

Hematopoietic induction and respecification of A-P identity by visceral endoderm signaling in the mouse embryo

Maria Belaoussoff, Sarah M. Farrington* and Margaret H. Baron†,§

Department of Molecular and Cellular Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA

*Current address: Department of Biology and Center for Cancer Research, Massachusetts Institute of Technology, 40 Ames Street, Cambridge, MA 02139, USA

†Current address: Departments of Medicine, Developmental and Molecular Biology, Ruttenberg Cancer Center, and Institute for Gene Therapy and Molecular Medicine, The Mount Sinai School of Medicine, New York, NY 10029, USA

§Author for correspondence (e-mail: mhbaron@msvax.mssm.edu)

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SUMMARY

The anteroposterior axis of the developing embryo becomes morphologically apparent at the onset of gastrulation with the formation of the primitive streak. This structure, where the first mesodermal cells arise, marks the posterior aspect of the embryo. To examine the potential role of non-mesodermal signals in specifying posterior (hematopoietic and endothelial) cell fates in the mouse embryo, we have devised a transgenic explant culture system. We show that interactions between primitive endoderm and adjacent embryonic ectoderm or nascent mesoderm are required early in gastrulation for initiation of hematopoiesis and vasculogenesis. Surprisingly, primitive endoderm signals can respecify anterior (prospective neural) ectoderm to a

posterior mesodermal fate, resulting in formation of blood and activation of endothelial markers. Reprogramming of anterior ectoderm does not require cell contact and is effected by stage-dependent, short-range, diffusible signal(s). Therefore, primitive endoderm signaling is a critical early determinant of hematopoietic and vascular development and plays a decisive role in anterior-posterior patterning during mouse embryogenesis.

Key words: Gastrulation, Hematopoiesis, Visceral endoderm, Vasculogenesis, Axis determination, Embryonic patterning, Cell signaling, Transgenic mice, Explant culture

INTRODUCTION

Interactions between neighboring tissues are required for morphogenesis and patterning throughout embryonic development. In the mouse, the extraembryonic (primitive or visceral) endoderm is strategically positioned for inductive interactions with the underlying ectoderm and later the mesoderm. Over the past few years, it has become clear that the primitive endoderm, a secretory epithelium, is involved in regulation of a number of early developmental processes (reviewed by Rossant, 1995). For example, a death signal emanating from the primitive (visceral) endoderm has been implicated in the process of cavitation, which transforms a solid mass of primitive ectoderm cells into a hollow tube shortly after implantation (Cocouvanis and Martin, 1995). Another role of the visceral endoderm (VE) is the promotion of growth and survival of the underlying ectoderm. Targeted mutagenesis of the *HNF-4* gene, a winged-helix transcription factor expressed in the visceral endoderm, results in extensive ectodermal cell death early in gastrulation (Chen et al., 1994). Mutations in the *Smad/DPC4* (Sirard et al., 1998) and *evx1* (Spyropoulos and Capecchi, 1994) genes affect visceral endoderm development and lead to the rapid degeneration of the ectoderm, providing further evidence that the visceral endoderm is required for ectoderm survival.

A number of recent studies suggest that the primitive endoderm is also involved in the patterning of anterior ectoderm, a tissue fated to give rise to neural structures (Lawson et al., 1991). Patterning of the anterior central nervous system was originally attributed to the derivatives of the mouse node, a structure equivalent to the *Xenopus* organizer. However, accumulating molecular evidence now indicates that the visceral endoderm is patterned along the anterior-posterior (A-P) axis prior to gastrulation (Belo et al., 1997; Biben et al., 1998; Rosenquist and Martin, 1995; Thomas and Beddington, 1996; Thomas et al., 1998). The *Cerberus-like* (*Cer-1*) (Belo et al., 1997; Biben et al., 1998) and *nodal* (Varlet et al., 1997) genes encode secreted proteins expressed in the anterior visceral endoderm and are likely to be involved in the patterning of the underlying anterior ectoderm.

Smad2, an intracellular effector in the TGF β and activin signaling pathway, has recently been shown to play a key role in specification of the anterior aspect of the embryo (Waldrip et al., 1998). This gene is widely expressed and regulates an extraembryonic signal, though the tissue of origin – primitive endoderm or extraembryonic ectoderm or both – has not yet been identified.

Patterning of the posterior ectoderm is less well understood. During gastrulation, extensive morphogenetic movements place a subset of nascent mesoderm cells derived

from the posterior primitive streak in close apposition with primitive endoderm in the extraembryonic region of the embryo. It is here, in what will become the visceral yolk sac, that the extraembryonic mesoderm gives rise to blood islands late in gastrulation. These structures contain clusters of hematopoietic (largely primitive erythroid) cells surrounded by endothelial cells (angioblasts) (Haar and Ackerman, 1971b) and are characteristic of nearly all vertebrate embryos. Blood islands gradually merge to form vascular channels and then discrete blood vessels within the yolk sac, which in the mouse eventually expands to surround the entire embryo proper (see Hogan et al., 1994). Primitive hematopoiesis, the formation of embryonic blood cells, and vasculogenesis, the de novo formation of blood vessels from endothelial cells, are therefore closely associated processes in the yolk sac and may arise from a common progenitor, the hypothetical 'hemangioblast' (reviewed by Flamme et al., 1997).

Although the mechanism by which posterior (blood and endothelial) cell fates are specified remains elusive, the juxtaposition of primitive endoderm and extraembryonic mesoderm in the yolk sac raises the possibility that interactions between these two tissue layers might be required for the formation of blood islands. Classical transplantation studies in chick embryos have implicated the primitive endoderm in the formation of blood and vascular tissue by extraembryonic mesoderm (Miura and Wilt, 1969; Wilt, 1965). Whether primitive endoderm is required for the development of hematopoietic and endothelial lineages in mammalian embryos is controversial (e.g. compare Bielinska et al., 1996; Palis et al., 1995).

To more definitively assess the possible role of primitive endoderm signaling in specifying posterior mesodermal (hematopoietic and angioblastic) cell fates in the mouse, we devised an explant culture system in which embryos carrying an ϵ -globin/*lacZ* reporter transgene are recombined with non-transgenic primitive endoderm. The transgenic ectoderms thus serve as a potential source of marked primitive erythroid cells. Embryonic ectoderm stripped of its primitive endoderm is unable to form blood cells or to activate endogenous embryonic globin genes in culture. Recombination of embryonic ectoderm with primitive endoderm results in the development of blood cells and activation of embryonic β -like globin genes, demonstrating that the primitive endoderm is necessary for embryonic hematopoiesis around the onset of gastrulation. Primitive endoderm signaling is required only during a narrow window of time, because formation of erythroid cells and expression of hematopoietic and vascular endothelial marker genes become autonomous to the mesoderm by midgastrulation. Remarkably, early gastrulation-stage primitive endoderm can respecify anterior ectoderm to develop into posterior (hematopoietic and angioblastic) cell lineages. Reprogramming of anterior ectoderm by primitive endoderm signaling occurs in the absence of extraembryonic ectoderm. Cells of the anterior ectoderm are normally fated to give rise to neural structures by midgastrulation but, when recombined with visceral endoderm, they adopt posterior mesodermal (hematopoietic and endothelial) cell fates. Trans-filter experiments demonstrate that the posteriorizing primitive endoderm signal(s) is(are) mediated by diffusible molecules. These

signals are still present late in gastrulation, though their activity is less potent, and they are no longer detectable at later developmental stages. Based on these findings, we conclude that the developmental program of anterior ectoderm is still quite plastic even by midgastrulation. Primitive endoderm signals play a crucial role in patterning the A-P axis of the embryo and in specification of posterior mesodermal (hematopoietic and endothelial) cell fates.

MATERIALS AND METHODS

Explant culture assay

Non-transgenic CD1 (Charles River Laboratories) or ICR (Taconic or Harlan) mice were used for these studies; transgenic mice were CD1. Noon of the day of detection of the copulation plug was taken as day 0.5 of gestation (0.5 days post coitum, dpc). Prestreak to early streak embryos were dissected at ~6.0-6.25 dpc, and embryos to be used for assays of anterior and posterior regions were dissected at ~6.75 dpc. Careful notes were kept on the apparent developmental stage of the embryos based on morphological landmarks (Downs and Davies, 1993) and, except where indicated, only embryos that developed to the desired stage were used. For most experiments, explants were cultured individually in the wells of 4-well (Nunc) or 24-well (Costar) plates. Culture times ranged from 2 to 4 days, as indicated. Explant culture medium was Dulbecco's Modified Eagle's Medium (DME) supplemented with 30% fetal bovine serum (FBS), 2 mM glutamine, 10 mM Hepes pH 7.4, 68 μ M α -methyl thioglycerol, penicillin (1,000 U/ml) and streptomycin (1,000 μ g/ml).

Embryos were enzymatically separated (Farrington et al., 1997) into ectodermal and visceral endoderm components. Ectodermal explants were then recombined with visceral endoderm in 5-10 μ l of collagen (rat tail, type I). After the collagen had formed a gel, medium was added to the well. Cultures were maintained at 37°C and 5% CO₂. Medium was changed after 24 and 48 hours.

Analysis of explants

RNA was harvested from individual explants at the times indicated using a small-scale method (Chomczynski and Sacchi, 1987) and analyzed for expression of various mouse genes by semiquantitative multiplex RT-PCR, as described previously (Farrington et al., 1997). Annealing temperature for all primer pairs was 55°C. Sequences for the following amplification primers can be obtained from the corresponding references: β -actin and AFP (Farrington et al., 1997), ϵ^{β} - and β maj-globin (Baron and Farrington, 1994), CD-34 (McClanahan et al., 1993) and α -cardiac myosin (Johansson and Wiles, 1995). Other oligonucleotide DNA primers were:

Brachyury (5'): 5'-GTCTTCTGGTTCCTCCGATGT-3'
 Brachyury (3'): 5'-CCAGGTGCTATATATTGCCT-3'
 Otx2 (5'): 5'-AGGAGCTGAGTCGCCACCTC-3'
 Otx2 (3'): 5'-GTAGCCCACGGAGGGATGCA-3'
 GATA-1 (5'): 5'-CAGCACTGGCCTACTACAG-3'
 GATA-1 (3'): 5'-TCAAGGTGTCCAAGAACGTG-3'
 flk-1 (5'): 5'-CCATACCGCCTCTGTGACTT-3'
 flk-1 (3'): 5'-ACACGATGCCATGCTGGTCA-3'
 PECAM-1 (5'): 5'-TGCGATGGTGTATAACGTCA-3'
 PECAM-1 (3'): 5'-GCTTGGCAGCGAAACACTAA-3'

Transgenic mice

Mice transgenic for a *lacZ* reporter linked to one of several human embryonic β -globin upstream regulatory sequences (Dyer et al., 1998; Trepicchio et al., 1993, 1994) were bred to homozygosity. Embryos from these animals served as a source of marked embryos in which the transgene was expressed only in primitive erythroid cells. These mouse lines will be described in detail elsewhere (M. B., L. Degenstein, E. Fuchs, and M. H. B., unpublished work).

Nucleopore filter assays

Sandwiches were prepared in which anterior ectoderm was placed into a ~10 μ l drop of collagen at the bottom of the well and covered with a piece of polycarbonate filter (Nucleopore, 0.1 μ m pore size) cut so that it was significantly larger than the explants. The filter was then overlaid with 3-4 visceral endoderm explants and the collagen was allowed to gel.

X-gal staining and cryosectioning

Transgenic explants were stained with X-gal using a standard protocol (Wassarman and DePamphilis, 1993). Stained explants were fixed and photographed as whole mounts or processed for cryosectioning (Ausubel et al., 1997). The sections were counterstained with Kernechtrot, mounted in Gelmount (Fisher) and photographed.

RESULTS

Explant culture assay for induction of hematopoiesis

To determine whether the primitive endoderm lineage is required for embryonic hematopoiesis (which at its earliest stages is largely erythropoietic), we devised an explant culture system in which ectoderm dissected from prestreak or early streak mouse embryos (6.0-6.25 dpc) was cultured in the presence or absence of visceral endoderm and analyzed for activation of a primitive erythroid reporter transgene (shown schematically in Fig. 1A) or of endogenous hematopoietic marker genes (Fig. 1B). For this assay, embryos were harvested at the prestreak to early streak stage, prior to the formation of morphologically detectable blood cells or their molecular markers. At this stage of development, embryonic (ϵ^y) globin RNA – a marker of primitive erythroblasts – is not detected by whole-mount in situ hybridization (not shown) or by RT-PCR (see below). Ectoderm (both embryonic and extraembryonic regions) and visceral endoderm layers were separated following brief enzymatic treatment of the embryos (Fig. 1) and were subsequently assayed for transgene (Fig. 1A) or endogenous gene (Fig. 1B) expression. Analysis of the expression of endoderm- and ectoderm-specific genes (Farrington et al., 1997) by RT-PCR confirmed that there was no cross-contamination of the separated tissues (data not shown).

To examine the pattern of globin gene activation in cultured whole embryos and isolated ectoderms, we took advantage of transgenic mouse lines carrying a *lacZ* reporter gene linked to human embryonic β -like (ϵ) globin upstream regulatory sequences (Trepicchio et al., 1993). These animals served as a source of marked embryos (shown at different stages in Fig. 2) in which the transgene is expressed only in nucleated, primitive erythroid cells (M. B. and M. H. B., unpublished data). Transgenic males bred to homozygosity were mated with non-transgenic females and embryos were harvested at 6.0-6.25 days. Whole embryos or ectoderms stripped of visceral endoderm were cultured individually for 48-72 hours, then fixed and stained with X-gal to monitor the generation of primitive erythroblasts. When cultured either on polycarbonate filters (Fig. 3A), in the wells of chambered slides (Fig. 3C), or in plastic dishes (not shown), whole embryos formed β -gal-positive blood islands. In contrast, β -gal staining was not detected in cultured ectoderms separated from visceral endoderm (Fig. 3B,D). These results suggest that embryonic

hematopoiesis is not autonomous to ectoderm (or intrinsic to the mesodermal cells arising from the primitive streak) but requires contact with or signals released from visceral endoderm.

Requirement of visceral endoderm signaling for embryonic hematopoiesis

To establish more directly that visceral endoderm is required for induction of hematopoiesis in the gastrulating embryo, tissue recombination experiments were performed. A semiquantitative RT-PCR protocol was used to assay for activation of the endogenous mouse embryonic β -like globin genes (Farrington et al., 1997). Ectoderm and visceral endoderm layers from individual embryos were cultured separately or in combination (see Fig. 1) for 2-4 days. RNA was prepared from individual explants and analyzed for ϵ^y - and β h1-globin (Shehee et al., 1989) gene activation using the RT-PCR assay.

Embryonic β -like globin gene expression was not detected in newly dissected 6.0-6.25 dpc whole embryos or isolated ectodermal layers (not shown). After 72 hours in culture, the mouse ϵ^y -globin gene was activated in whole embryos (Fig. 3E, lanes 4-6) but, in isolated ectoderm, little or no ϵ^y -globin transcription could be detected (lanes 7-12). In contrast, recombination of ectoderm with visceral endoderm from same stage embryos resulted in activation of ϵ^y -globin to levels comparable to those observed with whole embryos (lanes 13-17). Similar results (not shown) were obtained for a second mouse embryonic β -like globin gene, β h1 (Shehee et al., 1989). These observations establish that, around the onset of gastrulation, induction of primitive hematopoiesis is not autonomous to ectoderm (more specifically, nascent mesoderm cells arising from the primitive streak) and requires the presence of visceral endoderm.

To delineate the period during development when visceral endoderm is required for hematopoietic induction, we cultured ectoderm layers isolated from pre- to late-gastrulation-stage embryos. Developmental stage was identified on the basis of embryonic size and the presence of well-described morphological landmarks (Downs and Davies, 1993). By midgastrulation (midstreak stage), the proportion of globin-expressing ectoderms had increased to nearly 100% when mesectoderm from midstreak-stage embryos was cultured for 4 days (data not shown). These findings suggest that by this stage (~6.5-6.75 dpc), the ectoderm (or a small amount of closely associated nascent mesoderm) has already received a blood-inducing signal from the visceral endoderm. Therefore, blood formation is autonomous to mesectoderm by the midstreak stage, defining a narrow window of time during which the visceral endoderm is required for primitive hematopoiesis.

Respecification of anterior character by visceral endoderm

Lineage tracing experiments have shown that hematopoietic mesoderm arises from the posterior primitive streak (posterior mesoderm; reviewed by Tam and Behringer, 1997). The explants used in the experiments described above contained posterior ectoderm, which gives rise to blood cells and other derivatives of extraembryonic mesoderm. To determine whether visceral endoderm signaling can respecify ectoderm

that does not contain mesoderm and is not normally fated to express a posterior embryonic developmental program, we modified the explant culture assay. In place of prestreak or early streak embryonic ectoderm, we used anterior ectoderm from midstreak-stage embryos (~6.75 dpc). At this time during development, the anterior epiblast does not yet contain mesoderm (Beddington, 1982) and is fated to give rise to neurectoderm. It is therefore not expected to produce hematopoietic or vascular tissue in culture. Anterior and posterior aspects of the midstreak-stage embryo are easily distinguished by the presence of prominent mesodermal wings in the posterior region and by the primitive streak, which marks the posterior pole of the A-P axis (Downs and Davies, 1993).

Embryonic ectoderms stripped of visceral endoderm were dissected into mesoderm-free anterior pieces and posterior pieces with their associated mesodermal wings (Fig. 4A). To ensure that the anterior ectoderm pieces were free of contaminating mesoderm, uncultured anterior and posterior pieces were assayed for expression of *Brachyury*, an early mesodermal marker. *Brachyury* expression was confined to the posterior pieces (data not shown), confirming that the mesoderm remained associated with the posterior ectoderm. After 4 days in culture, *Brachyury* expression was undetectable in whole embryos and in both anterior and posterior pieces (not shown). The downregulation of *Brachyury* in cultured whole embryos and posterior pieces mimics the behavior of this gene in vivo.

Early in gastrulation, *Otx2* is expressed throughout the ectoderm (Simeone et al., 1992) but by the headfold stage becomes restricted to the anterior of the embryo (Acampora et al., 1995; Ang et al., 1994). If anterior ectoderm is respecified to a posterior fate when cultured with primitive endoderm, expression of *Otx2* should be downregulated in recombinants. Consistent with this prediction, *Otx2* was expressed at significant levels in both posterior and anterior pieces at the time of their dissection from 6.75 dpc embryos (Fig. 4B, TOP, lanes 3-14) but during culture was dramatically reduced or absent from posterior explants (BOTTOM, lanes 13-17) and recombinants (BOTTOM, lanes 4-7; the one positive recombinant, lane 3, showed little globin gene activation). In contrast, *Otx2* expression continued in isolated anterior explants (Fig. 4B, BOTTOM, lanes 8-12).

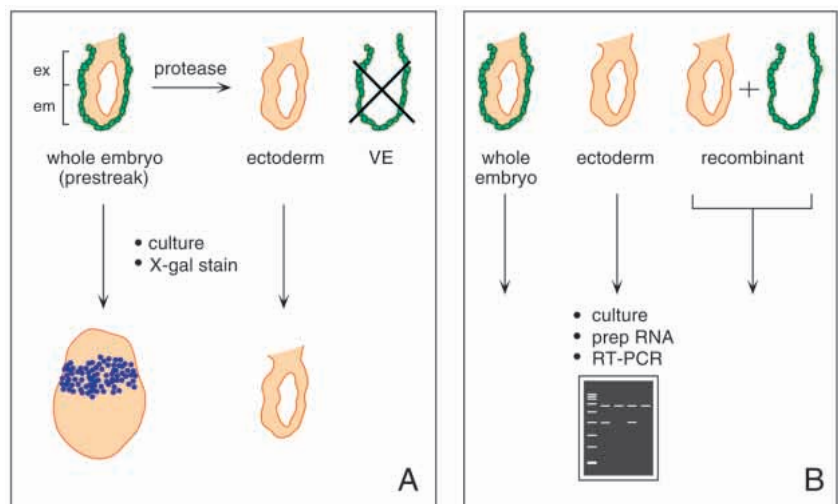
Respecification of anterior ectoderm to a hematopoietic cell fate by visceral endoderm

To confirm that dissected anterior and posterior ectodermal explants differ in their capacity to activate expression of ϵ^y -globin RNA during culture, as would be predicted from the lineage tracing studies (Tam and Beddington, 1987), an RT-PCR assay was performed. Abundant transcription of the endogenous ϵ^y -globin gene (correlating with the presence of posterior mesoderm) was detected in cultured posterior pieces (Fig. 4C, lanes 13-17), whereas cultured anterior explants lacked any detectable ϵ^y -globin gene expression (lanes 8-12). We next asked whether visceral endoderm can activate hematopoietic gene expression when cultured with anterior ectoderm. Recombination of anterior epiblasts with early or midstreak visceral endoderm resulted in the activation of ϵ^y -globin gene expression (Fig. 4C, lanes 3-7) to levels comparable to those observed in cultured posterior explants (lanes 13-17) or whole embryos (lane 19). The adult β major globin gene was weakly expressed in cultured posterior ectodermal pieces (lanes 13-17) and anterior ectoderm/VE recombinants (lanes 3-7), but not at all in anterior explants (lanes 8-12).

Globin RNA is a marker of differentiating erythroid cells. To determine whether markers of hematopoietic stem/progenitor cells are activated by primitive endoderm signals, we assayed for expression of CD-34 and GATA-1. As shown in Fig. 4C, both genes were activated in posterior ectodermal explants (lanes 13-17) and in anterior ectoderm/VE recombinants (lanes 3-7). Little or no expression of these markers was detected in visceral endoderm alone (lane 18) or in uncultured whole embryos (lane 20) from the same stage of development. The presence of visceral endoderm in recombinants did not result in promiscuous activation of mesoderm differentiation markers: cardiac myosin, which is expected to be expressed only in cardiac tissue (and therefore only at a later developmental stage), was not detected in anterior or posterior epiblast pieces or in recombinants during the first 4 days in culture (lanes 3-17), but was detected in a 10.5 dpc embryonic control (lane 2).

Together with the data shown in Fig. 4B, these results suggest that signals from primitive endoderm can respecify

Fig. 1. Experimental scheme for testing requirement for primitive endoderm in hematopoietic induction around the onset of gastrulation. Pre- or early-streak mouse embryos were separated into primitive endoderm (shown in green) and ectodermal portions and then cultured as explants as described in the text. After 2-4 days, explants were analyzed for activation of the *lacZ* transgene or of endogenous marker genes. (A) Comparison of human ϵ -globin/*lacZ* transgene activation in whole embryos or ectoderms stripped of surrounding primitive endoderm. Cultured transgenic explants were stained with X-gal to reveal formation of primitive erythroblasts. (B) Analysis of endogenous marker gene (e.g. *globin*) activation in whole embryos, ectodermal explants or ectoderm/VE recombinants by semiquantitative RT-PCR. Abbreviations: ex, extraembryonic ectoderm; em, embryonic ectoderm; VE, visceral endoderm.



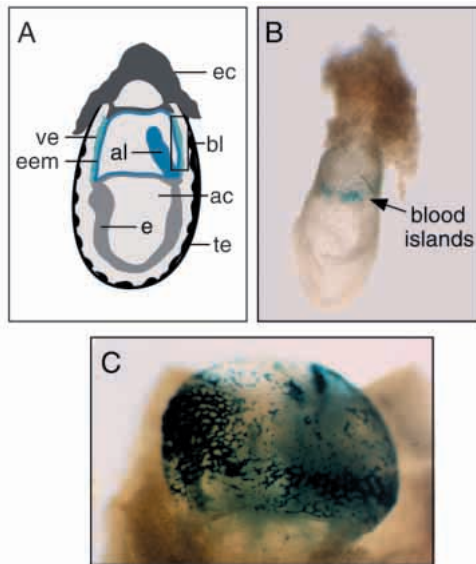


Fig. 2. *lacZ* expression in primitive erythroid cells of human ϵ -globin/*lacZ* transgenic mice. *lacZ* expression was revealed by blue staining and represents primitive erythroblasts. (A) Cartoon of cross section of a 7.5 dpc (late gastrulation stage) embryo. Boxed areas flanking the exocoelomic cavity indicate region of blood island formation (actually a ring, as shown in B). Abbreviations: ec, ectoplacental cone; bl, blood islands; ac, amniotic cavity; te, trophoblast; al, allantois; eem, extraembryonic mesoderm (shown in blue); ve, visceral endoderm (shown in green); e, embryonic ectoderm. (B) Bright-field photograph of an X-gal-stained 7.5 dpc transgenic embryo. Blood islands containing primitive erythroblasts and vascular endothelial cells are visible as a blue ring encircling the embryo at the level of the exocoelomic cavity. (C) Bright-field photograph of a transgenic embryo at 8.5 dpc. By this stage of development, blood islands have merged to form vascular channels, outlined here by the β -gal-positive erythroid cells, and the yolk sac now encloses nearly the entire embryo.

tissue (anterior ectoderm) that does not contain mesoderm and has no intrinsic potential to activate expression of markers of a posterior (hematopoietic) cell fate. These results are consistent with the conclusion drawn previously from transplantation experiments that the developmental fate of embryonic ectoderm is not prepatterned but can be influenced by local environment (Beddington, 1982). The reduction of *Otx2* expression and concomitant induction of hematopoietic marker genes in anterior ectoderm/VE recombinants suggest that anterior ectoderm can be respecified by the visceral endoderm to adopt a posterior fate.

Activation of vascular endothelial markers by visceral endoderm signals

An accumulating body of circumstantial evidence strongly suggests that the primitive hematopoietic and vascular endothelial lineages share a common progenitor termed the hemangioblast (reviewed recently by Flamme et al., 1997). For example, targeted mutagenesis of a number of mouse genes prevents the formation of both embryonic blood and endothelial cells in the yolk sac (discussed by Shalaby et al., 1997). The observation that 6.5 dpc primitive endoderm posteriorizes anterior ectoderm from midgastrulation-stage

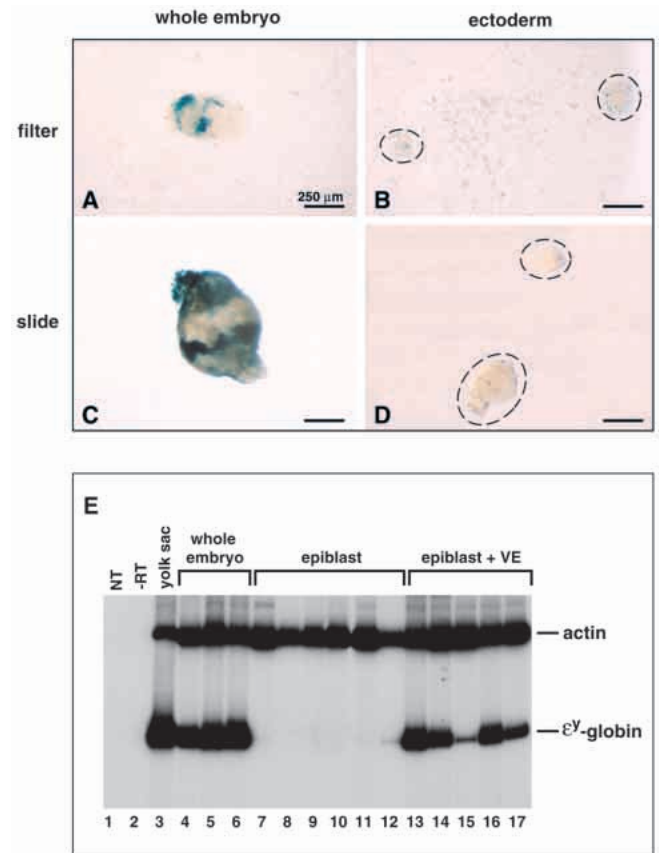
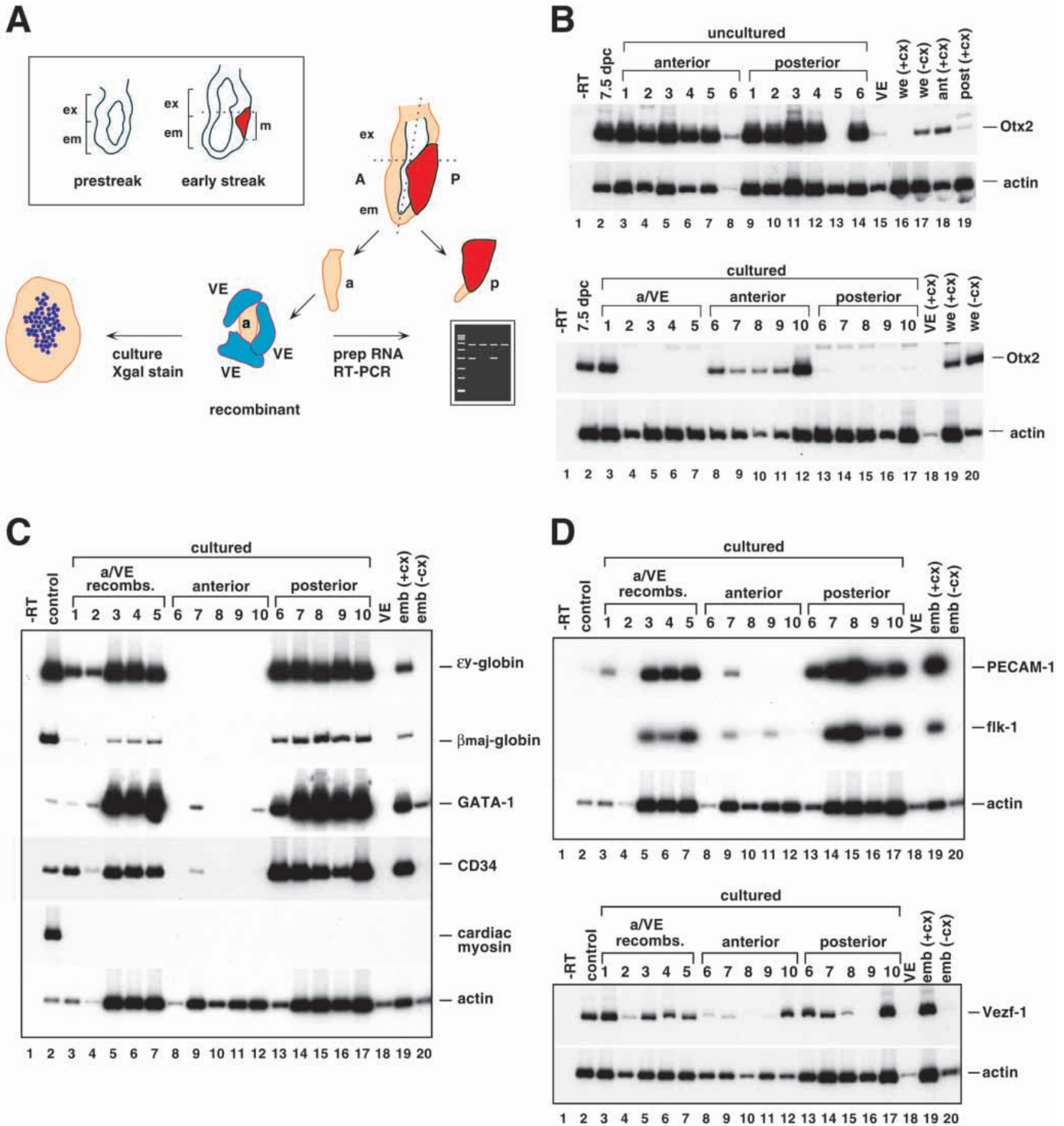


Fig. 3. Expression of transgenic and endogenous embryonic globin gene expression in explant cultures. (A-D) Expression of embryonic β -globin/*lacZ* transgene in explant cultures of whole embryos and separated ectoderm layers. Pre- or early-streak transgenic embryos were cultured whole or after removal of visceral endoderm. Dashed lines are drawn around the ectodermal explants, which would otherwise be difficult to see in the photograph. In the experiment shown here, explants were cultured either on polycarbonate filters (Nucleopore, 0.4 μ m) (A,B) or in the chambers of 8-well glass slides (C,D), with equivalent results. Scale bar, 250 μ m. (E) Primitive endoderm is required early in gastrulation for induction of hematopoiesis. Whole embryos, isolated ectodermal explants, or ectoderm/visceral endoderm (VE) recombinants were grown in collagen gels for 3 days. RNA was prepared from individual explants and assayed for endogenous mouse embryonic β -like (ϵ^y) globin gene expression using a semiquantitative RT-PCR protocol. Actin served as an internal control. NT, PCR reaction carried out in the absence of cDNA template. -RT, reverse transcription carried out in the absence of RNA template. yolk sac, RNA from 10.5 dpc whole yolk sac.

embryos (resulting in the formation of blood) prompted us to examine whether this tissue also has an effect on the development of endothelial cells.

Expression of the receptor tyrosine kinase gene *flk-1* is detected in blood islands as soon as they appear in the yolk sac (Yamaguchi et al., 1993). *Flk-1* is subsequently downregulated in differentiating blood cells but is maintained at high levels in endothelial cells. Analysis of explant cultures by RT-PCR showed that *flk-1* mRNA is expressed at high levels in posterior (Fig. 4D, lanes 13-17) and recombinant explants (lanes 3-7) but is almost undetectable in cultured anterior ectoderm (lanes



8-12). Similarly, *PECAM-1*, which encodes a surface glycoprotein involved in endothelial cell adhesion and is expressed later than *flk-1*, was also expressed in posterior explants (Fig. 4D, lanes 13-17) and recombinants (lanes 3-7). Little or no *PECAM-1* expression was detected in cultured anterior explants (lanes 8-12). Finally, expression of *Vezf-1* (*Vascular endothelial zinc finger-1*), a novel gene expressed in early endothelial cells (H. Stuhlmann, personal communication), was activated in posterior embryonic explants

(Fig. 4D, lanes 13-17) and recombinants (lanes 3-7) but was detected only at low levels in anterior explants (lanes 8-12).

The results shown in Fig. 4C,D suggest that primitive endoderm from early to midgastrulation-stage embryos can respecify cell fate in anterior ectoderm, resulting in the formation not only of blood cells but also of endothelial cells, a second posterior cell type. These findings are consistent with the hypothesis that the two lineages share a common progenitor.

Fig. 4. Respecification of anterior ectoderm by primitive endoderm. (A) Experimental scheme for separation of epiblast into anterior and posterior portions. Embryos were harvested during mid- to late gastrulation (for comparison, see cartoons of pre- and early-streak embryos shown in the box at upper left.) Primitive endoderm was enzymatically removed. Extraembryonic ectoderm was dissected away to leave the epiblast, which was transected into anterior and posterior portions. (B) Changes in *Otx2* expression during culture. (TOP) Individual anterior and posterior ectodermal regions were dissected from 6.75 dpc embryos and analyzed for *Otx2* expression by RT-PCR. (BOTTOM) Individual anterior and posterior ectodermal regions from 6.75 dpc embryos as well as anterior/VE recombinants were cultured for 4 days and then analyzed by RT-PCR. –RT, reverse transcription carried out in the absence of RNA template. 7.5 dpc, whole embryo harvested at 7.5 dpc. VE (+cx), cultured visceral endoderm. we (+cx), cultured whole embryo. we (–cx), uncultured whole embryo. YS (10.5 dpc), yolk sac isolated from 10.5 dpc embryo. (C) Primitive endoderm can respecify anterior embryonic ectoderm to express hematopoietic markers. Individual anterior and posterior ectoderms, recombinants, and whole embryos were cultured for 3 days and then analyzed by RT-PCR for expression of the genes indicated. Cardiac myosin served as a specificity control. –RT, reaction carried out in the absence of reverse transcriptase. Control PCR reactions were carried out for samples processed using a reverse transcription cocktail without reverse transcriptase and were negative (this figure and data not shown). (D) Respecification of anterior embryonic ectoderm by primitive endoderm to express endothelial markers. Anterior and posterior ectoderms, recombinants and whole embryos were cultured for 3 days and then analyzed by RT-PCR for expression of *flk-1*, *PECAM-1*, and *Vezf-1*.

Cell proliferation in anterior and posterior ectodermal explants

After 4 days in culture, posterior explants showed significant growth, whereas little or no increase in size was observed for mesoderm-free anterior explants (Fig. 5A). BrdU-labeling

experiments showed that, while tissue growth was significantly more robust for cultured posterior pieces, anterior/VE recombinants and whole embryos, cell proliferation could be detected in anterior pieces during the culture period (not shown). Cultured anterior and posterior embryo fragments were also clearly distinguishable by morphological criteria. After 3 or 4 days in culture, posterior explants contained clusters of round cells sitting above a layer of flattened epithelial cells. These round cells were evident even without X-gal staining of transgenic ectoderm explants and correspond to primitive erythroblasts (see below). In contrast, anterior epiblast explants retained their ectodermal character and lacked visible clusters of round cells. Anterior explants grown for as long as 9 days retained this morphology (not shown).

Mesodermal origin of hematopoietic cells in recombinant explants

To confirm that the hematopoietic development detected in recombinant explants reflected formation of primitive erythroblasts from cells of the anterior ectoderm and was not due to the presence of contaminating mesodermal cells in isolated visceral endoderm layers, we took advantage of the human *ϵ -globin/lacZ* transgenic mouse lines described earlier. As outlined in Fig. 4A, anterior epiblast pieces dissected from 6.75 dpc transgenic embryos were recombined with non-transgenic visceral endoderm in collagen droplets and cultured for 4 days. Consistent with the RT-PCR and X-gal staining results presented above, transgenic whole embryos or posterior pieces contained large numbers of β -gal-positive, round erythroid cells after 4 days (Fig. 5A and data not shown). In contrast, β -gal-positive cells were not detected in cultured transgenic anterior pieces, indicating the absence of blood cells (Fig. 5A). However, when transgenic anterior ectoderms were recombined with non-transgenic visceral endoderm, large

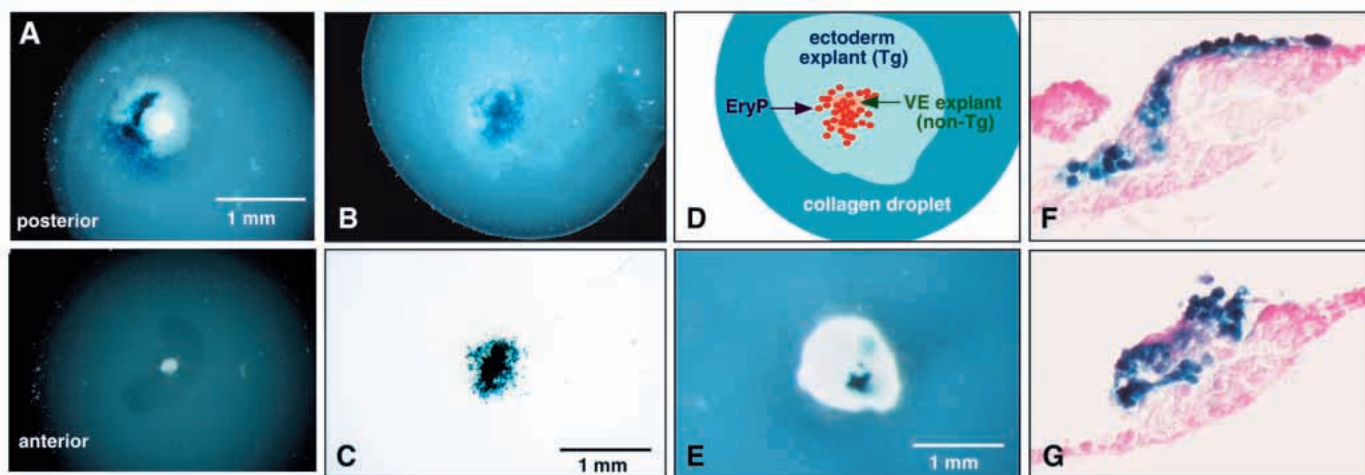


Fig. 5. Mesodermal origin of hematopoietic cells in recombinant explants. Cell proliferation in ectodermal explants. (A) Differences in tissue growth and formation of hematopoietic cells between posterior and anterior regions of the embryonic ectoderm. Cultured anterior and posterior portions of *lacZ* transgenic embryos harvested at midgastrulation and stained with X-gal. Scale bar, 1 mm. (B) Dark-field photomicrograph of recombinant containing transgenic epiblast and non-Tg VE. The recombinant shown here contained VE tissue from a total of three embryos. Scale bar, 1 mm. (C) Bright-field photomicrograph of recombinant shown in A. Scale bar, 1 mm. (D) Cartoon of recombinant shown in B. The anterior ectoderm explant (pale green) grew considerably during culture with VE (beige) from three embryos and produced numerous clusters of β -gal-positive cells (here shown in red). Tg, transgenic; VE, visceral endoderm; EryP, primitive erythroid cells. (E) Potency of primitive endoderm signaling activity decreases late in gastrulation. Dark-field photograph of a recombinant formed between anterior ectoderm (Tg) and non-transgenic VE from three late gastrulation-stage (7.5 dpc) embryos. Scale bar, 1 mm. (F,G) Frozen sections taken through recombinant. Frozen sections were prepared from X-gal-stained recombinants and counterstained to permit visualization of non-transgenic tissue.

numbers of β -gal-positive blood cells were detected in the recombinants (Fig. 5B,C; see also cartoon in 5D). This experiment confirmed that the erythroid cells present in cultured recombinants were derived from anterior ectoderm (transgenic) and not from the visceral endoderm (non-transgenic). Sectioning of stained recombinants confirmed that X-gal staining was confined to hematopoietic cells (Fig. 5F,G). Furthermore, β -gal-positive cells remained localized to an area immediately adjacent to visceral endoderm, suggesting either that primitive endoderm signaling to underlying ectoderm requires cell-cell contact or that the signal(s) are diffusible but act within short range.

In summary, these experiments demonstrate that primitive endoderm from early- or midstreak-stage embryos can respecify anterior ectoderm, a tissue destined to form neuroectoderm, to a hematopoietic fate characteristic of posterior mesectoderm. These experiments also demonstrate that extraembryonic ectoderm (which is removed from anterior ectoderm prior to culture) is not required for blood formation in visceral endoderm-reconstituted cultures.

The posteriorizing activity of the primitive endoderm is stage- and dose-dependent

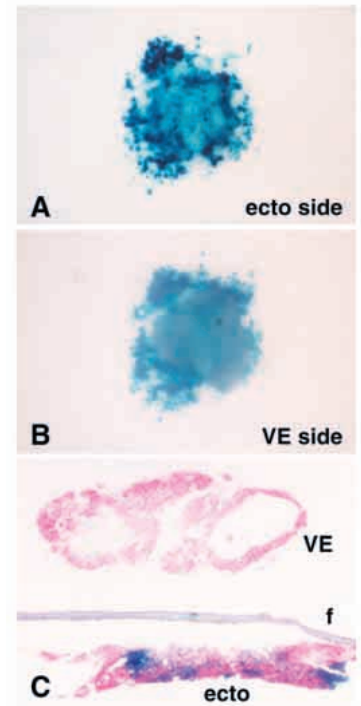
The recombination experiments described above reflect signaling activity by visceral endoderm from early- to midstreak-stage embryos. To determine whether the hematopoietic-determining activity of primitive endoderm persists at later stages of development, transgenic anterior ectoderm was recombined with non-transgenic visceral endoderm from day 7.5, 9.5, 10.5 or 11.5 embryos and assayed for blood formation by X-gal staining. Anterior ectoderm co-cultured with 7.5 dpc VE contained a few small clusters of β -gal-positive cells (Fig. 5E). However, by comparison with the numbers of stained cells observed for recombinants containing 6.5 dpc VE, the numbers of β -gal-positive cells in recombinants containing 7.5 dpc VE were dramatically reduced. Culture of VE from older (9.5-11.5 dpc) embryos with anterior ectoderm did not result in formation of blood, although ectodermal growth was promoted in these recombinants (not shown). Similar experiments in which we varied the number of visceral endoderms per recombinant from 1 to 3 indicated that induction of β -gal-positive cells by visceral endoderm signals is dose dependent. Thus, for example, many more X-gal-staining cells were observed when the recombinant contained 3 VEs (as in Fig. 5B) than when it contained only one.

The foregoing results indicate that primitive endoderm signals can reprogram anterior ectoderm in a dose-dependent manner to form hematopoietic cells. They are still present late in gastrulation (7.5 dpc), although their activity is less potent, but are no longer detectable at later developmental stages.

Activation of hematopoiesis by diffusible signals from the primitive endoderm

We next asked whether cell-cell contact is required for respecification of anterior ectoderm or whether posteriorizing VE signal(s) are diffusible. Transgenic anterior ectoderm and non-transgenic VE were cultured on opposite sides of a Nucleopore filter of pore size ($0.1 \mu\text{m}$) too small to permit the passage of individual cells. β -gal-positive erythroblasts were detected only on the side of the filter containing anterior

Fig. 6. Hematopoietic induction by VE is mediated by diffusible molecules. A Nucleopore filter of pore size ($0.1 \mu\text{m}$) too small to permit the passage of cells was interposed between one anterior ectoderm (transgenic, Tg) and VE (non-Tg) from three midstreak embryos to form a sandwich. The recombinant was cultured for 4 days and then stained with X-gal. (A) Bright-field photograph looking up at ectodermal face of filter. (B) Bright-field photograph looking down on VE face of filter. The VE pieces are now in focus and the β -gal-positive cells, located on the opposite side of the filter, appear blurry. (C) Frozen section through ectoderm-filter-VE sandwich. The apparent gap between the tissues and the filter (f) is a sectioning artifact: in the sandwich, anterior ectoderm (ecto) and VE were retained in close apposition to the filter by a drop of collagen.



ectoderm (Fig. 6A) and were absent from the VE side (Fig. 6B). This observation was reinforced by sectioning through an X-gal-stained ectoderm-filter-VE sandwich (Fig. 6C). Therefore, the reprogramming of cells in anterior ectoderm to a posterior fate is mediated by diffusible signaling molecule(s) from VE.

DISCUSSION

We have shown here that hematopoiesis and vascular development are not autonomous to nascent mesoderm in the mouse and that molecular determinants of these posterior embryonic lineages are present in primitive endoderm as early as 6.5 dpc (early gastrulation), at least 18 hours before blood cells are morphologically detectable. Moreover, the formation of these lineages requires signals from visceral endoderm for only a brief period (possibly as few as 12 hours) during gastrulation. The posteriorizing visceral endoderm signals are diffusible and stage-dependent and can act at short range to induce the formation of blood in pre- or early-gastrulation embryos. They can also respecify the developmental program in prospective neuroectoderm in the anterior regions of embryos in which gastrulation is already well underway.

Epithelial-mesenchymal interactions in hematopoiesis and vasculogenesis

Embryonic blood and endothelial cells form within the yolk sac blood islands by around 7.5 days of development in the mouse and arise from extraembryonic mesoderm (Haar and Ackerman, 1971a). Experiments carried out in the chick more than 30 years ago pointed to a role for primitive endoderm signaling in embryonic hematopoiesis and vasculogenesis (Miura and Wilt,

1969; Wilt, 1965). In contrast to the chick transplantation experiments, a report published while the present work was in progress concluded that the visceral endoderm is not required for blood island formation in the mouse embryo (Palis et al., 1995). However, those experiments were performed using 7.5 dpc embryos. At this stage of development, blood islands have already begun to form (our unpublished observations and those of Wong et al., 1985). In addition, our studies have established a requirement for visceral endoderm signals from ~6.0 to 6.5 dpc. Therefore, the mesodermal tissue explanted at 7.5 dpc (Palis et al., 1995) would already have received inductive signals from visceral endoderm. On the basis of the experiments reported here, we conclude that at least some aspects of embryonic hematopoiesis and vasculogenesis are conserved between mouse and chick.

Visceral endoderm and vasculogenesis

The common origin of blood and endothelial cells from extraembryonic mesoderm and the close temporal association of their development in the yolk sac blood islands led to the hypothesis that these two cell lineages share a common progenitor, the 'hemangioblast' (for reviews, see Flamme et al., 1997; Shalaby et al., 1997). In our studies, recombination of anterior ectoderm with visceral endoderm resulted not only in formation of primitive erythroblasts and activation of hematopoietic markers but also in the activation of three endothelial genes, *flk-1*, *Vezf-1* and *PECAM-1*. Our results demonstrate that the visceral endoderm can reprogram anterior ectoderm to form cells of both these lineages, perhaps by acting on a target hemangioblast. However, we emphasize that the evidence in support of the existence of this hypothetical cell remains circumstantial and awaits more definitive confirmation through lineage tracing studies.

Inductive signals from visceral endoderm are instructive

The pre- or early-gastrulation-stage ectoderms used in our initial recombinants were presumably poised for mesoderm induction or had already initiated this process. The results of those experiments indicated not only that the visceral endoderm is required for embryonic blood induction at these stages but that it may also be the source of an instructive signal. More definitive evidence of an instructive signal came from our surprising observation that primitive endoderm could reprogram mesoderm-free anterior ectoderm from midgastrulation-stage embryos to form hematopoietic and vascular tissue characteristic of posterior ectoderm. Anterior ectoderm co-cultured with visceral endoderm did not maintain its anterior character, as evidenced by the decrease in *Otx2* mRNA levels in recombinants compared with levels in cultured anterior ectoderm. The reduction in *Otx2* expression concomitant with the activation of blood and endothelial specific genes in recombinants suggest that visceral endoderm respecifies anterior ectoderm into posterior mesodermal cell fates via an instructive signal.

Anterior versus posterior determination in the epiblast

Recently, Robertson and colleagues reported that *Smad2* mutant embryos develop normal yolk sacs in the absence of any embryonic tissues (Waldrup et al., 1998). The *Smad2*

pathway thus plays a critical role in A-P axis determination, evidently by restricting the site of primitive streak formation. TGF β signals transmitted through *Smad2* act independently of mesoderm induction and are of extraembryonic origin, though whether they emanate from primitive endoderm or extraembryonic ectoderm or both is currently unknown (Waldrup et al., 1998).

Our findings are particularly striking in light of the *Smad2* mutant phenotype. Those results (Waldrup et al., 1998) indicate that a *Smad2*-regulated signal from the extraembryonic region of the embryo normally functions to confer molecular asymmetry to the epiblast by specifying the anterior pole. *Smad2* is genetically intact in the embryos used to create the recombinants in our experiments, yet its anteriorizing activity is apparently overcome when visceral endoderm is cultured with anterior ectoderm. The primitive endoderm thus is a source of potent signals that antagonize the *Smad2* pathway. Moreover, posterior fate in the epiblast is evidently not a default state but is regulated by visceral endoderm signal(s). Finally, extraembryonic ectoderm was removed from the transgenic embryos used as a source of marked anterior ectoderm in the present experiments. A simple explanation for our results which also takes into account the *Smad2* mutant phenotype is that *Smad2* signals critical for establishing anterior polarity function through extraembryonic ectoderm.

Regionalization of the visceral endoderm

During normal development, the anterior ectoderm is in direct contact with primitive endoderm. Accumulating molecular evidence suggests that, prior to and during gastrulation, the anterior visceral endoderm constitutes a signaling center that functions in maintenance of anterior character and head induction (Belo et al., 1997; Biben et al., 1998; Rosenquist and Martin, 1995; Thomas and Beddington, 1996; Thomas et al., 1998; Varlet et al., 1997). Yet, we have shown that when cultured in isolation with visceral endoderm, anterior ectoderm can be reprogrammed to posterior (hematopoietic and vascular) cell lineages. How can this apparent paradox be explained?

Our observations indicate that the signal(s) involved in induction of hematopoiesis and vasculogenesis early in gastrulation may normally be regionalized within the posterior visceral endoderm. If the signal is localized, our failure to detect blood formation in recombinants containing later stage visceral endoderm could be explained if this tissue was inadvertently taken from the wrong part of the embryo. Though there is clear evidence that some regulatory molecules (e.g. the secreted proteins Cer-1 and nodal and the transcription factors lim-1, gooseoid and HNF-3 β) are restricted in their expression at some point during development to the anterior primitive endoderm and are likely to be involved in the patterning of the underlying anterior ectoderm (Belo et al., 1997; Biben et al., 1998; Thomas and Beddington, 1996; Thomas et al., 1998; Varlet et al., 1997), specific expression in posterior primitive endoderm has not yet been reported for any molecule. Identification of the visceral endoderm signal and analysis of its developmental expression pattern will doubtless help to clarify this question.

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