The primitive streak gene *Mixl1* is required for efficient haematopoiesis and BMP4-induced ventral mesoderm patterning in differentiating ES cells

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Summary

The homeobox gene *Mixl1* is expressed in the primitive streak of the gastrulating embryo, and marks cells destined to form mesoderm and endoderm. The role of *Mixl1* in development of haematopoietic mesoderm was investigated by analysing the differentiation of ES cells in which GFP was targeted to one (*Mixl1*$^{GFP/w}$) or both (*Mixl1*$^{GFP/GFP}$) alleles of the *Mixl1* locus. In either case, GFP was transiently expressed, with over 80% of cells in day 4 embryoid bodies (EBs) being GFP^{+}. Up to 45% of *Mixl1*$^{GFP/w}$ day 4 EB cells co-expressed GFP and the haemangioblast marker FLK1, and this doubly-positive population was enriched for blast colony forming cells (BL-CFCs). *Mixl1*-null ES cells, however, displayed a haematopoietic defect characterised by reduced and delayed *Flk1* expression and a decrease in the frequency of haematopoietic CFCs. These data indicated that *Mixl1* was required for efficient differentiation of cells from the primitive streak stage to blood. Differentiation of ES cells under serum-free conditions demonstrated that induction of *Mixl1*- and *Flk1*-expressing haematopoietic mesoderm required medium supplemented with BMP4 or activin A. In conclusion, this study has revealed an important role for *Mixl1* in haematopoietic development and demonstrates the utility of the *Mixl1*$^{GFP/w}$ ES cells for evaluating growth factors influencing mesendodermal differentiation.

Key words: *Mixl1*, BMP4, Haematopoiesis, *Kdr*

Introduction

Gastrulation in vertebrates is a tightly coordinated process in which the pluripotent epiblast is converted into the three embryonic germ layers (Robb and Tam, 2004; Tam and Behringer, 1997; Tam et al., 2001; Tam et al., 2003). In mice, the earliest visible indicator of gastrulation is the formation of the primitive streak, a transient structure at the presumptive posterior of the embryo from which mesodermal and endodermal cells develop (Tam and Behringer, 1997). Several families of growth factors have been implicated in the regulation of gastrulation in the mouse, including fibroblast growth factors and members of the transforming growth factor β (TGFβ) superfamily (Conlon et al., 1994; Sun et al., 1999; Winnier et al., 1995).

Key targets of TGFβ signalling in gastrulating *Xenopus laevis* embryos include the Mix/Bix homeobox genes that regulate mesoderm and endoderm formation in response to *nodal/activin* and BMP4 (Rosa, 1989; Vize, 1996). *Mix.1* is induced by BMP4 and can ventralise mesoderm (Mead et al., 1996), while a number of other Mix/Bix genes induce endoderm (Henry and Melton, 1998; Latinkic and Smith, 1999; Lemaire et al., 1998; Tada et al., 1998). The two zebrafish *Mix*-related homeobox genes, *bon* and *mezzo* (*og9x* – Zebrafish Information Network) are immediate-early targets of *nodal* signalling that are transiently expressed in precursors of mesoderm and endoderm (Kikuchi et al., 2000; Poulain and Lepage, 2002; Trinh et al., 2003). The avian *Mix* gene is expressed in the epiblast and posterior marginal zone endoderm just prior to gastrulation and in the primitive streak, excluding Hensen's node (Peale et al., 1998; Stein et al., 1998). Similarly, expression of the single mouse Mix gene homologue, *Mixl1*, is restricted to the visceral endoderm of the pre-gastrulation embryo and the primitive streak (Pearce and Evans, 1999; Robb et al., 2000). Indeed, gene targeting has confirmed the important role that *Mixl1* plays during gastrulation. *Mixl1*-null mutants display an enlarged primitive streak and subsequently exhibit abnormalities in axial morphogenesis and formation of definitive endoderm that result in death at embryonic day (E) 8.5 (Hart et al., 2002).

In mice, the first mesoderm to emerge from the primitive streak migrates extra-embryonically and forms the blood islands of the yolk sac at E7-7.5 (Kinder et al., 1999). The first wave of haematopoiesis occurs concurrently with the formation of extra-embryonic vasculature, consistent with the development of these two lineages from a common progenitor (Keller et al., 1999; Lacaud et al., 2001). The in vitro differentiation of embryonic stem (ES) cells recapitulates many aspects of early haematopoietic development and represents a

valuable model system to study a process occurring at a relatively inaccessible period of embryogenesis (Dang et al., 2002; Desbaillets et al., 2000; Keller, 1995; Maye et al., 2000; Takahashi et al., 2003). Indeed, the *Flk1* (*Kdr* – Mouse Genome Informatics)-positive blast colony forming cell (BL-CFC) capable of giving rise to both haematopoietic and endothelial lineages was first isolated from embryoid bodies (EBs), thus providing tangible evidence for the existence of an haemangioblast (Choi et al., 1998; Kennedy et al., 1997; Nishikawa et al., 1998).

We have used mouse ES cell lines in which *Mixl1*-coding sequences on one (*Mixl1*$^{GFP/w}$) or both (*Mixl1*$^{GFP/GFP}$) alleles were replaced by the gene encoding green fluorescent protein (GFP) (Hart et al., 2002) to investigate the role of *Mixl1* in ventral mesoderm patterning and haematopoiesis. We have shown that a large proportion of differentiating *Mixl1*$^{GFP/w}$ ES cells transiently expressed both GFP and *Flk1* and that this doubly-positive population was enriched for BL-CFCs. However, in differentiating *Mixl1*-null ES cells, *Flk1* expression was delayed and reduced and the frequency of haematopoietic CFCs was decreased. Differentiation of ES cells under serum-free (SF) conditions demonstrated that induction of *Mixl1*- and *Flk1*-expressing haematopoietic mesoderm required medium supplemented with BMP4 or activin A. Therefore, this study has revealed an important role for *Mixl1* in haematopoietic development and demonstrated the utility of the *Mixl1*$^{GFP/w}$ ES cells for evaluating growth factors influencing mesendodermal differentiation.

Materials and methods

Generation of targeted ES cells

A cassette encoding enhanced GFP and a loxP-flanked neomycin resistance gene was knocked into the first exon of *Mixl1*by homologous recombination as described (Hart et al., 2002). Two karyotypically normal heterozygous ES lines (M147 and M114) were chosen for subsequent experiments. To generate the *Mixl1*-null line M916, a plasmid encoding cre recombinase (pMC1Cre, provided by K. Rajewsky) (Gu et al., 1994) was transiently transfected into the M147 line to remove the neomycin resistance cassette prior to a second round of gene targeting using the original *Mixl1* targeting vector. The *Mixl1*-null cell line M3C5 was created by targeting the wild-type allele of M114 with a *Mixl1*GFP targeting vector that incorporated a puromycin resistance cassette.

ES cell culture and differentiation

ES cells were cultured as described (Barnett and Köntgen, 2001) and differentiated using the method of Kennedy et al. (Kennedy et al., 1997). For differentiation of ES cells under SF conditions, ES cells were resuspended at 5000 cells/ml in modified Chemically Defined Medium (CDM) (Johansson and Wiles, 1995) comprising IMDM/Ham's F12 with Glutamax (Gibco) supplemented with 5 mg/ml bovine serum albumin (Sigma), 1 U/ml LIF (Chemicon), 4.5×10^{-4} M α-MTG (Sigma), a 1% Chemically-Defined Lipid Concentrate (Gibco), 1% Insulin-Transferrin-Selenium-X Supplement (Gibco) and antibiotics. Activin A (0.1-100 ng/ml) or BMP4 (0.5-20 ng/ml) (R&D Systems) were added at the time of cell plating. Cultures were maintained at 37°C in a humidified environment of 8% CO_2 in air. Phase-contrast and fluorescent microscopy images of EB cultures were acquired using a Zeiss Axiocam mounted on an Axiovert 200 microscope and processed with Axiovision software. Single optical sections of EBs were taken using a Leica confocal scanning microscope.

Flow cytometry

Embryoid bodies were dissociated to single cells using trypsin/EDTA (Gibco) containing 1% chicken serum (Hunter). Cells were resuspended in a block solution (phosphate-buffered saline supplemented with 2% FCS, 1% goat serum and 1% rabbit serum) and incubated with primary antibodies directed against E-cadherin (ECCD-2, Zymed), FLK1 (VEGF-R2, Ly-73) conjugated to phycoerythrin (PE) (Avas 12α1, BD Biosciences), Ter-119 (Ly-76) conjugated to PE (BD Biosciences) and CD34 (RAM34) conjugated to biotin (BD Biosciences). Anti E-cadherin antibodies were detected with either PE or allophycocyanin (APC)-conjugated anti-rat IgG (BD Biosciences) while biotinylated anti-CD34 antibodies were detected with streptavidin-conjugated PE or APC. Cells were analysed using a FACSCalibur (Becton Dickinson) running CellQuest software (Becton Dickinson). For cell-sorting and reculture experiments, differentiating *Mixl1*$^{GFP/w}$ EBs were dissociated, stained with antibodies against FLK1 and sorted according to GFP and FLK1 expression using a FACStar Plus (Becton Dickinson).

Haematopoietic colony forming assays

Haematopoietic colonies were generated by plating 2.5×10^{4}-10^{5} dissociated EB cells into 1.5 ml of 1% methylcellulose in IMDM supplemented with 10% FCS, 25% D4T endothelial cell conditioned medium, 25 μg/ml ascorbic acid, 2 mM L-glutamine (Kennedy et al., 1997). To assay BL-CFCs, methylcellulose cultures were supplemented with 5 ng/ml Vascular Endothelial Growth Factor (VEGF) (R&D systems) and 50 ng/ml Stem Cell Factor (SCF) (R&D systems). For detection of BL-CFCs and primitive erythroid colonies (EryP), the growth factor combination used was 5 ng/ml VEGF, 50 ng/ml SCF, 5 U/ml EPO (Janssen Cilag) and IL3 (1% of a supernatant from a cell line producing mIL3) (Karasuyama and Melchers, 1988). Colonies were scored after 5-7 days. Blast colonies were expanded in liquid culture supplemented with the same combination of growth factors and analysed after 7 days. Morphology was assessed by May-Grunwald-Giemsa staining of cytocentrifuge preparations and endothelial cells were identified by staining wells fixed with 4% paraformaldehyde (Sigma) with anti-PECAM1 antibodies (MEC13.3, BD Biosciences).

Gene expression analysis

Total RNA was prepared using an RNeasy kit (Qiagen) according to the manufacturer's instructions. DNase I treated samples were reverse transcribed using Superscript II (Invitrogen) and the resultant cDNA preparations standardized as described (Elefanty et al., 1997). Primer sequences and annealing temperatures are shown in Table 1. References for Brachyury, FLK1, βH1 globin and HPRT primer sequences can be found in Elefanty et al. (Elefanty et al., 1997). PCRs were performed for 30 cycles with reaction conditions as described (Elefanty et al., 1997). PCR products were analysed by electrophoresis on a 2% agarose gel.

Results

Transient expression of the primitive streak marker *Mixl1* during embryonic stem cell differentiation

Consistent with the findings of others (Lacaud et al., 2002; Mohn et al., 2003; Robertson et al., 2000), gene expression analysis of differentiating mouse ES cells demonstrated the progressive downregulation of stem cell genes such as *Rex1* (*Zfp42* – Mouse Genome Informatics), *Oct4* (*Pou5f1* – Mouse Genome Informatics), E-cadherin (*Cdh1* – Mouse Genome Informatics) and *Sox2* accompanied by the sequential acquisition of molecular markers of epiblast, primitive streak and mesoderm (Fig. 1). Expression of the epiblast marker Fgf5

Table 1. Oligonucleotide primers used for RT-PCR

Gene	5′ primer	3′ primer	T (°C)*	Size (bp)†
Oct4	CGTTCTCTTTGGAAAGGTGTTC	GAACCATACTCGAACCACATCC	55	320
Rex1	TGAAAGTGAGATTAGCCCCGAG	GTCCCATCCCCTTCAATAGCAC	55	933
E-cadherin	GCAGTCAGATCTCCCTGAGTTCGAG	CTACATACAAAGGTCACTCTAGCAAC	60	372
Sox2	ACGCAAAAACCGTGATGCCGAC	CGTTTGCCTTAAACAAGACCACG	55	624
Fgf5	TGTACTGCAGAGTGGGCATCGG	ACTTCTGCGAGGCTGCGACAGG	60	487
Brachyury	TGCTGCCTGTGAGTCATAAC	TCCAGGTGCTATATATTGCC	55	947
Goosecoid	GAGCAGCTGGCCAGGAAGGTGCAC	CAGCTAGCTCCTCGTTGCTTTCTC	60	323
Flk1	TAGGTGCCTCCCCATACCCTGG	TGGCCGGCTCTTTCGCTTACTG	60	398
βH1 globin	CTCAAGGAGACCTTTGCTTCA	AGTCCCCATGGACTCAAAGA	55	265
HPRT	GCTGGTGAAAAGGACCTCT	CACAGGACTAGAACACCTGC	55	249

*Annealing temperature.
†PCR product size.

peaked at day 2 followed by transient expression of the primitive streak genes brachyury, *Mixl1* and *Gsc* at days 3 and 4. Transcription of *Flk1*, an early marker of ventral mesoderm, was up regulated from day 3, and βH1 globin, which reflects the development of primitive erythroblasts, was expressed from day 4. Thus, the kinetics of *Mixl1* expression in differentiating ES cells was consistent with its restricted expression during primitive streak formation in vivo, and marked the period of transition between the epiblast and mesoderm.

Transient fluorescence of differentiating *Mixl1*$^{GFP/w}$ ES cells

ES cells in which GFP was expressed from one (*Mixl1*$^{GFP/w}$) or both (*Mixl1*$^{GFP/GFP}$) alleles of the *Mixl1* locus have been described (Hart et al., 2002). Previous analysis had shown that *Mixl1*$^{GFP/w}$ mice were phenotypically normal and that GFP expression was observed in the primitive streak, validating the use of GFP as a reporter of *Mixl1*-expressing cells (Hart et al., 2002). This analysis also confirmed that the neomycin resistance cassette did not affect the distribution of GFP in these animals or in the differentiating ES cells in vitro (data not shown).

Examination of differentiating *Mixl1*$^{GFP/w}$ ES cells revealed that most day 3 and 4 embryoid bodies (EBs) expressed GFP, coinciding with endogenous *Mixl1* expression (Figs 1, 2 and data not shown). Confocal images demonstrated that GFP was present in the outer, flattened endoderm-like cells and in the inner core of the EBs (Fig. 2C,D).

GFP expression during differentiation of *Mixl1*$^{GFP/w}$ cells was examined by flow cytometry. In the representative experiment shown in Fig. 3, GFP expressing cells were first evident at day 2.5 and their frequency rapidly increased to 43.5% at day 3 and peaked at 85.2% at day 4 of differentiation. Although *Mixl1* RNA was not detected after day 5 (Fig. 1), GFP expression did not wane until after day 6, consistent with the long half life of this reporter protein (Corish and Tyler-Smith, 1999). A similar time course of GFP expression was observed in two independent *Mixl1*$^{GFP/w}$ ES lines (data not shown).

Mixl1-null EBs displayed a similar profile of GFP

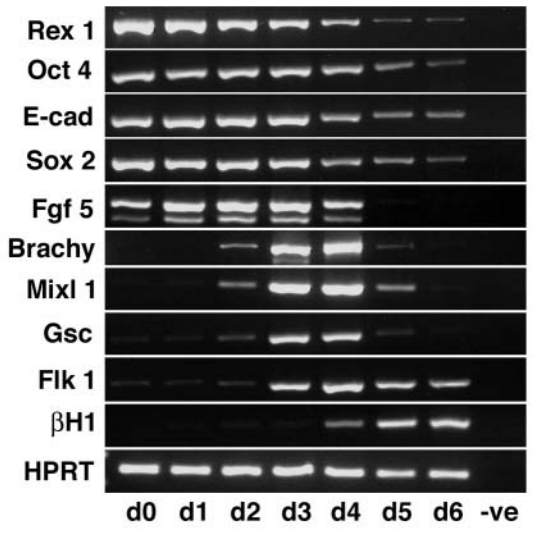

Fig. 1. Analysis of gene expression in differentiating ES cells showing the progressive loss of stem cell markers and the sequential acquisition of transcripts indicative of specific stages of embryonic development. RT-PCR on RNA isolated from undifferentiated ES cells (day 0) and EBs harvested at daily intervals (days 1-6) was performed using primers specific for the genes indicated. –ve, no template.

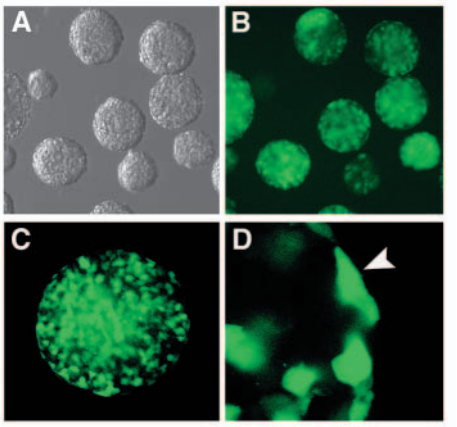

Fig. 2. GFP fluorescence of day 4 *Mixl1*$^{GFP/w}$ EBs. Low-magnification bright-field (A) and epifluorescence (B) images showing that most EBs contain GFP-positive cells. (C) Confocal image showing that GFP-positive cells are present both on the surface and distributed throughout the EB. (D) High magnification of the EB in C, showing GFP-positive cells located on the surface of the EB have a flattened morphology reminiscent of endoderm (arrowhead).

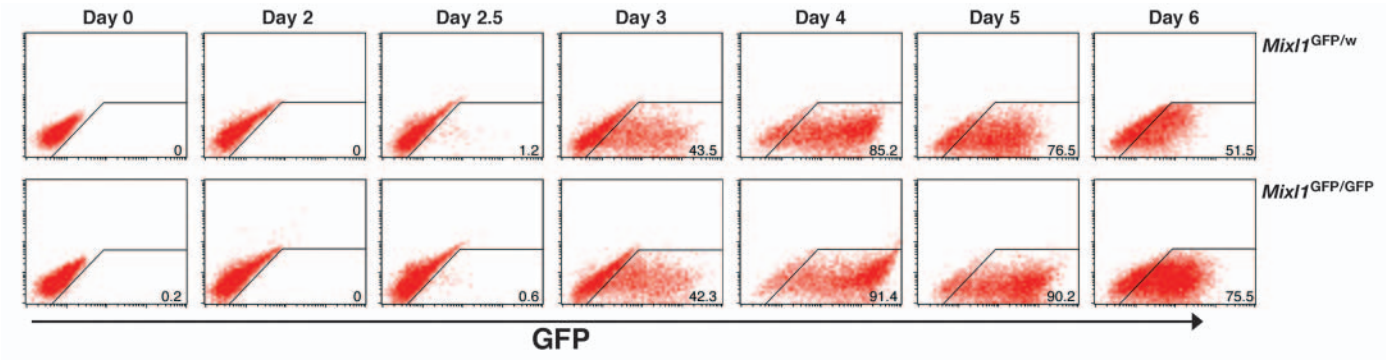

Fig. 3. Time course of GFP expression of in differentiating *Mixl1*$^{GFP/w}$ heterozygous and *Mixl*$^{GFP/GFP}$ null ES cells as determined by flow cytometry. Both lines initiated GFP expression at day 2.5 and peak expression was seen at day 4. The frequency of GFP-positive cells diminished more quickly in *Mixl1* heterozygous cells than in those deficient in *Mixl1*. The percentage of GFP-positive cells is shown in the lower right of each plot.

expression to heterozygous *Mixl1*$^{GFP/w}$ ES cells with the frequency of GFP-positive cells peaking at ~90% at day 4 (Fig. 3). A higher intensity and prolongation of GFP expression in *Mixl1*$^{GFP/GFP}$ EBs was consistently observed, probably reflecting the presence of two copies of GFP in the null cells.

GFP is co-expressed with the stem cell marker E-cadherin in *Mixl1*$^{GFP/w}$ and *Mixl1*$^{GFP/GFP}$ cells

Examination of the gene expression profile of differentiating ES cells demonstrated that day 3 EBs simultaneously expressed stem cell, primitive streak and mesodermal genes (Fig. 1). We used flow cytometry to correlate the expression of GFP with that of the stem cell marker E-cadherin (E-cad) and the ventral mesoderm marker FLK1 in differentiating *Mixl1*$^{GFP/w}$ and *Mixl1*$^{GFP/GFP}$ ES cells. In differentiating EBs from all *Mixl1* genotypes, E-cad was expressed in over 90% of cells up until day 2 of differentiation (Fig. 4A and data not shown), consistent with its expression in the inner cell mass, epiblast and primitive streak in vivo (Ciruna and Rossant, 2001; Huber et al., 1996). Between day 2 and day 4, the proportion of E-cad$^+$ cells fell to under 20%, reflecting loss expression in emerging mesoderm (Fig. 4B). The first cells expressing GFP were invariably E-cad$^+$, consistent with the expected phenotype of primitive streak cells (Ciruna and Rossant, 2001; Huber et al., 1996) (Fig. 4B). As differentiation progressed, an increasing proportion of GFP$^+$ cells lost E-cad expression, suggesting that they had 'passed through' the streak. There was no significant difference in the E-cad expression profiles between *Mixl1*$^{GFP/w}$ and *Mixl1*$^{GFP/GFP}$ EBs (Fig. 4A and data not shown).

The profile of *Flk1* expression was examined to determine the relationship between *Mixl1* expression and the earliest ventral mesoderm cells (Fig. 5A). Trace amounts of *Flk1* expression were first detected on day 2 of differentiation. In wild-type ES cells, the frequency of *Flk1*-expressing cells increased rapidly between day 3 and day 4 to ~70%, then fell to ~20% by day 6. In both *Mixl1*$^{GFP/w}$ and *Mixl1*$^{GFP/GFP}$ EBs, a small population of GFP$^-$FLK1$^+$ cells was consistently detected at day 2.5 (Fig. 5A). By day 3, differences between the *Flk1* expression of *Mixl1*$^{GFP/w}$ and *Mixl1*$^{GFP/GFP}$ lines became apparent, with *Mixl1*$^{GFP/GFP}$ EBs showing a reduced proportion of FLK1$^+$ and GFP$^+$FLK$^+$ cells. This difference was more evident at day 4 of differentiation. Although the frequency of *Flk1*$^+$ cells was similar in *Mixl1*$^{w/w}$ (69.5%) and *Mixl1*$^{GFP/w}$ (57.8%) EBs, the frequency of *Flk1*$^+$ cells was lower in *Mixl1*$^{GFP/GFP}$ EBs (28.2%) (Fig. 5A). Moreover, in the example shown, only 29% of GFP$^+$ cells from day 4 *Mixl1*$^{GFP/GFP}$ EBs expressed *Flk1* compared with 61% from *Mixl1*$^{GFP/w}$ EBs, suggesting that *Mixl1* was required to generate normal proportions of FLK1$^+$ cells (Fig. 5A). These observations were confirmed in experiments using independently derived *Mixl1* heterozygous and null ES cell clones (Fig. 5B,C). In wild-type and *Mixl1*$^{GFP/w}$ EBs, the

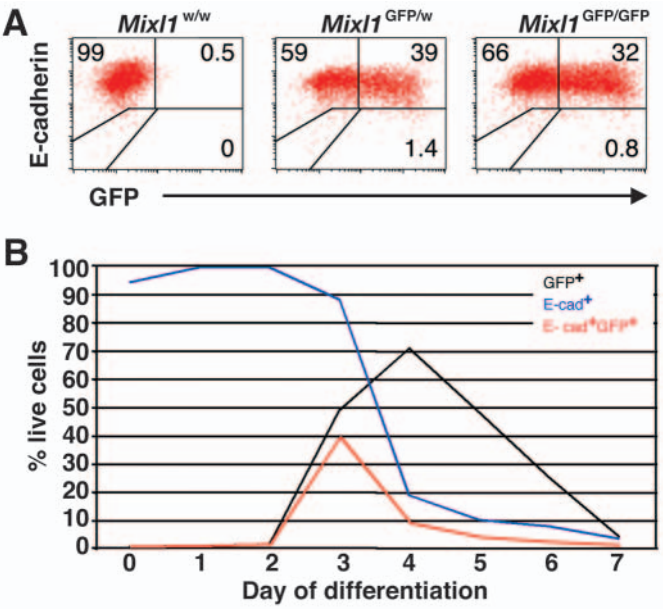

Fig. 4. Expression of E-cadherin and GFP in differentiating *Mixl1* wild-type (*Mixl1*$^{w/w}$), heterozygote (*Mixl1*$^{GFP/w}$) and null (*Mixl1*$^{GFP/GFP}$) ES cells. (A) Flow cytometric analysis of day 3 EBs showed that more than 98% of cells were E-cadherin positive and more than a third of these also expressed GFP from the *Mixl1* locus. (B) Time course of E-cadherin and GFP expression in differentiating *Mixl1*$^{GFP/W}$ ES cells. Initially, E-cadherin-positive (E-cad$^+$) stem cells differentiate into a transient double-positive population (E-cad$^+$ GFP$^+$) corresponding to cells at the primitive streak stage of development. After day 3, *Mixl1*-expressing (GFP$^+$) nascent mesoderm downregulated E-cadherin.

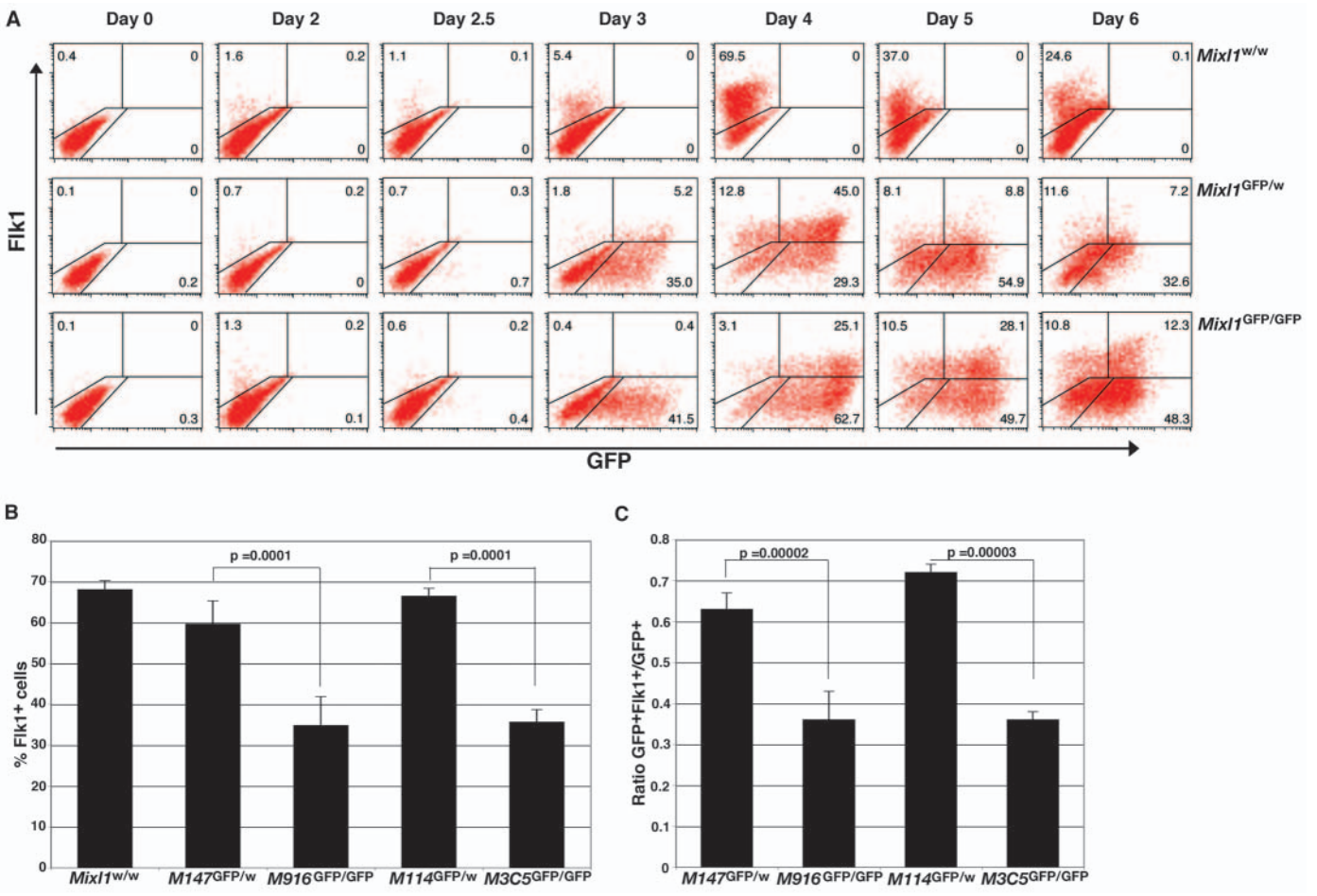

Fig. 5. Expression of FLK1 and GFP in differentiating *Mixl1* wild-type (w/w), heterozygote (GFP/w) and null (GFP/GFP) ES cells. (A) Time course of FLK1 and GFP expression showing that the onset of FLK1 expression is delayed and that the frequency of FLK1-positive cells was reduced in *Mixl1*-deficient ES cells. (B,C) Summary of data (n=3-6) demonstrating a lower percentage of FLK1$^+$ cells (B) and a reduction in the ratio of double-positive GFP$^+$FLK1$^+$ to GFP$^+$ cells (C) in day 4 EBs derived from two independent *Mixl1*-deficient ES cell lines compared with their heterozygote counterparts. The *Mixl1*-null lines M916 and M3C5 were derived from *Mixl1*-heterozygous ES cell lines M147 and M114, respectively. Mixl1$^{w/w}$ is the parental ES cell line. The error bars represent 1 s.d. and the P values indicated were derived using a two-tailed t-test.

frequency of *Flk1*-expressing cells declined after day 4. By comparison, the highest frequency of *Flk1*$^+$ cells in *Mixl1*-null EBs occurred 1 day later, at day 5 of differentiation (Fig. 5A).

Mixl1-null EBs have a reduced frequency of haematopoietic colony forming cells

In order to ascertain whether the abnormal *Flk1* expression profile in *Mixl1*$^{GFP/GFP}$ EBs foreshadowed a later haematopoietic defect, day 6 EBs were analysed for expression of the haematopoietic stem cell/endothelial marker CD34 and the late erythroid marker Ter119 (Fig. 6A). Although there was no significant difference in the frequency of CD34-expressing cells, EBs derived from *Mixl1*-null ES cells invariably contained very small numbers of Ter119-positive cells and did not undergo visible haemoglobinization, indicating reduced formation of terminally differentiated erythroid cells.

To determine whether the reduced ability of *Mixl1*-null EBs to generate haemoglobinised erythroid cells reflected an underlying defect in haematopoietic progenitors, the frequency of haematopoietic colony-forming cells (CFCs) was examined in day 4 EBs (Fig. 6B). In experiments using two independent *Mixl1*$^{GFP/w}$ and *Mixl1*$^{GFP/GFP}$ ES cell lines, we found that *Mixl1*-null EBs contained significantly fewer blast (BL)-CFCs and primitive erythroid progenitors (EryP) than did *Mixl1*-heterozygous EBs. This reduction in the frequency of CFCs was also evident in *Mixl1*-null EBs at day 3, and persisted in day 6 and day 7 EBs despite an increase in the frequency of FLK1$^+$ cells at these later time points (data not shown). These data confirmed that the absence of *Mixl1* impaired haematopoiesis in differentiating ES cells.

Blast colony forming cells are enriched in the GFP$^+$FLK1$^+$ population from day 4 *Mixl1*$^{GFP/w}$ EBs

In order to further explore the relationship between *Mixl1* expression and haematopoietic differentiation, the blast colony-forming ability of sorted fractions of anti-FLK-1 labelled *Mixl1*$^{GFP/w}$ day 3 and day 4 EBs were compared. FACS sorted GFP$^-$FLK1$^-$, GFP$^+$FLK1$^-$, GFP$^+$FLK1$^+$ and GFP$^-$FLK1$^+$ populations were cultured in methylcellulose in the presence or absence of vascular endothelial growth factor (VEGF) and stem cell factor (SCF) in order to detect haematopoietic blast colonies (Kennedy et al., 1997).

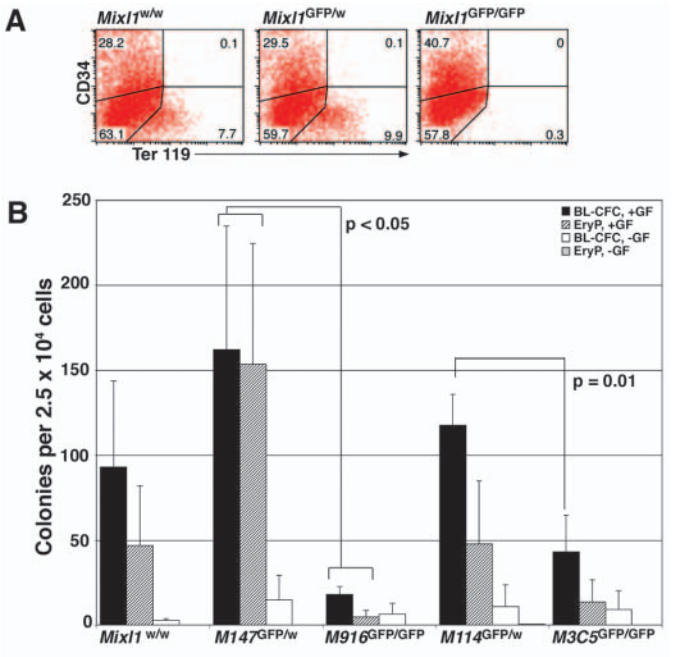

Fig. 6. Comparison of haematopoietic development from differentiating *Mixl1* (w/w), heterozygote (GFP/w) and null (GFP/GFP) ES cells. (A) Flow cytometric analysis of day 6 EBs showing *Mixl1*-deficient cells failed to efficiently generate TER119-positive erythroid precursors despite their ability to form significant numbers of CD34-positive cells. (B) Summary of methylcellulose culture experiments (n=3) indicating that *Mixl1*-deficient day 4 EBs contained significantly fewer blast colony-forming cells (BL-CFCs) and primitive erythroid precursors (EryP). Data were derived from experiments performed with two independent *Mixl1*-deficient ES cell lines and their wild-type and heterozygote counterparts. The *Mixl1*-null lines M916 and M3C5 were derived from *Mixl1*-heterozygous ES cell lines M147 and M114 respectively. Mixl1$^{w/w}$ is the parental ES cell line. The error bars represent 1 s.d. and the P values indicated were derived using a two-tailed t-test. GF, growth factors (VEGF, SCF, IL3, EPO). No EryP colonies were seen in the absence of growth factors (–GF).

Interestingly, BL-CFCs (Fig. 7A-D) were cultured from all sorted fractions from day 3 EBs, although the highest frequency was observed in the GFP$^+$ fractions (219 colonies/5×10^4 GFP$^+$FLK1$^-$ cells and 225/5×10^4 GFP$^+$FLK1$^+$ cells) (Fig. 7E). Irrespective of their population of origin, all the blast cell colonies displayed similar morphology, dependence on VEGF and SCF and ability to give rise to haematopoietic and adherent vascular cells. The endothelial nature of the adherent cells was verified by staining with antibodies to PECAM1 or FLK1 (see Fig. 7A-D; data not shown). Consistent with the transient nature of BL-CFCs (Kennedy et al., 1997), their frequency was considerably decreased in day 4 EBs (Fig. 7F). At this time, the majority of BL-CFCs were found in the GFP$^+$FLK1$^+$ fraction, although some were present in the GFP$^-$FLK1$^+$ population (Fig. 7F).

GFP$^+$ cells are generated until day 4 of differentiation and contain precursors for GFP$^+$FLK1$^+$ but not GFP$^-$FLK1$^+$ cells

In order to dissect the developmental relationship between the GFP- and FLK1-expressing cells and to rationalize the emergence of haematopoietic blast colonies from FLK$^-$ populations, sorted fractions taken at different time points were recultured and analysed. We wished to determine whether cells in the GFP$^+$FLK1$^-$ and GFP$^-$FLK1$^-$ fractions from early timepoints, in which we had already found BL-CFCs (Fig. 7E), could differentiate into FLK1$^+$ cells. This would allow us to postulate that a subset of the FLK1$^-$ cells from day 3 EBs cultured in methylcellulose in VEGF and SCF upregulated FLK1 and responded to the factors by forming BCs. At day 2.8, EBs contained only 12.0% GFP$^+$ cells and FLK1 expression was virtually absent (Fig. 8A). When overnight cultures of GFP positive and negative fractions were reanalysed, the GFP$^-$ cells had differentiated into GFP$^+$FLK1$^-$ (10.7%), GFP$^+$FLK1$^+$ (9.3%) and GFP$^-$FLK1$^+$ (25.7%) cells, and 22.7% of the initial GFP$^+$ cells had become GFP$^+$FLK1$^+$. These experiments demonstrated that day 2.8 GFP$^-$ cells had considerable differentiative capacity and that the GFP$^+$FLK1$^+$ population enriched for BL-CFCs could arise from GFP$^-$FLK1$^-$ or GFP$^+$FLK1$^-$ cells. GFP$^-$FLK1$^-$ cells from day 3 EBs also possessed the capacity to give rise to GFP$^-$FLK1$^+$, GFP$^+$FLK1$^-$ and GFP$^+$FLK1$^+$ cells (Fig. 8B). Interestingly, it appeared that some FLK1$^+$ cells arose directly from the GFP$^-$FLK1$^-$ population without passing through an intermediate GFP$^+$ stage. FLK1 expression was also observed in 6.3% of re-cultured GFP$^+$FLK1$^-$ cells, indicating the persistence of a subset of the GFP$^+$ cells with the ability to develop into GFP$^+$FLK1$^+$ precursors. Conversely, GFP$^-$FLK1$^+$ cells rarely turned on GFP expression. Consistent with the transient nature of FLK1 expression during EB differentiation (see Fig. 5A), the majority of recultured GFP$^+$FLK1$^+$ and GFP$^-$FLK1$^+$ populations actually lost FLK1 expression. Reanalysis of sorted day 4 EBs, revealed that the GFP$^-$FLK1$^-$population was no longer capable of differentiating into GFP$^+$ or FLK1$^+$ cells (Fig. 8C). One third of recultured GFP$^+$FLK1$^-$ cells lost GFP expression and this population displayed minimal ability to develop into new GFP$^+$FLK1$^+$ cells. Although most GFP$^+$FLK1$^+$ cells no longer expressed FLK1 after overnight culture, a distinct subset of GFP$^-$FLK1$^+$ cells was seen, indicating that some GFP$^-$FLK1$^+$ cells derived from GFP$^+$FLK1$^+$ precursors. This experiment also showed that ~70% of recultured GFP$^-$FLK1$^+$ cells lost FLK1 expression by day 5.

These data demonstrated that GFP$^+$ and FLK1$^+$ cells were only generated between d2.8 and day 4 of ES cell differentiation, the same time frame during which BL-CFCs develop from FLK1$^+$ precursors (Chung et al., 2002; Fehling et al., 2003). Although most of the BL-CFCs were cultured from GFP$^+$FLK1$^+$ cells in day 4 EBs, the considerable capacity of GFP$^-$FLK1$^-$ and GFP$^+$FLK1$^-$ cells at earlier time points to differentiate into FLK1$^+$ cells provided an explanation for our ability to derive VEGF-dependent BL-CFCs from all day 3 EB fractions (Fig. 7E) and reconciled our findings with data of others who had shown that BC-CFCs expressed FLK1 on their cell surface (Chung et al., 2002; Fehling et al., 2003).

BMP4 induces GFP and FLK1 expression and augments survival in *Mixl1*$^{GFP/w}$ ES cells differentiated in SF medium

Studies in *Xenopus laevis* demonstrated that Mix-family genes were directly induced by both activin and BMP4 (Mead et al., 1996; Rosa, 1989; Vize, 1996). Therefore, we examined the

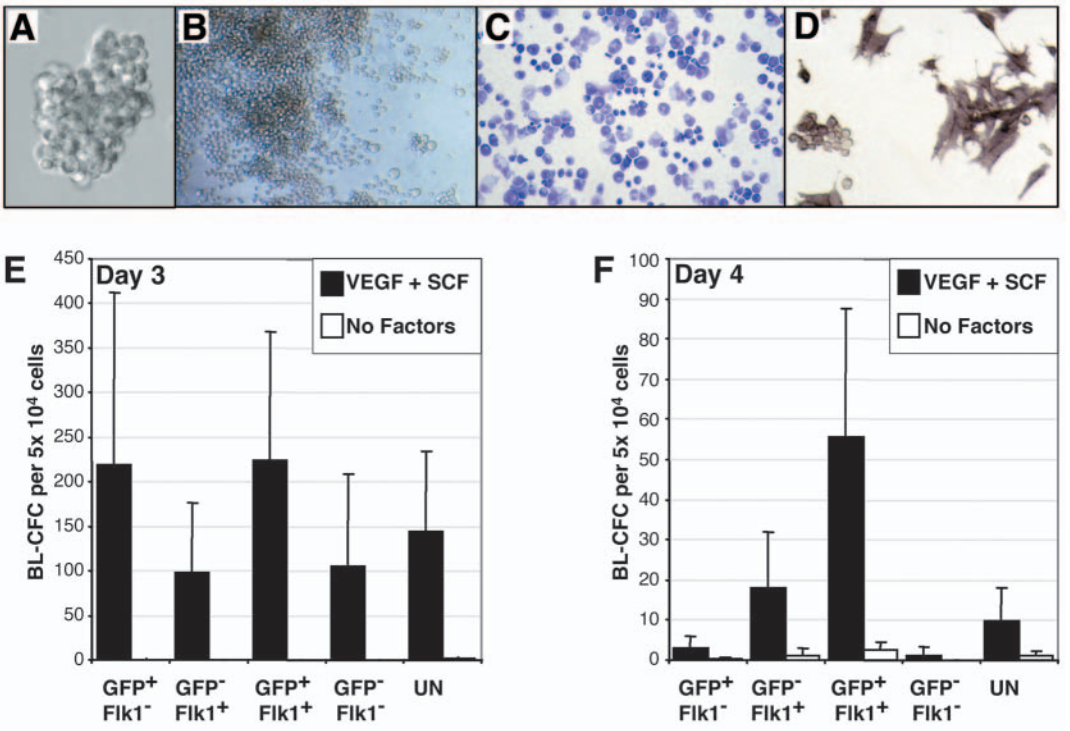

Fig. 7. (A-D) Blast colonies develop into haematopoietic and endothelial cells. Blast colonies (A), typically composed of 50-200 round cells emanating from a dense core, proliferated in liquid culture (B) supplemented by VEGF, SCF, IL3 and EPO to give haematopoietic cells of myeloid and erythroid lineages (C, May-Grunwald-Giemsa stained cytocentrifuge preparation) and adherent endothelial cells (D) visualized by antibodies to CD31. (E,F) Frequency of blast colony-forming cells (Blast-CFCs) in GFP^+FLK1^+, GFP^+FLK1^-, GFP^-FLK1^+, GFP^-FLK1^- populations of cells isolated from differentiating *Mixl1*$^{GFP/w}$ ES cells. (E) In day 3 EBs, Blast-CFCs were distributed throughout the four populations, reflecting the dynamic nature of the differentiation process at this time (see Discussion). (F) By day 4, the GFP^+FLK1^+ fraction contained the highest frequency and the majority of Blast-CFCs. UN, unsorted. Error bars represent 1 s.d. (n=3 for day 3; n=4 for day 4).

ability of these factors to induce GFP expression in *Mixl1*-heterozygous and *Mixl1*-null ES cells. ES cells differentiated in SF medium (Johansson and Wiles, 1995) in the absence of growth factor supplements failed to express GFP or FLK1, but lost expression of the stem cell marker E-cad (Fig. 9C,E and data not shown), consistent with a default to neurectodermal differentiation (Wiles and Johansson, 1999).

Supplementation of the SF medium with activin A at increasing concentrations resulted in a small, dose-dependent increase in the percentage of GFP-expressing cells in day 4 EBs (Fig. 9A). By contrast, BMP4 was far more potent at inducing GFP expression. More than 50% of *Mixl1*$^{GFP/w}$ EB cells exposed to 1 ng/ml BMP4 were GFP^+ at day 4 compared with a maximum of 10% GFP^+ cells seen when EBs were cultured in the presence of 100 ng/ml activin A (Fig. 9A,B).

Comparison of GFP induction in *Mixl1*$^{GFP/w}$ cells in serum containing and SF media revealed that the appearance of GFP-expressing cells was delayed in SF media supplemented with BMP4 (SF/BMP4) and that the peak percentage of GFP^+ cells was reduced (Fig. 9C). Interestingly, supplementation of SF medium with BMP4 significantly increased the viability of cells at day 4 and 5 of differentiation (Fig. 9D). Moreover, the augmentation of cell survival in SF medium by BMP4 occurred prior to the induction of GFP expression (data not shown).

Induction of GFP expression was examined in *Mixl1*$^{GFP/w}$ and *Mixl1*$^{GFP/GFP}$ EBs cultured in SF medium in the absence or presence of BMP4 (Fig. 9E). GFP expression was seen in 46% of *Mixl1*$^{GFP/w}$ and 55% of *Mixl1*$^{GFP/GFP}$ cells in day 4 EBs cultured in SF/BMP4. FLK1 expression was reduced and delayed in the *Mixl1*$^{GFP/GFP}$ cells, as observed in serum containing medium. In contrast to the results for serum-containing cultures in which the highest percentage of $FLK1^+$ cells coincided with maximal GFP expression (Fig. 5A), the frequency of $FLK1^+$ cells in SF/BMP4 medium continued to increase for at least 3 days after the peak in GFP expression (Fig. 9E). In day 4 EBs, clonogenic haematopoietic progenitor cells were ~30-fold less frequent in SF/BMP4 than in serum differentiated EBs, further indicating that the addition of 5 ng/ml BMP4 was insufficient to completely replicate the effects of serum (compare Fig. 6B with Fig. 9F). One tenth the number of CFCs were observed in the *Mixl*-null cell lines, consistent with the results obtained from experiments using serum containing medium (Fig. 6B). Despite the observation that, by day 7, *Mixl1*$^{GFP/w}$ and *Mixl1*$^{GFP/GFP}$ EBs contained a similar percentage of $FLK1^+$ cells, the frequency of CFCs was still lower in EBs derived from *Mixl1*-null cells (Fig. 9F). These data establish a link between activin A and BMP4 signalling and *Mixl1* expression in mammalian cells. Furthermore, analysis of *Mixl1*-null cells in SF medium demonstrates that *Mixl1* is required for normal BMP4-dependent expression of *Flk1* and development of haematopoietic CFCs.

Discussion

Mixl1 expression identifies primitive streak mesodermal progenitors

In accordance with the results of others (Keller et al., 1993; Lacaud et al., 2002; Robertson et al., 2000), we found that EB formation was characterised by the sequential expression of genes associated with the initial developmental stages of postimplantation embryos. The expression of stem cell genes

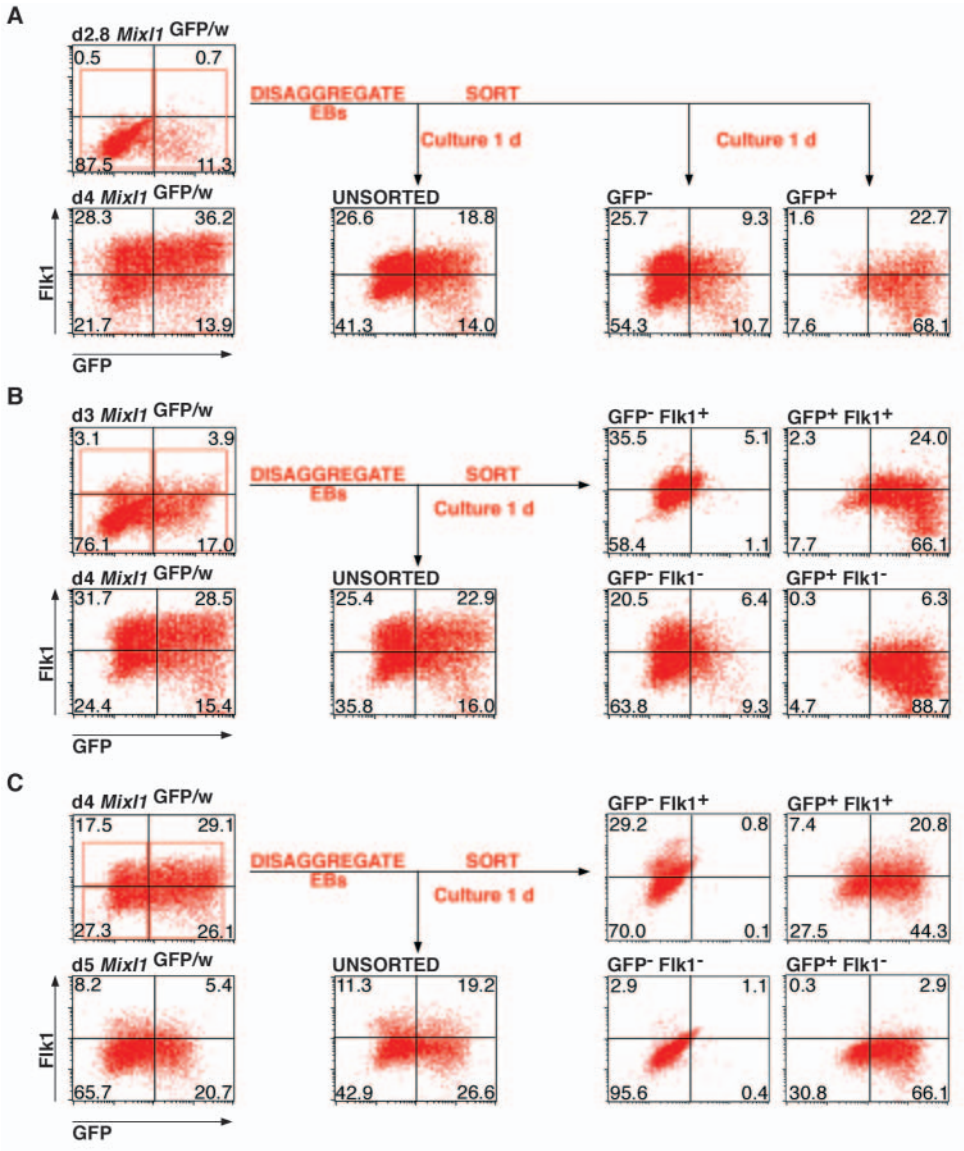

Fig. 8. Experiments examining the developmental relationship between GFP$^+$FLK1$^+$, GFP$^+$FLK1$^-$, GFP$^-$FLK1$^+$ and GFP$^-$FLK1$^-$ populations isolated from differentiating *Mixl1*$^{GFP/w}$ ES cells at day 2.8 (A), day 3 (B) and day 4 (C). At time points shown, a sample was removed from each culture and the EBs disaggregated. Cells were sorted into populations based on expression of GFP and FLK1 and recultured overnight. The following day, the sorted and unsorted populations, as well as EBs, from the continuing differentiation cultures were analysed for expression of GFP and FLK1.

was gradually lost as primitive streak genes, such as *Mixl1*, brachyury and goosecoid, were induced. The restricted expression of these genes between day 3 and day 4 indicated that the ES cells underwent a process of 'molecular gastrulation', during which genes normally expressed during embryonic gastrulation were transiently expressed. This was followed by the upregulation of genes associated with ventral mesoderm and haematopoiesis. The kinetics of GFP induction in differentiating *Mixl1*$^{GFP/w}$ EBs was consistent with the PCR gene expression data. There was a higher proportion of *Mixl1*-expressing cells in EBs (85% at day 4) compared with an embryo at the equivalent developmental stage (Hart et al., 2002). In fact, studies by Keller and colleagues (Fehling et al., 2003) demonstrated that a similarly high percentage of differentiating ES cells expressed another primitive streak gene, brachyury. Also consistent with the results of others (Chung et al., 2002; Kabrun et al., 1997; Nishikawa et al., 1998), we observed that a large percentage of differentiating ES cells (up to 70%) expressed the ventral mesoderm marker *Flk1*, consistent with the majority of primitive streak cells being directed towards the haematopoietic lineages. These data indicate that, in serum containing cultures, the differentiation of mouse ES cells is biased towards the formation of 'primitive streak' cells and, subsequently, ventral mesoderm.

The appearance of GFP$^+$ cells in *Mixl1*-null EBs mirrored that seen in EBs from the *Mixl1*-heterozygous line, consistent with the assumption that 'pre gastrulation' events would be unchanged in the absence of *Mixl1*. The higher peak percentage of GFP$^+$ cells and the prolongation of GFP expression seen in *Mixl1*-null cells could be explained by the presence of two GFP alleles. However, an alternative hypothesis is that these features represented an in vitro correlate of the expanded primitive streak and delayed egress of GFP$^+$ nascent mesendodermal cells observed in *Mixl1*-null embryos (Hart et al., 2002). Definitive exclusion of this scenario will require analysis of *Mixl1*-null ES cells that carry only one GFP allele.

In order to place *Mixl1* expression during ES cell differentiation into a developmental context, flow cytometry was used to correlate the expression of GFP in *Mixl1*$^{GFP/w}$ EBs with the expression of E-cadherin, a cell-adhesion molecule expressed in the epiblast of the mouse embryo (Burdsal et al., 1993). Experiments by Ciruna and Rosssant showed that expression of E-cadherin was downregulated as cells passed through the primitive streak and was lost by the time they underwent the epithelial mesenchymal transition required for migration from the streak (Ciruna and Rossant, 2001). Therefore, cells co-expressing E-cadherin and *Mixl1* during EB differentiation could be regarded as a primitive streak like population. Indeed, flow cytometric analysis of E-cadherin and GFP during differentiation of *Mixl1*$^{GFP/w}$ ES cells allowed the identification of cells reminiscent of the embryonic stages of epiblast (GFP$^-$E-cad$^+$), primitive streak (GFP$^+$E-cad$^+$) and nascent mesoderm (GFP$^+$E-cad$^-$). By day 4 of ES differentiation, most

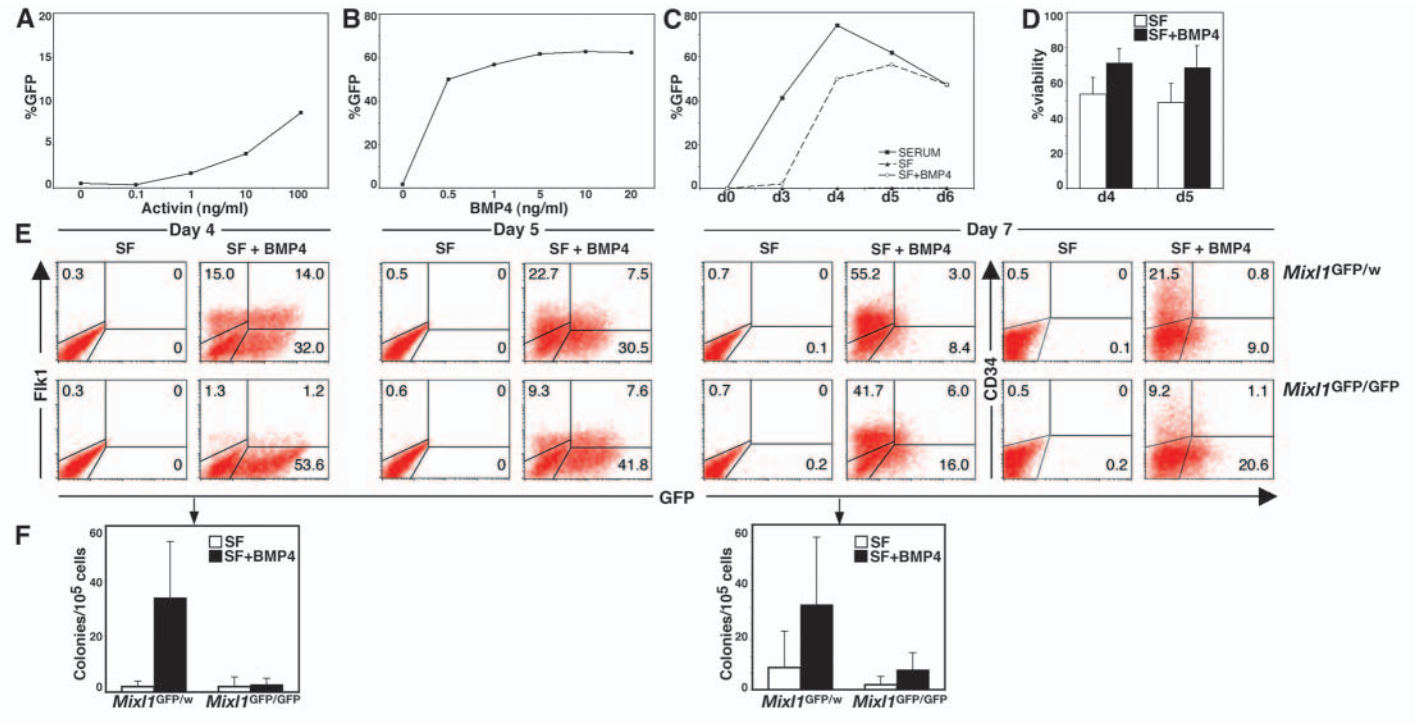

Fig. 9. Differentiation of *Mixl1* heterozygote (GFP/w) and null (GFP/GFP) ES cells in serum-free (SF) media supplemented with BMP4 or activin A. In SF media, activin A (A) was a weaker inducer of GFP expression in *Mixl1*$^{GFP/w}$ cells than BMP4 (B). In comparison with serum-induced differentiation (C), the magnitude of the response to SF+BMP4 media was less and GFP induction was delayed. There was no GFP expression in SF media alone. (D) BMP4 increased the percentage of viable cells isolated from day 4 and day 5 cultures of ES cells differentiated in SF media (P<0.01). Error bars represent 1 s.d. and P values were calculated using a two-tailed t-test (n=7). (E) The previously observed defect (see Fig. 5A) in the ability of *Mixl1*-null ES cells to generate FLK1$^+$ cells at day 4 was exacerbated in SF+BMP4 differentiation cultures. At d7, differences in frequency of FLK1-positive cells between the two cell lines had diminished and both cell lines were able to generate CD34-positive cells. (F) Summary of methylcellulose culture experiments showing that *Mixl1*-deficient ES cells generated fewer haematopoietic CFCs in SF+BMP4 differentiation cultures than their heterozygous counterparts.

GFP$^+$ cells expressed the haematopoietic marker *Flk1* and only ~20% of cells were still E-cad$^+$. In fact, as reported by Nishikawa et al. (Nishikawa et al., 1998), we found that FLK1$^+$ and E-cad$^+$ populations were mutually exclusive (data not shown).

Mixl1 is required for efficient haematopoiesis

In heterozygous *Mixl1*$^{GFP/w}$ ES cells, *Flk1* was expressed immediately following GFP and both genes were maximally expressed in day 4 EBs. In *Mixl1*-null cells, the onset of *Flk1* expression was delayed and, even though the frequency of FLK1$^+$ cells increased by day 4, normal haematopoiesis was not established, irrespective of whether the cells were differentiated in serum-based or SF medium (compare Fig. 5 with Fig. 9). This was most obviously evidenced by the failure of visible haemoglobinisation of day 7 *Mixl1*-null EBs. Also, the large reduction in the frequency of blast and erythroid colonies generated from day 4 *Mixl1*$^{GFP/GFP}$ EBs suggested that the haematopoietic defect could not simply be explained by the lower percentage of FLK1$^+$ cells at that time. This conclusion is consistent with analysis of *Flk1*-null ES cells showing that *Flk1* was not necessary for the development of haematopoietic cells in vitro (Ema et al., 2003; Schuh et al., 1999). These data indicated that *Mixl1* expression was required for the efficient generation of haematopoietic cells and placed *Mixl1* functionally upstream of a FLK1$^+$ haematopoietic precursor. Interestingly, recent studies showed that the haematopoietic defect associated with deficiency of the transcription factor *Scl* could only be rescued by expressing *Scl* by day 3 of ES cell differentiation, contemporaneous with the expression of *Mixl1* but antedating expression of *Flk1* (Endoh et al., 2002). Collectively, these data predict that the specification of haematopoietic precursors occurs early in gastrulation and requires the input of a number of transcription factors, including *Mixl1* and *Scl*.

The consequences of *Mixl1*-deficiency were reminiscent of the phenotype observed in the absence of *Fgfr1* or *Fgf8*, the predominant FGF family member expressed in the gastrulating embryo. Specifically, embryos lacking *Fgfr1* exhibited an enlarged primitive streak due to a failure of progenitor cell migration (Ciruna and Rossant, 2001; Ciruna et al., 1997; Deng et al., 1994; Yamaguchi et al., 1994) and *Fgfr1*-deficient ES cells displayed a marked impairment in haematopoietic colony formation in vitro (Faloon et al., 2000). Similarly, *Fgf8*-null embryos also displayed a cell migration defect in the primitive streak and showed evidence of perturbed haematopoiesis, with reduced expression of the erythroid and endothelial markers *Fog* and PECAM in the yolk sac (Sun et al., 1999). Collectively, these data speak to the requirement for both *Mixl1* expression and FGF signalling in normal streak morphogenesis and haematopoietic specification. As the expression patterns of *Fgf8* and *Mixl1* overlapped both in the primitive streak

(Crossley and Martin, 1995; Pearce and Evans, 1999; Robb et al., 2000) and in differentiating EBs (C. Hirst, A. Mossman and A.G.E., unpublished), we are investigating the possibility of a direct link between these two molecules.

Haemangioblasts arise from a *Mixl1*-expressing mesodermal progenitor

Primitive erythroid and endothelial cells arise from a common mesodermal precursor, the haemangioblast, most convincingly identified as a transient FLK1$^+$ population in day 2.75-4.00 EBs (Choi et al., 1998; Chung et al., 2002; Fehling et al., 2003; Kennedy et al., 1997; Nishikawa et al., 1998). Furthermore, Fehling et al. (Fehling et al., 2003) showed that in day 3.5 EBs, haemangioblasts arose exclusively from brachyury-positive precursors and that brachyury$^+$FLK1$^+$ cells from day 2.5 EBs were enriched for BL-CFCs. Given the overlapping expression of brachyury and *Mixl1* in differentiating ES cells (Kubo et al., 2004) (this study) and during embryogenesis, we anticipated that haemangioblasts would arise from a *Mixl1*-positive precursor and be enriched in GFP$^+$FLK1$^+$ cells from *Mixl1*$^{GFP/w}$ EBs. Indeed, when cells were sorted from day 4 *Mixl1*$^{GFP/w}$ EBs, most BL-CFCs were found in the GFP$^+$FLK1$^+$ fraction. Interestingly, some BL-CFCs were detected in the GFP$^-$FLK1$^+$ population from day 4 EBs. This might have represented an artefact of the sorting process, with some GFP-dull cells inadvertently included in the GFP$^-$FLK1$^+$ fraction. Alternatively, BL-CFCs did not express *Mixl1* and might have expressed alternate 'gastrulation' genes such as brachyury. Indeed, in ES cells in which GFP was targeted to the brachyury locus, essentially all the FLK1$^+$ cells were GFP$^+$ (Fehling et al., 2003).

The frequency of BL-CFCs was much higher in cells sorted from day 3 EBs but there was not a statistically significant enrichment in the GFP$^+$FLK1$^+$ population. Because overnight culture of sorted cells demonstrated a narrow window between day 2.8 and day 4, during which GFP$^-$FLK1$^-$ and GFP$^+$FLK1$^-$ cells could continue to differentiate, cells that fell into the either of these populations at the time of sorting at day 3 could have upregulated *Mixl1* and/or *Flk1* expression after seeding in methylcellulose and produced VEGF-dependent blast cell colonies. However, in other studies that compared the BL-CFC content of FLK1$^+$ and FLK1$^-$ populations sorted from day 3 EBs, a clearer enrichment of BL-CFC in the FLK1$^+$ cells was observed (Chung et al., 2002; Faloon et al., 2000; Fehling et al., 2003). It is possible that differences in the kinetics of FLK1 expression between the various ES cell lines used or differences in the ability of the methylcellulose cultures to support further differentiation may explain this discrepancy.

BMP4 and activin A induce *Mixl1* in vitro

We used a SF culture system in order to determine which growth factors regulate 'molecular gastrulation' and subsequent haematopoietic commitment in differentiating ES cells. In this context, the *Mixl1*$^{GFP/w}$ ES cells provided a simple means to identify live cells at the primitive streak stage by fluorescence microscopy and by flow cytometry. Previous studies in *Xenopus* have shown that *Mix.1*, the closest homologue to mammalian *Mixl1*, was induced by either activin A or BMP4 (Mead et al., 1996; Rosa, 1989). In fact, more recently, Kubo et al. (Kubo et al., 2004) used a two-step SF culture system to show that *Mixl1* RNA could be induced by activin A. In our culture system, although both activin and BMP4 induced GFP with similar kinetics in *Mixl1*$^{GFP/w}$ EBs, the percentage of GFP$^+$ cells was much greater in BMP4-treated cultures. The modest expression of *Mixl1* in response to activin A contrasted with the robust induction of brachyury GFP by this factor reported by Keller and colleagues (Fehling et al., 2003). Indeed, in their original description of the SF medium used in our studies, Johansson and Wiles demonstrated that brachyury was more readily induced by activin than by BMP4 (Johansson and Wiles, 1995). These data suggest that aspects of TGFβ signalling may be conserved between mouse ES cells and *Xenopus* embryos, in which activin induced both brachyury and *Mix.1*, and BMP4 treatment of animal pole cells induced *Mix.1* (Cunliffe and Smith, 1992; Mead et al., 1996; Smith et al., 1991).

The addition of BMP4 to ES cells differentiated in SF cultures led to an increase in total cell numbers and an improvement in cell viability that antedated *Mixl1* expression (Fig. 9D) (E.S.N. and A.G.E., unpublished), corresponding to the growth-promoting effect of BMP4 on epiblast cells prior to gastrulation (Beppu et al., 2000; Mishina et al., 1995; Winnier et al., 1995).

Park et al. (Park et al., 2004) recently described *Flk1* induction by BMP4 in a different SF medium. However, because brachyury was expressed in their EBs prior to the addition BMP4, it was unclear whether BMP4 induced mesoderm or simply acted to ventrally pattern existing mesoderm. We also observed the emergence of *Flk1*$^+$ ventrally patterned mesoderm in *Mixl1*$^{GFP/w}$ EBs cultured in SF/BMP4 medium. Our data showed unambiguously that induction of the primitive streak marker *Mixl1* was completely dependent on the inclusion of BMP4 in the culture medium, strengthening the link between BMP4 and mesoderm induction in mammalian cells suggested by Johansson and Wiles (Johansson and Wiles, 1995; Wiles and Johansson, 1997). Nevertheless, as others have shown (Park et al., 2004), additional growth factors, such as VEGF, are required to efficiently generate haematopoietic CFCs from BMP4-induced FLK1$^+$ ventral mesoderm.

In conclusion, we have shown that differentiating ES cells express *Mixl1* as they transit through a stage equivalent to the primitive streak of the gastrulating mouse embryo. The most primitive haematopoietic BL-CFCs arise from *Mixl1*-expressing cells and absence of *Mixl1* disrupts normal haematopoiesis. We have demonstrated that BMP4 augments survival, induces *Mixl1* expression and ventrally patterns mesoderm in differentiating EBs. Finally, the *Mixl1*$^{GFP/w}$ ES cell lines will be valuable tools in further elucidating the factors that regulate mesoderm and endoderm formation.

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