Erythropoiesis and Vasculogenesis in Embryoid Bodies Lacking Visceral Yolk Sac Endoderm

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During mouse embryogenesis the first hematopoietic and endothelial cells form in blood islands located between layers of visceral endoderm and mesoderm in the yolk sac. The role of visceral endoderm in primitive hematopoiesis and vasculogenesis is not well understood. We have assessed the consequences of a lack of visceral endoderm on blood cell and vessel formation using embryoid bodies derived from mouse embryonic stem (ES) cells deficient in GATA-4, a transcription factor expressed in yolk sac endoderm. When differentiated in vitro, these mutant embryoid bodies do not develop an external visceral endoderm layer. We found that Gata4−/− embryoid bodies, grown either in suspension or attached to a substratum, are defective in primitive hematopoiesis and vasculogenesis as evidenced by a lack of recognizable blood islands and vascular channels and a reduction in the expression of the primitive erythocyte marker ε-globin. Expression of the endothelial cell transcription factors Flk-1, Flt-1, and platelet-endothelial cell adhesion molecule (PECAM) was not affected in the mutant embryoid bodies. Gata4−/− ES cells retained the capacity to differentiate into primitive erythroblasts and endothelial cells when cultured in methylcellulose or matrigel. Analysis of chimeric mice, generated by injecting Gata4−/− ES cells into 8-cell stage embryos of ROSA26 transgenic animals, showed that Gata4−/− ES cells can form blood islands and vessels when juxtaposed to visceral endoderm in vivo. We conclude that the visceral endoderm is not essential for the differentiation of primitive erythrocytes or endothelial cells, but this cell layer plays an important role in the formation and organization of yolk sac blood islands and vessels.

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ONE OF THE first sites of hematopoiesis and blood vessel formation during mouse embryogenesis is the visceral yolk sac, an extramembrane tissue composed of both endodermal and mesodermal derivatives.1,2 Endodermal cells of the yolk sac arise from primitive endoderm,3 whereas yolk sac mesoderm cells descend from primitive ectoderm during gastrulation and migrate beneath the visceral endoderm between 7 and 7.5 days post-coitum (p.c.).4 Once subjacent to the visceral endoderm layer, these mesodermal cells proliferate and differentiate into blood islands, structures consisting of hematopoietic cells surrounded by a loose network of endothelial cells.4

Hematopoiesis in the yolk sac is closely tied to endothelial cell differentiation and blood vessel development.5 Studies in mouse,6 chick,7 and other species,8,9 suggest that a common precursor cell, the hemangioblast, gives rise to both erythrocytes and endothelial cells within blood islands. In the mouse, mutations that disrupt genes encoding proteins critical for vasculogenesis, such as fibronectin,10 α5-integrin,11 bone derived morphogenetic factor-4,12 transforming growth factor-β1,13 and vascular endothelial cell growth factor,14,15 and its receptors,16 also adversely affect primitive hematopoiesis, implying that these processes are closely linked.

Production of blood cells in yolk sac mesoderm is restricted to the erythroid and macrophage lineages.4,9,18 Yolk sac or “primitive” hematopoiesis generates large erythrocytes that remain nucleated throughout their lifespan and express the embryonic globins ε, δ, and ζ.4,18 At about 12 days p.c. this extraembryonic hematopoiesis gives way to intraembryonic “definitive” hematopoiesis, first in the fetal liver and later the bone marrow (BM) and spleen.4,9 Definitive hematopoiesis generates all hematopoietic lineages and produces small enucleated erythrocytes that contain the adult globins α, βα2, and ββ2.19,20 Knockout studies suggest that primitive and definitive erythropoiesis are controlled by distinct mechanisms.19,20 Inactivation of the genes encoding rbm2,21 SCL/tal-1,22,23 GATA-1,24,25 or GATA-227 disrupts both primitive and definitive erythropoiesis, whereas mutation of the factors c-myb,28 PU.1,29 EKLF,30,31 AML1,32 and the erythropoietin receptor33,34 affects only definitive erythropoiesis.19,20

Whether a common source of hematopoietic stem cells is used for primitive and definitive hematopoiesis is controversial.18 According to one hypothesis distinct populations of hematopoietic stem cells arise de novo in the yolk sac, liver, spleen, and BM.4,18 Another possibility is that hematopoietic stem cells are created only in the yolk sac or in an intraembryonic region termed the aorta-gonad-mesonephros and that these stem cells then migrate to the liver, spleen, and BM.4,18

The role of visceral yolk sac endoderm in the control of primitive erythropoiesis and vasculogenesis remains a subject of debate. Several lines of evidence suggest that the visceral endoderm can influence the differentiation of adjacent tissue, including the mesodermal precursors of yolk sac blood cells and vessels. Removal of endoderm from cultured explants of chick embryo35,36 or mouse yolk sac37 disrupts the formation of blood islands and vessels. Immortalized mouse yolk sac endoderm cell lines can support hematopoiesis.38,39 In vitro studies also suggest that visceral endoderm signals direct lumenization or cavitation in subjacent tissue.40 Embryoid bodies derived from mouse embryonic stem (ES) cells have been shown to be a useful in vitro model for the study of a variety of differentiation programs, including

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Submitted March 12, 1996; accepted June 19, 1996.

Supported by National Institutes of Health Grant No. HL52134, AHA Grant 94-605, the Monsanto/Searle-Washington University Biomedical Agreement, the Finnish Cultural Foundation, the Council for Tobacco Research, and the Paulo Foundation. D.B.W. is an Established Investigator of the American Heart Association.

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0006-4971/96/8810-0127$3.00/0
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Fig 1. Toluidine blue stained sections through 12 day wild type (a and c) and Gata4-/- (b and d) embryoid bodies grown in suspension culture. Note the presence of visceral endoderm, hematopoietic cells, and endothelial cell-lined vascular channels in the wild type embryoid bodies. In contrast, the Gata4-/- embryoid bodies lack visceral endoderm, blood cells, and vascular channels. Abbreviations: bi, blood island; cc, cyst cavity; e, endothelial cell; h, hematopoietic cells; m, mesothelium; ve, visceral endoderm. Bar = 50 μm in (a and b); bar = 10 μm in (c and d).

vasculogenesis \(^{41-44}\) and hematoPOIESIS. \(^{41,44-53}\) Recently we reported that ES cells homozygous deficient for GATA-4, a lineage restricted transcription factor expressed in yolk sac endoderm and a limited number of other tissues, \(^{54-58}\) display a specific block in visceral yolk sac endoderm formation when differentiated into embryoid bodies. \(^{59}\) Biochemical markers of visceral endoderm, including α-fetoprotein, hepatocyte nuclear factor-4, and oligosaccharide binding sites for Dolichos biflorus agglutinin (DBA), are absent from Gata4-/- embryoid bodies. \(^{60}\) Many other aspects of differentiation appear unperturbed in Gata4-/- embryoid bodies, making this an attractive in vitro system for assessing the role of yolk sac endoderm in primitive hematopoiesis and vasculogenesis. In this study we have examined the formation of blood cells and endothelium in visceral endoderm deficient Gata4-/- embryoid bodies. We show that the visceral endoderm layer is essential for organization of blood islands and vessels, but is not required for differentiation of primitive erythroblasts or endothelial cells.

MATERIALS AND METHODS

ES cell maintenance and differentiation. Wild type and Gata4-/- ES cells were prepared and maintained in an undifferentiated state on mitomycin C treated STO feeder layers. \(^{61}\) Before differentiation, the ES cells were passaged in the presence of leukemia inhibitory factor to remove feeder cells, as described previously. \(^{62}\) ES cells were differentiated into embryoid bodies using the suspension culture method of Doetschman et al. \(^{63}\) Alternatively, ES cells were grown on a substratum using a two-stage culture method similar to that reported elsewhere. \(^{64}\) ES cells were grown for 3 to 6 days in hanging drop suspension culture and were then transferred to gelatinized chamber slides, allowed to adhere, and maintained in culture for 3 to 6 additional days.

Histological analysis of embryoid bodies. Sectioning and toluidine blue staining of paraformaldehyde-fixed, resin-embedded embryoid bodies was performed as described. \(^{65}\) To visualize erythroid cells, embryoid bodies grown in suspension culture on a substratum were washed with phosphate-buffered saline (PBS), stained for 10 minutes with benzidine and H₂O₂, \(^{61}\) and then immediately photographed using an inverted microscope. Endothelial cells were stained by overnight incubation in media supplemented with 5 mg/mL acetylated low density lipoprotein labeled with 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (diI-Ac-LDL) (Biomedical Technologies, Inc, Stoughton, MA). \(^{64,64}\) Embryoid bodies labeled with diI-Ac-LDL were fixed for 10 minutes in 10% formalin in PBS, mounted in Mowiol, \(^{62}\) and photographed using a fluorescent microscope.

RNase protection analysis. RNA was isolated by the method of Chomczynski and Sacchi. \(^{65}\) RNase protection assays were performed with a commercially available kit (Ambion, Austin, TX) and 15 μg of total RNA. \(^{[32]P}\)Labeled antisense riboprobe recognizing various transcripts were prepared by in vitro transcription in the presence of [\(^{32}P\)JUTP (800 Ci/mmol, ICN) using the following plasmid templates, linearizing restriction endonucleases, and polymerases: c'-globin, plasmid SP56M, \(^{66}\) HnIII, SP6; β''''-globin, EcoRI-BamHI fragment encoding exons 1 and 2 of the gene in pBluescript KS, \(^{67}\) HindIII, T7; Flk-1, pBluescript subclone, \(^{67}\) HindIII, T3; Flk-1, 2.2 kb BamHI-Xba fragment of a full-length cDNA provided by M. Shibuya (University of Tokyo, Japan) and subcloned into pBluescript KS, BamHI-T3; PECAM, pBluescript KS subclone of the fourth Ig extracellular domain provided by S. Baldwin (Wistar Institute, Philadelphia, PA), Pst I, T7. Antisense β-actin probe was prepared according to the manufacturer’s recommendations (Ambion). Protected fragments were separated by polyacrylamide gel electrophoresis in 8 mol/L urea. \(^{66}\) The sizes of the protected RNA fragments (in nucleotides) were as follows: c'-globin, 207; β''''-globin, 209 and 145; Flk-1, 530; Flt-1, 220; PECAM, 199; and β-actin, 250.

Methylcellulose cultures. Single cell suspensions of 3,000 undifferentiated wild type or Gata4-/- ES cells were plated into 35 mm culture dishes in basic methylcellulose culture medium supplemented with erythropoietin (Epo) (3 U/mL) or complete media #3434 containing Epo (3 U/mL), kit ligand (KL) (5 U/mL), IL-3, IL-6, insulin, and transferrin (Stem Cell Technologies, Vancouver, Canada). Hemoglobinized (red) colonies were cyto centrifuged and stained with May-Grunwald-Giemsa (Sigma, St Louis, MO). Alternatively, wild type and Gata4-/- embryoid bodies were first grown in suspension culture for 3 to 7 days and then plated into 35 mm plates containing basic methylcellulose media. At days 8 and 10 of
Fig 2. Lineage tracing in chimeric mouse embryos. Chimeric embryos were prepared by introducing *Gata*4-/- ES cells into 8-cell stage embryos derived from a ROSA26 transgenic mouse line bearing a ubiquitously expressed β-galactosidase transgene. Shown are tissue sections through an 8.5 day p.c. high percentage *Gata*4-/- ES ↔ ROSA26 chimera stained with X-gal. In these sections the yolk sac endoderm is exclusively derived from β-galactosidase positive host cells, whereas blood cells and endothelial cells are derived from either ES cells or host cells. Abbreviations: bi, blood island; e, endothelial cell; ee, embryonic ectoderm; h, hematopoietic cell; ve, visceral endoderm. In (a) bar = 100 μm. In (b) bar = 10 μm.
differentiation, the individual embryoid bodies were removed from methylcellulose and stained with either benzidine \( \text{I} \) or fluorescein isothiocyanate (FITC)-labeled DBA (Sigma) \( \text{II} \) to visualize yolk sac endoderm.

Blood vessel outgrowth. Gata4-/- embryoid bodies grown for 5 days in suspension culture were transferred into 35 mm bacterial plates covered with a thick layer of matrigel basement membrane matrix (Becton Dickinson, Bedford, MA) prepared according to the manufacturer’s instructions. The gel was covered by 1.5 mL of the media used for culturing embryoid bodies, which was changed every 3 to 4 days. After 18 days of culture fresh media containing 10 mg/mL of dil-Ac-LDL was added. After overnight labeling this media was removed and the gel was covered by fresh media without dil-Ac-LDL. The embryoid bodies were photographed during the subsequent days using an inverted microscope and dark field or fluorescent optics.

Generation and analysis of chimeric mice. Established techniques were employed to produce chimeric mice for analysis.  \( \text{I} \) TgR(Rosa26)26Sort mice \( \text{II} \) hereafter referred to as "Rosa26 mice," which bear a ubiquitously expressed \( \beta \)-galactosidase transgene on a strain 129 background, were obtained from Jackson Labs. To generate the chimeric embryos, male mice homozygous for the Rosa26 transgene were bred with supraovulated C57BL/6J females. 8-Cell stage embryos were harvested at 2.5 days and injected with 2-4 Gata4-/- ES cells. The injected embryos were then implanted into the oviducts of pseudopregnant Swiss-Webster females. Embryos with attached yolk sacs were obtained at 8 to 9 days p.c. and subjected to whole mount staining for \( \beta \)-galactosidase with X-gal (Promega) as described. Chimeric embryos were identified by X-gal staining and then post-fixed in 4% paraformaldehyde, embedded in resin, sectioned, and lightly counterstained with hematoxylin-eosin.

RESULTS

Gata4-/- embryoid bodies grown in suspension culture lack morphologically recognizable blood islands and vessels. ES cells deficient in transcription factor GATA-4 display a defect in yolk sac endoderm formation when differentiated in vitro, though many other differentiation programs appear unperturbed in these mutant embryoid bodies. To assess how an absence of yolk sac endoderm affects in vitro erythropoiesis and vasculogenesis, we first examined histological sections of wild type and Gata4-/- embryoid bodies grown in suspension culture. An equivalent number of benzidine-stained cells were detected in wild type and Gata4-/- embryoid bodies between 8 and 11 days of differentiation using the whole mount technique (data not shown). However, differences were apparent when the wild type or Gata4-/- embryoid bodies were secondarily plated onto a substratum. For these experiments, embryoid bodies were grown initially in hanging drop suspension culture for either 3 days (before visceral endoderm differentiation) \( \text{I} \) or 6 days (after the onset of visceral endoderm differentiation). The embryoid bodies were then allowed to adhere to plastic surfaces and maintained in culture until day 9 to 10. In secondary platings of wild type embryoid bodies initially grown for 6 days in suspension culture, we observed patches of benzidine+ cells, reminiscent of blood islands, adjacent to small, endoderm-lined cysts (Fig 2b). We assumed that these small cysts reflect collapsed visceral endoderm-lined cysts that form during suspension culture. When wild type embryoid bodies were grown for only 3 days in suspension culture, an insufficient amount of time for visceral endoderm formation, and then plated onto a substratum for 6 more days of culture, cysts were not observed, and benzidine positive cells were fewer in number and more scattered in appearance (Fig 2a)

In secondary platings of Gata4-/- embryoid bodies that had been maintained for either 3 or 6 days in suspension culture, no endoderm-lined cysts formed, and benzidine positive cells were scarce and dispersed (Fig 2c and d). These findings of an altered benzidine staining pattern in secondary platings of Gata4-/- embryoid bodies support the notion of impaired or disorganized erythropoiesis in these visceral endoderm deficient bodies.
To visualize the distribution of endothelial cells within visceral endoderm deficient embryoid bodies, we labeled embryoid bodies growing in suspension culture or on a substratum with dil-Ac-LDL, a fluorescent macromolecule that undergoes endocytosis by the scavenger receptor on endothelial cells and macrophages.\(^{33,34}\) In wild type embryoid bodies grown in suspension culture, dil-Ac-LDL accumulated in linear structures beneath the visceral endoderm layer; based on their appearance and location, these linear structures were assumed to be vascular channels (Fig 3a). The dil-Ac-LDL also labelled isolated cells within the cores of the embryoid bodies, presumed to be macrophages or individual endothelial cells. Consistent with the histological findings described above, no dil-Ac-LDL accumulated beneath the surface of Gata4-/- embryoid bodies grown in suspension culture, suggesting an absence of vessel development in this region of the bodies (Fig 3b). Scattered cells in the cores of mutant embryoid bodies, again assumed to be endothelial cells and macrophages, did label with dil-Ac-LDL (not shown). In wild type embryoid bodies grown on a substratum, dil-Ac-LDL\(^+\) vascular channels were commonly seen next to small cysts (Fig 3c). In adherent Gata4-/- embryoid bodies, organized vascular channels were not evident, but isolated cells took up the dil-Ac-LDL (Fig 3d).

Taken together, the benzidine and dil-Ac-LDL labeling studies of embryoid bodies verified that blood island and vessel formation are disrupted in visceral endoderm deficient Gata4-/- embryoid bodies. At the same time, these experiments suggested that hematopoiesis and endothelial cell differentiation are not abolished in the mutant embryoid bodies.

Expression of hematopoietic and endothelial transcripts in Gata4-/- embryoid bodies. To further characterize differences between wild type and visceral endoderm deficient embryoid bodies, we compared the expression of hematopoietic and endothelial cell transcripts in suspension cultures of wild type or Gata4-/- embryoid bodies using RNase protection analysis. \(\beta\)-actin message was measured concurrently to control for yield, and assays were performed on two independently selected Gata4-/- lines to ensure consistent results.

\(\epsilon\)-Globin mRNA, a marker of primitive erythropoiesis, was detected in both wild type and Gata4-/- embryoid bodies, although differences were evident in the amount of expression of this transcript (Fig 4). In wild type embryoid bodies, \(\epsilon\)-globin mRNA was first detected at day 7 of differentiation, reached a maximum at day 10, and then declined over the ensuing 6 days of culture (Fig 4). A similar pattern of \(\epsilon\)-globin mRNA expression in differentiating embryoid bodies has been reported by other investigators.\(^{51,52}\) The onset of \(\epsilon\)-globin expression in embryoid bodies derived from the two Gata4-/- ES cell lines coincided with the start of \(\epsilon\)-globin expression in the wild type embryoid bodies. However, the amount of this transcript at day 7 of differentiation was markedly reduced in the mutant embryoid bodies; furthermore, the level of expression of \(\epsilon\)-globin remained low in the Gata4-/- embryoid bodies through day 16 of culture (Fig 4). In multiple experiments with two different Gata4-/- lines, total \(\epsilon\)-globin expression in Gata4-/- embryoid bodies averaged 18\% that in wild type cells. Therefore, we conclude that a lack of visceral endoderm significantly reduces, but does not eliminate, primitive erythropoiesis in embryoid bodies.

The onset of expression of \(\beta^{\text{maj}}\) globin mRNA, a marker of definitive erythropoiesis, coincided with that of \(\epsilon\)-globin. In wild type embryoid bodies this transcript was first evident at 6 to 7 days of differentiation, peaked at day 10, and declined thereafter (Fig 4). In contrast to the situation with \(\epsilon\)-globin, there was no reduction in the amount of \(\beta^{\text{maj}}\) globin expression at 6 to 7 days of differentiation in Gata4-/- embryoid bodies (Fig 4). At day 10 of differentiation, the mutant embryoid bodies showed a modest reduction in the level of
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Fig 4. RNase protection assays for hematopoietic transcripts in wild type and Gata4-l- embryo bodies differentiated for varying lengths of time in suspension culture. Two independently selected Gata4-l- ES cell clones, termed 9c and 10b, are shown. Note that expression of $\varepsilon$-$\alpha$-globin, normalized to $\beta$-actin, is markedly reduced in both Gata4-l- lines.

$\beta^{\text{emb}}$ globin expression compared to the wild type embryoid bodies (Fig 4); however, between days 6 and 16 the total $\beta^{\text{emb}}$ globin was found to be 90% that in wild type embryoid bodies. Thus, these RNase protection studies indicate that visceral endoderm deficiency reduces primitive erythropoiesis more than definitive erythropoiesis.

Transcripts of three genes expressed selectively in endothelial cells – flk-1, flt-1, and PECAM – were measured. Flk-1, which is expressed both in early endothelial cell progenitors (hemangioblasts) and well-differentiated endothelial cells,9 had a similar expression pattern in the wild type and Gata4-l- embryo bodies (Fig 5). This transcript was first detectable between 4 to 7 days of differentiation and persisted through day 23 of culture. Expression of Flt-1, an mRNA expressed slightly later in endothelial differentiation program than Flk-1,17 was indistinguishable in the wild type and Gata4-l- embryo bodies (Fig 5). Transcripts for PECAM, an adhesion molecule present on endothelial cells, platelets and certain other cell types,70,71 were detected throughout differentiation of both wild type and Gata4-l- embryo bodies (Fig 5). Thus, there were no significant differences in the expression of Flk-1, Flt-1, or PECAM between the wild type and Gata4-l- embryo bodies. We conclude that although Gata4-l- embryo bodies lack an organized vascular network at their surface, endothelial cell differentiation nevertheless occurs within these bodies. Because none of the endothelial markers we employed was specific for yolk sac endothelium, it is not possible to say whether a subset of endothelial cells unique to yolk sac vasculature is selectively affected in the mutant embryoid bodies.

Methylcellulose cultures. The deficiency in organized blood island formation in the mutant embryoid bodies and the RNase protection results prompted us to assess the capacity of Gata4-l- ES cells to differentiate into primitive and definitive erythrocytes in methylcellulose culture. We grew Gata4-l- embryo bodies in methylcellulose media supplemented with Epo alone to promote primitive erythropoiesis or the combination of Epo plus KL to facilitate definitive erythropoiesis.60 Gata4-l- ES cells grown for 8 to 9 days in methylcellulose supplemented with Epo developed into embryo bodies with hemoglobinized cells on their surface. Cyto centrifugation of these hemoglobinized embryo bodies revealed cells with morphological features typical of primitive erythroblasts (Fig 6a). Methylcellulose culture in the presence of Epo plus KL for 8 to 10 days resulted in embryo bodies covered with loose patches of hemoglobinized cells. Cyto centrifugation of these embryo bodies verified the presence of definitive erythroid cells (Fig 6b). Thus, methylcellulose assays confirmed that Gata4-l- ES cells have the capacity to differentiate in vitro into both primitive and definitive erythrocytes.

When single cell suspensions of undifferentiated wild type or Gata4-l- ES cells were plated into methylcellulose media in the absence of supplemental hematopoietic growth factors, benzidine staining of the resultant embryo bodies revealed scattered erythrocytes within these bodies (Fig 7a and b). We also observed similar surface benzidine staining of blood cells in wild type and mutant embryo bodies, which were transferred to methylcellulose after first being cultured in suspension (data not shown), suggesting that visceral endoderm may not develop in methylcellulose, even in cultures derived from wild type cells.

Because wild type and Gata4-l- ES cells exhibited similar capacities to differentiate into erythroid cells in methylcellulose but differences in their ability to form blood islands and $\varepsilon$-$\alpha$-globin in suspension culture, we compared the surface morphology of embryo bodies grown under these two conditions. The appearance of embryo bodies cultured in methylcellulose differed from those maintained in suspension culture. When grown in methylcellulose from single cell suspensions, neither wild type nor Gata4-l- embryo bodies developed surface visceral endoderm, as evidenced by histological sections (Fig 7c and d) and binding of FITC-labeled DBA (Fig 7e and f). Hence, culturing in methylcellulose effectively removes the influence of visceral endoderm on hematopoietic development, and consequently the phenotypes of wild type and Gata4-l- embryo bodies grown in methylcellulose are indistinguishable.

This suggests that defects in blood island formation noted in the mutant embryoid bodies grown in suspension culture are secondary to a lack of visceral endoderm rather than a cell autonomous defect in erythroid differentiation in Gata4-l- cells.

Endothelial cell outgrowth from embryo bodies. To show that Gata4-l- ES cells have the capacity to differentiate and organize into vessels under the proper external signals,
we grew mutant embryoid bodies for 5 days in suspension culture and then transferred the bodies to matrigel, a basement membrane matrix extracted from tumor cells that facilitates epithelial cell attachment, cell differentiation, and vascular outgrowth. Over the ensuing days of culture, the resultant embryoid bodies elaborated vascular processes that labeled with diI-Ac-LDL (Fig 8). Although vessel development in this experimental system is more characteristic of angiogenesis than vasculogenesis, these results nevertheless confirm that *Gata4-/-* embryoid bodies can form endothelial cell-lined vessels in the proper microenvironment. Furthermore, these findings suggest that an absence of visceral endoderm-derived basement membrane may contribute to

![Image of RNase protection assays for the endothelial cell markers FLK-1, FLT-1, and PECAM in *Gata4+/* and *Gata4--* embryoid bodies differentiated for varying lengths of time. *β*-actin message is included as a control.](image)

![Fig 5. The appearances of wild type and *Gata4-/-* embryoid bodies grown in methylcellulose are indistinguishable due to a lack of surface visceral endoderm formation. Methylcellulose cultures of wild type (a) or *Gata4-/-* (b) embryoid bodies grown in the absence of supplemental growth factors. Bars = 100 μm. Toluidine blue stained histological sections through day 8 methylcellulose cultures of wild type (c) or *Gata4-/-* (d) embryoid bodies, revealing a lack of visceral endoderm or vascular channels. Bars = 10 μm. (e) FITC-DBA binding to the surface of day 10 wild type embryoid bodies grown in methylcellulose, showing a lack of surface visceral endoderm staining; (f) FITC-DBA binding to suspension culture of wild type embryoid bodies showing the presence of abundant visceral endoderm. Bars = 100 μm.](image)

### Fig 6. Cyto centrifugation of *Gata4-/-* embryoid bodies grown in methylcellulose in the presence of Epo alone (a) or Epo + KL (b). Bar = 20 μm.

![Image of cyto centrifugation of *Gata4-/-* embryoid bodies grown in methylcellulose in the presence of Epo alone (a) or Epo + KL (b).](image)
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Fig 8. Vessel outgrowth from a Gata4-/- embryoid body grown in matrigel. This embryoid body was grown in suspension culture for 5 days and then transferred to matrigel for an additional 13 days. The embryoid body was labeled with dl-Ac-LDL to visualize endothelial cells. The embryoid body was photographed under (a) brightfield and (b) fluorescence optics. Note the outgrowth of endothelial cells. Bars = 100 μm.

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The lack of vessel development in Gata4-/- embryoid bodies grown in suspension culture or on a substratum.

Analysis of mouse embryo chimeras. To verify that GATA-4 deficient ES cells have the capacity to organize into yolk sac blood cells and vessels in the presence of visceral endoderm, we prepared and analyzed Gata4-/- chimeric mouse embryos. Gata4-/- ES cells were injected into 8-cell stage embryos derived from ROSA26 mice, a transgenic line in which the β-galactosidase gene is ubiquitously expressed throughout development.69 The injected embryos were implanted into pseudopregnant females. Embryos and attached yolk sacs were harvested at 8 to 9 days p.c. and stained for β-galactosidase activity using X-gal. Host-derived cells in the chimeric embryos could be readily distinguished from ES cell descendants on the basis of β-galactosidase expression (Fig 9a and b). Whereas visceral yolk sac endoderm cells in the Gata4-/- ES → ROSA26 chimeric animals were consistently derived from the ROSA26 host cells, hematopoietic and endothelial cells in the yolk sac of mosaic animals often did not express β-galactosidase and were therefore derived from ES cells (Fig 9a and b). In control experiments we verified that nonchimeric ROSA26 embryos exhibited uniform expression of β-galactosidase in blood cells and vessels of the yolk sac (data not shown). On the basis of these findings we conclude that Gata4-/- ES cells can differentiate into normal appearing blood islands and vessels when juxtaposed to visceral endoderm in vivo.

DISCUSSION

The contribution of extraembryonic endoderm to yolk sac hematopoiesis and vasculogenesis has been the subject of study and debate for decades.5,8,35,39,44 Gata4-/- embryoid bodies, which lack visceral endoderm, provide a novel and powerful system to assess the role of this cell layer in the regulation of yolk sac hematopoiesis and blood vessel development. We found that organized blood islands and vascular channels are absent from the surface of Gata4-/- embryoid bodies grown in suspension culture, whereas these blood islands and vessels are readily observed on the surface of normal embryoid bodies. Defects in blood island formation and vessel development were also manifest in Gata4-/- embryoid bodies grown on a substratum. The level of ε-globin mRNA was markedly reduced in suspension cultures of Gata4-/- embryoid bodies, indicating that visceral endoderm is required for optimal primitive erythropoiesis in vitro. Visceral endoderm may be an important component of the hematopoietic microenvironment in embryoid bodies. Another possibility is that visceral endoderm is critical for organizing and retaining blood islands at the surface of embryoid bodies in suspension culture. In the absence of a surface endoderm layer, primitive erythroblasts that do form may be shed into the medium. Despite a lack of morphologically recognizable vascular channels, Flk-1, Flt-1, and PECAM transcripts were detected within Gata4-/- embryoid bodies, indicating that endothelial differentiation can proceed in the absence of visceral endoderm. Studies of blood and vessel formation in methylcellulose, matrigel, and chimeric mice verified that Gata4-/- ES cells retain the capacity to differentiate into primitive erythrocytes and endothelial cells and to organize blood islands and vessels when provided with the proper microenvironment. We conclude that visceral endoderm participates in development and organization of blood islands and vessels in vitro, but this cell layer is not essential for primitive erythroblast or endothelial cell differentiation.

These results are strikingly similar to findings obtained using cultured explants of chick35,36 and mouse.72 Studies with chick embryo explants showed that the endoderm layer is required for the normal formation of blood islands by mesodermal cells.35,36 When the endoderm layer was stripped from the mesoderm, no endothelium developed in the mesodermal layer, erythrocyte formation was modestly reduced, hemoglobin synthesis was delayed 1 to 2 days, and erythropoiesis took place in small clusters of cells scattered through the mesodermal layer rather than in blood islands.72 Analogous explant studies performed on mouse yolk sac37 showed that visceral endoderm is essential for the formation of blood vessels in 7.5 day p.c. yolk sac mesoderm explants. However, explanted mouse yolk sac mesoderm cells were observed to undergo primitive erythropoiesis in the absence of either visceral endoderm or a vascular network. Based on these findings, Palis et al77 concluded that if the visceral yolk sac endoderm has an inductive role in mouse blood cell development, it must occur before 7.5 days p.c., the time at which the endoderm and mesoderm layers were separated for explant analysis. Our in vitro embryoid body model argues that yolk sac endoderm is not required for induction of primitive hematopoiesis, even early in development (ie, before 7.5 days p.c.). Collectively, explant studies in chick and mouse and our studies of differentiating embryoid bodies suggest that endoderm is essential for development of the vascular network within the mesodermal layer, but primitive erythropoiesis can proceed in the absence of endoderm, al-
heit in a more disorganized fashion. The means by which visceral endoderm influences vasculogenesis and hematopoiesis is unknown, although several potential mechanisms exist. \(^{37-39}\) Visceral endoderm may produce soluble growth factors or cell surface ligands/receptors that participate in hematopoiesis or vasculogenesis. \(^{38,39}\) Extracellular matrix synthesized by visceral endoderm may facilitate blood island and vessel organization. \(^7\) Alternatively, visceral endoderm may transport maternal proteins/nutrients that aid blood cell and vessel development. \(^{12,2}\)

While embryoid bodies are a useful model for the study of many differentiation programs, the limitations of this system should be kept in mind. Numerous reports have documented that embryoid body differentiation in suspension culture recapitulates events in yolk sac development, including differentiation of visceral endoderm, \(^{31}\) formation of vascular channels, \(^{2,24-26}\) and temporal expression of embryonic and adult globins. \(^{32,47}\) However, embryoid bodies do not express all markers present in the intact mouse yolk sac, \(^{39}\) suggesting this in vitro model may not reproduce all aspects of yolk sac development. Both in vitro and in vivo studies suggest that the hematopoietic potential of ES cells is limited. \(^{48,56,57,6}\) It is possible that primitive erythroblasts within ES cell-derived embryoid bodies are not subject to the same positive or negative regulatory signals as in vivo. Consequently, the embryoid body system may over or underemphasize the role of visceral endoderm in primitive erythropoiesis. However, the striking similarities between our embryoid body findings and results obtained with tissue explants \(^{52,57}\) suggest that the embryoid body system is a reasonable model.

Through knockout studies in mice, a number of genes participating in yolk sac hematopoiesis and vasculogenesis have been identified. Some of these genes disruptions affect just hematopoiesis, \(^{22,23,30,56}\) others mainly disturb vascular development, \(^{17,77,79}\) and still others affect both processes. \(^{5,11,12,14}\) Additional factors influencing yolk sac vasculogenesis and hematopoiesis are likely to emerge in the coming years. \(^{Gata4-/-}\) embryoid bodies will provide a useful visceral endoderm-free system in which to study the effects of growth or differentiation factors normally elaborated by visceral endoderm, including substances that affect primitive hematopoiesis and vasculogenesis.

**ACKNOWLEDGMENT**

We thank the laboratories of Stuart Orkin, Scott Baldwin, M. Shibuya, and Tim Ley for providing RNase protection probes. The \(^{Gata4-/-}\) ES lines were prepared in collaboration with Claire Soudais, Celeste Simon, and Jeff Leiden of the University of Chicago, and we are indebted to the assistance and generosity of these investigators. We thank Jeff Gordon and members of his laboratory for suggestions regarding the \(^{ROSA26}\) chimeras, and Craig MacArthur, Jonathan Gitlin, and Greg Longmore for critiquing the report.

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