Step-wise divergence of primitive and definitive haematopoietic and endothelial cell lineages during embryonic stem cell differentiation

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Abstract

Background: The developmental processes leading from the mesoderm to primitive and definitive haematopoietic and endothelial lineages, although of great importance, are still poorly defined. Recent studies have suggested a model in which common precursors give rise to endothelial progenitors and haematopoietic progenitors, the latter subsequently generating both primitive and definitive haematopoietic lineages. However, this model is contradicted by findings that suggest the emergence of haematopoietic cells from the endothelial lineage.

Results: We found sequential steps in the differentiation of FLK1⁺ mesoderm into haematopoietic and endothelial lineages in an in vitro differentiation system of embryonic stem (ES) cells: (i) the GATA-1⁺ subset of FLK1⁺ mesodermal cells loses the capacity to give rise to endothelial cells and is restricted to primitive erythroid, macrophage and definitive erythroid progenitors; (ii) the remaining GATA-1⁻ cells give rise to VE-cadherin⁺ endothelial cells; and subsequently (iii) multiple definitive haematopoietic progenitors and endothelial cells branch off from a subset of VE-cadherin⁺ cells.

Conclusions: These observations strongly suggest that the divergence of primitive and multilineage definitive haematopoietic/endothelial lineages occurs first, and then multilineage definitive haematopoietic progenitors arise from VE-cadherin⁺ endothelial cells in the development of haematopoietic and endothelial cells.

Introduction

During vertebrate development, haematopoiesis occurs in successive waves at distinct anatomical sites. In the developing mouse embryo, the formation of blood begins in the extra-embryonic yolk sac at day 7.5 of gestation (Moore & Metcalf 1970). Aggregates of mesodermal cells form blood islands; endothelial cells differentiate from the edges of the aggregates, while primitive erythrocytes arise from the central region. Following the process known as primitive haematopoiesis, the major haematopoietic site shifts to the foetal liver at day 10 of gestation, and thereafter to the bone marrow at days 15–16 of gestation (termed definitive haematopoiesis) (Ogawa et al. 1988; Delassus & Cumano 1996). Definitive haematopoietic progenitors and stem cells have been found in distinct sites in the embryo, including the umbilical and vitelline arteries, the para-aortic splanchnopleure and aorta-gonado-mesonephros (AGM) region (Muller et al. 1994; Cumano et al. 1996; Medvinsky & Dzierzak 1996). Histological analyses revealed that clusters of haematopoietic cells are found associated with the endothelium on the ventral floor of the dorsal aorta (Garcia Porrero et al. 1995) at developmental stages when multipotent haematopoietic progenitors with long-term repopulating activity appear in the AGM region.

The close temporal and spatial associations between the development of haematopoietic and endothelial cell lineages have raised the hypothesis that the two lineages are derived from a common progenitor, the haemangioblast. A number of findings provide support for the existence of such a common precursor. First, several studies have demonstrated that the haematopoietic and
endothelial lineages share the expression of genes such as CD34 (Young et al. 1995), FLK1 (Eichmann et al. 1997) and TIE2 (Hagamuchi et al. 1999). Second, gene-targeting studies have revealed a complete absence of embryonic haematopoietic and endothelial lineages in the embryos lacking flk-1 (Shalaby et al. 1997). While these findings are consistent with the concept of a haemangioblast, no direct evidence has been reported for the existence of a bipotential cell that gives rise to only haematopoietic and endothelial cell lineages in developing embryos. The difficulty in collecting sufficient numbers of cells from early embryos has hampered access to the early precursors for haematopoietic and endothelial cell lineages.

This hurdle can be overcome with the use of mouse embryonic stem (ES) cells as a model for looking at the early events during the development of haematopoietic and endothelial cells. Attempts to detect early haematopoietic progenitors in the ES cell in vitro differentiation system pointed to a common precursor for primitive and definitive haematopoietic and endothelial cells (Kennedy et al. 1997; Choi et al. 1998). These findings suggested a model in which common precursors give rise to endothelial progenitors and haematopoietic progenitors, the latter subsequently generating both primitive and definitive haematopoietic lineages (Keller et al. 1999). However, this model is contradicted by recent findings which suggested the emergence of haematopoietic cells from the endothelial lineage (Nishikawa et al. 1998b).

Dissection of these processes requires characterization of the developmental potential of cells at successive intermediate stages. Taking advantage of the differential expression of cell surface markers on precursor populations, fluorescence activated cell sorting (FACS) can be utilized to progressively separate differentiated cells. This approach was employed by Faloon et al. (2000) to isolate FLK1+ cells that give rise to primitive, definitive haematopoietic and endothelial lineages. Thus, to define the diverging points of primitive, definitive and endothelial lineages, it is important to focus on the early differentiation processes of FLK1+ cells. To date, however, there are no studies documenting the cell surface markers expressed on the early FLK1+ cells destined for primitive, definitive haematopoietic or endothelial lineages. Since several transcription factors are thought to regulate the process of fate determination, they should be useful to dissect early mesoderm. Green fluorescent protein (GFP) reporter gene linked to a tissue-specific promoter of such transcription factor genes should facilitate the dissection of early FLK1+ cells.

One candidate promoter for this purpose is the haematopoietic-specific regulatory element of the transcription factor GATA-1. The transcription factor GATA-1 is a central regulator for erythroid gene transcription (Weiss & Orkin 1995; Orkin & Zon 1997) that has been shown to be expressed in the extraembryonic mesoderm prior to the establishment of the blood islands (Silver & Palis 1997). Recently, Onodera et al. (1997) identified a regulatory element that directs primitive and definitive haematopoietic-specific expression of GATA-1. This promoter has been demonstrated to be sufficient to recapitulate the expression of GATA-1 in extraembryonic mesodermal tissue as well as in erythroid cells (Onodera et al. 1997). The use of this element in a reporter gene would be useful for tracing haematopoietic cell development from the mesoderm.

In this study, we established ES cell clones stably transfected with the GFP reporter gene under the control of a haematopoietic-specific GATA-1 regulatory element (Onodera et al. 1997) and analysed the developmental processes in haematopoietic and endothelial lineages using OP9 stromal cells (Nakano et al. 1996). Through this approach, we show the sequential steps leading from mesoderm to haematopoietic and endothelial cell lineages: (i) a population characterized by the expression of GATA-1 diverges from FLK1+ mesoderm and gives rise to primitive erythroid, macrophage and definitive erythroid lineages; (ii) the remaining GATA-1− subset of FLK1+ mesoderm that does not have the developmental potential to give rise to primitive erythroid, macrophage and definitive erythroid lineages; (iii) multiple definitive haematopoietic and endothelial cell lineages branch off from the VE-cadherin+ cells.

Results

Expression of GATA-1 in extraembryonic mesoderm

Onodera et al. (1997) previously generated a transgenic (Tg) mouse which harbours the 3.9 kb of 5′ flanking sequence for IE exon through to the GATA-1 translational initiation codon ligated to the LacZ gene. This Tg mouse has been demonstrated to exhibit LacZ expression in 7.0–7.5 dpc embryos. In order to investigate whether or not GATA-1 expression can further dissect mesoderm, we analysed LacZ activity in 7.5 dpc embryos. As previously demonstrated (Onodera et al. 1997), a ring of LacZ expression was observed in the proximal region of the yolk sac (data

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Genes to Cells (2001) 6, 1113–1127 © Blackwell Science Limited
To determine the cellular component exhibiting LacZ activity, Tg mouse embryos were analysed histologically. LacZ expression was detected in the vast majority of haematopoietic cells in blood islands (Fig. 1Aa). It is also noteworthy that the extraembryonic mesodermal component also exhibited LacZ staining (Fig. 1Ab,d). In contrast, no LacZ activity was observed in the embryonic body (data not shown).
not shown). To verify LacZ activity in the extraembryonic mesodermal component, sections were stained with anti-FLK1 mAb, because FLK1 is expressed predominantly in extraembryonic mesodermal cells in 7.5 dpc embryos (Kataoka et al. 1997). Of 170 FLK1+ cells in the mesodermal component (Fig. 1Ac,e), 19% were LacZ+, suggesting that the extraembryonic mesoderm can be further divided by GATA-1 expression at the early stages of haematopoietic differentiation. Interestingly, of 568 LacZ+ haematopoietic cells in the blood islands, 31% were FLK1+ cells. This indicates that FLK1 is expressed in a part of the primitive erythroid progenitors as previously reported (Kabrun et al. 1997). Taken together, these findings strongly suggest that a part of FLK1+ extraembryonic mesoderm in the yolk sac express GATA-1.

Expression of the GATA-1 promoter-driven GFP during ES cell differentiation

The next question is the developmental potential of the LacZ-positive or -negative subset of FLK1+ mesodermal cells. However, it has been extremely difficult to collect a sufficient number of cells for fate analyses from the 7.5 dpc embryos of the Tg mouse. To overcome this problem, we established ES cell clones stably transfected with the GFP reporter gene under the same GATA-1 promoter.

To monitor GATA-1 expression during ES cell differentiation, we used the in vitro differentiation induction system on the OP9 stromal cell layer (Nakano et al. 1994). By RT-PCR, we found that GATA-1 was not expressed in undifferentiated ES cells but became up-regulated as these cells differentiated. Low levels of GATA-1-specific mRNA were detectable by day 4 of differentiation and increased over the next 12 h (Fig. 1B). The up-regulation of GATA-1 expression between days 4 and 5 of differentiation is consistent with findings in previous studies (Robertson et al. 2000). FACS analyses showed that the GFP+ cells were first detectable in day 4.0 cells; almost all GFP+ cells were FLK1+ at this stage (Fig. 1Db). Within the following 12 h of differentiation, the number of GFP+ cells increased to constitute ≈4–8% of the differentiated ES cells, with approximately half of them being FLK1+ (Fig. 1Dc). This increase in reporter expression between days 4 and 4.5 of differentiation is consistent with the observed increase in GATA-1 mRNA (Fig. 1B). After day 5.5 of differentiation, a considerable fraction of GFP+ cells no longer express FLK1 (Fig. 1Dd). The accuracy of the GFP expression in correlating with endogenous GATA-1 expression was confirmed by immunocytochemistry in day 4.5 sorted cells. 86% of day 4.5 GFP+ cells (n = 400) were positively stained with anti-GATA-1 mAb, compared to 0.5% of GFP− cells (n = 400) (Fig. 1C). In addition, GATA-1 mRNA was only detected in the GFP+ cell fraction by RT-PCR (Fig. 2B). These results demonstrate that GFP expression under the
GATA-1 promoter is tightly correlated with the expression of endogenous GATA-1 gene.

The above data suggest that GATA-1/GFP cells differentiate from FLK1<sup>+</sup> cells in the ES cell culture. To examine this possibility, we purified FLK1<sup>+</sup>GFP<sup>+</sup>, FLK1<sup>+</sup>GFP<sup>−</sup> and FLK1<sup>−</sup>GFP<sup>+</sup> fractions (Fig. 1De,g and i) at day 4.0 of differentiation by sorting, and recultured them under the same conditions. After 12 h, cultured cells were subjected to FACS analyses. Both the FLK1<sup>+</sup>GFP<sup>+</sup> and FLK1<sup>−</sup>GFP<sup>+</sup> cells were induced from the day 4.0 FLK1<sup>+</sup>GFP<sup>−</sup> fraction (Fig. 1Df). It is of note that a considerable fraction of the FLK1<sup>−</sup> cells rapidly lost the expression of FLK1, as was previously demonstrated (Nishikawa et al. 1998a). Recently Yamashita et al. (2000) demonstrated that this FLK1<sup>−</sup> population contains smooth muscle actin<sup>+</sup> mesenchymal cells. On the other hand, the FLK1<sup>−</sup>GFP<sup>−</sup> cells were induced from the day 4.0 FLK1<sup>+</sup>GFP<sup>−</sup> fraction (Fig. 1Dh). The double negative cells induced from the day 4.0 FLK1<sup>+</sup>GFP<sup>−</sup> fraction contained mature primitive erythrocytes (data not shown). This observation is consistent with the down-regulation of the LacZ gene in mature primitive erythrocytes in the Tg mouse (Onodera et al. 1997).

GFP<sup>+</sup> cells were not induced from the day 4.0 FLK1<sup>−</sup>GFP<sup>−</sup> fraction (Fig. 1Dj). When FLK1<sup>+</sup>GFP<sup>−</sup> cells were sorted at day 4.5 or 5.0 of differentiation and recultured, no induction of GFP<sup>+</sup> cells was observed (Fig. 1Dk, l; data not shown). These results suggest that GATA-1 expressing cells are induced from the FLK1<sup>−</sup> cells and then differentiate into FLK1<sup>−</sup>GATA-1<sup>+</sup> cells.

To define the developmental status of the GFP<sup>+</sup> cells, day 4.5 cells were analysed by FACS for the expression of VE-cadherin, CD45 and Ter119, known to be present in endothelial and haematopoietic cells, respectively. FACS analyses of day 4.5 cells showed that GFP<sup>+</sup> cells did not co-express these lineage markers (Fig. 2A). We then fractionated day 4.5 cells into FLK1<sup>+</sup>GFP<sup>−</sup>, FLK1<sup>+</sup>GFP<sup>−</sup> and FLK1<sup>−</sup>GFP<sup>+</sup> populations by FACS (Fig. 2B) and performed RT-PCR for the detection of marker genes known to be expressed in mesodermal and haematopoietic precursors. These included Brachyury (indicative of mesoderm), GATA-1, and βH1 and β-major globins (definitive markers of erythroid development). As shown in Fig. 2C, Brachyury mRNA was detected in all fractions tested, although levels were higher in the FLK1<sup>+</sup> fractions. The GATA-1, βH1 and β-major genes were not detected in the FLK1<sup>−</sup>GFP<sup>−</sup> fraction (Fig. 2C).
globin mRNAs were only detected in the GFP+ populations. Therefore, our findings indicate that FLK1+ mesodermal cells diverge into a GATA-1-positive and -negative subset and that GATA-1 expressing subset encompasses developmental process ranging from mesoderm to the early stages of haematopoietic differentiation.

**Successive development of primitive and definitive haematopoietic lineages from separate mesodermal progeny**

The next series of experiments were aimed at examining the haematopoietic potential of GFP+ mesodermal progeny. For this, fractions from day 4.5 cells (Fig. 2B) were co-cultured with OP9 stromal cells in the presence of erythropoietin (Epo). The first wave of erythropoiesis appeared in the FLK1+ GFP+ and FLK1− GFP+ fractions within 24 h, thus at day 5.5 from initial induction of differentiation. All time points mentioned in this paper refer to culture period commencing from the initiation of differentiation culture. Almost all erythroid cells observed at day 8 were large-nucleated cells morphologically identical to primitive erythrocytes (EryP) (Fig. 3A, Ba and b). Occasionally, only a small number of erythroid cells were detected in the culture of the FLK1+ GFP− fraction. Because of their low number, it is unclear if they represent contaminants from the GFP+ fractions or true GFP− cells expressing endogeneous GATA-1. It should be noted that EryD could only be detected at day 8 in cultures containing FLK1+ GFP+ fractions, although their incidence was very low (less than 1/200). This finding suggests that the primitive and initial wave of definitive haematopoiesis arises in parallel from the GATA-1 expressing subset of the FLK1+ mesoderm. Subsequently, the second wave of erythropoiesis appeared around day 10 in FLK1+ GFP+ and FLK1− GFP+ cultured fractions but not in the FLK1− GFP+ fraction. Erythroid cells from the second wave consisted of small-nucleated or enucleated mature blood cells which were morphologically identical to definitive erythrocytes (EryD) (Fig. 3A, Bc and d). EryP and EryD were not induced from the FLK1− GFP− fraction (data not shown; Figs 4B, C). The addition of blocking antibody to c-Kit (Ack2, Nishikawa et al. 1991) completely abolished the second wave of erythropoiesis (Fig. 3A), indicating that c-Kit signalling is necessary for the second wave of erythropoiesis. Results of RT-PCR analyses of the day 8 and 14 erythrocytes for globin gene expression are shown in Fig. 3C; βH1 and β-major globin mRNA were detected in day 8 erythrocytes induced from both the FLK1+ GFP+ and FLK1− GFP+ fractions. As definitive erythrocytes do not arise from cultures of FLK1− GFP+ fractions, the β-major globin expression is suggested to originate from the primitive erythroid lineage cells. This interpretation is supported by a recent study which demonstrated β-major globin expression in the primitive erythroid lineage (Palis et al. 1999). On the other hand, high levels of β-major globin mRNA were found, while βH1 mRNA was not detectable in day 14 erythrocytes induced from the
FLK1\(^+\) GFP\(^-\) and FLK1\(^+\) GFP\(^+\) fractions. Collectively, these results indicate that the first and the second waves of haematopoiesis represent primitive and definitive haematopoiesis, respectively.

**Hematopoietic cell development from GATA-1 expressing progeny of FLK1\(^+\) mesoderm**

Since the expression of GATA-1 in the FLK1\(^+\) mesoderm correlated with the first wave of haematopoiesis, we examined the GFP\(^+\) progeny of FLK1\(^+\) mesodermal cells for the presence of hematopoietic progenitors, as schematically outlined in Fig. 4A. Day 4.5 cells were sorted into FLK1\(^+\) GFP\(^-\), FLK1\(^+\) GFP\(^+\), FLK1\(^-\) GFP\(^+\) and FLK1\(^-\) GFP\(^-\) fractions (Fig. 2B) and plated into methylcellulose cultures containing different combinations of cytokines including SCF. The incidence of colony-forming cells in the FLK1\(^+\) GFP\(^+\) and FLK1\(^-\) GFP\(^+\) fraction was 11% and 26%, respectively, whereas that in the FLK1\(^+\) GFP\(^-\) fraction was very low (0.3%) (Figs 4B, C). The FLK1\(^-\) GFP\(^-\) fraction did not show CFU-C activity (Figs 4B, C). These results indicate that only the GATA-1\(^+\) progeny from the FLK1\(^+\) mesoderm contain hematopoietic progenitors capable of generating colonies in response to cytokines at this stage. Virtually all (97%) of the progenitors were Epo responsive and gave rise to small colonies of large nucleated erythroid cells which were morphologically identical to EryP (Fig. 4B), expressing βH1 globin (data not shown). Macrophage progenitors (CFU-M) and early EryD progenitors (BFU-E) were also found in the FLK1\(^+\) GFP\(^+\) fraction, exclusively (Fig. 4C). However, the incidence of CFU-M and BFU-E in the FLK1\(^+\) GFP\(^+\) fraction was 0.6% and 0.4%, respectively (Fig. 4C), and was much lower than that of EryP progenitors. These findings suggest that nascent GATA-1 expressing cells which have diverged from the FLK1\(^+\) mesoderm are already biased towards haematopoietic, particularly primitive erythroid lineages, while the remaining GATA-1\(^-\) FLK1\(^+\) mesodermal cells do not yet have the potential for cytokine-stimulated colony production.

Although the presence of EryD progenitors together with EryP in the FLK1\(^+\) GFP\(^+\) fraction suggested the existence of common hematopoietic precursors for primitive and definitive erythroid lineages, we did not observe any colonies containing both primitive and definitive haematopoietic cells (Figs 4B, C). We also examined the differentiation of FLK1\(^+\) GFP\(^+\) cells at the single cell level. When individual FLK1\(^+\) GFP\(^+\) cells (n = 572) were seeded on to an OP9 stromal cell layer in microtitre wells with SCF, IL-3 and Epo by single-cell deposition, no well in which both primitive and definitive hematopoietic colonies had arisen was
observed. This finding is consistent with the absence of CFU-Cs containing primitive and definitive haematopoietic cells (Fig. 4B). This indicates that primitive and definitive haematopoietic cells diverge at an early stage in the initial wave of haematopoiesis.

**Differentiation of VE-cadherin**<sup>+</sup> endothelial cells from FLK1<sup>+</sup> mesoderm

Our previous studies using the *in vitro* ES cell differentiation system suggested that a subset of VE-cadherin<sup>+</sup> endothelial cells are endowed with haematopoietic potential (Nishikawa *et al*. 1998a; Ogawa *et al*. 1999). To investigate the processes of the second wave of definitive haematopoiesis (Fig. 3A), we examined a potential of sorted fractions giving rise to VE-cadherin<sup>+</sup> endothelial cells. For this, day 4.5 cells were fractionated (Fig. 2C) and re-cultured with OP9 stromal cells. FACS analysis of cells after an additional 2 days of culture revealed that VE-cadherin<sup>+</sup> cells were induced from the FLK1<sup>+</sup>GFP<sup>−</sup> and FLK1<sup>+</sup>GFP<sup>+</sup> fractions (Fig. 5Aa, b), though a considerable portion of the FLK1<sup>+</sup> cells rapidly lost their FLK1 expression, as previously described. The formation of endothelial cell colonies was confirmed by immunohistochemical staining of whole cultures with an anti-VE-cadherin mAb (Fig. 5B). Both the FLK1<sup>+</sup>GFP<sup>−</sup> and FLK1<sup>+</sup>GFP<sup>+</sup> fractions gave rise to endothelial cell colonies. The incidences of colony forming cells in the FLK1<sup>+</sup>GFP<sup>−</sup> and FLK1<sup>+</sup>GFP<sup>+</sup> fractions were 4.2% and 1.8%, respectively (Fig. 5C). Colony formation was rare in the FLK1<sup>+</sup>GFP<sup>+</sup> fraction (1 : 2000 cells; Fig. 5C). Comparable results were obtained with an anti-PECAM-1 antibody (data not shown). These findings indicate the following: (i) VE-cadherin<sup>+</sup> endothelial cells differentiate from FLK1<sup>+</sup> mesoderm after the divergence of GATA-1 expressing cells; and (ii) some of the GATA-1 expressing progeny of FLK1<sup>+</sup> mesoderm also can generate VE-cadherin<sup>+</sup> endothelial cells.

**Hematopoietic cell development from VE-cadherin**<sup>+</sup> cells

To investigate whether the VE-cadherin<sup>+</sup> cells induced...
from FLK1$^+$ GFP$^-$ and FLK1$^+$ GFP$^+$ fractions have the potential to give rise to definitive haematopoietic cells, cells from 2-day cultures of day 4.5 FLK1$^+$ GFP$^-$ and FLK1$^+$ GFP$^+$ cells were fractionated into VE-cadherin$^+$ and VE-cadherin$^-$ populations by FACS. Reanalysis of the sorted VE-cadherin$^+$ cells revealed that these population did not express GFP (Fig. 5Ac,d). The sorted cells were transferred on to OP9 stromal cell layers and maintained for 7 days in methylcellulose medium containing recombinant IL-3, Epo, G-CSF and SCF. One in 40 cells and 1 in 50 cells from the VE-cadherin$^+$ fractions derived from the FLK1$^+$ GFP$^-$ and FLK1$^+$ GFP$^+$ cells, respectively, gave rise to haematopoietic cell clusters (Fig. 6A). In contrast to the VE-cadherin$^+$ fractions, the VE-cadherin$^-$ fractions contained much fewer haematopoietic cell progenitors (less than 1 in 250 cells; Fig. 6A). These results demonstrated that VE-cadherin$^+$ fractions derived from FLK1$^+$ mesoderm and GATA-1 expressing progeny of FLK1$^+$ mesoderm contained haematopoietic progenitors.

To further characterize the haematopoietic cells obtained from the VE-cadherin$^+$ fractions (Fig. 5Ac,d), sorted cells were cultured for 7 additional days on OP9 stromal cell layers in the presence of SCF, IL-3, Epo and G-CSF. Similar frequencies of Mac-1$^+$, Gr-1$^+$ and Ter119$^+$ cells were detected in both cultures by FACS analyses (Fig. 6Ba, b, c and d). The addition of SCF, FL and IL-7 induced CD19$^+$ B cells from these VE-cadherin$^+$ cells (Fig. 6Be, f). May–Giemsa staining showed that the cultured cells contained haematopoietic cells such as definitive erythrocytes, granulocytes, macrophages, megakaryocytes and lymphocytes (data not shown). The in vitro colony-forming activity of VE-cadherin$^+$ cells was assayed in methylcellulose cultures containing SCF, IL-3, Epo and G-CSF. The frequency of CFU-C was 1.9% and 1.7% in the FLK1$^+$ GFP$^-$ and FLK1$^+$ GFP$^+$ fractions, respectively. Macrophage, granulocyte/macrophage, erythroid and multiple myeloid progenitors were induced in the cultures initiated from both fractions (Fig. 6C). However, EryP progenitors were not detected in either culture. Together, these results indicate that multilineage definitive haematopoietic progenitors differentiate from VE-cadherin$^+$ cells.

To determine if single VE-cadherin$^+$ cells give rise to endothelial and definitive haematopoietic cells, we next performed single cell deposition. When 190 single VE-cadherin$^+$ cells were seeded on to OP9 stromal cell layers in microtitre wells containing SCF, IL-3, Epo and G-CSF by single-cell deposition, small colonies of PECAM-1$^+$ endothelial cells were found in 15 (FLK1$^+$ GFP$^-$) and 13 (FLK1$^+$ GFP$^+$) wells (Fig. 7A, Ba). Hematopoietic cell colonies were detected in two of the PECAM-1$^+$ endothelial cell$^+$ wells in both cultures (Fig. 7A, Bb). May–Giemsa staining showed that the cultured cells contained haematopoietic cells such as granulocytes, monocytes/macrophages and erythroid cells (Fig. 7Bc,d). These results demonstrate that definitive haematopoietic and endothelial cells diverge from VE-cadherin$^+$ cells.

**Discussion**

We demonstrated that extraembryonic mesodermal
cells can be further divided by GATA-1 expression (Fig. 1A). In mouse embryos, FLK1 is first expressed in the proximal lateral mesoderm (Yamaguchi et al. 1993; Kataoka et al. 1997). Considering that proximal lateral mesodermal cells give rise to extraembryonic and lateral mesoderm, our finding indicates that only a subset of cells in the extraembryonic mesoderm express GATA-1 during the differentiation of proximal lateral mesodermal cells. In an in vitro differentiation system of ES cells, a subset of the FLK1<sup>+</sup> cells also expressed GATA-1 prior to the expression of endothelial or haematopoietic lineage specific markers such as VE-cadherin, CD45 and Ter119 (Fig. 2A). Similar to the proximal lateral mesoderm, which generates haematopoietic and endothelial lineages, FLK1<sup>+</sup> VE-cadherin<sup>−</sup> CD45<sup>−</sup> and Ter119<sup>−</sup> cells induced from ES cells also give rise to haematopoietic and endothelial lineages (Nishikawa et al. 1998a; Hirashima et al. 1999; Ogawa et al. 1999). Thus, the ES-derived FLK1<sup>+</sup> cells most likely represent proximal lateral mesodermal cells. Therefore, the presence of FLK1<sup>+</sup> GATA-1<sup>+</sup> cells in this culture system indicates that the process to generate GATA-1-positive and -negative mesoderm is recapitulated in ES cell differentiation culture. Expression of Brachyury, a molecular marker for mesoderm (Wilkinson et al. 1990) in GATA-1 expressing cells (Fig. 2B) is consistent with the idea that these GATA-1<sup>+</sup> cells represent mesodermal cells.

Next, the FLK1<sup>+</sup> GATA-1<sup>+</sup> cells induced from ES cells were a transient population and differentiated into the FLK1<sup>+</sup> GATA-1<sup>+</sup> cells (Fig. 1D), indicating that the FLK1<sup>+</sup> GATA-1<sup>+</sup> cells most likely represent the immediate progeny of FLK1<sup>+</sup> GATA-1<sup>+</sup> cells. Our progenitor assay demonstrated that primitive erythroid progenitors were found exclusively in the FLK1<sup>+</sup> GATA-1<sup>+</sup> and FLK1<sup>+</sup> GATA-1<sup>−</sup> fractions. These findings thus indicate that the differentiation from FLK1<sup>+</sup> mesodermal cells to GATA-1<sup>+</sup> progeny correlates well with primitive haematopoiesis in the extraembryonic mesoderm. Indeed, the FLK1<sup>+</sup> GATA-1<sup>+</sup> extraembryonic mesodermal cells and FLK1<sup>+</sup> GATA-1<sup>+</sup> primitive erythroid cells were observed in blood islands of the Tg mouse embryos (Fig. 1A).

In addition to primitive erythropoiesis, the initial wave of definitive haematopoietic progenitors, predominantly consisting of definitive erythroid and macrophage progenitors was detected exclusively in the FLK1<sup>+</sup> GATA-1<sup>+</sup> fraction (Fig. 4B). The appearance of definitive haematopoietic progenitors together with primitive erythroid progenitors suggests that the first wave haematopoiesis arising from the FLK1<sup>+</sup> mesoderm is not entirely restricted to a primitive erythroid lineage. This notion is supported by a recent study which demonstrated the appearance of definitive erythroid and macrophage progenitors within the yolk sac at a stage of primitive erythropoiesis prior to the establishment of circulation in mouse embryos (Palis et al. 1999). Thus, these findings demonstrate that a population with the capacity to give rise to primitive erythropoiesis and initial definitive haematopoiesis diverges first from differentiating FLK1<sup>+</sup> mesoderm.

The identification of definitive erythroid and macrophage progenitors together with primitive erythroid progenitors may suggest the presence of common haematopoietic precursors for primitive and definitive lineages within the FLK1<sup>−</sup> mesoderm. While we have not succeeded in detecting such cells, common haematopoietic precursors have been identified in developing embryoid bodies (Kennedy et al. 1997). The basis for this difference might be related to the fact that our analyses were carried out on the later stages of ES cell differentiation than that of the former study. In any case, the lack of single FLK1<sup>+</sup> GATA-1<sup>−</sup> cells which generate primitive erythrocytes and definitive haematopoietic cells simultaneously (Fig. 4) demonstrates that the divergence between the primitive erythrocytoid lineage and initial wave of definitive haematopoietic lineage is completed at this stage.

While a subset of FLK1<sup>+</sup> mesoderm gave rise to GATA-1<sup>+</sup> progeny, the majority of FLK1<sup>+</sup> mesoderm differentiated without GATA-1 expression (Fig. 1B) and did not exhibit CFU-C activity (Fig. 4). This finding suggests that the GATA-1<sup>−</sup> progeny of the FLK1<sup>+</sup> mesoderm have lost or do not possess the capacity to give rise to primitive erythropoiesis and the initial wave of definitive haematopoiesis. However, the FLK1<sup>+</sup> GATA-1<sup>−</sup> fraction gave rise to VE-cadherin<sup>+</sup> cells (Fig. 5). VE-cadherin is one of the functional molecules of endothelial cells (Breier et al. 1996; Matsuyoshi et al. 1997). In an ES cell in vitro differentiation system, VE-cadherin<sup>+</sup> cells give rise to VE-cadherin<sup>+</sup> FLK1<sup>+</sup> CD31<sup>+</sup> sheet-like structures which incorporate Ac-LDL, confirming their endothelial identity (Hirashima et al. 1999; Ogawa et al. 1999). Thus, these ES-derived VE-cadherin<sup>+</sup> cells most likely represent endothelial cells. It should be noted that the FLK1<sup>+</sup> GATA-1<sup>−</sup> fraction could give rise to both VE-cadherin<sup>+</sup> endothelial cells and haematopoietic progenitors (Fig. 5). However, the subsequent FLK1<sup>−</sup> GATA-1<sup>−</sup> population could no longer give rise to endothelial progenitors and were restricted to primitive erythrocyte fates (Figs 4 and 5). These findings indicate that
In conclusion, we have shown the sequential steps leading from mesoderm to haematopoietic and endothelial cell lineages: mesodermal precursors give rise to primitive erythroid cells and VE-cadherin

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cells, the latter subsequently generating both definitive haematopoietic and endothelial cells. Access to these populations should help to elucidate the molecular mechanisms underlying the developmental process of the haematopoietic system.

Experimental procedures

Monoclonal antibodies (mAbs)

The mAbs AVAS12 (anti-FLK1) (Kataoka et al. 1997), and VECD1 (anti-VE-cadherin) (Matsuyoshi et al. 1997) were purified from hybridoma culture supernatants on a protein G-Sepharose column (Pharmacia, Uppsala, Sweden). These mAbs were labelled with allopheocyanin (APC) by standard methods. The biotin-conjugated mAbs 30-F11 (anti-CD45), M1/70 (anti-Mac-1) and Ter119, and phycoerythrin (PE) conjugated 1D3 (anti-CD19) and RB6-8C5 (anti-Gr-1), and PECAM-1 (anti-CD31) were purchased from PharMingen (San Diego, CA). The mAbs N1 (sc-266) and N6 (sc-265) (anti-PECAM-1 (anti-CD31) and RB6-8C5 (anti-Gr-1)), and PECAM-1 (anti-CD31) were purchased from Dako Corporation (CA). The APC or PE-conjugated strepavidin was purchased from Molecular Probes.

Analysis of the transgenic mouse embryos

For whole mount X-Gal staining, embryos were removed at 7.5 days post-coitus (dpc). Whole mount X-Gal staining was performed as previously described (Onodera et al. 1997). Tissue fixation procedures of X-Gal stained embryos were principally the same as described (Yoshida et al. 1993). Fixed specimens were embedded in polyester wax and sectioned by RM2145 (Leica, Heerbrugg, Switzerland) at 4 μm. Sections were dewaxed and washed with PBS. Non specific binding was blocked with PBS containing 1% skim milk (Becton Dickinson, Sparks, MD) for 60 min. For immunostaining, sections were first incubated with the AVAS12 or N6 (sc-265) mAb overnight, washed three times, and then incubated for 2 h with biotin-conjugated anti-rat IgG (H+L) (Bio Source International, Camarillo, CA). After three washes with PBS, sections were incubated with strepavidin-conjugated Texas Red (Molecular Probes, Eugene, OR) or extravidin-conjugated FITC (Sigma, St. Louis, MO) for 30 min and washed again three times.

Vectors

The reporter gene construct was generated by the insertion of a genomic promoter fragment of mouse GATA-1 extending from a BamHI site (3.9 kbp 5′ to the IE exon) to the NotI site in the second exon (Onodera et al. 1997) together with the polyadenylation consensus sequence of pSVB (Clontech, Palo Alto, CA) into the pBluescript KS (+) plasmid (Stratagene, La Jolla, CA). A NotI-Sall fragment containing green fluorescent protein (GFP) cDNA in pCMX-SAH/Y145F plasmid was then introduced into the SpeI site of this plasmid intermediary. The final construct was referred to as pIE3.9intGFP.

Electroporation and selection procedure

CCE ES cells were electroporated with linearized pIE3.9intGFP and pEF-MC1neo and then selected for resistance to G418 (300 μg/mL). Embryoid bodies (EBs) were generated from resistant ES cell clones using standard protocols as previously described (McClanahan et al. 1993). Growing EBs were monitored by fluorescence microscopy using an FITC filter set (Zeiss, Jena, Germany), and reporter gene integration in the selected clones was confirmed by PCR of the genomic DNA. The primers used were: GFP-S primer, 5′-AGCAAGG CGAGGAGCTGTTACC-3′; GFP-AS primer, 5′-TGCC GTCGTCCTTTGAAAGATG-3′. To avoid artefacts due to G418 selection, three independent clones (no. 6, no. 49 and no. 58) were examined. No significant differences between selected clones and the parental cell line in regard to differentiation were observed.

Cell culture

Culture of CCE ES cells and OP9 stromal cells was performed as previously described (Nishikawa et al. 1998a).

Cell staining and sorting

Cell staining and sorting was performed as previously described (Ogawa et al. 1999). Cells were gated to exclude OP9 stromal cells by a difference in forward and side scatter. Immunostaining of cultured cells by anti-GATA1 mAb or anti-embryonic haemoglobin was performed as described (Nishikawa et al. 1998a).

In vitro differentiation of ES cells

The induction of ES cell differentiation was carried out as previously described (Nakano et al. 1994). Transfected ES cells were seeded on to confluent OP9 cell layers in six-well plates (Becton Dickinson Labware, Bedford, MA) at a density of 10^4 cells per well. α-MEM (Gibco BRL) supplemented with 10% FCS (Gibco BRL), and 50 μmol/L 2ME in the absence of LIF was used as culture media. The induced cells were harvested in cell dissociation buffer (Gibco BRL) and analysed by FACS Vantage. Harvested cells were fractionated by cell sorting and cultured for subsequent induction of haematopoietic or endothelial cells. For the induction of erythroid/ myeloid cells, sorted cells were transferred on to fresh OP9 cells at a density of 2 × 10^3 cells per well in 12-well plates (Becton Dickinson) and incubated in induction media supplemented with a mixture of recombinant growth factors containing 200 U/mL murine interleukin-3 (IL-3), 2 U/mL human erythropoietin (Epo), 100 ng/mL human granulocyte colony-stimulating factor (G-CSF), and 100 ng/mL murine c-Kit ligand (SCF). For the induction of B-lymphoid cells, sorted cells were cultured with OP9 stromal cells at a density of 3–5 × 10^3 cells per well in the presence of 100 ng/mL SCF, 100 ng/mL human Flt3/Flk-2 ligand (FL) and 200 U/mL human interleukin-3 (IL-3) and 2 U/mL human interleukin-6 (IL-6).
Total RNA was prepared from 104 sorted cells or cultured cells using Isogen (Nippon Gene, Toyama, Japan). RNA was reverse-transcribed with Superscript II reverse transcriptase (Gibco BRL) and oligo dT primers (Gibco BRL) according to the manufacturer’s instructions. PCR assays were performed in reaction mixtures containing 1 U ExTaq Buffer (Takara Shuzo, Osaka, Japan), 200 μM dNTPs (Pharmacia), 25 U/mL ExTaq polymerase (Takara Shuzo), several dilutions of cDNA, and 2 μM/L of oligonucleotide primers. Sequences of primers are described elsewhere: Brachyury (Keller et al. 1993); GATA-1, βH1 and β-major globins, and GAPDH (Ogawa et al. 1999). Amplification of the cDNA was performed using Peltie thermal cycler (MJ Research Inc., Watertown, MA) on a regimen of 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min for 30 cycles. RT-PCR products were electrophoresed through 1% agarose gel and analysed by staining with ethidium bromide.

Acknowledgements

We thank Drs M Evans, K. Umesono and N. Matsuyoshi for generous gifts of CCE cell line, pCMX-SAH/Y145F plasmid and VECD1 MoAb, respectively. We also thank Dr Ruth Yu for a critical reading of the manuscript. This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (nos 07CE2005, 12215071 and 12670301), The Cell Science Research Foundation and Japanese Society for the Promotion of Science ‘Research of Future’ Program.

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Yoshida, H., Nishikawa, S., Okamura, H., Sakakura, T. &


Received: 31 August 2001
Accepted: 5 October 2001