

Acceleration of mesoderm development and expansion of hematopoietic progenitors in differentiating ES cells by the mouse *Mix*-like homeodomain transcription factor

Stephen Willey, Angel Ayuso-Sacido, Hailan Zhang, Stuart T. Fraser, Kenneth E. Sahr, Matthew J. Adlam, Michael Kyba, George Q. Daley, Gordon Keller, and Margaret H. Baron

The cellular and molecular events underlying the formation and differentiation of mesoderm to derivatives such as blood are critical to our understanding of the development and function of many tissues and organ systems. How different mesodermal populations are set aside to form specific lineages is not well understood. Although previous genetic studies in the mouse embryo have pointed to a critical role for the homeobox gene *Mix-like* (*mMix*) in gastrulation, its function in mesoderm development remains unclear.

Hematopoietic defects have been identified in differentiating embryonic stem cells in which *mMix* was genetically inactivated. Here we show that conditional induction of *mMix* in embryonic stem cell–derived embryoid bodies results in the early activation of mesodermal markers prior to expression of *Brachyury/T* and acceleration of the mesodermal developmental program. Strikingly, increased numbers of mesodermal, hemangioblastic, and hematopoietic progenitors form in response to premature activation of

mMix. Differentiation to primitive (embryonic) and definitive (adult type) blood cells proceeds normally and without an apparent bias in the representation of different hematopoietic cell fates. Therefore, the mouse *Mix* gene functions early in the recruitment and/or expansion of mesodermal progenitors to the hemangioblastic and hematopoietic lineages. (Blood. 2006;107:3122-3130)

© 2006 by The American Society of Hematology

Introduction

The formation of mesoderm is a pivotal process in the normal development of major tissues and organs of the mammalian embryo, including the hematopoietic, cardiovascular, and musculoskeletal systems. During early development, mesoderm is also a crucial component of extraembryonic structures such as the yolk sac, allantois, and placenta.^{1,2} Mesoderm begins to form shortly after implantation during a process known as gastrulation.³⁻⁶ Fate maps suggest that different mesodermal derivatives arise in an orderly manner from patterning of cell populations within distinct posteroanterior regions of the streak.^{7,8} The most posterior region of the primitive streak is fated to form the extraembryonic mesoderm, while successively more anterior derivatives give rise to lateral, paraxial, and axial mesoderm. Despite intense effort, the mechanisms underlying the orderly allocation of mesoderm to its various lineages remain obscure.⁹

A number of major signaling pathways, including transforming growth factor β (TGF β), fibroblast growth factor,¹⁰ and Wnt, have been shown to play central roles during gastrulation.^{4,6} While the functional relationships among these pathways in the induction and patterning of mesoderm remain to be defined, their activities are likely to be integrated, at least in part, through the downstream activities of nuclear transcription factors. Members of the *Mix/Bix*

family of paired class homeobox genes are direct or indirect targets of the TGF β family members Nodal/activin¹¹⁻²⁵ and BMP4.^{26,27} *Xenopus Mix.1* (*Xmix.1*), the founding member of the *Mix/Bix* family, has been reported to ventralize mesoderm.²⁶ Several other *Xenopus* and zebrafish *Mix/Bix* genes have been implicated in the formation of endoderm or mesendoderm.^{16,18-20,22,24,25,28}

Although *Mix*-related genes have been identified in all eukaryotic species analyzed,²⁹ only a single *Mix* gene has been found in mouse, chick, and humans.²⁹⁻³³ The mouse *Mix-like* gene (here termed *mMix*; also known as *Mml* or *Mixl1*) is expressed in the posterior visceral endoderm prior to gastrulation and later in the primitive streak and nascent mesoderm.³²⁻³⁴ The significance of this gene in gastrulation was revealed by analysis of *mMix*-deficient mouse embryos, which display numerous mesodermal and endodermal defects and arrest by embryonic day 9.5 (E9.5).³⁵ For example, the primitive streak is enlarged, a heart tube is absent and, in some mutants, the allantois is abnormally large.³⁵

In vitro systems have provided important insights into the processes involved in germ layer formation and specification and complement genetic studies in the animal. Embryonic stem (ES) cells have gained increasing attention as an approach to exploring the induction and differentiation of mesoderm, endoderm, and

From the Departments of Medicine, Gene and Cell Medicine, and Oncological Sciences and Molecular, Cellular and Developmental Biology, Mount Sinai School of Medicine, New York, NY; Center for Developmental Biology, University of Texas Southwestern Medical Center, Dallas; and Division of Hematology/Oncology, Children's Hospital, Boston, MA.

Submitted October 17, 2005; accepted December 8, 2005. Prepublished online as *Blood* First Edition Paper, January 10, 2006; DOI 10.1182/blood-2005-10-4120.

Supported by grants from the National Institutes of Health (NIH; RO1 HL62248, DK52191, and EB02209) (M.H.B.).

S.W. and A.A.-S. contributed equally to this paper.

The online version of this article contains a data supplement.

Reprints: Margaret H. Baron, Mt Sinai School of Medicine, Box 1079, 1425 Madison Ave, Rm 11-70B, New York, NY 10029-6574; e-mail: margaret.baron@mssm.edu.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

© 2006 by The American Society of Hematology

ectoderm in culture. Under appropriate conditions, ES cells form structures known as embryoid bodies (EBs) that can differentiate along a number of distinct lineages representing each of the 3 embryonic germ layers.³⁶ The temporal appearance of hematopoietic and endothelial progenitors and activation of mesodermal, hematopoietic, and endothelial genes in developing EBs has been well characterized and mimics that observed during normal embryogenesis.³⁷⁻⁴⁰ Indeed, cells with the properties of the hemangioblast, a common progenitor for hematopoietic and endothelial cells, were first identified in the ES cell system^{37,38} and were later shown to be present in gastrulating mouse embryos.⁴¹

During the differentiation of ES cells to EBs, activation of *mMix* begins just prior to the appearance of hemangioblasts and precedes formation of primitive and definitive hematopoietic cell types.³⁴ *Mix* is expressed in a population of Brachyury/T⁺Flk1⁺ cells from EBs that contain hemangioblasts³⁴; conversely, hemangioblasts are enriched in *mMix*-expressing cell populations.²⁷ A function for *mMix* in blood cell development is strongly suggested by the recent demonstration of hematopoietic defects in *mMix*-deficient embryoid bodies.²⁷

To evaluate the response to *mMix* expression during the earliest stages of ES cell differentiation, we have generated ES cell lines in which *mMix* can be rapidly induced with doxycycline (DOX). Here we report that early up-regulation of *mMix* accelerates the mesoderm developmental program. The numbers of mesodermal, hemangioblastic, and hematopoietic progenitors are significantly increased, as evidenced by formation in clonogenic assays of transitional, blast, and hematopoietic colonies. Moreover, analysis of marker gene and cell surface protein expression and colony and cell morphologies indicates that these progenitors differentiate normally. We propose that the mouse *Mix* gene functions early in the recruitment and/or expansion of mesodermal progenitors to the hemangioblastic and hematopoietic lineages.

Materials and methods

Generation of plox-mMix targeting vector and inducible mMix ES cell lines

A FLAG-tagged mMix cDNA²⁹ was subcloned into the plox targeting vector⁴² to create plox-mMix, which was coelectroporated with pSalk-Cre into Ainv 15 ES cells.⁴² Drug-resistant targeted clones were identified by Southern blotting and polymerase chain reaction (PCR). Clonal isolates were cultured in the presence or absence of 1 μ g/mL DOX (Sigma, St Louis, MO; no. D9891) for 48 hours, and cell lysates were analyzed by immunoblotting^{34,43} using an antibody against the FLAG epitope (anti-FLAG M2; Sigma; no. 3165). ES cell clones were expanded and differentiated essentially as described.⁴⁴ Transgene expression was induced 24 hours after plating in EB culture (day 1) by the addition of DOX to a final concentration of 0.1 μ g/mL. The medium was changed on day 3, and fresh DOX was added. The experiments included in this report were repeated from 2 to 5 times.

RNA preparation and RT-PCR

Total cellular RNA was prepared from 0.5×10^7 to 1.0×10^7 cells using TRIzol (Invitrogen, Carlsbad, CA; no. 15596). To eliminate contaminating genomic DNA, the initial RNA pellet was resuspended in ribonuclease (RNase)-free deionized water and incubated with deoxyribonuclease (DNase) I (2 to 4 U/ μ L; Roche, Indianapolis, IN; no. 776-785) for 30 minutes at 37°C in the buffer supplied by the manufacturer. The sample was then extracted with phenol-chloroform and precipitated in ethanol in the presence of glycogen carrier.⁴⁵ Conditions for radiolabeled, semiquantitative reverse transcription (RT)-PCR and primer sequences were as de-

scribed.^{43,46,47} Additional primer sequences (annealed at 55°C to 58°C) were as follows: T (forward): 5'-GTCTTCTGGTTCTCCGATGT-3' (545 bp product), T (reverse): 5'-CTACTCTAAGGCAACAAGGG-3'; c-kit (forward): 5'-TGCTCTCCAGTTCCCTGC-3' (765 bp product), c-kit (reverse): 5'-TTCAGGGACTCATGGGCTCA-3'; Rex1 (forward): 5'-GGTGCGAGAAGAGCTTTATTC-3' (438-bp product), Rex1 (reverse): 5'-CTGCCAAAGTTGGCCATTC-3'.

For each experiment, controls were performed in which reverse transcriptase was omitted from the cDNA reaction mixture and template DNA was omitted from the PCR mixture. For quantitative real-time PCR (QRT-PCR), gene-specific primers and 5'-FAM (carboxyfluorescein)-Black Hole Quencher (FAM:BHQ) probe(s) (Table S1, available on the *Blood* website; see the Supplemental Table link at the top of the online article) corresponded to 3'-untranslated regions. They were designed using Primer3 software⁴⁸ and synthesized at the Hartwell Center at St Jude Children's Research Hospital (Memphis, TN). Briefly, 1 to 5 μ g RNA was converted into cDNA, and a series of diluted samples were used for 40-cycle PCR in TaqMan Universal PCR Master Mix (containing AmpliTaq Gold enzyme; ABI; no. 4304437) in an ABI (Perkin-Elmer-Applied Biosystems, Foster City, CA) Prism 9700 instrument. Reactions (20 μ L total) contained 1 μ L cDNA, 6 μ M each primer, and 4 μ M probe and were run using the default ABI 7900 program. To generate a standard curve for comparison of mRNA levels in different samples, multiple dilutions of the control cDNA sample, spanning at least 3 orders of magnitude, were prepared. The equation describing the plot of threshold cycle, C_t , versus log concentration was used to determine relative amounts of mRNA in experimental samples. Using the optimized conditions and threshold values, individual samples were analyzed in triplicate using the probe of interest and an internal control expected to be unchanged between samples. Three different internal controls were used: glyceraldehyde-3-phosphate-dehydrogenase (*Gapdh*), glucose phosphate isomerase-1 (*Gpi-1*), and metallothionein-2 (*Mt-2*). From the C_t values, the relative transcript concentration was calculated and normalized to that of the internal control. The maximum expression data point was adjusted to 100. Data are shown for samples normalized to *Gapdh*, but results were comparable when analysis was performed using *Gpi-1* or *Mt-2* alone or a combination of all 3 controls.

Assays for hematopoietic progenitors and blast colonies

Blast and transitional colony assays⁴⁴ and clonogenic assays for primitive erythroid progenitors^{43,45} were performed as described previously. Definitive hematopoietic progenitors were assayed in Methocult (StemCell Technologies, Vancouver, BC, Canada).⁴⁵ Cytospin preparations and May-Grünwald Giemsa staining were as described.^{43,45}

Fluorescence activated cell sorting analysis

At the indicated times in culture, EBs were dispersed using 0.05% trypsin/EDTA (Gibco Invitrogen, Grand Island, NY). Single-cell suspensions containing 1×10^5 to 3×10^5 cells were stained with antibodies in phosphate-buffered saline (PBS) containing 3% FCS and 0.1% sodium azide. Antibodies used were biotinylated anti-rat Flk1 (subclone M218) and phycoerythrin (PE)-conjugated anti-c-kit (clone 2B8; BD Pharmingen, San Diego, CA; no. 553355). Flk1 monoclonal antibody was affinity purified using protein G-Sepharose and biotinylated using the Fluoreporter Biotin-XX Protein Labeling Kit (Molecular Probes, Eugene, OR; no. F2610). Flk1 expression was detected using allophycocyanin (APC)-conjugated streptavidin (BD Pharmingen). Flow cytometry was performed using a FACScalibur instrument (Becton Dickinson [BD], San Jose, CA). The cytometer was calibrated against unstained and single stained cells. Dead cells were excluded by forward and side scatter or according to propidium iodide uptake.

Immunocytochemistry

Single-cell suspensions of embryoid bodies were prepared by treatment with trypsin. Cells were plated on gelatin-treated coverslips and fixed in paraformaldehyde (1% in PBS) for 10 minutes and permeabilized in methanol for 30 minutes prior to incubation in blocking solution (PBS

containing 1% bovine serum albumin; Sigma; no. A9647). Anti-FLAG M2 mouse monoclonal antibody (Sigma; no. F3165) was used at a concentration of 5 $\mu\text{g}/\text{mL}$. Alexa Fluor 488–conjugated rabbit antimouse secondary antibody (Molecular Probes; no. A11122) was used at a dilution of 1:500 in blocking solution. Coverslips were mounted on microscope slides using Vectashield Mounting Medium with Dapi (Vector Laboratories, Burlingame, CA; H-1200).

Microscopy

Fluorescently labeled cells were visualized using a Zeiss Axioplan-2 microscope with a 40 \times Plan-NEOFLUAR/0.75 NA objective and appropriate filter sets (Chroma Technology, Rockingham, VT). Images were captured with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI). Bright-field images of embryoid bodies were acquired in AxioVision (Carl Zeiss Microsystems, Thornwood, NY) using an Axiocam MRC camera mounted on a Zeiss Axiovert 25 microscope or a Leica MZ12 stereo dissecting microscope.

Results

Transient expression of *mMix* early in differentiating ES cells

Under appropriate culture conditions, murine ES cells form “embryoid bodies” (EBs) whose development recapitulates a number of aspects of early embryonic development, including differentiation along mesodermal, endodermal, and ectodermal lineages.³⁶ We had previously reported that *mMix* is transiently expressed in embryoid bodies derived from the CCE ES cell line.³⁴ In preparation for generation of the inducible system discussed in the next section, which is based on the E14 line of ES cells,⁴² we also examined the time course of expression of *mMix* and other genes in differentiating E14 EBs. The patterns of transcription in E14 EBs were very similar to those observed for CCE EBs. For example, *Fgf4*, which encodes a growth factor that plays a role in epithelial-mesenchymal interactions throughout development, is expressed in the inner cell mass (ICM) of the blastocyst, in ES cells, in the primitive streak of the gastrulating embryo and, later, in certain mesodermal populations.⁴⁹ In differentiating EBs, levels of *Fgf4* mRNA decrease steadily as ES cells (day 0 in Figure 1A) differentiate. Transient expression of *mMix* and *T* was detected

early in embryoid body differentiation³⁴ (Figure 1A and data not shown; note peak at days 3 and 4). The initiation of *Flk1* transcription overlapped with peak *mMix* and *T* expression (day 3) and continued after the down-regulation of these genes. Progenitors for primitive and definitive hematopoietic cell types formed by day 6 and days 8 to 10, respectively (not shown). Therefore, *mMix* is turned on just prior to and peaks around the time of activation of *Flk1* and formation of cells with hematopoietic and endothelial potential. It is silenced before their differentiation in EBs.

Conditional expression of *mMix* in ES cell–derived embryoid bodies

Gain-of-function studies have been a classical approach in developmental biology to understand gene function and complement loss-of-function studies. Using a system developed for DOX-inducible expression of exogenous genes in differentiating ES cells,⁴² we generated a panel of ES cell lines (here termed *i-Mix*) in which FLAG-tagged *mMix* is induced in response to DOX via activation of a reverse tet-transactivator protein (rtTA; Figure 1B). This system allows consistent and reversible induction of a conditional reporter, and the uninduced cells provide an ideal control.⁴² Because all cell lines are derived by Cre-mediated recombination of the cDNA of interest into the same locus,⁴² position effects are not a problem. Candidate *i-Mix* lines were screened for inducible expression of FLAG-*mMix* protein (see “Materials and methods”). Three clones (termed 1, 2, and 10) expressed the transgene and displayed similar levels of FLAG-*mMix* protein expression, growth and induction kinetics, and expression of cell surface *Flk1* and *c-kit* (Figure 5). Clone 2 was chosen for further analysis. As shown in Figure 1C–D, FLAG-*mMix* protein was detected in the presence but not in the absence of DOX and could be detected at DOX concentrations as low as 0.025 to 0.10 $\mu\text{g}/\text{mL}$ (Figure 1C). Expression of FLAG-*mMix* protein was nuclear (Figure 1D), as expected from our earlier studies.³⁴ Subsequent studies were performed using DOX at 0.1 $\mu\text{g}/\text{mL}$.

Mesoderm formation is accelerated in response to *mMix*

As a first step in examining the events following premature activation of *Mix*, a time course experiment was performed in which *i-Mix* ES cells were plated in the absence of leukemia inhibitory factor (LIF) or feeder cells. DOX was added to half of

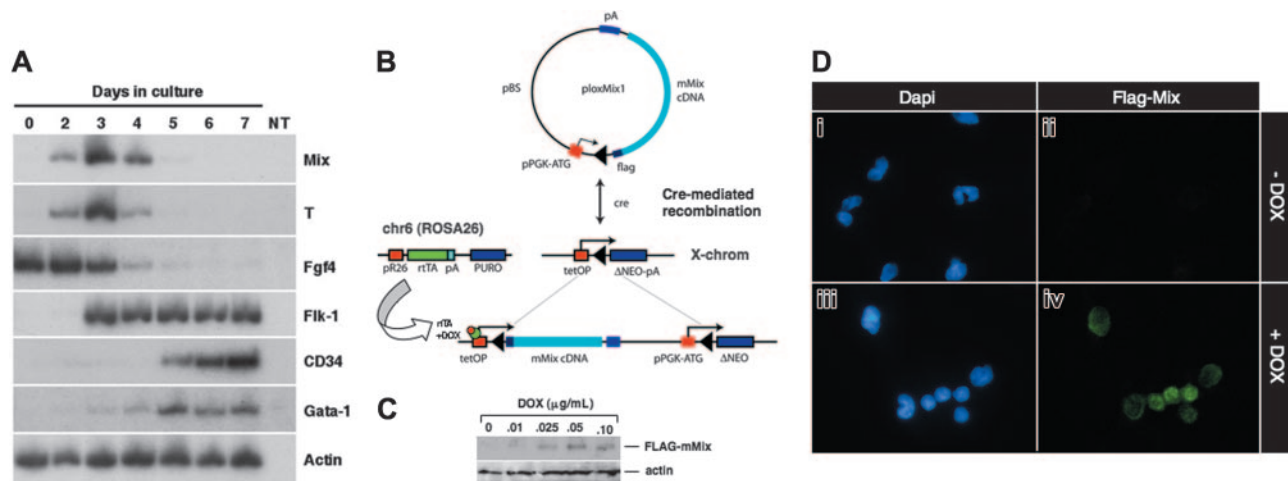


Figure 1. Mouse *Mix* is activated early during ES cell differentiation and is conditionally expressed in *i-Mix* ES cell lines. (A) RT-PCR analysis of *mMix* expression in EBs (E14 line). Controls (-RT, -DNA) were routinely performed. NT indicates minus template control. (B) Strategy for conditional expression of *mMix* in ES cells. The vector plox-Mix1, encoding amino-terminal FLAG-tagged *mMix*, was inserted into the X chromosome using Cre-mediated recombination.⁴² In the presence of DOX, rTA protein expressed from the ROSA26 locus on chromosome 6 (chr6) can bind to a tetracycline operator (tetOP), and expression of *mMix* is activated. (C) Western blot analysis of 1 of the 3 *i-mMix* ES cell lines (clone 2) cultured at different concentrations of DOX. Actin was used as a loading control. (D) Nuclear expression of FLAG-tagged *mMix* protein in cells from induced *i-Mix* EBs. Magnification, $\times 40$.

the cultures 24 hours after plating (day 1.0), and EBs were harvested at 3- to 4-hour intervals beginning at day 2.0 after plating of ES cells (shown schematically in Figure 2A). Total cellular RNA was isolated and analyzed by semiquantitative RT-PCR (Figure 2B). Samples were normalized for expression of β -actin.^{43,46,47}

As anticipated, the zinc finger transcription factor *Rex1* was down-regulated both in the presence and absence of DOX. In DOX-induced EBs, down-regulation of *Rex1* was slightly delayed (Figure 2B [compare lanes 2-5, -DOX, with lanes 12-15, +DOX] and Figure 2C; see "Discussion"). In contrast, conditional activation of *mMix* resulted in premature induction of the mesodermal program of development, as reflected in the early activation of the pan-mesodermal marker *T* (Figure 2B, compare lanes 3-6 with lanes 13-17).

In light of our previous finding³⁴ that *mMix*, *T*, and *Flk1* are coexpressed in a subpopulation of EB cells in which a green fluorescent protein (*Gfp*) gene was targeted to the *T* locus,⁴⁰ we assayed for *Flk1* (an early hemangioblast marker) and *c-kit*, a marker of hematopoietic stem cells that is also expressed, at low levels, on EB cells with hemangioblast potential.⁴⁰ Activation of *Flk1* and *c-kit* was also accelerated (Figure 2B, compare lanes 2-7 with lanes 12-17). *c-kit* is expressed in undifferentiated E14 ES cells (as we expected; Figure 2B, lane 1⁴⁰). Upon initiation of differentiation, *c-kit* was transiently down-regulated (Figure 2B, lanes 2 and 3) and was activated again around days 2.6 to 3.0 (Figure 2B, lanes 4 and 5). In the presence of DOX, *c-kit* expression was easily detected as early as day 2 (Figure 2B, compare lanes 2-4, -DOX, with lanes 12-14, +DOX); whether down-regulation of *c-kit* was blocked between 24 and 48 hours by early induction of *Mix* was not determined. *T*, *Flk1*, and *c-kit* were also down-regulated more rapidly at later stages of EB differentiation in the presence than in the absence of DOX (Figure 2B,

compare lanes 10 and 11 with lanes 20 and 21), suggesting that conditional activation of *mMix* resulted in acceleration of mesodermal development.

Mesodermal gene activation following induction of *i-Mix* was examined more systematically using quantitative real-time PCR (QRT-PCR). For these studies, expression was normalized to *Gapdh* (Figure 2C), *Gpi-1*, and/or *Mt-2* (see "Materials and methods"). Delayed down-regulation of *Rex1* in response to *mMix* was again observed (Figure 2C), confirming the semiquantitative RT-PCR result (Figure 2B). Four genes that are expressed in the primitive streak were all activated prematurely in the induced *i-Mix* EBs: *gsc*,⁵⁰ *T*,^{51,52} endogenous *mMix*,^{32,34} and *Wnt3a*.^{53,54} *Goosecoid* (*gsc*), which in the mouse embryo is first expressed early in gastrulation, in the emerging primitive streak,⁵⁰ was activated shortly after induction of *mMix* and nearly 24 hours earlier than in uninduced EBs (Figure 2C). In the frog, *gsc* is a direct target of *XMix.1*²⁸; its rapid activation in response to *mMix* suggests that it may be a direct *Mix* target in the mouse embryo as well. Transient expression of *T*, endogenous *mMix*, and *Wnt3a* followed activation of *gsc* (Figure 2C). Markers of several types of embryonic mesoderm were also activated earlier in the induced *i-Mix* EBs (Figure 2C): lateral (*Twist*⁵⁵), axial (*EphA4* and *Foxa2/Hnf3 β* ⁵⁶⁻⁵⁹), paraxial (*Tbx-6* and *Mox1*^{60,61}), and cardiac (*Nkx2.5*⁶²). These mesodermal genes were activated in an orderly fashion, following up-regulation of *gsc*, *T*, and endogenous *mMix*.

Accelerated and enhanced activation of hemangioblastic and hematopoietic developmental programs

During our initial characterization of the *i-Mix* ES cell lines, we noticed that the DOX-treated EBs became brightly pigmented by days 6 and 7 (reflecting hemoglobin production in erythroid cells),

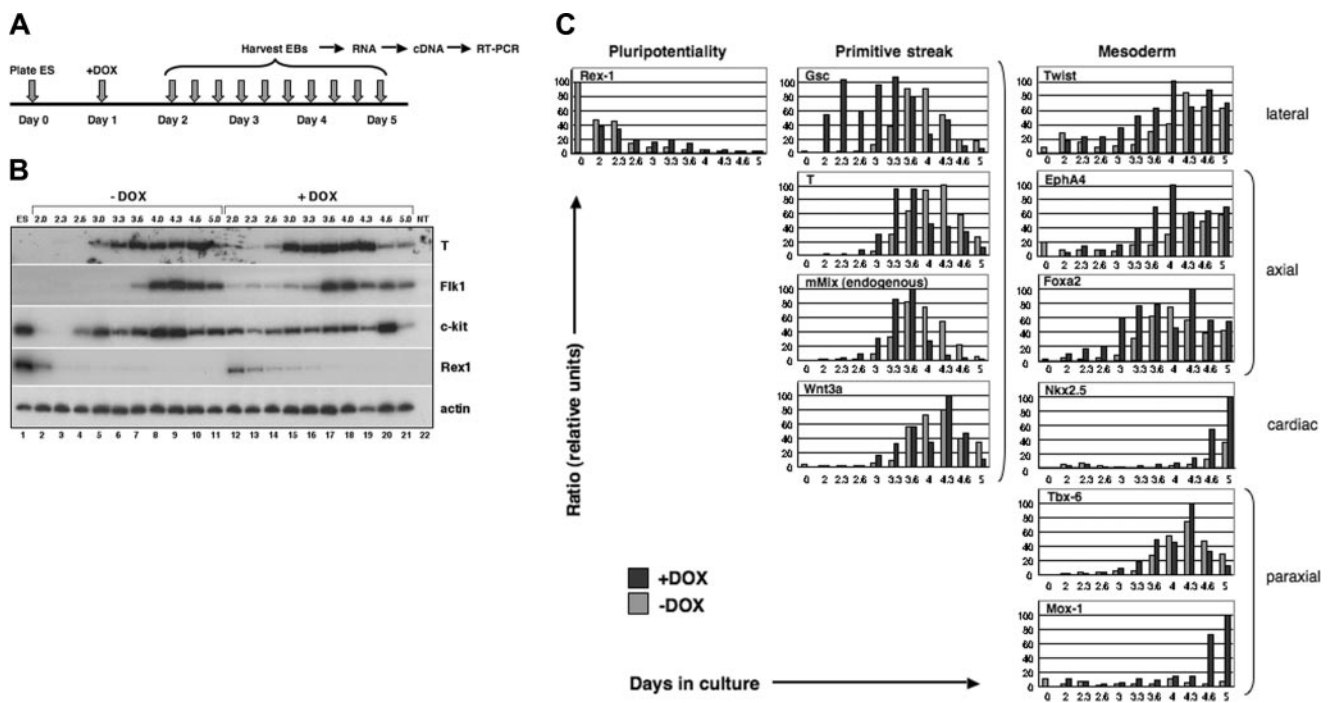


Figure 2. The mesodermal developmental program is accelerated in response to *mMix*. (A) General experimental protocol for induction and analysis of *i-Mix* EBs cultured with or without DOX. DOX (0.1 μ g/mL) was added on day 1 after plating under differentiation conditions. (B) Semiquantitative RT-PCR analysis of gene expression in *i-Mix* EBs cultured with or without DOX. EBs were harvested on days 2.0 (lanes 2, 12), 2.3 (lanes 3, 13), 2.6 (lanes 4, 14), 3.0 (lanes 5, 15), 3.3 (lanes 6, 16), 3.6 (lanes 7, 17), 4.0 (lanes 8, 18), 4.3 (lanes 9, 19), 4.6 (lanes 10, 20), and 5.0 (lanes 11, 21). NT (lane 22) indicates minus template control. Expression of *T* was transient, preceding activation of *Flk1* and *c-kit*. The differentiation program in DOX-induced *i-Mix* cells was accelerated (see text). (C) Quantitative real-time RT-PCR analysis of primitive streak and early mesodermal gene expression in *i-Mix* EBs cultured with or without DOX. Expression was normalized to that of a housekeeping gene (for this figure, *Gapdh*) and expressed as a ratio (see "Materials and methods").

at least 24 hours earlier than untreated EBs (Figure 3). By day 8, the uninduced EBs showed equivalent pigmentation. Therefore, we investigated the formation of hemangioblastic and hematopoietic mesoderm in greater detail.

Expression of a panel of markers for hemangioblast (Figure 4, left panels) and hematopoietic (Figure 4, right panels) development was examined using QRT-PCR. Early activation of *Flk1* and *c-kit* was again detected in this analysis. *Runx1* is expressed in yolk sac mesoderm prior to the formation of blood islands and in EB-derived blast colony-forming cells (BL-CFCs), the in vitro equivalent of the hemangioblast.⁶³⁻⁶⁵ It encodes the DNA-binding subunit of a core binding factor transcriptional regulator,⁶⁶ is a marker of long-term repopulating hematopoietic stem cells,⁶⁷ and is essential for hematopoietic commitment of hemangioblasts.⁶⁵ *Scf/tal*, a member of the basic helix-loop-helix family of transcription factors, functions somewhat later than *Runx1* or *Flk1*.^{68,69} *Flk1* and *Tal1* are thought to act in a combinatorial manner to regulate cell fate choice in the early embryo to the hematopoietic, endothelial, and smooth muscle lineages.⁶⁸ All 4 genes (*Runx1*, *Flk1*, *c-kit*, and *Scf/tal*) were prematurely activated in response to induction of *mMix*. Two homeobox genes that play pivotal roles early in hematopoiesis, *Cdx4* and *Hoxb4*, were also induced by *mMix* (Figure 4). *Cdx4*, a caudal class homeobox gene, regulates the specification of hematopoietic (but not endothelial) progenitors.⁷⁰ *Hoxb4* has been implicated in hematopoietic stem cell proliferation⁷¹⁻⁷⁴ and confers engraftment potential on ES and yolk sac progenitor cells.⁴² In keeping with its demonstrated genetic position with respect to *Hoxb4*,⁷⁰ activation of *Cdx4* preceded that of *Hoxb4* in induced *i-Mix* EBs (Figure 4). *Gata1* encodes a zinc finger transcription factor that is essential for normal erythroid development,⁷⁵ while SY-globin (*Hbb-y*) is a marker of differentiated primitive erythroblasts. Both genes were activated much earlier and to equivalent (if not higher) levels when *i-Mix* EBs were cultured in the presence rather than in the absence of inducer (Figure 4), consistent with the observed acceleration of hemoglobin pigmentation in DOX-treated EBs (Figure 3). In agreement with these data, a cDNA microarray analysis of an independent set of induced and uninduced *i-Mix* EBs revealed that *Gata1*, *eY-globin*, *βmaj-globin*, *α-globin*, and *Pecam-1* (the last of which is expressed

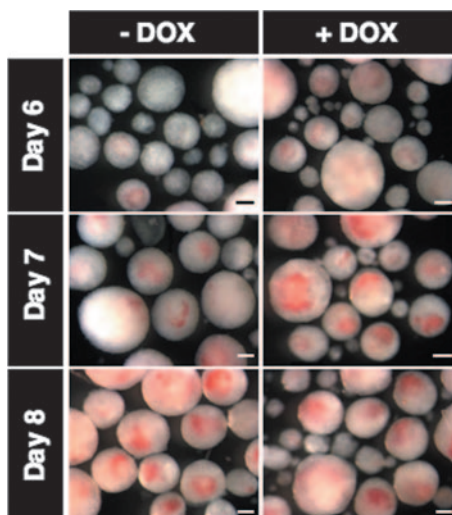


Figure 3. Development of hemoglobin pigment is accelerated in response to *mMix*. Representative photomicrographs are shown for *i-Mix* EBs cultured for 6, 7, or 8 days in the presence (+DOX) or absence (-DOX) of doxycycline. Scale bar, 200 μ m.

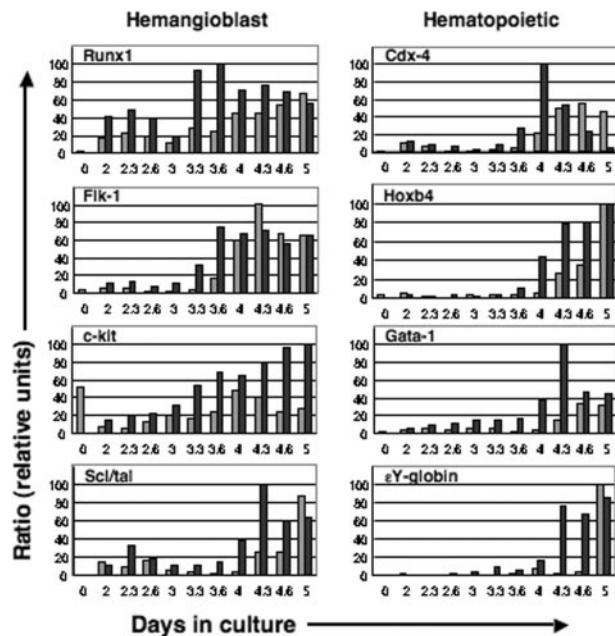


Figure 4. Activation of the hemangioblastic and hematopoietic developmental programs is accelerated and enhanced in response to *mMix*. Quantitative real-time RT-PCR analysis of gene expression in *i-Mix* EBs cultured with (■) or without (□) DOX. Markers of the hemangioblast and genes involved in hematopoiesis were activated in response to induced *mMix*. Expression was normalized to that of a housekeeping gene (for this figure, *Gapdh*) and expressed as a ratio. For additional details, see Figure 2.

on hematopoietic stem cells, erythroid and endothelial progenitors, and mature endothelial cells⁷⁶) were all transcribed at significantly higher levels in response to conditional activation of *mMix* (day 4; M.A. Dyer, S.W., K.E.S. and M.H.B.; unpublished data, June 2004).

Accelerated and enhanced formation of *Flk1*⁺ and *c-kit*⁺ cell populations

The premature activation of *Flk1* and *c-kit* genes in response to induced *mMix* led us to evaluate the cell surface expression of *Flk1* and *c-kit* protein using flow cytometry (fluorescence-activated cell sorting [FACS]). In DOX-treated EBs, accelerated and enhanced formation of 3 populations of cells was observed, beginning on day 2 (24 hours after addition of DOX; not shown) and most evident on day 3 (48 hours after DOX) (Figure 5; Table 1): (i) *Flk1*⁺ (about 4-fold increase in DOX-treated versus untreated EBs); (ii) *Flk1*⁺*c-kit*⁺ (more than 4-fold increase); and (iii) *c-kit*^{hi} (about 8-fold increase). Between days 3 and 5, a dramatic decrease in the number of *Flk1*⁺ cells was observed in DOX-treated EBs (from about 11% to about 3%), whereas this population continued to expand in the uninduced EBs (from about 3% to about 10%). During this period, in both induced and uninduced EBs, increased numbers of *Flk1*⁺*c-kit*⁺ (about 7% to about 13% +DOX; 0.9% to about 10% -DOX) and *c-kit*^{hi} (about 23% to about 31% +DOX; about 5% to about 10% -DOX) cell subsets were found. Therefore, in response to induction of *mMix*, formation of all 3 populations occurred more rapidly, with the most dramatic acceleration measured for *Flk1*⁺ single-positive cells. The *Flk1*⁺ and *c-kit*^{hi} populations have been shown to have hemangioblastic and hematopoietic potentials, respectively.^{39,77} The *Flk1*⁺*c-kit*⁺ double-positive cell population has hematopoietic potential (Marion Kennedy and G.K., manuscript in preparation).

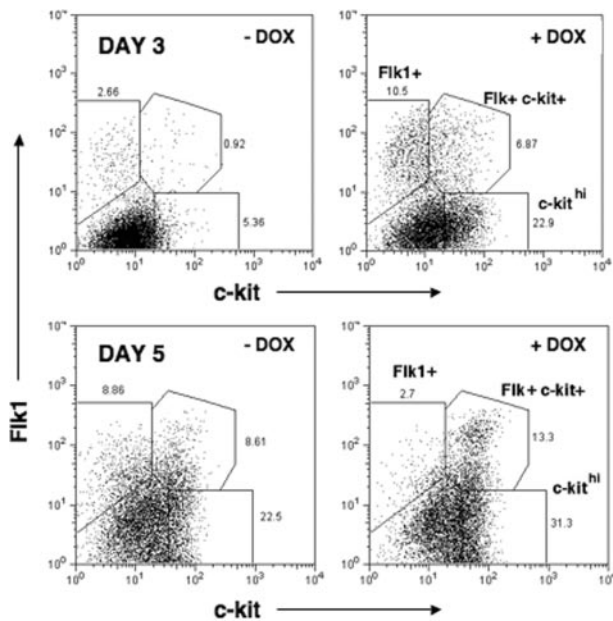


Figure 5. Formation of Flk1⁺ and c-kit⁺ cell populations is accelerated and enhanced in response to mMix. FACS analysis of cell surface Flk1 and c-kit protein expression in i-Mix EBs cultured in the presence or absence of DOX for 3 or 5 days (DOX added on day 1). Single-cell suspensions were labeled with anti-Flk1 and -c-kit sera and analyzed by FACS. Parental (wild-type) ES and uninduced (-DOX) i-Mix cells produced comparable numbers of Flk1⁺, c-kit^{hi}, and Flk1⁺c-kit⁺ double-positive cells (not shown). This experiment was repeated 3 times with similar results.

Mesodermal, hemangioblastic and hematopoietic potentials of i-Mix EBs

To evaluate the mesodermal, hemangioblastic, and hematopoietic potentials of i-Mix EBs, single-cell suspensions were prepared from EBs harvested after 3, 4, or 5 days and replated in methylcellulose under blast colony or hematopoietic conditions. Colonies were scored on the days indicated (Figure 6). Very strikingly, over all times in culture evaluated (Figure 6 and data not shown), progenitors for each of the hematopoietic lineages formed in consistently larger numbers in the DOX-induced than in uninduced EBs. Therefore, not only was activation of the hematopoietic program accelerated in response to mMix, but the numbers of progenitors were expanded. Transitional mesodermal progenitors of hemangioblasts³⁸ and blast hemangioblast³⁷ colonies formed earlier and in larger numbers in DOX-treated versus untreated secondary cultures (Figure 6A). These colony types were much less abundant by day 5, reflecting their development to hematopoietic and endothelial stem/progenitor cells.^{37,38,40} Both primitive (erythroid [EryP]; megakaryocyte) and definitive hematopoietic progeni-

Table 1. Quantitation of FACS analysis of Flk1 and c-kit expression on cells from i-Mix EBs cultured with or without DOX

	-DOX, %	+DOX, %	Ratio, +DOX/-DOX
Day-3 EBs			
Flk1 ⁺	2.7	10.5	3.9
c-kit ^{hi}	5.4	22.9	4.2
Flk1 ⁺ c-kit ⁺	0.9	6.9	7.7
Day-5 EBs			
Flk1 ⁺	8.9	2.7	0.3
c-kit ^{hi}	22.5	31.2	1.4
Flk1 ⁺ c-kit ⁺	8.6	13.3	1.5

The percentages of cells in each of the 3 populations gated in Figure 4 are displayed here with the ratio (+DOX/-DOX).

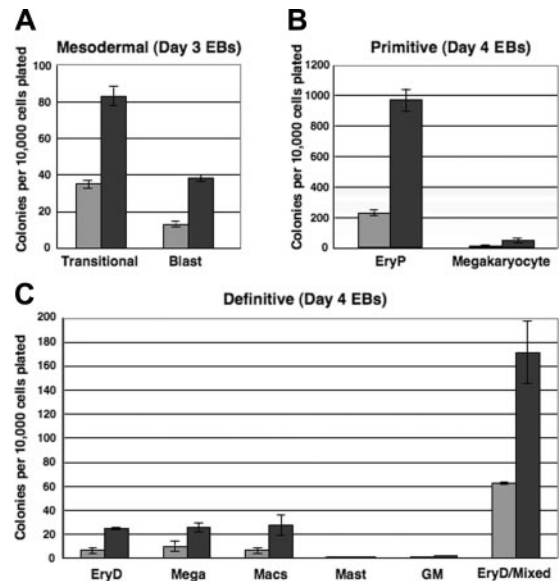


Figure 6. Mesodermal and hematopoietic progenitor potentials of ES cells are increased in response to mMix. i-Mix EBs were cultured in the presence (■) or absence (□) of DOX, harvested on the indicated days, and dispersed to single-cell suspensions. Cells were plated in methylcellulose progenitor assays in triplicate, and colonies were scored as indicated. Colony counts are expressed as mean ± SEM per 10 000 cells plated. (A) Mesodermal progenitors (transitional and blast colonies). EBs were harvested on days 3 and 4 and plated in methylcellulose blast colony cultures. Colonies were scored after 4 days. (B) Primitive erythroid and megakaryocyte colonies. EBs were harvested on day 4 or 5, plated in primitive hematopoietic progenitor cultures, and colonies were scored after 4 or 6 days. (C) Definitive hematopoietic colonies. EBs were harvested on day 4 or 5, plated in Methocult, and scored on day 8 or 10.

tors were found in higher numbers in the DOX-induced cultures (Figure 6B-C). Thus, formation of mesodermal, hemangioblastic, and primitive and definitive hematopoietic progenitors reflected the gene and cell surface protein expression patterns observed for the i-Mix EBs. The primitive and definitive erythroid colonies (EryP and EryD, respectively) that formed after replating of both induced and uninduced EB cells were red, as expected. Conditional activation of mMix did not appreciably alter hematopoietic differentiation: The primitive and definitive colonies from DOX-treated and untreated EBs were morphologically identical.

To determine whether conditional activation of mMix influences cell fate decisions within the hematopoietic lineage, the data from Figure 6B-C were replotted in stacked column form to emphasize the representation of each lineage among colonies scored (Figure 7). The frequencies of hematopoietic colony types were very similar for induced and uninduced EBs, indicating that conditional expression of mMix does not bias development toward specific hematopoietic lineages.

Discussion

Gastrulation is a complex process in which changes in the adhesive and migratory properties of cells within the epiblast lead to the ingression of ectodermal cells through the primitive streak and their eventual recruitment to the mesodermal germ layer. The mouse *Mix-like* gene is essential for normal gastrulation,³⁵ though its specific function(s) in this process remains to be worked out. The embryonic stem (ES) cell differentiation system recapitulates a number of aspects of early mouse embryogenesis, including formation of a variety of mesodermal lineages under appropriate

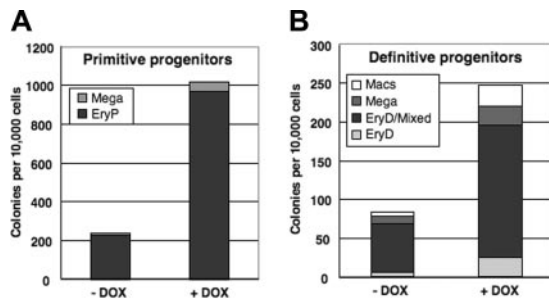


Figure 7. Conditional activation of *mMix* does not alter cell fate decisions within the hematopoietic lineage. (A) Primitive hematopoietic (EryP and megakaryocyte) colonies formed in methylcellulose progenitor assays upon replating of day 4 *i-Mix* EBs cultured in the presence or absence of DOX. The colonies were scored after 6 days. (B) Definitive hematopoietic colonies formed upon replating in methylcellulose of day 4 *i-Mix* EBs cultured in the presence or absence of DOX. The colonies were scored after 8 days.

conditions in culture.⁴⁰ We have developed a model for conditional expression of *mMix* in differentiating ES cells. Our gain-of-function studies reveal that, while *mMix* is apparently not required for the induction of mesoderm (as suggested by the observed expression of *Brachyury/T* and *Nodal* in *mMix*-deficient embryos³⁵), it is sufficient to accelerate the formation of mesoderm prior to expression of *T* in ES cell–derived embryoid bodies. Gene transcriptional analysis indicated a rapid and ordered progression of mesoderm development upon conditional expression of *mMix*. For example, activation of primitive streak genes was followed by up-regulation of markers of lateral, axial, and paraxial mesoderm. Similarly, expression of hemangioblast markers was detected very early and preceded that of hematopoietic genes. To our knowledge, these studies represent the first demonstration that conditional expression of a single gene is sufficient to accelerate development in the embryoid body system.

Interestingly, down-regulation of *Rex1* (*Zfp-42*) was slightly delayed in DOX-induced EBs. *Rex1* is expressed in the ICM of preimplantation embryos, trophoblast, spermatocytes, and ES cells.^{78,79} Although *Rex1*, like *Oct3/4* and *Nanog*, is considered a marker of stem cell pluripotency,⁸⁰ its expression is not restricted to cells of the ICM and ES cells, as initially believed.^{78,79} RNA and protein have been detected in more restricted progenitor cell populations as well, including CD34⁺ cells and multilineage progenitors from adult bone marrow or cord blood,^{81–83} spermatocytes prior to maturation to spermatozoa,⁷⁹ and an ES line committed to the endothelial lineage.⁸⁴ *Rex1* is downstream from and is regulated by *Oct4*.^{85,86} The persistence of *Rex1* expression suggests the interesting possibility that *Rex1* may have a previously unappreciated function in commitment of at least some populations of early mesoderm. Whether *mMix* and *Rex1* are expressed in the same or overlapping cell populations is currently under investigation.

The changes in response to early activation of *mMix* were not solely a reflection of altered developmental timing, because significantly increased numbers of mesodermal progenitors with hemangioblastic and hematopoietic potentials were found. Thus, the mouse *Mix-like* gene can regulate mesodermal development. Notably, in the mouse embryo, commitment to hemangioblasts is initiated prior to or during the emergence of their mesodermal progenitors from the posterior streak,⁴¹ where *mMix* expression is first detected within the epiblast (*mMix* is not expressed in the yolk sac^{32–34}).

Loss-of-function studies have demonstrated that *mMix* is required for normal development of the node, notochord, axial mesoderm, allantois, and heart.³⁵ Although blood islands appar-

ently form in the yolk sac,³⁵ *mMix*-null mutant EBs do show hematopoietic abnormalities. Analysis of *mMix*-deficient embryos has not yet been reported but should reveal whether more subtle defects (eg, reduced numbers of primitive erythroid progenitors) are present in these mutants.

Previous studies in the frog and zebrafish have established *Mix/Bix* genes as determining factors in endoderm formation.^{16,18–20,22,24,28} Chimera analysis has revealed a crucial role for *mMix* in endoderm differentiation in the mouse embryo.³⁵ However, *mMix* expression has not been detected in definitive endoderm.³⁴ Therefore, *mMix* may have a non-cell-autonomous role in mesoderm that serves to modulate endodermal differentiation, or it may function cell-autonomously within a transient population of mesendodermal progenitors.³⁴ When ectopically expressed in frog embryos, the mouse *Mix* gene was found to activate endodermal but not mesodermal markers.³⁴ The human *HMIXL* gene could activate *Xenopus* α T4-globin in animal cap assays if basic fibroblast growth factor (bFGF) were used to induce mesoderm,⁸⁷ indicating that *HMIXL* could drive mesoderm to a hematopoietic fate.

One model that could be envisioned to explain the results of our conditional activation studies is that *mMix* controls cell fate choice in mesendoderm, forcing the formation of mesoderm at the expense of endoderm in the DOX-treated EBs. However, even when mesoderm formation was accelerated, a later wave of endodermal gene expression (*Foxa2/Hnf3 β* , *Sox17*, *Gata4*, and *Hnf4*; A.A.-S., S.W., K.E.S., and M.H.B.; unpublished data, February 2004) was still observed. These findings, together with gene targeting analyses that demonstrated formation of mesoderm and definitive endoderm in the absence of *mMix*,³⁵ would suggest that *mMix* does not regulate a simple mesoderm versus endoderm fate decision during gastrulation.

Conditional activation of *mMix* in differentiating ES cells led to rapid up-regulation of *gsc*. In the frog, *gsc* is a direct target of *XMix.1* and represses transcription of the *Brachyury/T* homolog, *Xbra*.²⁸ *XMix.1* is therefore thought to function indirectly in the negative regulation of *Xbra*.²⁸ Genetic ablation of *mMix* in the mouse resulted in an expanded domain of *T* expression, again suggesting direct or indirect suppression.³⁵ However, in differentiating *i-Mix* ES cells, induction of *mMix* was followed by early activation of *T*. Like all of the known vertebrate members of the *Mix/Bix* family, the mouse *Mix* protein contains a highly conserved region of polar and acidic residues toward the carboxy terminus. This region has the potential to form an amphipathic helix (a characteristic of many transcriptional activators) and is part of a larger region that can activate transcription in yeast 2-hybrid assays.²⁹ It is entirely possible that, like many other transcription factors, *mMix* can function as an activator or a repressor depending on the cellular context (eg, through differential recruitment of coactivators and corepressors). Indeed, microarray analysis of induced and uninduced *i-Mix* EBs revealed dynamic changes in gene expression (both up- and down-regulation) within 24 hours of addition of DOX (M.A. Dyer, S.W., K.E.S., and M.H.B.; unpublished data, June 2004). The inducible cell lines described here will provide a useful model for the identification of *mMix* target genes.

We conclude that induction of *mMix* results in the increased recruitment or expansion of mesodermal progenitors for hemangioblastic and hematopoietic lineages. The accelerated expression of markers of other types of early mesoderm suggests the possibility that the representation of other mesodermal lineages might also be increased in response to *mMix*, given appropriate conditions for their differentiation in culture. To clarify this issue, we have initiated a systematic analysis of the effect of induction of *mMix* at

different times during EB development. An understanding of mesoderm lineage specification and differentiation at the earliest stages of embryoid body development in culture may provide valuable insights into the corresponding processes in the embryo and will be crucial for the directed differentiation of progenitors for cell-based therapies in humans.

References

- Rossant J, Tam PPL. *Mouse Development: Patterning, Morphogenesis, and Organogenesis*. San Diego: Academic Press; 2002.
- Epstein CJ, Erickson RP, Wynshaw-Boris A, eds. *Inborn Errors of Development: The Molecular Basis of Clinical Disorders of Morphogenesis*. New York, NY: Oxford University Press; 2004.
- Tam PL, Behringer RR. Mouse gastrulation: the formation of a mammalian body plan. *Mech Dev*. 1997;68:3-25.
- Lu CC, Brennan J, Robertson EJ. From fertilization to gastrulation: axis formation in the mouse embryo. *Curr Opin Genet Develop*. 2001;11:384-392.
- Tam PP, Gad JM, Kinder SJ, Tsang TE, Behringer RR. Morphogenetic tissue movement and the establishment of body plan during development from blastocyst to gastrula in the mouse. *Bioessays*. 2001;23:508-517.
- Shen MM, Pedersen RA. Developmental origins of the mammalian body plan. In: Epstein CJ, Erickson RP, Wynshaw-Boris A, eds. *Inborn Errors of Development: The Molecular Basis of Clinical Disorders of Morphogenesis*. New York, NY: Oxford University Press; 2004:53-66.
- Lawson KA, Meneses JJ, Pedersen RA. Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. *Development*. 1991;113:891-911.
- Tam PPL, Beddington RSP. The formation of mesodermal tissues in the mouse embryo during gastrulation and early organogenesis. *Development*. 1987;99:109-126.
- Kinder SJ, Loebel DA, Tam PP. Allocation and early differentiation of cardiovascular progenitors in the mouse embryo. *Trends Cardiovasc Med*. 2001;11:177-184.
- Sun X, Meyers EN, Lewandoski M, Martin GR. Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo. *Genes Dev*. 1999;13:1834-1846.
- Rosa FM. Mix.1, a homeobox mRNA inducible by mesoderm inducers, is expressed mostly in the presumptive endodermal cells of *Xenopus* embryos. *Cell*. 1989;57:965-974.
- Vize P. DNA sequences mediating the transcriptional response of the Mix.2 homeobox gene to mesoderm induction. *Dev Biol*. 1996;177:226-231.
- Huang H-C, Murtaugh LC, Vize PD, Whitman M. Identification of a potential regulator of early transcriptional responses to mesoderm inducers in the frog embryo. *EMBO J*. 1995;14:5965-5973.
- Chen X, Rubock MJ, Whitman M. A transcriptional partner for MAD proteins in TGF- β signaling. *Nature*. 1996;383:691-696.
- Chen X, Weisberg E, Fridmacher V, Watanabe M, Naco G, Whitman M. Smad4 and Fast-1 in the assembly of activin responsive factor. *Nature*. 1997;389:85-89.
- Mead PE, Kelley CM, Hahn PS, Piedad O, Zon LI. SCL specifies hematopoietic mesoderm in *Xenopus* embryos. *Development*. 1998;125:2611-2620.
- Ecochard V, Cayrol C, Rey S, et al. A novel *Xenopus* mix-like gene milk involved in the control of the endomesodermal fates. *Development*. 1998;125:2577-2585.
- Henry GL, Melton DA. Mixer, a homeobox gene required for endoderm development. *Science*. 1998;281:91-96.
- Lemaire P, Darras S, Caillol D, Kodjabachian L. A role for the vegetally expressed *Xenopus* gene Mix.1 in endoderm formation and in the restriction of mesoderm to the marginal zone. *Development*. 1998;125:2371-2380.
- Tada M, Casey ES, Fairclough L, Smith JC. Bix1, a direct target of *Xenopus* T-box genes, causes formation of ventral mesoderm and endoderm. *Development*. 1998;125:3997-4006.
- Alexander J, Stainier DY. A molecular pathway leading to endoderm formation in zebrafish. *Curr Biol*. 1999;9:1147-1157.
- Kikuchi Y, Trinh LA, Reiter JF, Alexander J, Yelon D, Stainier D. The zebrafish *bonnie* and *clyde* gene encodes a Mix family homeodomain protein that regulates the generation of endodermal precursors. *Genes Dev*. 2000;14:1279-1289.
- Reiter JF, Kikuchi Y, Stainier D. Multiple roles for Gata5 in zebrafish endoderm formation. *Development*. 2001;128:125-135.
- Poulain M, Lepage T. Mezzo, a paired-like homeobox protein is an immediate target of Nodal signalling and regulates endoderm specification in zebrafish. *Development*. 2002;129:4901-4914.
- Trinh LA, Meyer D, Stainier DY. The Mix family homeodomain gene *bonnie* and *clyde* functions with other components of the Nodal signaling pathway to regulate neural patterning in zebrafish. *Development*. 2003;130:4989-4998.
- Mead PE, Brivanlou IH, Kelley CM, Zon LI. BMP-4-responsive regulation of dorsal-ventral patterning by the homeobox protein Mix.1. *Nature*. 1996;382:357-360.
- Ng ES, Azzola L, Sourris K, Robb L, Stanley EG, Elefanty AG. The primitive streak gene *Mix1* is required for efficient haematopoiesis and BMP4-induced ventral mesoderm patterning in differentiating ES cells. *Development*. 2005;132:873-884.
- Latinkic BV, Smith JC. Goosecoid and mix.1 repress Brachyury expression and are required for head formation in *Xenopus*. *Development*. 1999;126:1769-1779.
- Sahr K, Dias D, Sanchez R, et al. Structure, upstream promoter region, and functional domains of a mouse and human Mix paired-like homeobox gene. *Gene*. 2002;291:135-147.
- Peale FV, Sugden L, Bothwell M. Characterization of CMIX, a chicken homeobox gene related to the *Xenopus* gene mix1. *Mech Dev*. 1998;75:167-170.
- Stein S, Roeser T, Kessel M. CMIX, a paired-type homeobox gene expressed before and during formation of the avian primitive streak. *Mech Dev*. 1998;75:163-165.
- Pearce JH, Evans MJ. Mml, a mouse Mix-like gene expressed in the primitive streak. *Mech Dev*. 1999;87:189-192.
- Robb L, Hartley L, Begley CG, et al. Cloning, expression analysis, and chromosomal localization of murine and human homologues of a *Xenopus* mix gene. *Dev Dyn*. 2000;219:497-504.
- Mohn D, Chen S, Dias D, et al. The mouse Mix gene is activated early during differentiation of ES and F9 stem cells and induces endoderm in frog embryos. *Dev Dyn*. 2003;226:446-459.
- Hart AH, Hartley L, Sourris K, et al. Mix1 is required for axial mesoderm morphogenesis and patterning in the murine embryo. *Development*. 2002;129:3597-3608.
- Keller G. Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Genes Dev*. 2005;19:1129-1155.
- Choi K, Kennedy M, Kazarov A, Papadimitriou JC, Keller G. A common precursor for hematopoietic and endothelial cells. *Development*. 1998;125:725-732.
- Robertson SM, Kennedy M, Shannon JM, Keller G. A transitional stage in the commitment of mesoderm to hematopoiesis requiring the transcription factor SCL/tal-1. *Development*. 2000;127:2447-2459.
- Keller GA, Webb S, Kennedy M. Hematopoietic development of ES cells in culture. In: Klug CA, Jordan CT, eds. *Hematopoietic Stem Cell Protocols*. Totowa, NJ: Humana Press; 2001:209-230.
- Fehling HJ, Lacaud G, Kubo A, et al. Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation. *Development*. 2003;130:4217-4227.
- Huber TL, Kouskoff V, Fehling HJ, Palis J, Keller G. Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. *Nature*. 2004;432:625-630.
- Kyba M, Perlingeiro RCR, Daley GQ. HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell*. 2002;109:29-37.
- Dyer MA, Farrington SM, Mohn D, Munday JR, Baron MH. Indian hedgehog activates hematopoiesis and vasculogenesis and can respectively prospective neuroectodermal cell fate in the mouse embryo. *Development*. 2001;128:1717-1730.
- Kennedy M, Keller GM. Hematopoietic commitment of ES cells in culture. *Methods Enzymol*. 2003;365:39-59.
- Baron MH, Mohn D. Mouse embryonic explant culture system for analysis of hematopoietic and vascular development. In: Baron MH, ed. *Developmental Hematopoiesis: Methods and Protocols*. Vol 105. Totowa, NJ: Humana Press; 2005:231-256.
- Farrington SM, Belaousoff M, Baron MH. Winged-Helix, Hedgehog and Bmp genes are differentially expressed in distinct cell layers of the murine yolk sac. *Mech Dev*. 1997;62:197-211.
- Belaousoff M, Farrington SM, Baron MH. Hematopoietic induction and respecification of A-P identity by visceral endoderm signaling in the mouse embryo. *Development*. 1998;125:5009-5018.
- Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol*. 2000;132:365-386.
- Niswander L, Martin GR. Fgf-4 expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development*. 1992;114:755-768.
- Blum M, Gaunt SJ, Cho KWY, et al. Gastrulation in the mouse: the role of the homeobox gene goosecoid. *Cell*. 1992;69:1097-1006.
- Herrmann BG, Kispert A. The T genes in embryogenesis. *Trends Genet*. 1994;10:280-286.
- Kispert A, Herrmann BG. Immunohistochemical analysis of the Brachyury protein in wild-type and

- mutant mouse embryos. *Dev Biol.* 1994;161:179-193.
53. Liu P, Wakamiya M, Shea MJ, Albrecht U, Behringer RR, Bradley A. Requirement for Wnt3 in vertebrate axis formation. *Nat Genet.* 1999;22:361-365.
 54. Takada S, Stark K, Shea M, Vassileva G, McMahon J, McMahon A. Wnt-3a regulates somite and tailbud formation in the mouse embryo. *Genes Dev.* 1994;8:174-189.
 55. Wolf C, Thisse C, Stoetzel C, Thisse B, Gerlinger P, Perrin-Schmitt F. The M-twist gene of *Mus* is expressed in subsets of mesodermal cells and is closely related to the *Xenopus X-twi* and the *Drosophila twist* genes. *Dev Biol.* 1991;143:363-373.
 56. Nieto MA, Gilardi-Hebenstreit P, Chamay P, Wilkinson DG. A receptor protein tyrosine kinase implicated in the segmental patterning of the hindbrain and mesoderm. *Development.* 1992;116:1137-1150.
 57. Ang S-L, Wierda A, Wong D, et al. The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/forkhead proteins. *Development.* 1993;119:1301-1315.
 58. Weinstein DC, Ruiz i Altaba A, Chen WS, et al. The winged-helix transcription factor HNF-3 β is required for notochord development in the mouse embryo. *Cell.* 1994;78:575-588.
 59. Dufort D, Schwartz L, Harpal K, Rossant J. The transcription factor HNF3 β is required in visceral endoderm for normal primitive streak morphogenesis. *Development.* 1998;125:3015-3025.
 60. Chapman DL, Agulnik I, Hancock S, Silver LM, Papaioannou VE. Tbx6, a mouse T-Box gene implicated in paraxial mesoderm formation at gastrulation. *Dev Biol.* 1996;180:534-542.
 61. Candia AF, Hu J, Crosby J, et al. Mox-1 and Mox-2 define a novel homeobox gene subfamily and are differentially expressed during early mesodermal patterning in mouse embryos. *Development.* 1992;116:1123-1136.
 62. Lints TJ, Parsons LM, Hartley L, Lyons I, Harvey RP. Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. *Development.* 1993;119:419-431.
 63. North T, Gu TL, Stacy T, et al. Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. *Development.* 1999;126:2563-2575.
 64. Lacaud G, Kouskoff V, Trumble A, Schwantz S, Keller G. Haploinsufficiency of Runx1 results in the acceleration of mesodermal development and hemangioblast specification upon in vitro differentiation of ES cells. *Blood.* 2004;103:886-889.
 65. Lacaud G, Gore L, Kennedy M, et al. Runx1 is essential for hematopoietic commitment at the hemangioblast stage of development in vitro. *Blood.* 2002;100:458-466.
 66. Speck NA, Stacy T, Wang Q, et al. Core-binding factor: a central player in hematopoiesis and leukemia. *Cancer Res.* 1999;59(suppl 7):1789s-1793s.
 67. North TE, de Bruijn MF, Stacy T, et al. Runx1 expression marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo. *Immunity.* 2002;16:661-672.
 68. Ema M, Faloon P, Zhang WJ, et al. Combinatorial effects of Flk1 and Tal1 on vascular and hematopoietic development in the mouse. *Genes Dev.* 2003;17:380-393.
 69. Park C, Afrikanova I, Chung YS, et al. A hierarchical order of factors in the generation of FLK1- and SCL-expressing hematopoietic and endothelial progenitors from embryonic stem cells. *Development.* 2004;131:2749-2762.
 70. Davidson AJ, Ernst P, Wang Y, et al. Cdx4 mutants fail to specify blood progenitors and can be rescued by multiple hox genes. *Nature.* 2003;425:300-306.
 71. Sauvageau G, Thorsteinsdottir U, Eaves C, et al. Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. *Genes Dev.* 1995;9:1753-1765.
 72. Antonchuk J, Sauvageau G, Humphries RK. HOXB4 overexpression mediates very rapid stem cell regeneration and competitive hematopoietic repopulation. *Exp Hematol.* 2001;29:1125-1134.
 73. Bjornsson JM, Larsson N, Brun AC, et al. Reduced proliferative capacity of hematopoietic stem cells deficient in Hoxb3 and Hoxb4. *Mol Cell Biol.* 2003;23:3872-3883.
 74. Brun AC, Bjornsson JM, Magnusson M, et al. Hoxb4-deficient mice undergo normal hematopoietic development but exhibit a mild proliferation defect in hematopoietic stem cells. *Blood.* 2004;103:4126-4133.
 75. Cantor AB, Orkin SH. Hematopoietic development: a balancing act. *Curr Opin Genet Dev.* 2001;11:513-519.
 76. Baumann CI, Bailey AS, Li W, Ferkowicz MJ, Yoder MC, Fleming WH. PECAM-1 is expressed on hematopoietic stem cells throughout ontogeny and identifies a population of erythroid progenitors. *Blood.* 2004;104:1010-1016.
 77. Chung YS, Zhang WJ, Arentson E, Kingsley PD, Palis J, Choi K. Lineage analysis of the hemangioblast as defined by FLK1 and SCL expression. *Development.* 2002;129:5511-5520.
 78. Hosler BA, LaRosa GJ, Grippo JF, Gudas LJ. Expression of REX-1, a gene containing zinc finger motifs, is rapidly reduced by retinoic acid in F9 teratocarcinoma cells. *Mol Cell Biol.* 1989;9:5623-5629.
 79. Rogers MB, Hosler BA, Gudas LJ. Specific expression of a retinoic acid-regulated, zinc-finger gene, Rex-1, in preimplantation embryos, trophoblast and spermatocytes. *Development.* 1991;113:815-824.
 80. Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med.* 2004;10:55-63.
 81. Goolsby J, Marty MC, Heletz D, et al. Hematopoietic progenitors express neural genes. *Proc Natl Acad Sci U S A.* 2003;100:14926-14931.
 82. D'Ippolito G, Diabira S, Howard GA, Menei P, Roos BA, Schiller PC. Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. *J Cell Sci.* 2004;117:2971-2981.
 83. Baal N, Reisinger K, Jahr H, et al. Expression of transcription factor Oct-4 and other embryonic genes in CD133 positive cells from human umbilical cord blood. *Thromb Haemost.* 2004;92:767-775.
 84. Yin Y, Que J, Teh M, Cao WP, El Oakley RM, Lim SK. Embryonic cell lines with endothelial potential: an in vitro system for studying endothelial differentiation. *Arterioscler Thromb Vasc Biol.* 2004;24:691-696.
 85. Ben-Shushan E, Thompson JR, Gudas LJ, Bergman Y. Rex-1, a gene encoding a transcription factor expressed in the early embryo, is regulated via Oct-3/4 and Oct-6 binding to an octamer site and a novel protein, Rox-1, binding to an adjacent site. *Mol Cell Biol.* 1998;18:1866-1878.
 86. Du Z, Cong H, Yao Z. Identification of putative downstream genes of Oct-4 by suppression-subtractive hybridization. *Biochem Biophys Res Commun.* 2001;282:701-706.
 87. Guo W, Chan AP, Liang H, et al. A human Mix-like homeobox gene MIXL shows functional similarity to *Xenopus Mix.1*. *Blood.* 2002;100:89-95.