The allantois and chorion, when isolated before circulation or chorio-allantoic fusion, have hematopoietic potential

Brandon M. Zeigler1, Daisuke Sugiyama1*, Michael Chen1, Yalin Guo1, Karen M. Downs2† and Nancy A. Speck1,†

The chorio-allantoic placenta forms through the fusion of the allantois (progenitor tissue of the umbilical cord), with the chorionic plate. The murine placenta contains high levels of hematopoietic stem cells, and is therefore a stem cell niche. However, it is not known whether the placenta is a site of hematopoietic cell emergence, or whether hematopoietic cells originate from other sites in the conceptus and then colonize the placenta. Here, we show that the allantois and chorion, isolated prior to the establishment of circulation, have the potential to give rise to myeloid and definitive erythroid cells following explant culture. We further show that the hematopoietic potential of the allantois and chorion does not require their union, indicating that it is an intrinsic property of these tissues. These results suggest that the placenta is not only a niche for, but also a source of, hematopoietic cells.

KEY WORDS: Placenta, Allantois, Chorion, Hematopoiesis, Mouse

INTRODUCTION

Hematopoietic cells develop from mesoderm located in multiple sites in the conceptus, beginning in the mouse at the mid-primitive-streak stage (~7.0 days postcoitum (dpc)) (Palis et al., 1999). The sites of hematopoietic cell emergence in mammals have been defined using hematopoietic progenitor assays or, in the case of hematopoietic stem cells, by transplantation into newborn, irradiated, or otherwise compromised adult mice (for reviews, see Melchers, 1979; Ottersbach and Dzierzak, 2005) is simply a site of hematopoietic cell colonization and is not an intrinsic source of hematopoietic cells (Houssaint, 1981). However, it is not known whether the placenta, whose contribution to mammalian fetal hematopoiesis was more recently appreciated (Alvarez-Silva et al., 2003; Gekas et al., 2005; Melchers, 1979; Ottersbach and Dzierzak, 2005) is simply a site of colonization (like the fetal liver), or whether it is also a source of hematopoietic cells. Determining whether any tissue is truly a site of hematopoietic cell emergence is complicated by the fact that hematopoietic cells circulate throughout the conceptus. Thus, in this study, we have investigated the hematopoietic potential of the two individual components of the murine placenta, the allantois and the chorion, prior to establishment of vascular continuity within the conceptus.

The chorion arises during gastrulation (~7.0-7.25 dpc) by displacement of extra-embryonic mesoderm onto the roof of the newly formed exocoelomic cavity (Snell and Stevens, 1966). It is a bilayered tissue of dual origin, composed of extra-embryonic mesoderm derived from trophectoderm and extra-embryonic mesoderm derived from proximal epiblast (Gardner, 1983; Kinder et al., 1999; Lawson et al., 1991) (Fig. 1A). The ectodermal component of the chorion differentiates into syncytio- and cytotrophoblasts, which mediate fetal/maternal exchange.

The murine allantois is thought to be composed wholly of extra-embryonic mesoderm (Duval, 1891) derived from proximal epiblast that is transformed into mesoderm via passage through the posterior primitive streak (Beddington, 1982; Copp et al., 1986; Kinder et al., 1999; Lawson et al., 1991; Tam and Beddington, 1987). The allantois appears slightly later than the chorion during gastrulation, and emerges first as a bud emanating from the posterior region of the streak (~7.25 dpc). The bud enlarges within the exocoelomic cavity by a combination of mitosis, continued cellular addition from the primitive streak and distal cavitation (Brown and Papaioannou, 1993; Downs and Bertler, 2000; Ellington, 1985; Tam and Beddington, 1987). Ultimately, the allantois elongates far enough to make contact with the chorionic mesoderm, and fuses with it [6-8 somite pairs (s), ~8.5 dpc]. Chorio-allantoic union is mediated by the maturity of the allantois (Downs and Gardner, 1995), and requires both the cell-surface molecule vascular cell adhesion molecule 1 (VCAM1) on the outer mesothelial surface of the allantois (Gurtner et al., 1995; Kwee et al., 1995) and its counter receptor α4 integrin, on chorionic mesoderm (Yang et al., 1995). Together the allantois and chorion give rise to the labyrinth region of the placenta.

Shortly after the allantoic bud is formed, allantoic mesoderm vascularizes de novo (~7.5 dpc). Vascularization occurs with distal-to-proximal polarity, spreading toward the posterior end of the primitive streak (Downs et al., 1998). At about the same time, vasculogenesis takes place in the yolk sac blood islands and heart region, and spreads toward the posterior streak. By 4-6 s, the three major circulatory systems unite near the base of the allantois,
forming a vascular continuum throughout the conceptus (Downs, 1998; Inman and Downs, 2006). By 8 s (~8.5-8.75 dpc), primitive erythroid cells can be seen freely circulating within the allantois (Downs et al., 1998).

Although erythroid cells could be detected in the murine allantois in early somite pair conceptuses, it was not entirely clear whether or not they originated from the allantois. In avian embryos, the apparently prevascularized allantois appears to be a hematopoietic tissue and contains blood-island-like clusters of hematopoietic cells (Caprioli et al., 1998; Caprioli et al., 2001). Dieterlen-Liévre and colleagues also showed that the avian allantois could contribute extensively to hematopoietic and endothelial cells in the adult bone marrow following engraftment into the coelom of host embryos, and thus has the potential to form definitive (adult) blood (Caprioli et al., 1998). In contrast, when headfold-stage murine allantoises were labeled intrinsically with lacZ and extrinsically with tritiated thymidine, and then grafted into the exocoelomic cavity of the host fetus, no labeled host-derived erythroid cells were detected in the host allantoises following 24 hours of whole embryo culture (Downs et al., 1998). Furthermore, those explants that were free-floating in the exocoelom or fused only with the chorion and did not exhibit vascular continuity with the fetus and/or yolk sac were entirely devoid of benzidine-positive cells, suggesting that, in the mouse conceptus, the allantois is not a source of erythroid cells (Downs et al., 1998). However, some data suggested that the murine allantois might have erythropoietic potential: 5-10% of allantoises explanted between the neural plate and 4 s stages (~7.5-8.25 dpc), or cultured in isolation for 24 hours exhibited a constant but relatively small population of erythroid cells, the origin of which has never been accounted for (Downs et al., 1998).

The aforementioned studies of the murine allantois were carried out during only a short period (24 hours), and early markers of hematopoietic cells were not examined. In light of recent experiments suggesting that hematopoietic cells emerge from the placenta several days later (Álvarez-Silva et al., 2003; Gekas et al., 2005; Ottmersbach and Dzierzak, 2005), we undertook a series of experiments to examine the hematopoietic potential of the allantois and the chorion prior to their fusion and, more importantly, prior to the vascular link-up between the umbilical, yolk sac and cardiovascular systems in the conceptus. First, we followed the expression of Runx1 in both the intact conceptus and in explant cultures. Runx1 is a transcription factor required for hematopoietic stem cell formation, and is also an early marker for sites of hematopoietic cell emergence in the embryo (Cai et al., 2000; Clau-Uitz et al., 2000; Kalev-Zylinski et al., 2002; Lacaud et al., 2002; North et al., 2002; North et al., 1999; Okuda et al., 1996; Wang et al., 1996). Runx1 expression has been documented in the placenta (Ottmersbach and Dzierzak, 2005), at the site of chorio-allantoic fusion (North et al., 1999) and in the chorion prior to fusion (Lacaud et al., 2002), but has not been reported in the pre-fusion allantois. Second, we cultured allantoises and chorions on OP9 stromal cells in the presence of hematopoietic cytokines, or as explants on plastic followed by methyccellulose colony forming assays, to determine their hematopoietic potential.

Here we show that both the allantois and chorion, having been isolated from early headfold-stage conceptuses before establishment of the circulation, express Runx1 and exhibit hematopoietic potential following explant culture. Furthermore, we show that this hematopoietic potential is not dependent on chorio-allantoic fusion, indicating that it is an intrinsic property of both tissues.

**MATERIALS AND METHODS**

**Mouse husbandry**

All mice were maintained in light-reversed conditions (lights off: 12:00/ lights on: 0:00). Runx1−/− deficient explants were generated by mating Runx1+/− females (Wang et al., 1996) with Runx1+/+ males (North et al., 1999) (formal designations of the Runx1+/+ and Runx1+/− alleles are Runx1+/+ and Runx1+/−, respectively). For marking experiments, B6.Cg-Tg(Actb-Bgeo/GFP)21Lbe/J (Jackson Laboratory, Bar Harbor, ME), Tg(Ly6a/GFP) (Ottersbach and Dzierzak, 2005), or Runx1+/+ male mice were bred to female C57BL/6J, C57BL/6J × 129S1/SVIm F1, or B6CBAFI/J mice (Jackson Laboratory). We identified female mice in estrus, mated them 5 minutes before the beginning of the dark cycle and then examined them 4 hours later for the presence of a vaginal plug. Dissections, staining and morphological scoring were performed as previously described (Downs, 2006; Downs and Davies, 1993; Downs and Harmann, 1997; Downs et al., 2004). All mouse procedures were approved by our Institutions’ Animal Care and Use Committees.

**Embryo dissection**

We isolated allantoises by mouth aspiration (Downs, 2006) (Fig. 2A), and in some cases subdivided the allantoises into distal, mid and proximal thirds with glass scalps.

To isolate the chorion, the Reichert’s membrane was removed and the embryonic and visceral endoderm was labeled by submerging the intact conceptus for 1 minute in AlexaFluor594-conjugated Concanavalin A (ConA 594) [5 mg/ml in phosphate-buffered saline (PBS)] (Molecular Probes/Invitrogen, Carlsbad, CA) (Fig. 2B). The mesoderm lining the exocoelomic cavity was labeled by injecting AlexaFluor488-conjugated Concanavalin A (ConA 488) into the exocoelomic cavity using a mouth-held microcapillary pipette. Tissues were digested using pancreatin/trypsin (Downs, 2006) for 10 minutes at 4°C and separated with the aid of glass scalps and a mouth aspirator. Separation of the chorion from visceral endoderm was confirmed by the absence of ConA 594 and the presence of ConA 488 by fluorescence stereomicroscopy (Leica, Ernst-Leitz-Strasse, Germany). Once this technique was established, we labeled only the endoderm by dipping the conceptus into ConA 488 and removing the ConA 488-positive tissue from the chorion.

We isolated PAS as described by Godin et al. (Godin et al., 1993). We isolated embryonic ectoderm by incubating headfold-stage conceptuses in ConA 488 to label the anterior head endoderm (Fig. 2C). A transverse cut was made below the amnion using a glass scalpel to separate the extra-embryonic from embryonic components of the conceptus. A sagittal cut was then made through the epiblast; the anterior portion of the epiblast was saved. The tissues were digested in trypsin/pancreatin at 4°C for 10 minutes and then dissected using a glass needle and mouth aspirator to separate the anterior neuro-ectoderm and mesoderm from the anterior head endoderm.

**Explant cultures for Runx1 and Ly6A expression**

Allantoises and chorions were cultured as explants directly on plastic (24 Well Cell Culture Cluster, Corning, Corning, NY) or in roller drum suspensions for 48 hours (Downs, 2006). Most material was at headfold stage, with a minor 1-2 s component. We fixed the explants in 4% paraformaldehyde for 2 hours on ice; they were then washed with PBS and stained for 6 hours with β-galactosidase activity (Miles et al., 1997). The genotype of explants from Runx1+/+ × Runx1+/+ matings was determined by PCR from corresponding epiblast DNA using previously described primers and amplification conditions (North et al., 1999).

**OP9 explant cultures**

Individual allantois, chorion, PAS, visceral endoderm and neuroectoderm explants were added to a freshly prepared layer of OP9 stromal cells in single wells of 12-well plates and grown in 1 ml α-MEM (Gibco/Invitrogen) containing 20% fetal bovine serum supplemented with stem cell factor (SCF; 50 ng/ml), interleukin 3 (IL3, 5 ng/ml), interleukin 6 (IL6, 5 ng/ml), Fli-3 ligand (Flt3-L, 10 ng/ml), granulocyte colony stimulating factor (G-CSF, 5 ng/ml), granulocyte macrophage colony stimulating factor (GM-CSF, 5 ng/ml) (all cytokines from R&D Systems, Minneapolis, MN) and 2-mercaptoethanol (2-ME, 1 × 10−5 M) (Sigma) at 37°C in 5% CO2 (Nishikawa et al., 1998) to
encourage the proliferation and differentiation of myeloid lineage cells. Half of the medium and cytokines were replaced every other day. Explant cultures were analyzed for Dil-Ac-LDL (Biomedical Technologies, Stoughton, MA) uptake or CD45 expression after 7 days of culture. Fluorescence activated cell sorting (FACS) assays were performed after 14 days of explant culture.

Flow cytometry
Single-cell suspensions were prepared by incubating the explants for 10 minutes in 500 μl of Cell Dissociation Buffer (Gibco/Invitrogen) at 37°C followed by passage through a 40-μm strainer. Explant cell suspensions at a concentration of 2×10^6 cells per ml were treated with Fc blocking serum (BD Pharmingen, San Diego, CA) for 20 minutes at 4°C then incubated with CD45-PerCP (30-F11), Mac-1-PE (M11/70), c-kit-FITC (2B8), or Gr-1-FITC (RB6-85C) (BD Pharmingen or eBioscience, San Diego, CA), washed and re-suspended in 200 μl FACS buffer (BioSure, Grass Valley, CA). Dead cells were excluded with TO-PRO-3 (Molecular Probes/Invitrogen), and cells were analyzed on a FACS Calibur flow cytometer (Becton Dickinson, Rockville, MD) with FlowJo software (Tree Star Inc., Ashland, OR).

Progenitor assays
Two-day explant cultures were performed by plating six conceptus equivalents of allantoises or chorions in a single well of a 24-well plate containing 500 μL of 50% rat serum/DMEM (Dowds et al., 2001). The medium was replaced after 24 hours and at 48 hours the explants were washed in PBS, then incubated in pre-heated (37°C) Dispase II (Roche, Basel, Switzerland) for 25 minutes and mechanically dissociated using a pipette. Cells were collected by centrifugation and cultured in 3.3 ml of 3434 Methocult (StemCell Technologies, Vancouver, Canada). Colonies were counted and selected 7 days later.

Five-day explant cultures were performed by plating six conceptus equivalents of allantoises or chorions on OP9 cells in a single well of a 24-well plate in the presence of 50 ng/ml SCF, 10 ng/ml Flt3-L, 4 U/ml equivalents of allantoises or chorions on OP9 cells in a single well of a 24-well plate. Cells were collected by centrifugation and cultured in 3.3 ml of 3434 Methocult (StemCell Technologies, Vancouver, Canada). Colonies were scored and harvested 10 days later.

β-Globin expression analysis
RNA was extracted from individual burst-forming unit erythroid (BFU-E) or colony-forming unit granulocyte-macrophage (CFU-GM) colonies, adult mouse bone marrow, and ~9.0 dpc mouse yolk sac using an RNA extraction kit (Qiagen, Valencia, CA). β-Globin (ey) primers (5′-TGT CCT CTG CCT CTG CCA TAA 3′ and 5′-AGC GGA GAC ACA AGA GGA TTG CTC 3′) (Fiering et al., 1995), β-major globin primers (5′-CTG ACA GAT GCT CTC TTG GG-3′ and 5′-CAG AAA CCC CAG AAA CAG CAG-3′) and Hprt primers (5′-GGT GGG GAA GAC GAG CTC TCC 3′ and 5′-CAG AGG ACT AGA ACA CTT GC-3′) were used to analyze globin expression (Keller et al., 1993). PCR was performed for 29 cycles: 94°C, 30 seconds; 57°C, 30 seconds; 72°C, 45 seconds. Histological analysis
Conceptuses were assayed for β-galactosidase activity (Miles et al., 1997), and 5 μm paraffin sections were either counterstained with Nuclear Fast Red (Vector Labs, Burlingame, CA) or left unstained. Staining for β-galactosidase was performed for 6 hours at 37°C, unless otherwise noted in the figure legends. We examined five conceptuses at bud stages, eight at headfold stages, 17 at 1-16 s and two at 9.5 dpc.

Explants from OP9 cultures were incubated in 1 μl/ml Dil-Ac-LDL for 4 hours at 37°C, fixed in 4% paraformaldehyde, and then incubated first with CD45-biotinylated antibody (BD Pharmingen) overnight at 4°C and then with Pacific Blue-conjugated streptavidin (Molecular Probes/Invitrogen) for 90 minutes at room temperature. Explants were observed by fluorescence stereomicroscopy and photographed using a monochrome digital camera (Roper Scientific, Tucson, AZ).

Single-cell suspensions of explants (1×10^4 cells) or individually selected colonies were centrifuged onto slides (14 g, 8 minutes), dried for 3 hours at room temperature and stained by May-Grünewald Giemsa.

RESULTS
Runx1 is expressed in the allantois and the chorion
We examined the expression of Runx1 in the intact conceptus prior to and shortly after chorio-allantoic fusion (~7.5-9.25 dpc) (Fig. 1). We and others previously reported that at stages prior to fusion, Runx1 is expressed in yolk sac visceral endoderm (North et al., 1999), the vascular and hematopoietic components of yolk sac blood islands (Lacauda et al., 2002; North et al., 1999), and in chorionic mesoderm (Lacauda et al., 2002). We observed Runx1 expression, as visualized by a lacZ 'knock-in' allele (North et al., 1999), in these same locations (Fig. 1B). Runx1 expression in the chorion was first seen as punctate blue staining in some mesodermal cells beginning at the early bud stage (not shown), and was intense in most chorionic mesodermal cells by the late headfold stage (Fig. 1B). Chorionic ectoderm was negative at all stages examined. In the allantois, we found convincing blue stain within the proximal allantois and amnion where these two tissues join, as well as faint blue stain within the distal portion of the allantois, including the outer layer of mesothelium in some specimens (not shown). After chorio-allantoic fusion (12 s, ~9.0 dpc), Runx1 expression was seen within internal cells at the chorio-allantoic interface (Fig. 1C-E), at external sites where the allantois intersects the chorion (Fig. 1E, blue arrows) and also in a small cluster of cells within the base of the allantois (Fig. 1E,F). At later times (~16 s, ~9.25 dpc), Runx1 expression was seen both at the plane of chorio-allantoic fusion (Fig. 1G) and in endothelial cells lining the proximal allantoic vasculature (Fig. 1G,H) but not in the mid-region of the allantois (Fig. 1G). Runx1 expression in allantoic mesothelium was clearly seen in the 9.5 dpc conceptus (Fig. 1J). By ~10.5 dpc, the umbilical artery, which forms from the allantois, expressed Runx1 throughout its length both in endothelial cells and in intra-aortic hematopoietic clusters (Fig. 1K,L) as described previously (North et al., 1999). In previous studies with the same Runx1(2) allele, Ottersbach and Dzierzak (Ottersbach and Dzierzak, 2005) showed that by ~11.0 dpc, Runx1 is expressed in the labyrinth region of the placenta in endothelial cells, in cells underlying the endothelium and in cells within the circulation.

Faint, punctuate blue staining was observed in both endodermal and mesodermal components of the visceral yolk sac as previously described (North et al., 1999) (not shown), and in extra-embryonic visceral endoderm overlying the chorionic ectoderm ['distal' extra-embryonic endoderm or 'ectoplacental' endoderm (Duval, 1891)] (Fig. 1B).

Runx1 is expressed in both the allantois and the chorion, independent of their union
Although our results of histology clearly revealed Runx1 expression in the chorion prior to chorio-allantoic fusion, allantoic mesothelial expression was much less intense. In addition, once chorio-allantoic fusion has occurred it is no longer possible to determine whether the Runx1+ cells at the fusion junction originated from the allantois, from the chorion, or from both tissues. Therefore, to confirm that both the allantois and chorion express Runx1, we isolated both tissues prior to fusion, cultured them in vitro as explants and analyzed the explants for Runx1 expression.
Fig. 1. Runx1 expression before and after chorio-allantoic fusion. (A) Schematic diagram of an early headfold (EHF) stage mouse embryo, showing locations of the ectoplacental cavity (epc), chorion (ch), exocoelomic cavity (ecc), amnion (am), amniotic cavity (ac), embryo (emb), yolk sac blood islands (bi) and allantois (al). Shown in color and labeled are both embryonic and extra-embryonic endoderm layer (end), mesoderm layer (mes) and ectoderm layer (ect). The conceptus is surrounded by embryonic (emb), yolk sac (ys) and ectoplacental (ep) visceral endoderm (vis end). The chorion consists of chorionic mesoderm (ch mes) and ectoderm (ch ect), the latter of which is derived from trophectoderm. The anterior (a) and posterior (p) boundaries of the primitive streak (ps) are indicated. Compass shows anterior (ant), posterior (pos), proximal (pro) and distal (dis) directions for the extra-embryonic compartment. (B) Sagittal section of an EHF-stage Runx1lz/+ conceptus showing Runx1 expression (blue) in the chorionic (ch) mesoderm (black arrow) and blood islands (bi). The blue arrow indicates area of punctate blue staining in ectoplacental visceral endoderm. β-Galactosidase staining was performed for 18 hours. (C) Runx1 expression (arrowheads) at the chorio-allantoic fusion junction in a 12 s Runx1lz/+ conceptus. (D) Detail of Runx1 expression from boxed region in C. (E) Runx1 expression in a 12 s Runx1lz/+ conceptus at the plane of chorio-allantoic fusion (black arrow), at the junctions between the allantois and chorion (blue arrows) and in a cluster of cells within the base (boxed region). β-Galactosidase staining was performed for 18 hours. (F) Detail of Runx1 expression from boxed region in E. (G) Runx1 expression in the distal region (arrowhead) and within the allantoic base (box) in a 16 s Runx1lz/+ conceptus. (H) Detail of Runx1 expression (boxed region in G) in putative endothelial cells (arrowheads) lining a blood vessel within the allantoic base. (I) Section through placenta of a 9.5 dpc Runx1lz/+ embryo showing Runx1+ cells in allantoic mesothelium (arrow). (J) Detail of boxed region from I. (K) Runx1 expression in endothelium and umbilical artery hematopoietic clusters in a 10.5 dpc Runx1lz/+ conceptus. (L) Whole mount showing Runx1 expression in the chorionic disk (cd) and along the length of the umbilical artery (ua) of a 10.5 dpc Runx1lz/+ conceptus.
As previously defined, the boundary of the allantois with the posterior primitive streak was taken as the site of insertion of the allantois with the amnion and yolk sac (Downs and Harmann, 1997). When aspirated via a mouth-held microcapillary pipette, the entire allantois can be removed from these insertion sites. We aspirated allantoises from early headfold to 1-2 s conceptuses with a glass pipette (Fig. 2A). Following aspiration, we carefully trimmed several cell layers from the base of the allantois with a glass scalpel to ensure that the allantoises were not contaminated with yolk-sac-derived cells. Allantoises from Runx1lz/+ conceptuses were then cultured for 48 hours on plastic, after which time the explants were assayed for β-galactosidase activity. We estimated that 48-hour explants were the equivalent of ~9.75 dpc (~20 s), as allantoises appear to have an internal timing mechanism revealed by their normal behavior even when removed from contact with the primitive streak (Downs and Gardner, 1995; Downs et al., 2004; Downs et al., 2001). Plated allantoic explants gave rise to Runx1+ cells (Fig. 3A).

Runx1+ cells included cells that remained within the explant, as well as mesenchymal-like cells that had crawled off the explant (Fig. 3A). These motile cells were previously shown by limited fate mapping to originate within the allantoic mesothelium (Downs et al., 2004), confirming that at least some of the Runx1+ cells came from the mesothelial component of the allantois.

To rule out the possibility that Runx1 expression in the explants was induced nonspecifically through contact with the plastic surface of the culture well, we suspended explanted allantoises in roller tubes and cultured them in vitro for 48 hours (equivalent to ~9.75 dpc). Runx1 expression was observed in the spheres that formed under these culture conditions (Fig. 3C). Sectioning through the spheres revealed that Runx1 was expressed in the outer one to two cell layers around the surface of the spheres (Fig. 3C), consistent with expression in mesothelium. To discover which region of the allantois gave rise to Runx1+ cells, we also subdivided headfold-stage allantoises into distal, mid and proximal thirds and cultured
these pieces separately as 24- or 48-hour explants on tissue culture plastic. All three pieces of the allantois gave rise to Runx1+ cells (not shown), indicating that Runx1 expression may ultimately initiate throughout the length of the allantois.

To assess whether the chorion also expressed Runx1, we isolated choriots and cultured them as explants for 48 hours on tissue culture plastic (Fig. 3B). Runx1+ cells persisted in chorionic explants, and most had crawled off the explant. As we did not separate chorionic mesoderm from ectoderm, we do not know which chorionic component gave rise to the Runx1+ cells in the explant culture, but given that chorionic mesoderm is Runx1+, and that mesoderm is characteristically migratory, we suspect that the plated Runx1+ population was derived from choronic mesoderm.

In addition to Runx1, we also analyzed the expression of another hematopoietic marker, Sca1, as visualized with a Ly6a/GFP transgene (de Bruijn et al., 2002; Ottmersbach and Dzierzak, 2005) in allantoises and choriorts before and after explant culture. Ottmersbach and Dzierzak (Ottmersbach and Dzierzak, 2005) reported that Ly6a/GFP is expressed in the extra-embryonic ectoderm and ectoplacental cone beginning in pre-streak-stage conceptuses. Consistent with their findings, we observed Ly6a/GFP expression in the freshly isolated chorion but not in the allantois (Fig. 3D). After 24 hours of explant culture, both the allantois and the chorion expressed Ly6a/GFP (Fig. 3D).

Thus, on the basis of these observations, we conclude that Runx1, one of the earliest markers of definitive hematopoiesis, is expressed in both the allantois and in the chorionic mesoderm. Furthermore, Runx1 expression in the allantois and the chorion is independent of fusion and is therefore intrinsic to these two tissues.

**The allantois and chorion have hematopoietic potential**

We next assessed the hematopoietic potential of allantoises and choriorts isolated from early headfold to 1-2 s conceptuses, before a circulatory continuum is established between the major vascular systems in the conceptus. We first cultured allantoic and chorionic explants on OP9 stromal cells (Suzuki and Nakano, 2001) in the presence of hematopoietic cytokines that support the growth and differentiation of myeloid lineage cells (IL3, IL6, G-CSF, GM-CSF). The allantois and chorion from wild-type conceptuses gave rise to abundant round nonadherent cells (Fig. 4C,E) at efficiencies that were equivalent to those seen with the PAS, an established hematopoietic territory (Table 1). In contrast, allantoises and choriorts isolated from Runx1-deficient conceptuses failed to yield round nonadherent cells (Fig. 4D,F), consistent with the strict requirement for Runx1 in establishing definitive hematopoiesis (Okuda et al., 1996; Wang et al., 1996).

To demonstrate that the nonadherent cells were derived from the conceptus and not a maternal source, we isolated allantoises and choriorts from conceptuses derived from crossing a transgenic β-actin/GFP male mouse with a wild-type female mouse. In both allantois and chorion cultures the nonadherent cells were GFP+, indicating that they were derived from the conceptus and not from the mother (Fig. 4G,I). Some of the nonadherent GFP+ cells also expressed the pan-leukocyte marker CD45, and many appeared to be macrophages based on their ability to uptake Dil-Ac-LDL (Fig. 4H,J).

Runx1 is required for the emergence of CD45+ cells, including yolk sac macrophages, but it is not required for primitive erythropoiesis (Lacaud et al., 2002; Li et al., 2006; Mukouyama et al., 2000; Okuda et al., 1996; Wang et al., 1996). Cells expressing CD45, c-kit, or both antigens were detected in nonadherent cells collected from both the allantois and chorion explants from wild-type conceptuses (Fig. 4K), while cells expressing these markers were absent in cultures from Runx1-deficient conceptuses. Many of the CD45+ cells generated from allantois and chorion explants cultured in SCF, IL3, IL6, Flt3-L, G-CSF and GM-CSF expressed either or both the myeloid cell markers Gr-1 and Mac-1 (Fig. 5A), and cytospin preparations accordingly revealed myeloid lineage cells in the explants (Fig. 5B).

As a second approach we performed hematopoietic progenitor assays following 5 days of OP9 explant culture in the presence of SCF, Flt-3 ligand and EPO, while omitting G-CSF, GM-CSF, IL6 and IL3 in order to preferentially encourage the proliferation of erythroid progenitors. We found that granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM), granulocyte-macrophage (CFU-GM) and erythroid (BFU-E) progenitors were present in the explant cultures (Fig. 5C). Hematopoietic progenitors were also found in explants following 2 days of culture on plastic in 50% rat serum (Fig. 5C); they appeared at the same time and under the same conditions as the expression of Runx1 in the explants (Fig. 3A,B).

We analyzed β-globin gene expression in individual colonies by RT-PCR to determine whether the BFU-E colonies contained primitive or definitive erythroid cells. The erythroid colonies expressed β-major globin but not εy, the latter of which is a specific marker of primitive, yolk-sac-derived erythroid cells (Brotherton et al., 1979; Wang et al., 1983), indicating that the BFU-E colonies consisted of definitive erythroid cells.

**DISCUSSION**

The chorio-allantoic placenta of eutherian mammals is a composite of two organs: the umbilical cord and the chorionic disk. Two progenitor tissues of the placenta, the allantois and the chorion, are initially physically well separated in the conceptus, and then fuse to form the chorionic labyrinth, where exchange between the fetus and mother takes place. Recent studies have suggested that the murine placenta is an enriched source of hematopoietic progenitors, including CFU-GM, CFU-GEMM, BFU-E and high-proliferation-potential colony-forming cells (Alvarez-Silva et al., 2003). By 11.0 dpc, the placenta is also a source of long-term repopulating hematopoietic stem cells (Gekas et al., 2005; Ottmersbach and Dzierzak, 2005). However, whether these cells were derived from other hematopoietic sites within the conceptus, such as the yolk sac and aorta-gonad-mesonephros (AGM) region, or from the allantois and/or chorion themselves was not known.

In this study, we have demonstrated that both the allantois and the chorion have hematopoietic potential. The potential of both tissues was revealed after culturing them as explants on OP9

---

**Table 1. Efficiency at which OP9 explant cultures yielded hematopoietic cells (HC)**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Stage</th>
<th>Number of explants</th>
<th>% yielding HC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allantois</td>
<td>EHF†</td>
<td>17</td>
<td>77</td>
</tr>
<tr>
<td>Allantois</td>
<td>LHF†</td>
<td>22</td>
<td>90</td>
</tr>
<tr>
<td>Allantois</td>
<td>1-2 s</td>
<td>8</td>
<td>100</td>
</tr>
<tr>
<td>Chorion</td>
<td>EHF to 1-2 s</td>
<td>27</td>
<td>90</td>
</tr>
<tr>
<td>PAS§</td>
<td>EHF to 1-2 s</td>
<td>22</td>
<td>96</td>
</tr>
<tr>
<td>Embryonic ectoderm¶</td>
<td>EHF to 1-2 s</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

*Cultures included SCF, Flt3-L, IL3, IL6, G-CSF, GM-CSF and 2-ME, and were scored for the presence of abundant nonadherent cells as seen in Fig. 4C,E.
†Early headfold stage.
‡Late headfold stage.
¶Isolation of the embryonic ectoderm is described in the Materials and methods (see also Fig. 2C).
stromal cells in the presence of hematopoietic cytokines, or by CFU-C assays following a 2 day explant culture on plastic. Explants formed myeloid lineage cells and progenitors for definitive erythroid cells whereas Runx1-deficient explants did not. Thus, our results strongly suggest that Runx1 expression identifies hematopoietic progenitor cells within both the allantois and chorion of the intact conceptus.

Downs and colleagues originally showed that the murine allantois produced very few, if any, erythroid cells when grafted into the exocoelomic cavity or into the allantois of unlabeled host conceptuses (Downs et al., 1998; Downs and Harmann, 1997). However, ectopically transplanted allantoic cells from the distal, middle and proximal portions of the allantois did contribute extensively to the endothelium of the dorsal aorta and the

---

**Fig. 4. The pre-fusion allantois and chorion have hematopoietic potential.** (A) Explant of ConA 488 (red) labeled chorion following 48 hours of culture on OP9 stromal cells. ConA 488 positive cells that crawled off the explant are boxed. (B) Explant of ConA 594 labeled visceral endoderm (ve) after 48 hours on OP9 cells. (C) Allantois cultured for 7 days on OP9 cells in the presence of SCF, IL3, IL6, Flt3-L, G-CSF, GM-CSF and 2-ME. Note the presence of abundant, nonadherent round cells. (D) Allantois OP9 culture from a Runx1 deficient (Runx1<sup>ld/l</sup>) conceptus. No nonadherent round cells are present. (E) Chorion OP9 explant culture, same conditions as in panel C. (F) Chorion OP9 culture from a Runx1 deficient (Runx1<sup>ld/l</sup>) conceptus. (G) Allantois OP9 culture from a β-actin/GFP transgenic conceptus, showing that nonadherent cells originate from the conceptus. (H) Allantois OP9 culture from β-actin/GFP transgenic conceptus, stained with DiI-Ac-LDL (red) and anti-CD45 (blue). GFP<sup>+</sup> DiI-Ac-LDL<sup>+</sup> cells are yellow and GFP<sup>+</sup> CD45<sup>+</sup> cells are cyan. (I) Chorion OP9 culture from a β-actin/GFP transgenic conceptus. (J) Chorion OP9 culture from a β-actin/GFP transgenic conceptus, labeled with DiI-Ac-LDL and anti-CD45 as in panel H. (K) Representative FACS analysis of three pooled allantoises or chorions from each type of conceptus. The experiment was performed three times, with a total of ten ++ and seven Runx1<sup>ld/l</sup> allantoises and chorions.
Fig. 5. Myeloid and definitive erythroid potential of allantois and chorion explants. (A) FACS analysis of ten pooled allantois and nine pooled chorion explants following culture for 14 days on OP9 stromal cells in the presence of SCF, IL3, IL6, Flt3-L, G-CSF, GM-CSF and 2-ME. n_experiments=4, n_allantois=32, n_chorion=25. (B) May-Grunwald Giemsa stained nonadherent cells isolated from allantois and chorion explants cultured on OP9 cells under the conditions described in A. a-h, allantois explants; i-p, chorion explant cultures; a-c, i-n, monocytes; d-e, macrophages; f-h, o-p, myelocytes. (C) Methylcellulose colony forming assays from the allantois and chorion. Six conceptus equivalents of allantoises and chorions were cultured together as explants, disaggregated and plated in methylcellulose. The top three panels are representative colonies. (Bottom) May-Grunwald Giemsa-stained cytospin preparations of individual colonies from the allantois and chorion cultures. e, erythocyte; m, myeloid cell; bar=10 μm. Colony numbers per six conceptus equivalents (±s.e.m. in parentheses) following 2 days of explant culture in the presence of 50% rat serum on plastic, or after 5 days of explant culture on OP9 cells in the presence of SCF, Flt3-L and EPO were averaged from three independent experiments. (D) RT-PCR for globin gene expression (εγ and βmajor). BFU-E or CFU-GM represents individual colonies from either the allantois or chorion methylcellulose cultures. Yolk sac was isolated from ~9.0 dpc conceptuses as a positive control for εγ expression, and bone marrow was used as a negative control.
mesenchyme directly adjacent to it within the PAS of the host embryo (Downs and Harmann, 1997). The PAS is an established intra-embryonic hematopoietic territory and, between the headfold and 10 s stages, contains multipotent hematopoietic activity that could be revealed following explant culture on stromal cells (Cumano et al., 1996; Cumano et al., 2001). In retrospect, the extensive contribution of the allantois to the dorsal aorta and peri-aortic mesenchyme upon transplantation (Downs and Harmann, 1997) is consistent with the hematopoietic potential demonstrated in vitro in the current study. Importantly, the hematopoietic potential of the allantois was demonstrated here in all three allantoic subregions of early headfold stage conceptuses, before vascular continuity between the allantois and yolk sac was achieved. Therefore the hematopoietic progenitors must have arisen de novo from the allantois and not via the circulation from the yolk sac or the embryo proper.

Although we did not separate the chorioclastic mesoderm from ectoderm, we suspect, based on our results of β-galactosidase staining, that the Runx1+ mesodermal component is the source of the hematopoietic cells supplied to the chorion. The site within the allantois that gives rise to hematopoietic cells is not clear; however, as Runx1+ cells were located on the outer mesothelial surface, which mediates chorio-allantoic union, it is likely that the allantoic mesothelium is involved, with Runx1+ endothelial cells within the base of the allantois being a second potential source.

Very little is known about either allantoic mesothelium or chorioclastic mesoderm other than that they mediate chorio-allantoic fusion (Downs and Gardner, 1995) via VCAM1 on the allantoic mesothelium (Gurtner et al., 1995; Kwee et al., 1995) and its counter receptor α4 integrin on chorioclastic mesoderm (Yang et al., 1995). Once the allantois fuses with the chorion, it is not known whether the fusion surfaces of the allantois and chorion break down, persist or differentiate into other cell types (Downs, 2002). Based on findings presented here, we suggest that cells from one or both of these fusing surfaces persist. In this way, Runx1+ hematopoietic cells derived from allantoic mesothelium and/or chorioclastic mesoderm may then enter the allantoic vasculature during or after its penetration into the chorionic plate. One interesting observation is that Runx1 expression in the chorioclastic mesoderm and at the site of intersection between the allantois and the chorion very closely resembles that of α4 integrin, both temporally and spatially (Downs, 2002). Both α4 integrin and Runx1 are expressed on all long-term repopulating hematopoietic stem cells in the embryo (Gribi et al., 2006; North et al., 2002), lending support to the notion that the Runx1+ and α4 integrin+ cells at the chorio-allantoic fusion junction ultimately contribute to hematopoiesis in the placenta.

One of the issues raised by these studies concerns the location of the source of the signal (or signals) that induces the hematopoietic program in the allantois and chorion. Studies in both avian and murine conceptuses clearly show that the induction of the hematopoietic program in mesoderm requires a signal from endoderm. For example, in the mammalian yolk sac, visceral endoderm provides soluble signals that are required for the hematopoietic program in mesoderm. For example, in the mammalian yolk sac, visceral endoderm provides soluble signals that are required for the hematopoietic program in mesoderm. For example, in the mammalian yolk sac, visceral endoderm provides soluble signals that are required for the hematopoietic program in mesoderm. For example, in the mammalian yolk sac, visceral endoderm provides soluble signals that are required for the hematopoietic program in mesoderm.

Within it endoderm derived from the primitive hindgut (Caprioli et al., 1998). However, whereas the allantois of many eutherian mammals exhibits an endodermal component (Mossman, 1937), the allantois of the house mouse is thought to consist entirely of extra-embryonic mesoderm that contains within it a core of Brachyury-positive cells and is surrounded by a mesoderm-derived mesothelial layer (Duval, 1891; Inman and Downs, 2006; Mossman, 1937). A re-examination of the murine allantois for an endodermal component using modern techniques and markers may be warranted.

The distal tip of the allantois is the ‘oldest’ allantoic tissue, having emerged first from the primitive streak (Downs et al., 2004; Kinder et al., 1999; Lawson et al., 1991), whereas the cluster of cells within the base of the allantois emerges later (Kinder et al., 1999). We hypothesize that signals separated either temporally or spatially may activate Runx1 expression in the distal portion of the allantois and within the base. It is tempting to speculate that, as the mesoderm at these two sites originates within the primitive streak over a prolonged period (6.75–8.5 dpc) (Beddington, 1982; Copp et al., 1986; Downs, 2002; Kinder et al., 1999; Lawson et al., 1991; Tam and Beddington, 1987), these signals may be encountered at different times in the primitive streak. However, as so little is known about the role of the streak in specification of allantoic mesoderm (Downs et al., 2004), further experimentation is needed to address this notion. Moreover, as so little is known about chorioclastic mesoderm and ectoderm, the signals responsible for Runx1 expression in the chorioclastic mesoderm are completely unclear at this time.

In summary, our findings strongly suggest that the placenta itself is a hematopoietic organ. To what extent hematopoietic activity emerges de novo from the placenta, or comes from other sites of hematopoietic emergence awaits further investigation.

We thank Elaine Dzierzak for providing the Tg(Ly6a/GFP) mice and Yuko Fujiwara for sending them. We thank Xiao Hu and Steve Fiering for providing the yolk sac cDNA, and both Steve Fiering and Jim Palis for their helpful advice. We acknowledge Françoise Dieterlen-Lievre for her pioneering work on the hematopoietic potential of the avian allantois. This work was supported by Public Health Service grant ROI CA58343 to N.A.S., RO1 HD042706 to K.M.D. B.M.Z. is supported by T32 AI 07519.

References
probed before circulation in mouse, is restricted to caudal extraembryonic splanchnopleura. Cell 86, 907-916.


