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The Role of Meis1 in Hematopoietic Development

Mi Cai
Washington University in St. Louis

WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences

Molecular Cell Biology

Dissertation Examination Committee:
Kenneth M. Murphy, Chair
David C. Beebe
Deepta Bhattacharya
Raphael Kopan
Kristen Kroll
David M. Ornitz

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By

Mi Cai

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ABSTRACT OF THE DISSERTATION

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Previous work has identified *Mesp1* as an important regulator of the epithelial-mesenchymal transition (EMT) and of cardiovascular cell fate in differentiating embryonic stem cells (ESCs). To understand the molecular mechanisms underlying the actions of *Mesp1,* we sought to identify transcription targets of *Mesp1*. *Mesp1* rapidly induced expression of *PDGFR*α in differentiating ESCs and directly bound to evolutionary conserved E-boxes within the *PDGFR*α promoter. This result suggested that *PDGFR*α could be a direct target of *Mesp1*. However, we found that *PDGFR*α was not sufficient for the induction of EMT in ESCs or the induction of $Flk1^+$ mesoderm, but that it may play a role rather in the survival of *Mesp1*-induced mesodermal cells.

Although a clear role for *Mesp1* in EMT and cardiovascular differentiation has been established, its function in hematopoietic development is still unclear. Previous lineage tracing demonstrated that *Mesp1* activity labeled endothelial cells of embryonic dorsal aorta, which recently was shown to give rise to definitive hematopoietic progenitors. This suggested the potential that *Mesp1* activity in endothelium might

influence subsequent hematopoietic development. Although *in vitro* studies indicated that *Mesp1* acted to suppress emergence of hematopoietic progenitors, we made the surprising observation in lineage tracing analysis of *Mesp1* that all adult hematopoietic progenitors and mature lineages were efficiently labeled by *Mesp1*-Cre, and further that *Mesp1* was necessary for hematopoietic differentiation of ESCs. In examining the downstream targets of *Mesp1* in ESC-derived endothelial cells, we identified myeloid ecotropic viral integration site 1 (*Meis1*).

Meis1 forms a heterodimer with Pbx1 that augments Hox-dependent gene expression. In addition, *Meis1* has been associated with leukemogenesis and hematopoietic stem cell self-renewal. In examining potential roles of *Meis1* in hematopoietic development, we identified two independent actions. One activity regulated cellular proliferation of early hematopoietic progenitors. The second activity was involved the fate choice between erythroid and megakaryocyte lineages. First, we found that endogenous *Mesp1* indirectly induces *Meis1* and *Meis2* in endothelial cells derived from embryonic stem (ES) cells. Overexpression of *Meis1* and *Meis2* greatly enhanced the formation of hematopoietic colonies from ES cells, with the exception of erythroid colonies, by maintaining hematopoietic progenitor cells in a state of proliferation. Second, overexpression of *Meis1* repressed the development of early erythroid progenitors, acting *in vivo* at the megakaryocyte-erythroid progenitor (MEP) stage to skew development away from erythroid generation and toward megakaryocyte development. This previously unrecognized action of *Meis1* may explain the embryonic lethality observed in *Meis1*$^{-/-}$ mice that arises from failure of lymphatic-venous separation, and which can result as a consequence of defective platelet generation. These results

show that *Meis1* exerts two independent functions, with its role in proliferation of hematopoietic progenitors acting earlier in development from its influence on the fate choice at the MEP between megakaryocytic and erythroid development.

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Finally, I would like to dedicate this thesis to my parents and my husband Peng Lin for their love.

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CHAPTER 1: INTRODUCTION

The embryonic stem cell model system

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of blastocyst-stage embryos.[1,2] These cells have two unique characteristics. First, they can be maintained and expanded as pure populations of undifferentiated cells in culture. Second, they are pluripotent and possess the capacity to generate every cell type in the body. The pluripotent nature of mouse ES cells was formally demonstrated by their ability to contribute to all tissues of adult mice, including the germ line, following their injection into host blastocysts.[3] In addition to their developmental potential *in vivo*, ES cells display a remarkable capacity to form differentiated cell types in culture. Under appropriated conditions, ES cells will differentiate and generate progeny consisting of derivatives of three embryonic germ layers: mesoderm, endoderm, and ectoderm.[4,5] Studies during the past 30 years have led to the development of appropriate culture conditions and protocols for the generation of a broad spectrum of lineages.

The ability to derive multiple lineages from ES cells opens exciting new opportunities to model embryonic development *in vitro* for studying the events regulating the earliest stages of lineage induction and specification. Findings from several different studies have demonstrated striking parallels between the ES cell model and the early embryo. In addition, ES cell model enable us to access cells at different stages of development and to use a combination of genetic, molecular biology and tissue culture tools to study developmental processes when such studies are difficult in the mouse embryo and impossible in the human embryo. Mesoderm-derived lineages, including the hematopoietic, vascular, and cardiac, are among the easiest to generate from ES cells and have been studied in considerable detail. In addition to providing a model of early

development, the ES cell differentiation system is viewed as a novel and unlimited source of cells and tissues for transplantation for the treatment of a broad spectrum of diseases.

The role of Mesp1 in embryogenesis and mouse ESC differentiation

Mesp1, a basic helix-loop-helix transcription factor, is one of the earliest markers for the vertebrate cardiovascular development.[6] It is transiently expressed in the primitive steak at the onset of gastrulation (E6.5) and in the prospective cardiac mesoderm and is then rapidly downregluated after E7.5.[7] Lineage tracing studies suggest that *Mesp1* is expressed by almost all precursors of the cardiovascular system.[8] *Mesp1*-deficient mice show aberrant heart morphogenesis that resulted in cardia bifida.[8] Recently, by taking advantage of the *in vitro* ESC differentiation model, our lab and other two groups demonstrate that *Mesp1* acts as a master regulator during cardiovascular specification (Figure 1.1).[9-11] Transient *Mesp1* expression in ESCs is sufficient to promote a restricted set of cardiovascular fates including cardiomyocytes, smooth muscle cells and endothelial cells. Our group also suggested *Mesp1* induces features of EMT and *snai1* in differentiating ESCs, consistent with a role in gastrulation.[12] In addition, Bondue et al. found *Mesp1* induction leads to a transient inhibition of apoptosis of differentiating ESCs.[9]

Although a clear role for *Mesp1* in cardiovascular differentiation and EMT has been established, its function in hematopoietic development is still unclear. Our *in vitro* studies indicated that *Mesp1* acted to suppress emergence of hematopoietic progenitors, although we did not establish if this action occurred *in vivo*. Previous lineage tracing demonstrated that *Mesp1* activity labeled endothelial cells of embryonic dorsal aorta,

which recently was shown to give rise to definitive hematopoietic progenitors. This suggested the potential that *Mesp1* activity in endothelium might influence subsequent hematopoietic development.

It is important to identify transcriptional targets of *Mesp1* to understand its actions. The effect of *Mesp1* on myocardial differentiation was ascribed to the ability of *Mesp1* to directly transactivate *Dkk1* expression.[11] However, our group showed *Dkk1* alone was not sufficient for the observed *Mesp1* effects, and Bondue et al. further clarified that *Dkk1* was not a direct target of *Mesp1* by chromatin immunoprecipitation (ChIP) in differentiating ESCs.[9,10] Instead, based on evidence from ChIP assays, Bondue et al. suggested that *Mesp1* directly activated the core cardiac transcription factors *Hand2*, *Nkx2*.5, *Myocd* and *Gata4*.[9] However, these genes were not upregulated until 24 or 48 hours after *Mesp1* induction based on our microarray expression data. Instead, we found a group of genes which were induced by *Mesp1* within 6 or 12 hours.[10] Since direct *Mesp1* targets should be induced earlier than indirect targets, these genes may represent additional direct targets of *Mesp1*.

PDGFRα and its developmental functions

PDGFRα is one of those genes which were induced by *Mesp1* within 6 hr. PDGFRs and their ligands (PDGFs) have served as prototypes for receptor tyrosine kinase and growth factor function for a long time. In higher vertebrates, there are two PDGF receptors, PDGFRα and PDGFRβ, which form both homo- and hetero-dimers. The cellular processes that depend on PDGFR signaling include cell survival, cell proliferation and directed cell migration, and signaling through PI3K-Akt downstream

appears to be critical for these processes.[13] Embryogenesis studies suggest an evolutionary conserved role for PDGFRα signaling during gastrulation of a variety of organisms, including frog, zebrafish, and sea urchin.[14-17] In *Xenopus* embryos, the involuting mesodermal cells fail to migrate beneath the blastocoel roof ectoderm but undergo apoptosis upon PDGFRα inhibition. Rescue from apoptosis did not rescue the migration defect, suggesting that PDGFRα signaling independently controls both processes.[18] Recently, PDGFRα signaling is shown to control the migration of mesoderm cells during chick gastrulation.[19] Both expression of a dominant-negative PDGFRα and depletion of endogenous PDGFRα ligands inhibit the migration of mesoderm cells after their ingression through the primitive streak.

Since the double *Mesp1/2* null mice exhibit a similar defect in gastrulation which is the mesodermal cells fail to migrate out from the primitive streak,[12] it is possible that *PDGFRα* acts downstream of *Mesp1* in mouse gastrulation. Although *PDGFA* is expressed in the epiblast and *PDGFRα* is expressed in the nascent mesoderm during mouse gastrulation, a role for *PDGFRα* signaling in mouse gastrulation is not apparent.[20,21] *PDGFA* and *PDGFRα*-null mouse embryos nevertheless show severe impairment of early mesenchymal derivatives in both embryo and extraembryonic tissues and die during gestation.[22] However, the gastrulation defects in *PDGFRα*-null mice might be compensated by *PDGFRβ* or other factors. A recent paper shows that *Mesp1*-cre-mediated conditional knockout mice of both *PDGFRα* and *PDGFRβ* don't result in an observable cardiac phenotype.[23] However, as we have shown *PDGFRα* is an early target of *Mesp1* and therefore some *PDGFRα* might have been transcribed before the *Mesp1*-cre-mediated deletion occurs, the early gastrulation defects can be masked. Due

to the complexity and technique difficulties of *in vivo* study, ES cell differentiation model might be a good alternative for us to understand *PDGFR*α and its transcriptional and functional relationships with *Mesp1*.

Hemogenic endothelium

During embryonic development, hematopoietic stem cells (HSCs), which give rise to adult blood system, and endothelial cells, which line blood vessels, both form from the mesodermal germ cell layer; but exactly how is debatable. Two theories have been proposed. One is that both hematopoietic and endothelial cells arise from a mesoderm-derived common precursor called a hemangioblast. The other theory proposes that HSCs derive from a subset of early endothelial cells known as hemogenic endothelium. Using time-lapse imaging with live markers and genetic analysis of differentiating ESCs, two groups revealed that hematopoietic cells were generated from hemogenic endothelium *in vitro*.[24,25] Moreover, recent studies using *in vivo* imaging directly observed that HSCs emerging from aortic endothelium during both zebrafish and mouse development.[26-28] These studies clearly demonstrated the presence of hemogenic endothelium in mouse embryo and it is the origin of HSCs. However, the molecular mechanism underlying the transition from hemogenic endothelium to nascent HSCs remains unclear.

Meis1 protein and its regulation

Meis1 was identified as a common site of proviral integration by the ecotropic virus in BXH-2 mice that promoted myeloid leukemias.[29,30] Meis1 belongs to the TALE class of homeodomain transcription factors characterized by a three amino acid loop

extension between the α-helices within its homeodomain (Figure 1.2A). Meis1 interacts with other homeodomain proteins,[31] in particular Pbx1,[32] forming a heterodimer that recognizes DNA (Figure 1.2B). The Meis1 protein contains a domain that recognizes wild-type Pbx proteins, but not chimeric Pbx1 proteins formed by translocations such as the E2a-Pbx1 oncoprotein.[33] The Meis1/Pbx dimer cooperatively associates with Hox homeodomain proteins, and *in vitro* interaction between Meis1, Hoxa9 and Pbx proteins can occur in the absence of DNA.[34] In addition to its Pbx interaction motif (PIM),[33] Meis1 also contains a carboxyl-terminal (C-terminal) region that is required for leukemia induction.[35] This C-terminal region of Meis1 contains transcriptional activity regulated by protein kinase A (PKA) that appears dependent upon the co-activator of cAMP response element-binding protein (CREB), CREB-binding protein (CBP).[36] Thus, Meis1 appears to augment Hox transcription factor activity and can be regulated by extracellular signaling cues.

The role of meis1 in leukemogenesis

Initial analysis of *Meis1* focused on its role in leukemic transformation. Acute myeloid leukemia (AML) induced by *Hoxa9* was significantly accelerated by co-expression with *Meis1*, but not by co-expression with *Pbx1b*.[37] A cellular action of *Meis1* appeared to be the suppression of differentiation and the promotion of proliferation in a system of cytokine driven *Hoxa9*-immortalized cells.[38] A Hoxa9 chimeric fusion protein, NUP98-Hoxa9, independently induced a silent pre-leukemic phase of disease which was accelerated by Meis1, suggesting that Meis1 augments the activities of the Hoxa9-dependent transformational event.[39] Interactions between Meis1 and Hoxa9 also

occur in a model of leukemia induced by rearrangements of the *MLL/ALL1* gene, which represents approximately 20% of acute lymphoblastic leukemias and 5-6% of AML.[40] In this setting, *Meis1* is an essential, rate limiting regulator of the development of MLL-dependent leukemias.[40,41]

The role of Meis1 in hematopoiesis

Studies based on *Meis1* overexpression initially suggested a role in regulation of proximodistal limb axis development.[42] However, studies based on targeted disruption of *Meis1* in mice observed more substantial defects in hematopoiesis, angiogenesis and eye development.[43-45] Complete elimination of *Meis1* by gene targeting caused death between embryonic day 11.5 and 14.5.[44] While definitive myeloerythroid lineages are present in *Meis1*$^{-/-}$ embryos, the total numbers of colony-forming cells are significantly reduced. Similar defects were observed when *Meis1* was targeted by a strategy that potentially generated a dominant negative protein, but in this case defects were also observed in the developing eye, with partially duplicated retinas and smaller lenses.[43] This latter effect potentially could represent interference with the normal actions of *Meis2*, rather than *Meis1*, since *Meis2* has been demonstrated to regulate the expression of *Pax6*, a pivotal regulator of eye development.[46] *Meis2* has been shown to maintain retinal progenitor cells in a state of rapid proliferation, at least in part through regulation of cell cycle machinery including *cyclin D1*.[47]

Early embryonic lethality due to *Meis1* deficiency was initially thought to result from hemorrhage secondary to vascular defects,[43,44] but subsequent studies demonstrated that the absence of platelets in *Meis1*$^{-/-}$ embryos leads to a failure in separation of

lymphatic vessels during embryonic angiogenesis,[45] as platelets are critical in mediating separation of the venous and lymphatic systems.[48,49] Further studies have recognized additional defects in heart development in *Meis1*$^{-/-}$ embryos.[50]

While *Meis1* promotes leukemogenesis and normal hematopoiesis by modulating self-renewal of progenitor-like cells, the basis for this action as well as the defect in platelet development in *Meis1*$^{-/-}$ mice are still incompletely understood.

The transcriptional targets of Meis1

The oncogenicity of *Meis1* has been linked to its transcriptional activation of downstream effectors *Flt3, Cd34, Erg, c-Myb, Trib2* and *Ccl3*.[51-53] *CyclinD* has also been shown as a direct target of *Meis1* in leukemogenesis.[54] However, only a few of transcriptional targets of *Meis1* involved in normal hematopoiesis have been identified. Studies in zebrafish showed that *Meis1* and *Pbx* act upstream of *gata1* to regulate primitive hematopoiesis.[55,56] Megakaryocytic gene *PF4* and HSC-specific gene *Hif-1*α have been shown to be direct targets of *Meis1* in normal hematopoiesis.[57,58] Although a very recent study has mapped the genome-wide DNA binding sites of *Meis1* in hematopoietic progenitor cells using ChIP-seq,[59] none of these targets has been functionally validated. Therefore, identifying transcriptional targets of *Meis1*might be important for the understanding of its actions during both leukemogenesis and normal hematopoiesis.

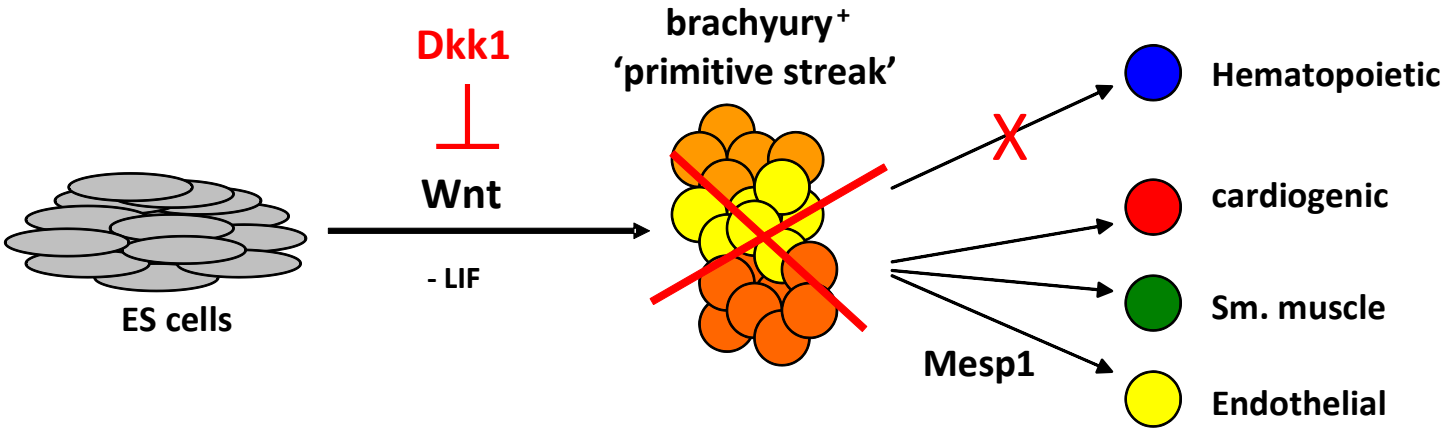

Figure 1.1 The role of *Mesp1* in ES cell differentiation.

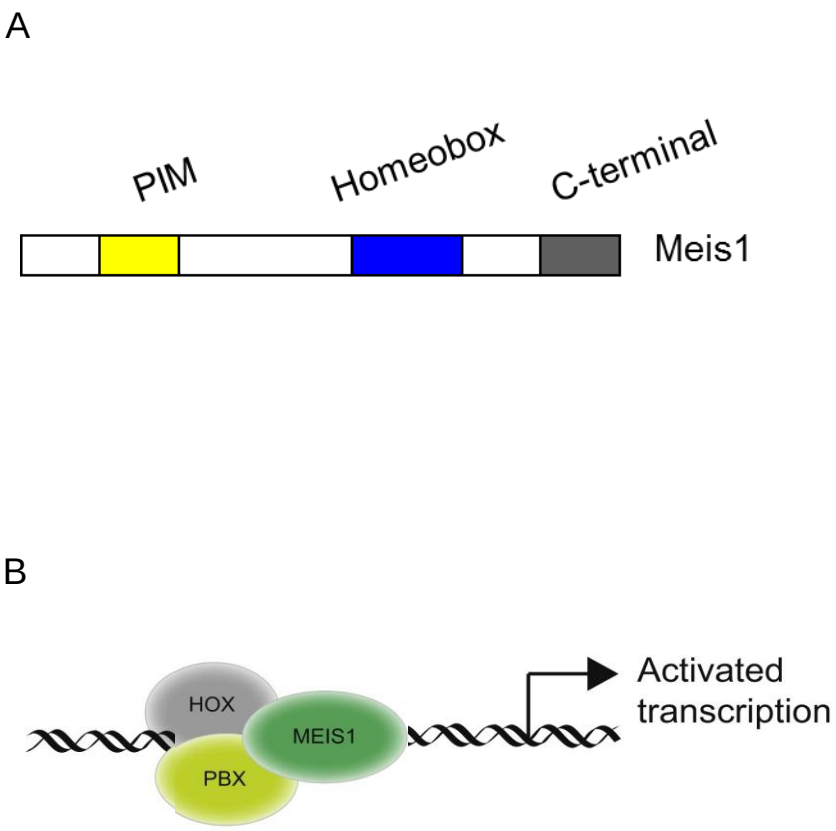

Figure 1.2 The protein structure and binding properties of Meis1 protein.

CHAPTER 2: EXPERIMENTAL PROCEDURES

Mouse ES cell generation and embryoid body (EB) differentiation

To generate inducible ES cell lines (A2lox.Mesp1, A2lox.Mesp2, A2lox.PDGFRα, A2lox.Meis1, A2lox.Meis2, A2lox.Plac8, A2lox.Serpinb2, A2lox.Meis1a, A2lox.Meis2(2a), A2lox.Meis2(4a), and A2lox.HoxA9), individual cDNAs were amplified from EB RNA using gene-specific primers (Table 2.1) and cloned into the p2lox targeting vector.[60] Site specific recombination into A2lox ESCs was performed using co-transfected Cre recombinase (Figure 2.1), and all A2lox ESC lines were maintained as described.[10]

To generate inducible ES cell lines (A2lox.Plac8*tet*Serpinb2, A2lox.Meis1*tet*HoxA9, A2lox.HoxA9*tet*Meis2, A2lox.bio-Mesp1*tet*BirA), an inducible, dual-expression vector was prepared by cloning a cDNA (*Serpinb2*, *HoxA9*, *BirA* or *Meis2*) into the vector pTet-CMV_{min}pA-zeocin (Dr. Jonathan Green, Washington University School of Medicine), transferring this Tet-CMV_{min} promoter-cDNA-pA cassette into the p2lox targeting vector downstream of the original p2lox pA site, and followed by inserting a second cDNA (*Plac8*, *Meis1*, *Mesp1* or *HoxA9*) into the resulting plasmid, allowing simultaneous dox induction of both cDNAs in transfected A2lox ESCs.

For differentiation, ESCs were plated in suspension in Petri dishes at 1.5×10^4 cells/ml in Iscove's modified Dulbecco's medium (IMDM) with 10% FCS, nonessential amino acids, L-glutamine, Na Pyruvate, Pen/Strep, and 2-mercaptoethanol as described,[10] and supplemented where indicated with Dkk1 or SC-51322 (Enzo Life Sciences). Gene expression was induced by addition of doxycycline (250-500 ng/ml).

Generation of recombinant Dkk1-his

A PCR product generated using the primers 5′*Hind III* Dkk1 (CCAAAGCTTCGGAGATGATGGTTGTGTG) and 3′*Age I* Dkk1 (GCAACCGGTGTGTCTCTGGCAGGTGTGGA) and cDNA from day 4 embryoid bodies was digested with *Hind III* and *Age I* and ligated into *Hind III*- and *Age I*-digested pcDNA4-myc-hisA to generate a C-terminal 6His tag in frame with full length Dkk1. The resulting plasmid, pcDNA-Dkk1-his, was transfected into 293F/T cells (Invitrogen) using Ca_2PO_4 precipitation. Supernatants from transfected cells were adjusted to pH 8.0 by the addition of 1/3 volume of 1× Ni-NTA binding buffer and then purified on Ni-NTA His•Bind resin (Novagen). Purified Dkk1-his was dialyzed against two changes of PBS, and was shown to consist predominantly of a closely spaced doublet (M_r=35×10^3) that was recognized by an antibody to penta-His (Qiagen) on Western analysis. Activity of purified Dkk1-his was confirmed by ability to inhibit SUPER8×TOPFlash reporter activity. Dkk1-his and commercially available Dkk1 were further demonstrated to display no substantial cytotoxic effects.

Gene expression analysis

RNA from FACS-sorted cell populations was extracted using RNeasy kits (Qiagen, Germantown, MD), and cDNA was synthesized using Superscripts III (Invitrogen, Carlsbad, CA). Quantitative RT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) and a StepOne Plus Real Time PCR System (Applied Biosystems) with Intron-spanning, gene-specific primers shown in Table 2.1. Large-scale gene expression analysis of A2lox.Mesp1 samples was done

using Affymetrix MOE430_2.0 arrays as described.[10] Data were normalized and modeled using DNA-Chip Analyzer/dChip. For A2lox.Meis1 and A2lox.Meis2 samples, gene expression analysis was done using Affymetrix Mouse Gene 1.0 ST arrays, and data was normalized and modeled using ArrayStar. The microarray datasets were deposited in the NCBI GEO database under accession numbers GSE34537 (A2lox.Mesp1 data), GSE34543 (A2lox.Meis1 data), and GSE34541 (A2lox.Meis2 data).

Luciferase assay

293T cells were transfected with firefly luciferase reporter constructs containing a minimal CMV promoter (CMVmini), or enhancers/promoters for *Epha4*, *Nkx2*.5, *Myocd*, *Meis1* or *Meis2* cloned upstream of CMVmini using primers indicated in Table 2.1, along with expression vectors for *Mesp1* and *E47*, either separately or together. Cells were harvested 24 hr later and lysed. Luciferase activity was determined from 20 μl of cell extract in triplicate, using the luciferase assay substrate with an Opticomp II automated luminometer (MGM Instruments, Hamden, Conn.), reading relative light activity for 20 sec. Firefly luciferase activity was normalized using co-transfected Renilla luciferase construct (prL-CMV) to account for possible differences in cell density and transfection efficiency.

EMSA

A2lox.bio-Mesp1*tet*BirA ESCs were differentiated in the presence of Dkk1, with or without Dox (250 ng/ml) treatment on day 2. After 36 hr, cells were harvested and nuclear extract was prepared. Complementary double-stranded oligonucleotides (Table

2.2) containing the evolutionary conserved E-boxes within *PDGFR*α promoters were radiolabeled with [α-^{32}P] dCTP as previously described.[61] Radiolabeled probe (5.0×10^4 cpm) was incubated for 30 min on ice or at room temperature in binding reaction buffer[61] with 5 μg of whole cell extract and 1 μg of poly (dI-dC). For antibody supershifts in EMSAs, whole cell extract was incubated with anti-biotin antibody on ice for 15 min before adding radiolabeled probes as indicated. The final reaction mixture was electrophoresed through a nondenaturing 5% polyacrylamide gel at 150 V for 2 h at room temperature in 0.4× TBE buffer (l× TBE is 89 mM Tris [pH 8.2], 89 mM boric acid, and 2 mM EDTA).

MicroRNA-mediated knockdown

Four miR30-based shRNA (Ptger3mir1-4) cassettes, described in Table 2.3, directed against the target gene *Ptger3* were cloned into vector pcDNA3.1 zeo/(+) Vav1 mir-shRNA (from Dr. Andrew Shaw, Washington University School of Medicine, St. Louis, MO) using *Xho I* and *EcoR I*, and then subcloned into p2lox.CAGGFP.MCS.pA, a vector expressing GFP, at the *Nhe I* and *EcoR V* sites in the 3' UTR downstream of the GFP coding exon. A2lox ES cell lines with constitutive expression of shRNA targeting *Ptger3* were generated. The extent of knockdown of the *Ptger3* was assessed by quantitative RT-PCR.

ES/OP9 co-culture

Day-6 EBs were trypsinized to single cell suspensions and plated on a monolayer of irradiated OP9-GFP cells[62] at a density of 100,000 cells/ml in IMDM with 10% FCS,

nonessential amino acids, L-glutamine, Na Pyruvate, Pen/Strep, 2-mercaptoethanol and cytokines (100 ng/ml recombinant murine (rm) stem cell factor (SCF), 40 ng/ml rm thrombopoietin (TPO), 40 ng/ml rm vascular endothelial growth factor (VEGF), 5% Flt3 ligand conditioned medium (Flt3L), 10 ng/ml rm interleukin-3 (IL-3), and 20 ng/ml rm IL-6). All cytokines are from PeproTech.

FACS analysis and sorting

EBs or ES/OP9 co-cultures dissociated by trypsin, or blood cells collected from mouse bone marrow (BM), spleen or peripheral blood were treated with Fc block (BD Biosciences Pharmingen) on ice for 5 min and then stained with antibodies. Primary antibodies: PE α-Tie2 (1 μg/ml, TEK4), peridininchlorophyll protein (PerCp)-Cy5.5 α-CD16/32 (1 μg/ml, 93), PE α-Flk1 (1 μg/ml, Avas12a1), PE-Cy7 α-CD41 (1 μg/ml, eBioMWReg30), allophycocyanin (APC) α-PDGFRα (1 μg/ml, APA5), APC α-Flk1 (1 μg/ml, Avas12a1), APC α-CD150 (1 μg/ml, mShad150), APC α-AA4.1 (1 μg/ml, AA4.1), APC α-CD42d (1 μg/ml, 1C2), APC-eFluor 780 α-c-kit (1 μg/ml, 2B8), APC-eFluor 780 α-CD45.2 (1 μg/ml, 104), eFluor 450 α-B220 (1 μg/ml, RA3-6B2), and eFluor 450 α-CD105 (1 μg/ml, MJ7/18) (eBioscience), PE α-CD71 (1 μg/ml, C2), PE-Cy7 α-Sca1 (1 μg/ml, D7), PE-Cy7 α-Mac1 (1 μg/ml, M1/70), APC α-Gr1 (1 μg/ml, RB6-8C5), SA/PE-Cy7 (BD Biosciences Pharmingen), V500 α-B220 (1 μg/ml, RA3-6B2, BD Hrizon), biotin α-mE-cadherin (1.25μg/ml, R&D Systems), PE or APC α-hCD4 (1 μg/ml, Invitrogen). Data were acquired on a FACS Canto II (BD Biosciences) and analyzed using FlowJo 7.6.5 (Tree Star). A2lox.Mesp1 cells were sorted based on Flk1 and Tie2 expression by using a MoFlo cytometer (Dako North America).

A2lox.Meis1 or A2lox.Meis2 cells were sorted on the FACS Aria II (BD Biosciences) based on levels of CD41 expression.

Colony-forming assay

Day-6 EBs were dissociated by trypsin and added to MethoCult GF M3434 methylcellulose-based medium (StemCell Technologies, Vancouver, BC) for hematopoietic colonies. Methylcellulose suspension cultures were supplemented with doxycycline where indicated. All colonies were counted on day 6 of methylcellulose culture. Megakaryocyte progenitors were examined using MegaCult-C collagen-based medium supplemented with 50 ng/mL rm TPO, 10 ng/mL rm IL-3, 20 ng/mL rm IL-6, and 50 ng/mL rm IL-11, following the protocol provided by the vendor (StemCell Technologies, Vancouver, BC). Megakaryocytic colony formation was assessed after growth for 6 days by dehydrating, fixating, and staining the slides with acetylthiocholine iodide (Sigma-Aldrich) and Harris hematoxylin (Sigma-Aldrich) counterstain. The acetylthiocholine iodide-stained colonies were counted. Bright field images were captured by a Nikon Eclipse TS100 microscope with an Optronics 60800 camera, and imported into MagnaFire 2.0 software.

Retrovirus production and cell infection

Meis1a, *Meis1b* and *Meis2(2b)* were subcloned from p2lox constructs into IRES-human CD4 (hCD4)-retrovirus (RV)[63] with *EcoR I.* BM cells were collected from 8- to 10-week-old 129S6/SvEvTac mice (Taconic, Germantown, NY) and c-kit$^+$ progenitor cells were enriched with CD117 MicroBeads as described by the vendor (Miltenyi

Biotec). c-kit$^+$ BM progenitors were expanded in IMDM medium with 10% FCS, 100 ng/mL rm SCF and 50 ng/mL rm TPO overnight, and then infected with retroviral supernatant in the presence of polybrene (2 μg/ml) by spin infection.

BrdU labeling

Bromodeoxyuridine (BrdU) was added to ES/OP9 co-cultures 3 hours before harvesting the cells for FACS analysis. The percentage of BrdU-positive cells among CD41$^+$ cells was assessed by cell-surface marker staining followed by cell permeabilization and APC α-BrdU antibody staining (BD Biosciences Pharmingen).

Apoptosis assay

To analyze the degree of apoptosis of CD41$^+$ cells, ES/OP9 co-cultures were disaggregated and stained with CD41 antibody on ice for 30 minutes. After one wash in 1×Binding Buffer (BD Biosciences), per 1×10^5 cells were stained with 5 μl PE-Annexin V (BD Biosciences Pharmingen) and 5 μl cell viability dye 7-amino-actinomycin D (7-AAD) for 15 minutes at room temperature. The proportion of cells in different quadrants was determined by FACS.

BM transplantation

24 hours after infection, c-kit-enriched BM progenitor cells were washed in 1×PBS and transplanted by retro-orbital injection into 129S6/SvEvTac recipients that had been sub-lethally irradiated at 600 cGy.

BM chimeras

BM cells were collected from 8- to 10-week-old *Plac8*$^{-/-}$ or wild type C57BL/6J background littermate control mice (Jackson Laboratory; CD45.2^{+}) and transplanted by retro-orbital injection with competitor marrow (B6.SJL-*Ptprc*^{a}/BoyAiTac; Taconic; CD45.1^{+}) at 1:1 ratio into B6.SJL-*Ptprc*^{a}/BoyAiTac recipients that had received 1,100 cGy. Donor-derived cells in BM (CD45.2^{+}) were analyzed 4 weeks after transfer by FACS analysis.

Mice

Mesp1-Cre mice were obtained from the Riken BioResource Center as cryo-preserved embryos.[6] Heterozygous *Mesp1*$^{Cre/+}$ mice were bred to homozygous Rosa26-CAG-STOP-eGFP$^{+/+}$ reporter mice. To generate Rosa26-CAG-STOP-eGFP$^{+/+}$ reporter mice, the plasmid pCAG-Cre:GFP (Addgene plasmid 13776)[64] was digested with *EcoR I* and *Sal I* and the CAG-promoter containing 1718 bp fragment was cloned bluntly into the *Pac I* site of a modified version of pROSA26-1 (Addgene plasmid 11739).[65] The resulting plasmid was digested using *Pvu I* and the gel purified 13366 bp fragment was electroporated into the 129SvEv ES cell line, EDJ 22 (ATCC number: SCRC-1021). Targeted clones were identified by Southern blot analysis using probes cloned from isogeneic genomic ES cell DNA using oligonucleotides 5'-CTCACTCAGCCCGCTGCCCGAG and 5'-CTCCCGCCAGAGTCCCGATCCCC (probe A), and 5'-GGCTTGTTGGTTCCATACATCTACTGG and 5'-GATCAAGATGAAGGAAGAGACCCTCC (probe B). Mice were generated by

injecting targeted ES cells into C57BL/6J blastocysts and maintained on a 129 background after transmission of the targeted allele through the male germ line.

Statistical analysis

Error bars in all panels represent standard deviation (SD). Paired student's *t*-tests were performed to calculate *P* values and are indicated in the graph.

Table 2.1 Oligonucleotide primers used in this study

Cloning Primers	
Name	Sequence
Meis1a_F*	GAAGTAGGAAGGGAGCCAGAGAGG
Meis1a_R*	CACCATTGTAGACAACGCATATTCCC
Meis1b_F*	GAAGTAGGAAGGGAGCCAGAGAGG
Meis1b_R*	CACCATTGTAGACAACGCATATTCCC
Meis2(2a)_F†	TACGACACATCCAGGAGTTTATTG
Meis2(2a)_R†	GTGTGTTTCCTTCTTCCTTGAGTT
Meis2(2b)_F†	TACGACACATCCAGGAGTTTATTG
Meis2(2b)_R†	GTGTGTTTCCTTCTTCCTTGAGTT
Meis2(4a)_F†	TACGACACATCCAGGAGTTTATTG
Meis2(4a)_R†	GTGTGTTTCCTTCTTCCTTGAGTT
Mesp1_F	AATGGTCAGGCCTCCGTTGC
Mesp1_R	TGTCCCCTCCACTCTTCAGGCA
Mesp2_F	GGTCCAGCTTCCCAGAGTCA
Mesp2_R	GGTGCAGGTAAGGCCATATTA
PDGFRα_F	GGAAGGACTGGAAGCTTGG
PDGFRα_R	GCTCTAGAAGGTTATCCCGAGGAGGC
Plac8_F1	TTTTGAGACCTCGCATCGAA
Plac8_R1	TCTTGCCATCCAGCTCCTTA
Serpinb2_F	AGCTGTGTAGAGGATTGAAACAATG
Serpinb2_R	GCTACAAAACCTCATGTGGAAATAG
HoxA9_F	GCGCCGGCAACTTATTAG
HoxA9_R	ACAGAGGGAGACGGACAGTC
Meis1_P_F	CGGGATCCTTTTTCTCTGGAACTGGGAGC
Meis1_P_R	GAAGATCTGCGTGTGTAAAGTGTGTGTTG
Meis2_P_F	CGGGATCCTGTACCGTACTTTCCCTGTGG
Meis2_P_R	GAAGATCTCCAAACCAAGGAGACTTCTC
Epha4_Enh_F	CTGACGTTGCCCTTGACTAAG
Epha4_Enh_R	AGGCATAGGCAGTACACTTTC
Nkx2.5_Enh_F	AAGCACGGGCCAGGCCAA
Nkx2.5_Enh_R	TGCCTTTTAAAGACTTGGTGC
Myocd_Enh_F	TCCTGTGTTTTCTGACTGGGT
Myocd_Enh_R	GGCTACTTGACTTTTCTGCAG
Quantitative RT-PCR Primers	
Name	Sequence
Plac8_F2	ATTTGTAGTAAGACTCAACCCCAGAC
Plac8_R2	CAGACAACACTCATTCATGTCAG
Ptger3_F	GGGATCATGTGTGTGCTGTC
Ptger3_R	AGCAGATAAACCCAGGGATC
Gapdh_F	TGCCCCCATGTTTGTGATG

Gapdh_R	TGTGGTCATGAGCCCTTCC
Mesp1_F	AGAATCGTGGGACCCATCGTTC
Mesp1_R	ATTGTCCCCTCCACTCTTCAGGC
Mesp2_F	CCACTGAACCTGCAGAGCTGACTAAAG
Mesp2_R	AGATAAAGGCACTTCCAAGGC
Meis1a_F	TCCAGCATCTAACACACCCTTAC
Meis1a_R	CATTCCACTCATAGGTCCTGG
Meis1b_F	TCCAGCATCTAACACACCCTTAC
Meis1b_R	GGCATACTTTGCAGCCCTGG
Meis2(2a)_F	AAATCGAGCAGTGAGCCAAG
Meis2(2a)_R	CGAAGGTTACATATAGTGCCACTG
Meis2(2b)_F	AAATCGAGCAGTGAGCCAAG
Meis2(2b)_R	GGCATGCTCTGCAAACCTGCA
Meis1_F	TCCAGCATCTAACACACCCTTAC
Meis1_R	AAAACCTCCCATTGGCTGTC
Meis2_F	ACAACAGCAGTGAGCAAGGC
Meis2_R	GGGTACGGGTGTGTGAGATG

* *Meis1a* and *Meis1b* were cloned using the same primers and distinguished by sequencing.

† *Meis2(2a)*, *Meis2(2b)*, and *Meis2(4a)* were cloned using the same primers and distinguished by sequencing.

Table 2.2 EMSA probes

Name	Sequence
Epha4 E3_F	GAGGTGGGCACATTTGTCCAAAA
Epha4 E3_R	GCCATTTTGGACAAATGTGCCCA
PDGFRα E1_F	GCTATTTACTTTAAGCAAATGATTAGTTTT
PDGFRα E1_R	TCGAAAAACTAATCATTTGCTTAAAGTAAA
PDGFRα E2_F	TCGACCCCACCCCATCTGGTTTGCT
PDGFRα E2_R	GGGGAGCAAACCAGATGGGGTGGGG
PDGFRα E3_F	CCTGAAGGAACATGTGGAAGTAATAGT
PDGFRα E3_R	GCTCACTATTCATTCCACATGTTCCTT
PDGFRα E4_F	TGTGAAGTTACCATTTGTGGGAGGA
PDGFRα E4_R	GTTCTCCTCCCACAAATGGTAACTT
PDGFRα E5_F	TACCAGAAGAGCAACTGGGTGCT
PDGFRα E5_R	GTATCGAGCACCCAGTTGCTCTT
PDGFRα E6_F	GGAAACCTTTAGCAAATGTTTGTTAAT
PDGFRα E6_R	GATCATTAACAAACATTTGCTAAAGGT
PDGFRα E7_F	TGTCACACATGGAAACCTTTAGCAAATGTT
PDGFRα E7_R	TCGAAACATTTGCTAAAGGTTTCCATGTGT
PDGFRα E8_F	CTTTGCCCAACCATTTGCTTGCCTG
PDGFRα E8_R	GGAGCAGGCAAGCAAATGGTTGGG
PDGFRα E9_F	TGCAGGCAAGGCAGATGCTTTG
PDGFRα E9_R	ACCCAGCAAAGCATCTGCCTTG
PDGFRα E10_F	AGGGACTCCAACATCTGGTTGCCG
PDGFRα E10_R	GATGCGGCAACCAGATGTTGGAGT
PDGFRα E11_F	CTCCGAAGCCACAGCTGTGAGCTGGG
PDGFRα E11_R	GCTTCCCAGCTCACAGCTGTGGCTT
PDGFRα E12_F	AATGACAAACACATTTGGCCT
PDGFRα E12_R	CACTCGAGGCCAAATGTGTTTGT
PDGFRα E13_F	CGCATTCCAGCAACTGGGATTTGAGG
PDGFRα E13_R	GAAATTCCTCAAATCCCAGTTGCTGGAA
PDGFRα E14_F	TCTCAGGTCGCAGTTGAAAACAA
PDGFRα E14_R	TGCATTGTTTTCAACTGCGAC

Table 2.3 MicroRNA knockdown sequences

	mir30-based shRNA cassettes
Name	Sequence
Ptger3mir1	TGCTGTTGACAGTGAGCGAGGAGAAGTTTAGCTAAAGACATA GTGAAGCCACAGATGTATGTCTTTAGCTAAACTTCTCCCTGCC TACTGCCTCGGA
Ptger3mir2	TGCTGTTGACAGTGAGCGAGGTTGAGCAATGCAAGACACATA GTGAAGCCACAGATGTATGTGTCTTGCATTGCTCAACCGTGC CTACTGCCTCGGA
Ptger3mir3	TGCTGTTGACAGTGAGCGATGCCAAGAAGGTATACAGTACTA GTGAAGCCACAGATGTAGTACTGTATACCTTCTTGGCACTGC CTACTGCCTCGGA
Ptger3mir4	TGCTGTTGACAGTGAGCGAAGGGAAGGATGACTGAGTATTTA GTGAAGCCACAGATGTAAATACTCAGTCATCCTTCCCTGTGC CTACTGCCTCGGA

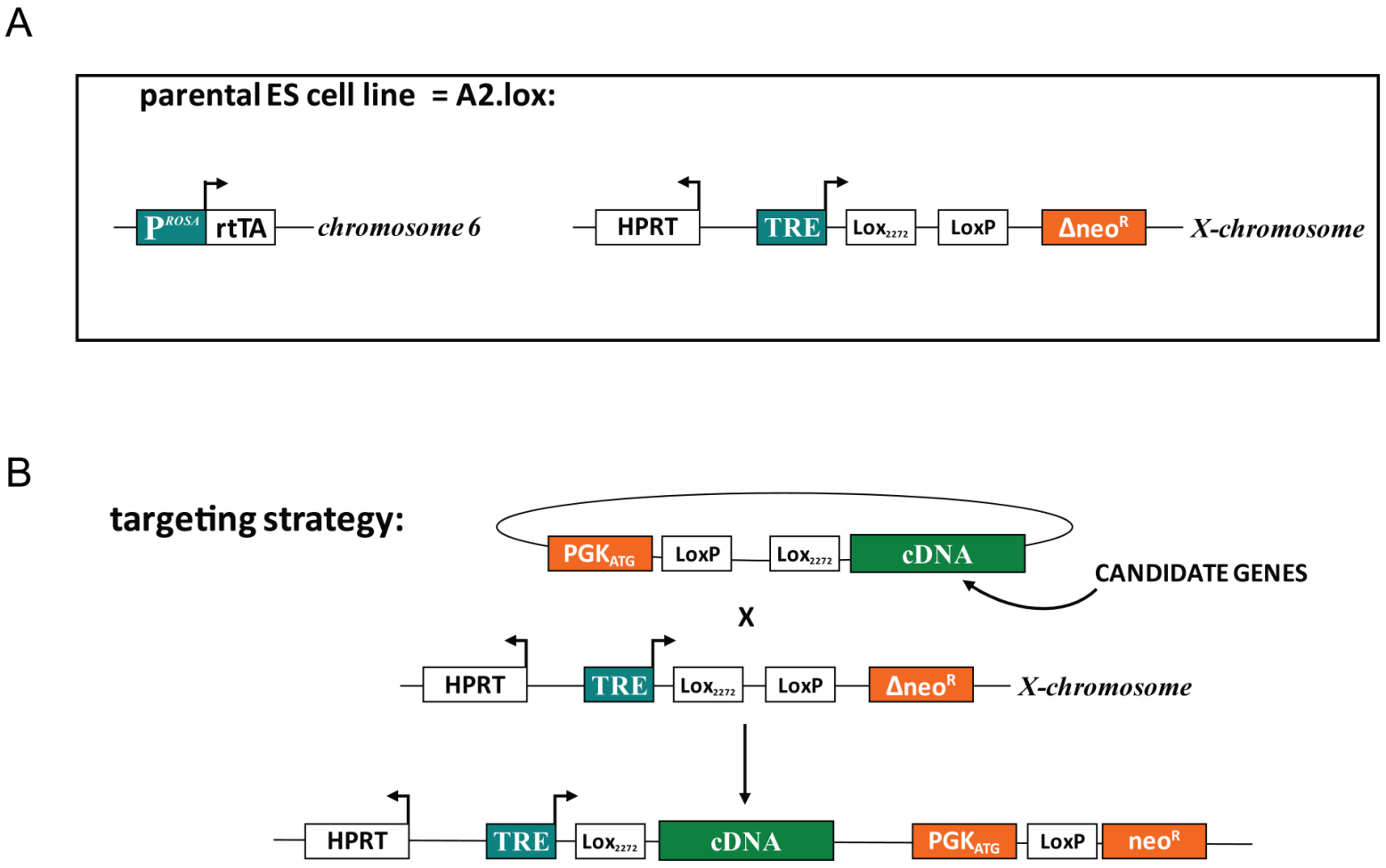

Figure 2.1 Schematic of p2lox targeting strategy. Plasmid and cells are from Michael Kyba; figure adapted from M. Kyba and R.C. Lindsley.

CHAPTER 3: IDENTIFICATION OF TRANSCRIPTONAL TARGETS OF *MESP1*

Mesp2 has similar actions to Mesp1 on induction of mesoderm markers and induction of EMT

Since *Mesp2* can compensate for migratory defects in *Mesp1*-deficient embryos,[12] we asked if *Mesp2* can induce mesoderm and EMT in differentiating ESCs (Figure 3.1). Dox treatment of A2lox.*Mesp2* cells induced expression of Flk1 and PDGFRα (Figure 3.1A), downregulated E-cadherin (Figure 3.1A), and induced *Snai1* expression in Dkk1 treated cultures (Figure 3.1B). Thus, the ability of differentiating *Mesp1*-deficient ESCs to express *Snai1* and undergo EMT may be due to compensation by *Mesp2*.

Identification of early transcriptional targets of Mesp1 activity

We used reporter analysis to ask if *Nkx2.5* or *Myocd* were direct targets of *Mesp1* (Figure 3.2A). Activation of the *Epha4* enhancer required both *Mesp1* and *E47*, as expected.[66] However, cardiac-specific enhancers for *Nkx2.5*[67] and *Myocd*[68] were not activated by cotransfection of *Mesp1* alone or with *E47*, indicating they may not be direct targets of *Mesp1*. However, other enhancer regions of *Nkx2.5*[69] or *Myocd* might be responsive to *Mesp1*, or other factors not present in 293T cells may be required in order for *Mesp1* to act.

PDGFRα is induced rapidly by Mesp1 in a dose-dependent manner

Since direct *Mesp1* targets should be induced earlier than indirect targets, we characterized *Mesp1*-induced gene expression after 6, 12, and 24 hr of dox treatment on day 2 of differentiation in DKK1-treated cultures (Figure 3.2B-C). After 6 hr, 41 genes were induced >3-fold; after 12 hr, 152 genes were induced; and after 24 hr, >500 genes

were induced. Among these genes, the expression of *PDGFRα* increased progressively from 14-fold induction at 6 hr to more than 200-fold induction at 24 hr following *Mesp1* induction (Figure 3.3A). The protein level of PDGFRα is also upregulated at the early time points of Dox treatment in a dose-dependent manner (Figure 3.3B). Consistent with these overexpression studies, *PDGFRα* expression is downregulated in differentiating *Mesp1*-deficient ESCs (data not shown).

Mesp1 binds to evolutionary conserved sites on the promoter region of PDGFRα

The rapid and robust induction of *PDGFRα* by *Mesp1* in differentiating ES cells strongly suggested that it might be a direct *Mesp1* target gene. Further studies were performed to test this hypothesis. Two *PDGFRα* transcripts (NM 011058 and NM 001083316), which have different promoters and 5' UTRs but share the same protein-coding sequence, have been reported by NCBI. They were both induced in differentiating A2lox.Mesp1 ESCs treated with Dox except transcript NM 011058 was more abundant and showed a higher induction (data not shown). Therefore, NM 011058 might be a major *PDGFRα* transcript induced by *Mesp1* and was focused in the following studies.

Eleven evolutionary conserved *Mesp1* responsive elements (E-boxes) within the 15-kb *PDGFRα* (NM 011058) promoter were identified (Figure 3.4A). To test whether *Mesp1* directly binds to these E-boxes, a dox-inducible, dual-expression A2lox.bio-Mesp1*tet*BirA ES cell line was generated. In this cell line, *Mesp1* fused with a short 'biotinylation peptide' is induced by dox and serves as an *in vivo* substrate for *Escherichia coli* biotin holoenzyme synthetase (*BirA*) which is simultaneously induced

by dox. The binding abilities of these E-boxes to biotinylated *Mesp1* in differentiating A2lox.bio-Mesp1*tet*BirA ESCs treated with dox were tested by EMSA. Two E-boxes (E4 and E8) were found to bind to biotinylated-*Mesp1* and the bands were super-shifted by anti-biotin antibody (Figure 3.4B). An evolutionary conserved E-box (E12) within the promoter of *PDGFRα* (NM 001083316) was found to bind to biotinylated-*Mesp1* as well. These E-boxes share the same consensus sequence CAAATG, which is the same as the sequence published for Epha4 E3.[66]

PDGFRα may be necessary for survival of Mesp1-induced mesodermal cells

Although *PDGFRα* is robustly induced by *Mesp1*, it's unclear what the functional roles of *PDGFRα* are and why *Mesp1* induces it during ES cell differentiation. Although *PDGFRα* has only been used as an early mesoderm marker in ESC studies, it plays important roles in a lot of biological processes, such as mesodermal cell migration and survival during gastrulation, and EMT in various types of cancer.[15,16,20,70,71] Therefore, we hypothesize that *PDGFRα* might be required for *Mesp1* to induce EMT, mesoderm, and/or to support mesodermal cell survival during ES cell differentiation.

To test the role of *PDGFRα* during ES cell differentiation, AG1296, a kinase inhibitor specific for *PDGFR*, was added to differentiating A2lox.Mesp1 ES cells with or without dox treatment. In a dose-dependent manner, AG1296 blocked the induction of early mesoderm markers (Flk1 and PDGFRα) in both untreated A2lox.Mesp1 ES cells and cells treated with a low dose of Dox (Figure 3.5A). However, I also noticed that less live cells were detected by FACS analysis if the cells were treated with a higher dose of AG1296 (Figure 3.5B), which suggested AG1296 might inhibit cell survival. The

repression on early mesoderm induction might be a secondary effect of the death of nascent mesodermal cells.

PDGFRα is not sufficient for Mesp1 to induce EMT or mesoderm lineages

To test whether *PDGFRα* is sufficient for any of *Mesp1*'s effects during ES cell differentiation, gain-of-function studies were performed. Induction of *PDGFRα* in ES cells had no effects on Flk1 induction or E-cad downregulation in the presence or absence of Dkk1 on both day 4 and day 5 of differentiation (Figure 3.6A-B). Addition of PDGFRα ligand PDGF-AA had little effects on Flk1 induction or E-cad downregulation in differentiating A2lox.PDGFRα ESCs either in the presence of absence of Dox (data not shown). These data suggested that *PDGFRα* is not sufficient to induce EMT and mesoderm lineages as *Mesp1*.

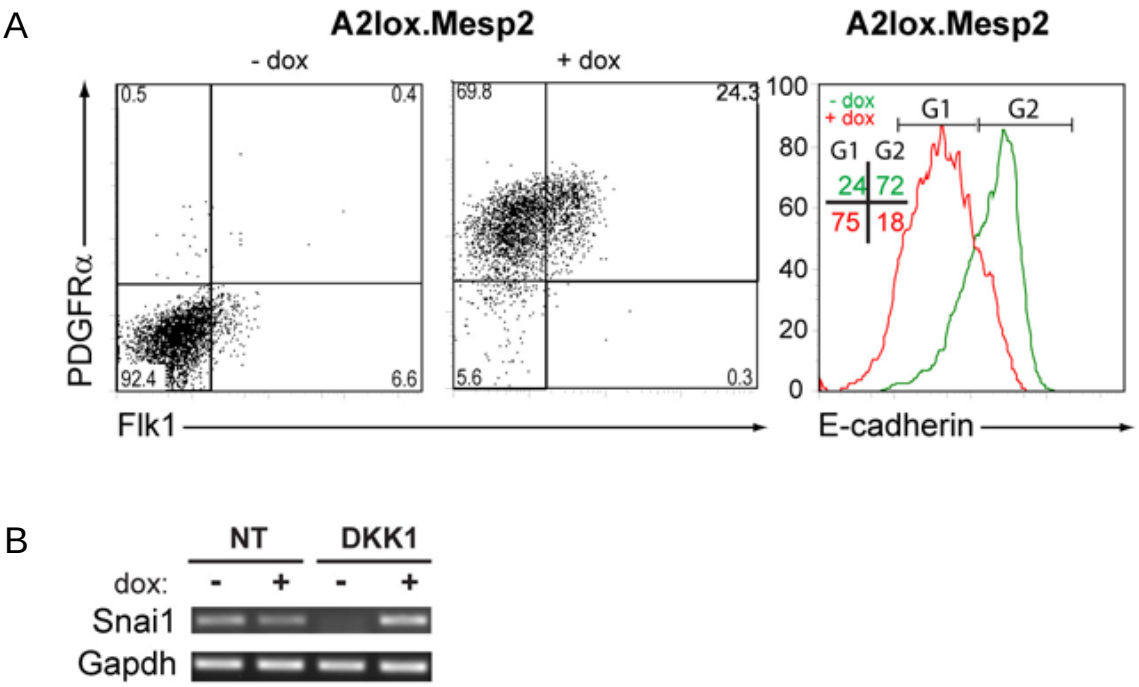

Figure 3.1 ***Mesp2*** **has similar actions to** ***Mesp1*** **on induction of mesoderm markers and induction of EMT.** A2lox.Mesp2 ES cells were differentiated in SCM for 4 days with Dkk1 added on day 2, and with or without dox added on day 2. (A) Cells were analyzed for PDGFRα, Flk1, and E-cadherin expression on day 4 by flow cytometry. (B) *Snai1* and *Gapdh* expression in differentiated A2lox.Mesp2 cells was analyzed on day 4 by RT-PCR.

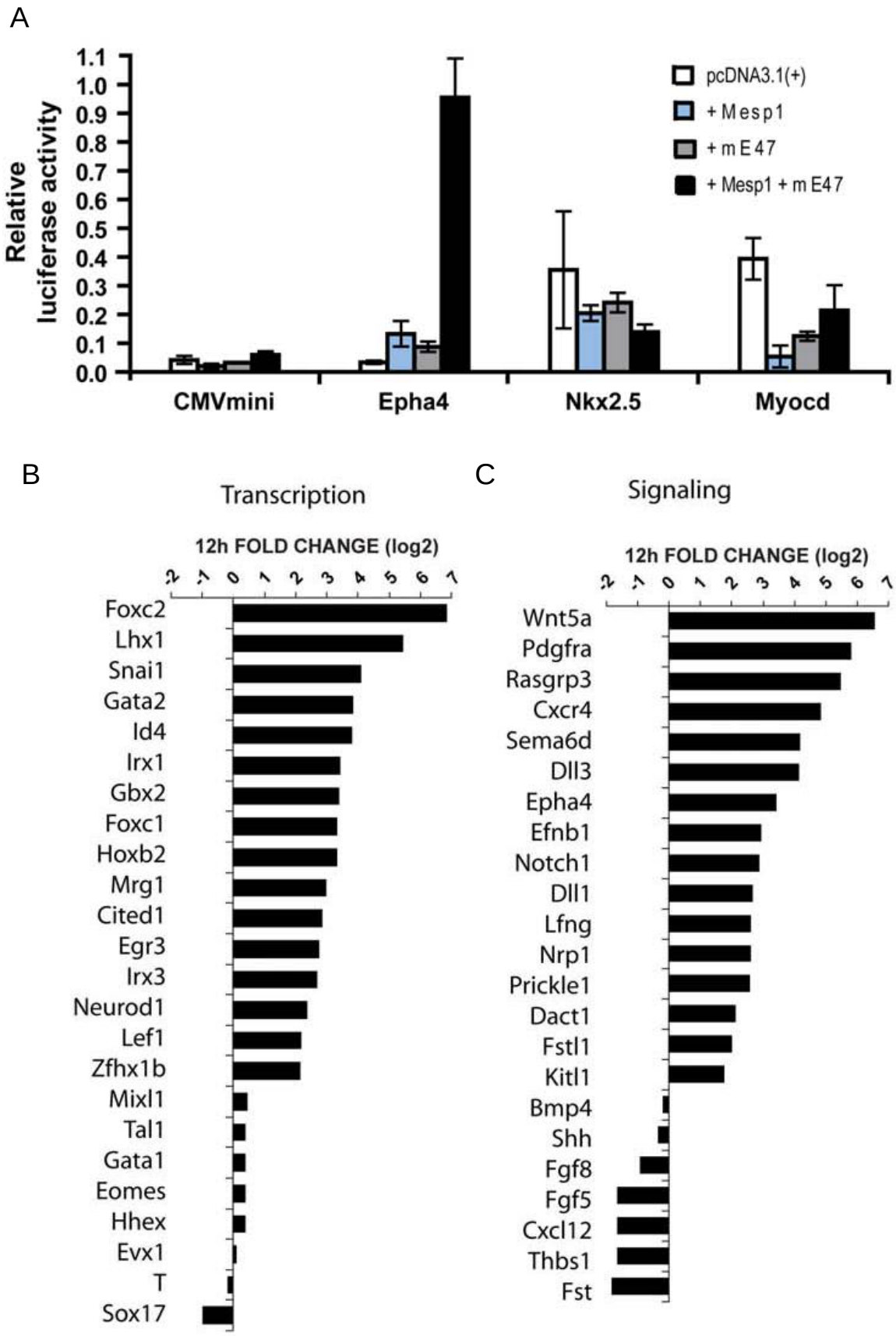
A
Relative luciferase activity
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
pcDNA3.1(+)
+ Mesp1
+ mE47
+ Mesp1 + mE47
CMVmini
Epha4
Nkx2.5
Myocd
B
Transcription
12h FOLD CHANGE (log2)
-2 -1 0 1 2 3 4 5 6 7
Foxc2
Lhx1
Snai1
Gata2
Id4
Irx1
Gbx2
Foxc1
Hoxb2
Mrg1
Cited1
Egr3
Irx3
Neurod1
Lef1
Zfhx1b
Mixl1
Tal1
Gata1
Eomes
Hhex
Evx1
T
Sox17
C
Signaling
12h FOLD CHANGE (log2)
-2 -1 0 1 2 3 4 5 6 7
Wnt5a
Pdgfra
Rasgrp3
Cxcr4
Sema6d
Dll3
Epha4
Efnb1
Notch1
Dll1
Lfng
Nrp1
Prickle1
Dact1
Fstl1
Kitl1
Bmp4
Shh
Fgf8
Fgf5
Cxcl12
Thbs1
Fst

Figure 3.2 ***Mesp1*** **rapidly induces expression of regulatory genes.** (A) 293T cells were transfected with firefly luciferase reporter constructs containing a minimal CMV promoter (CMVmini), or enhancers for *Epha4*, *Nkx2*.5, or *Myocd* cloned upstream of CMVmini, along with expression vectors for *Mesp1* and *E47*, either separately or together. Firefly luciferase activity was normalized using co-transfected Renilla luciferase construct (prL-CMV). (B, C) A2lox.*Mesp1* ES cells were differentiated in the presence of Dkk1 without or with addition of dox on day 2. Gene expression was evaluated by microarray. Shown is the log2 of the fold induction of selected transcription factors (B) and signaling pathway genes (C), comparing samples treated with Dkk1 and dox for 12 hours to samples treated only with Dkk1 during differentiation.

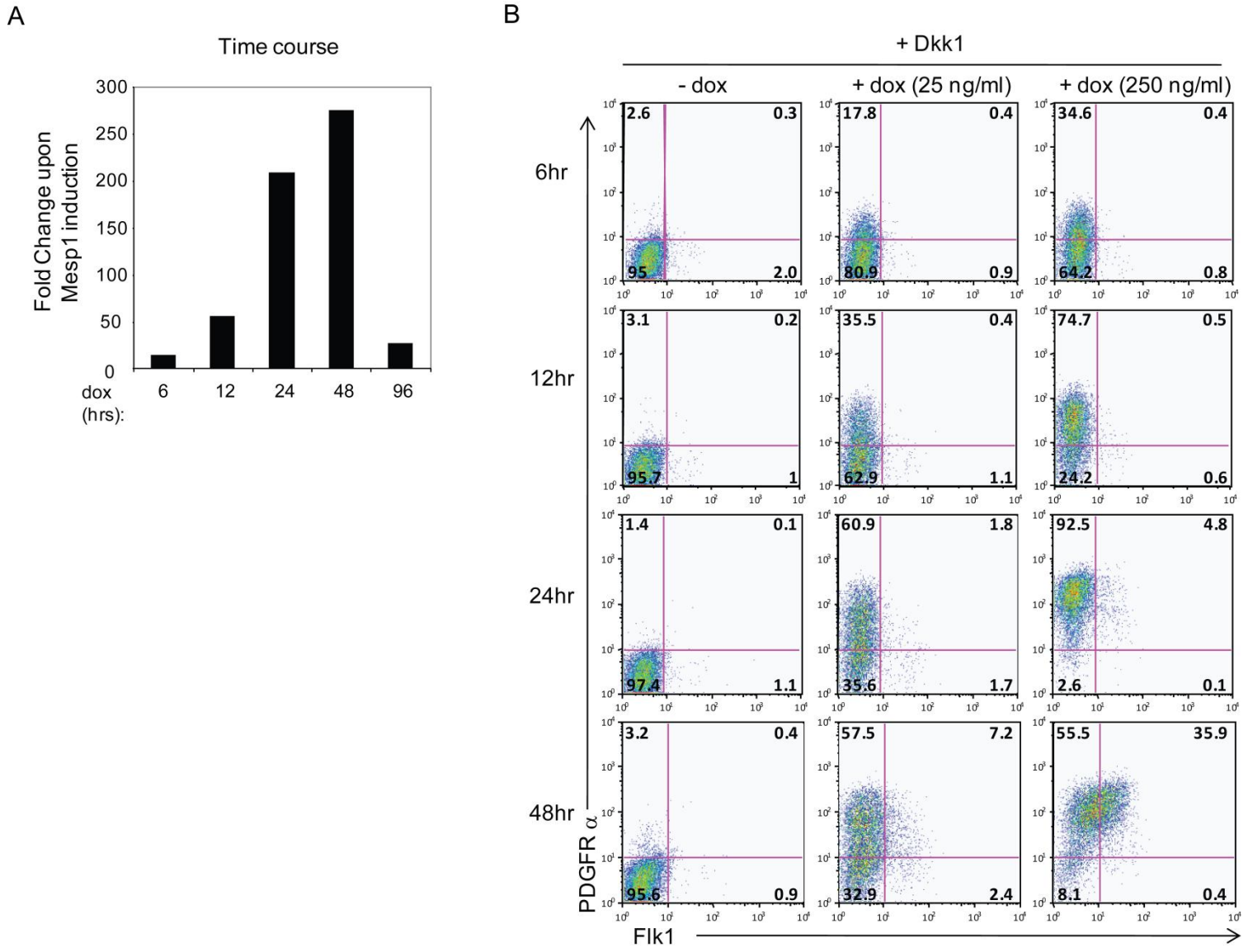
A
Time course
Fold Change upon Mesp1 induction
300
250
200
150
100
50
0
dox (hrs):
6
12
24
48
96
B
+ Dkk1
- dox
+ dox (25 ng/ml)
+ dox (250 ng/ml)
6hr
12hr
24hr
48hr
2.6
0.3
95
2.0
17.8
0.4
80.9
0.9
34.6
0.4
64.2
0.8
3.1
0.2
95.7
1
35.5
0.4
62.9
1.1
74.7
0.5
24.2
0.6
1.4
0.1
97.4
1.1
60.9
1.8
35.6
1.7
92.5
4.8
2.6
0.1
3.2
0.4
95.6
0.9
57.5
7.2
32.9
2.4
55.5
35.9
8.1
0.4
PDGFR α
Flk1

Figure 3.3 *Mesp1* induces *PDGFRα* rapidly and in a dose-dependent manner. (A) A2lox.Mesp1 ESCs were differentiated in the absence or presence of Dkk1 from days 2-6, either with or without 250 ng/ml dox from day 2-4. *PDGFRα* Gene expression was analyzed using microarray 6, 12, 24, 48, and 96 hr after *Mesp1* induction. Shown is the fold increase of *PDGFRα* in cells treated with Dkk1 and dox compared to time-matched controls treated with Dkk1 only. (B) A2lox.Mesp1 ESCs were differentiated as in (A) except the dose of dox was titrated. Cells were analyzed by flow cytometry for PDGFRα and Flk1 expression.

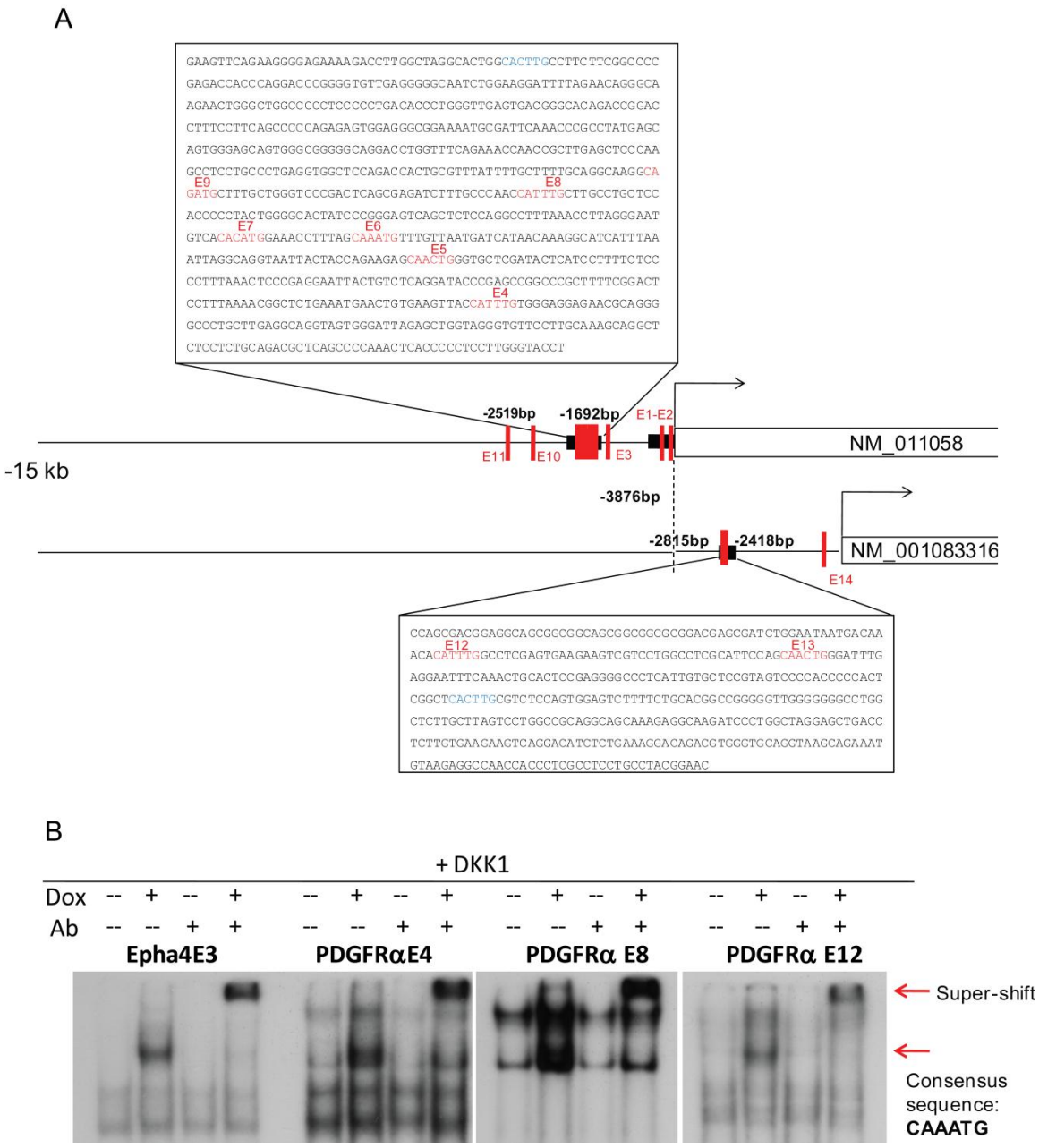

Figure 3.4 *Mesp1* directly binds to the *PDGFRα* promoter *in vitro*. (A) A schematic of the evolutionary conserved E-boxes identified within the promoters of the two *PDGFRα* transcripts. The arrow indicates transcription start site for the specific transcript of *PDGFRα*. Core evolutionary conserved regions (ECR) and E-boxes between the mouse and human are indicated as the solid black boxes and the red lines, respectively. They are identified using the ECR Browser tool on the website: http://www.dcode.org/. (B) *Mesp1* binds to E4 and E8 within the promoter of transcript NM_011058 and E12 within the promoter of transcript NM_001083316. A2.bio-Mesp1*tet*BirA ESCs were differentiated in the presence of Dkk1, with or without Dox

(250 ng/ml) treatment on day 2. After 36 hr, cells were harvest and nuclear extract was prepared for EMSA. Radiolabeled E4, E8 and E12 probes are incubated with nuclear extract from cells treated with both Dkk1 and dox or with Dkk1 only, either in the absence or presence of anti-biotin antibody. *Epha4* E3 serves as a positive control. The red arrow indicates the super-shifted bands.

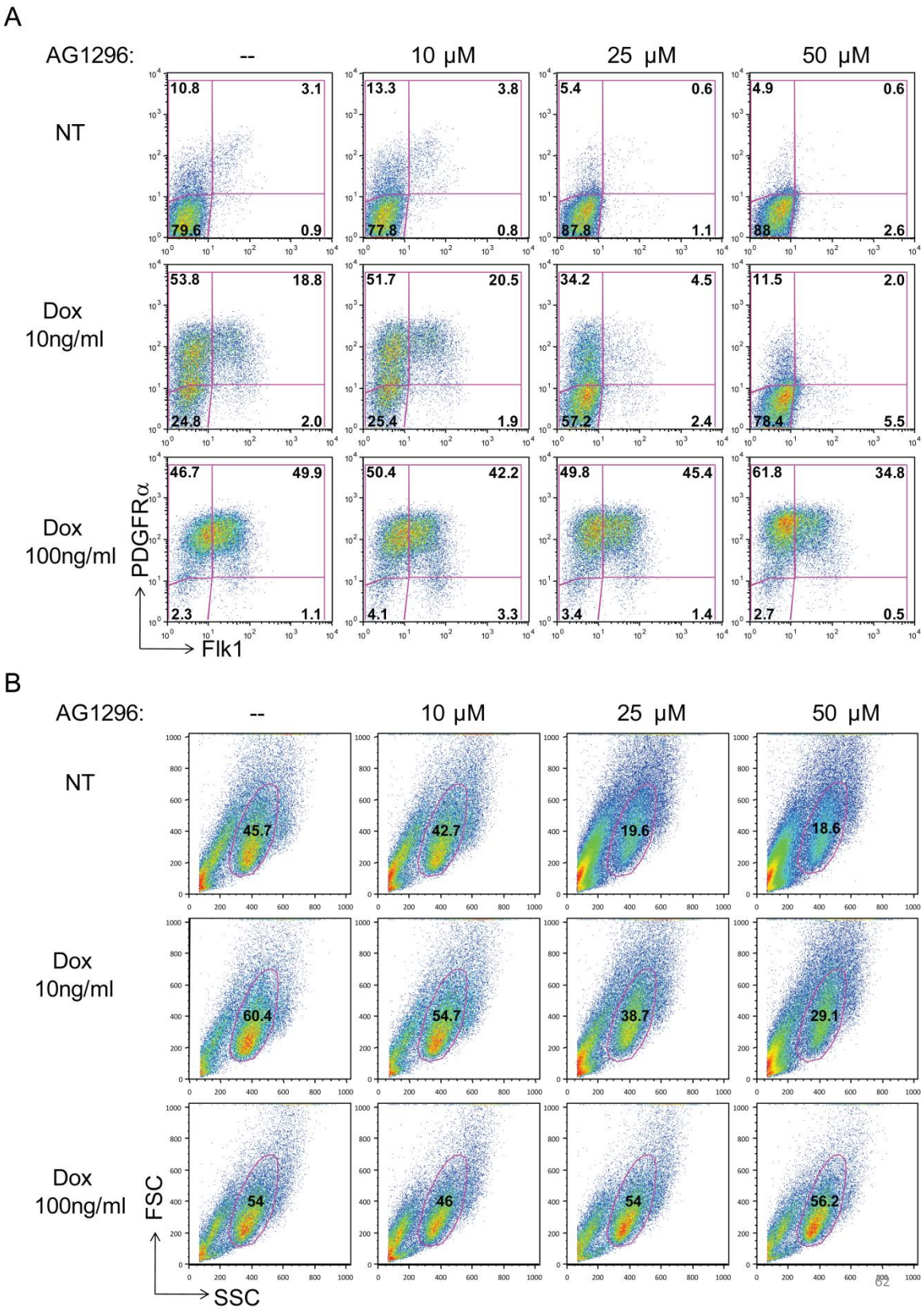
A
AG1296:
--
10 μM
25 μM
50 μM
NT
Dox 10ng/ml
Dox 100ng/ml
PDGFRα
Flk1
10.8
3.1
79.6
0.9
13.3
3.8
77.8
0.8
5.4
0.6
87.8
1.1
4.9
0.6
88
2.6
53.8
18.8
24.8
2.0
51.7
20.5
25.4
1.9
34.2
4.5
57.2
2.4
11.5
2.0
78.4
5.5
46.7
49.9
2.3
1.1
50.4
42.2
4.1
3.3
49.8
45.4
3.4
1.4
61.8
34.8
2.7
0.5
B
AG1296:
--
10 μM
25 μM
50 μM
NT
Dox 10ng/ml
Dox 100ng/ml
FSC
SSC
45.7
42.7
19.6
18.6
60.4
54.7
38.7
29.1
54
46
54
56.2

Figure 3.5 AG1296 blocked the induction of Flk1 and PDGFRα in a dose-dependent manner in untreated A2lox.Mesp1 ESCs or cells treated with a low dose of dox. (A-B) A2lox.Mesp1 ESCs were differentiated without dox treatment (NT) or with 10ng/ml or 100ng/ml dox treatment from days 2-4, in the absence (--) or presence of an increasing dose of AG1296. On day 4, cells were analyzed by flow cytometry for Flk1, and PDGFRα expression. (A) The induction of Flk1 and PDGFRα was inhibited in NT or in 10ng/ml of dox-treated A2lox.Mesp1 ESCs by AG1296 in a dose-dependent manner. Numbers indicate the percentage of live-gated cells within each quadrant. (B) Less live cells were present in NT or in 10ng/ml of dox treated A2lox.Mesp1 ESCs with a higher dose of AG1296, based on the FSC and SSC gating of all cells.

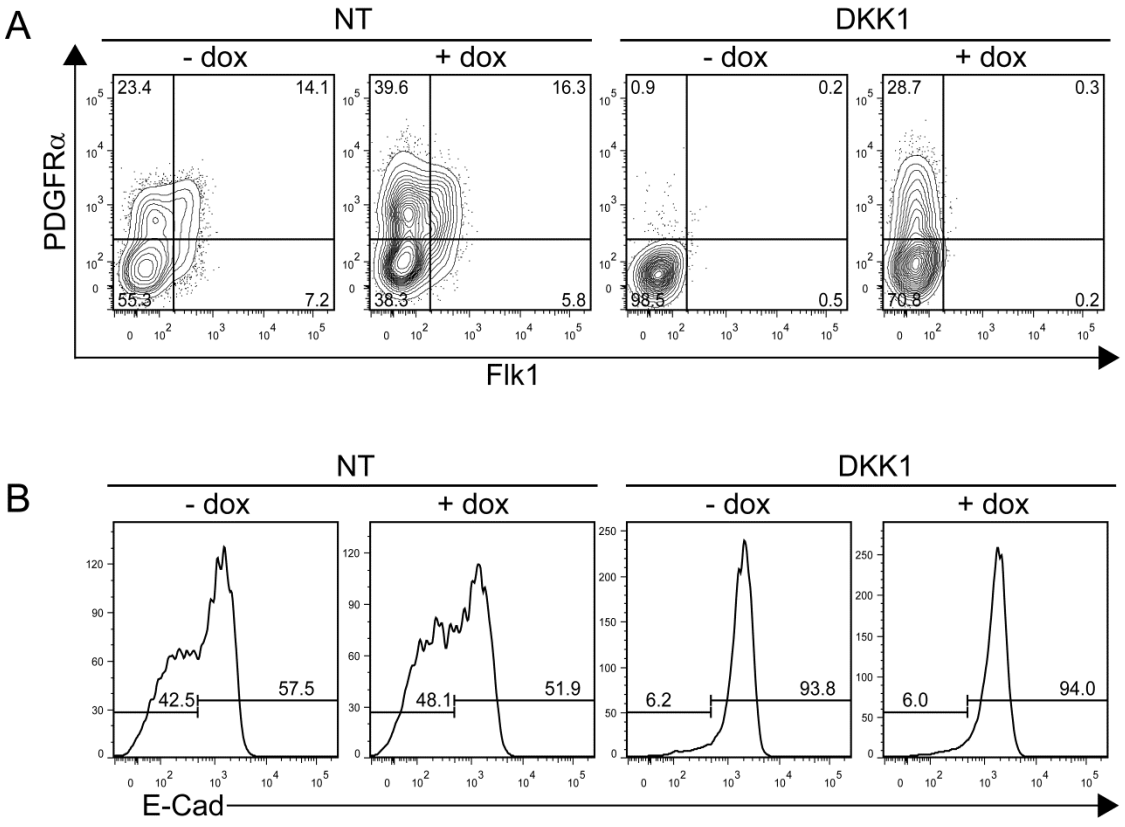

Figure 3.6 *PDGFRα* is not sufficient to induce EMT or Flk1$^+$ mesoderm.
A2lox.PDGFRα ESCs were differentiated as EBs without dox treatment (- dox) or with 250ng/ml dox treatment (+ dox) from days 2-4, in the absence (NT) or presence (Dkk1) of Dkk1. On day 5, cells were analyzed by flow cytometry for Flk1, PDGFRα and E-cadherin expression.

CHAPTER 4: *MESP1* LABELS ALL HEMATOPOIETIC LINEAGES AND INDIRECTLY INDUCES *MEIS1* IN ESC-DERIVED ENDOTHELIUM

Mesp1 labels all hematopoietic lineages

Previously, we reported that the transcription factor *Mesp1* regulates the epithelial-mesenchymal transition (EMT) and promotes the cell fates of cardiomyocytes, smooth muscle, and vascular endothelium from differentiating embryonic stem (ES) cells.[10] *Mesp1* also reduces the *in vitro* development of hematopoietic lineages from ES cells, although we did not establish if this action occurred *in vivo*. Since hematopoietic stem cells (HSC) were shown to derive from hemogenic endothelium,[26-28] and *Mesp1* regulates development of endothelium, we wondered if *Mesp1* might influence gene expression related to hematopoietic development.

Previous fate mapping studies showed that *Mesp1* is expressed in the precursors of the cardiovascular system including endothelium, endocardium, myocardium, and epicardium,[12] but did not carefully examine tracing of hematopoietic cells. We therefore carried out fate mapping using Cre recombinase expressed by the *Mesp1* locus with a modified ROSA-GFP reporter locus (Figure 4.1A-B). Unexpectedly, we found that hematopoietic cells were efficiently labeled by Mesp1-Cre, with different efficiencies between individual animals ranging from 10% to as high as 99% of cells. Within a given individual, the same percentage of hematopoietic cells was labeled by ROSA-GFP across all hematopoietic lineages (Table 4.1). In particular, HSCs were labeled at the same frequency as multi-potent progenitors (MPP), the megakaryocyte progenitors (MkP), granulocyte-macrophage progenitors (GMP), and mature lineages derived from these progenitors. Although the basis for individual animal variation is still unclear, these results suggested that *Mesp1* might be expressed in some fraction of hematopoietic progenitors, possibly hemogenic endothelium.

Mesp1 is necessary for hematopoietic differentiation of ESCs

Indeed, examination of *Mesp1*-deficient ES cells showed reduced numbers of developing $Tie2^+$ endothelial cells and $CD41^+$ hematopoietic progenitors (Figure 4.2A) as well as reduced hematopoietic development as assessed by methylcellulose assays (Figure 4.2B). *Mesp1* lineage tracing previously identified that *Mesp1* labels the endothelium of the dorsal aorta in the embryo. Since *Mesp1* is not expressed in adult and mature hematopoietic lineages (Figure 4.1C), these results suggest a potential role of *Mesp1* in optimal development of hemogenic endothelium.

Mesp1 indirectly induces Meis1 and Meis2 in ESC-derived endothelium

To test this, we purified $Flk1^+$ $Tie2^+$ endothelium derived from ES cell differentiation as described[10] (Figure 4.2C) and used microarray expression analysis to identify genes strongly induced by *Mesp1* (Figure 4.2D). The transcription factor *Snai1* was strongly induced by *Mesp1*, as we previous reported.[10] Unexpectedly, *Mesp1* also induced expression of *Tlx1*, required for spleen development,[72] and both *Meis1* associated with both hematopoietic and endothelial development,[43,44] and *Meis2*.[47] However, *Mesp1* did not globally induce genes associated with hematopoietic development, since it inhibited expression of *Fli1*,[73] *Etv2*,[74] *Runx1*,[75] *Cdx2*,[76] and *Myb*[77] (Figure 4.2D). We confirmed that *Meis1* and *Meis2* are indeed regulated by *Mesp1*, since both are reduced in expression in ES cells that are deficient for *Mesp1* (Figure 4.2E). However, the regulation of *Meis1* and *Meis2* may be indirect, since *Mesp1* could be demonstrated to activate the *Epha4* enhancer, but not the *Meis1* or *Meis2* promoter/enhancer regions (Figure 4.2F).

Table 4.1 Hematopoietic lineages labeled by Mesp1-Cre

Bone Marrow Progenitors

	HSC	MPP	MkP	GMP	Pre GM	Pre MegE	Pre CFU-E	CFU-E
Mice#1	20.5%	25.5%	26.9%	26.1%	25.5%	28.3%	23.7%	25.1%
Mice#2	31.3%	44.1%	38.1%	43.5%	47.8%	41.1%	35.8%	41.3%
Mice#3	10.8%	9.5%	10.4%	11.2%	11.3%	11.6%	9.7%	11.5%

Spleen

	Lymphoid		Myeloid						Meg	Erythroblast			
	$B220^+$	$CD3^+$	Granulocyte	Monocyte	Inflammatory DC	$CD4^+$ DC	$CD8^+$ DC	pDC	$CD41^+$	Pro	Baso	Poly	Ortho
Mice#1	14.4%	20.4%	25.4%	24.3%	19.6%	17.2%	20.0%	25.0%	14.8%	17.4%	9.1%	12.4%	1.3%
Mice#2	26.1%	37.6%	36.4%	38.0%	32.0%	31.8%	32.3%	36.4%	31.4%	33.6%	20.7%	14.0%	1.0%
Mice#3	9.7%	14.4%	10.3%	10.1%	9.3%	9.5%	9.1%	10.5%	10.4%	10.3%	9.7%	1.7%	0.1%

Peripheral Blood

	Lymphocyte			Granulocyte
	$B220^+$	$CD4^+$	$B220^- CD4^-$	
Mice#4	14.5%	19.3%	17.0%	16.6%
Mice#5	20.5%	21.4%	19.2%	20.2%
Mice#6	97.9%	97.0%	94.0%	99.7%

DC indicates dendritic cell; and pDC, plasmacytoid dendritic cell.

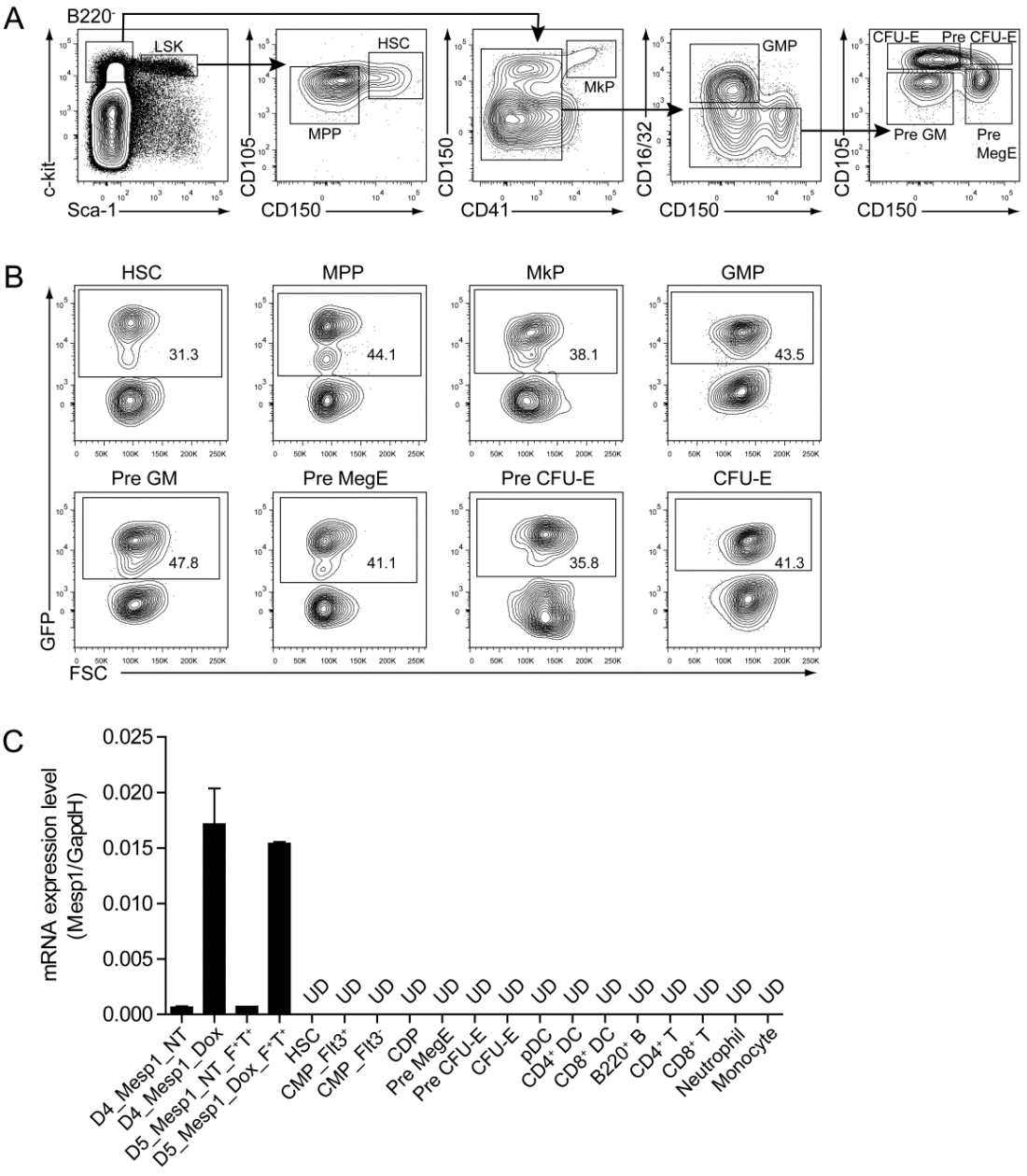

Figure 4.1 ***In vivo*** **lineage tracing of myeloerythroid progenitors by Mesp1-Cre in mouse bone marrow.** Murine bone marrow cells collected from adult $Mesp1^{Cre/+}$ Rosa26-CAG-STOP-$eGFP^{+/+}$ mice were analyzed by FACS for GFP expression in various myeloerythroid progenitors. (A) Bone marrow cells were stained with antibodies to Sca1, c-kit, B220, CD41, CD105, CD150, and CD16/32. Shown is the gating scheme used to identify progenitor populations LSK, HSC, MPP, MkP, GMP, CFU-E, Pre CFU-E, Pre GM, and Pre MegE.[78] (B) Shown are two-parameter contours for GFP expression and forward scatter (FSC) for each indicated progenitor population from an individual $Mesp1^{Cre/+}$ Rosa26-CAG-STOP-$eGFP^{+/+}$ mouse. Numbers indicate the percentage of cells within the indicated gates. (C) Listed hematopoietic subsets were sort-purified from 129S6/SvEV wild-type mice, harvested for RNA, and analyzed for the expression

of *Mesp1* by quantitative RT-PCR (n=3). RNA isolated from D4 A2lox.Mesp1 EBs with or without dox treatment, and from $Flk1^{+}$ $Tie2^{+}$ cells sorted from D5 A2lox.Mesp1 EBs with or without dox treatment were used as positive controls.

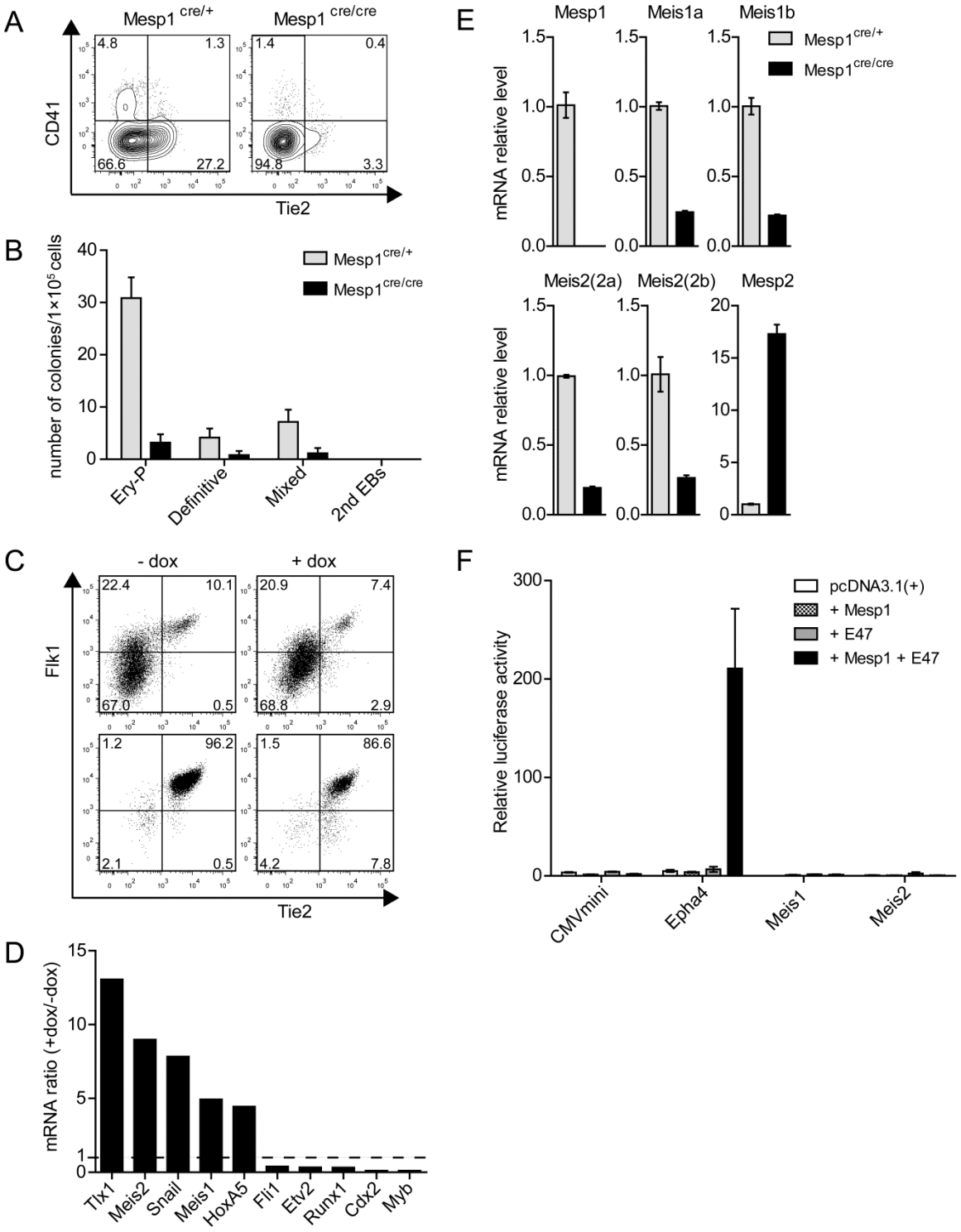

Figure 4.2 ***Mesp1*** **induces a subset of hematopoietic-associated transcription factors in ES cell-derived hemogenic endothelium.** (A) ES cells derived from control heterozygous Mesp1$^{cre/+}$ mice (Mesp1$^{cre/+}$) or homozygous Mesp1-deficient Mesp1$^{cre/cre}$ mice (Mesp1$^{cre/cre}$) as described previously[24] were differentiated as embryoid bodies (EBs) for 6 days and analyzed by FACS. Shown are two-parameter histograms for expression

of CD41 and Tie2. Numbers indicate the percent of cells in the indicated quadrant. (B) ES cells described in (A) were cultured as EBs for 6 days before plating in methylcellulose media with cytokines as described in Methods. Hematopoietic colonies were quantitated after 6 days growth on methylcellulose based on morphologies. Data represent the average of three experiments. Error bars represent SD. (C) ES cells harboring a doxycyline (dox)-inducible *Mesp1* gene (A2lox.Mesp1) were differentiated as embryoid bodies (EBs) for 5 days in the absence (-) or presence (+) of dox from day 2 to day 4. $Flk1^{+}$ $Tie2^{+}$ cells comprising between 5 and 10% of the population (Presort) were purified by cell sorting (Postsort). (D) Microarray analysis of transcription factors associated with hematopoietic development. Expression of the indicated genes is shown as a ratio of expression values by dox-treated endothelial cells relative to untreated cells. (E) Cells described in (A) were cultured as EBs for 5 days and total RNA was isolated to detect the expression levels of the indicated genes by quantitative RT-PCR using primers described in Table 2.1. (F) 293T cells were transfected with firefly luciferase reporter constructs containing a minimal CMV promoter (CMVmini), CMVmini with *Epha4* enhancer (Epha4), or 1kb upstream promoter/enhancer regions for *Meis1* (Meis1) and *Meis2* (Meis2). These were co-transfected along with expression vectors for *Mesp1* and *E47*, either separately or together as indicated. Luciferase activity was normalized using co-transfected Renilla luciferase construct (prL-CMV). Shown is the normalized luciferase for the indicated constructs. Bars represent the SD of triplicate determinations.

CHAPTER 5: DUAL ACTIONS OF *MEIS1* INHIBIT ERYTHROID PROGENITOR DEVELOPMENT AND SUSTAIN GENERAL HEMATOPOIETIC CELL PROLIFERATION

Meis1 and Meis2 promote hematopoietic colony formation from ES cells in vitro

To determine the activity of these *Mesp1*-induced genes in differentiating endothelium, we generated ES cells with inducible *Meis1* or *Meis2* expression and examined their differentiation *in vitro* by colony formation assay (Figure 5.1). Induction of *Meis1* caused a 5- to 6-fold increase in the numbers and size of definitive and mixed hematopoietic colonies (Figure 5.1A-B), but had little effect on primitive erythroid colonies. *Meis2* also markedly increased numbers of definitive hematopoietic colonies (Figure 5.1A) and increased their size (Figure 5.1B). In collagen-based media containing thrombopoietin (TPO), induction of either *Meis1* or *Meis2* caused more than 4-fold increase in the numbers of pure megakaryocyte colonies, and also increased the mixed megakaryocyte colonies (Figure 5.1C-D). To facilitate analysis of hematopoietic progenitors, we differentiated ES cells in liquid culture as embryoid bodies (EBs), and in co-culture with OP9 cells,[62] with or without cytokines (Figure 5.2A). We determined that doxycycline induced approximately a 4-fold increase in *Meis1* expression over the endogenous *Meis1*, and more than 30-fold increase in *Meis2* (Figure 5.2B). When examined in EBs, *Meis1* caused only a slight increase in expression of CD41, a marker for the earliest hematopoietic progenitor[79] on day 9 of differentiation, but this effect was lost by day 12 (Figure 5.2C). When examined in ES cells co-cultured with OP9 cells alone, *Meis1* produced a larger induction of CD41 on day 9, which again was lost by day 12. However, in ES cells co-cultured with OP9 and cytokines, *Meis1* caused a robust induction of CD41 in more than 50% of cells on day 9, and this effect persisted to day 12. Therefore, we used these last conditions to further study *Meis1* and *Meis2* in regulating hematopoietic differentiation.

Meis1 increases most hematopoietic progenitors but inhibits early erythroid progenitors

Previous studies suggested that *Meis1* augments the proliferative potential of various progenitor cells.[38,47] Initially we expected that *Meis1* and *Meis2* might increase all hematopoietic progenitors. Induction of *Meis1* or *Meis2* by doxycycline on day 7 and 8 increased the differentiation of $CD71^{-}$ $CD41^{+}$ hematopoietic progenitors by 50% to 200% (Figure 5.3A). However, both factors decreased the number of early $CD71^{+}$ erythroid progenitors, consistent with the observed decrease in numbers of primitive erythroid colonies in methylcellulose assays caused by *Meis1* and *Meis2* (Figure 5.1A).

Meis1 and *Meis2* caused a substantial increase of the numbers and maintenance of $CD41^{+}$ hematopoietic progenitors on day 12 of differentiation (Figure 5.3B). *Meis1* and *Meis2* induced the formation of two populations of $CD41^{+}$ cells. One population expressed intermediate levels of CD41 ($CD41^{int}$), and the second expressed high levels ($CD41^{hi}$). The $CD41^{int}$ cells induced by *Meis1* and *Meis2* were negative for expression of CD42d, a component of the von Willebrand factor receptor expressed by platelets. Notably, $CD41^{hi}$ cells co-expressed CD42d, suggesting they are megakaryocytic precursors. $CD41^{int}$ cells expressed a low frequency of c-kit or AA4.1/CD93, an early hematopoietic progenitor marker, but expressed a higher frequency of CD45, a definitive hematopoietic marker (Figure 5.3C). Notably, $CD41^{hi}$ cells induced by *Meis1* were largely negative for CD45, consistent with the lack of CD45 expression on megakaryocytic precursors. *Meis1* induced the development of macrophages compared to control cultures (Figure 5.3C lower panels). These results identify two distinguishable effects of *Meis1* in differentiating ES cells. First, *Meis1* increases the numbers of

hematopoietic progenitors and maintains their persistence in culture. Second, and unexpectedly, *Meis1* skews hematopoietic differentiation by suppressing erythroid while enhancing megakaryocytic progenitor differentiation.

Meis1 maintains proliferation of hematopoietic progenitors

The above actions of *Meis1* could result either from increased proliferation or decreased cell death of hematopoietic progenitors. To distinguish these possibilities, we determined effect of *Meis1* and *Meis2* on the total number of $CD41^+$ hematopoietic progenitors (Figure 5.4A). Both *Meis1* and *Meis2* markedly increased the total accumulation of $CD41^+$ cells in culture, ranging from a 4-fold increase to more than a 10-fold increase in total cells (Figure 5.4A). This indicates that the increase in percentages of $CD41^+$ populations seen earlier (Figure 5.3) represents an increase in total $CD41^+$ cells. We next measured proliferation directly using BrdU incorporation *in vitro* (Figure 5.4B). *Meis1* and *Meis2* induced only slight increases in the rate of proliferation of $CD41^+$ cells on day 8, two days after transfer into OP9 cultures (Figure 5.4B upper panels). However, *Meis1* and *Meis2* caused the maintenance of cell proliferation on day 11 (Figure 5.4B). $CD41^+$ cells in which *Meis1* or *Meis2* was not induced showed a marked decrease in proliferation at this time, with only 20% of cells incorporating BrdU with a 3-hour pulse. However, induction of *Meis1* or *Meis2* caused a rapid rate of proliferation to be maintained even on day 11 (Figure 5.4B lower panels). This result is consistent with *Meis1* and *Meis2* being able to maintain progenitor cells in a proliferative state, as has been described previously.[38,47]

Increased cell numbers could conceivably arise from decreased apoptosis of $CD41^+$ cells induced by *Meis1*. To test this, we stained differentiating ES cells in OP9 cultures with Annexin V and 7-AAD (Figure 5.4C). First, $CD41^+$ cells undergoing apoptosis (stained with Annexin V but not 7-AAD) were rare in ES/OP9 co-cultures, being less than 5% either with or without induction of *Meis1* or *Meis2*. Secondly, the induction of *Meis1* or *Meis2* had no effect on the percentage of Annexin V^+ cells, indicating little effect of these factors on apoptosis of proliferating $CD41^+$ progenitors.

Isoform-specific repression of in vivo erythroid progenitor development

Since *Meis1* and *Meis2* have multiple isoforms,[44] we wished to test their actions in regulating erythroid and megakaryocyte development. *Meis1* has two naturally occurring isoforms, *Meis1a* and *Meis1b* (Figure 5.5A), formed by an alternative splicing of different terminal exons generating proteins with divergent C-terminal sequences. *Meis2* has at least six reported isoforms generated through alternative splicing (Figure 5.5A). We first tested whether the alternative isoforms of *Meis1* and *Meis2* had similar effects on increasing $CD41^{int}$ hematopoietic progenitors (Figure 5.5B). Notably, *Meis1a*, *Meis2(2a)*, and *Meis2(4a)* each substantially increased the number of $CD41^{int}$ hematopoietic progenitors on day 9 and day 12 relative to controls, indicating that these isoforms act similarly to *Meis1b* and *Meis2(2b)* tested earlier (Figure 5.3B). Interestingly, *Meis1* and *Meis2* appear to have different expression patterns *in vivo*. *Meis1* is expressed in hematopoietic progenitors, while *Meis2* is expressed in the nervous system.[80] We find evidence for differential capacities of *Meis1* and *Meis2* isoforms to suppress erythroid differentiation and promote megakaryocytic progenitors *in vivo* (Figure 5.6). First we

used CD71 and CD41 expression to examine early erythroid progenitors from control BM or from BM transduced with retroviruses expressing *Meis1a*, *Meis1b*, or *Meis2(2b)* (Figure 5.6A). As a control, BM cells transduced with an empty retrovirus showed no difference in the extent of erythroid progenitor development compared to non-transduced BM within the same animal (Figure 5.6A left panel). As before, we found that expression of *Meis1b* substantially reduced the population of $CD71^+$ erythroid precursors, and increased the population of $CD41^+$ $CD71^-$ megakaryocyte progenitors. Notably, *Meis1a* caused a greater reduction in development of erythroid progenitors, and a stronger enhancement of $CD41^+$ megakaryocyte progenitors compared to *Meis1b*. In contrast, *Meis2(2b)* appeared weaker than *Meis1b* in these actions.

We repeated this analysis using CD105 and CD150 expression to distinguish earlier stages of erythroid and megakaryocyte differentiation. $CD150^+$ $CD105^-$ population represents both the megakaryocyte-erythroid progenitor (MEP) and the committed megakaryocyte progenitor (MkP). As the MEP differentiates towards the erythrocyte lineage, CD105 expression is induced, followed by loss of CD150 expression. In this system, again bone marrow cells transduced with an empty retrovirus showed very little effect on the inhibition of erythrocyte differentiation. In contrast, *Meis1b* substantially reduced the size of the $CD105^+$ $CD150^-$ population of erythroid progenitors. Again, the *Meis1a* isoform was even more robust in extinguishing erythroid progenitor differentiation, and the *Meis2(2b)* isoform was much weaker than *Meis1b* or *Meis1a*.

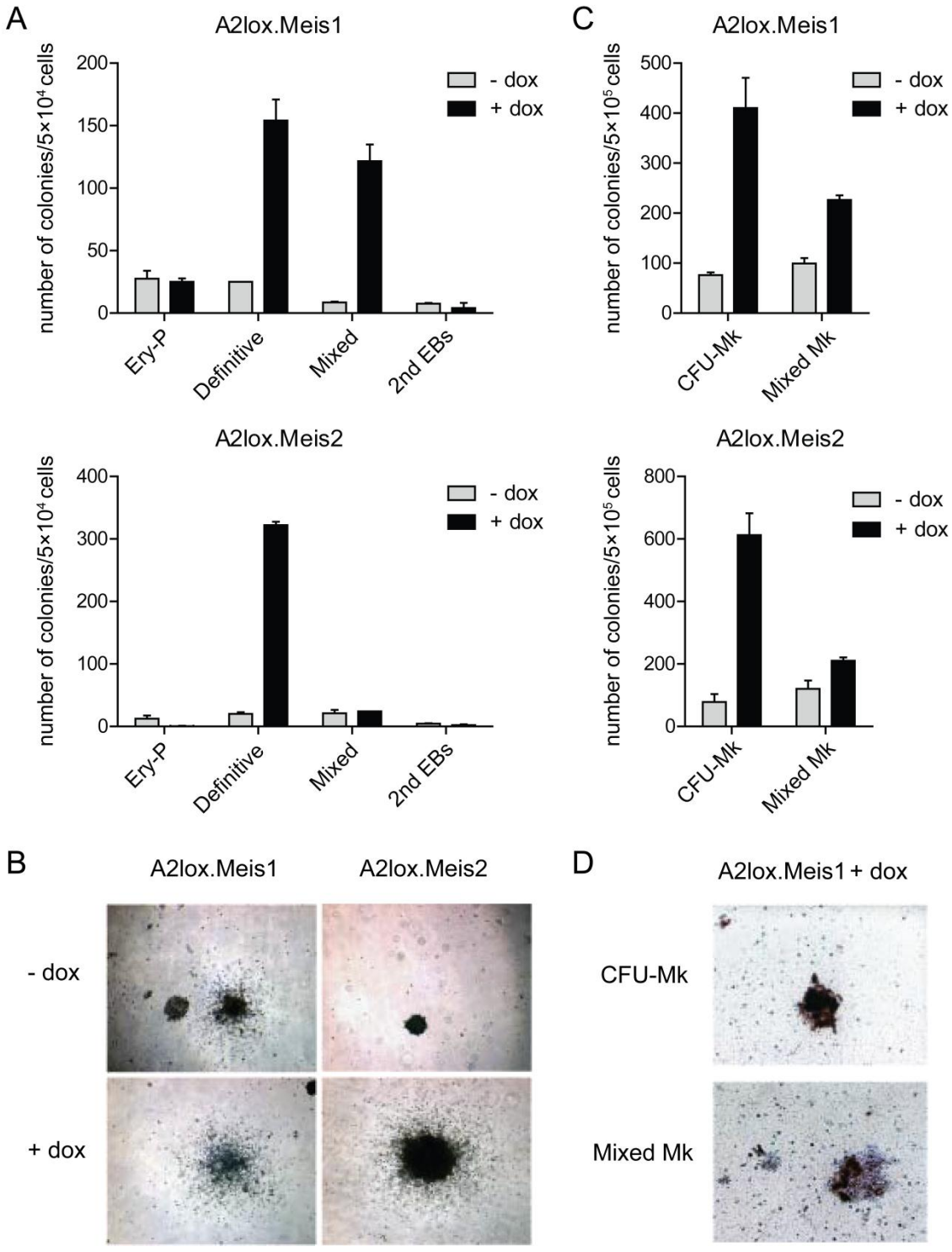

Figure 5.1 ***Meis1*** **and** ***Meis2*** **increase the numbers of ES cell-derived definitive hematopoietic colonies in semisolid media.** (A) ES cells with dox-inducible *Meis1* (A2lox.Meis1) or *Meis2* (A2lox.Meis2) were differentiated as embryoid bodies (EBs) for 6 days before plating in methylcellulose media with cytokines as described in "Methods".

Hematopoietic colonies were quantitated after 6 days growth on methylcellulose based on morphologies. Data represent the average of three experiments. Error bars represent SD. (B) Bright field microscopy of definitive hematopoietic colonies derived from A2lox.Meis1 or A2lox.Meis2 ES cells with (+) or without (-) treatment with doxycycline (dox). Original magnification ×40. (C) A2lox.Meis1 or A2lox.Meis2 EBs were dissociated on day 6 after differentiation and plated in MegaCult-C media. After another 6 days, megakaryocyte colony formation was visualized by acetylthiocholine iodide and Harris hematoxylin counterstain, and CFU-Mk and mixed Mk colonies were quantitated. Data represent the average of four experiments. Error bars represent SD. (D) Bright field microscopy of CFU-Mk and mixed Mk colonies derived from A2lox.Meis1 ES cells in the presence of dox. Original magnification ×100. CFU-Mk appeared brown, since murine megakaryocytes express acetylcholinesterase, producing brown precipitate. Mixed Mk colonies were distinguished by the presence of non-megakaryocytic cells within brown-staining cell clusters.

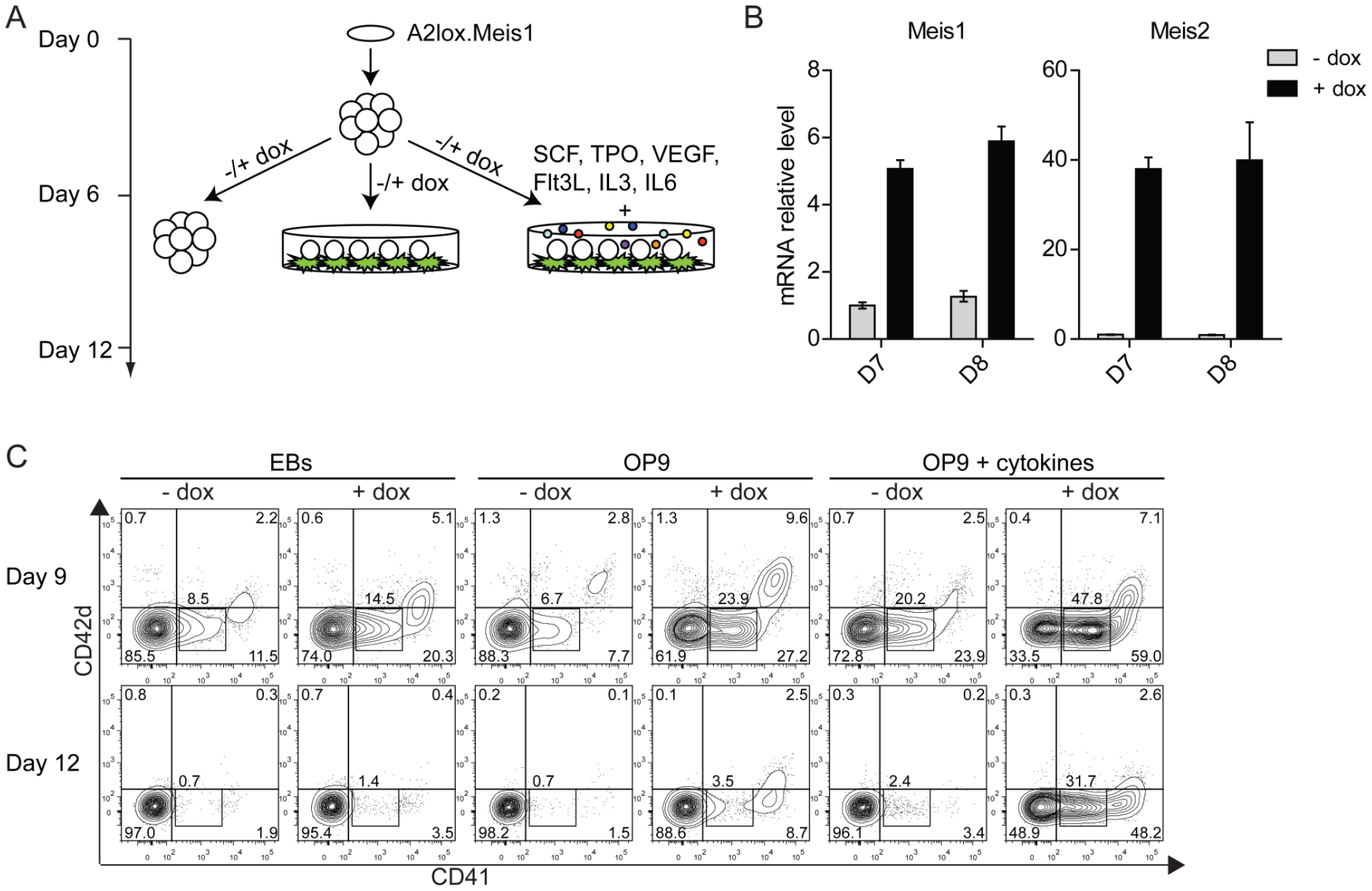
A
Day 0
Day 6
Day 12
A2lox.Meis1
-/+ dox
-/+ dox
-/+ dox
SCF, TPO, VEGF,
Flt3L, IL3, IL6
+
B
Meis1
Meis2
- dox
+ dox
mRNA relative level
D7
D8
D7
D8
C
EBs
OP9
OP9 + cytokines
- dox
+ dox
- dox
+ dox
- dox
+ dox
Day 9
Day 12
CD42d
CD41

Figure 5.2 *Meis1* maintains CD41^{+} hematopoietic progenitors from ES cells co-cultured with OP9 and hematopoietic cytokines. (A) A2lox.Meis1 ES cells were differentiated as embryoid bodies (EBs) for 6 days. On day 6, EBs were left intact, or dissociated and co-cultured with an OP9-GFP cell monolayer in the absence (OP9) or presence (OP9 + cytokines) of the cytokines stem cell factor (SCF), thrombopoietin (TPO), vascular endothelial growth factor (VEGF), Flt3 ligand (Flt3L), interleukin-3 (IL-3) and IL-6 (IL-6) as described in "Methods". Doxycycline (dox) was added as indicated on day 6 and replenished every other day until day 12. (B) A2lox.Meis1 and A2lox.Meis2 ES cells were differentiated as embryoid bodies (EBs) for 6 days and plated on OP9 cells with cytokines described above in (A). Levels of *Meis1* and *Meis2* were determined by quantitative RT-PCR on day 7 or day 8 as indicated in the presence or absence of doxycycline (dox) administered on day 6. (C) On day 9 and 12, cells from each differentiation condition were analyzed by FACS for expression of CD41 and CD42d. Numbers indicate the percentage of cells in the indicated gates.

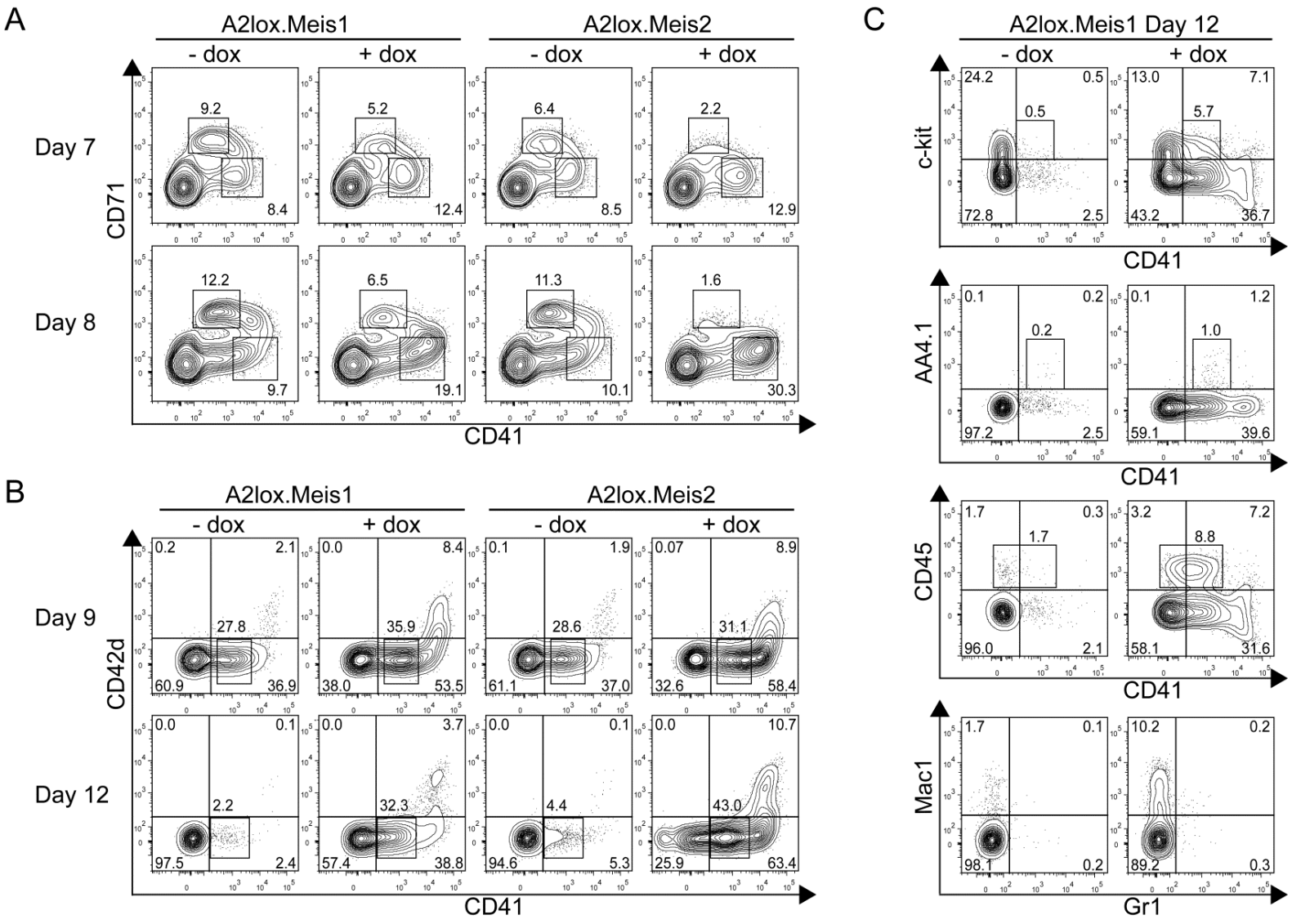
A
A2lox.Meis1
A2lox.Meis2
- dox
+ dox
Day 7
Day 8
CD71
CD41
9.2
8.4
5.2
12.4
6.4
8.5
2.2
12.9
12.2
9.7
6.5
19.1
11.3
10.1
1.6
30.3
B
Day 9
Day 12
CD42d
0.2
2.1
27.8
60.9
36.9
0.0
8.4
35.9
38.0
53.5
0.1
1.9
28.6
61.1
37.0
0.07
8.9
31.1
32.6
58.4
0.0
0.1
2.2
97.5
2.4
0.0
3.7
32.3
57.4
38.8
0.0
0.1
4.4
94.6
5.3
0.0
10.7
43.0
25.9
63.4
C
A2lox.Meis1 Day 12
c-kit
AA4.1
CD45
Mac1
Gr1
24.2
0.5
0.5
72.8
2.5
13.0
7.1
5.7
43.2
36.7
0.1
0.2
0.2
97.2
2.5
0.1
1.2
1.0
59.1
39.6
1.7
0.3
1.7
96.0
2.1
3.2
7.2
8.8
58.1
31.6
1.7
0.1
98.1
0.2
10.2
0.2
89.2
0.3

Figure 5.3 *Meis1* and *Meis2* inhibit erythroid progenitor differentiation but increase CD41^{+} hematopoietic progenitors differentiation from ES cells cultured on OP9 with hematopoietic cytokines. (A) A2lox.Meis1 or A2lox.Meis2 ES cells were differentiated as EBs for 6 days before plating on OP9-GFP cell monolayers and cytokines, and treated with (+) or without (-) doxycycline (dox) every 2 days until day 12. On day 7 and 8, cells were analyzed by FACS for CD71 and CD41 expression. Data shown are for cells gated for negative expression of GFP to exclude OP9 cells from the analysis. Numbers indicate the percentage of cells within each quadrant. (B) Cells were treated as in panel A and analyzed on day 9 and 12 for expression of CD42d and CD41. (C) Cells were treated as in panel A and analyzed on day 12 for expression of c-kit, AA4.1, CD45, Mac1, and Gr1.

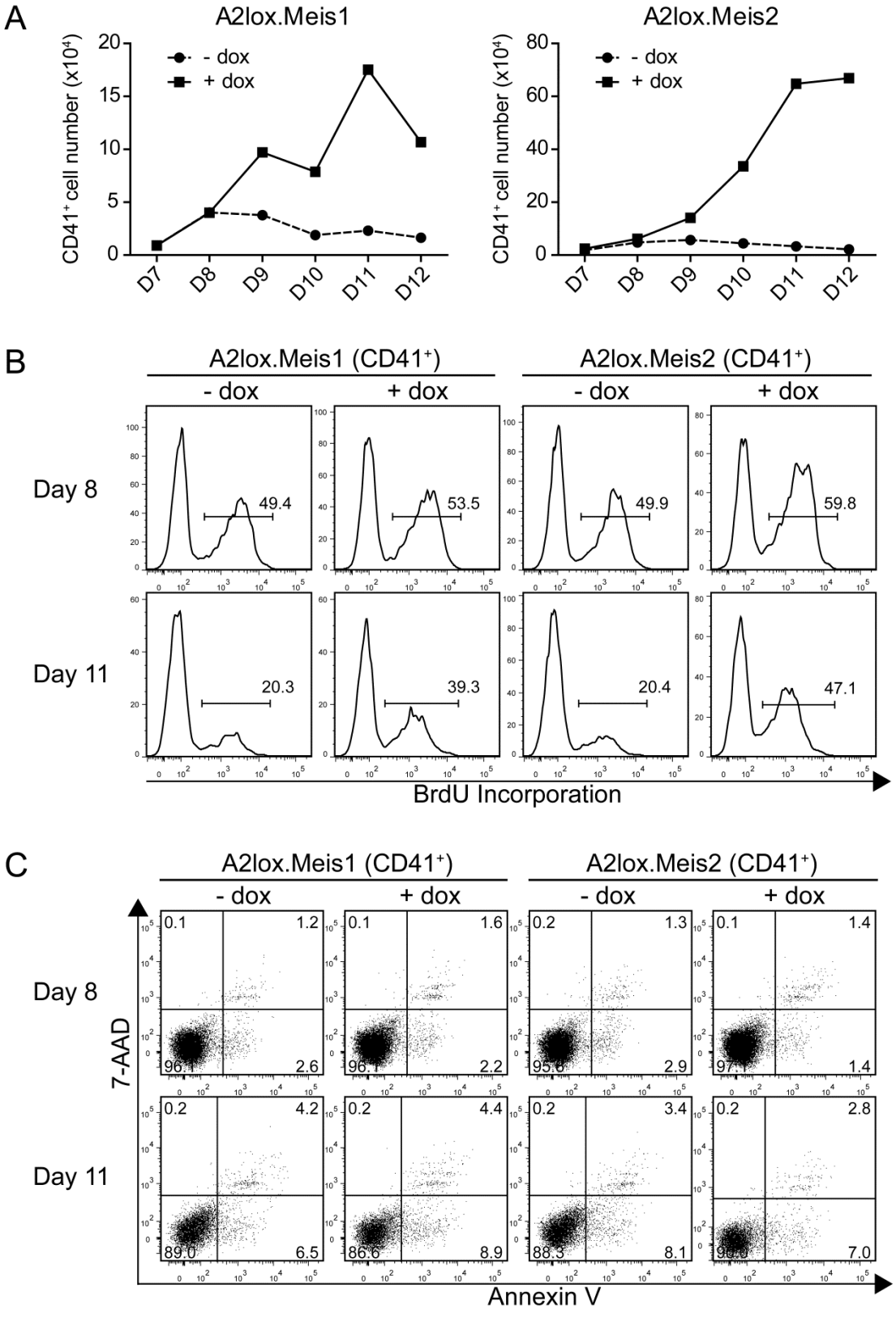
A
A2lox.Meis1
A2lox.Meis2
- dox
+ dox
CD41+ cell number (x10⁴)
D7
D8
D9
D10
D11
D12
B
A2lox.Meis1 (CD41+)
A2lox.Meis2 (CD41+)
Day 8
Day 11
49.4
53.5
49.9
59.8
20.3
39.3
20.4
47.1
BrdU Incorporation
C
7-AAD
Annexin V
0.1
1.2
96.1
2.6
0.1
1.6
96.1
2.2
0.2
1.3
95.6
2.9
0.1
1.4
97.1
1.4
0.2
4.2
89.0
6.5
0.2
4.4
86.6
8.9
0.2
3.4
88.3
8.1
0.2
2.8
90.0
7.0

Figure 5.4 *Meis1* and *Meis2* maintain the proliferation of CD41$^+$ hematopoietic progenitors from ES cells cultured on OP9 with hematopoietic cytokines. (A) A2lox.Meis1 (left panel) or A2lox.Meis2 (right panel) ES cells were differentiated as in Figure 5.3A and analyzed for total cell numbers and CD41 expression on the indicated days. Absolute CD41$^+$ cell numbers were determined from the product of total cell counts and the percent of CD41$^+$ expressing cells determined by FACS. (B) Cells differentiated as in panel A were pulsed with BrdU for 3 hours on day 8 or 11 and analyzed by FACS for CD41 expression and BrdU incorporation. Numbers indicate the percentage of BrdU$^+$ CD41$^+$ cells. (C) Cells differentiated as in panel A were analyzed by FACS for staining with Annexin V and 7-AAD to label apoptotic cells and with anti-CD41 antibody to label hematopoietic progenitors. Numbers indicate the percentage of CD41$^+$ cells within each quadrant.

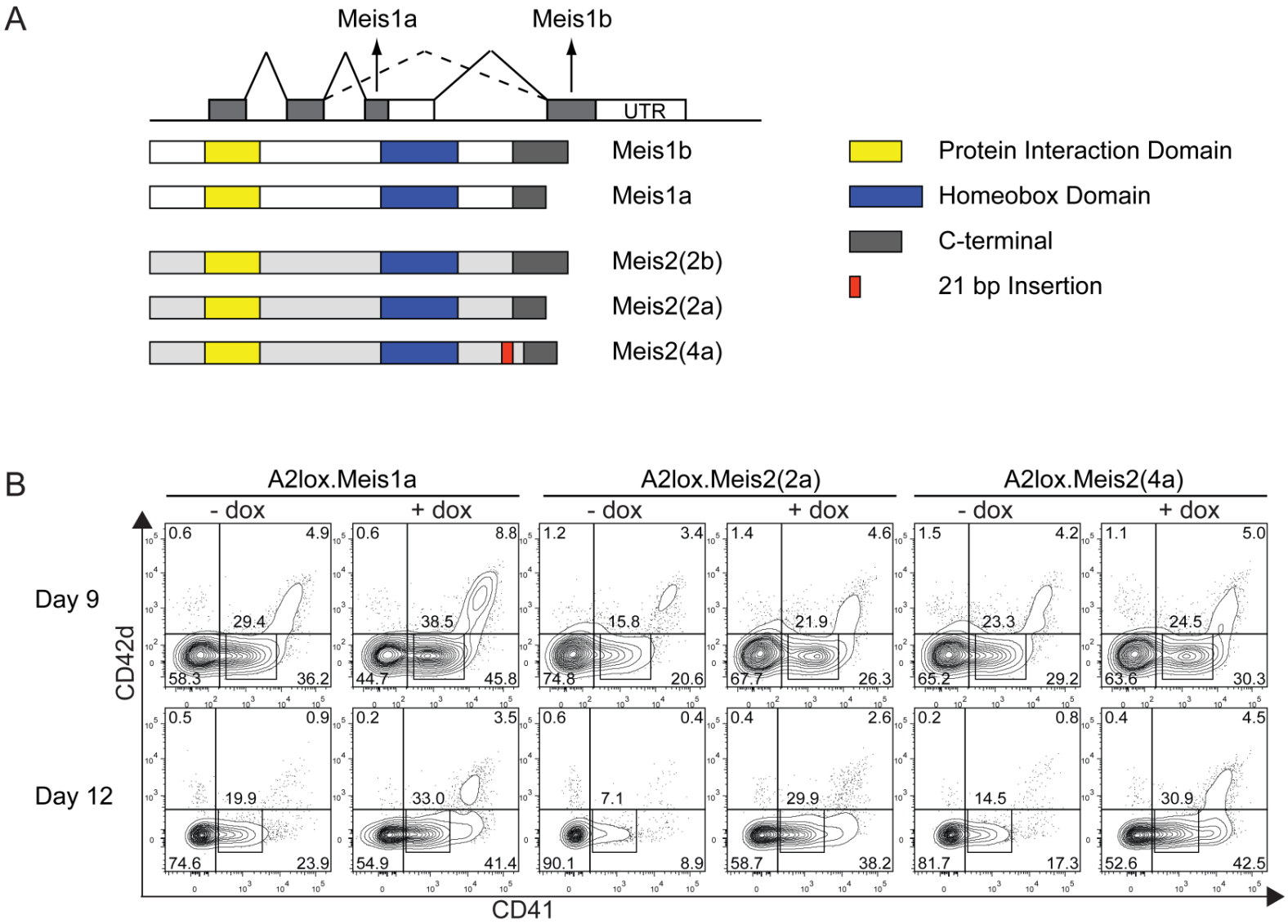
A
Meis1a
Meis1b
UTR
Meis1b
Meis1a
Meis2(2b)
Meis2(2a)
Meis2(4a)
Protein Interaction Domain
Homeobox Domain
C-terminal
21 bp Insertion
B
A2lox.Meis1a
A2lox.Meis2(2a)
A2lox.Meis2(4a)
- dox
+ dox
Day 9
Day 12
CD42d
CD41
0.6 4.9 29.4 58.3 36.2
0.6 8.8 38.5 44.7 45.8
1.2 3.4 15.8 74.8 20.6
1.4 4.6 21.9 67.7 26.3
1.5 4.2 23.3 65.2 29.2
1.1 5.0 24.5 63.6 30.3
0.5 0.9 19.9 74.6 23.9
0.2 3.5 33.0 54.9 41.4
0.6 0.4 7.1 90.1 8.9
0.4 2.6 29.9 58.7 38.2
0.2 0.8 14.5 81.7 17.3
0.4 4.5 30.9 52.6 42.5

Figure 5.5 Isoforms of *Meis1* and *Meis2* maintain CD41^{+} hematopoietic progenitors differentiated from ES cells. (A) Shown is the domain structure of *Meis1* and *Meis2* isoforms. *Meis1a* and *Meis1b* differ in the carboxyl-terminal (C-terminal) region due to alternative splicing. Similarly, the C-terminal regions of *Meis2(2a)* and *Meis2(4a)* differ from *Meis2(2b)*. (B) ES cells with dox-inducible *Meis1a* (A2lox.Meis1a), *Meis2(2a)* (A2lox.Meis2(2a)), or *Meis2(4a)* (A2lox.Meis2(4a)) were differentiated and analyzed as in Figure 5.3A.

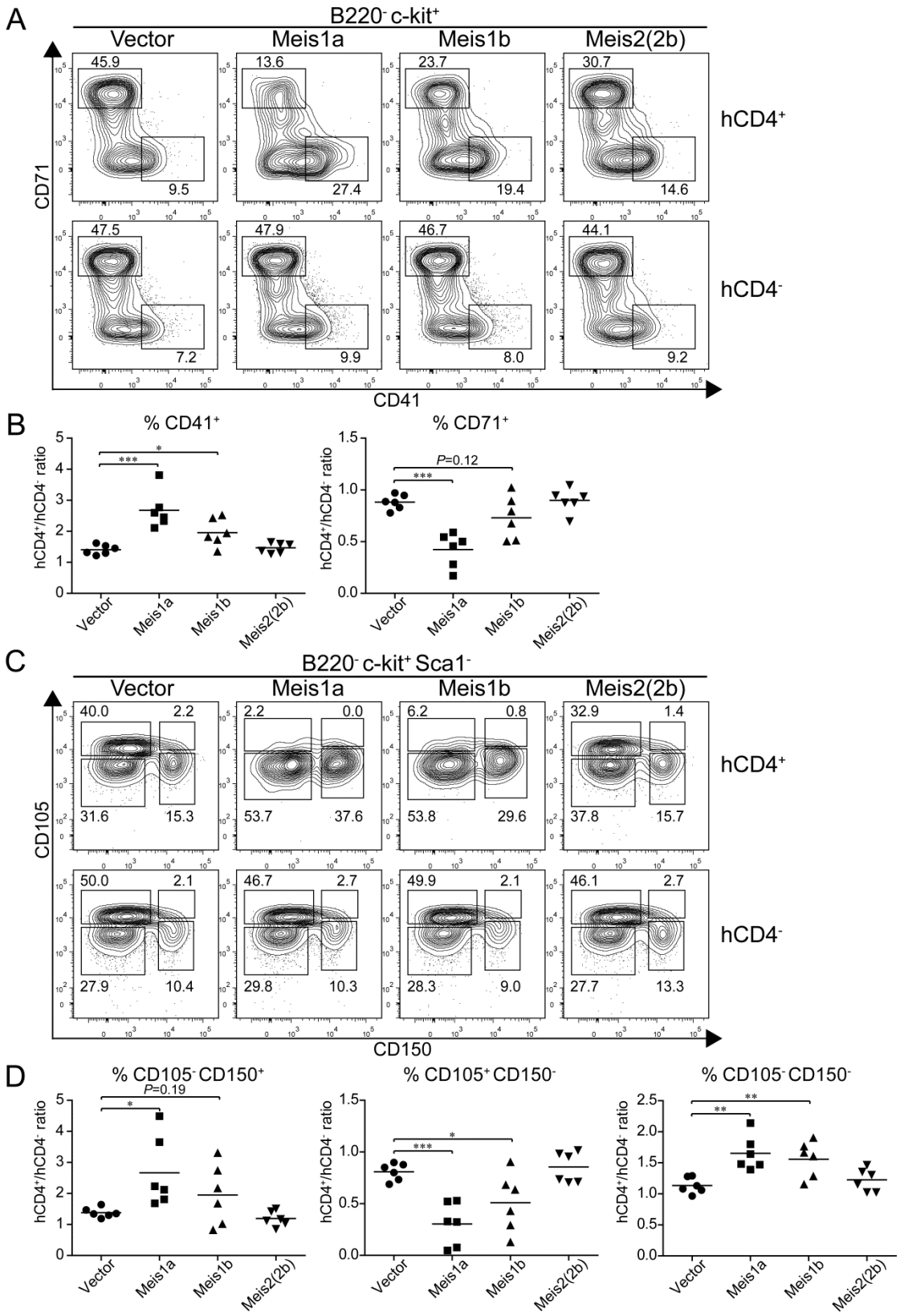
A
B220- c-kit+
Vector
Meis1a
Meis1b
Meis2(2b)
45.9
9.5
13.6
27.4
23.7
19.4
30.7
14.6
47.5
7.2
47.9
9.9
46.7
8.0
44.1
9.2
hCD4+
hCD4-
CD71
CD41
B
% CD41+
% CD71+
hCD4+/hCD4- ratio
***
*
P=0.12
Vector
Meis1a
Meis1b
Meis2(2b)
C
B220- c-kit+ Sca1-
40.0
2.2
31.6
15.3
2.2
0.0
53.7
37.6
6.2
0.8
53.8
29.6
32.9
1.4
37.8
15.7
50.0
2.1
27.9
10.4
46.7
2.7
29.8
10.3
49.9
2.1
28.3
9.0
46.1
2.7
27.7
13.3
CD105
CD150
D
% CD105- CD150+
% CD105+ CD150-
% CD105- CD150-
P=0.19
**

Figure 5.6 *Meis1a* and *Meis1b* inhibit the *in vivo* erythroid potential of the megakaryocyte-erythroid progenitor (MEP). Stem/progenitor-cell-enriched bone marrow cells from donor mice were transduced with a control vector (IRES-hCD4) or a retroviral construct expressing *Meis1a* (Meis1a-IRES-hCD4), *Meis1b* (Meis1b-IRES-hCD4) or *Meis2(2b)* (Meis2(2b)-IRES-hCD4) as indicated. The transduced bone marrow progenitor cells were transplanted into sub-lethally irradiated recipients and bone marrow was analyzed 4 to 6 weeks later. (A) Bone marrow was analyzed for expression of hCD4, B220, c-kit, CD71 and CD41. Shown are two-color contours of CD71 and CD41 expression for cells gated as $B220^-$ $c\text{-}kit^+$. Numbers indicate the percentage of cells in the indicated gates. (B) The percentage of megakaryocytic progenitors ($CD41^+$) or erythroid progenitors ($CD71^+$) as in panel A was determined for both transduced ($hCD4^+$) cells and non-transduced ($hCD4^-$) cells within the same recipient. Shown is the ratio for each vector for megakaryocytic progenitors ($CD41^+$) or erythroid progenitors ($CD71^+$) as indicated. Each dot represents data from an individual recipient (n = 6). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. (C) Bone marrow from recipients described in panel A was analyzed for expression of hCD4, B220, c-kit, Sca1, CD105 and CD150. Shown are two-color contours of CD105 and CD150 expression for cells gated as $B220^-$ $c\text{-}kit^+$ $Sca1^-$. (D) The percentage of Pre MegE and MkP ($CD105^-$ $CD150^+$), CFU-E ($CD105^+$ $CD150^-$), or GMP and Pre GM ($CD105^-$ $CD150^-$) progenitors was determined for both the transduced ($hCD4^+$) and non-transduced ($hCD4^-$) bone marrow cells within the same recipient. Shown is the ratio of $hCD4^+$ to $hCD4^-$ progenitors for each construct and cell population. Each dot represents data from one recipient mouse (n = 6). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

CHAPTER 6: IDENTIFICATION OF TRANSCRIPTIONAL TARGETS AND BINDING PARTNERS OF *MEIS1*

Identification of gene targets of Meis1

Having identified two distinct effects of *Meis1*, we wished to determine their mechanisms. *Meis1* might promote megakaryocyte-specific transcription factors or suppress genes associated with erythrocyte development. In regulating hematopoietic progenitor proliferation, *Meis1* might directly induce genes associated with cell cycle, as reported for the induction of *cyclin* D,[47,54,81] or be involved in expressing growth factor receptors that could support progenitor proliferation. To distinguish these possibilities, we used microarray analysis to compare the various populations of cells developing in ES/OP9 co-cultures in the presence and absence of *Meis1* and *Meis2* induction (Figure 6.1). To identify proximal gene targets of *Meis1*, we first needed to identify the critical period of time *in vitro* during which *Meis1* exerts its effects on proliferation and differentiation. Thus, we carried out a temporal analysis of *Meis1's* actions on hematopoietic cell development (Figure 6.1A). Optimal effects of *Meis1* on inducing CD41 were observed when *Meis1* was induced on either day 4 or day 6 of ES cell differentiation, but this effect was substantially reduced when induction of *Meis1* was delayed until day 8 of differentiation (Figure 6.1B). Induction after day 8 led to essentially a loss of any activity of *Meis1* induction on hematopoietic progenitor development.

Therefore, to identify proximal targets of *Meis1* in differentiating ES cells, we induced *Meis1* on day 6, and purified populations of $CD41^-$ and $CD41^+$ cells 24 hours after *Meis1* induction (day 7), and $CD41^-$, $CD41^{int}$, and $CD41^{hi}$ populations 48 hours after induction of *Meis1* (day 8) (Figure 6.2A) and analyzed them by microarray (Figure 6.2B-C). Notably, induction of *Meis1* and *Meis2* substantially decreased a number of

erythrocyte-specific genes, including hemoglobin alpha (*Hba-a1/2*) and glycophorin A (*Gypa*), both 24 hours and 48 hours after induction (Figure 6.2B). In addition, a number of other genes associated with erythroid development were repressed by the induction of *Meis1* and *Meis2* (Table 6.1). Expression of these erythroid genes was evident in $CD41^+$ populations, but absent from the $CD41^-$ populations, as expected. Thus, *Meis1* appears to suppress erythrocyte gene expression within $CD41^+$ hematopoietic progenitors, consistent with inhibition of $CD71^+$ erythroid progenitors (Figure 5.3A) and reduced primitive erythroid colony formation (Figure 5.1A). This may be related to the requirement for *Meis1* in platelet development, given that *Meis1*$^{-/-}$ embryos die of vascular defects secondary to the absence of platelets.[43-45]

We also identified a few genes that were strongly induced by *Meis1* and *Meis2* (Figure 6.2C). Among these, placenta-specific 8 (*Plac8*), and serine peptidase inhibitor, clade B, member 2 (*Serpinb2*) were strongly induced primarily in $CD41^{hi}$ cells on day 8, but induced by lesser amounts on day 7. Notably, the prostaglandin E receptor 3 (*Ptger3*) was induced by *Meis1* specifically in $CD41^+$ cells both on day 7 and on day 8. We verified that the known target of *Meis1*, the platelet factor 4 (*Pf4*) was also induced by *Meis1*, and specific to $CD41^{hi}$ cells on day 8, consistent with its selective expression in megakaryocytes and platelets (Figure 6.2C). The induction of *Plac8* and *Ptger3* by *Meis1* and *Meis2* was confirmed by quantitative RT-PCR (Figure 6.2D).

Plac8 and Serpinb2

Next we turned to test the functional role of these three candidates in regulating hematopoietic cell progenitor proliferation. Among these candidates, *Ptger3* appeared to

be conceivably related to hematopoietic progenitor proliferation, since its ligand prostaglandin E2 can expand hematopoietic stem cells in the setting of transplantation.[82,83] However, *Plac8* and *Serpinb2* might conceivably regulate cell proliferation or survival. *Plac8* reportedly supports cell proliferation and survival by modulating the Akt-Mdm2 pathway and P53 level,[84] while *Serpinb2* may enhance tumor cell survival.[85] We tested *Plac8* and *Serpinb2* for their ability to influence accumulation of $CD41^+$ cells in ES cell differentiation cultures using a doxycycline inducible system (Figure 6.3). However, induction of *Plac8* or *Serpinb2* alone or in combination showed no effect on the accumulation of $CD41^+$ cells or on the expression of CD42d either on day 9 or day 12 in ES/OP9 co-cultures. *Plac8*$^{-/-}$ mice are able to generate normal hematopoietic cell lineages, and only a slight influence on neutrophils function in an infectious model system has been reported.[86] Conceivably, *Plac8* might provide a proliferative advantage to hematopoietic progenitors that was obscured in the setting of the knockout. To test this notion, we generated mixed bone marrow (BM) chimeras from wild type and *Plac8*$^{-/-}$ donor mice (Figure 6.4). We compared the ability of *Plac8*$^{-/-}$ BM progenitors to compete with wild-type progenitors across a wide range of stages of hematopoietic development (Figure 6.4A). The ratio of chimerism observed for HSCs was similar to the ratio in all subsequent stages of myeloerythroid differentiation, indicating that loss of *Plac8* does not influence progenitor proliferation relative to wild-type progenitors. Thus, *Plac8* may be a target of *Meis1* but does not appear to mediate its effects on hematopoietic progenitor proliferation.

Ptger3

Meis1 induced expression of *Ptger3* in $CD41^{+}$ cells on day 7, and in $CD41^{hi}$ cells on day 8 (Figure 6.2C). *Ptger3* is expressed in the MkP at high levels relative to other hematopoietic progenitors (Figure 6.5A). *Meis1* expression is high in the MkP, but is also expressed in other hematopoietic progenitors except for erythroid precursors (Figure 6.5B). To test whether signaling through this receptor could mediate *Meis1*'s effect on hematopoietic progenitor proliferation, we inhibited prostaglandin E receptor in the presence or absence of *Meis1* induction in differentiating ES cells (Figure 6.6). In the absence of a prostaglandin E receptor antagonist SC-51322, both *Meis1* and *Meis2* induction caused a robust increase in the number of $CD41^{int}$ and $CD41^{hi}$ cells on day 12, as expected (Figure 6.6A-C). However, in the induced conditions, increasing doses of antagonist caused a statistically significant reduction in the $CD41^{int}$ population generated by overexpression of *Meis1* and *Meis2* (Figure 6.6B-C). The effect of this inhibition is more evident in conditions in which *Meis1* or *Meis*2 is induced by doxycycline relative to the inhibition in the presence of endogenous levels of Meis1 or Meis2 (Figure 6.6A-C). This may indicate that the actions of *Meis1* and *Meis2* in promoting hematopoietic progenitor expansion may depend upon signaling through the *Ptger3*. However, the inability of several shRNAs to significantly reduce endogenous *Ptger3* expression in ES cells prevents definitive determination of the requirement for this candidate in mediating the effects of *Meis1* (Figure 6.7).

HoxA9 may not be the right partner for Meis1 in differentiating ESCs

Previous studies showed Meis1 interacts with Hoxa9 to accelerate leukemic transformation,[37] and *Hoxa9* also modulates *Meis1* to influence normal hematopoiesis.[87] However, we found co-expression of *Hoxa9* with *Meis1* or *Meis2* did not enhance proliferation of $CD41^+$ hematopoietic progenitors derived from ES/OP9 co-cultures (Figure 6.8), suggesting either that Hoxa9 is not a binding partner of Meis1 during *in vitro* hematopoietic differentiation of ES cells, or that Hoxa9 is not present in limiting amounts in our system.

Table 6.1 Erythroid genes down-regulated by *Meis2*

Gene Symbol	Fold decrease (+dox/-dox)		
	D7_CD41^{+}	D8_CD41^{int}	D8_CD41^{hi}
Hba-a1/2	-3.37	-6.33	-20.15
Hbb-y	-2.22	-5.31	-12.12
Hba-x	-1.85	-3.19	-12.01
Hbb-b1/2	-1.66	-2.75	-4.77
Hbb-bh1	-1.36	-2.36	-3.95
Gypa	-2.45	-5.72	-7.05
Kel	-2.37	-1.40	-1.83
Rhag	-1.80	-2.25	-3.02
Tfrc	-1.68	-1.53	-2.98
Cpox	-2.50	-2.11	-3.00
Spna1	-2.17	-2.22	-4.50
Slc25a37	-1.63	-2.32	-4.26
Nfe2	-2.07	-1.85	-2.14

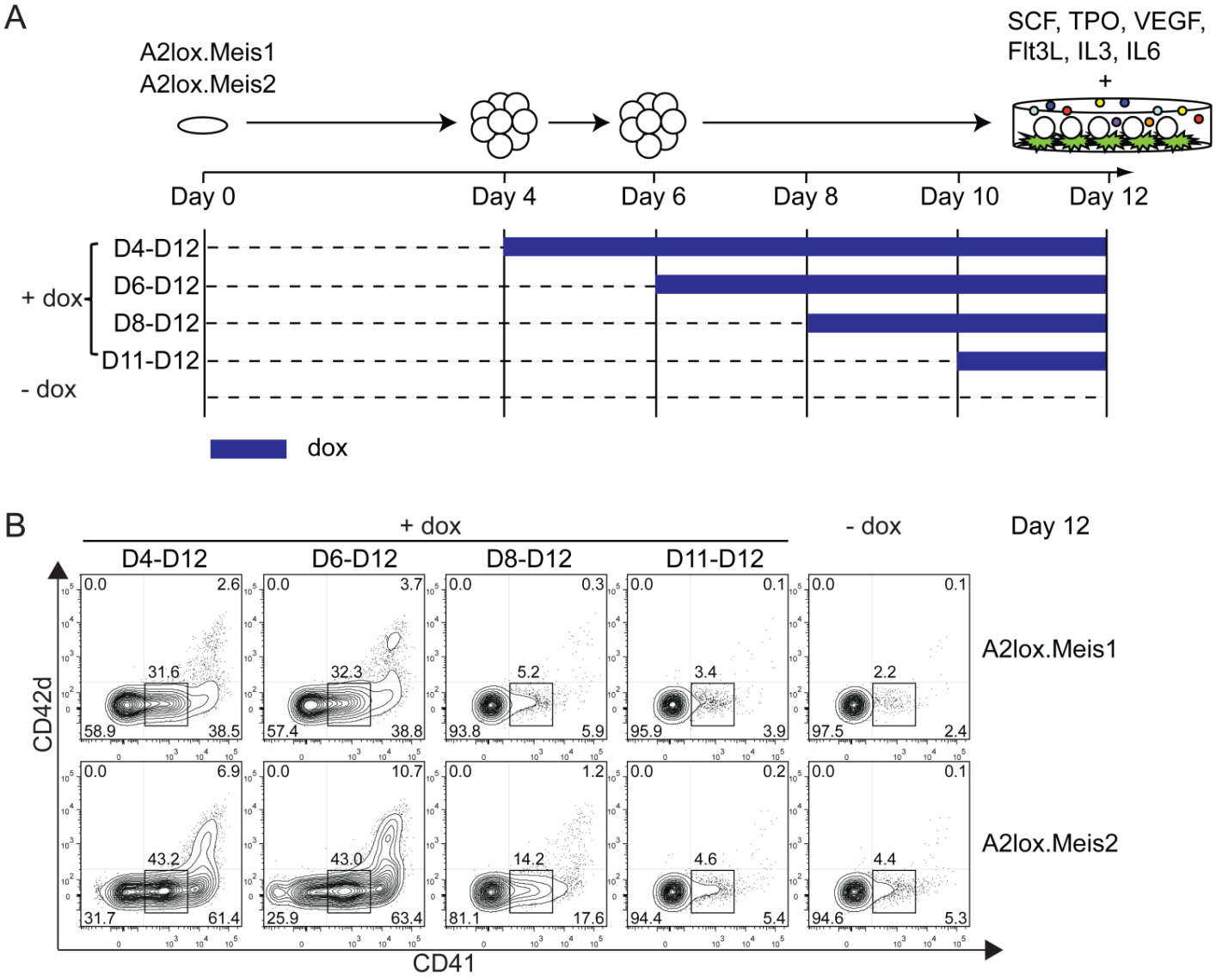
A
A2lox.Meis1
A2lox.Meis2
SCF, TPO, VEGF,
Flt3L, IL3, IL6
+
Day 0
Day 4
Day 6
Day 8
Day 10
Day 12
+ dox
D4-D12
D6-D12
D8-D12
D11-D12
- dox
dox
B
+ dox
- dox
Day 12
D4-D12
D6-D12
D8-D12
D11-D12
A2lox.Meis1
A2lox.Meis2
CD42d
CD41
0.0
2.6
31.6
58.9
38.5
0.0
3.7
32.3
57.4
38.8
0.0
0.3
5.2
93.8
5.9
0.0
0.1
3.4
95.9
3.9
0.0
0.1
2.2
97.5
2.4
0.0
6.9
43.2
31.7
61.4
0.0
10.7
43.0
25.9
63.4
0.0
1.2
14.2
81.1
17.6
0.0
0.2
4.6
94.4
5.4
0.0
0.1
4.4
94.6
5.3

Figure 6.1 Induction of *Meis1* or *Meis2* before day 8 of differentiation is required to maintain CD41^{+} hematopoietic progenitors derived in ES/OP9 co-cultures. (A) A2lox.Meis1 or A2lox.Meis2 ES cells were differentiated as EBs for 6 days before plating on OP9-GFP monolayers and cytokines as described in Figure 5.2A. Doxycycline (dox) was added on day 4, 6, 8, or 10 as indicated and replenished every other day until day 12. (B) A2lox.Meis1 or A2lox.Meis2 ES cells from the conditions indicated in panel A were analyzed on day 12 by FACS for CD41 and CD42d expression.

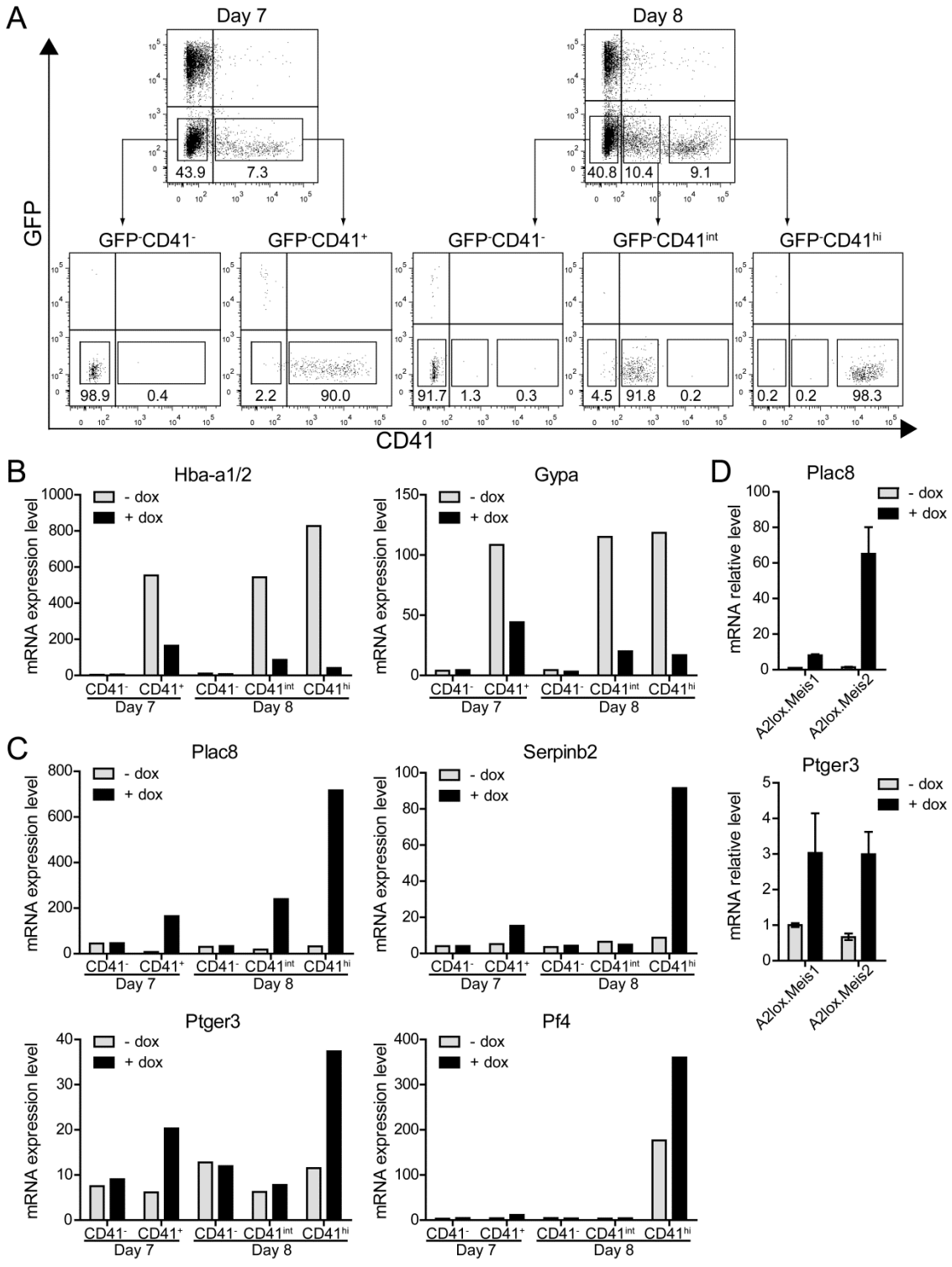

Figure 6.2 Identification of *Meis1* and *Meis2* target genes. (A) A2lox.Meis1 or A2lox.Meis2 ES cells were differentiated as in Figure 5.3A with or without treatment with doxycycline, and were purified by cell sorting on day 7 or 8 into the indicated populations based on levels of CD41 expression; $CD41^-$, $CD41^+$ (day 7) and $CD41^-$,

CD41^{int}, and CD41^{hi} (day 8). (B-C) Gene expression of A2lox.Meis2-derived populations with (+) or without (-) treatment by doxycycline (dox) was determined by microarray analysis. (B) Hemoglobin alpha (*Hba-a1/2*) and glycophorin A (*Gypa*) expression is shown for the indicated populations treated with (+) or without (-) dox. (C) Expression of placenta-specific 8 (*Plac8*), serine peptidase inhibitor, clade B, member 2 (*Serpinb2*), prostaglandin E receptor 3 (*Ptger3*) and platelet factor 4 (*Pf4*) is shown for the indicated populations and conditions. (D) Expression of *Plac8* and *Ptger3* by CD41^{hi} day 8 cells treated with (+) or without (-) dox as described in panel A was determined by quantitative RT-PCR.

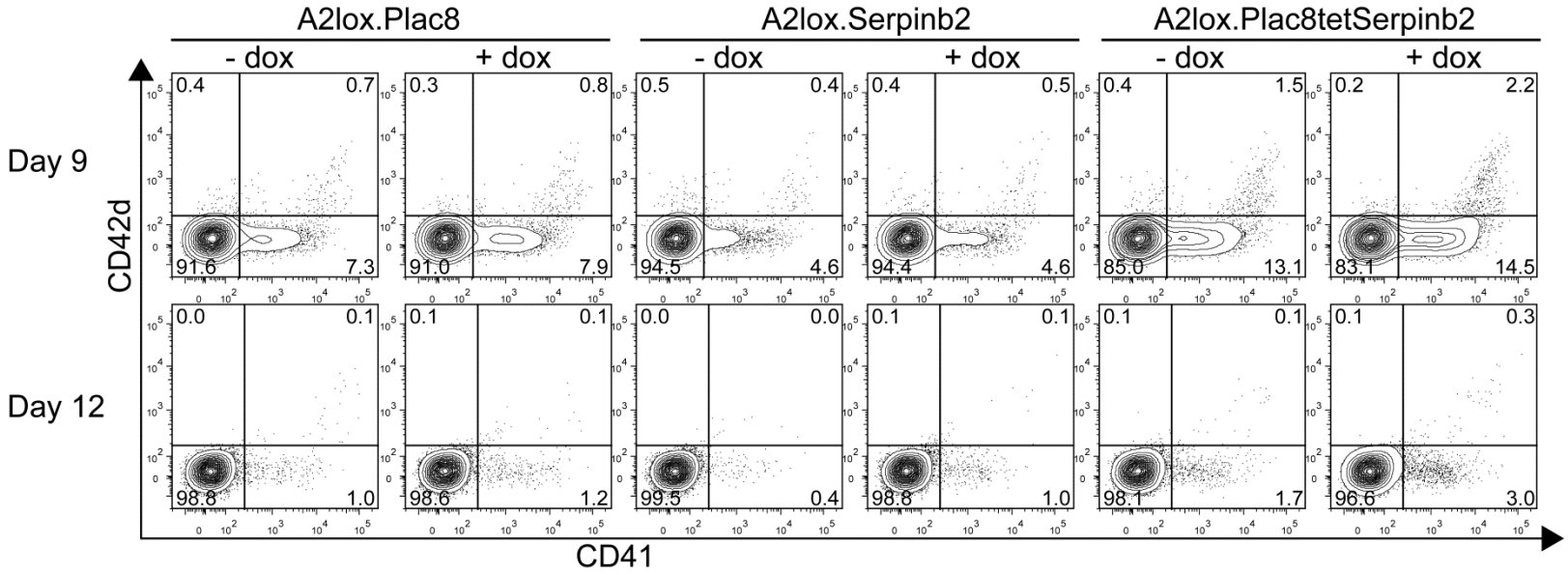

Figure 6.3 *Plac8* and *Serpinb2* are not sufficient to maintain CD41$^+$ hematopoietic progenitors derived in ES/OP9 co-cultures. ES cells with dox-inducible *Plac8* (A2lox.Plac8), *Serpinb2* (A2lox.Serpinb2), or both (A2lox.Plac8tetSerpinb2) were differentiated as EBs for 6 days and co-cultured with OP9-GFP monolayers and cytokines as in Figure 5.2A. Doxycycline (dox) was added on day 6 and replenished every other day until day 12. On day 9 and 12, cells were analyzed by FACS for CD41 and CD42d expression.

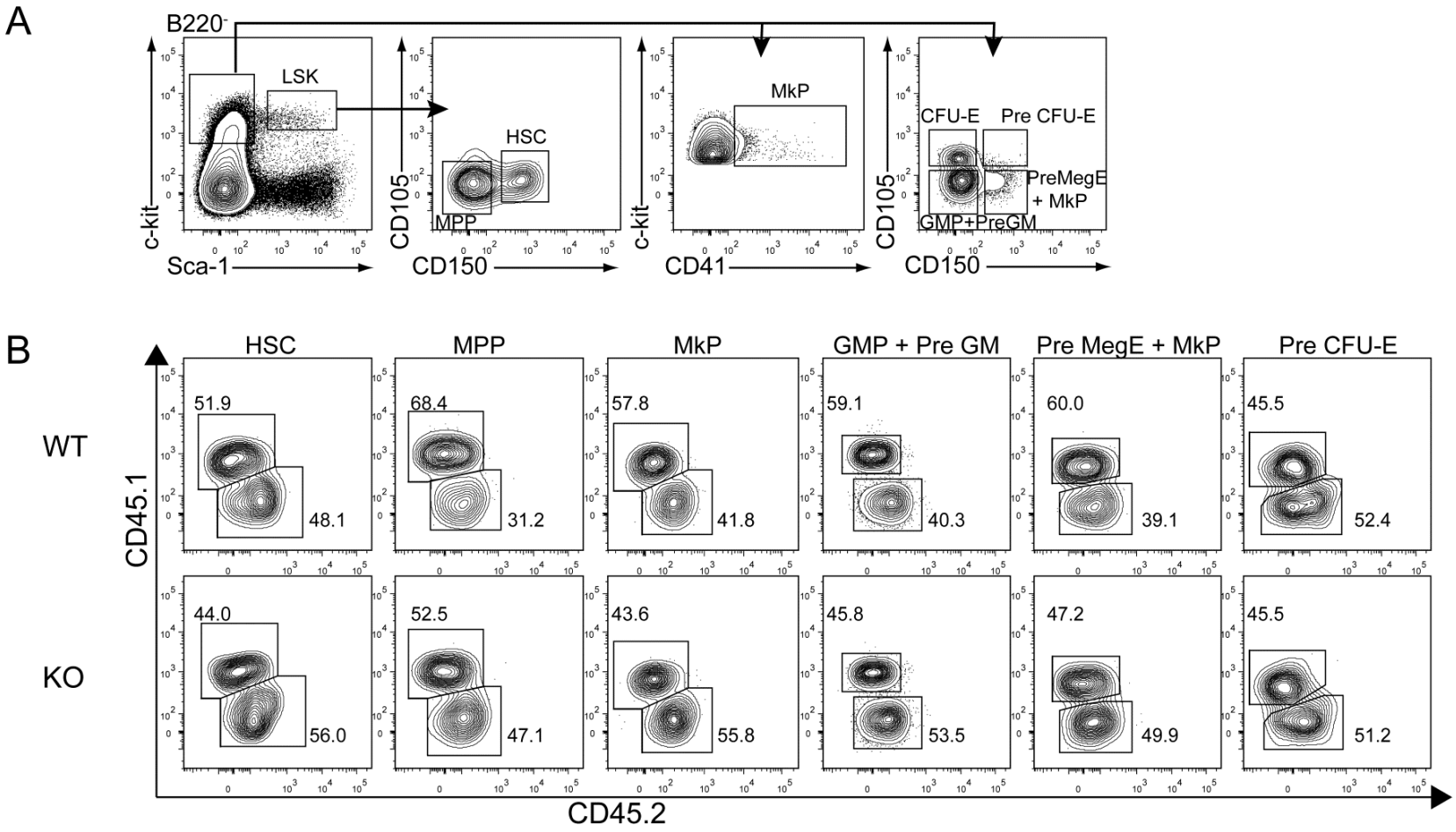

Figure 6.4 ***Plac8*** **is dispensable for normal** ***in vivo*** **hematopoietic progenitor proliferation.** CD45.2^{+} bone marrow cells isolated from *Plac8*$^{-/-}$ mice (KO) or wild-type (WT) littermate control mice were mixed with CD45.1^{+} competitor bone marrow cells from B6.SJL-*Ptprc*^{a}/BoyAiTac at 1:1 ratio and transplanted into lethally irradiated B6.SJL-*Ptprc*^{a}/BoyAiTac recipients. Bone marrow from recipients was analyzed by FACS after 4 weeks. (A) Cells were stained with antibodies against Sca1, c-kit, B220, CD41, CD105,

CD150, CD45.1 and CD45.2. Shown are two-color histograms and gating scheme used to identify the populations indicated in supplemental Figure 1. (B) Cells from recipients were stained as in panel A. Shown are two-color histograms for CD45.1 and CD45.2 expression gated on the indicated populations as shown in panel A. Numbers are the percentage of cells in the indicated gates.

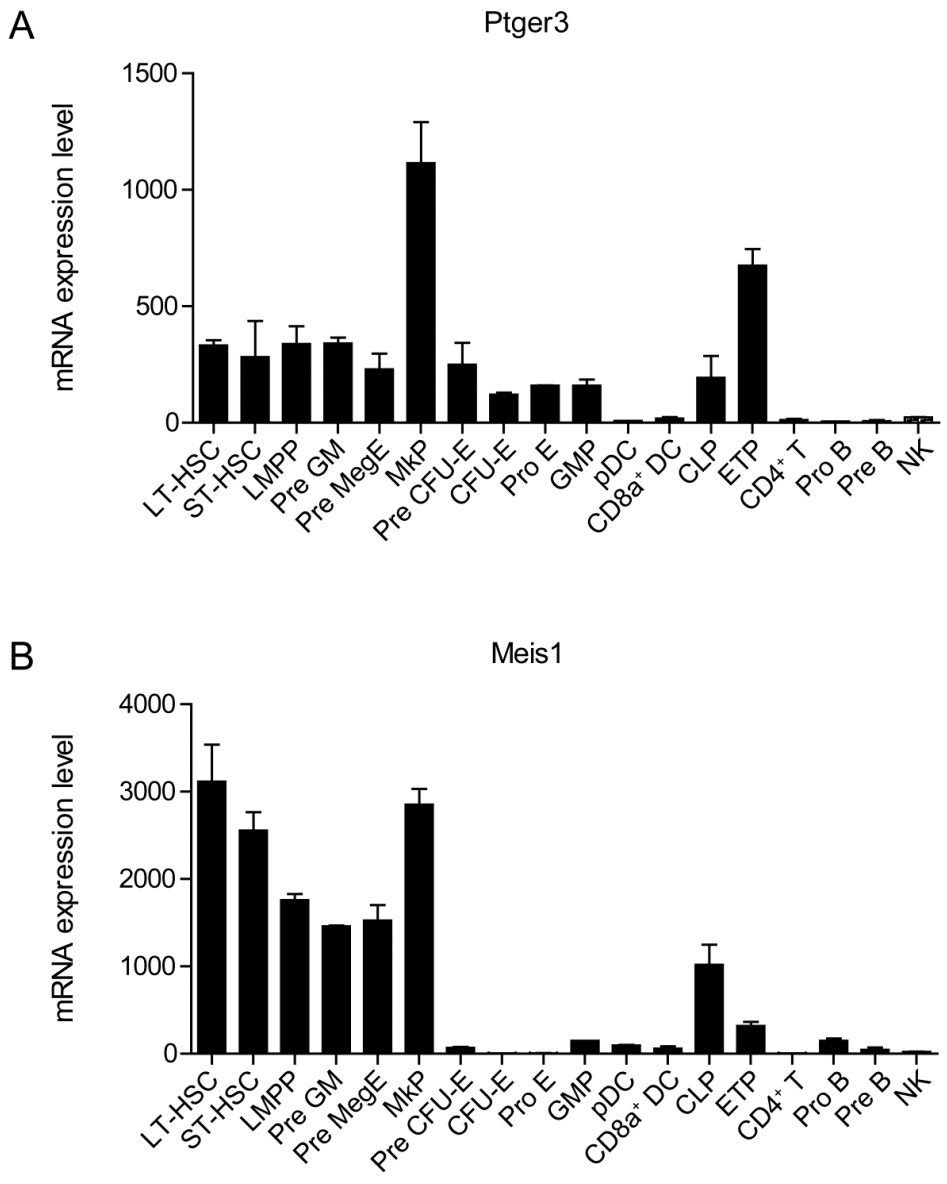

Figure 6.5 Expression pattern of *Ptger3* and *Meis1* in adult hematopoietic subsets. Expression values are shown for *Ptger3* (A) and *Meis1* (B) for each indicated cell population derived from microarray analysis.[6] Data are assembled from two to four replicate arrays.

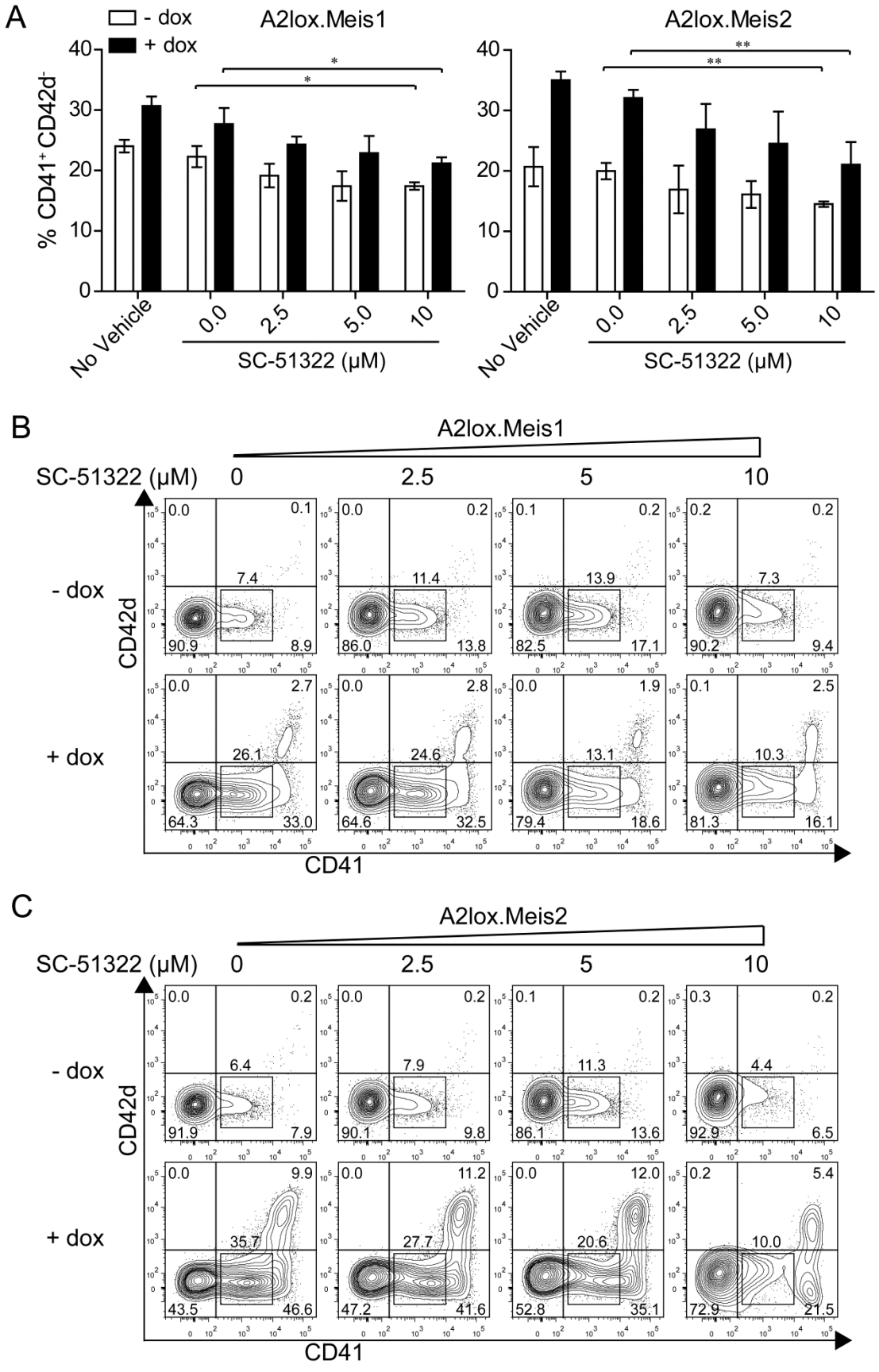
A
- dox
+ dox
A2lox.Meis1
A2lox.Meis2
% CD41+ CD42d-
No Vehicle
0.0
2.5
5.0
10
SC-51322 (μM)
B
A2lox.Meis1
SC-51322 (μM)
- dox
+ dox
CD42d
CD41
C
A2lox.Meis2
SC-51322 (μM)
- dox
+ dox
CD42d
CD41

Figure 6.6 *Meis1* and *Meis2* induce CD41^{int} hematopoietic progenitors through *Ptger3* signaling pathway. (A) A2lox.Meis1 or Meis2 ES cells were differentiated as described in Figure 5.3A in the presence of the indicated concentration of SC-51322 added on day 6 and replenished daily. On day 9 cells were harvested and analyzed by FACS for expression of CD42d and CD41. Cells are gated as GFP-negative to exclude OP9 cells. Numbers indicate the percentage of CD41^{+} CD42d^{-} cells developing in the indicated conditions from three independent experiments. Bars indicate the SD. * indicates $P<0.05$ and ** indicates $P<0.01$ from a paired students *t*-test. (B) A2lox.Meis1 ES cells were differentiated as in Figure 5.3A with the indicated concentration of SC-51322 added on day 6 and replenished each day until day 12. On day 12, cells were analyzed by FACS for expression of CD42d and CD41. Shown are data for GFP-negative cells to exclude analysis of OP9 cells. Numbers indicate the percentage of cells in the indicated gates. (C) A2lox.Meis2 ES cells were differentiated and analyzed as in panel A.

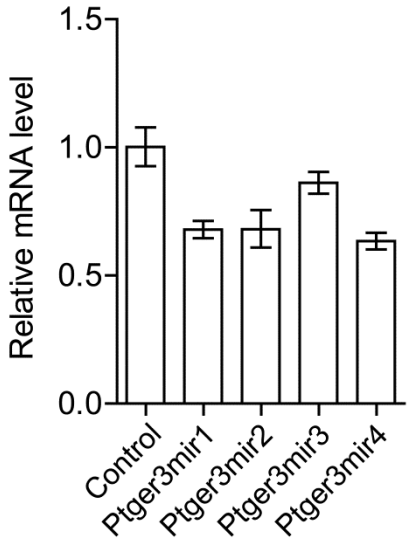

Figure 6.7 Lack of extinction of *Ptger3* mRNA after miR-based shRNA expression. A2lox ES cell lines with constitutive expression of shRNA targeting *Ptger3* were differentiated and co-cultured with OP9 as described in Figure 5.3. On day 8, ESCs with (Ptger3mir1-4) or without (Control) shRNA expression were collected and total RNA was isolated. The extent of knockdown of the *Ptger3* was assessed by quantitative RT-PCR.

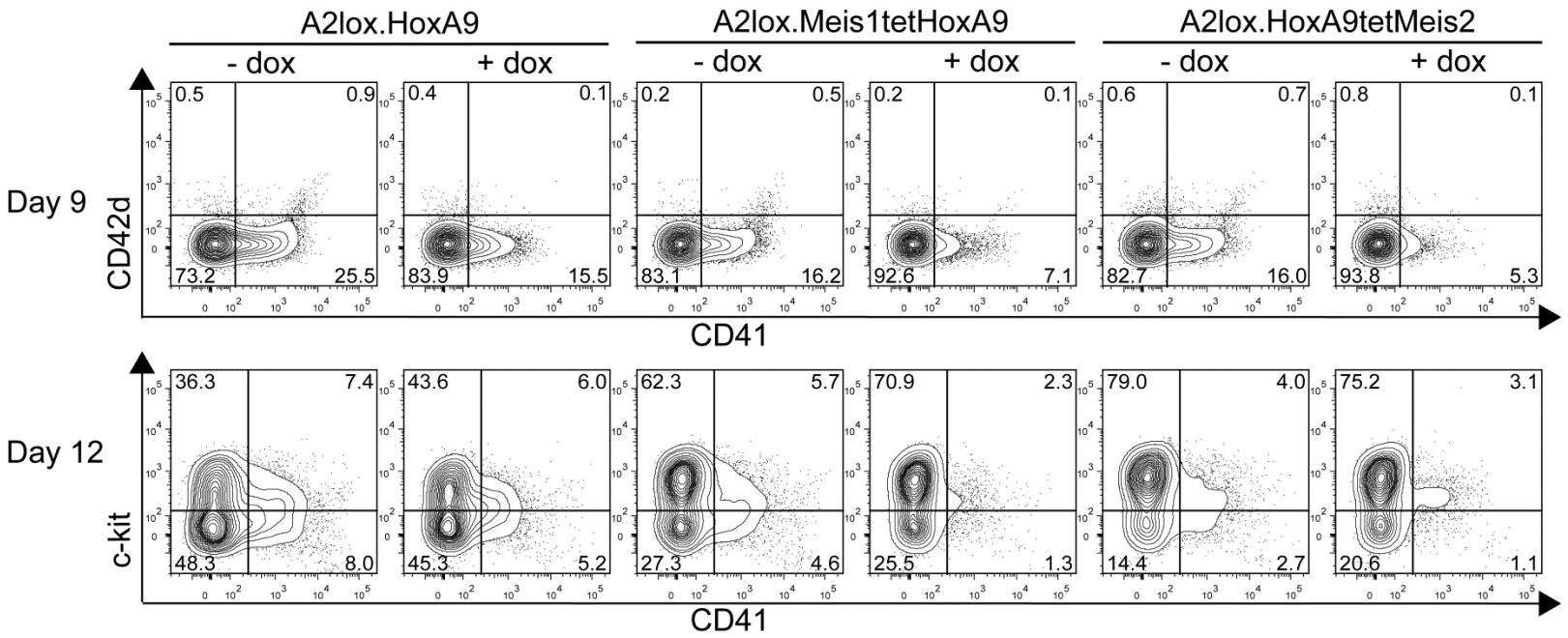

Figure 6.8 *HoxA9* is insufficient for maintaining CD41$^+$ hematopoietic progenitors differentiated from ES cells either alone or in combination with *Meis1* or *Meis2*. A2lox.HoxA9 ESCs containing dox-inducible *HoxA9*, A2lox.Meis1*tet*HoxA9 ESCs containing simultaneously dox-inducible *Meis1* and *HoxA9*, and A2lox.HoxA9*tet*Meis2 ESCs containing simultaneously dox-inducible *Meis2* and *HoxA9* were differentiated as in Figure 6.3. On day 9, cells were analyzed by FACS for CD41 and CD42d expression. On day 12, cells were analyzed by for CD41 and c-kit expression.

CHAPTER 7: DISCUSSION

Correlation of in vivo and in vitro actions of Mesp1 and Mesp2 activity

Mesp1 was cloned by subtractive hybridization from the posterior primitive streak of 7.5 day embryos.[7] *Mesp2*, a closely related homolog located near the *Mesp1* gene, was cloned by homology to *Mesp1*.[88] *Mesp1* is required for normal heart development, and *Mesp1*$^{-/-}$embryos display early lethality.[8] In *Mesp1*$^{-/-}$ mice in which β Gal is targeted to the *Mesp1* locus, β Gal-expressing cells accumulate in the primitive streak.[8] However,*Mesp1*$^{-/-}$ embryos do generate cardiac mesoderm, although their heart tubes fail to fuse, leading to cardia bifida and embryonic death.[8] *Mesp1*$^{-/-}$ embryos also show increased and prolonged expression of *Mesp2* in the primitive streak, suggesting that *Mesp2* may compensate for migratory defects, but not for heart development seen in *Mesp1*$^{-/-}$ embryos.[12] In *Mesp1*$^{-/-}$*Mesp2*$^{-/-}$ double-deficient embryos, there is an accumulation of nonmigrating cells in the primitive streak and complete failure to form cardiac mesoderm.[12] Thus, *Mesp1*and *Mesp2* may share potential transcriptional targets, consistent with finding common induction of *Snai1*, but private functions may arise from expression at distinct sites and times in the embryo, and verifying targets of *Mesp1* in vivo may require examination of *Mesp1*$^{-/-}$*Mesp2*$^{-/-}$ embryos.

Transcriptional targets of Mesp1

Our previous study identified *Snai1* and *Myocd* as early transcriptional targets of *Mesp1*, representing plausible links to EMT and cardiovascular commitment, although we have not determined whether these are direct *Mesp1* targets.[10] In this study, we sought to identify the direct downstream targets of *Mesp1* to reveal the mechanisms by which *Mesp1* acts. We found that *Nkx2.5* and *Myocd* were not direct targets of *Mesp1*

using reporter analysis. We also tested *PDGFRα*, which was induced rapidly by *Mesp1*in differentiating ESCs, and found *Mesp1* directly bound to evolutionary conserved E-boxes within the *PDGFRα* enhancer/promoter. This result suggested that *PDGFRα* could be a direct target of *Mesp1*. However, we found that *PDGFRα* was not sufficient for the induction of EMT in ESCs or the induction of $Flk1^{+}$ mesoderm, but that it may play a role rather in the survival of *Mesp1*-induced mesodermal cells. Identifying the mechanisms by which *Mesp1* acts may be relevant to understanding cardiac development and eventual applications to regenerative therapy.

Mesp1 and hematopoietic differentiation

Previous work documented primarily a cardiovascular pattern of lineage tracing, with endothelium of the dorsal aorta also being labeled, where the definitive HSCs are derived.[89] Our previous study emphasized that the effect of *Mesp1* in ES cultures was predominantly to restrict fate to the smooth muscle and cardiovascular fates. However, we clearly see labeling of mature hematopoietic lineages in a strongly mosaic pattern, an indication that some mature cells derive from HSCs with a history of significant *Mesp1* expression in their progenitors. This mosaic pattern of labeling in Mesp1-Cre mice suggests the possibility of different sources of HSCs that give rise to the labeled mature blood cells. Given recent progress in distinguishing HSCs of yolk sac from HSCs derived from AGM, it will be interesting to see if *Mesp1*-traced blood tracks the actual origin of *Mesp1*-labelled HCS that generate the labeled mature lineages in our study.

Using *Mesp1*-deficient ES cells, we showed *Mesp1* is necessary for normal hematopoietic differentiation of ESCs. In examining the downstream targets of *Mesp1* in

ESC-derived endothelial cells, we identified myeloid ecotropic viral integration site 1 (*Meis1*). Using reporter assays, we further suggested that *Mesp1* activated *Meis1* in an indirect manner.

The role of Meis1 in hematopoietic development

Meis1 was initially discovered as a common virus integration site for myeloid leukimias,[29] and much subsequent analysis has associated its actions with leukemic transformation.[37,41,90] However, the role of *Meis1* in normal hematopoiesis is not well studied. Our study identified two distinct roles of *Meis1* during normal hematopoiesis based on analysis of *Mesp1*-deficient ES cells and on analysis of overexpression of *Meis1* and *Meis2* (Figure 7.1). First, we observed a robust action of *Meis1* to maintain the proliferative state of early hematopoietic progenitors. Although previous study reported a reduction of HSC population in the fetal liver of *Meis1*$^{-/-}$ mice,[43,44] suggesting *Meis1* may play an important role in hematopoietic stem cell self-renewal, there is no direct evidence that *Meis1* acts on HSC proliferation. Our study demonstrated that *Meis1* maintains a proliferative state in CD41^{+} hematopoietic progenitors by directly measuring their BrdU incorporation, which was associated with an increase of hematopoietic progenitors accumulating in ES cell/OP9 co-cultures (Figure 5.4). Thus, our analysis extends our understanding of how *Meis1* promotes HSC self-renewal, unifying its action with those described for *Meis2* in regulating retinal progenitor cell proliferation during eye development.[47,81]

Second, our study shows that *Meis1* actively suppresses erythroid progenitor differentiation while promoting megakaryocyte progenitor development. This previously

unrecognized action is very likely to underlie the defect in platelet development that has been described as a basis for embryonic lethality in *Meis1*$^{-/-}$ mice.[45] Our *in vivo* analysis of bone marrow progenitors suggests that *Meis1* regulated the early lineage decision choice at the stage of the megakaryocyte-erythroid progenitor (MEP). In summary, our study demonstrates dual actions of *Meis1*, distinguishing its later action on lineage specification from its earlier role in HSC self-renewal.

Transcriptional targets of Meis1

In addition, our study identified novel gene targets of *Meis1* to explain its actions during normal hematopoiesis. We identified four potential candidates, *Plac8, Ptger3, Serpinb2*, and *Pf4*, based on specific and robust induction by *Meis1* in CD41^{+} hematopoietic progenitors. The platelet-specific gene *Pf4*/*Cxcl4* is a chemokine-like protein and a known target of *Meis1*.[57] We tested whether the other three candidates were sufficient or required for *Meis1*'s actions. *Plac8* reportedly supports cell proliferation and survival by decreasing p53 via the AKT-Mdm2 pathway.[84] However, we found it was neither sufficient to replace *Meis1* for promoting hematopoietic cell progenitor proliferation, nor necessary for maintaining *in vivo* hematopoietic stem cell proliferation in a mixed bone marrow chimera setting. Likewise, we found *Serpinb2* was insufficient either alone or in combination with *Plac8* for maintaining the persistence of hematopoietic progenitors in ES/OP9 co-culture as *Meis1*. Several reports have demonstrated that *Meis1* can regulate progenitor cell proliferation through influencing the expression of cell cycle components including *cyclin D1* and *cyclin D3*.[47,54,81] However, our gene expression analysis identified no cell cycle components to be substantially

altered by *Meis1* in $CD41^{+}$ hematopoietic progenitors. Instead, our study suggests a novel mechanism by which *Meis1* influences progenitor cell proliferation through inducing a receptor for the prostaglandin signaling pathway. We found that *Ptger3*, one of the four G-protein coupled receptors for prostaglandin E2, could potentially mediate increased hematopoietic progenitor proliferation, since inhibition of prostaglandin signaling blocked *Meis1*'s effect on proliferation of hematopoietic progenitors.

Our study also identified that *Meis1* robustly repressed a number of erythroid-specific genes including *Hba-a1/2* and *Gypa*, consistent with an inhibitory role in erythroid differentiation. At present, we do not distinguish between direct or indirect actions of *Meis1* on these target genes. *Meis1* might directly recruit transcriptional co-repressors to the target loci, or could induce repressors that can inhibit expression of these erythroid-specific genes. In addition, other transcription factors such as *Klf1* and *Fli1* have been reported to be critical regulators of erythroid and megakaryocyte development.[91,92] It will be interesting to examine the transcriptional hierarchy of *Meis1* and these other factors in erythroid cell and megakaryocyte development. A dissection of actions of *Meis1* between early HSC proliferation and later erythroid and megakaryocyte lineage specification should help in understanding the important functions of *Meis1* during normal hematopoiesis.

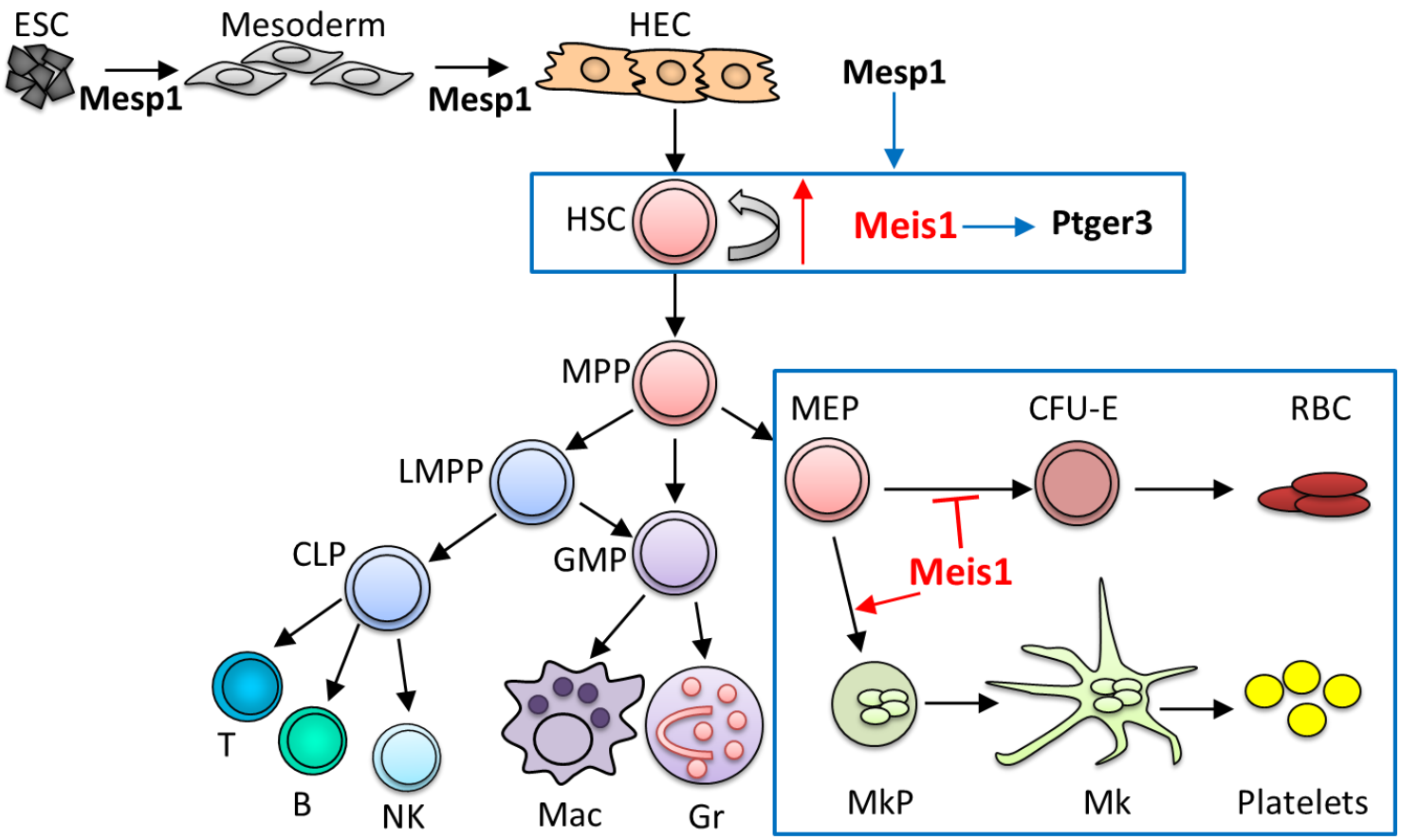

Figure 7.1 The role of *Meis1* during hematopoietic development

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