Acceleration of Mesoderm Development and Expansion of Hematopoietic Progenitors in Differentiating ES Cells by the Mouse Mix-Like Homeodomain Transcription Factor

Running title: Mouse Mix-like and hematopoietic development

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Abstract

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 *Brachury/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.
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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 postero-anterior 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 \(\beta\) (TGF\(\beta\)), 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\(\beta\) family members Nodal/activin\(^11^,^25\) 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\textsuperscript{29}, only a single *Mix* gene has been found in mouse, chick and humans\textsuperscript{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\textsuperscript{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 E9.5\textsuperscript{35}. For example, the primitive streak is enlarged, a heart tube is absent and, in some mutants, the allantois is abnormally large\textsuperscript{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 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 three embryonic germ layers\textsuperscript{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\textsuperscript{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\textsuperscript{37,38} and were later shown to be present in gastrulating mouse embryos\textsuperscript{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
Mix is expressed in a population of Brachyury/T+Flk1+ cells from EBs that contains hemangioblasts; conversely, hemangioblasts are enriched in mMix-expressing cell populations. A function for mMix in blood cell development is strongly suggested by the recent demonstration of hematopoietic defects in mMix-deficient embryoid bodies.

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 upregulation 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 indicate 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 cDNA was subcloned into the plox targeting vector to create plox-mMix, which was co-electroporated with pSalk-Cre into Ainv 15 ES cells. 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 μg/ml doxycycline (DOX, Sigma #D9891, St. Louis, MO) for 48 hr and cell lysates were analyzed by immunoblotting using an antibody against the FLAG epitope (anti-FLAG M2, Sigma #3165). ES cell clones were
expanded and differentiated essentially as described\(^44\). Transgene expression was induced 24 hr after plating in EB culture (day 1) by the addition of DOX to a final concentration of 0.1 \(\mu\)g/ml. The medium was changed on day 3 and fresh DOX was added. The experiments included in this report were repeated from two to five times.

**RNA preparation and RT-PCR**

Total cellular RNA was prepared from 0.5-1.0 \(\times 10^7\) cells using TRIzol (Invitrogen #15596). To eliminate contaminating genomic DNA, the initial RNA pellet was resuspended in ribonuclease (RNase)-free deionized water and incubated with deoxyribonuclease (DNase) I (Roche #776-785, 2-4 U/\(\mu\)L) for 30 min at 37\(^\circ\)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, semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and primer sequences were as described\(^43,46,47\). Additional primer sequences (annealed at 55-58\(^\circ\)C) were as follows:

T (forward): 5'- GTCTTCTGGTTCTCCGATGT-3' (545 bp product)
T (reverse): 5'- CTACTCTAAGGCAACAAGGG -3'

C-kit (forward): 5'- TGTCTCTCCAGTTTCCCTGC -3' (765 bp product)
C-kit (reverse): 5'- TTCAGGGACTCATGGGCTCA -3'

Rex1 (forward): 5'-GGCTGCGAGAAGAGCTTTATTC -3' (438 bp product)
Rex1 (reverse): 5'- CTGCCAAAG TTG GCC ATTC -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) (Supplementary Table 1)
corresponded to 3'-untranslated regions. They were designed using Primer3 software\textsuperscript{48} and synthesized at the Hartwell Center at St. Jude Children's Research Hospital (Memphis, TN). Briefly, 1-5 micrograms of RNA was converted into cDNA and a series of diluted samples were utilized for 40-cycle PCR in TaqMan® Universal PCR Master Mix (containing AmpliTaq Gold enzyme; ABI #4304437) in an ABI (Perkin-Elmer-Applied Biosystems, Inc.) Prism 9700 instrument. Reactions (20 μL total) contained 1 μL cDNA, 6 μmol each primer, and 4 μmol 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\textsubscript{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\textsubscript{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 three controls.

\textbf{Assays for hematopoietic progenitors and blast colonies}

Blast and transitional colony assays\textsuperscript{44} and clonogenic assays for primitive erythroid progenitors\textsuperscript{43,45} were performed as described previously. Definitive hematopoietic progenitors were assayed
in Methocult® (Stem Cells Technologies, Vancouver)⁴⁵. Cytospin preparations and May-Grunwald Giemsa staining were as described⁴³,⁴⁵.

**FACS analysis**

At the indicated times in culture, EBs were dispersed using 0.05% trypsin/EDTA (Gibco Invitrogen, Grand Island, NY). Single cell suspensions containing 1-3 x 10⁵ 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, #553355). Flk1 monoclonal antibody was affinity purified using Protein G-Sepharose and biotinylated using the Fluoreporter Biotin-XX Protein Labeling Kit (Molecular Probes, Eugene, Oregon, cat. #F2610). Flk1 expression was detected using allophycocyanin (APC)-conjugated streptavidin (Pharmingen). Flow cytometry was performed using a FACS calibur 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 min and permeabilized in methanol for 30 min prior to incubation in blocking solution (PBS containing 1% bovine serum albumin, Sigma A9647). Anti-FLAG M2 mouse monoclonal antibody (Sigma, F3165) was used at a concentration of 5 μg/ml. Alexa Fluor 488-conjugated rabbit anti-mouse secondary antibody (Molecular Probes #A11122, Eugene, OR) 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, H-1200).

**Microscopy**

Fluorescently labeled cells were visualized using a Zeiss Axioplan-2 microscope with a 40X Plan-NEOFLUAR/0.75 NA objective and appropriate filter sets (Chroma Technology Corp., 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\textsuperscript{36}. We had previously reported that \textit{mMix} is transiently expressed in embryoid bodies derived from the CCE ES cell line\textsuperscript{34}. In preparation for generation of the inducible system discussed below, which is based on the E14 line of ES cells\textsuperscript{42}, we also examined the time course of expression of \textit{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, \textit{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 then, later, in certain mesodermal populations. In differentiating EBs, levels of Fgf4 mRNA decrease steadily as ES cells (day 0 in Fig. 1A) differentiate. Transient expression of mMix and T was detected early in embryoid body differentiation (Fig. 1A and data not shown; note peak at day 3-4). The initiation of Flk1 transcription overlapped with peak mMix and T expression (day 3) and continued after the downregulation of these genes. Progenitors for primitive and definitive hematopoietic cell types formed by day 6 and 8-10, 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 doxycycline-inducible expression of exogenous genes in differentiating ES cells, we generated a panel of ES cell lines (here termed i-Mix) in which FLAG-tagged mMix is induced in response to doxycycline (DOX) via activation of a reverse tet-transactivator protein (rtTA; Fig. 1B). This system allows consistent and reversible induction of a conditional reporter and the uninduced cells provide an ideal control. Because all cell lines are derived by Cre-mediated recombination of the cDNA of interest into the same locus, position effects are not a problem. Candidate i-Mix lines were screened for inducible expression of FLAG-mMix protein (see 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 (see below). Clone 2 was chosen for further analysis. As shown in Fig. 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 0.025-0.10 µg/ml (Fig. 1C). Expression of FLAG-mMix protein was nuclear (Fig. 1D), as expected from our earlier studies.34 The studies reported below were performed using DOX at 0.1 µg/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 the cultures 24 hr after plating (day 1.0) and EBs were harvested at 3-4 hr intervals beginning at day 2.0 after plating of ES cells (shown schematically in Fig. 2A). Total cellular RNA was isolated and analyzed by semi-quantitative RT-PCR (Fig. 2B). Samples were normalized for expression of β-actin.43,46,47

As anticipated, the zinc finger transcription factor Rex1 was downregulated both in the presence and absence of DOX. In DOX-induced EBs, downregulation of Rex1 was slightly delayed (Fig. 2B,C; compare lanes 2-5, -DOX, and lanes 12-15, +DOX; 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 (Fig. 2B, compare lanes 3-6 with lanes 13-17).

In light of our previous finding34 that mMix, T and Flk1 are co-expressed in a subpopulation of EB cells in which a green fluorescent protein (Gfp) gene was targeted to the T locus40, 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 potential40. Activation of Flk1 and c-kit was also accelerated (Fig. 2B, compare lanes 2-7 with lanes 12-17). c-kit is
expressed in undifferentiated E14 ES cells (as we expected, lane 1). Upon initiation of
differentiation, c-kit was transiently downregulated (lanes 2,3) and was activated again around
day 2.6-3.0 (lanes 4,5). In the presence of DOX, c-kit expression was easily detected as early as
day 2 (compare lanes 2-4, minus DOX, with lanes 12-14, +DOX); whether downregulation of c-
kit was blocked between 24 and 48 hr by early induction of Mix was not determined. T, Flk1 and
c-kit were also downregulated more rapidly at later stages of EB differentiation in the presence
than in the absence of DOX (Fig. 2B, compare lanes 10,11 with lanes 20,21), suggesting that
conditional activation of mMix resulted in acceleration of mesodermal development.

Mesodermal gene activation following induction of iMix was examined more systematically
using real-time PCR (QRT-PCR). For these studies, expression was normalized to Gapdh (Fig.
2C), Gpi-1 and/or Mt-2 (see Materials and Methods). Delayed downregulation of Rex1 in
response to mMix was again observed (Fig. 2C), confirming the semi-quantitative RT-PCR
result (Fig. 2B). Four genes that are expressed in the primitive streak were all activated
prematurely in the induced i-Mix EBs: gsc50, T51,52, endogenous mMix32-34, and Wnt3a53,54.
Gooseoid (gsc), which in the mouse embryo is first expressed early in gastrulation, in the
emerging primitive streak50, was activated shortly after induction of mMix and nearly 24 hr
earlier than in uninduced EBs (Fig. 2C). In the frog, gsc is a direct target of XMix.i28; 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
(Fig. 2C). Markers of several types of embryonic mesoderm were also activated earlier in the
induced i-Mix EBs (Fig. 2C): lateral (Twist55), axial (EphA4 and Foxa2/Hnf3β56-59), paraxial
(Tbx-6 and Moxi60,61), and cardiac (Nkx2.562). These mesodermal genes were activated in an
orderly fashion, following upregulation 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 day 6-7 (reflecting hemoglobin production in erythroid cells), at least 24 hr earlier than untreated EBs (Fig. 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 (Fig. 4, left panels) and hematopoietic (Fig. 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. It encodes the DNA-binding subunit of a Core Binding Factor transcriptional regulator, is a marker of long-term repopulating hematopoietic stem cells, and is essential for hematopoietic commitment of hemangioblasts. Scl/tal, a member of the basic-helix-loop-helix family of transcription factors, functions somewhat later than Runx1 or Flk1. 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. All four genes (Runx1, Flk1, c-kit, and Scl/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 (Fig. 4). Cdx4, a caudal class homeobox gene, regulates the
specification of hematopoietic (but not endothelial) progenitors. Hoxb4 has been implicated in hematopoietic stem cell proliferation and confers engraftment potential on ES and yolk sac progenitor cells. In keeping with its demonstrated genetic position with respect to Hoxb4, activation of Cdx4 preceded that of Hoxb4 in induced i-Mix EBs (Fig. 4). Gata1 encodes a zinc-finger transcription factor that is essential for normal erythroid development, while εγ-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 than in the absence of inducer (Fig. 4), consistent with the observed acceleration of hemoglobin pigmentation in DOX-treated EBs (Fig. 3). In agreement with these data, a cDNA microarray analysis of an independent set of induced and uninduced i-Mix EBs revealed that Gata-1, εγ-globin, βmaj-globin, α-globin, and Pecam-1 (the last of which is expressed on hematopoietic stem cells, erythroid and endothelial progenitors, and mature endothelial cells) were all transcribed at significantly higher levels in response to conditional activation of mMix (day 4; our unpublished data).

**Accelerated and enhanced formation of Flk1- and c-kit-positive 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 three populations of cells was observed, beginning on day 2 (24 hr after addition of DOX; not shown) and most evident on day 3 (48 hr post DOX) (Fig. 5 and Table 1): (i) Flk1+ (~4-fold increase in DOX-treated versus untreated EBs); (ii) Flk+c-kit+ (>4-fold increase); and (iii) c-kit~hi (~8-fold increase). Between days 3 and 5, a dramatic decrease in the number of Flk+ cells was observed.
in DOX-treated EBs (from ~11% to ~3%), whereas this population continued to expand in the uninduced EBs (from ~3% to ~10%). During this time period, in both induced and uninduced EBs, increased numbers of Flk+c-kit+ (~7% to ~13% +DOX; 0.9% to ~10% -DOX) and c-kit^{hi} (~23% to ~31% +DOX; ~5% to ~10% -DOX) cell subsets were found. Therefore, in response to induction of mMix, formation of all three 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 Flk^{+}c-kit^{+} double-positive cell population has hematopoietic potential (M. Kennedy and G. Keller, in preparation).

**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 (Fig. 6). Very strikingly, over all times in culture evaluated (Fig. 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 hemangioblasts^{38} and blast hemangioblast^{37} colonies formed earlier and in larger numbers in DOX-treated versus untreated secondary cultures (Fig. 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 progenitors were found in higher numbers in the DOX-induced cultures (Fig. 6B,C). Thus, formation of mesodermal,
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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 Fig. 6B,C were re-plotted in stacked column form to emphasize the representation of each lineage among colonies scored (Fig. 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 ingress 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 remain 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 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 embryos\textsuperscript{35}, 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 upregulation 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, downregulation of *Rex1 (Zfp-42)* was slightly delayed in DOX-induced EBs. *Rex-1* is expressed in the inner cell mass (ICM) of preimplantation embryos, in trophectoderm, spermatocytes, and ES cells\textsuperscript{78,79}. Although *Rex-1*, like *Oct3/4* and *Nanog*, is considered a marker of stem cell pluripotency\textsuperscript{80}, its expression is not restricted to cells of the ICM and ES cells, as initially believed\textsuperscript{78,79}. RNA and protein have been detected in more restricted progenitor cell populations as well, including CD34\textsuperscript{+} cells and multilineage progenitors from adult bone marrow or cord blood\textsuperscript{81-83}, in spermatocytes prior to maturation to spermatozoa\textsuperscript{79}, and in an ES line committed to the endothelial lineage\textsuperscript{84}. Rex-1 is downstream from and is regulated by Oct-4\textsuperscript{85,86}. The persistence of *Rex1* expression suggests the interesting possibility that *Rex-1* may have a previously unappreciated function in commitment of at least some populations of early mesoderm. Whether *mMix* and *Rex-1* 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, as significantly increased numbers of mesodermal progenitors with
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hemangioblastic and hematopoietic potentials were found. Thus, the mouse Mix-like gene can regulate mesodermal development. It is worth noting that, in the mouse embryo, commitment to hemangioblasts is initiated prior to or during the emergence of their mesodermal progenitors from the posterior streak, where \( mMix \) expression is first detected within the epiblast (\( mMix \) is not expressed in the yolk sac).

Loss of function studies have demonstrated that \( mMix \) is required for normal development of the node, notochord, axial mesendoderm, allantois and heart. Though blood islands apparently form in the yolk sac, \( mMix \) null mutant EBs do show hematopoietic abnormalities. Analysis of \( mMix \)-deficient embryos has not yet been reported but should reveal whether more subtle defects (e.g. reduced numbers of primitive erythroid progenitors) are present in these mutants.

Previous studies in the frog and zebrafish had established Mix/Bix genes as determining factors in endoderm formation. Chimera analysis has revealed a crucial role for \( mMix \) in endoderm differentiation in the mouse embryo. However, \( mMix \) expression has not been detected in definitive endoderm. 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. When ectopically expressed in frog embryos, the mouse Mix gene was found to activate endodermal but not mesodermal markers. The human \( HMIXL \) gene could activate Xenopus \( \alpha T4 \)-globin in animal cap assays if basic fibroblast growth factor (bFGF) was used to induce mesoderm, 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/Hnf3β, Sox17, Gata4, and Hnf4; A. A.-S., S.W., K.E.S. and M.H.B., unpublished data) 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 upregulation of gsc. In the frog, gsc is a direct target of XMix.1 and represses transcription of the Brachyury/T homologue, Xbra28. XMix.1 is, therefore, thought to function indirectly in the negative regulation of Xbra28. Genetic ablation of mMix in the mouse resulted in an expanded domain of T expression, again suggesting direct or indirect suppression35. 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 two-hybrid assays29. It is entirely possible that, like many other transcription factors, mMix can function as an activator or a repressor depending on the cellular context, e.g. 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 hr of addition of DOX (M.A. Dyer, S.W., K.E.S., and M.H.B., unpublished data). The inducible cell lines described here will provide a useful model for the identification of mMix target genes.
We conclude that induction of \textit{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 \textit{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 \textit{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.

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References


Table 1

Table 1. Quantitation of FACS analysis of Flk1 and c-kit expression on cells from i-Mix EBs cultured with or without DOX. The percentages of cells in each of the three populations gated in Figure 4 are displayed here with the ratio (±DOX).

<table>
<thead>
<tr>
<th></th>
<th>Day 3 EBs</th>
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<th>Day 5 EBs</th>
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<tr>
<td></td>
<td>- DOX</td>
<td>+ DOX</td>
<td>Ratio (± DOX)</td>
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Figure Legends

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, minus template control. (B) Strategy for conditional expression of mMix in ES cells. The vector plox-Mix1, encoding amino-terminal FLAG tagged mMx, was inserted into the X-chromosome using Cre-mediated recombination. In the presence of DOX, rtTA 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 one of the three 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 mMx protein in cells from induced i-Mix EBs. Magnification, 40x.

Figure 2. The mesodermal developmental program is accelerated in response to mMx. (A) General experimental protocol for induction and analysis of i-Mix EBs cultured +/- DOX. DOX (0.1 µg/ml) was added on day 1 after plating under differentiation conditions. (B) Semi-quantitative RT-PCR analysis of gene expression in i-Mix EBs cultured +/-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), 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 +/-DOX. Expression was normalized to that of a housekeeping gene (for this figure, Gapdh) and expressed as a ratio (see Materials and Methods).
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 microns.

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 +/-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 legend to Fig. 2.

Figure 5. Formation of Flk1-positive and c-kit-positive 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 (WT) ES and uninduced (-DOX) i-Mix cells produced comparable numbers of Flk+, c-kit^hi and Flk^+ c-kit^+ double-positive cells (not shown). This experiment was repeated three times with similar results.

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 ± S.E.M. per 10,000 cells plated. (A) Mesodermal progenitors (transitional and blast colonies). EBs were harvested on day 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 days 8 or 10.

**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.
FIGURE 1
FIGURE 2

(A) Schematic diagram showing the experimental setup for the experiment.

(B) Western blot analysis showing protein expression under different conditions.

(C) Graphs showing the expression levels of different genes over time in culture.
FIGURE 3
FIGURE 4

[Diagram showing gene expression levels over days in culture for Hemangioblast and Hematopoietic cells with +DOX and -DOX conditions.]
FIGURE 5
FIGURE 6

A) Mesodermal (Day 3 EBs)  
B) Primitive (Day 4 EBs)  
C) Definitive (Day 4 EBs)
FIGURE 7
Acceleration of mesoderm development and expansion of hematopoietic progenitors in differentiating ES cells by the mouse mix-like homeodomain transcription factor

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