Vascularization in the murine allantois occurs by vasculogenesis without accompanying erythropoiesis

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SUMMARY

The aim of this study was to determine whether the blood vessels of the murine allantois are formed by vasculogenesis or angiogenesis. Morphological analysis revealed that differentiation of allantoic mesoderm into an outer layer of mesothelium and an inner vascular network begins in the distal region of the allantois, which is most remote from other tissues, as early as the late neural plate stage (approximately 7.75 days postcoitum). Nascent blood vessels were not found in the base of the allantois until 4-somite pairs had formed in the fetus (approximately 8.25 days postcoitum), and vascular continuity with the yolk sac and fetus was not present until the 6-somite-pair stage (approximately 8.5 days postcoitum). Immunohistochemical analysis demonstrated that *flk-1*, a molecular marker of early endothelial cells, is expressed in significantly more distal than basal core cells in the early allantois and never in mesothelium. Furthermore, synchronous grafting of donor yolk sac containing blood islands into blood islands of headfold-stage host conceptuses provided no evidence that the yolk sac contributes endothelial cells to the allantois. Finally, when removed from conceptuses and cultured in isolation, neural plate and headfold-stage allantoises formed a conspicuous vascular network that was positive for Flk-1. Hence, the vasculature of the allantois is formed intrinsically by vasculogenesis rather than extrinsically via angiogenesis from the adjacent yolk sac or fetus.

Whether allantoic vasculogenesis is associated with erythropoiesis was also investigated. Benzidine-staining in situ revealed that primitive erythroid cells were not identified in the allantois until 6-somite pairs when continuity between its vasculature and that of the yolk sac was first evident. Nevertheless, a small number of allantoises removed from conceptuses at a considerably earlier stage were found to contain erythroid precursor cells following culture in isolation. To determine whether such erythroid cells could be of allantoic origin, host allantoises were made chimeric with *lacZ*-expressing donor allantoises that were additionally labeled with [3H]methyl thymidine. Following culture and autoradiography, many *lacZ*-expressing benzidine-stained cells were observed in donor allantoises, but none contained silver grains above background. Moreover, no cells of donor allantoic origin were found in the fetus or yolk sac. Hence, vasculogenesis seems to be independent of erythropoiesis in the allantois and to involve a distal-to-proximal gradient in differentiation of allantoic mesoderm into the endothelial cell lineage. Furthermore, this gradient is established earlier than reported previously, being present at the neural plate stage.

Key Words: Allantois, Endothelial cell, Erythropoiesis, *flk-1*, In vitro, Mesothelium, Mouse, Placenta, Transplantation, Vasculogenesis, Yolk sac

INTRODUCTION

The chorioallantoic placenta of eutherian mammals is critical for fetal development and growth during gestation. While much effort has been devoted to investigating the roles of the mature placenta, study of differentiation of two of its component structures, the allantois and the chorion, has so far largely been neglected.

The murine allantois is derived from the proximal epiblast (primitive ectoderm) of the early mouse gastrula, the same embryonic progenitor tissue that gives rise to the blood islands of the visceral yolk sac, the chorionic mesoderm, the amnion and, possibly, the germline (Gardner et al., 1985; Lawson et al., 1991; Lawson and Pedersen, 1992; Lawson and Hage, 1994; reviewed in Downs, 1998). At the onset of gastrulation, cells from the proximal epiblast enter the posterior primitive streak and emerge as extraembryonic mesoderm (Lawson et al., 1991). This mesoderm lines the exocoelomic cavity and also forms the entire allantois (Jolly and Férester-Tadié, 1936; Bonnevie, 1950; Lawson et al., 1991; Lawson and Pedersen, 1992; Lawson and Hage, 1994). By approximately 7.5 days postcoitum (dpc), the contribution of extraembryonic mesoderm to the lining of the exocoelom ceases and cells newly emerging from the primitive streak are channeled almost exclusively into the

allantois (Beddington, 1982; Copp et al., 1986; Tam and Beddington, 1987) whose size is also increased by mitosis and cavitation (Ellington, 1985). As it enlarges, the allantois acquires selective adhesiveness for the chorion (Downs and Gardner, 1995) and, following attachment to the latter at between 4- and 6-somite pairs (approximately 8.25-8.5 dpc), the allantois becomes conspicuously vascularized throughout. This is the prelude to the allantois forming the vascular link between the placenta and fetus via a single umbilical artery and vein.

Several studies suggest that vascularization of the allantois occurs before it fuses with the chorion and therefore does not depend on contact with this polar trophectoderm derivative (Ellington, 1985; Downs and Gardner, 1995). However, at present it is not clear whether the vasculature of the allantois is formed by the extension of blood vessels that are formed elsewhere (angiogenesis, reviewed in Risau, 1997) or by differentiation of endothelial cells that originate within it (vasculogenesis, Sabin, 1920; reviewed in Risau and Flamme, 1995). The proximity of the base of the allantois to the vasculogenic visceral yolk sac (Haar and Ackerman, 1971; Kaufman, 1992) suggests that its vasculature could form by angiogenesis, particularly as the allantois contains no endoderm, which has been proposed to be requisite for vasculogenesis (reviewed in Risau and Flamme, 1995). However, according to histological observations in the rat, vascular channels first differentiate distally rather than basally in the mesodermal core of the allantois (Ellington, 1985). These observations support a vasculogenic rather than an angiogenic origin for the allantoic vasculature in rodents, as do the results of grafting murine allantoic cells into the fetus (Downs and Harmann, 1997). Ectopically grafted allantoic cells showed a strong propensity to integrate into the dorsal aorta, a structure that has been shown to form by vasculogenesis in the avian embryo (Pardanaud et al., 1987; Coffin and Poole, 1988).

The fact that vasculogenesis and hematopoiesis often occur together raises the further question of whether the murine allantois is, like the visceral yolk sac, also a source of primitive erythrocytes (Metcalf and Moore, 1971). At present, the evidence is confusing. The erythroid cells that first appear in the basal region of the allantois as endothelial cells differentiate there were presumed by Ellington (1985) to originate from the visceral yolk sac. However, the results of orthotopic grafting experiments suggested that at least some erythropoietic cells might be of allantoic origin (Downs and Harmann, 1997). Earlier gene expression studies had demonstrated that both *flk-1* and *TGFβ1*, which are required for vasculogenesis and primitive erythropoiesis (Shalaby et al., 1995; Dickson et al., 1995) were expressed in the murine allantois as well as the yolk sac (Yamaguchi et al., 1993; Akhurst et al., 1990). However, no cells that were positive for EKLF, one of the first erythroid transcription factors expressed in the yolk sac (Southwood et al., 1996), were found in approximately 8-8.25 dpc headfold-to early-somite-stage murine allantoises.

The aims of the present study were to determine whether the vasculature of the murine allantois forms by vasculogenesis or angiogenesis and whether the allantois is erythropoietic. Using a combination of morphological analysis, microsurgical manipulation, immunohistochemistry and histochemistry, and both embryo and tissue culture, we have found that the mesodermal core of the murine allantois engages in vasculogenesis that is not accompanied by erythropoiesis.

MATERIALS AND METHODS

Mouse strains, recovery, staging, culture and scoring of whole conceptuses

Both donor and host conceptuses were of the same genetic background (C57Bl/6×CBA), but the donors were made heterozygous for a *lacZ* transgene (ROSA26*, see Friedrich and Soriano, 1991; Zambrowicz et al., 1997) as described by Downs and Harmann (1997).

Mice were maintained under a 12-hour light/dark cycle. Dissection, staging, culture and scoring of conceptuses were as previously described (Downs and Davies, 1993; Downs and Gardner, 1995; Downs and Harmann, 1997). Culture periods were typically 24 hours but varied for some experiments (Figs 1, 4, 5; Tables 2, 3). Most embryos destined for culture or supplying allantoises were initially at headfold stages (exceptions are Fig. 7, Table 4C). Abnormal conceptuses were not included in the analyses (two unfused allantoises/173 operated conceptuses, 1.2%; one blistered epithelium/105 unoperated control conceptuses, 1.0%; Tables 2, 5).

The circulation was scored by examining conceptuses immediately following removal from the incubator. It was recorded as negative if no movement of red blood cells was observed after 15 seconds, as weak if some movement of red blood cells was seen through the yolk sac, and as vigorous if red blood cells coursed throughout the conceptus.

In all experiments, unoperated host and/or donor conceptuses were cultured alongside the experimental ones. As reported previously (Downs and Gardner, 1995; Downs and Harmann, 1997), there were no significant differences in development between experimental and control conceptuses.

Culture of allantoises in isolation

Prefusion allantoises were aspirated using mouth-held, hand-pulled capillaries (Downs and Harmann, 1997). Glass scalpels (Beddington, 1987) were used to divide some allantoises into the basal third and distal two-thirds, and to separate postfusion allantoises from the chorion and embryo following removal of the yolk sac. Individual allantoises or their subregions were cultured in 0.5 ml of medium either suspended in tubes (Downs and Gardner, 1995) or plated in untreated wells of 24-well tissue-culture dishes (Falcon, No. 3047). Isolated yolk sac blood islands cultured in suspension served as positive controls for benzidine staining.

After culture and benzidine staining (see below), explants were postfixed in 4% paraformaldehyde for 1-2 hours at 4°C and either viewed in the plastic wells on an inverted compound microscope (Nikon), gently squashed onto slides as previously described (Downs and Harmann, 1997) or prepared for histology (below).

Histological preparation of conceptuses and allantoic explants

Material was either fixed for 1-3 hours in Bouin's fluid at room temperature (Figs 1, 2) or, if it was to be immunostained (below) or X-gal-stained (Downs and Harmann, 1997), in 4% paraformaldehyde at 4°C for 2 hours (Figs 3, 4, 5C, 8, 9; Tables 2, 3, 5). Subsequent preparation for histology was as described by Kaufman (1992). Most specimens were sectioned at 4 μm (exception, Fig. 3) before being stained with nuclear fast red or hematoxylin alone, or with hematoxylin/eosin.

Benzidine-staining was done prior to fixation either using a full-strength solution (Downs and Harmann, 1997; 10 minutes: Table 5, Figs 8, 9; 30 minutes: Table 4, Figs 1, 7), or a dilute solution (20% of full-strength; 30 minutes: Tables 2, 3, Figs 1A, 4, 5) often followed by fixation and X-gal staining. Full-strength benzidine for 10 minutes enabled identification of most *lacZ/+* hemoglobin-synthesizing cells (Downs and Harmann, 1997) in both ex vivo and cultured conceptuses: although dilute benzidine did not (Fig. 1A), it did ensure that all *lacZ*-expressing donor cells entering the allantois from the yolk sac would be identified.

Localization of Flk-1 protein by immunohistochemistry

Conceptuses were fixed in 4% paraformaldehyde, prepared for wax histology, and sectioned at 6 μm. Immunostaining for Flk-1 (Santa Cruz Biotechnology, Inc.) was carried out according to the supplier's recommendations. Antibodies against Flk-1 (10:1; polyclonal antiserum raised in rabbit) were prebound with immunizing peptide (Santa Cruz Biotechnology, Inc.) in 1.5% goat serum for 3 hours and applied in place of primary antibodies. Primary antibodies were omitted as a second control.

For immunohistochemistry, allantoises were plated on untreated sterile 8-well plastic tissue-culture chamber slides (Nunc) containing 0.4 ml of culture medium, cultured for 24 hours and rinsed three times in PBS followed by ice-cold methanol for 5 minutes. All subsequent steps were carried out at room temperature as described above for Flk-1 immunohistochemistry in histological sections. Allantoises were counterstained in hematoxylin, then rinsed in tap water and coverslipped with water-based mounting medium (Lerner Laboratories, Pittsburgh, PA).

Quantification of *flk-1* expression

Every sagittal section containing the allantois was photographed on 35 mm color slide film, developed and scanned for each of twelve conceptuses. Adobe Photoshop was used to construct a grid in which the base of the allantois was defined (Downs and Harmann, 1997), the proximodistal axis was projected from the basalmost boundary to the tip of the longest allantoic projection and the axis was subdivided into three equal regions. The grid was superimposed over each successive serial section by orienting the base as described above. All sections were printed and every Flk-1-positive cell fragment was counted per region with simultaneous verification in the compound microscope. The number of Flk-1-positive cell fragments was calculated as the percentage of the total number of cell fragments per allantoic region.

Yolk sac transplantation

Glass scalpels were used to remove the entire circumferential strip of yolk sac containing blood islands from 1 or 2 donor conceptuses per experiment. Each strip was then cut into smaller pieces measuring approximately 269.7 μm (s.d.±46.3)×156.5 μm (s.d.±50.0) (*n*=111) in dissection medium. A single piece of donor yolk sac was aspirated into a glass capillary so that its mesodermal surface was in contact with the capillary and placed into the hole made in an intact host yolk sac (Downs and Gardner, 1995) at the level of anterior or posterior blood islands so that it extended through the yolk sac's full thickness. The site of transplantation of donor blood islands was not made to correspond with their anterior or posterior origin.

Allantoic grafting, autoradiography and scoring donor cells

Manipulations, labeling in [³H]methyl thymidine and culture were carried out as previously described (Downs and Gardner, 1995). Controls for each experiment were: (1) uptake controls (Beddington, 1981, 1982), (2) cultured unoperated donor conceptuses to ascertain the amount of thymidine-labeling in donor-derived benzidine-positive cells, and (3) cultured unoperated host conceptuses to estimate background levels of silver grains over benzidine-positive cells.

For grafting, labeled donor allantoises were aspirated following removal of the ectoplacental cone and chorion and introduced via anterior yolk sac puncture, above the blood islands, base first, into the exocoelomic cavity of unlabeled host conceptuses whose own allantoises had been removed through this site.

Preparation of sections for autoradiography was as previously described (Downs and Gardner, 1995). Slides were exposed in light-tight boxes at 4°C for 25-27 days. Benzidine-positive cells were scored as donor-derived if their cytoplasm was either blue throughout or contained spots of X-gal precipitate. The number of silver grains contained within all doubly stained X-gal- and benzidine-positive cell

fragments was counted for each grafted conceptus and the average taken for each experiment.

Student's *t*-test

Data in Tables 1, 2, 4, 5 and Fig. 1 were analyzed for statistical significance (*P*≤0.05) using a two-way Student's *t*-test (Mini-Tab) with a 95% confidence interval and equal variances assumed.

RESULTS

Vascularization and time of appearance of erythroid cells in the allantois

The headfold-stage allantois was found to exhibit a precise topographical arrangement of cells (Downs and Harmann, 1997), with those in the distal region being the most restricted in developmental potency and those in the proximal region adjacent to the nascent fetus, the least restricted.
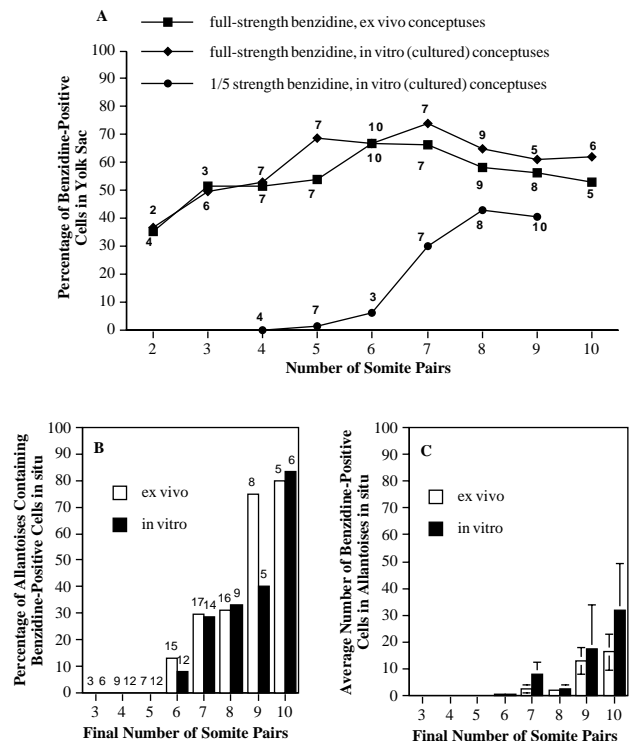

Fig. 1. Benzidine-staining in yolk sacs and allantoises of ex vivo and cultured conceptuses. (A) The percentage of deeply stained benzidine-positive cells in yolk sacs was calculated as a function of numbers of somite pairs from a minimum of two experiments (ordinals at each point are total numbers of conceptuses). *P*≥0.051 for ex vivo and cultured conceptuses in full-strength benzidine. 3.1% of cells in yolk sac blood islands in ex vivo conceptuses at headfold stages were benzidine-positive (five conceptuses examined). (B) Bar graph comparing percentages of benzidine-stained cells in the allantois and reported as a function of the final number of somite pairs for ex vivo (white) and cultured conceptuses (black). Numbers of conceptuses examined are indicated at the left (ex vivo) and right (in vitro) of each somite-pair on the abscissa or at the top of each vertical bar. (C) Average number of benzidine-positive cells in allantoises (including negative conceptuses) is shown as a function of the final number of somite pairs. Error bars represent the standard error of the mean. *P*≥0.24 for average numbers of benzidine-positive cells in allantoises of ex vivo and cultured conceptuses.

Vascularization might therefore be expected to begin in the distal region of the allantois and then extend proximally. Accordingly, such a sequential pattern of differentiation of the allantois should be evident both morphologically and at the molecular level.

The allantois was examined in conventional histological sections of conceptuses that were freshly recovered from the uterus beginning at the neural plate stage, when it is an early bud, and at successive stages thereafter (Fig. 2). Particular attention was paid to the following: (i) conversion of the outer cells into a mesothelium, (ii) the appearance of a vascular scaffold within the mesodermal core, (iii) the spatial relationship between the base of the allantois and the nascent yolk sac blood islands, (iv) the timing of amalgamation of allantoic, yolk sac and fetal blood vessels, and (v) the timing of appearance of benzidine-positive cells within the allantois.

The distalmost outer cells of the allantoic bud were the first to undergo morphological differentiation by flattening to form a single layer of squamous mesothelium (compare Fig. 2A and B). This morphological change spread proximally (Fig. 2A-D) and appeared to be completed by the 4-somite stage when the free surface of the allantois was completely enveloped by typical mesothelium (Fig. 2G).

A similar distal-to-proximal sequence was evident by the morphological differentiation into a rudimentary vasculature of the mesodermal core of the allantois. Most, if not all, core cells were initially round (Fig. 2A,B). Then, as headfolds appeared in the embryo, cells in the distal third of the allantois showed signs of flattening (Fig. 2C,D) followed by coalescence during very early somite stages (Fig. 2E) and then the formation of delicate vascular channels (Fig. 2F). This morphological sequence was reiterated in the middle third of the allantois between the late headfold and 3-somite-pair stages (Fig. 2D and data not shown), but was not conspicuous in the basal third until 6-somite pairs (Fig. 2I). However, by 4-somite pairs, a very small nascent blood vessel was invariably found in the base of the allantois at its junction with the yolk sac (Fig. 2G). By 6-somite pairs, this vessel had amalgamated with others that had spread toward the base of the allantois from the yolk sac blood islands and the fetus (Fig. 2H,I). By 8-somite pairs, movement of red blood cells into the basal region of the allantois from the yolk sac was discernible in some specimens (Fig. 2J).

Between the neural plate and early headfold stages, there appeared to be an intimate relationship between the thickened mesoderm of the prospective yolk sac blood islands and that of the nascent allantois, suggesting possible intermingling of cells between them (Fig. 2A-C). By the late headfold and early somite stages, discrete clusters of blood islands had formed in the yolk sac, which were typically well separated from the base of the allantois by a well-defined layer of mesothelium

(Fig. 2D,G). Some erythropoietic cells in the yolk sac were benzidine-positive in whole-mount and histological preparations as early as the headfold stages (legend to Fig. 1A).

The earliest time of appearance of benzidine-positive cells in the allantois was investigated both in freshly recovered conceptuses and, in anticipation of grafting experiments, those
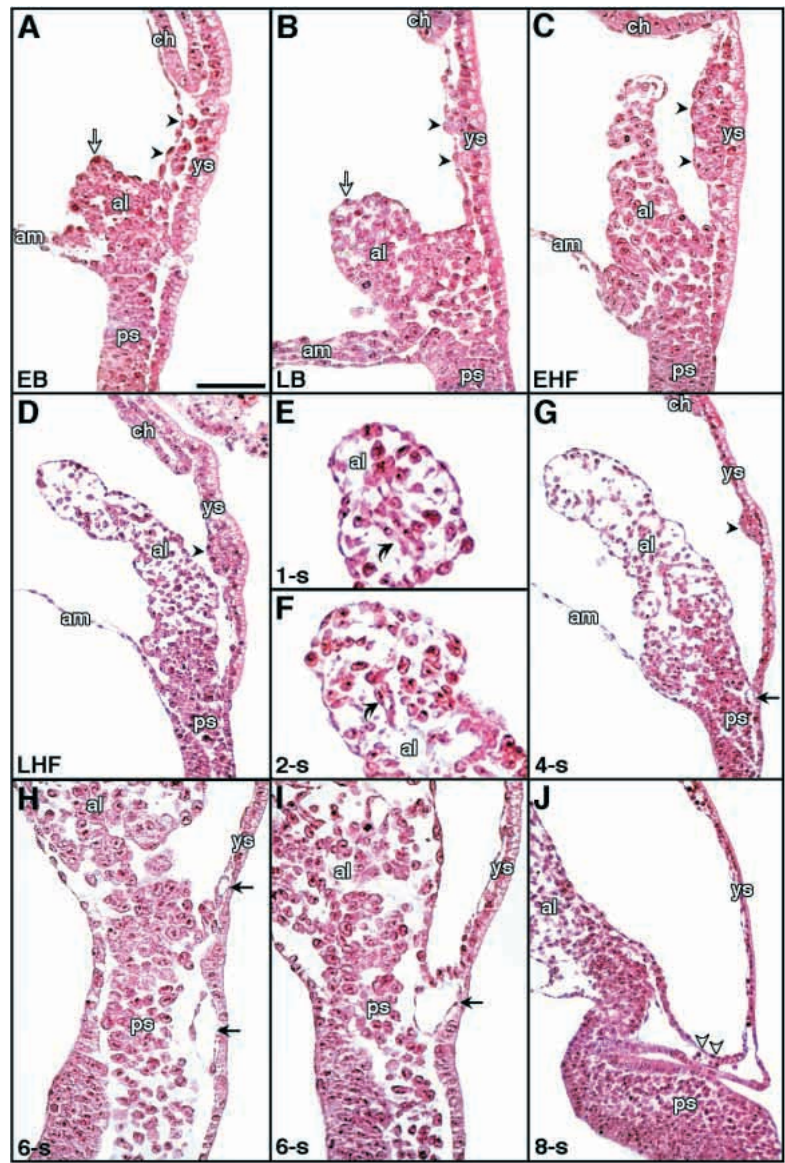

Fig. 2. Morphological vascularization in the developing allantois. Morphological vascularization was examined in allantoises in at least five freshly recovered conceptuses from three experiments for each stage of development. Whole (A-D, G-J) or distal allantoic regions (E,F) are shown. (A) Neural plate/early bud stage (EB). (B) Neural plate/late bud stage (LB). (C) Early headfold stage (EHF). (D) Late headfold stage (LHF). (E) 1-somite pair (1-s). (F) 2-somite pairs (2-s). (G) 4-somite pairs (4-s). (H-I) 6-somite pairs (6-s) showing yolk sac and fetal vessels (arrows, H) amalgamating with the allantoic vessel (arrow, I). (J) 8-somite pairs (8-s). Abbreviations: al, allantois; am, amnion; ch, chorion; ps, posterior primitive streak region; ys, yolk sac. White arrow in A,B, mesothelium. Arrowheads in A-D,G, yolk sac blood islands. Curved arrows in E,F, flattening (E) and coalescing (F) core cells. Open arrowheads in J, red blood cells. Scale bar in A, 50 μm (A,B); 52 μm (C); 74 μm (D); 39 μm (E); 37 μm (F); 84 μm (G); 42 μm (H,I); 86 μm (J).

that had been cultured from headfold stages (Fig. 1B). Modest numbers (<10) of benzidine-positive cells appeared in a small percentage of allantoises as early as 6-somite pairs (Fig. 1C) and gradually increased in abundance thereafter. Slightly more benzidine-positive cells were found in cultured conceptuses than in ex vivo conceptuses at most somite stages, but the difference was not significant ($P \geq 0.24$). Thus whole embryo culture does not appear to perturb the timing of either globin expression in the erythroid cells of the yolk sac (legend to Fig. 1A) or the appearance of globin-positive cells in the allantois.

Localization of Flk-1 protein in the developing allantois

Previous results demonstrated that Flk-1 mRNA was present in the allantois (Yamaguchi et al., 1993; Dumont et al., 1995). Here, immunohistochemistry was used to determine the pattern of localization of Flk-1 protein in the developing allantois.

At no stage was expression of *flk-1* observed in the mesothelium of the allantois (Fig. 3A-G) but, in all allantoises examined, the core mesoderm contained Flk-1-positive cells. At the neural plate and headfold stages, the percentage of positive core cells was highest in the distal third of the allantoic bud, significantly lower in the basal third and intermediate in the middle third (Fig. 3A,B; Table 1). Onset of *flk-1* expression began as an intracellular spot that did not always coincide with localization of Flk-1 in the plasma membrane (Fig. 3A,B). By 3- to 4-somite pairs, expression of *flk-1* had become abundant in the basal third of the allantois (Fig. 3C-E) and was invariably expressed in the nascent allantoic blood vessel found at the allantoic/yolk sac junction at 4-somite pairs (data not shown). By 6-somite pairs, Flk-1 protein was abundant in flattened cells that formed obvious vascular channels throughout the allantois (Fig. 3F) and it continued to be expressed in such nascent vessels as late as 10-somite pairs (Fig. 3G).

Grafting headfold-stage yolk sacs containing blood islands

To investigate whether the yolk sac can vascularize the allantois, blood island-containing pieces of yolk sac from *lacZ*/+ donor conceptuses were grafted into anterior or posterior blood islands of similarly staged hosts (Table 2; Fig. 4A). Following culture, control and grafted conceptuses were scored for a yolk sac circulation (Fig. 5A,B). After staining for *lacZ* activity, the grafted conceptuses were examined grossly for the location of the grafts (Fig. 4B). They were then processed and examined histologically to check whether the grafts had integrated into the host yolk sac (Table 2; Fig. 4C) and

to obtain a definitive count of the number of somite pairs. Integration of the grafts was presumed to have occurred if the donor yolk sac mesoderm and visceral endoderm were continuous with those of the host so that donor cells could gain access to the fetus and allantois (Table 2). In all cases, the numbers of cells dispersed from the site of the graft were recorded in three sites: the yolk sac, the fetus and the allantois (Figs 4D, 5C).
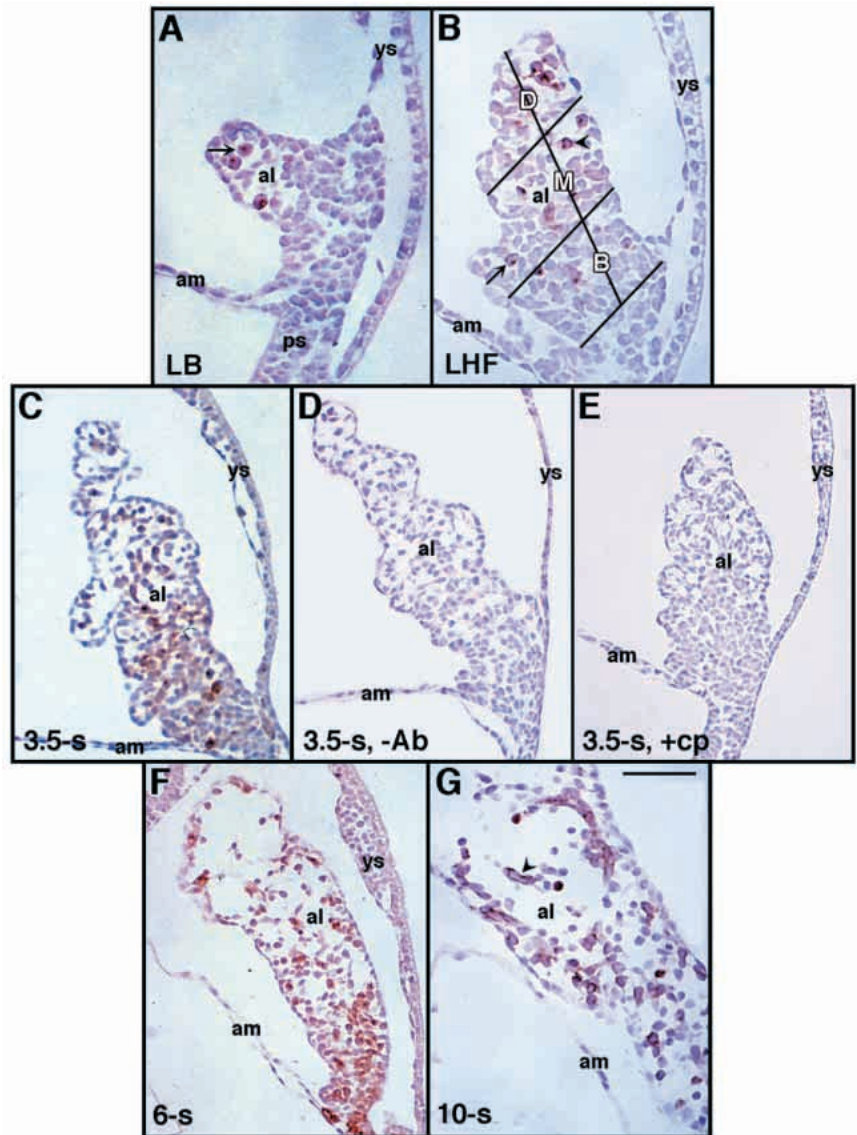

Fig. 3. Localization of Flk-1 protein in the developing allantois by immunohistochemistry. At least six conceptuses from three experiments were analyzed for each developmental stage; each experiment contained the controls described in Materials and Methods. (A) Late allantoic bud (LB), neural plate stage. (B) Late headfold stage (LHF) showing an example of the grid dividing the allantois into three regions. (C) 3.5-somite pairs (3.5-s). (D) 3.5-s minus primary antibody. (E) 3.5-s in which the antibody against Flk-1 was prebound with control peptide. (F) 6-somite pairs (6-s). (G) 10-somite pairs (10-s). Arrows in A,B indicate cells containing an internal spot of Flk-1 without concomitant localization in the plasma membrane. Arrowheads in B,G indicate cells exhibiting Flk-1 staining in the plasma membrane which, in G, is found in coalescing cells of nascent vascular channels. Abbreviations as in Fig. 2; in addition: B, basal third, M, middle third; D, distal third. Scale bar in G: 40 µm (A); 43 µm (B); 67 µm (C); 72 µm (D,E); 78 µm (F); 54 µm (G).

Table 1. Average numbers of Flk-1-positive cell fragments in subregions of late neural plate and headfold allantoises

Number of conceptuses	Stage	Average number of cell fragments counted per subregion (±s.d.)			Average percentage of Flk-1-positive cell fragments per subregion (±s.d.)		
		B	M	D	B	M	D
6	LB	356.8 (±114.4)	261.2 (±90.3)	151.7 (±64.3)	2.4 (±0.8)	7.8 (±2.9)	11.4 (±4.0)
6	HF	977.7 (±182.7)	564.7 (±106.5)	239.3 (±69.4)	5.2 (±3.1)	11.8 (±4.9)	17.5 (±5.8)

All Flk-1-positive cell fragments were counted for each allantoic subregion (Fig. 3B), basal (B), middle (M) and distal (D) thirds, in neural plate/late bud (LB) and headfold (HF) stage allantoises and taken as the number of immunopositive cell fragments over the total number of cell fragments for that region ($n \geq 5$ experiments). Results of Student's *t*-test (significant differences are in boldface): LB stage: for B versus D, **P=0.003**; B versus M, **P=0.002**; M versus D, P=0.10.
 HF stage: for B versus D, **P=0.001**; B versus M, **P=0.02**; M versus D, P=0.09.

By 5-somite pairs, slight movement of red blood cells could be discerned in the yolk sac of a small minority of conceptuses (Fig. 5A). A vigorous circulation was evident in some conceptuses with 7- to 8-somite pairs and in nearly all those with 10-somite pairs (Fig. 5A,B). Contribution of donor-derived endothelial cells to the host was slight and was restricted to the yolk sac in the immediate vicinity of the grafts (data not shown). Contribution of donor-derived cells to the host at sites far removed from the donor graft was invariably intravascular and therefore presumed to be hematopoietic in nature. In most cases where donor cells had dispersed, a pulsatile circulation was evident. In the absence of a discernible circulation, 11% of yolk sacs and 3% of fetuses contained donor cells (Table 3). However, there were considerably fewer dispersed donor cells in conceptuses without a circulation than in those with a slight or vigorous one (Table 3). Notably, yolk-sac-derived cells were identified in the allantois only in host conceptuses with an unequivocal circulation. Thus, the timing of initial dissemination of donor cells into hosts was 6-somite pairs for the yolk sac, 8-somite pairs for the fetus and 10-somite pairs for the allantois (Fig. 5C).

Table 2. Summary of grafts of donor yolk sac containing blood islands to host yolk sac blood islands

	Number and position of donor yolk sac grafts in host conceptuses	
	A	P
Total number of conceptuses grafted	85	56
Number of abnormal conceptuses	1	1
Number of unproductive grafts	12	6
Number of grafts exhibiting fusion with chorion and/or non-blood islands	13	8
Total number of conceptuses scored	59	41

The total number of headfold-stage host conceptuses grafted with yolk sac containing blood islands from donor conceptuses of the same stage and their anterior (A) or posterior (P) position into host yolk sac blood islands are shown (*n*=14 experiments). The number of abnormal conceptuses, unproductive grafts and those grafts that did not remain within yolk sac blood islands are indicated. These three categories were not included in the final analysis. The final number of conceptuses containing productive grafts is shown on the last line. The average number of donor yolk sac-derived cells (not shown) was compared for the anterior and posterior sites of grafting and found to be not signficant for either donor cell infiltration into the allantois (*P*=0.33) or into the fetus (*P*=0.10).

Culture of allantoises in isolation

Allantoises were explanted from host or donor conceptuses at the headfold-stage to determine whether the allantois could become vascularized following its isolation from all neighboring tissues. Isolation was by aspiration, either laterally through the yolk sac blood islands or, to avoid possible contamination with erythropoietic cells, dorsally via the opening created by removing the chorion and ectoplacental cone. Once isolated, allantoises were plated individually on plastic or cultured in suspension.

Plated allantoises formed criss-crossing networks of putative vascular channels within 24 hours (Fig. 6A; Table 4A). Those cultured in suspension rounded up to an extent that vascular channels were not discernible even after gentle squashing on microscope slides (Downs and Harmann, 1997). Nevertheless, these also invariably exhibited signs of vascularization when examined after sectioning (Fig. 6B; Table 4B). The endothelial nature of the putative vascular network was confirmed by its specific reactivity with the anti-Flk-1 serum (Fig. 6C-F, and data not shown).

Whole allantoises were then isolated from younger neural plate/late allantoic bud-stage conceptuses, i.e., approximately

Table 3. Summary of the distribution of dispersed yolk sac-derived donor cells in the yolk sac, fetus and allantois of grafted host conceptuses as a function of host circulation

Circulation	No circulation	Weak circulation	Vigorous circulation
Total number of conceptuses	31	20	49
Average number of donor cells in host yolk sac (% positive)	5.2±2.5 (35.5)	47.8±17.3 (60.0)	540±107 (95.9)
Average number of donor cells in host fetus (% positive)	0.6±0.5 (9.7)	2.9±1.1 (35.0)	220.6±52.1 (81.6)
Average number of donor cells in host allantois (% positive)	0.0 (0)	1.4 (5.0)	35.4±11.6 (57.1)

Negative conceptuses were included in the calculation of the average number of donor-derived cells for each location. The percentage of conceptuses containing donor-derived cells is shown in parentheses for each category of circulation. All donor cells were intravascular.

Table 4. Summary of vascularization and benzidine-staining in allantoises grown in isolation

| | | | | Site of removal of allantois | | | |
| | | | | EPC region | | Yolk sac blood islands | |
Expt. set	No. expts.	Genotype	Stage	Vascularization	RBCs*	Vascularization	RBCs*
A	7	+/+	HF	33/33	1/33 (6)	27/27	0/27
	4	lacZ/+	HF	12/12	0/12	14/14	0/14
Totals A	7	+/+, lacZ/+	HF	45/45	1/35	41/41	0/41
B‡	5	+/+	HF	14/14	1/25 (20§)	12/12	2/16 (5, 61)
	4	lacZ/+	HF	5/5	2/15 (38; 268§)	5/5	0/14
Totals B	5	+/+, +/lacZ	HF	19/19	3/40	17/17	2/30
Totals, A & B (%)	12	+/+ and lacZ/+	HF	64/64 (100%)	4/85 (4.7%)	58/58 (100%)	2/71 (2.8%)
C	5	+/+ and lacZ/+	LB	10/10 (100%)	1/10 (10) (10.0%)	9/9 (100%)	0/9 (0)

Allantoises were removed from either wild-type (+/+) or lacZ-expressing (lacZ/+) conceptuses and placed directly onto tissue culture plastic (experimental sets A and C) or in suspension (experimental set B). Following culture, allantoises in A and C were stained in benzidine and examined for both vascularization and the presence of benzidine-positive cells. Allantoises in B were either squashed to discover benzidine-positive cells or prepared for histology so that both vascularization and benzidine-staining could be observed (Materials and Methods). For each experiment, allantoises were removed via the region previously occupied by the ectoplacental cone (EPC) and chorion or through yolk sac blood islands. HF, headfold-stage; LB, neural plate/late allantoic bud stage.

*Parentheses for experimental sets A-C contain the number of benzidine-positive cells (RBCs, red blood cells) observed in each positive allantois.

‡In Experimental Set B, the average (±s.d.) and median diameter of 25 explants was 333.5±46.2 μm.

§The benzidine-positive cells in these two allantoises were examined by light microscopy following histological preparation and were all found to be nucleated.

Results of Student's t-tests for benzidine-positive cells in sets A and B: (1) plated allantoises (A) versus allantoises cultured in suspension (B): $P=0.12$; (2) allantoises removed via ectoplacental cone versus removal through the yolk sac blood islands: $P=0.41$; (3) allantoises of genotype (lacZ/+) versus wild-type allantoises: $P=0.21$.

10 hours before the 1- to 2-somite stage when yolk sac vascularization is thought to get underway (Kaufman, 1992). Such early allantoises also invariably underwent vascularization in culture (Table 4C).

Finally, because it appears less differentiated than the remainder as judged by morphology and molecular differentiation (Figs 2C,D, 3A,B) and by tests of developmental potency (Downs and Harmann, 1997), the basal third of some allantoises isolated at the headfold stage was cultured separately from the distal two-thirds. In each of three experiments in which basal and distal regions of either three or four allantoises were cultured, both formed a similar vascular network to whole allantoises that were cultured simultaneously (data not shown).

Erythroid cells in allantoises cultured in isolation

The foregoing results support the conclusion that the allantois becomes vascularized by vasculogenesis rather than through extension of endothelium from the yolk sac or fetus. Benzidine-positive cells were detectable in the allantois as early as 6-somite pairs. However, because as many as half of erythroid cells might go undetected by benzidine staining prior to 6-somite pairs (see Fig. 1A), erythroid precursor cells may be present in the allantois even earlier. In order that allantoic erythroid precursors would have time to mature into globin-positive cells that would then be detectable with benzidine, a time-course study was carried out.

Overall, fewer than 5% of cultured headfold-stage allantoises contained any benzidine-positive cells (Table 4). Furthermore, the proportion of positive specimens did not differ significantly according to the route of their removal, whether they were of donor or host genotype, or whether they were cultured plated or in suspension (see footnotes to Table 4). 37 of the headfold-stage allantoises that were cultured in

suspension were examined histologically and found to be well-vascularized; amongst two benzidine-positive allantoises, all of the benzidine-positive cells were contained within vascular channels and were nucleated (legend to Table 4B, and data not shown), and were therefore not of maternal origin.

Amongst allantoises removed between the neural plate/late bud and 4-somite-pair stages, a small percentage (2.6-9.1%) contained benzidine-stained cells following culture (Fig. 7). Amongst those removed between 5- and 7-somite pairs, 25-36.8% of allantoises contained benzidine-stained cells; this developmental period coincided with when the allantoic vasculature appeared to be amalgamating with that of the yolk sac and fetus (Fig. 2) and a circulation was visible in a small percentage of conceptuses (Fig. 5A,B). Over 80% of allantoises removed at or beyond 8-somite pairs exhibited benzidine-positive cells after culture and corresponded with the time when some conceptuses exhibited a vigorous circulation (Fig. 5A,B) and erythroid cells were evident in the base of the allantois by morphology (Fig. 2J). Of 25 benzidine-positive allantoises that had been cultured at different developmental stages and examined by histology, all were well-vascularized and all of the benzidine-stained cells contained within the vasculature were nucleated, again establishing that they were not of maternal origin (data not shown).

Allantoic grafting

To determine whether erythroid cells uncovered within the allantois originated there, entire headfold-stage allantoises from donor conceptuses labeled with [3H]methyl thymidine were introduced into the exocoelom of similarly staged host conceptuses from which the resident allantois had been removed.

Histological examination after culture of grafted hosts revealed that all the allantoic grafts became well-vascularized
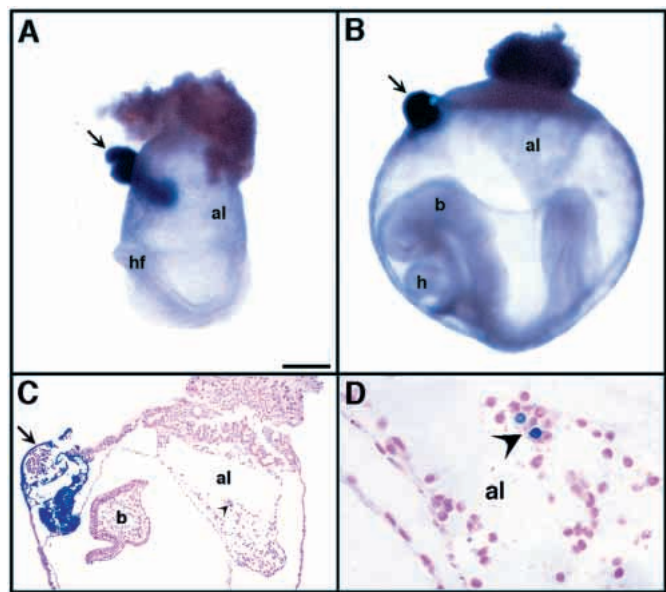
Fig. 4. Yolk sac transplantation. (A) Anterior placement of donor headfold-stage yolk sac (arrow) into the blood islands of a headfold-stage host conceptus. (B) Location of the donor-derived graft (arrow) in this 10-somite pair grafted host conceptus following 21 hours in culture. (C) Micrograph of an anteriorly grafted host conceptus containing ten somite pairs. Arrow, donor graft; arrowhead, two donor-derived cell fragments in the host allantois. (D) Higher magnification of donor-derived cells (arrowhead) from (C). Scale bar in D: 300 μm (A); 400 μm (B); 190 μm (C); 15 μm (D).
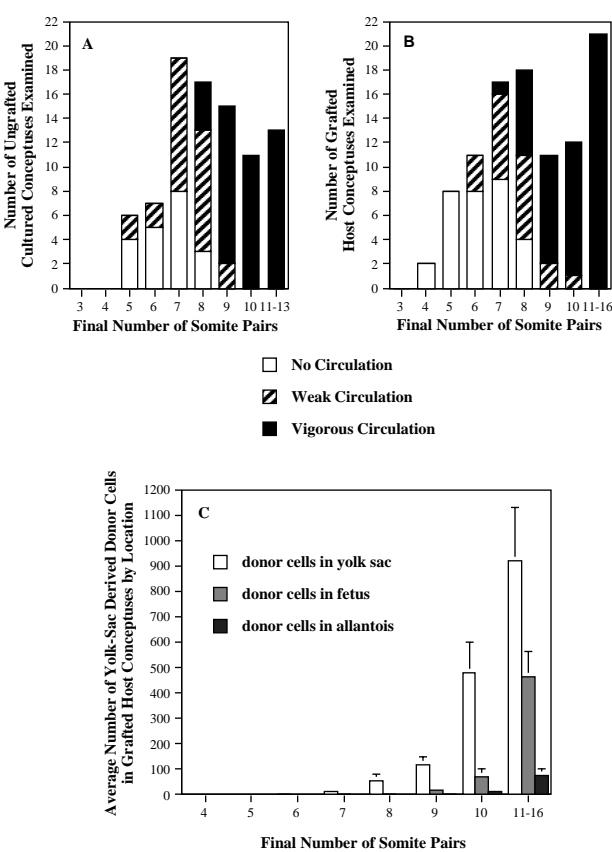

Fig. 5. (A) Timing of onset of yolk sac circulation in cultured and grafted conceptuses. Stacked column graph indicates the timing of appearance of the yolk sac circulation in (A) unoperated cultured conceptuses and (B) grafted conceptuses as a function of final numbers of somite pairs. (C) Average number of dispersed yolk sac-derived cells in grafted host conceptuses of B. Bar graph indicates the average number of donor-derived yolk sac cells in host yolk sacs, fetuses and allantoises as a function of the final number of somite pairs of grafted host conceptuses. The incidence of negative sites was included in the calculations. Error bars, standard error of the mean.

(Fig. 8) and could be assigned to one of five categories according to their relationship with exocoelomic tissues of the host (Table 5A). Unlike grafts of distal halves, which rarely attached to the host allantoic stump (Downs and Gardner, 1995), whole donor allantoises most often attached during culture with the remnant host allantois, thereby forming a chimeric allantois in which the donor portion fused with the chorion (Table 5A; Fig. 8A). In only two of the categories were benzidine-stained cells found in the grafts (Table 5A; Fig. 8B,C). The most common category was where the graft had attached to the remnant of the allantois as well as to the chorion of the host. Donor and host allantoic cells were often well-mixed at the site of union (16/32, Table 5A; Fig. 8B). In the second category, the graft had attached to the yolk sac of the host (Table 5A; Fig. 8C). An average of 92.8 and 136.2 doubly labeled benzidine/X-gal-positive cell fragments per chimera were found within the vascular channels of donor grafts from categories 1 and 2, respectively (Table 5A; Fig. 8B). In both of these categories of grafts, other round cells were observed within the donor vasculature. These were assumed to be erythroid precursors which had yet to synthesize hemoglobin (examples in Figs 8C, 9B-D).

In contrast with the foregoing two categories of grafts, which we considered 'productive' because the presence of erythroid cells suggested that the graft had established vascular continuity with the host, benzidine-positive or precursor erythroid cells were not observed in the three other categories. These were therefore considered 'unproductive', as the absence of hematopoietic cells suggested a lack of vascular continuity with the host (Table 5A; Fig. 8D-F). In the first of

these, the donor allantois had attached to both the host allantois and chorion (6/32, Table 5A), but a sharp mesothelial border was observed over much or all of the site of attachment (Fig. 8D), suggesting limited or no cellular passage between donor and host allantoises. In the last two categories, the donor allantois had either fused with the chorion alone (3/32, Table 5A; Fig. 8E) or had remained free-floating in the exocoelom (3/32, Table 5A; Fig. 8F).

To check that the doubly stained X-gal- and benzidine-positive cells in the grafts were of donor origin, grain counts were made on the sections. We first established that the majority of allantoic cells exhibited heavy labeling in uptake controls for [^{3}H]methyl thymidine; amongst six conceptuses scored in three experiments, 91.9% of cells were labeled (Fig. 9A; the limit of the base of the allantois was as previously described (Downs and Harmann, 1997)). We then calculated the average number of silver grains in benzidine-positive cell fragments in cultured donor and host conceptuses (Fig. 9B-D; Table 5B). In contrast with labeled donor controls, host benzidine-positive cell fragments displayed between 27 and 34
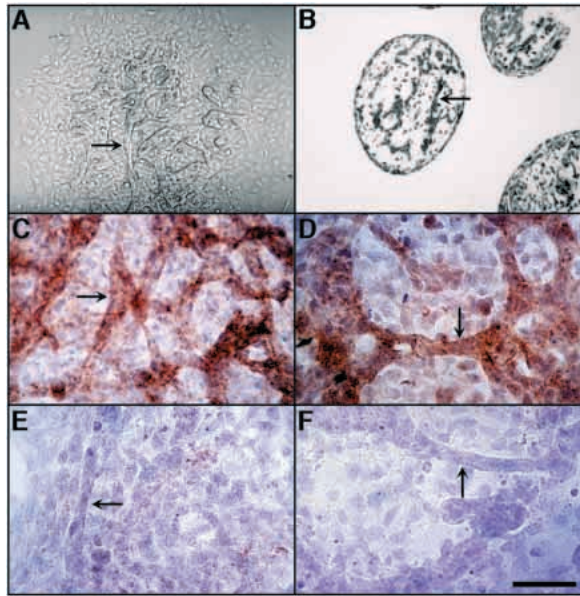
Fig. 6. Allantoises cultured in isolation and immunostained for Flk-1. (A) Headfold-stage allantois plated onto tissue culture plastic and cultured for 24 hours. (B) Example of section through allantoises cultured in suspension for the same time period. (C-F) Flk-1 immunohistochemistry on plated allantoises. Immunohistochemical analyses of allantoic cultures with primary antibody concentrations of either 1 µg/ml (C) or 0.2 µg/ml (D) show *flk-1* expression specific to the vascular channels. This staining is not seen when blocked by a Flk-1 control peptide against 0.2 µg/ml antibody to Flk-1 (E) or if no antibody is applied (F). Arrows in all panels indicate sites of putative vascularization. Scale Bar in F: 100 µm (A); 84 µm (B); 72 µm (C-F).

Fig. 7. Percentage of allantoises containing benzidine-positive cells after culture in suspension. Whole allantoises were examined following culture for the presence of benzidine-stained cells. The percentage of allantoises containing benzidine-positive cells is indicated as a function of the developmental stage when allantoises were removed from conceptuses; ordinal numbers above each vertical bar are the numbers of allantoises examined. Abbreviations on abscissa: LB, neural plate/late bud stage; HF, headfold stages; 1-11, 1- to 11-somite pairs.

times fewer grains (Table 5B; Fig. 9B-D). We interpreted this level as equivalent to background. Over all three experiments, the average number of silver grains per benzidine-positive cell fragment for all donor grafts was no higher than 0.25 (Table 5B; Fig. 8B, insert), similar in two experiments to background levels. In experiment 2, the number of silver grains in grafts was significantly lower than in the controls (Table 5B) but is likely attributable to having counted many fewer donor- than host-derived benzidine-positive cells.

DISCUSSION

Vascularization in the murine allantois occurs by vasculogenesis that is not accompanied by erythropoiesis

It has long been known that a major function of the murine allantois is to lay down the umbilical vasculature critical for fetal survival and development. What is not known is how the allantois accomplishes this. Results described here demonstrate that the murine allantois vascularizes intrinsically rather than by angiogenesis involving the yolk sac or fetus and that allantoic vasculogenesis is not accompanied by erythropoiesis.

A vasculogenic mechanism for the initial formation of blood vessels in the allantois is supported by several observations. The first is that the morphological sequence of de novo vascularization began in the region of the allantois most remote
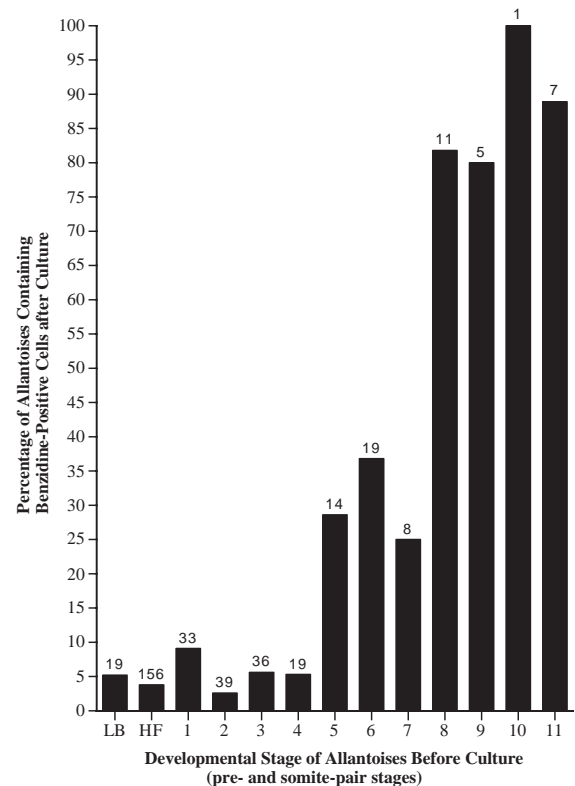
from the fetus and yolk sac. Round core cells in the distal mesoderm of the allantoic rudiment began to flatten at the late neural plate stage (approximately 7.75 dpc; Figs 2, 3) and then coalesced to form delicate blood vessels obvious by 2-somite pairs. This sequence was reiterated in the allantoic midregion before spreading to the base so that a small blood vessel was invariably present at the junction of the allantois with the yolk sac by 4-somite pairs. The vasculature of the yolk sac and fetus both originated at sites that are remote from the allantois, namely the blood islands and the cardiac region, respectively (data not shown; Kaufman, 1992), before converging on the basal allantoic blood vessels at 6-somite pairs (approximately 8.5 dpc; Fig. 2H,I).

Expression of *flk-1*, a tyrosine kinase receptor for vascular endothelial growth factor (VEGF) and a marker of early endothelial cells (Terman et al., 1991, 1992; Matthews et al., 1991; Sarzani et al., 1992; Millauer et al., 1993; Yamaguchi et al., 1993; Shalaby et al., 1995) closely followed the morphological gradient of vascularization, being observed first in the distal region of the allantois (Fig. 3A,B; Table 1) and then spreading toward its base (Fig. 3C).

Grafting of yolk sac blood islands produced no evidence that the yolk sac contributes endothelial cells to the allantoic

Table 5A. Summary of allantoic grafting

Experiment Number	Number of grafted conceptuses	Mean number of final somite pairs (±s.d.)	Median number of final somite pairs	Numbers and categories of attachment of donor allantoic grafts to components of the exocoelom (total number of benzidine-positive cell fragments of apparent donor origin)				
				Productive		Unproductive		
				1 ch+al	2 ys	3 ch+al	4 ch	5 free-floating
1	8	15.0 (±1.1)	15.0	4 (324)	2 (174)	1 (0)	0	1 (0)
2	12	13.3 (±1.5)	13.0	6 (510)	2 (371)	3 (0)	0	1 (0)
3	12	12.8 (±1.8)	14.0	6 (651)	0	2 (0)	3 (0)	1 (0)
1-3	32	13.5 (±1.7)	14.0	16 (1485)	4 (545)	6 (0)	3 (0)	3 (0)

Headfold-stage donor (*lacZ/+*) allantoises that were also labeled with [³H]methyl thymidine were grafted into host conceptuses which were then cultured and scored (see Materials and Methods). 'Productive' grafts were those in which benzidine-positive cells were found in the donor graft: (1) Donor allantois attached to the host chorion and allantois (ch+al), 1/16 donor allantoises also showed evidence of attachment to the host amnion; and (2) donor graft attached to yolk sac (ys); 1/4 donor allantoises associated with host yolk sac alone, 1/4 grafts was associated with the host allantois and 2/4 grafts were associated with both the host chorion and the allantois. 'Unproductive' grafts were those in which no benzidine-positive cells were found in the donor grafts; (3) donor allantois attached to the host chorion and allantois (ch+al); (4) donor allantois fused with the chorion alone (ch); and (5) donor allantois free-floating and not attached to any component of the exocoelom. The total number of headfold-stage conceptuses fixed immediately after labeling in tritium and examined histologically was thirteen; the total numbers of unoperated cultured host conceptuses was seventeen and unoperated labeled donor conceptuses was twelve.

Table 5B. Average number of silver grains per benzidine-positive cell fragment in allantoic grafting experiments

Experiment number	Number of donor and grafted conceptuses	Average number of silver grains per benzidine-positive cell fragment (±s.d.) (total number benzidine-positive cell fragments counted per category)			Total number benzidine/ X-gal positive cell fragments (range)
		1. Cultured donor conceptuses	2. Cultured host conceptuses	3. Grafted donor allantoises	Grafted donor allantoises
1	2, 6	9.4 (±1.9) (112)	0.28 (±0.09) (1155)	0.25 (±0.09) (498)	498 (6-239)
2	4, 8	8.4 (±1.4) (411)	0.30 (±0.07) (2266)	0.19 (±0.09) (881)	881 (13-358)
3	4, 6	9.0 (±2.9) (338)	0.34 (±0.13) (1439)	0.19 (±0.11) (651)	651 (1-295)

Three allantoic grafting experiments were scored for the average number of silver grains per benzidine-positive cell fragment in cultured (1) donor conceptuses, (2) host conceptuses and (3) donor allantoises of grafted hosts after autoradiography (Materials and Methods). At least 100 cell fragments per unoperated donor conceptus (six sections over three slides per donor) were scored. In category (3), grain counts were made over benzidine-positive cells of apparent donor origin in the grafted allantois. No significant differences were found between benzidine-positive cells located in the yolk sac, allantois or fetus of either host or donor conceptuses. The 20 embryos comprising the productive attachment categories in Table 5A are reported here. Grain numbers in benzidine-positive cells in both cultured host conceptuses and within the grafted donor cells were no higher than and ranged from 0-3 grains/benzidine-positive cell, whereas in the cultured donor conceptuses, the numbers of silver grains within benzidine-positive cells ranged from 0-30. Although all productive grafts contained round cells of apparent donor origin within the donor vasculature, none of these exhibited silver grains above background. Further, no silver grains above background were found in any host cells contained outside the graft. The P-value for comparisons between unoperated cultured donor-derived cells with hosts or grafted allantoic cells was <0.0000 for each of the three experiments whereas comparisons between the ratios of silver grains/benzidine-Xgal-positive cells of host conceptuses with donor grafted cells were generally not significantly different within each of the three experiments ($P=0.68$, Expt.1; $P=0.01$, Expt. 2; $P=0.05$, Expt. 3) but see Results for explanation.

vasculature. Endothelial cells from donor grafts were always found in the immediate vicinity of the blood islands but never near the base of the allantois. Moreover, all allantoises became vascularized following isolation at the late neural plate and headfold stages (Table 4; Fig. 6), considerably earlier than the one-to-two somite stage when the yolk sac is thought to vascularize (Kaufman, 1992), and their nascent vascular channels expressed *flk-1* (Fig. 6C,D). The basal allantoic subregion, which appeared relatively undifferentiated in potency tests (Downs and Harmann, 1997) and by morphology and molecular criteria (Figs 2A-D, 3A,B), also vascularized (described in Results).

We also obtained evidence that, despite containing

benzidine-positive cells relatively early in its development, the murine allantois is not intrinsically erythropoietic. Small numbers of benzidine-positive cells were found in allantoises in situ starting at 6-somite pairs, which corresponded with when the major vasculatures united (Fig. 2H,I) and when some conceptuses exhibited a pulsatile circulation (Fig. 5A,B). Thereafter, the number of benzidine-positive cells in both in situ and explanted allantoises correlated with the proportion of conceptuses with a pulsatile circulation (Figs 1B, 5A,B, 7).

Hence, the benzidine-stained cells in the allantois likely came from the yolk sac or fetus. This conclusion is supported by the further finding that integrated grafts of yolk sac blood islands contributed hematopoietic cells to allantoises beginning

at 10-somite pairs (Fig. 5C). That they did not do so earlier could be because they constituted too small a fraction of the total mass of blood islands for their red blood cells to feature among the small number colonizing allantoises prior to 10-somite pairs (Fig. 1B). Another possibility that cannot be discounted at present is that some allantoic erythroid cells came from the fetus. For example, the floor of the dorsal aorta in the chick reportedly produces hematopoietic cells (Pardanaud et al., 1996), and the AGM region of the mouse fetus does so as early as 10-somite pairs (Godin et al., 1993, 1995) and robustly by 10 dpc (approximately 31- to 40-somite pairs, Medvinsky et al., 1993; Medvinsky and Dzierzak, 1996). Perhaps a small number of aorta-derived blood cells gained entry to the allantois when it attained vascular union with the fetus (Fig. 2H,I).

Curiously, there was a small number of allantoises isolated prior to 6-somite pairs that yielded erythroid cells after culture (<10%; Table 4; Fig. 7). These benzidine cells were nucleated and therefore not of maternal origin. Furthermore, since their incidence was not significantly higher in allantoises removed via the yolk sac rather than the chorionic route (Table 4), their presence is unlikely to be due to contamination from blood islands. Cultured allantoises removed at 5-somite pairs that developed erythroid cells may have been infiltrated with hematopoietic cells from the yolk sac or fetus as a result of precocious union of vasculatures (Fig. 2G-I). The proportion of allantoises isolated before this that yielded benzidine-positive cells following culture was consistently low (Fig. 7). That there were any positive cases among such early isolates may reflect the proximity of the allantoic rudiment to the nascent yolk sac blood islands (Fig. 2A-C). One possibility that this offers is exchange of cells between the blood islands and the allantois, which would cease once the blood islands became more defined and spatially remote from the base of the allantois (Fig. 2C,D,G). Another is that the yolk sac endoderm may be close enough to induce occasional allantoic mesodermal cells to become erythropoietic. It is not clear, however, whether endoderm induces associated mesoderm to form erythroid cells (Miura and Wilt, 1969, 1970; Pardanaud et al., 1989) and, if so, whether allantoic mesoderm is competent to respond.

Yet further support for an extrinsic origin of allantoic erythroid cells was obtained from grafts of headfold stage allantoises, which failed to reveal benzidine-positive or erythroid precursor cells of donor origin, either within or outside of the grafts (Table 5B; Fig. 8). Moreover, hematopoietic cells were altogether absent from those grafted allantoises that had little-to-no detectable access to the vitelline or fetal circulations (Table 5A), i.e., grafts that were free-floating in the exocoelom (Fig. 8F), fused only with the chorion (Fig. 8E) or in which a thin cellular barrier occluded the site of union between donor and host allantoises (Fig. 8D). In this last category, reformation of the mesothelium may have taken place on the remnant allantois prior to attachment with the donor allantois or, alternatively, the donor allantois may have been positioned such that the distal mesothelium fused with the basal remnant of the host allantois. Such non-integrated grafts resembled the great majority of headfold stage allantoises cultured in isolation in containing delicate blood vessels devoid of hematopoietic cells (Table 4; Figs 6B, 7).

The results of preliminary experiments using only *lacZ* as a marker of donor cells in allantoic grafting experiments suggested that the allantois may be intrinsically erythropoietic because some of the benzidine-positive cells in the grafts appeared blue (data not shown; Fig. 8B,C). However, where donor cells were labeled additionally with tritiated thymidine, none of the blue benzidine-positive cells exhibited silver grains above background (Table 5; Fig. 8). One explanation for this disparity is that the boundary between host erythroid and *lacZ*/+ donor endothelial cells is not adequately resolved in conventional histological sections. Another is that if cell death occurs during allantoic morphogenesis, released β-galactosidase might have been taken up by surrounding erythroid cells via fluid phase endocytosis or a receptor-mediated process. For example, certain lysosomal enzymes such as β-glucuronidase are secreted from various types of cell into interstitial fluid which, provided that the enzyme is glycosylated with mannose-6-phosphate, can be picked up by other cells via a mannose-6-phosphate receptor (Feder, 1976; Stahl et al., 1976a,b, 1978; Schlesinger et al., 1978; reviewed by Hille-Rehfeld, 1995). Lastly, during regression and remodeling, dead β-galactosidase-containing cells may have accumulated within extracellular allantoic spaces, including blood vessel lumena, then becoming associated with host erythroid cells. The extracellular spaces of allantoises did clearly contain X-gal-stained material (Figs 8C, 9C). Conventional staining with hematoxylin and eosin of both freshly recovered and cultured conceptuses demonstrated this material to be highly eosinophilic and associated with mixed-sized hematoxylin-stained granules (data not shown), diagnostic of dying cells (Coucouvanis and Martin, 1995). This lends credence to the notion that some host erythroid cells appeared blue in donor grafts through intimate association with the cytoplasm of endothelial cells during vascular remodeling, an idea supported by previous results in the rabbit corpus luteum and chick limb bud (Latker, 1980; Latker et al., 1986).

Distal-to-proximal sequence of allantoic vasculogenesis

The observed spatiotemporal sequence in morphological and molecular differentiation in the allantois accord with the results of earlier heterotopic transplantations at headfold stages (Downs and Harmann, 1997), which revealed that distal cells were more restricted in potency than basal ones. Our present findings show that such regional differentiation is established even earlier than previously reported, being already evident at the late neural plate stage.

The heterotopic grafting experiments suggested that the distal and midallantoic regions were also not developmentally equivalent. Although cells from both regions colonized the large fetal vasculature, distal cells did so much less readily, often being extruded from the fetus. This suggested that the distal allantoic region either contained a lower proportion of endothelial cells than the midregion or that some distal cells expressed additional proteins that impaired their integration.

Quantification of Flk-1 along the distoproximal axis of headfold-stage allantoises revealed that, although the distal region contained fewer cells than the midregion, its percentage of *flk-1*-expressing cells was invariably somewhat higher (Table 1). Differences in the frequency of heterotopic integration of distal versus midallantoic grafts may therefore relate to differentiation of mesothelium rather than to initiation of endothelium formation via *flk-1* expression. Formation of

Fig. 8. Attachment categories of allantoic grafts.
(A) 12-somite-stage operated conceptus that had
undergone allantoic grafting at the headfold stage prior
to culture and attached to both the host allantoic
remnant and chorion. Due to the intensity of staining
in nuclear fast red in this early specimen, benzidine-
positive cells, if present, were not visible in the donor
allantois. (B) Attachment of the donor allantois to the
host chorion and remnant allantois. Insert: High
magnification of benzidine-positive cells from another
example of category 1 chimera, Table 5A. Open
arrows point to red blood cells that appear to contain
X-gal precipitate. (C) Attachment of the donor
allantois to the host chorion, the remnant allantois and
the yolk sac. Open arrows as in B; closed arrowhead
points to non-benzidine-positive hematopoietic cell
contained within a vascular channel at the point of
union between the donor allantois and the host yolk
sac. Curved arrow points to extracellular X-gal-stained
material. (D) Attachment of the donor allantois to the
host chorion and the remnant allantois; the solid arrow
points to a fairly extensive barrier at the point of
attachment between the donor and host allantoises,
whereas the open arrowhead indicates a small region
of presumed free cellular passage between donor and
host allantoises. (E) Fusion of the donor allantois with
the host chorion alone. (F) Free-floating donor
allantois. Abbreviations: b, brain; ch, chorion; dal,
donor allantois; hal, host allantois; ys, yolk sac. Scale
bar in F: 260 µm (A); 80 µm (B), 19 µm (insert in B);
70 µm (C); 104 µm (D,E); 96 µm (F).
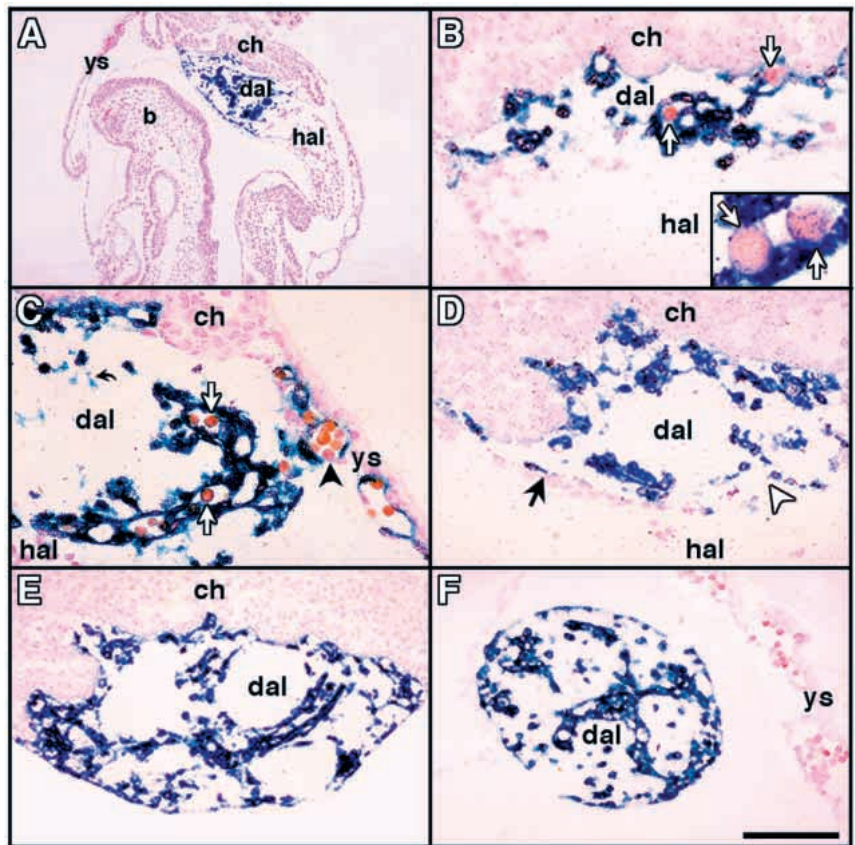
mesothelium begins distally (Fig. 2A-C) and may be associated
with changes in adhesive properties ultimately required for
chorioallantoic fusion.

Does the mesothelium play a role in allantoic vasculogenesis?

It has been proposed that endoderm provides stimuli required
to induce adjacent mesoderm to differentiate into the
endothelial cell lineage (Wilt, 1965; Pardanaud et al., 1989;
reviewed in Risau and Flamme, 1995). How then to explain
our conclusion that the murine allantois, which does not
contain endoderm, undergoes vasculogenesis?

It is unlikely that the mechanism of allantoic vasculogenesis
is unique given that the murine allantois expresses genes
known to be required for yolk sac vasculogenesis (Akhurst et
al., 1990; Dickson et al., 1995; Yamaguchi et al., 1993;
Dumont et al., 1995). An intriguing possibility is that
mesothelium serves as the allantoic equivalent of yolk sac
endoderm, providing inducing factors necessary to convert
core allantoic mesoderm into a vascular plexus.

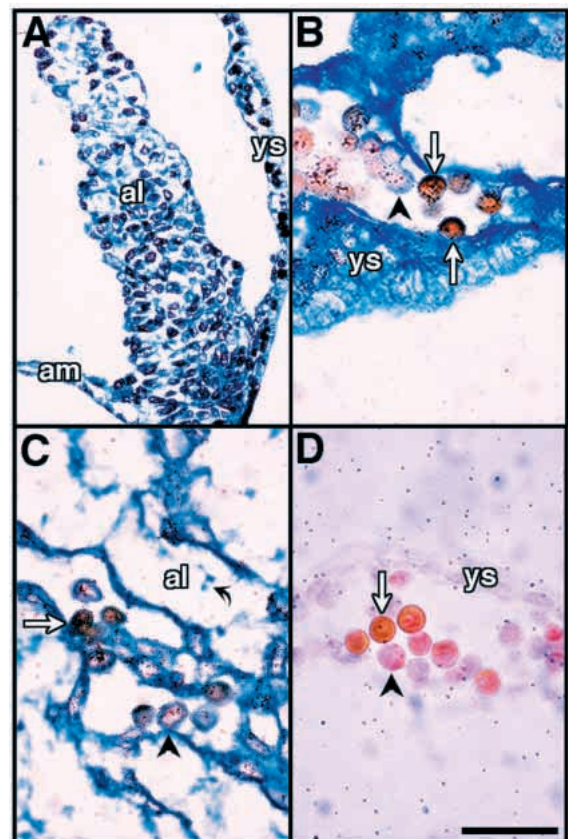Little is known about allantoic mesothelium other than its

Fig. 9. Controls for allantoic grafting. Photomicrographs showing
silver grains in: (A) the allantois of an uptake control; (B) benzidine-
positive cells (open arrows) and non-benzidine-positive
hematopoietic cells (closed arrowheads) in the yolk sac of a cultured
donor conceptus; (C) the allantoic vasculature of a donor conceptus
(arrows and arrowheads same as in (B); and (D) the yolk sac of an
operated host conceptus. Curved arrow in C points to extracellular X-
gal. Abbreviations as in Figs 2, 8. Scale bar in D: 60 µm (A); 29 µm
(B-D).

role in initiating attachment of the allantois to the chorion during the first steps of placental ontogeny (Ellington, 1985; Downs and Gardner, 1995). Chorioallantoic attachment is dictated by the developmental state of the allantois and may be mediated by an internal timing mechanism (Downs and Gardner, 1995; reviewed in Downs, 1998), possibly linked in the allantois to expression of *VCAM1*, whose protein product binds to the α4-integrin receptor, localized to chorionic mesothelium (Kwee et al., 1995; Gurtner et al., 1995; Yang et al., 1995).

We have found that mesothelium first forms in the distalmost region of the allantois from where it then spreads basally. Its formation appears to coincide with, or possibly precede, the distal-to-proximal spread of morphological vascularization within core mesoderm. The mesothelium finally envelops the base of the allantois at 4-somite pairs, which is also when blood vessels are first recognizable there. Intriguingly, our data show that Flk-1 is localized only to core allantoic mesoderm and never to the mesothelium, whereas Dumont et al. (1995) reported expression of VEGF in the mesothelium. Thus, induction of mesoderm may occur via a paracrine mechanism that involves VEGF and its receptor, Flk-1, localized respectively to yolk sac endoderm and mesoderm (Dumont et al., 1995; reviewed in Klagsbrun and D'Amore, 1996). Together, these observations suggest a new function for allantoic mesothelium in development, namely that it may induce vasculogenesis within core mesoderm.

CONCLUDING REMARKS

Two major facets of development of the murine allantois that are essential for its role in mediating exchange between the placenta and the fetus are fusion with the chorion (Downs and Gardner, 1995) and differentiation of a vasculature. According to the present finding, allantoic vascularization occurs via vasculogenesis rather than angiogenesis. Unlike the chick (Caprioli et al., 1998), vasculogenesis in the mouse allantois is not associated with erythropoiesis. In accordance with previous suggestions, *flk-1* is probably involved in the early signaling events of vasculogenesis (Shalaby et al., 1995). However, in the case of the murine allantois, the presence of Flk-1 is evidently not identifying a population of bipotential hemangioblasts destined to establish both endothelial and hematopoietic cell lineages (Sabin, 1920; Shalaby et al., 1997) because, if it were, all allantoises cultured in isolation would have been expected to be benzidine-positive (Figs 3, 7; Table 3). The overwhelming majority of allantoises removed between the neural plate and 4-somite-pair stages and then cultured in isolation were devoid of benzidine-positive cells. Lastly, our observation that the allantois vascularizes with similar kinetics in cultured conceptuses as in those freshly recovered from the uterus and that it also does so rapidly following isolation suggests that the allantois may be a valuable system for elucidating the early steps of vasculogenesis.

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