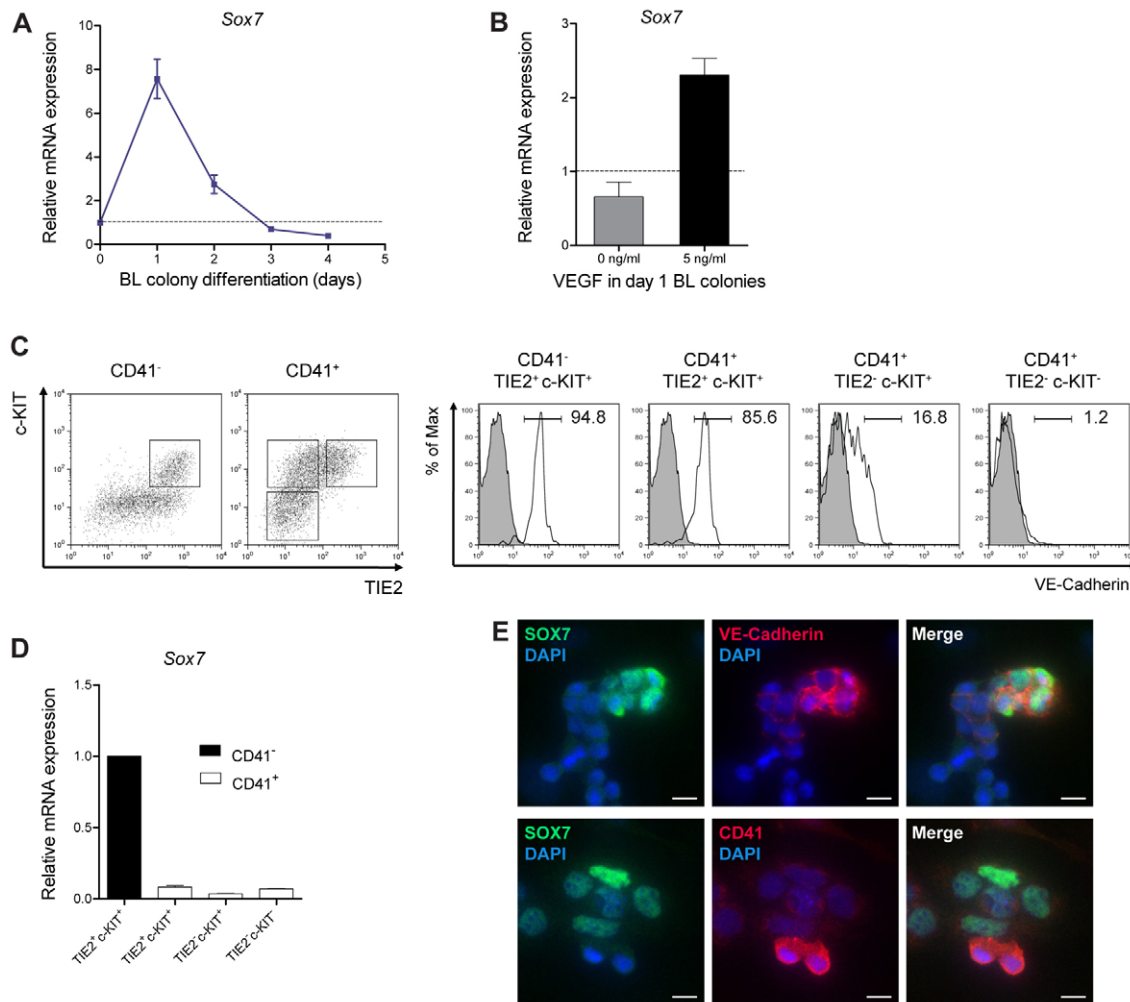
**Fig. 1. SOX7 is expressed in the haemogenic endothelium.** (**A**,**B**) qPCR data showing the fold changes in *Sox7* transcript levels normalised against β-actin in blast colonies relative to freshly isolated FLK1^{+} cells. (A) Expression pattern of *Sox7* during 4 days of blast colony differentiation. (B) Expression levels of *Sox7* in FLK1^{+} cells differentiated for 24 hours in the presence or absence of VEGF. Data are mean±s.e.m. (*n*=3). (**C**) Left: representative FACS plots of TIE2 and c-KIT expression profiles in CD41^{−} and CD41^{+} cells at day 2 of blast colony differentiation; gates define TIE2 and c-KIT subpopulations used subsequently. Right: representative FACS histograms showing the percentage of VE-cadherin^{+} cells in the indicated CD41/TIE2/c-KIT subpopulations of day 2 of blast colony differentiation (line histogram: stained cells; filled histogram: unstained control cells). (**D**) qPCR analysis of *Sox7* transcript levels normalised against β-actin in TIE2/c-KIT subpopulations of CD41^{+} cells (white bars) relative to CD41^{−} TIE2^{+} c-KIT^{+} cells (black bar) at day 2 of blast colony differentiation; data are mean±s.e.m. (*n*=3). (**E**) Immunofluorescence analysis shows co-staining of SOX7 with VE-cadherin (upper panels) but not with CD41 (bottom panels) in day 2 blast colony cells. Data represent one of at least three independent experiments. Scale bars: 10 μm.

## The continued expression of *Sox7* impairs the generation of mature blast colonies

To understand the functional role played by SOX7 during blast colony formation, we took advantage of a previously described mouse ES cell line containing a doxycycline inducible *Sox7* cDNA, hereafter referred to as iSOX7 (Gandillet et al., 2009). The enforced expression of *Sox7* in this cell line can be monitored by the bi-cistronic transcription and translation of a GFP reporter. This ES cell line allowed us to counteract the bona fide transient expression of *Sox7* during haematopoietic development and to assess the impact of maintaining the expression of this transcription factor beyond its normal time frame. FLK1^{+} cells isolated from day 3 iSOX7 EBs were cultured with or without doxycycline and analysed for morphological differences induced by maintaining the expression of *Sox7*. In agreement with previous data on the morphological changes associated with blast colony differentiation (Lancrin et al., 2009), iSOX7 FLK1^{+} cells cultured without doxycycline differentiated into colonies composed of endothelial adherent cells after 36 hours in culture. By 48 hours, haematopoietic non-adherent cells began to emerge at the edge of the clusters and 12 hours later (60 hours), these cells had proliferated and detached from the compact core of the colonies. At this time-point, blast colonies composed of blood precursors surrounding endothelial cores had reached their full maturation stage (Fig. 2A). Colonies cultured in the presence of doxycycline similarly appeared as clusters of adherent cells at early stages of differentiation. However, after 60 hours of culture, the majority of those colonies had not acquired the typical mature morphology observed in the control culture. Haematopoietic cells expressing CD41 were generated at the surface of the colonies but failed to be released from the clusters (Fig. 2A,B).
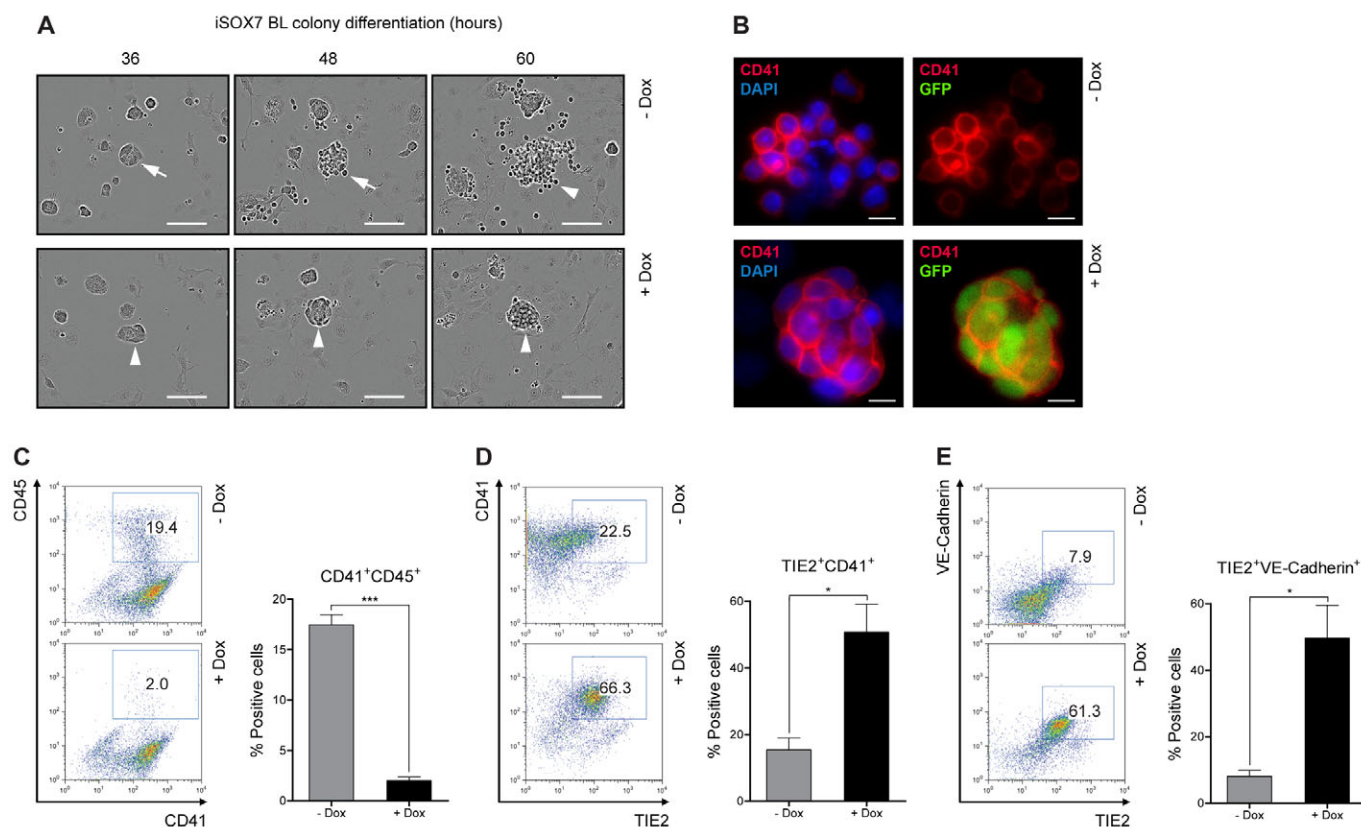
**Fig. 2. The continued expression of *Sox7* impairs the maturation of blast colonies.** (**A**) Phase-contrast microscopy of iSOX7 blast colonies cultured for the indicated times; representative morphologies of colonies cultured in the presence (arrows) or absence (arrowheads) of doxycycline (Dox) are shown. (**B**) Immunofluorescence analysis of CD41 in day 3 iSOX7 blast colonies; the expression of GFP results from the enforced bicistronic transcription of *Sox7* and *GFP* cDNAs. Data represent one of at least three independent experiments. (**C-E**) Representative FACS plots show the percentage of (C) CD41+ CD45+, (D) TIE2+ CD41+ and (E) TIE2+ VE-cadherin+ cells in day 3 iSOX7 blast colonies; bars represent mean±s.e.m. (*n*=3); *$P<0.05$; ***$P<0.001$. Scale bars: 100 μm in A; 10 μm in B.

The morphological changes induced by enforced SOX7 expression on the differentiation of FLK1+ cells led us to investigate the impact that continued SOX7 expression had on the generation of haematopoietic precursors. Once committed, CD41 blood progenitors initiate their lineage specification and upregulate the pan-haematopoietic marker CD45. In the blast colony assay, this is represented by a gradual emergence of a double-positive population of cells. By day 3 of blast differentiation, the frequency of CD41+ CD45+ cells generated in colonies cultured with doxycycline was nearly ten times lower than in those cultured in the absence of doxycycline (Fig. 2C), suggesting that the continued expression of *Sox7* halted the maturation of blood precursors. To further characterise this phenotypical blockage in haematopoietic differentiation, we next investigated the expression of CD41 in combination with TIE2, which is normally downregulated in blood progenitors as they emerge from blast colonies. Surprisingly, the frequency of TIE2+ CD41+ present in iSOX7 blast colonies cultured with doxycycline was nearly threefold higher than in control cultures (Fig. 2D), suggesting that *Sox7*-enforced expression impaired the endothelial to haematopoietic immunophenotypical transition that naturally accompanies the maturation of blast colonies. In this context, we also analysed the expression of the vascular-specific cadherin VE-cadherin in combination with TIE2 to further confirm the maintenance of an endothelial signature induced by the enforced expression of *Sox7*.

Similar to TIE2+ cells, the frequency of VE-cadherin-expressing cells decreased in differentiating blast colonies. However, iSOX7 blast colonies cultured with doxycycline for 3 days accumulated cells expressing these two endothelial markers and contained approximately sevenfold more TIE2+ VE-cadherin+ cells than control colonies (Fig. 2E).

The immunophenotypical defects induced by the enforced expression of *Sox7* complement the morphological arrest in blast development described above. Hence, the continued expression of *Sox7* appears to inhibit the full differentiation of blast colonies, maintaining them in an immature endothelial-like state. In order to assess whether the transcriptional program reflected this haematopoietic blockage, we analysed the expression kinetics of *Pu.1* (*Sfpi1* – Mouse Genome Informatics). This gene encodes a transcription factor involved in the specification of lymphoid and myeloid cells (reviewed by Rosenbauer and Tenen, 2007), and its expression is a powerful indicator of the emergence of haematopoietic cells in blast colonies (Hoogenkamp et al., 2009). Similar to the control cultures, *Pu.1* remained undetectable at day 1 in iSOX7 cells maintained with doxycycline. However, the strong upregulation in *Pu.1* expression observed from day 2 onwards in control cultures was dramatically impaired by *Sox7*-enforced expression, with seven times lower transcript levels by the end of the differentiation assay (Fig. 3A). An overall decrease in PU.1 protein levels was also observed in day 4 blast colony cells (Fig.
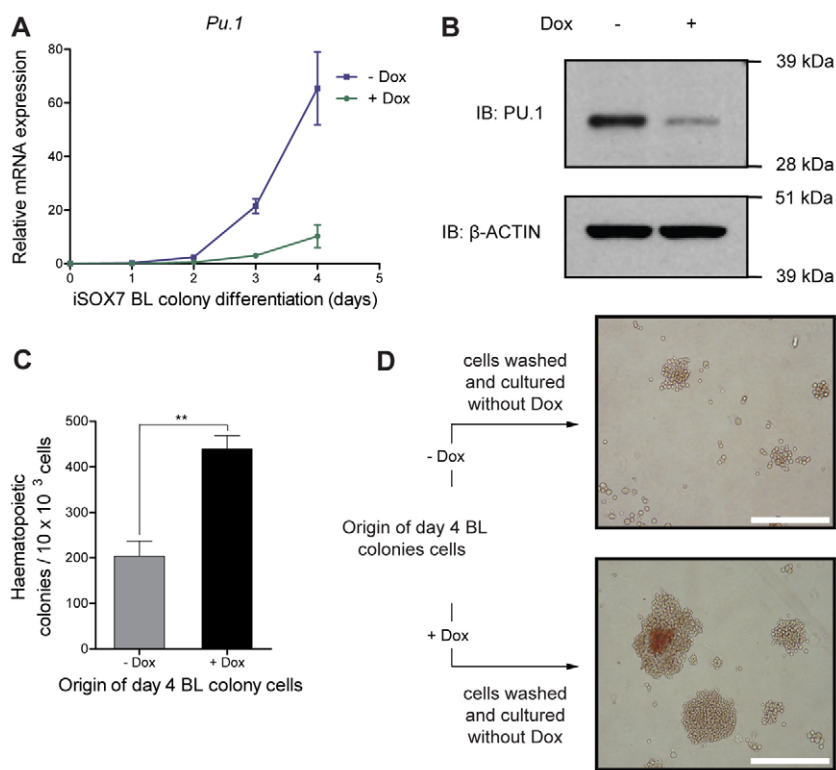
**Fig. 3. Enforced expression of *Sox7* blocks the maturation of haematopoietic progenitors.** (**A**) qPCR analysis of fold changes in *Pu.1* transcript levels normalised against β-actin during iSOX7 blast colony differentiation relative to freshly isolated FLK1^{+} cells; data are mean±s.e.m. (*n*=3). (**B**) Representative western blot analysis of PU.1 in iSOX7 blast colonies at day 4 of differentiation; β-actin was used as loading control. (**C,D**) Day 4 iSOX7 blast colony cells cultured in the presence or absence of Dox were washed and replated in secondary semi-solid cultures without Dox. (C) Mean number of haematopoietic colonies counted after 7 days of culture ±s.e.m. (*n*=3); **P<0.01. (D) Phase-contrast microscopy of representative haematopoietic colonies in semi-solid cultures. Data represent one of at least three independent experiments. Scale bars: 500 μm.

3B). The accumulation of endothelial-related cell surface markers and blockage in haematopoietic maturation induced by the enforced expression of *Sox7* suggests that the haematopoietic potential of blood precursors might be maintained within the developing blast colonies. To test this hypothesis, iSOX7 blast colony cells maintained in the presence or absence of doxycycline for 4 days were cultured in haematopoietic differentiation assays. At collection, control and test cultures were washed and then replated in semi-solid conditions or in OP9/OP9-DL1 co-cultures without doxycycline. Interestingly, cells derived from doxycycline-containing cultures generated twice the number of haematopoietic colonies relative to the control cultures in semi-solid assays (Fig. 3C). Furthermore, the former colonies were not only composed of greater number of cells than the latter, but they were also more diverse in nature. Doxycycline-derived semi-solid cultures contained both myeloid and mixed myeloid/erythroid colonies, whereas control cultures were composed only of myeloid colonies (Fig. 3D). The haematopoietic potential of iSOX7 blast colonies cultured with doxycycline was thus rather similar to that observed in early wild-type colonies, which is then reduced with differentiation (supplementary material Fig. S4). Finally, iSOX7 precursors derived from doxycycline cultures generated increased frequencies of B and T lymphocytes than control cultures when further differentiated on OP9 or OP9-DL1 stromal cells respectively (supplementary material Fig. S5).

Altogether, these data show that the continued expression of *Sox7* during the differentiation of FLK1^{+} cells impaired the formation of morphologically mature blast colonies, blocking the differentiation of CD41^{+} precursors into CD45-expressing cells and promoting the accumulation of an intermediate TIE2^{+} VE-cadherin^{+} CD41^{+} endothelial-like cell populations. Moreover, the continued expression of *Sox7* led to reduced levels of the transcription factor PU.1 and resulted in the maintenance of blood precursors with an enhanced potential to generate haematopoietic cells.

## Enforced expression of *Sox7* maintains the haemogenic endothelium

Given that *Sox7* is endogenously expressed in the transient haemogenic endothelium population present in developing blast colonies and that enforcing its expression beyond its normal time frame maintained an endothelial cell population, we postulated that the differentiation blockage could result from the maintenance of haemogenic endothelium progenitors. To explore this idea, *Sox7*-enforced expression experiments were carried out in haemogenic endothelium cells to assess specifically whether SOX7 could maintain this population. Therefore, CD41^{−} TIE2^{+} c-KIT^{+} haemogenic endothelium cells were isolated from day 2 iSOX7 blast colonies and cultured in conditions that allow the generation of CD41^{+} blood progenitors, hereafter referred to as haemogenic endothelium culture (Fig. 4A). Upon 2 days in culture, control cells had downregulated the expression of *Sox7*, to levels three times lower than in freshly isolated haemogenic endothelium cells (Fig. 4B), paralleling the loss of expression observed in differentiating blast colonies. Simultaneously, the frequency of cells presenting a TIE2^{+} c-KIT[+] immunophenotype was reduced in these day 2 haemogenic endothelium cultures (Fig. 4C). By contrast, culturing iSOX7 haemogenic endothelium cells in the presence of doxycycline resulted in a threefold increase in *Sox7* transcript levels (Fig. 4B), and a frequency of TIE2^{+} c-KIT^{+} cells that was double that in control cultures (Fig. 4C). The maintenance of the TIE2^{+} c-KIT^{+} cells in haemogenic endothelium cultures overexpressing *Sox7* suggests an arrest in haemogenic endothelium differentiation. Thus, we next assessed whether this effect was accompanied by a reduction in the percentage of CD41^{+} precursors generated by the CD41^{−} TIE2^{+} c-KIT^{+} haemogenic endothelium. Indeed, the frequency of CD41^{+} cells present in iSOX7 haemogenic endothelium cultures maintained with doxycycline for 2 days was approximately three times lower than in the absence of doxycycline (Fig. 4D). These results show that the differentiation
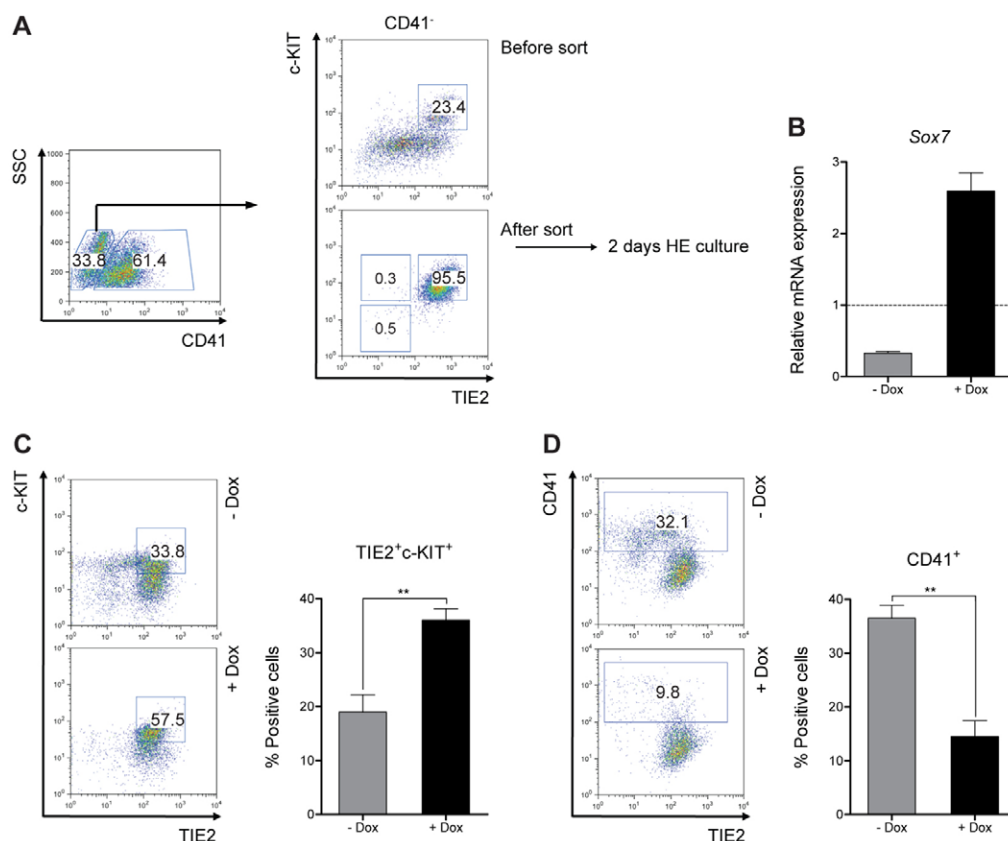
**Fig. 4. SOX7 sustains the haemogenic endothelium immunophenotype.** (**A**) Gating strategies used to isolate CD41− TIE2+ c-KIT+ cells from day 2 iSOX7 blast colonies; the efficiency of the separation is shown; isolated cells were maintained in haemogenic endothelium cultures for 2 days. (**B**) qPCR analysis of *Sox7* transcript levels in day 2 iSOX7 haemogenic endothelium cultures relative to freshly isolated CD41− TIE2+ c-KIT+ cells; bars represent mean±s.e.m. (*n*=3). (**C,D**) Representative FACS plots show the percentage of (C) TIE2+ c-KIT+ and (D) CD41+ cells after differentiating iSOX7 CD41− TIE2+ c-KIT+ progenitors in haemogenic endothelium cultures for 2 days; bars represent mean±s.e.m. (*n*=3); **P<0.01.

of haemogenic endothelium cells into CD41+ precursors was severely affected by *Sox7*-enforced expression and suggest that this transcription factor might maintain the haemogenic endothelium cell population.

## VE-cadherin is a transcriptional target of SOX7 at the onset of haematopoietic development

The expression of VE-cadherin in embryonic haematopoietic precursors has been widely associated with its expression in the haemogenic endothelium (Eilken et al., 2009; Fraser et al., 2002; Nishikawa et al., 1998a; Nishikawa et al., 1998b; Zovein et al., 2008). In light of these studies, understanding the regulation of VE-cadherin expression is of crucial importance to gain insight into the poorly explored molecular mechanisms that drive haemogenic endothelium specification from the haemangioblast. In order to shed light on the transcriptional activity of SOX7, we focused on the expression of VE-cadherin, a potential target of SOX7 at the onset of haematopoiesis. Interestingly, lentivirus-mediated overexpression of VE-cadherin during blast colony development decreased the frequency of CD41+ CD45+ haematopoietic cells (supplementary material Fig. S6), resembling the blockage in differentiation induced by the continued expression SOX7. In addition, we have shown here that similar to SOX7, VE-cadherin is expressed in the haemogenic endothelium and is downregulated in CD41+ blood precursors during blast colony development (Fig. 1). Hence, we first sought to explore the impact of overexpressing *Sox7* on VE-cadherin expression at these two stages of haematopoiesis. Maintaining iSOX7 haemogenic endothelium cells with doxycycline neither increased the levels of VE-cadherin transcripts (Fig. 5A) nor altered the frequency of VE-cadherin+ cells in culture (Fig. 5B). However, iSOX7 CD41+ cells isolated from day 5 EBs and cultured in conditions that permit a full

haematopoietic development, showed a 20-fold increase in the expression of VE-cadherin when cultured with doxycycline (Fig. 5C) and resulted in the emergence of VE-cadherin+ cells (Fig. 5D). The different effect that the enforced expression of *Sox7* had on the transcription of VE-cadherin in the haemogenic endothelium and in CD41+ cells is likely to reflect the distinct basal expression level of VE-cadherin in these two populations. Importantly, this finding demonstrates that SOX7 can promote the ectopic expression of VE-cadherin in blood precursors. Finally, we generated a doxycycline-inducible *Sox7* knockdown ES cell line to understand whether SOX7 is implicated in establishing the onset of VE-cadherin expression (supplementary material Fig. S7A,B). We observed that disrupting *Sox7* expression at the first day of blast colony differentiation resulted in lower levels of VE-cadherin transcripts (supplementary material Fig. S7B), and in a decrease of the frequency of VE-cadherin+ cells (supplementary material Fig. S7C). Altogether, these data further suggest that *VE-cadherin* might lie downstream of the transcriptional activity of SOX7.

The promoter of mouse VE-cadherin has been well described as a region of ~2.5 kb from the transcription start site (TSS), sufficient to drive the transcription of this gene in most endothelial cells (Gory et al., 1999). In order to determine whether SOX7 can bind and activate the promoter of VE-cadherin, we first set out to map motifs in its promoter sequence that could be recognised by SOX7. Given that no SOX7 positional weight matrix has been described to date, we performed a motif search analysis with the SOX5 matrix annotated in the JASPAR database of vertebrate transcription factor profiles (Bryne et al., 2008), as this is a fair representation of the binding-site motif common to all SOX proteins. With this approach, three SOX-binding sites were identified within the mouse VE-cadherin promoter, hereafter referred to as SBS1, SBS2 and SBS3 (supplementary material Fig.
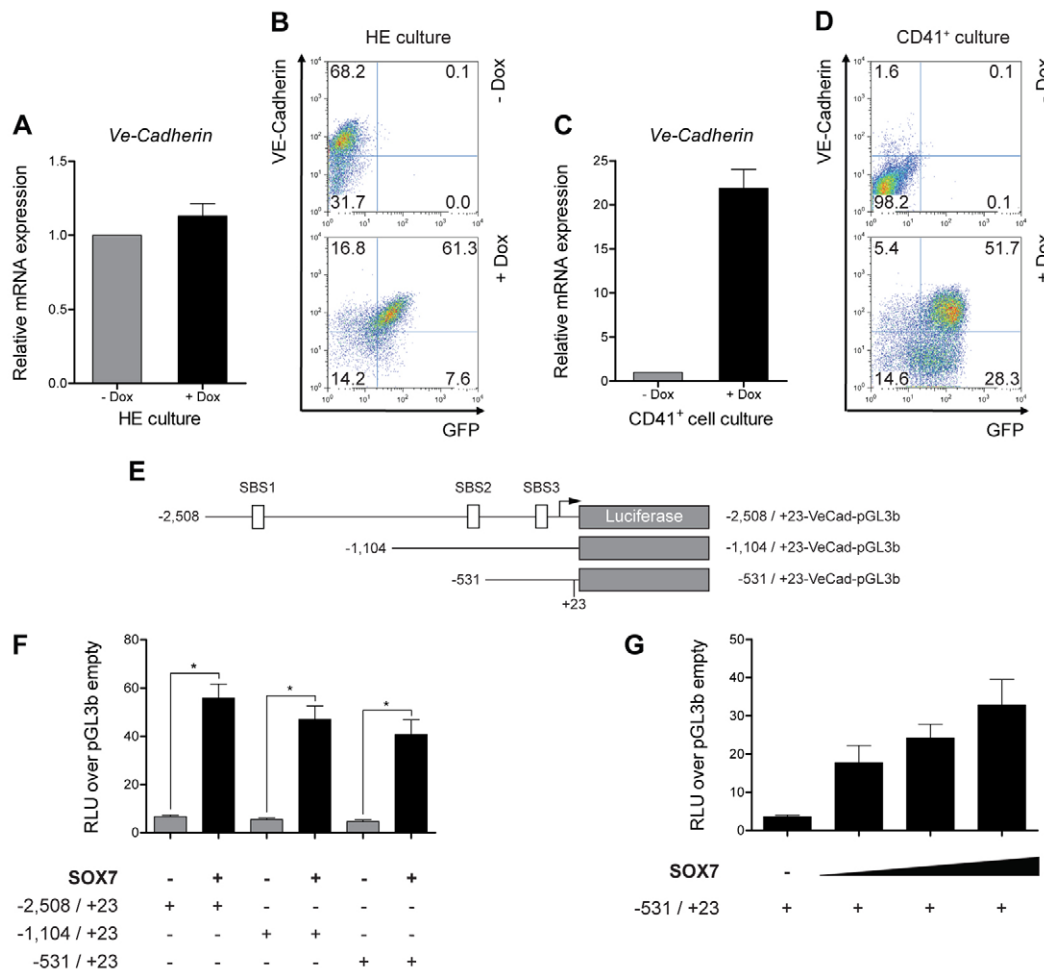
**Fig. 5. SOX7 activates the promoter of VE-cadherin.** (**A**) qPCR analysis of VE-cadherin expression levels normalised against β-actin in iSOX7 CD41− TIE2+ c-KIT+ cells isolated from day 2 blast colonies and maintained for 2 days in haemogenic endothelium cultures; bars represent mean±s.e.m. (*n*=3). (**B**) Representative FACS plots show the percentage of GFP+ and VE-cadherin+ cells in iSOX7 haemogenic endothelium cultures. (**C**) qPCR analysis of VE-cadherin expression levels normalised against β-actin in iSOX7 CD41+ CD34− blood precursors isolated from day 5 EBs and differentiated in haematopoietic cultures for 24 hours; bars represent mean±s.e.m. (*n*=3). (**D**) Representative FACS plots show the percentage of GFP+ and VE-cadherin+ cells in iSOX7 haematopoietic cultures. (B,D) The presence of GFP+ cells in the iSOX7 cultures treated with doxycycline (Dox) results from the enforced bicistronic transcription of *Sox7* and *GFP* cDNAs. (**E**) Schematic diagram depicts the full-length and deletion versions of the VE-cadherin promoter; numbers indicate positions relative to the transcription start site. (**F,G**) Luciferase assays using HEK293 cells co-transfected with the indicated VE-cadherin promoter constructs and pcDNA3 expression plasmids; activity of (F) the indicated VE-cadherin promoter constructs in the presence of pcDNA3-empty or pcDNA3-SOX7 and (G) –531/+23-VeCad-pGL3b in the presence of pcDNA3 empty or increasing concentrations of pcDNA3-SOX7; bars represent mean luciferase intensity relative to pGL3b-empty ±s.e.m. (*n*=3); *P<0.05; RLU, relative luciferase units.

S8A and Table S2). To explore whether SOX7 regulates the activation of VE-cadherin through these SBS motifs, we generated different reporter plasmids containing either the full-length or truncated VE-cadherin promoter fragments upstream of the luciferase gene (VeCad-pGL3b). The full-length promoter construct included a region from position –2508 to +23 relative to the TSS and encompassed SBS1, SBS2 and SBS3, whereas the short forms of this plasmid were truncated at positions –1104 or –531 and lacked SBS1 or contained only SBS3, respectively (Fig. 5E). We then performed transient transactivation assays by co-transfecting the reporter constructs into HEK293 cells in combination with an expression plasmid containing the coding sequence of SOX7. Interestingly, the presence of SOX7 induced an eightfold increase in luciferase levels regardless of the length of the promoter region used, demonstrating not only that the VE-cadherin promoter was activated by SOX7, but also that the most proximal region studied,

containing only SBS3, was sufficient for this effect (Fig. 5F). In addition, increasing concentrations of SOX7 led to a gradual increment in luciferase activity of the shortest construct, revealing a dose-dependent transcriptional activity mediated by SOX7 on this section of the promoter (Fig. 5G).

In light of these observations, we predicted that SOX7 could possibly bind directly to and exert its transcriptional activity through SBS3. In fact, SBS3 is the only SOX motif conserved across mammalian species in the promoter of VE-cadherin (supplementary material Fig. S8B). Therefore, we focused on this motif and undertook a series of experimental assays to assess the interaction of SOX7 with SBS3. First, four nucleotide substitutions were introduced by directed mutagenesis in the plasmid that contained the full-length VE-cadherin promoter, abrogating the consensus SOX core motif in SBS3 and generating a mutant version of the reporter construct (SBS3M; Fig. 6A). SBS3M was then transfected into
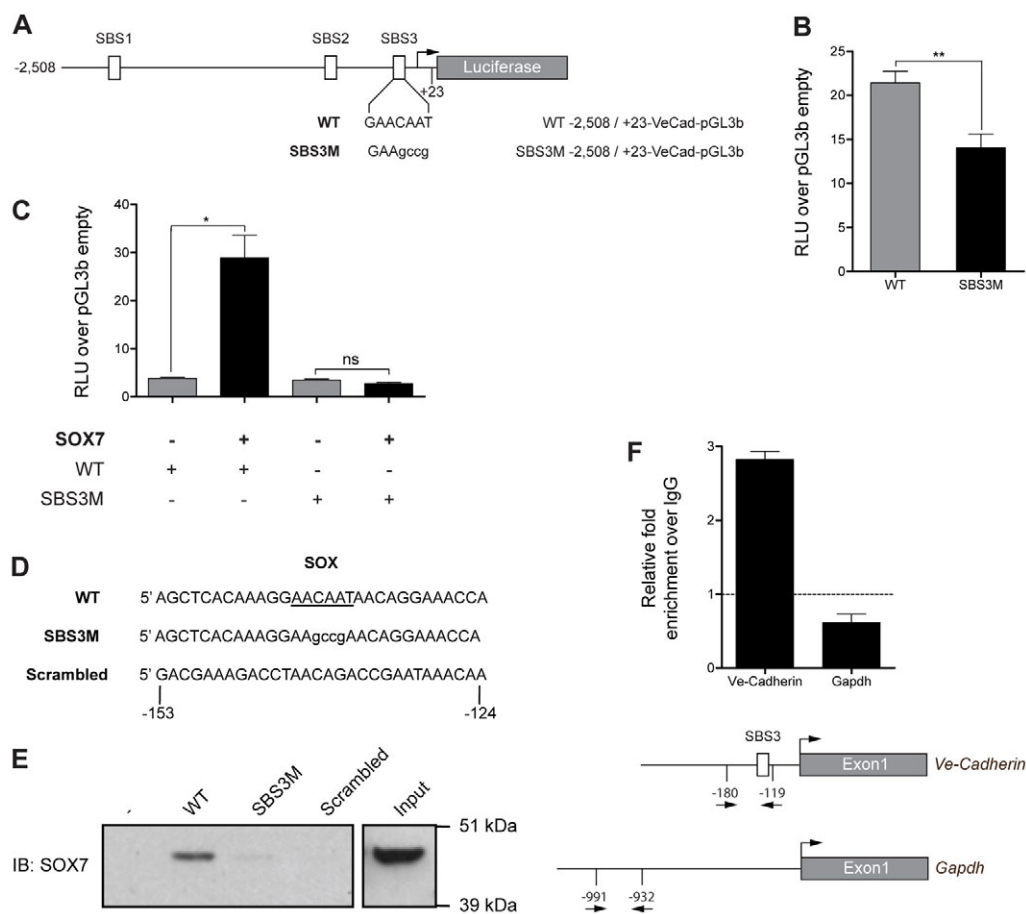
**Fig. 6. SOX7 binds the promoter of VE-cadherin.** (**A**) The wild-type (WT) SOX DNA-binding sequence and SBS3M mutant version; numbers indicate positions relative to the transcription start site (TSS). (**B,C**) Luciferase activity of wild type and SBS3M in (B) bEnd.3 cells and (C) HEK293 cells in the presence of pcDNA3-empty or pcDNA3-SOX7; bars represent the mean luciferase intensity relative to pGL3b-empty ±s.e.m. ($n$=3); *$P$<0.05, **$P$<0.01 ns, not significant; RLU, relative luciferase units. (**D**) Sequence of biotinylated probes used in pull-down assays; underlined nucleotides correspond to SBS3; numbers indicate the relative positions to the VE-cadherin TSS. (**E**) Representative western blot shows the binding of SOX7 to SBS3; nuclear protein extracts of MS1 cells were incubated with the indicated probe labelled beads; IB (immunoblotting) shows the levels of SOX7 present in pull-down eluates; input is 20% of total nuclear protein extracts used in the pull-down assays. (**F**) ChIP assay shows the fold enrichment in the occupancy of SOX7 on the promoter regions of VE-cadherin and *Gapdh* in day 4 EB cells; bars represent mean±s.e.m. ($n$=3); scheme represents the regions upstream of VE-cadherin and *Gapdh* analysed in ChIP assays (arrows indicate primer positions relative to the TSS).

bEnd.3 cells to test its luciferase activity relative to the wild-type VE-cadherin promoter construct in an endothelial cell context. The mutation in SBS3 caused a reduction in the activity of the full-length promoter, revealing that in bEnd.3 cells the integrity of the motif is relevant for the full activity of the promoter of VE-cadherin (Fig. 6B). To define whether the disruption of SBS3 impairs the transactivation of the VE-cadherin promoter by SOX7, HEK293 cells were co-transfected with SOX7 together with either the wild-type or SBS3M promoter constructs. Interestingly, the mutation in SBS3 caused a drastic reduction in luciferase levels of the full-length construct. Whereas wild-type luciferase levels were increased by approximately eightfold in the presence of SOX7, the SBS3M construct did not respond to the transcription factor (Fig. 6C), extending the notion that the integrity of SBS3 is necessary to the SOX7-mediated activation of VE-cadherin promoter.

If SBS3 is directly responsible for the transcriptional stimulation of the VE-cadherin promoter by SOX7 in HEK293 cells, it is likely that the transcription factor physically binds this motif. To verify this assumption, we first carried out biotin pull-down experiments by incubating nuclear protein extracts, obtained from SOX7-expressing MS1 cells, with a 30 bp biotinylated dsDNA probe corresponding to SBS3 and its flanking regions within the VE-cadherin promoter (wild type). Alternatively, we also used a probe containing the same four-nucleotide substitution in SBS3 used in the mutant luciferase reporter construct (SBS3M), and a scrambled version of the wild-type sequence as a control (Fig. 6D). After pulling-down the probes with streptavidin-coated magnetic beads, we observed SOX7 binding the wild-type biotinylated probe but not its scrambled version. Strikingly, disruption of the SOX-binding motif in SBS3M strongly reduced the binding of SOX7 (Fig. 6E). Finally, to gain insight into the potential interaction of endogenous SOX7 with SBS3 in differentiated ES cells, ChIP assays were performed on day 4 EB-derived chromatin samples. An enriched occupancy of SOX7 on the promoter of VE-cadherin was observed in the region of SBS3, but not in a control region in the locus of *Gapdh* (Fig. 6F).

In summary, these data demonstrate that SOX7 can enhance the expression of VE-cadherin by binding to the most proximal SOX-binding motif of its promoter, hence identifying VE-cadherin as a transcriptional target of SOX7.

DEVELOPMENT

## DISCUSSION

The initial observations reported by early 20th-century embryologists of the close association between nascent blood precursors and endothelial cells pioneered a fascinating line of research that led to the accumulation of recent findings on haemangioblast and haemogenic endothelium development (Jordan, 1917; Sabin, 1920; Murray, 1932; Huber et al., 2004; Eilken et al., 2009; Lancrin et al., 2009; Bertrand et al., 2010; Boisset et al., 2010; Kissa and Herbomel, 2010). In the present work, we exploited the ES cell-derived blast colony model system to characterise and investigate the role of the transcription factor SOX7 in early embryonic haematopoiesis. During blast colony maturation, *Sox7* undergoes a rapid but transient upregulation at the onset of haemangioblast development. A closer look revealed that this upregulation of *Sox7* is mediated by the addition of VEGF to blast colony cultures and that *Sox7* expression is restricted to cells with haemogenic endothelium properties. CD41$^+$ cells emerging from the haemogenic endothelium downregulate *Sox7*, a finding consistent with the decline in *Sox7* expression observed as maturing blast colonies generated blood precursors. In fact, *Sox7* was previously demonstrated to be expressed in vertebrate endothelial cells (Young et al., 2006; Sakamoto et al., 2007; Takahashi et al., 2007; Cermenati et al., 2008; Pendeville et al., 2008) and to act downstream of the VEGF signalling pathway in the regulation of vasculature development during zebrafish ontogeny (Pendeville et al., 2008). Therefore, SOX7 is likely to be part of the molecular program that is intrinsic to the specification of the endothelial precursor populations. The functional characterisation of embryonic haemogenic endothelium populations was led by studies that took advantage of properties that commonly define endothelial cells. These included the detection of blood cells that derive from precursors capable of acetyl-LDL uptake (Jaffredo et al., 1998), as well as the isolation of haematopoietic progenitors solely based on the presence of VE-cadherin and lack of CD45 expression (Nishikawa et al., 1998a; Nishikawa et al., 1998b; Fraser et al., 2002; Fraser et al., 2003). It is now well established that the acquisition of CD41 expression is a milestone in the commitment of embryonic blood progenitors from the haemangioblast. In this context, the transition of haemogenic endothelium cells into CD41-expressing blood cells entails the rapid loss of endothelial identity that is marked by dramatic morphological changes (Eilken et al., 2009; Lancrin et al., 2009; Boisset et al., 2010; Kissa and Herbomel, 2010). In the work presented here, we enforced the expression of *Sox7* at discrete stages of haematopoietic development to understand the impact of counteracting its expression dynamics on the generation of blood precursors. The overexpression of *Sox7* in maturing blast colonies,

and more specifically in haemogenic endothelium cultures, is sufficient to arrest their haematopoietic differentiation and to sustain the otherwise transient expression of endothelial cell surface markers. Thus, it is tempting to propose a model in which SOX7 acts upstream of endothelial-related genes in the haemogenic endothelium before the haematopoietic differentiation has been initiated. Thereafter, the downregulation of this transcription factor is necessary for the dynamic process of blood precursor emergence from the haemogenic endothelium.

VE-cadherin was first identified as a membrane protein exclusively expressed in endothelial cells (Breier et al., 1996). The targeted disruption of this locus results in severe intra- and extra-embryonic vascular plexus malformations and ultimately leads to a lethal arrest in development by E11.5 (Gory-Fauré et al., 1999). Although no defects in yolk sac haematopoiesis were reported in *VE-cadherin*$^{-/-}$ embryos, a growing body of evidence has demonstrated that embryonic haematopoietic precursors are derived from VE-cadherin-expressing cells. This has been proven not only for the earliest blood cells generated within the yolk sac, but also for intra-embryonic tissues in different models of haematopoietic differentiation (Nishikawa et al., 1998a; Nishikawa et al., 1998b; Fraser et al., 2002; Zovein et al., 2008; Eilken et al., 2009). The kinetics of VE-cadherin expression during blast colony development follows the overall downregulation of endothelial markers (supplementary material Fig. S1), a characteristic aspect of embryonic haematopoietic differentiation. This expression pattern was counteracted by the overexpression of *Sox7*, as we have shown via gain-of-function experiments. The role of VE-cadherin in endothelial cell junction maintenance (reviewed by Vestweber, 2008) points to the possible role of VE-cadherin in maintaining the adherence of immature blast colony clusters prior to blood precursor emergence, even possibly the adherence of haemogenic endothelium cells in embryonic sites of haematopoietic formation. Thus, the fact that blast colony cells transduced with VE-cadherin-expressing lentivirus generate lower frequency of CD41$^+$ CD45$^+$ progenitors raises an attractive hypothesis, in which the maintenance of VE-cadherin expression and function in emerging blood cells could in part mediate the differentiation arrest induced by the enforced expression of SOX7.

A few sequence-specific transcription factors are known to bind the VE-cadherin promoter and to activate its expression. These include SCL, LMO2 and members of the ETS and FOX family of proteins (Lelièvre et al., 2000; Deleuze et al., 2007; Birdsey et al., 2008; De Val et al., 2008). Although the role of SOX7 in vasculature development has been demonstrated (reviewed by Francois et al., 2010), its downstream targets remain so far unknown. We carried out a series of biochemical assays to
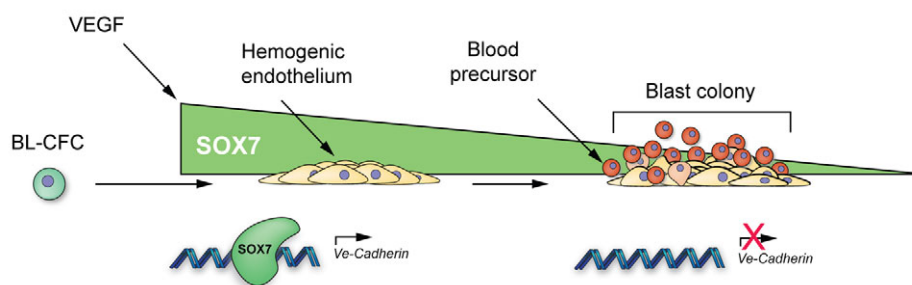


**Fig. 7. SOX7 regulates VE-cadherin in the haemogenic endothelium.** The proposed transcriptional control of VE-cadherin by SOX7 during haemangioblast/blast colony-forming cell (BL-CFC) differentiation. SOX7 lies downstream of the VEGF signalling pathway and is expressed in the transient population of haemogenic endothelium cells, where it activates the transcription of VE-cadherin. Upon differentiation, SOX7 is downregulated in haematopoietic precursors in which VE-cadherin expression is switched off.

demonstrate that SOX7 bound the promoter of VE-cadherin and enhanced its transcription. We have also shown that the knockdown of *Sox7* in blast colony cells reduces VE-cadherin expression. These findings strongly suggest that VE-cadherin is a direct target of SOX7 at the onset of haematopoietic specification (Fig. 7). Yet this does not demonstrate that SOX7 is the only SOX F member crucial for the bona fide transcription of VE-cadherin. In fact, SOX7 and SOX18 are well described to play redundant and balanced roles during cardiovascular and lymphatic development, including in the regulation of VE-cadherin expression in zebrafish embryos (Zhang et al., 2005; Cermenati et al., 2008; Herpers et al., 2008; Pendeville et al., 2008; Hosking et al., 2009). Moreover, SOX18 has been directly implicated in microvasculature stability (Downes et al., 2009) and in the regulation of the transcription of *Cldn5* and *Vcam1*, genes encoding key players in endothelial function (Hosking et al., 2004; Fontijn et al., 2008). Similar to *Sox7*, *Sox18* is expressed at early stages of haematopoiesis and its overexpression results in an arrest in development that resembles, to some extent, the effects mediated by SOX7 (Serrano et al., 2010). Therefore, we cannot rule out that SOX18 equally regulates the transcription of VE-cadherin at the onset of blood differentiation. Alternatively, SOX7 could have a differential activity that would depend on the type of endothelium, i.e. it would be crucial for the expression of VE-cadherin in haemogenic endothelium cells but not in non-haemogenic endothelium cells. Therefore, further SOX7 loss-of-function studies will be fundamental to clarify this issue.

### Competing interests statement
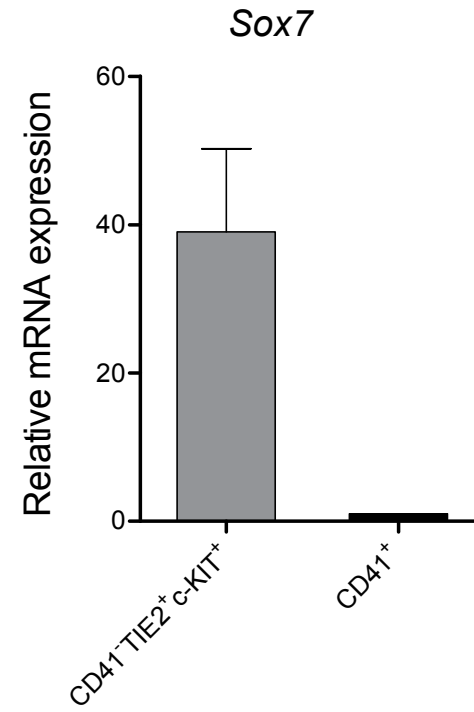The authors declare no competing financial interests.

### Supplementary material
Supplementary material available online at
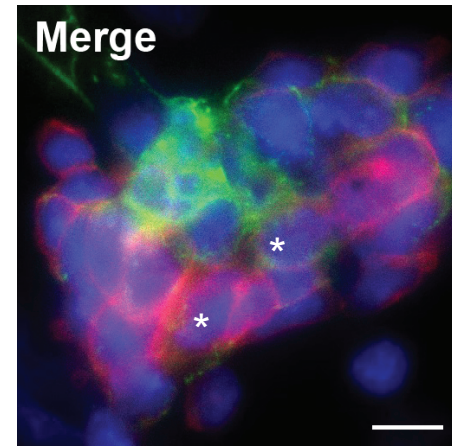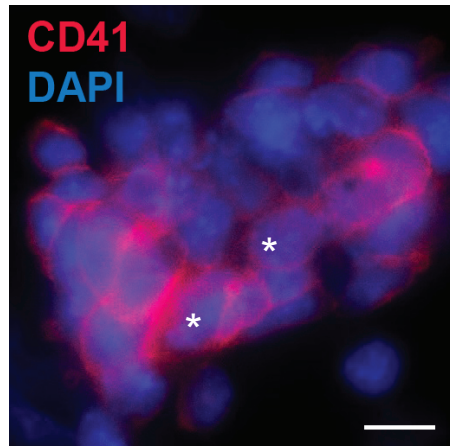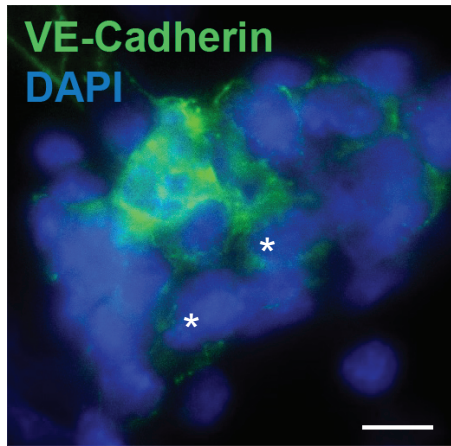http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.071282/-/DC1
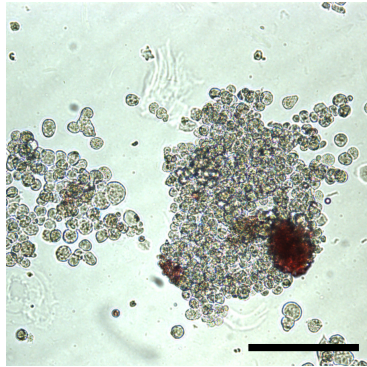
### References
**Bertrand, J. Y., Chi, N. C., Santoso, B., Teng, S., Stainier, D. Y. R. and Traver, D.** (2010). Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature* **464**, 108-111.

**Birdsey, G. M., Dryden, N. H., Amsellem, V., Gebhardt, F., Sahnan, K., Haskard, D. O., Dejana, E., Mason, J. C. and Randi, A. M.** (2008). Transcription factor Erg regulates angiogenesis and endothelial apoptosis through VE-cadherin. *Blood* **111**, 3498-3506.

**Boisset, J. C., van Cappellen, W., Andrieu-Soler, C., Galjart, N., Dzierzak, E. and Robin, C.** (2010). In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature* **464**, 116-120.

**Bowles, J., Schepers, G. and Koopman, P.** (2000). Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. *Dev. Biol.* **227**, 239-255.

**Breier, G., Breviario, F., Caveda, L., Berthier, R., Schnurch, H., Gotsch, U., Vestweber, D., Risau, W. and Dejana, E.** (1996). Molecular cloning and expression of murine vascular endothelial- cadherin in early stage development of cardiovascular system. *Blood* **87**, 630.

**Bryne, J. C., Valen, E., Tang, M. H., Marstrand, T., Winther, O., da Piedade, I., Krogh, A., Lenhard, B. and Sandelin, A.** (2008). JASPAR, the open access database of transcription factor-binding profiles: new content and tools in the 2008 update. *Nucleic Acids Res.* **36**, D102-D106.

**Cermenati, S., Moleri, S., Cimbro, S., Corti, P., Del Giacco, L., Amodeo, R., Dejana, E., Koopman, P., Cotelli, F. and Beltrame, M.** (2008). Sox18 and Sox7 play redundant roles in vascular development. *Blood* **111**, 2657-2666.

**Chen, M. J., Yokomizo, T., Zeigler, B. M., Dzierzak, E. and Speck, N. A.** (2009). Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. *Nature* **457**, 887-891.

**Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J. C. and Keller, G.** (1998). A common precursor for hematopoietic and endothelial cells. *Development* **125**, 725-732.

**Chung, Y. S., Zhang, W. J., Arentson, E., Kingsley, P. D., Palis, J. and Choi, K.** (2002). Lineage analysis of the hemangioblast as defined by FLK1 and SCL expression. *Development* **129**, 5511-5520.

**de Felipe, P., Martin, V., Cortes, M. L., Ryan, M. and Izquierdo, M.** (1999). Use of the 2A sequence from foot-and-mouth disease virus in the generation of retroviral vectors for gene therapy. *Gene Ther.* **6**, 198-208.

**De Val, S., Chi, N. C., Meadows, S. M., Minovitsky, S., Anderson, J. P., Harris, I. S., Ehlers, M. L., Agarwal, P., Visel, A., Xu, S. M. et al.** (2008). Combinatorial regulation of endothelial gene expression by ets and forkhead transcription factors. *Cell* **135**, 1053-1064.

**Deleuze, V., Chalhoub, E., El-Hajj, R., Dohet, C., Le Clech, M., Couraud, P. O., Huber, P. and Mathieu, D.** (2007). TAL-1/SCL and its partners E47 and LMO2 up-regulate VE-cadherin expression in endothelial cells. *Mol. Cell. Biol.* **27**, 2687-2697.

**Downes, M., Francois, M., Ferguson, C., Parton, R. G. and Koopman, P.** (2009). Vascular defects in a mouse model of hypotrichosis-lymphedema-telangiectasia syndrome indicate a role for SOX18 in blood vessel maturation. *Hum. Mol. Genet.* **18**, 2839-2850.

**Dull, T., Zufferey, R., Kelly, M., Mandel, R. J., Nguyen, M., Trono, D. and Naldini, L.** (1998). A third-generation lentivirus vector with a conditional packaging system. *J. Virol.* **72**, 8463-8471.

**Eilken, H. M., Nishikawa, S. I. and Schroeder, T.** (2009). Continuous single-cell imaging of blood generation from haemogenic endothelium. *Nature* **457**, 896-900.

**Faloon, P., Arentson, E., Kazarov, A., Deng, C. X., Porcher, C., Orkin, S. and Choi, K.** (2000). Basic fibroblast growth factor positively regulates hematopoietic development. *Development* **127**, 1931-1941.

**Fehling, H. J., Lacaud, G., Kubo, A., Kennedy, M., Robertson, S., Keller, G. and Kouskoff, V.** (2003). Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation. *Development* **130**, 4217-4227.

**Fontijn, R. D., Volger, O. L., Fledderus, J. O., Reijerkerk, A., de Vries, H. E. and Horrevoets, A. J.** (2008). SOX-18 controls endothelial-specific claudin-5 gene expression and barrier function. *Am. J. Physiol. Heart Circ. Physiol.* **294**, H891-H900.

**Francois, M., Koopman, P. and Beltrame, M.** (2010). SoxF genes: Key players in the development of the cardio-vascular system. *Int. J. Biochem. Cell Biol.* **42**, 445-448.

**Fraser, S. T., Ogawa, M., Yu, R. T., Nishikawa, S., Yoder, M. C. and Nishikawa, S. I.** (2002). Definitive hematopoietic commitment within the embryonic vascular endothelial-cadherin(+) population. *Exp. Hematol.* **30**, 1070-1078.

**Fraser, S. T., Ogawa, M., Yokomizo, T., Ito, Y., Nishikawa, S. and Nishikawa, S. I.** (2003). Putative intermediate precursor between hematogenic endothelial cells and blood cells in the developing embryo. *Dev. Growth Differ.* **45**, 63-75.

**Gandillet, A., Serrano, A. G., Pearson, S., Lie-A-Ling, M., Lacaud, G. and Kouskoff, V.** (2009). Sox7-sustained expression alters the balance between proliferation and differentiation of hematopoietic progenitors at the onset of blood specification. *Blood* **114**, 4813-4822.

**Gilham, D. E., Lie, A. L. M., Taylor, N. and Hawkins, R. E.** (2010). Cytokine stimulation and the choice of promoter are critical factors for the efficient transduction of mouse T cells with HIV-1 vectors. *J. Gene Med.* **12**, 129-136.

**Gory, S., Vernet, M., Laurent, M., Dejana, E., Dalmon, J. and Huber, P.** (1999). The vascular endothelial-cadherin promoter directs endothelial-specific expression in transgenic mice. *Blood* **93**, 184-192.

**Gory-Fauré, S., Prandini, M. H., Pointu, H., Roullot, V., Pignot-Paintrand, I., Vernet, M. and Huber, P.** (1999). Role of vascular endothelial-cadherin in vascular morphogenesis. *Development* **126**, 2093-2102.

**Herpers, R., van de Kamp, E., Duckers, H. J. and Schulte-Merker, S.** (2008). Redundant roles for sox7 and sox18 in arteriovenous specification in zebrafish. *Circ. Res.* **102**, 12-15.

**Hoogenkamp, M., Lichtinger, M., Krysinska, H., Lancrin, C., Clarke, D., Williamson, A., Mazzarella, L., Ingram, R., Jorgensen, H., Fisher, A. et al.** (2009). Early chromatin unfolding by RUNX1: a molecular explanation for differential requirements during specification versus maintenance of the hematopoietic gene expression program. *Blood* **114**, 299-309.

**Hosking, B., François, M., Wilhelm, D., Orsenigo, F., Caprini, A., Svingen, T., Tutt, D., Davidson, T., Browne, C., Dejana, E. et al.** (2009). Sox7 and Sox17 are strain-specific modifiers of the lymphangiogenic defects caused by Sox18 dysfunction in mice. *Development* **136**, 2385-2391.

**Hosking, B. M., Wang, S. C. M., Downes, M., Koopman, P. and Muscat, G. E. O.** (2004). The VCAM-1 gene that encodes the vascular cell adhesion molecule is a target of the Sry-related high mobility group box gene, Sox18. *J. Biol. Chem.* **279**, 5314-5322.

Huber, T. L., Kouskoff, V., Fehling, H. J., Palis, J. and Keller, G. (2004). Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. *Nature* **432**, 625-630.

Iacovino, M., Chong, D., Szatmari, I., Hartweck, L., Rux, D., Caprioli, A., Cleaver, O. and Kyba, M. (2010). HoxA3 is an apical regulator of haemogenic endothelium. *Nat. Cell Biol.* **13**, 72.

Jaffredo, T., Gautier, R., Eichmann, A. and Dieterlen-Lièvre, F. (1998). Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. *Development* **125**, 4575-4583.

Jaffredo, T., Nottingham, W., Liddiard, K., Bollerot, K., Pouget, C. and de Bruijn, M. (2005). From hemangioblast to hematopoietic stem cell: an endothelial connection? *Exp. Hematol.* **33**, 1029-1040.

Jordan, H. E. (1917). Aortic cell clusters in vertebrate embryos. *Proc. Natl. Acad. Sci. USA* **3**, 149-156.

Kanai-Azuma, M., Kanai, Y., Gad, J. M., Tajima, Y., Taya, C., Kurohmaru, M., Sanai, Y., Yonekawa, H., Yazaki, K., Tam, P. P. L. et al. (2002). Depletion of definitive gut endoderm in Sox17-null mutant mice. *Development* **129**, 2367-2379.

Kennedy, M., Firpo, M., Choi, K., Wall, C., Robertson, S., Kabrun, N. and Keller, G. (1997). A common precursor for primitive erythropoiesis and definitive haematopoiesis. *Nature* **386**, 488-493.

Kennedy, M., D'Souza, S. L., Lynch-Kattman, M., Schwantz, S. and Keller, G. (2007). Development of the hemangioblast defines the onset of hematopoiesis in human ES cell differentiation cultures. *Blood* **109**, 2679-2687.

Kim, I., Saunders, T. L. and Morrison, S. J. (2007). Sox17 dependence distinguishes the transcriptional regulation of fetal from adult hematopoietic stem cells. *Cell* **130**, 470-483.

Kissa, K. and Herbomel, P. (2010). Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature* **464**, 112-115.

Kyba, M., Perlingeiro, R. C. and Daley, G. Q. (2002). HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell* **109**, 29-37.

Lam, E. Y., Hall, C. J., Crosier, P. S., Crosier, K. E. and Flores, M. V. (2010). Live imaging of Runx1 expression in the dorsal aorta tracks the emergence of blood progenitors from endothelial cells. *Blood* **116**, 909-914.

Lancrin, C., Sroczynska, P., Stephenson, C., Allen, T., Kouskoff, V. and Lacaud, G. (2009). The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. *Nature* **457**, 892-895.

Lelièvre, E., Mattot, V., Huber, P., Vandenbunder, B. and Soncin, F. (2000). ETS1 lowers capillary endothelial cell density at confluence and induces the expression of VE-cadherin. *Oncogene* **19**, 2438-2446.

Murakami, A., Shen, H., Ishida, S. and Dickson, C. (2004). SOX7 and GATA-4 are competitive activators of Fgf-3 transcription. *J. Biol. Chem.* **279**, 28564-28573.

Murray, P. D. F. (1932). The development in vitro of the blood of the early chick embryo. *Proc. R. Soc. Lond.* **111**, 497-521.

Nishikawa, S. I., Nishikawa, S., Hirashima, M., Matsuyoshi, N. and Kodama, H. (1998a). Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. *Development* **125**, 1747-1757.

Nishikawa, S. I., Nishikawa, S., Kawamoto, H., Yoshida, H., Kizumoto, M., Kataoka, H. and Katsura, Y. (1998b). In vitro generation of lymphohematopoietic cells from endothelial cells purified from murine embryos. *Immunity* **8**, 761-769.

Pearson, S., Sroczynska, P., Lacaud, G. and Kouskoff, V. (2008). The stepwise specification of embryonic stem cells to hematopoietic fate is driven by sequential exposure to Bmp4, activin A, bFGF and VEGF. *Development* **135**, 1525-1535.

Pendeville, H., Winandy, M., Manfroid, I., Nivelles, O., Motte, P., Pasque, V., Peers, B., Struman, I., Martial, J. A. and Voz, M. L. (2008). Zebrafish Sox7 and Sox18 function together to control arterial-venous identity. *Dev. Biol.* **317**, 405-416.

Rosenbauer, F. and Tenen, D. G. (2007). Transcription factors in myeloid development: balancing differentiation with transformation. *Nat. Rev. Immunol.* **7**, 105.

Sabin, F. R. (1920). Studies on the origin of blood-vessels and of red blood-corpuscles as seen in the living blastoderm of chicks during the second day of incubation. *Contrib. Embryol. Carnegie Inst. Washington* **9**, 215-262.

Sakamoto, Y., Hara, K., Kanai-Azuma, M., Matsui, T., Miura, Y., Tsunekawa, N., Kurohmaru, M., Saijoh, Y., Koopman, P. and Kanai, Y. (2007). Redundant roles of Sox17 and Sox18 in early cardiovascular development of mouse embryos. *Biochem. Biophys. Res. Commun.* **360**, 539-544.

Serrano, A. G., Gandillet, A., Pearson, S., Lacaud, G. and Kouskoff, V. (2010). Contrasting effects of Sox17- and Sox18-sustained expression at the onset of blood specification. *Blood* **115**, 3895-3898.

Sroczynska, P., Lancrin, C., Pearson, S., Kouskoff, V. and Lacaud, G. (2009). In vitro differentiation of mouse embryonic stem cells as a model of early hematopoietic development. *Methods Mol. Biol.* **538**, 317-334.

Sun, D., Melegari, M., Sridhar, S., Rogler, C. E. and Zhu, L. (2006). Multi-miRNA hairpin method that improves gene knockdown efficiency and provides linked multi-gene knockdown. *Biotechniques* **41**, 59-63.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K. and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861-872.

Takash, W., Cañizares, J., Bonneaud, N., Poulat, F., Mattéi, M. G., Jay, P. and Berta, P. (2001). SOX7 transcription factor: sequence, chromosomal localisation, expression, transactivation and interference with Wnt signalling. *Nucleic Acids Res.* **29**, 4274-4283.

Vestweber, D. (2008). VE-cadherin: the major endothelial adhesion molecule controlling cellular junctions and blood vessel formation. *Arterioscler. Thromb. Vasc. Biol.* **28**, 223-232.

Vogeli, K. M., Jin, S.-W., Martin, G. R. and Stainier, D. Y. R. (2006). A common progenitor for haematopoietic and endothelial lineages in the zebrafish gastrula. *Nature* **443**, 337-339.

Wegner, M. (1999). From head to toes: the multiple facets of Sox proteins. *Nucleic Acids Res.* **27**, 1409-1420.

Young, N., Hahn, C. N., Poh, A., Dong, C., Wilhelm, D., Olsson, J., Muscat, G. E. O., Parsons, P., Gamble, J. R. and Koopman, P. (2006). Effect of disrupted SOX18 transcription factor function on tumor growth, vascularization, and endothelial development. *J. Natl. Cancer Inst.* **98**, 1060-1067.

Zhang, C., Basta, T. and Klymkowsky, M. W. (2005). SOX7 and SOX18 are essential for cardiogenesis in Xenopus. *Dev. Dyn.* **234**, 878-891.

Zovein, A. C., Hofmann, J. J., Lynch, M., French, W. J., Turlo, K. A., Yang, Y., Becker, M. S., Zanetta, L., Dejana, E., Gasson, J. C. et al. (2008). Fate tracing reveals the endothelial origin of hematopoietic stem cells. *Cell Stem Cell* **3**, 625-636.

Zufferey, R., Dull, T., Mandel, R. J., Bukovsky, A., Quiroz, D., Naldini, L. and Trono, D. (1998). Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J. Virol.* **72**, 9873-9880.
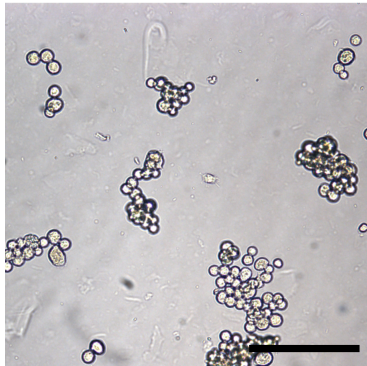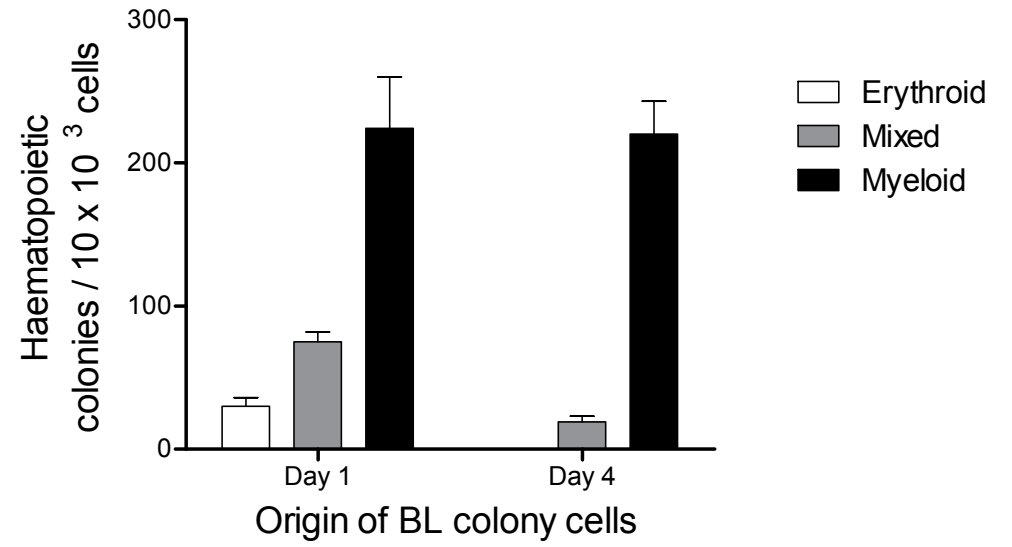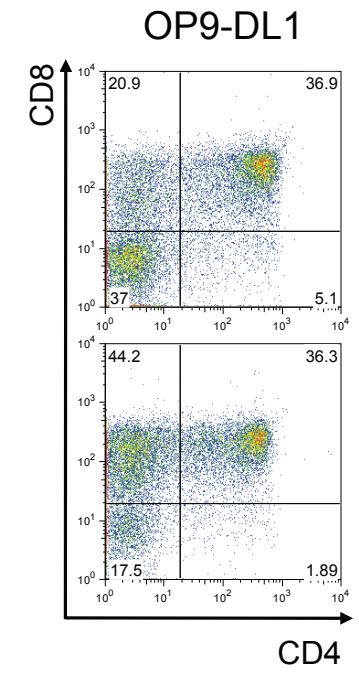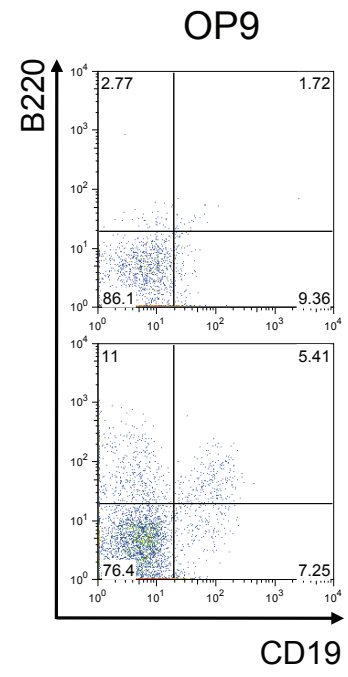
**A**

E7.5 embryos



Sort CD41$^+$ cells

Sort
CD41$^-$TIE2$^+$c-KIT$^+$ cells

**B**

*Sox7*

**A**

Day 1

Day 4

**B**

**A** *Sox7* shRNA

1  2  3

dTomato

**B**

*Sox7*

Relative mRNA expression

- Dox    + Dox

BL colony culture

*Ve-Cadherin*

Relative mRNA expression

- Dox    + Dox

BL colony culture

**C**

- Dox

30.3

+ Dox

16.6

% of Max

0  $10^2$  $10^3$  $10^4$  $10^5$         0  $10^2$  $10^3$  $10^4$  $10^5$

VE-Cadherin

## Table S1. Primers

| Primer | Sequence | Application (EUP No.) |
|---|---|---|
| β-Actin – For. | TGACAGGATGCAGAAGGAGA | Gene expression (106) |
| β-Actin – Rev. | CGCTCAGGAGGAGCAATG | Gene expression (106) |
| *Gapdh* promoter – For. | TAGGAGACACTGCCCCTTTC | ChIP (45) |
| *Gapdh* promoter – Rev. | CCCTCACGTCCCAACTCTC | ChIP (45) |
| *Pu.1* – For. | GGAGAAGCTGATGGCTTGG | Gene expression (94) |
| *Pu.1* – Rev. | CAGGCGAATCTTTTTCTTGC | Gene expression (94) |
| *Sox7* – For. | CAGCAAGATGCTGGGAAAG | Gene expression (97) |
| *Sox7* – Rev. | TGCATCATCCACATAGGGTCT | Gene expression (97) |
| *VE-cadherin* – For. | TCATCAAACCCACGAAGTCC | Gene expression (42) |
| *VE-cadherin* – Rev. | GGTCTGTGGCCTCAATGTAGA | Gene expression (42) |
| *VE-cadherin* promoter – For. | CACCGTAGGGCTTGCCTAT | ChIP (27) |
| *VE-cadherin* promoter – Rev. | TCGGGATGGTTTCCTGTTAT | ChIP (27) |
| *VE-cadherin* SBS3M promoter – For. | AGCTCACAAAGGAAgccgAACAGGAAACCA | Directed mutagenesis |
| *VE-cadherin* SBS3M promoter – Rev. | TGGTTTCCTGTTcggcTTCCTTTGTGAGCT | Directed mutagenesis |
| *VE-cadherin* MluI-3,039 promoter – For. | GAGAACGTGCGAGGGGAAGGAGGAGAAAGA | PCR amplification |
| *VE-cadherin* MluI-1,104 promoter – For. | GAGAACGCGTCACTCTGGGGGTCTGTCATT | PCR amplification |
| *VE-cadherin* MluI-531 promoter – For. | GAGAACGCGTCCCGACTCTGAGAACTCCAC | PCR amplification |
| *VE-cadherin* XhoI+23 promoter – Rev. | GAGACTCGAGCAGAAGGCTCTCTCCACTGC | PCR amplification |
| *VE-cadherin* CDS BamHI – For. | GGATCCAAGATCAGCTCCTCCACGAA | PCR amplification |
| *VE-cadherin* CDS XhoI – Rev. | CTCGAGGATGATGAGTTCCTCCTGGGG | PCR amplification |

Lowercase indicates nucleotide substitutions.
EUP, Exiqon universal probe; For., forward; Rev., reverse.

**Table S2. The promoter of *VE-cadherin* contains three SBSs**

| Name | Score | Start | End | Strand | Predicted site sequence |
|------|-------|-------|-----|--------|------------------------|
| SBS1 | 10.075 | −1895 | −1890 | + | AACAAT |
| SBS2 | 7.562 | −554 | −549 | − | ATTGTC |
| SBS3 | 9.767 | −141 | −136 | + | AACAAT |

Motif search analysis of SOX5 sites in the mouse VE-cadherin 2500 bp promoter using JASPAR database reveals the presence of three SBSs; the position of the predicted site sequence relative to the TSS is shown.