Indian hedgehog activates hematopoiesis and vasculogenesis and can respecify prospective neurectodermal cell fate in the mouse embryo

Michael A. Dyer1,2, Sarah M. Farrington3,*, Deanna Mohn1, James R. Munday1 and Margaret H. Baron1,3,4,‡

1Department of Medicine, Mount Sinai School of Medicine, New York, NY 10029, USA
2Department of Genetics, Harvard Medical School, Boston, MA 02115, USA
3Department of Molecular and Cellular Biology, The Biological Laboratories, Harvard University, Cambridge, MA 02138, USA
4Department of Biochemistry and Molecular Biology, Derald Ruttenberg Cancer Center, and Institute for Gene Therapy and Molecular Medicine, Mount Sinai School of Medicine, New York, NY 10029, USA

*Present address: Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
‡Author for correspondence (e-mail: margaret.baron@mssm.edu)

SUMMARY

During gastrulation in the mouse, mesoderm is induced and patterned by secreted signaling molecules, giving rise first to primitive erythroblasts and vascular endothelial cells. We have demonstrated previously that development of these lineages requires a signal(s) secreted from the adjacent primitive endoderm. We now show that Indian hedgehog (Ihh) is a primitive endoderm-secreted signal that alone is sufficient to induce formation of hematopoietic and endothelial cells. Strikingly, as seen with primitive endoderm, Ihh can respecify prospective neural ectoderm (anterior epiblast) along hematopoietic and endothelial (posterior) lineages. Downstream targets of the hedgehog signaling pathway (the genes encoding patched, smoothened and Gli1) are upregulated in anterior epiblasts cultured in the presence of Ihh protein, as is Bmp4, which may mediate the effects of Ihh. Blocking Ihh function in primitive endoderm inhibits activation of hematopoiesis and vasculogenesis in the adjacent epiblast, suggesting that Ihh is an endogenous signal that plays a key role in the development of the earliest hemato-vascular system. To our knowledge, these are the earliest functions for a hedgehog protein in post-implantation development in the mouse embryo.

Key words: Hedgehog, Hematopoiesis, Vasculogenesis, Gastrulation, Axis determination, Mouse embryo

INTRODUCTION

Morphogenesis and patterning of the vertebrate embryo are dependent upon finely orchestrated interactions between neighboring tissues. In the pre-gastrulation mouse embryo, the epiblast is situated adjacent to an outer layer of primitive (visceral) endoderm, a secretory epithelium that plays a number of crucial regulatory roles during early postimplantation development (reviewed by Rossant, 1995). For example, cavitation, growth and survival of the ectoderm require primitive endoderm signals (reviewed by Belaoussoff et al., 1998; Coucouvanis and Martin, 1999; Rossant, 1995). Patterning of the anterior epiblast, which will form parts of the central nervous system, is initiated by spatially regulated gene expression in the overlying visceral endoderm (VE; for recent reviews, see Beddington and Robertson, 1999; Viebahn, 1999). In the mouse, the anterior visceral endoderm (AVE) can be distinguished only at the molecular level. Patterned expression of a variety of genes, including Otx2, Lim1, cerberus-like, Hesx1 and Nodal is detected in the AVE well before formation of the primitive streak at the most posterior aspect of the epiblast (reviewed by Beddington and Robertson, 1999).

Patterning of the posterior epiblast is only poorly understood. During gastrulation, when the body plan of the embryo is established (reviewed by Tam and Behringer, 1997), expression of Brachyury (T) (Herrmann, 1991; Kispert and Herrmann, 1994), Nodal (Conlon et al., 1994), Wnt5 (Liu et al., 1999) and eomesodermin (Russ et al., 2000) becomes confined to posterior ectoderm, marking the prospective mesoderm prior to formation of the primitive streak. Bone morphogenetic protein 4 (BMP4), secreted first by extra-embryonic ectoderm (Coucouvanis and Martin, 1999; Waldrip et al., 1998) and later in the allantois and extra-embryonic mesoderm at the posterior of the primitive streak (Lawson et al., 1999; Winnier et al., 1995), appears to play a role in induction of mesoderm and formation of hematopoietic and vascular endothelial cells, though it is not absolutely essential for these processes to occur (Lawson et al., 1999; Winnier et al., 1995).

In nearly all vertebrate embryos, the first site of hematopoiesis and vasculogenesis is the yolk sac, an extra-embryonic membrane that serves a placental function prior to the establishment of the embryonic circulation (for reviews, see Belaoussoff et al., 1998; Farrington, 1996; Rossant, 1995). Yolk sac morphogenesis begins at gastrulation when mesodermal cells migrate into the extra-embryonic region and
line the exocoelomic cavity. Here, the extra-embryonic mesoderm and primitive endoderm layers form a bilaminar tissue which eventually comes to surround the entire embryo proper (Hogan et al., 1994). By late gastrulation, the yolk sac contains morphologically identifiable ‘blood islands’ in which clusters of primitive erythroid cells are surrounded by a layer of vascular endothelial cells (for reviews, see Baron, 2001; Keller et al., 1999). Shortly thereafter, endothelial cells from neighboring blood islands coalesce to form vascular channels and then a network of discrete blood vessels. Hematopoiesis occurs only transiently in the blood islands of the yolk sac and is restricted predominantly to the production of large nucleated primitive erythroblasts. Later in development, hematopoiesis moves to various sites within the embryo proper (reviewed by Dzierzak, 1999; Keller et al., 1999).

Within the exocoelomic cavity, extra-embryonic mesoderm comes in contact with three different lineages (embryonic and extra-embryonic ectoderm, and VE); however, only the mesoderm cells adjacent to visceral endoderm will form endothelial cells (angioblasts) and the first hematopoietic cells of the embryo. To determine whether the primitive endoderm lineage is required for embryonic hematopoiesis and vasculogenesis, we devised an explant culture system in which cells of the embryo. To determine whether the primitive endothelial cells (angioblasts) and the first hematopoietic mesoderm cells adjacent to visceral endoderm will form and extra-embryonic ectoderm, and VE); however, only the comes in contact with three different lineages (embryonic and extra-embryonic ectoderm, and VE); however, only the mesoderm cells adjacent to visceral endoderm will form endothelial cells (angioblasts) and the first hematopoietic cells of the embryo. To determine whether the primitive endoderm lineage is required for embryonic hematopoiesis and vasculogenesis, we devised an explant culture system in which ectoderm dissected from pre- or early-gastrulation stage mouse embryos was cultured in the presence or absence of visceral endoderm and analyzed for activation of a primitive erythroid gene in primitive erythroid cells or a \(\text{-globin-lacZ} \) (Belaoussoff et al., 1998). The \(\text{-globin-KGF} \) transgenic line was generated using a construct analogous to one of the \(\text{-globin-lacZ} \) lines and contains the human \(\text{-globin} \) minimal promoter and a truncated LCR (Trepicchio et al., 1993). Both transgens are expressed exclusively in primitive erythroid cells. The \(\text{Kdr}^{tm1Jrt} \) mouse line has been described (Shalaby et al., 1995) and was obtained from Jackson Laboratories. The \(\text{Runc}1/cbf42-lacZ \) knockout line (North et al., 1999) was generously provided by N. Speck. Nontransgenic mice were all CD1 or ICR (Belaoussoff et al., 1998).

**MATERIALS AND METHODS**

**Mouse lines**

The \(\text{-globin-lacZ} \) transgenic mouse lines have been described (Belaoussoff et al., 1998). The \(\text{-globin-KGF} \) transgenic line was generated using a construct analogous to one of the \(\text{-globin-lacZ} \) lines and contains the human \(\text{-globin} \) minimal promoter and a truncated LCR (Trepicchio et al., 1993). Both transgens are expressed exclusively in primitive erythroid cells. The \(\text{Kdr}^{tm1Jrt} \) mouse line has been described (Shalaby et al., 1995) and was obtained from Jackson Laboratories. The \(\text{Runc}1/cbf42-lacZ \) knockout line (North et al., 1999) was generously provided by N. Speck. Nontransgenic mice were all CD1 or ICR (Belaoussoff et al., 1998).

**Explant culture assays**

Explant cultures were performed as described previously (Belaoussoff et al., 1998). Anterior and posterior ectoderm pieces from the same epiblast were cultured in individual wells of a four-well (Nunc) tissue culture dish or an eight-chamber microscope slide (Nunc). The posterior epiblasts served as controls for the viability of the dissected pieces; embryos were excluded from the analysis if the posterior epiblast failed to proliferate and activate hematopoiesis in culture. Careful notes were kept on the apparent developmental stage of the embryos, based on morphological landmarks (Downs and Davies, 1993), and only embryos that developed to the desired stage were used. For co-culture experiments, anterior epiblasts were transferred onto END-2 monolayers.

**Cell culture**

END-2 cells were a gift from Drs E. Coucouvanis and C. Mummery. LM-‘TK’ cells were purchased from the American Type Culture Collection. Isolation and culture of mouse embryo fibroblasts (MEF) were described previously (Baron and Maniatis, 1986). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS, HyClone). END-2-conditioned medium was prepared after 48 hours culture of irradiated monolayers (6000 rad from \(\text{Cs}^{137} \) source) in explant culture medium (Belaoussoff et al., 1998) and used without further dilution.

**Recombinant proteins**

For early experiments (see, for example, Fig. 3), bacterially expressed N-terminal SHH-N protein (6×His-tagged) (Bumcrot et al., 1995) in 20 mM Tris-HCl pH 7.6, 250 mM NaCl, 5% glycerol, 1 mM DTT was generously provided by D. Bumcrot and A. McMahon. Protein was diluted to 1 μg/μl in 10 mg/ml bovine serum albumin (Stem Cells Technology) just before addition to explant culture medium. SHH-N protein was added at various concentrations (0.25 μg/ml to 5 μg/ml) to explant culture medium (Belaoussoff et al., 1998). Medium was changed after one day and fresh SHH-N protein was added. Explants were harvested after 4 days. For later experiments, bacterially...
expressed recombinant human IHH-N, SHH-N and DHH-N (Desert hedgehog; all cleaved using enterokinase to remove the 6xHis tag, Williams et al., 1999) in 5 mM sodium phosphate pH 5.5, 150 mM NaCl, 0.5 mM DTT were available from Biogen. We did not observe significant differences in activity between His-tagged and untagged hedgehog proteins. Protein was added directly to the medium as before. Alternatively, heparin-acryl beads (Sigma, H5263). 125-250 µm in diameter, were washed in phosphate-buffered saline (PBS) and incubated for 1 hour on ice in a drop (2-5 µl) of hedgehog protein at different dilutions of a 2-4 mg/ml stock, as indicated in the text. As controls, heparin-acryl beads were soaked in the hedgehog protein storage buffer. Two beads incubated with hedgehog protein or buffer were juxtaposed with each anterior ectodermal explant, in a 5 µl droplet of collagen I (Belaussoff et al., 1998). For all experiments, culture medium was changed each day and explants were harvested after 1 or 4 days.

**Analysis of explants**

To monitor the generation of primitive erythroblasts, transgenic explants were fixed and stained with X-gal for expression of lacZ as described (Desert hedgehog; all cleaved using enterokinase to remove the 6xHis tag, Williams et al., 1999; Farrington et al., 1997). Annealing temperature for all primer pairs was 55°C. Primer sequences for all but the following five genes can be found elsewhere (Belaussoff et al., 1998; Farrington et al., 1997).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gli1</td>
<td>5' - AGT GTC TTG CCA CGC CCA -3'</td>
<td>5' - CAG GCT CAA TGC CAC -3'</td>
</tr>
<tr>
<td>Ptch1</td>
<td>5' - AGC AGA AGA ACA CGG -3'</td>
<td>5' - CAG ACA AGA ACA AGA -3'</td>
</tr>
<tr>
<td>Ptch2</td>
<td>5' - AGC AGA AGA ACA CGG -3'</td>
<td>5' - CAG ACA AGA ACA AGA -3'</td>
</tr>
<tr>
<td>Shh</td>
<td>5' - AGA TGG AGC TCG CCA -3'</td>
<td>5' - CGA GCA TGC CCA -3'</td>
</tr>
<tr>
<td>Ptch</td>
<td>5' - AGA TGG AGC TCG CCA -3'</td>
<td>5' - CGA GCA TGC CCA -3'</td>
</tr>
</tbody>
</table>

For each experiment, controls were routinely performed in which reverse transcriptase was omitted from the cDNA reaction mixture and in which template DNA was omitted from the PCR mixture.

**Antibodies and protein kinase A (PKA) agonists**

Monoclonal antibody SEI (Ericson et al., 1996) recognizes Shh and Ihh and, to a lesser extent, Dhh (Wang et al., 2000), and blocks the activities of these proteins. It was obtained from Biogen (Cambridge, MA) or purchased from the Developmental Studies Hybridoma Bank (DSHB, Iowa) and used at concentrations ranging from 46 to 236 µg/ml, as indicated in the figure legends. Anti-Myc (9E10) and anti-LAMP1 (H4B4 or CD107b, a lysosomal membrane glycoprotein) were used as controls and were also purchased from DSHB. Rabbit polyclonal antisera raised against SHH, IHH and DHH peptides were generous gifts from Biogen. Anti-tubulin was purchased from Sigma. For antibody blocking experiments involving anterior ectoderm/VE recombinants, VE pieces were soaked in 5E1 antibody for 1 hour on ice prior to formation of the recombinant. Embryo culture medium containing 5E1 antibody was added and replaced daily, for up to 4 days. At the end of the culture period, tissues were stained with X-gal and scored according to the following criteria: (1) for each recombinant, the matching posterior ectoderm showed evidence of significant cell proliferation and stained heavily for β-galactosidase; and (2) the recombinant explant appeared healthy and also showed evidence of cell proliferation. Recombinants that did not fulfill both criteria were not included in the data summarized in the text. For experiments involving activation of PKA, forskolin and 1,9-dideoxyforskolin (an inactive derivative) were purchased from Calbiochem and dibutyryl CAMP was ordered from Sigma.

**Immunocytochemistry**

Embryo cryosections or cultured cells were fixed in paraformaldehyde (4% in PBS), washed and treated with hydrogen peroxide (1% in PBS) prior to incubation in blocking solution (PBS containing 0.1% Triton X-100 and 2% normal serum (Vector Laboratories). Normal donkey serum was used for the monoclonal antibodies and normal goat serum was used for the rabbit polyclonal antibodies. For immunostaining, primary antibodies were used at a concentration of 10 µg/ml. Anti-KHL serum (Pharmingen) was used as control IgG. Biotin-conjugated secondary antibodies (Vector Laboratories) were used at a dilution of 1:500 in blocking solution. Following secondary antibody binding, sections or cells were incubated first with an avidin-biotin-peroxidase complex (Vectastain ABC, Vector Laboratories) and then with FITC tyramide (synthesized according to the method of Bobrow et al., 1991) or Cy3 tyramide (NEN) as described (see http://axon.med.harvard.edu/~cepko/protocol/mike/; Bobrow et al., 1991). For nuclear staining, DAPI was added to the final wash solution at 0.0005%.

**Microscopy**

Fluorescently labeled cells were visualized using a Zeiss Axioplan-2 microscope with 10×, 20× and 40× Plan NEOFLUAR objectives or a 100× Plan APOCHROMAT objective with adjustable iris. Images were captured with a SPOT digital camera (Diagnostic Instruments). Confocal microscopy was performed using a Leica DM-RBE microscope equipped with a TCSNT true confocal scanner. Embryos and explant cultures were photographed on a Leica MZ12 dissecting microscope fitted with a 1.0× Planapo lens and a Sony DVL-970MD digital camera.

**Clonogenic assays for primitive erythroid progenitor cells**

Pools (10-30) of mid-streak embryos cultured for 2 days (Belaussoff et al., 1998) were washed in PBS and incubated for 3-5 minutes at 37°C in 0.25% trypsin-EDTA (Mediatech). Cells were dispersed by gentle passage four times through a 20G syringe needle and were then counted. Approximately 20,000-50,000 cells were plated in 1 ml of a medium similar to that reported for culture of primitive erythroid colonies from embryoid bodies (Kennedy et al., 1997): Iscove’s Modified Dulbecco’s Medium (IMDM) containing methylcellulose (55%), plasma-derived serum (PDS, 10%, Animal Technologies), PFHMI (5%, Gibco-BRL), penicillin-streptomycin, L-glutamine (2 mM), α-thioglycerol (0.6 mM, Sigma) and recombinant human erythropoietin (4 U/ml, Epogen, Amgen).

**Cytospin preparations**

Cells from cultured embryos were dispersed as described above, and cells from headfold stage embryos were dispersed by treatment with collagenase (0.15% in PBS containing 20% FBS) at 37°C for 30 minutes with occasional gentle passage through a 20G syringe needle. In some experiments, dispersed cells were deposited onto Superfrost Plus® slides (VWR) by cytocentrifugation (Shandon Cytospin 3) at 114 g for 4 minutes. Slides were stained with X-gal as described above.
Ihh null mutant embryos

Mice mutant for Ihh were generated at the Transgenic Mouse Facility at Mount Sinai School of Medicine by microinjection of Ihh<sup>+/−</sup> embryonic stem cells (St-Jacques et al., 1999) into C57Bl/6 blastocysts. Germline chimeras were initially back-crossed onto a C57Bl/6 background and then onto a CD-1 background. Approximately 50% of null mutant embryos died between days 9.5 and 10.5 on this background, about 24 hours earlier than reported for other backgrounds (St-Jacques et al., 1999).

RESULTS

Induction of embryonic hematopoiesis by secreted molecules from a primitive endodermal cell line

In the mouse embryo, hematopoietic induction and reprogramming of anterior ectoderm by primitive VE signaling is mediated by diffusible molecules (Belaussoff et al., 1998). This conclusion, revealed by epiblast complementation assays, was based on the observations that pre- or early-gastrulation stage epiblasts stripped of surrounding VE were unable to activate hematopoiesis unless recombined with VE tissue and that VE could reprogram cell fate in the anterior epiblast from neuroectodermal to hematopoietic and endothelial lineages. To identify the inductive signaling molecule(s) from VE, we turned to the END-2 cell line which was isolated from differentiating embryonal carcinoma cells and shown to express a variety of VE markers (Mummery et al., 1991). Conditioned medium was prepared from irradiated END-2 cell monolayers and its activity was tested in an epiblast complementation assay (Fig. 1A). Epiblasts stripped of visceral endoderm were unable to activate expression of the e-globin gene, a marker of primitive erythroid cells (Fig. 1B, lanes 6,8,9,15-18). The explant analyzed in lane 7 was taken from a developmentally more advanced embryo that had begun to form ‘mesodermal wings’ (Belaussoff et al., 1998; Downs and Davies, 1993) and therefore served as a positive control. To distinguish between END-2 and VE cultures we compared expression of the various hedgehog pathway genes in anterior and posterior epiblast pieces from later, mid-gastrulation stage embryos, total cellular RNA was prepared from individual, intact embryos and examined for expression of each of the hedgehog genes using the semi-quantitative RT-PCR assay. Only Ihh was expressed at this stage of development (Fig. 2A, lanes 1-3).

We next asked whether hedgehog receptors and downstream effectors are present in embryonic cells adjacent to the VE layer (the source of Ihh signaling) during early gastrulation. Binding of hedgehog proteins to the seven-pass membrane receptor patched (Ptc) results in the activation of signaling by a second transmembrane protein, smoothened (Smo) (reviewed in Ingham, 1998a; Ingham, 1998b; Johnson and Scott, 1998). Ptc is a target of hedgehog signaling; increased levels of patched protein are thought to sequester hedgehog at the cell surface, thereby limiting its range of action (reviewed by McMahon, 2000). The function of a second vertebrate patched protein, Ptc2, is not yet known (Carpenter et al., 1998; Morinaga et al., 1998b). Hedgehog signaling is transduced through the Gli family of zinc-finger transcription factors (reviewed in Altaba, 1999; Aza-Blanc and Kornberg, 1999). After enzymatic separation of tissues, total cellular RNA was isolated from individual epiblasts and VE tissues and analyzed for expression of Ptc, Ptc2, Smo and Gli1 using the RT-PCR assay (Farrington et al., 1997). Expression of all three genes encoding membrane components of the hedgehog signaling pathway (Fig. 2B, lanes 1-3) as well as a transcription factor (Gli1, not shown) was detected in epiblasts but was absent from VE (lane 4).

Because anterior epiblast (prospective neurectoderm) explants can be reprogrammed when recombined with VE tissue, we compared expression of the various hedgehog pathway genes in anterior and posterior epiblast pieces from later, mid-gastrulation stage embryos. Ptc, Ptc2 and Smo expression was detected throughout the epiblast (Fig. 2C, compare lanes 1-4). Similar results were obtained for the downstream effector Gli1. Primitive endoderm signaling not only induces hematopoietic development in epiblasts but can also reprogram cell fate in anterior ectoderm (Belaussoff et al., 1998), a region of the epiblast that normally forms neural ectoderm. We therefore asked whether END-2 cells can reprogram anterior epiblast explants in culture. To distinguish between END-2 and ectoderm cells, anterior epiblasts were isolated from transgenic embryos carrying a human e-globin-lacZ gene which is expressed only in primitive erythroid cells (Fig. 1E; Belaussoff et al., 1998). These genetically marked explants were placed on top of END-2 cell monolayers and co-cultured for 3 or 4 days. Whereas all of the transgenic anterior epiblasts cultured alone (on plastic, Fig. 1F; n=15) or on monolayers of fibroblasts (mouse embryo fibroblasts, MEF, Fig. 1F; and L cells, not shown; n=10) spread out over the surface and failed to activate the lacZ reporter gene, nearly 90% (14/16) of epiblasts co-cultured with END-2 cells remained intact, proliferated, and formed large numbers of β-galactosidase-positive primitive erythroblasts.

Expression of hedgehog genes and components of the hedgehog signaling pathway in embryos

Both Ihh and Dhh are expressed in mature yolk sac of the mouse embryo but in different (visceral endoderm and extra-embryonic mesoderm, respectively) tissue layers (Farrington et al., 1997). To determine whether both genes are also expressed in gastrulation stage embryos, total cellular RNA was prepared from individual, intact embryos and examined for expression of each of the hedgehog genes using the semi-quantitative RT-PCR assay. Only Ihh was expressed at this stage of development (Fig. 2A, lanes 1-3).

We had previously reported that Ihh but not Shh is expressed in visceral endoderm of gastrulation-stage embryos and mature yolk sacs (Farrington et al., 1997). Yolk sac expression of Desert hedgehog (Dhh) is confined to the mesodermal layer (Farrington et al., 1997). Ihh is therefore a candidate hematopoiesis-inducing signal from visceral endoderm and could be responsible for the activity detected in END-2-conditioned medium. To determine which Ihh is expressed in END-2 cells, we analyzed total cellular END-2 RNA by RT-PCR. Ihh transcripts were detected by this assay (Fig. 1C, lanes 3,4). Surprisingly, Dhh and Shh were also expressed in these cells at significant levels (lanes 5,6 and 7,8, respectively). To determine whether all three hedgehog genes are produced by these cells, we used specific antisera prepared from chemically synthesized Shh, Dhh and Ihh peptides, and a monoclonal antibody (5E1) raised against Shh (Ericson et al., 1996) that also recognizes Ihh (Vortkamp et al., 1996; Wang et al., 2000) and, to a lesser extent, Dhh (Wang et al., 2000). All three hedgehog proteins were detected in END-2 cells by immunostaining with these antibodies (Fig. 1D and data not shown).
Indian hedgehog in hematovascular development

Fig. 1. Hematopoietic induction and hedgehog expression by a visceral endoderm-like cell line. (A) Experimental scheme for testing ability of END-2 cell conditioned medium (CM) to substitute for visceral endoderm (VE) in activation of hematopoiesis. (B) END-2 conditioned medium induces primitive hematopoiesis in epiblasts stripped of VE. Pre- or early-gastrulation stage embryos stripped of VE were cultured for up to 4 days in the presence (+VE CM) or absence (−VE CM) of CM prepared from END-2 cells. Explanted epiblasts were harvested and total cellular RNA was isolated and analyzed for expression of the murine embryonic β-like globin gene (ε3, here termed ε) using an RT-PCR assay. Samples of cDNA were normalized for expression of actin prior to analysis of ε-globin gene expression. Most of the epiblasts cultured without CM failed to activate a marker of primitive erythropoiesis (the ε-globin gene), while most of the epiblasts cultured in the presence of CM did activate the gene. The explant analyzed in lane 7 was taken from a developmentally more advanced embryo that had already formed ‘mesodermal wings’ (Belaoussoff et al., 1998; Downs and Davies, 1993) and therefore served as a positive control. (C) Hedgehog genes are expressed by END-2 cells. Total cellular RNA was prepared from END-2 cells and analyzed in duplicate for expression of hedgehog genes using an RT-PCR assay. Transcripts from each of the three hedgehog genes were detected. In this preparation, Shh RNA levels (lanes 7, 8) were significantly higher than those of Ihh (lanes 3, 4) or Dhh (lanes 5, 6). Actin controls are shown. Amplification product sizes were (in base pairs): Ihh (222), Dhh (308) and Shh (333). (D) Immunofluorescence analysis of hedgehog protein expression in END-2 cells. (E) Dark-field photograph of headfold-stage embryos from homozygous ε-globin-lacZ transgenic mice. Transgene expression was detected by staining with X-gal. The transgene is expressed exclusively in primitive erythroid cells. (F) Close-up image of a representative area of the transgenic keratin for illustration purposes. Anterior epiblasts (dissected as indicated in diagram to left, after removal of VE layer) from ε-globin-lacZ transgenic embryos were cultured on plastic or on monolayers of END-2 or MEF cells for 4 days, and were then stained with X-gal. Matched posterior pieces are shown. Cells from both anterior and posterior epiblasts spread out over the surface of the plastic or MEF monolayer; the anterior epiblast explants remained intact and both stained intensely for β-galactosidase activity. (Although the photograph gives the impression that MEFs are inhibitory for posterior epiblasts, in other experiments transgene activation was robust.) Anterior and posterior epiblast explants cultured on END-2 monolayers remained intact and both stained intensely for β-galactosidase. Like visceral endoderm tissue, END-2 cells can activate hematopoiesis in prospective neural ectoderm. a, anterior; p, posterior. Scale bars: 10 μm for D; 100 μm for F (explant co-cultures).
These findings indicate that the posterior region of the epiblast, which is fated to form cells of the primitive hematopoietic and vascular endothelial lineages (discussed by Belaoussoff et al., 1998), is competent to respond to hedgehog signaling. Moreover, the anterior epiblast, which can be reprogrammed by VE signals, is also competent to respond to hedgehog activity.

Spatial expression of Ihh in primitive endoderm

We next asked whether expression of Ihh protein is regionalized within the VE layer. This question is pertinent because during normal development, the anterior epiblast is in direct contact with primitive endoderm, yet this tissue does not normally form hematopoietic or endothelial cells. To explain this apparent paradox, we had previously suggested that the signal(s) involved in induction of hematopoiesis and vasculogenesis early in gastrulation may normally be regionalized within the posterior visceral endoderm (Belaoussoff et al., 1998). Immunostaining of cryosections from gastrulating embryos revealed graded expression of Ihh protein from proximal to distal: Ihh is expressed throughout the proximal ~60% of the visceral endoderm layer but not in ectoderm (lane 4). A similar pattern of expression was detected for Shh (not shown). Ptc, Ptc2 and Smo RNAs were expressed throughout the epiblast, at somewhat higher levels in posterior than anterior. Samples of cDNA were normalized for expression of actin prior to analysis for expression of other genes. Control cDNA reactions without reverse transcriptase, –RT (lane 5).

Activation of primitive hematopoiesis by hedgehog protein

The visceral endoderm-like END-2 cells express many of the same genes as VE but were derived from embryonal carcinoma cells rather than from normal tissue. We therefore sought more definitive evidence to support roles for hedgehog signaling in activation of hematopoiesis in the early embryo using recombinant hedgehog proteins.

The active form of hedgehog is the N-terminal (HH-N) peptide. Because Shh and Ihh proteins have similar biological properties (see Vortkamp et al., 1996; Yang et al., 1998) and only recombinant (human) SHH-N protein was available to us when these studies were initiated, we tested the ability of SHH-N to substitute for VE signals to activate embryonic β-like globin gene expression in epiblasts. Using a range of SHH-N concentrations shown to be effective in other in vitro assays, we found that the endogenous e-globin gene was activated in SHH-N-treated epiblasts (Fig. 3). As demonstrated previously (Belaoussoff et al., 1998), epiblasts stripped of visceral endoderm did not express the embryonic β-like globin gene, e, during culture (lanes 1-5).
Respecification of developmental cell fate by hedgehog protein

Two hallmarks of primitive (visceral) endoderm signaling are its requirement for activation of hematopoiesis around the onset of gastrulation and its ability to instruct the development of primitive erythroid and endothelial cells (Belaoussoff et al., 1998). To determine whether hedgehog protein satisfies the second criterion for the VE signal, we therefore asked whether it can, like visceral endoderm, respecify the anterior epiblast. In pilot experiments (not shown), we found that addition of any of the three hedgehog proteins (SHH, IHH or DHH) to the culture medium stimulated cellular outgrowth and formation of primitive erythroblasts within explanted anterior epiblasts. Because Ihh is the biologically relevant hedgehog protein in gastrulation stage mouse embryos, we examined its activity in greater detail.

Heparin-acrylic beads were incubated with recombinant human Ihh (IHH-N) protein at low (0.2-0.4 mg/ml) or high (1.0-5.0 mg/ml) concentration or in buffer alone. Beads were co-cultured in collagen droplets with anterior epiblasts isolated from embryos carrying the human \(\varepsilon\)-globin-lacZ (\(\beta\)-galactosidase) transgene (Belaoussoff et al., 1998). Explants were then stained with X-gal to determine whether the transgene was activated and whether primitive erythroblasts had formed. As shown in Fig. 4A, anterior epiblasts failed to activate the \(\varepsilon\)-globin-lacZ transgene (0/7) when cultured with

Fig. 3. N-terminal Sonic hedgehog protein (SHH-N) can substitute for visceral endoderm to activate embryonic globin gene expression in explanted epiblasts. Epiblasts stripped of visceral endoderm were treated with SHH-N at 0.25 (lanes 6-9), 1 (lanes 10-13) or 5 \(\mu\)g/ml (lanes 14-17), as indicated, or with buffer alone (lanes 1-5). SHH-N activated endogenous mouse \(\varepsilon\)-globin gene expression. YS (lane 18), 10.5 day yolk sac control. Samples of cDNA were normalized for expression of actin prior to analysis for \(\varepsilon\)-globin gene expression. In this experiment, the actin and \(\varepsilon\)-globin genes were amplified in the same reaction but panels shown were from different exposures (shorter for actin) of the autoradiograph.

Fig. 4. (A) Recombinant human Ihh protein (IHH-N) can respecify the anterior epiblast from a neuroectodermal to a hematopoietic cell fate. Anterior epiblasts from mid-gastrulation stage embryos failed to activate the \(\varepsilon\)-globin-lacZ transgene in primitive erythroblasts when cultured with heparin-acrylic beads soaked in buffer alone (buffer control, a; ectoderm indicated by black arrow) but showed significant tissue growth as well as marked activation of the transgene when cultured with two IHH-N-soaked beads (c, beads indicated by white arrows; shown here for IHH-N at 1 mg/ml). Matched posterior control explants (b,d) are shown. (B) Cytospin preparation of dispersed cells from 4 day cultures of mid-streak \(\varepsilon\)-globin-lacZ transgenic embryos (left) and freshly dissected headfold stage \(\varepsilon\)-globin-lacZ transgenic embryos (right). The \(\beta\)-galactosidase-positive cells from cultured explants are indistinguishable from those from whole embryos. (C) Cells from cultured explants form primitive erythroid colonies (Ery\(^P\)) in secondary cultures. We have not yet optimized these secondary cultures but find that, under these conditions, 15-20 Ery\(^P\) colonies form per mid-streak embryo. Giemsa staining of dispersed cells from Ery\(^P\) colonies revealed morphology characteristic of primitive erythroblasts. Pigmented primitive erythroid colonies from \(\varepsilon\)-globin-lacZ transgenic explants stain with X-gal (not shown). Embryos from an analogous transgenic line in which a KGFP reporter was substituted for the lacZ gene (\(\varepsilon\)-globin-KGFP) form green fluorescent primitive erythroid cells (shown for headfold stage embryo, \(\varepsilon\)-GFP, lower left). As expected, cultured explants from this line form hemoglobinized primitive erythroid colonies (lower middle panel; scale bar, 100 \(\mu\)m) containing green fluorescent primitive erythroblasts (dispersed cells from colony, lower right). Scale bars: in A, 500 \(\mu\)m for A; in B, 10 \(\mu\)m for B; in C (top), 10 \(\mu\)m; in C (bottom), 25 \(\mu\)m.
beads soaked in buffer alone but showed significant tissue growth as well as marked activation of the transgene when co-cultured with beads incubated with IHH-N protein at 5 mg/ml (75%, 9/12, P<0.005) or at 1 mg/ml (40%, 4/10). Lower concentrations of IHH-N (0.2-0.4 mg/ml) activated the transgene with lower efficiency (18%, 3/16). As expected, the β-galactosidase positive cells are morphologically identical to those from later, headfold stage (~8.5 day) embryos (Fig. 4B). DNA synthesis was also stimulated in IHH-N-treated anterior epiblasts, as indicated by markedly increased [3H]-thymidine uptake (not shown). Matched, untreated posterior ectoderm (epiblast) controls (see Materials and Methods) are shown.

To confirm that functionally differentiated primitive erythroblasts form in this culture system, cells from explants cultured for two days were dispersed by treatment with trypsin and plated in secondary clonogenic cultures. Primitive erythroid (EryP) colonies were easily identified morphologically and by their red pigmentation (Fig. 4C). Dispersed cells from these colonies showed characteristic staining with May-Grunewald Giemsa (Fig. 4C) and expressed the lacZ transgene (not shown). Analogous experiments were performed using embryos from a transgenic mouse line in which the lacZ gene was replaced with a KGFP reporter (e-globin-KGFP; embryo marked ‘e-GFP’). The EryP colonies formed in secondary cultures, and their constituent cells, showed green fluorescence, as expected (Fig. 4C).

Endothelial cell markers were also activated in anterior epiblast-VE recombinants (Belaoussoff et al., 1998). Moreover, a lacZ reporter that partially replaces the AML1 gene Runx1/cbfa2 (North et al., 1999; Wang et al., 1996) and that is expressed in the early vascular network of the yolk sac (Fig. 5A, left) was activated in adherent cells of anterior epiblast-VE recombinants (Fig. 5A, right). Development of endothelial sprouts was also evident in these explants (arrows). To determine whether endothelial cells form in response to signal transduction by Ihh, anterior epiblasts were isolated from heterozygous embryos in which the Flik gene was partially replaced by a promoterless lacZ gene (Kdr tm1Jrt). The lacZ reporter in these embryos was expressed in endothelial cells of the yolk sac (Shalaby et al., 1995) and, as expected, cultured mid-streak embryos from this line form vascular networks of β-galactosidase-positive endothelial cells (Fig. 5B). At early times during culture of anterior epiblasts, β-galactosidase-positive cells formed and in some cases appeared to migrate towards and over the IHH-N-soaked beads (Fig. 5C, anterior 24 hour sample). Explants cultured for 4 days showed intense staining for β-galactosidase (matched posterior shown).

Some of the stained cells in the central portion of the explant may be progenitors of both hematopoietic and vascular endothelial cells (the hypothetical ‘hemangioblast’), but the staining pattern of the explant and morphology of individual stained cells observed more peripherally are clearly distinct from those seen with the e-globin-lacZ transgenic explants. Together, these data indicate that endothelial cells, as well as primitive hematopoietic cells, form in response to Ihh signaling.

**Activation of early and late markers of hematopoietic and endothelial developmental programs by Ihh**

Production of blood and endothelial cells within hedgehog-treated anterior ectoderm presumably is preceded by mesoderm induction, formation of hemangioblasts, then hematopoietic stem cells and angioblasts, and differentiation to...
primitive erythroblasts and endothelial cells. Each of these processes is associated with characteristic changes in gene expression. To assess such changes in hedgehog-treated ectoderm, we used the RT-PCR assay. All cDNA samples were normalized for expression of actin. To ensure that the reactions were not saturating, cycle number was carefully titrated for each primer pair (Farrington et al., 1997).

As expected (Belauossouff et al., 1998), the mesodermal marker T was strongly expressed early in IHH-N-treated anterior epiblasts, within 24 hours of culture, before activation of the endogenous mouse e-globin gene, and was downregulated by 4 days (not shown). In gastrulating embryos, expression of the Bmp4 is first detected in the extra-embryonic ectoderm (Waldrip et al., 1998) and then in the posterior epiblast and nascent mesoderm (Lawson et al., 1999; Winnier et al., 1995). Bmp4 functions in mesoderm formation and patterning (for a more detailed discussion, see Lawson et al., 1999; Winnier et al., 1995). On genetic backgrounds where Bmp4-null mutants survive beyond gastrulation, both hematopoiesis and vasculogenesis are greatly reduced, though not abolished (Winnier et al., 1995). Moreover, bone morphogenetic proteins are downstream of hedgehog genes in at least some systems (for a review, see Hammerschmidt et al., 1997). We therefore reasoned that the Bmp4 gene might be upregulated in response to Ihh signaling. As shown for two of the embryos from Fig. 5A (lanes marked *, see actin controls in Fig. 6A), exposure to Ihh protein resulted in upregulation of Bmp4 expression (Fig. 6B, compare buffer controls, lanes 1 and 3, with Ihh-treated samples, lanes 5 and 7).

Two genes expressed in hematopoietic stem/progenitor cells were also activated following treatment with IHH-N-beads: Flk1 (Fig. 4B and 6B) and CD34 (Fig. 6B; actin controls shown in Fig. 6A). These genes are also expressed in early endothelial cells (angioblasts) and may be markers of the ‘hemangioblast’; Flk1 continues to be transcribed at high levels in endothelial cells but is downregulated in primitive erythroblasts (Yamaguchi et al., 1993). Finally, transcription of two genes expressed in mature endothelial cells, PECAM and vWF, was activated in response to Ihh (lanes 5 and 7) but was not detected in untreated samples (Fig. 6B, lanes 1 and 3). As observed for recombinants formed between anterior ectoderm and VE (Belauossouff et al., 1998), reprogramming of gene expression was not promiscuous: the gene encoding α-cardiac myosin, which is activated much later in development and only in cardiac tissue, was not transcribed either in treated or untreated explants (not shown). Taken together, these results are consistent with the induction of mesoderm, formation of hemangioblasts, hematopoietic stem cells, and angioblasts, and differentiation of primitive erythroid and endothelial cells in hedgehog-treated ectoderm explants.

Upregulation of Ptc and Gli1 is considered to be indicative of a response by target cells to hedgehog signaling (Ingham, 1998b). Therefore, we analyzed expression of Ptc, Ptc2, Smo and Gli1, genes involved in the hedgehog signaling pathway, in control and IHH-N-treated ectoderm pieces. Transcription of receptor complex genes Ptc and Smo (Fig. 6B, lanes 5 and 7) and of the downstream effector gene Gli1 (not shown) was stimulated in response to Ihh. No change in expression was observed for Ptc2. Dhh, which was not detected in whole embryos (Fig. 2A), was activated during culture of both anterior and posterior ectoderms and to somewhat higher levels in posterior than anterior ectoderm (Fig. 6B – compare lanes 1, 3, 5 and 7 with lanes 2, 4, 6 and 8).

During normal embryonic development or when anterior epiblasts are cultured alone for 1 or 4 days, expression of the anterior neural genes Otx2 (Ang et al., 1994) and Vax1 (Hallonet et al., 1999) increases (Fig. 6C, lanes 1-6). In contrast, when anterior epiblasts are exposed to IHH-N protein or cultured as recombinants with VE, the expression of both genes is downregulated (compare lanes 7.8 with 9,10; lanes 11,12 with 13,14). These findings suggest that IHH-N or VE signals can respecify anterior neur ectodermal cell fate, consistent with our previous study (Belauossouff et al., 1998).

**Ihh is an endogenous inducer of hematopoietic and vasculogenic mesoderm**

The ability of secreted signals from visceral endoderm to activate hematopoietic and vascular development prompted us to ask whether visceral endoderm activity could be inhibited by the 5E1 monoclonal antibody which blocks Shh activity (Ericson et al., 1996) and also cross-reacts with Ihh (Wang et al., 2000). Intact pre- or early-gastrulation stage embryos (containing both ectoderm and primitive endoderm layers) were cultured alone or in the presence of control IgG or 5E1 monoclonal antibody and then analyzed for activation of the endogenous e-globin gene using the RT-PCR assay (Fig. 7A). By comparison with embryos cultured alone (lanes 1-3, 10-13) or in the presence of control IgG (lanes 4-6, 14-17), all of which activated the e-globin gene, most of the embryos cultured in the presence of the 5E1 blocking antibody (lanes 7-9, 18-21) expressed this gene at much lower or undetectable levels, in each of two experiments.

To determine whether the reprogramming activities of visceral endoderm are also mediated by Ihh, anterior ectoderm-visceral endoderm recombinants (Belauossouff et al., 1998) were cultured in the presence of 5E1 (anti-hedgehog) or control IgG or an unrelated monoclonal antibody (Fig. 7B). Recombinants were prepared from e-globin-lacZ transgenic anterior epiblasts and nontransgenic visceral endoderm; posterior epiblasts were also cultured, without antibody, as controls for the tissue viability of each dissected embryo. Each recombinant was scored for proportion of X-gal-staining cells (high, low, or undetectable). Representative examples of control IgG and 5E1 monoclonal antibody-treated recombinants are shown in Fig. 7B along with their matched posterior epiblasts. Nearly all (83%, 20/24) of the control recombinants stained intensely for β-galactosidase, and a much smaller number stained weakly (only a few stained cells) or not at all (4/24). In contrast, only 39% (12/31) of the recombinants cultured in the presence of the 5E1 antibody showed intense staining. The results of these experiments are supported by experiments with the PKA agonists forskolin and dibutyryl cAMP, which inhibit hedgehog signaling by increasing PKA activity (Hammerschmidt et al., 1996; Li et al., 1995). Both inhibitors blocked reprogramming of anterior epiblasts at concentrations of 5 μM or 0.5 mM, respectively, while an inactive forskolin derivative (1,9-dideoxy-forskolin) had no effect (data not shown).

**DISCUSSION**

Members of the hedgehog family of signaling molecules have
been shown to play important roles in control of cell fate and cell proliferation in a variety of developing systems (for a review, see Ingham, 1998b; Johnson and Scott, 1998). Ihh has key functions in the development of bone and cartilage (reviewed by Karp et al., 2000) and in pancreatic morphogenesis (Hebrok et al., 2000). A role for Ihh in the development of the mammary gland has been suggested based on analysis of its pattern of expression in pregnant and lactating mice that are haploinsufficient for \( \text{Ptch} \) (Lewis et al., 1999).
Flk1 angioblasts (activation of markers of hematopoietic stem cells and anterior epiblast by IHH-N protein is associated with signal in mesodermal patterning. Reprogramming of the vascular cell types. Therefore, Ihh may act as an instructive tissue that would normally not produce hematopoietic or endoderm and to respecify the developmental program of hematopoiesis and vasculogenesis in the absence of visceral hedgehog protein alone is sufficient to activate embryonic cells and their progenitors; ability to form primitive erythroid colonies in secondary clonogenic cultures; and ability to form vascular networks. In every case, the explants behave in exactly the same way as normal embryos when recombined with VE or exposed to hedgehog protein. We show that hedgehog protein alone is sufficient to activate embryonic hematopoiesis and vasculogenesis in the absence of visceral endoderm and to respecify the developmental program of a tissue that would normally not produce hematopoietic or vascular cell types. Therefore, Ihh may act as an instructive signal in mesodermal patterning. Reprogramming of the anterior epiblast by IHH-N protein is associated with activation of markers of hematopoietic stem cells and angioblasts (Flikl and CD34) and markers of primitive hematopoietic (ε-globin) and vascular endothelial (PECAM and vWF) cells. The mesoderm-patterning activity of IHH-N and visceral endoderm may be specific to hematopo-vascular mesoderm, as the gene for α-cardiac myosin was not activated during culture of anterior epiblasts with IHH-N protein (not shown) or with VE tissue (Belaoussoff et al., 1998). During culture of anterior epiblasts with IHH-N protein, downstream targets of the hedgehog signaling pathway (Ptc, Smo and Gli1) are upregulated. Furthermore, our data strongly suggest that Ihh is an endogenous activator of these processes during mouse development: inhibition of hedgehog signaling in pre- or early-gastrulation stage embryos or in recombinants formed between anterior epiblasts and visceral endoderm resulted in a marked reduction or abrogation of primitive hematopoietic development. We therefore conclude that the inductive activity of visceral endoderm is mediated, at least in part, by Ihh, and suggest that, in vivo, Ihh functions in patterning of nascent mesoderm at the posterior primitive streak and/or of extra-embryonic mesoderm. This idea is supported by a recent report suggesting a role for Ihh in hematopoietic and vascular induction in developing embryoid bodies (Maye et al., 2000). To our knowledge, the hematovo-vascular inducing activity of Ihh is the earliest known function for a hedgehog protein in the development of the post-implantation embryo.

**Targets of Ihh signal transduction**

The results summarized here indicate that cells within the epiblast are targets for Ihh signaling from visceral endoderm. It is worth noting that while Ptc RNA is restricted to hedgehog target cells, Ptc2 is coexpressed with hedgehog genes in some tissues (Motoyama et al., 1998a; Motoyama et al., 1998b; St-Jacques et al., 1998). However, our findings demonstrate that, at least during gastrulation, both Ptc1 and Ptc2 are expressed in the target and not in the signaling (VE) tissue. Ptc2 was not upregulated during treatment with IHH-N, in agreement with a previous report that its transcription is independent of hedgehog signaling (St-Jacques et al., 1998). Activation of Smo in response to IHH-N during explant culture raises the possibility that it may be a hedgehog protein target gene. Finally, although Dhh is not expressed in the early gastrulation stage embryo, its autonomous upregulation during culture of anterior epiblasts (Fig. 6B) suggests that it may be activated in vivo well before gonadal development begins (Bitgood et al., 1996).
A role for Ihh in anteroposterior axis determination

Considerable evidence now exists for a role for the anterior primitive endoderm (AVE) in establishing the anteroposterior axis of the mammalian embryo and for the development of anterior structures such as the forebrain (Beddington and Robertson, 1999; Viebahn, 1999). We have shown previously that patterning of the posterior epiblast is also controlled by primitive endoderm signaling (Belaoussoff et al., 1998). The experiments reported here raise the possibility that Ihh signaling plays a role in A-P axial patterning. Although production of Ihh protein is not restricted to the posterior aspect of the gastrulating embryo, its expression is graded from proximal to distal (Fig. 2D). Therefore, Ihh protein emitted by visceral endoderm could reach the most posterior (i.e. proximal) region of the primitive streak, from which hematopoietic mesoderm arises, at levels sufficient to influence cell fate specification. At comparable levels in the proximal anterior epiblast, in contrast, Ihh would not normally induce hematopoietic and endothelial development. It might do so, however, if expressed ectopically at much higher levels in this region of the embryo. Alternatively or in addition, Ihh patterns mesoderm as it reaches the extra-embryonic (proximal) region of the embryo, where Ihh expression is highest.

An extra-embryonic signal regulated by the transcriptional coactivator Smad2 confers molecular asymmetry to the epiblast by specifying the anterior pole and by restricting the site of primary streak formation (Waldrup et al., 1998). We propose that one function of Ihh is to help antagonize the Smad2 pathway in the posterior and/or extra-embryonic region of the embryo. Such a role would provide at least a partial explanation for the ability of primitive endoderm to reprogram the anterior epiblast in recombinant explants.

A role for the AVE in inhibiting activation of hematopoietic and vascular development in the anterior epiblast appears very unlikely. If the AVE antagonized formation of these cell types, then genes such as e-globin, Flk1 and Runx1/Cbfa2 should be activated upon removal of VE from anterior epiblasts. However, no activation of these or any other genes investigated in these studies was detected in anterior epiblasts stripped of VE. We have also found, in preliminary studies, that either anterior or posterior VE can activate hematopoiesis in anterior epiblasts, suggesting that the signal(s) is(are) not strongly regionalized in an anteroposterior direction.

Finally, anti-hedgehog activity such as that produced by Hip (Chuang and McMahon, 1999) in the anterior epiblast or a mouse homologue of Dispatched (Burke et al., 1999) in the AVE could in principle result in local inhibition of Ihh, restricting its inductive activity and, thus, the formation of hematopoietic mesoderm to the posterior epiblast.

Cellular proliferation in response to Ihh signaling

Hedgehog signaling stimulates cellular proliferation in a variety of systems (e.g. see Ahlgren and Bronner-Fraser, 1999; St-Jacques et al., 1999). Treatment of anterior epiblasts with Ihh-N clearly promotes a proliferative response. However, proliferation per se is not sufficient to activate hematopoiesis and vasculogenesis in Ihh-N-treated ectoderm. This conclusion is based on the following observations: (1) e-globin-lacZ transgenic anterior epiblasts co-cultured on fibroblast monolayers or treated with epidermal growth factor showed significant cell proliferation but did not form β-galactosidase-positive erythroblasts (Fig. 1F and data not shown); and (2) incubation of anterior epiblasts with the hedgehog blocking antibody 5E1 inhibited formation of erythroid cells but not cell proliferation (Fig. 7B). The antibody blocking experiments suggest that visceral endoderm provides a signal(s) in addition to Ihh that can stimulate cell proliferation.

Models for induction of hematopoiesis and vasculogenesis by Ihh

Does Ihh function directly in the patterning of hemato-vascular mesoderm or are other, downstream signals involved? We envision the following three models for the function of Ihh in early hematopoietic and vascular patterning:

Model 1: VE-secreted Ihh plays a direct role in the induction and patterning

Ihh alone is sufficient for these activities but is not essential in vivo, either because of functional redundancy with Dhh or because another molecule produced in the embryo or supplied maternally can largely compensate for the absence of Ihh at this early developmental stage.

Model 2: VE-secreted Ihh induces a second signal, such as Bmp4, which induces and patterns hematopoietic mesoderm

Ihh-N induces expression of Bmp4 in anterior epiblasts, and gene targeting of Bmp4 results in defects in hematopoietic and vascular development on some genetic backgrounds (Winnier et al., 1995). Therefore, Bmp4 could mediate the hedgehog signaling activities observed in the present study. Whether or not Bmp4 is a downstream component of this pathway will require further investigation.

Model 3: VE-secreted Ihh first induces endoderm, which then provides the actual hematopoietic mesoderm-inducing signal, either Bmp4 or another as yet unidentified molecule

Ihh induces visceral endoderm differentiation of F9 teratocarcinoma cells (Becker et al., 1997) and may induce or maintain endoderm differentiation in vivo, although it is apparently not essential for this process (St-Jacques et al., 1999). A model in which endoderm induced by Ihh is an intermediate step in its effect on ectoderm has been proposed for an embryonic carcinoma cell system (Maye et al., 2000). We do not favor this model but at present we cannot rule it out.

Functional redundancy of Hedgehog proteins and compensatory mechanisms

A role for Ihh in the patterning of hematopoietic mesoderm is implied by its ability to induce – directly or indirectly – the development of hematopoietic and vascular endothelial cells in the epiblast. However, analysis of mice deficient for Ihh (St-Jacques et al., 1999) indicates that this gene is not essential for these processes and that another hedgehog protein or a distinct pathway must at least partially compensate for its absence. The formation of blood and endothelial cells must occur early and rapidly in the developing embryo, and multiple regulatory pathways might provide a failsafe mechanism to ensure the survival of the conceptus past implantation.

Mice homozygous for a null mutation in Ihh do not
demonstrate obvious defects in formation of primitive erythroblasts or vascular endothelium, although approximately 50% of the null mutant embryos die around day 9.5-10.5 (our results) or day 10.5-11.5 (St-Jacques et al., 1999) of embryogenesis. We are currently investigating whether Ihh deficiency results in more subtle, quantitative defects in hematopoietic or vascular development (including delayed hemato-vascular development) by careful analysis of the null mutant embryos and through in vitro differentiation of Ihh null mutant embryonic stem cells (J. M. D., M. A. D., U. Chen, B. St-Jacques, A. McMahon, H. Kronenberg and M. H. B., unpublished). Our findings that another family member, Dhh, is activated in the embryo during gastrulation (Fig. 6B) and is expressed in the mature yolk sac mesoderm (Farrington et al., 1997) suggests that this protein might compensate, at least in part, for the function of Ihh in these early processes. Both Ihh and Dhh function would be inhibited by the 5E1 antibody (Wang et al., 2000), which we show blocks hematopoietic induction in epiblasts and respecification of the anterior epiblast (Fig. 7). In vivo, Dhh may serve to maintain the mesodermal patterning initiated by Ihh. Such an activity would help to explain our earlier observation that the requirement for primitive endoderm signaling in hematopoietic and vascular development is confined to a short period of time during gastrulation (Belauossouf et al., 1998) and would explain why Ihh alone is sufficient but not essential for induction of hematopoietic mesoderm. In any case, the abundance of Ihh expressed in the mature yolk sac mesoderm (Farrington et al., 1999) demonstrates obvious defects in formation of primitive endodermal signaling in the gastrulating embryo. It our assay all argue strongly that Ihh is a major component of Ihh alone is sufficient but not essential for induction of gastrulation (Belaoussof et al., 1998) and would explain why Ihh is activated in the embryo during gastrulation (Fig. 6B) and is expressed in the mature yolk sac mesoderm (Farrington et al., 1997) suggests that this protein might compensate, at least in part, for the function of Ihh in these early processes. Both Ihh and Dhh function would be inhibited by the 5E1 antibody (Wang et al., 2000), which we show blocks hematopoietic induction in epiblasts and respecification of the anterior epiblast (Fig. 7). In vivo, Dhh may serve to maintain the mesodermal patterning initiated by Ihh. Such an activity would help to explain our earlier observation that the requirement for primitive endoderm signaling in hematopoietic and vascular development is confined to a short period of time during gastrulation (Belauossouf et al., 1998) and would explain why Ihh alone is sufficient but not essential for induction of hematopoietic mesoderm. In any case, the abundance of Ihh expressed in the mature yolk sac mesoderm (Farrington et al., 1999) demonstrates obvious defects in formation of primitive endodermal signaling in the gastrulating embryo. It will be of obvious interest to examine the development of compound Ihh-Dhh mutants.

We are grateful to P. Tam, D. Sassoon, E. Robertson, A. McMahon, V. Ambros, K. Williams and B. St-Jacques for helpful discussions, and to P. Tam, V. Ambros, D. Sassoon, D. Weinstein, and T. Lufkin for insightful comments on the manuscript. We thank M. Minou and A. Corcoran for technical assistance; K. Kelley for performing injections of Ihh-null mutant ES cells into blastocysts and the e-globin-KGFP transgene into oocytes; and M. Belauossouf for contributions to early phases of this work. We thank E. Coucouvanis and C. Mummery for END-2 cells, A. McMahon for recombinant SHH-N protein used during the early phases of this work, K. Williams (Biogen) for recombinant hedgehog proteins and antisera; and N. Speck for the Ranxl/chfia2-lacZ mouse line. This project was supported in part by grants to M. H. B. from the NIH (GM42413 and DK52191) and the Vincent and Ellen Cavallo Family Philanthropic Fund. M. A. D. is a Revson Fellow of the Mount Sinai School of Medicine.

REFERENCES


