Developmental potency of the murine allantois

Karen M. Downs* and Christine Harmann

Department of Anatomy, 1300 University Avenue, University of Wisconsin-Madison Medical School, Madison, Wisconsin 53706, USA

*Author for correspondence (e-mail: kdowns@facstaff.wisc.edu)

SUMMARY

The murine allantois is the future umbilical component of the placenta. The base of the allantois is also thought to contain the future germ line. We have examined the fate and developmental potency of cells within the murine allantois during gastrulation.

lacZ-expressing headfold-stage allantoises (approximately 8.0 days postcoitum; dpc) were subdivided into three proximodistal regions and transplanted into three sites in synchronous non-transgenic host embryos: the primitive streak at the level of prospective paraxial mesoderm, the primitive streak at the level of lateral plate mesoderm, and the base of the allantois. After 23 hours in culture, operated conceptuses were examined histologically for contribution of donor allantoic cells to the conceptus. None of the allantoic regions contributed to paraxial mesoderm when placed into the fetus, but all three colonized the endothelium and adjacent mesenchyme of the dorsal aorta. The mid-region was most efficient at colonizing endothelium, whereas the base was the only allantoic region to exhibit relative pluripotency, colonizing several derivatives of all three primary germ layers.

Differences in the state of differentiation along the proximodistal axis of the allantois were further borne out when the three allantoic regions were placed into the base of the allantois of host conceptuses. Striking differences were observed in final position along the proximodistal axis of the host allantois. Most grafted cells translocated distally from the base; however, basal donor allantoic cells translocated typically only as far as the host’s mid-region, whereas donor allantoic tip cells typically returned to the tip, often colonizing the choioallantoic fusion junction. Together, our data reveal that the headfold-stage allantois may contain a proximodistal gradient of differentiation, and raise intriguing questions about how this gradient was established and the role it plays in umbilical vasculogenesis.

Key words: allantois, mouse, lateral plate mesoderm, paraxial mesoderm, potency, extraembryonic mesoderm, primordial germ cells, angioblasts, vasculogenesis, erythropoiesis

INTRODUCTION

Extraembryonic mesoderm is the first mesoderm formed during murine gastrulation. Epiblast cells from the proximal embryonic region of the egg cylinder translocate into the nascent posterior primitive streak and emerge as a bulging mesodermal mass at the posterior embryonic-extraembryonic junction (Lawson et al., 1991). By the late streak stage (Downs and Davies, 1993), this mesodermal mass develops coalescing lacunae which ultimately form the exocoelomic cavity whose major components, the amnion, choioallantoic placenta, and yolk sac, will support the fetus throughout the remainder of gestation.

The allantois develops from a small bud of extraembryonic mesoderm emanating from the posterior streak at the angle between the yolk sac and amnion in the early neural plate stage (approx. 7.5 days post coitum, dpc; Downs and Davies, 1993; reviewed by Downs, 1997). The allantoic bud projects into the exocoelomic cavity and enlarges by a combination of distal cavitation and mitosis (Ellington, 1985). During outgrowth, the allantois consists of an outer layer of mesothelium surrounding an inner core of mesoderm. Distal allantoic mesoderm is loosely scattered, while proximal allantoic mesoderm is densely packed and continuous with the posterior streak. The allantois eventually meets the chorion and attaches to it. This first step in placental morphogenesis is mediated by the mesothelial surfaces of the allantois and chorion (Ellington, 1985; Downs and Gardner, 1995). Fusion occurs shortly thereafter and the allantois becomes overtly vascularized, thereby providing the vital umbilical linkage between fetus and mother.

Because little is known about the extraembryonic mesoderm of the allantois, we initiated a study designed to address whether it is a unique mesoderm or whether it bears resemblance to embryonic mesoderm. The possibility that cells of the allantois might even be pluripotent, and thus able to contribute to other germ layers, was considered. The headfold-stage allantois was selected for this study, the rationale being that two of its regions appear to have different functions: the tip is involved in chorioallantoic fusion (Downs and Gardner, 1995) and the base may induce or protect presumptive primordial germ cells (PGCs) from differentiation (McLaren, 1983, 1992). To determine the developmental potential of the allantois, the allantois of lacZ-expressing transgenic mouse
embryos (Friedrich and Soriano, 1991; see Methods) was subdivided into three regions, tip, middle third, and base, at the headfold stage (approx. 8.0 dpc, Downs and Davies, 1993).

The three allantoic regions were transplanted individually into each of three sites in similarly-staged conceptuses: (1) the base of the allantois (called 'orthotopic' and 'heterotopic' grafts, Beddington, 1981, 1982), (2) the prospective lateral plate mesoderm of the fetus ('heterotopic' grafts), and (3) the prospective paraxial mesoderm of the fetus ('heterotopic' grafts). After 23 hours of whole embryo culture, operated conceptuses were analyzed for colonization patterns of the donor tissue in order to discover whether donor allantoic cells conformed to the normal fate of these regions, in which case they would be pluripotent, or whether they contributed to one or a small number of cell types, in which case they would be restricted, or possibly even determined for those lineages at the time of transplantation.

MATERIALS AND METHODS

Mouse strains

The lacZ transgene of ROSA26 (Friedrich and Soriano, 1991) was made hemizygous on the C57BL/6 (BL/6) genetic background by backcrossing ROSA26 lacZ/+ hemizygotes to BL/6 for 8 generations (generous gift from Dr K. Gould and Professor W. Dove, University of Wisconsin-Madison Medical School). Hemizygous lacZ+/+ BL/6 males and females were subsequently intercrossed to obtain homozygous lacZlacZ BL/6 females. Females were judged homozygous if they transmitted the transgene to all of at least 15 progeny. At n=15, the probability that at least one parental female is lacZ+/+ is equal to 0.003 (Professor Robert Wardrop, UW-Madison, Department of Statistics). Homozygous lacZlacZ BL/6 females were then crossed with male mice of the CBA strain (Jackson Laboratories). Hemizygous lacZ+/+ males and females (both now BL/6 × CBA) were subsequently intercrossed to obtain a homozygous breeding strain, designated ROSA26+ of genetic background (BL/6 × CBA). Donor embryos for all experiments were obtained by mating F1 hybrid females (C57BL/6 × CBA; Jackson Laboratories) with homozygous ROSA26+ lacZlacZ (C57BL/6 × CBA) males. Host embryos were non-transgenic F2s of hybrid F1 (C57BL/6 × CBA) matings.

Mice were kept in 12-hour light-reversed conditions (dark period: 13.00-1.00 hours). Pregnant females were killed by cervical dislocation without prior anesthesia, and the uterine horns dissected into phosphate-buffered saline (PBS; Sigma).

Removal and dissection of conceptuses

Host conceptuses were dissected at 10.00 am on the day of the experiment (approximately 7.75 days postcoitum; dpc). In most experiments, only neural plate/late allantoic bud and very early headfold-stage conceptuses were selected as hosts (Fig. 1A; Downs and Davies, 1993; Downs and Gardner, 1995) to ensure that by the time of transplantation several hours later, most embryos would be at the headfold stage. Conceptuses were paired, and the ectoplacental cone of one member of each pair was trimmed with scissors-motion of two 28 gauge syringe needles. Trimming the ectoplacental cone enabled the identification of each conceptus following transplantation and culture (Lawson et al., 1991). Pairs of host conceptuses were then placed in culture medium and maintained in the incubator (Downs and Gardner, 1995) until transplantation.

Culture of allantoises in isolation

Allantoises of lacZ+/+ hemizygous genotype were removed through two vitelline sites (Fig. 2): (1) through non-blood island tissue slightly distal to the level of the site of insertion of the allantois with the amnion, or (2) directly through visible yolk sac blood islands. Individual allantoises were then placed in 0.5 ml of culture medium (Downs and Gardner, 1995) and cultured in roller culture for 24 or 40-43 hours. After culture, each allantois was stained in benzidine for 30 minutes to detect hemoglobin-expressing cells (see Histochemical Analysis, below) and fixed briefly in 4% paraformaldehyde for 1 hour at 4°C before gently squashing each allantois on a microscope slide for visualization and counting of benzidine-positive cells.

Transplantation

The basal portion of the allantois was taken as the site of insertion of the amnion (Oźdzenski, 1967; Fig. 2). Headfold-stage donor allantoises were removed by capillary (inner diameter 90 μm) aspiration by piercing the yolk sac just distal to the site of insertion of the allantois with the amnion (Fig. 2). Allantoises in which basal cells were not sufficiently removed from attachment to the posterior streak after allantoic aspiration were discarded. Whole allantoises which exhibited distinct basal and distal regions were then rinsed in PBS, and placed individually in trypsin/pancreatin for 5 minutes on ice (Hogan et al., 1994), after which the tip, middle third, or base was excised using glass needles (Beddington, 1987; Fig. 2). Donor tissue from the embryonic regions included both epiblast and primitive streak but not visceral endoderm.

Transplantations were carried out either as a series of orthotopic grafts only (Table 1A), or as a series of heterotopic grafts (Table 1B) which also included a second set of control orthotopic grafts to the base of the allantois. The following host sites were targeted: (1) the base of the allantois, (2) the mid-primitive streak at the level of prospective lateral plate mesoderm, and (3) the more anterior primitive streak at the level of prospective paraxial mesoderm (Fig. 3). Transplantations of the mid- and distal allantoic regions to the base of the allantois were not strictly orthotopic, and therefore they were called ‘heterotopic’ grafts. The reason for grafting into the allantois was to control for contribution to the allantois in all experiments where allantoic tissue was placed into the fetus (Table 1, Experimental Set B). Every experiment included unoperated transgenic conceptuses and several unoperated transgenic conceptuses in order to compare the extent of development between donor and host embryos (see Morphology of Cultured Conceptuses, below), and to ensure appropriate benzidine and X-gal staining (see Histochemical Analysis).

At the time of transplantation, donor tissue was placed in a drop of dissection medium on a glass slide from which the single chamber was removed, leaving behind a silicon gasket (Nunc). The tissue was then cut further into clumps of approximately 10-30 cells. Every effort
Fig. 2. Schematic diagram of the allantois and delineation of the base at the level of insertion of the amnion. The site of insertion of the amnion (AM, thick arrow) delineated the base of the allantois from the subjacent primitive streak (PS). The solid diagonal line indicates the basalmost portion of the allantois taken for this study (Oźdzenski, 1967). The allantois was subdivided into three regions for transplantation (dotted lines): the base, the mid-region and the tip. The numbers 1 and 2 indicate yolk sac (YS) sites through which the allantois was removed for the experiments described in Table 2. Site 2 contains blood islands whereas site 1 is typically free of blood islands observable at the level of the light microscope. Site 1 was the principal site of yolk sac puncture through which the allantois was removed for all of the transplantation experiments described. The bold line surrounding the allantois and which is continuous with the yolk sac and amnion represents the mesodermal lining of the exocoelom.

was made to inject all fragments from the desired region; this was successful most of the time. Pairs of recipient conceptuses to be injected were placed in a second drop of dissection medium. Transplantations were carried out in a Nikon Diaphot inverted microscope with bright-field and DIC optics. Two Leitz micromanipulation arms were used to manipulate holding and microinjection pipettes. Holding pipettes (OD 1.00 mm, ID 0.75 mm) were hand-pulled and heat-polished (Beddington, 1987) on a de Fonbrune microforge to an inner diameter of 0.05 mm. Microinjection pipettes were pulled on an electrode puller (Sutter Instruments, Novato, CA), broken to an ID of 0.03 mm on the microforge, and heat-polished. Holding and microinjection pipettes were mounted on microelectrode holders (World Precision Instruments) and contained either 0.1 M KCl and silicon oil (holding pipettes) or dissection medium and silicon oil (microinjection pipettes). The electrode holders were connected to Hamilton syringes of 500 μl (holding pipettes) or 100 μl (microinjection pipettes).

All experimental embryos were photographed at the time of transplantation using a video monitor attached to a TV screen (Sony) and printer loaded with black-and-white heat-sensitive paper (Sony) in order to verify retrospectively initial embryonic stage and the site of injection.

Morphology of cultured conceptuses

Following culture, each conceptus was scored for the following morphological features (Brown, 1990): chorioallantoic fusion, yolk sac circulation, heart beat, turning, extent of development of the gut, brain, heart, and numbers of somite pairs (Fig. 1C). None of the conceptuses were grossly deficient in any of these features, but a few exhibited an obvious lack of growth and organization and were judged abnormal (0.65% total cultured conceptuses). The extent of development of unoperated hosts and transgenic conceptuses was also compared in order to verify that the donor and host conceptuses developed synchronously. In this analysis, a Student’s t-test revealed no significant differences between development of headfold-stage donor and host conceptuses (scoring based on Brown, 1990; n = 18 (lacZ+/−), n = 38 (+/++; P = 0.25, equal variances assumed, CI = 95.0%).

Histochemical analysis and scoring grafted cells

After culture, conceptuses and whole allantoises cultured in isolation were immediately stained in benzidine to identify host hemoglobin-synthesizing red blood cells: 20 μl 30% H2O2 (Sigma) was added to 1 ml benzidine stock solution (benzidine stock solution: 50 ml 0.5 M glacial acetic acid plus 100 mg benzidine; Sigma); 100 μl of the benzidine/H2O2 mixture was added to 400 μl PBS to produce a working solution. 100 μl of the working solution was added to 300 μl dissection medium which contained the conceptuses (Downs and Gardner, 1995) and this was kept at room temperature for 5-10 minutes, after which the yolk sac was pierced and the conceptuses stained for 10-20 minutes more. Conceptuses and allantoises were then rinsed twice in PBS and fixed for 2-2.5 hours at 4°C in 4% paraformaldehyde. All cells of ROSA26 lacZ/+ hemizygous donor headfold-stage conceptuses were blue after staining in X-gal, both before (Fig. 1B) and after culture (data not shown), with the exception of an occasional (<0.01%) benzidine-positive donor cell (see below). However, fixation periods of 5 hours or more compromised the ubiquity of X-gal staining in some cell lineages, particularly yolk sac endoderm, the chorion, and trophectoderm giant cells overlying the ectoplacental cone. No background staining for lacZ activity was found in non-transgenic wildtype hosts either before or after culture (not shown). Fixed conceptuses were rinsed 3 times in PBS (20 minutes each) and stained in X-gal (Sigma) at 37°C for at least 15 hours (Sanes et al., 1986). Postfixation was not necessary for the retention of X-gal. Conceptuses were examined for the presence of transgenic cells before processing for wax histology by conventional means.

Conceptuses were sectioned at 4 μm thickness (Reichert-Jung Autocut), and the sections dried at 42°C for at least 7 hours. Slides were dewaxed by conventional means, and counterstained in Nuclear Fast Red (Sigma; MacGregor et al., 1995) for 5 seconds to 1 minute before mounting in Permount. Nuclei of transgenic donor cells were counted in every section and locations noted. Operated conceptuses that contained ≥5 incorporated cells were scored as chimeric.

Only red blood cells stained pale yellow to deep orange if they contained hemoglobin (Fig. 4A). When co-stained with X-gal, the overwhelming majority of transgenic red blood cells contained significant amounts of X-gal precipitate; some red blood cells were speckled blue, and very infrequently (<0.01%), a hemoglobin-positive cell appeared negative (Fig. 4B). In the absence of benzidine, however, all transgenic red blood cells stained blue with X-gal (data not shown).

RESULTS

Transplantation was used to determine the developmental potency of the headfold-stage allantois. Donor allantoises of hemizygous lacZ/+ genotype were subdivided into three regions (Fig. 3): base, middle-third and tip. Each region was transplanted into one of three sites in each host conceptus: the base of the allantois, the primitive streak at the level of prospective lateral plate mesoderm (LPM) or the primitive streak at the level of prospective paraxial mesoderm (PM). These
Orthotopic grafts of the region of the primitive streak that gives rise to lateral plate mesoderm (Fig. 3B,D) resulted in colonization of a wide range of tissues (Fig. 6). Contribution to non-mesodermal tissues likely reflects the fact that the primitive streak was not separated from epiblast (Tam and Beddington, 1987), as it was not known whether the allantois would give rise to derivatives of all three primary germ layers rather than to mesoderm alone. Therefore, in the event that it did, we wished to see the full colonization profile in the controls for this prospective region.

Orthotopic transplantations of the region of the conceptus giving rise to paraxial mesoderm exhibited the lowest rate of chimerism (33.3%, Table 1A) of all orthotopic grafts. However, this number was not significantly different from the overall rate of chimerism for the other two sites. Because transplantations to this site contained a similar number of grafted cells as the other two transplantation sites, the smaller number of chimeras found at the level of prospective paraxial mesoderm is likely due to a technical limitation. Overlap between the holding and injection pipettes (Fig. 3E) was apparently significant enough to result in unstable placement of donor tissue and subsequent loss of the grafted cells to the amniotic cavity or culture medium (Table 1B; compare Fig. 3E with Fig. 3C,D). Nevertheless, we found that this staggered arrangement of micromanipulation pipettes was superior to a symmetric one for this region (data not shown). Colonization patterns in chimeric embryos included paraxial mesoderm, as previously described (Tam and Beddington, 1987; Fig. 7).

**Developmental potency of the allantois**

Transplantation of the allantois into the base of host allantoises

Given the array of potentially colonizable tissues at each of the three transplantation sites, a series of experiments designed to determine the developmental potential of the allantois was carried out (Table 1B). Orthotopic and heterotopic transplantations of the allantois into the base of host allantoises were used to verify accuracy of graft placement by demonstrating within each experiment that allantoic cells contributed to the allantois when placed there. In addition, they provided further data on the fate of the basal allantoic cells, and on the state of differentiation of the allantoic mid-region and tip (see below).

When each of the allantoic regions was placed into the base of the allantois of a host (Fig. 3C), grafted allantoic cells were well-incorporated in the allantois and were often distributed amongst two of the three allantoic regions delineated in this study: base, mid-region and distal third (Fig. 5). Grafted allantoic cells were typically well-spread across the width of the host allantois in sagittal sections (data not shown). Several representative examples of donor allantoic cell distribution in host conceptuses are shown in Fig. 8A,B.

Of potential significance was our finding that when placed into the base of the allantois, donor allantoic cells from all three allantoic regions were conspicuously absent in the fetus, either as integral tissue components or contained within the fetal vasculature. Rather, most donor allantoic grafts contributed cells only to the allantois or, in a minority of cases, to the yolk sac (Fig. 5; see below). Moreover, only 8.2% of all donor grafts contributed cells to the base of the allantois. This observation suggests that normal cell movement within the allantois is from proximal-to-distal. Of the chimeras which contained a few

---

**Orthotopic control transplantations**

To obtain a profile of the potentially colonizable tissues for each of the three transplantation sites, a set of five orthotopic experiments was carried out, using similar numbers of conceptuses (Table 1A). The number of chimeras obtained after grafts at the orthotopic embryonic sites was within the range of that previously reported (5-12 chimeras; Tam and Beddington, 1987). Embryos receiving grafts from the base of the allantois exhibited the highest rate of chimerism, whereas those receiving grafts at the level of paraxial mesoderm were least efficiently colonized.

Orthotopic transplantations of the base of the allantois were carried out to reveal the fate of cells in the base of the allantois. All orthotopically placed cells from the base of the allantois contributed exclusively to the allantois. None were found in the fetus (Fig. 5A).
Developmental potency of the murine allantois
donor cells in the base, colonization by these donor cells was found in the nascent allantoic vasculature (data not shown).

In four chimeras, some donor allantoic cells contributed to the endothelium of the immediately adjacent omphalomesenteric artery (Fig. 5A,B). Because the donor allantois was always at the headfold stage, it is unlikely that yolk sac cells had infiltrated the allantois at this early time and returned to the yolk sac after grafting (M. Blahnik, S. Gifford, and K. Downs, unpublished data). Rather, because the overwhelming majority of grafts contributed exclusively to the allantois (45/49), we believe that contribution to the yolk sac vasculature may have been due to contamination of the yolk sac by grafted cells when the injection pipette was withdrawn from the conceptus, or possibly by displacement of part of the graft to the yolk sac during subsequent culture. This interpretation is based on one instance where a small cluster of unincorporated cells (chimera number 3, Fig. 5B) was found adherent to the outside of the yolk sac at the level of the site of injection into the base of the allantois.

Striking differences were found in the pattern of colonization of the host allantois by each of the three donor allantoic regions (compare Figs 5A-C; summarized in Fig. 5D). In particular, although all three allantoic regions had been placed into the base of the allantois (Fig. 3C), cells derived from the donor mid-region and distal third made significant contributions to the chorioallantoic fusion junction of the host (Fig. 9), with cells from the tip contributing the highest number. In contrast, donor basal cells rarely translocated that far, typically ending up in the host allantoic mid-region where the y contributed predominantly to the nascent endothelial network (Figs 5A,D, 9).

In three chimeras, nine benzidine-positive cells of donor origin were identified amongst the benzidine-positive cells of the allantois (Figs 4C, 5A,B). This was tantalizing, because it suggested that the allantois, like the yolk sac, may be intrinsically both erythropoietic and vasculogenic. On the other hand, it could mean that a few donor allantoic cells had been contaminated by yolk sac blood islands or, less likely, induced to form red blood cells during removal through the erythropoietic yolk sac.

To distinguish between these possibilities, allantoises of lacZ+/ genotype were cultured in isolation for 24 and 40-43 hours (Table 2). Allantoises were removed through the yolk sac, either just above the level of the base of the allantois (Site 1, Fig. 2), as had been done for all the grafts reported here, or directly through more distally situated yolk sac blood islands (Site 2, Fig. 2).

In isolation, allantoises lost their rod-like shape and were round, suggesting that the unique projectile-like morphology of the allantois is a result of molding of the allantois by its

<table>
<thead>
<tr>
<th>Expt set</th>
<th>Site of injection</th>
<th>Transplanted tissue</th>
<th>No. expts</th>
<th>No. embs. injected</th>
<th>No. grafts (%)</th>
<th>No. grafts unincorp. (%)</th>
<th>No. chimeras (%)</th>
<th>Mean no. grafted cells ±s.e.m.</th>
<th>Type of tissue colonized</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>O-Base</td>
<td>Base of allantois</td>
<td>5</td>
<td>20</td>
<td>3 (15.0)</td>
<td>3 (15.0)</td>
<td>14 (70.0)</td>
<td>182.6±46.2 (115.5)</td>
<td>Allantois</td>
</tr>
<tr>
<td></td>
<td>O-LPM</td>
<td>Prospective LPM</td>
<td>5</td>
<td>19</td>
<td>4 (21.1)</td>
<td>5 (26.3)</td>
<td>10 (52.6)</td>
<td>192.5±68.7 (100.5)</td>
<td>Multiple</td>
</tr>
<tr>
<td></td>
<td>O-PM</td>
<td>Prospective PM</td>
<td>5</td>
<td>21</td>
<td>6 (28.6)</td>
<td>8 (38.1)</td>
<td>7 (33.3)</td>
<td>145.9±61.3 (73)</td>
<td>Multiple</td>
</tr>
<tr>
<td>B</td>
<td>Base of allantois</td>
<td></td>
<td>4</td>
<td>15</td>
<td>5* (33.3)</td>
<td>3 (20.0)</td>
<td>7 (46.7)</td>
<td>442.0±304.0 (185.0)</td>
<td>Allantois</td>
</tr>
<tr>
<td></td>
<td>Middle third</td>
<td></td>
<td>4</td>
<td>21</td>
<td>2† (9.5)</td>
<td>3 (14.3)</td>
<td>16 (76.2)</td>
<td>607.0±165.0 (471.0)</td>
<td>Allantois</td>
</tr>
<tr>
<td></td>
<td>Tip of allantois</td>
<td></td>
<td>4</td>
<td>14</td>
<td>1 (7.1)</td>
<td>1 (7.1)</td>
<td>12 (85.7)</td>
<td>208.4±82.6 (102)</td>
<td>Allantois</td>
</tr>
<tr>
<td>Prospect. LPM</td>
<td></td>
<td>Base of allantois</td>
<td>4</td>
<td>17</td>
<td>7 (41.2)</td>
<td>6 (35.3)</td>
<td>4 (23.5)</td>
<td>697.0±402.0 (452.0)</td>
<td>Multiple</td>
</tr>
<tr>
<td></td>
<td>Middle third</td>
<td></td>
<td>4</td>
<td>22</td>
<td>3 (13.6)</td>
<td>7 (31.8)</td>
<td>12 (54.5)</td>
<td>230.8±60.0 (175)</td>
<td>Endothelia, mesenchyme</td>
</tr>
<tr>
<td></td>
<td>Tip of allantois</td>
<td></td>
<td>4</td>
<td>18</td>
<td>2 (11.1)</td>
<td>11 (61.1)</td>
<td>5 (27.8)</td>
<td>199.0±139.0 (87)</td>
<td>Endothelia, mesenchyme</td>
</tr>
<tr>
<td>Prospect. PM</td>
<td></td>
<td>Base of allantois</td>
<td>4</td>
<td>19</td>
<td>7 (36.8)</td>
<td>10 (52.6)</td>
<td>2 (10.5)</td>
<td>68.0±48.0 (87)</td>
<td>Endothelia, mesenchyme</td>
</tr>
<tr>
<td></td>
<td>Middle third</td>
<td></td>
<td>4</td>
<td>21</td>
<td>7 (33.3)</td>
<td>8 (38.1)</td>
<td>6 (28.6)</td>
<td>176.7±75.7 (130)</td>
<td>Endothelia, mesenchyme</td>
</tr>
<tr>
<td></td>
<td>Tip of allantois</td>
<td></td>
<td>3</td>
<td>13</td>
<td>1 (7.7)</td>
<td>8 (61.5)</td>
<td>4 (30.8)</td>
<td>85.0±43.2 (62)</td>
<td>Endothelia, mesenchyme</td>
</tr>
</tbody>
</table>

In Experimental Set A, orthotopic (O) transplantations were carried out to three sites: the base of the allantois, the prospective lateral plate mesoderm (LPM), and the prospective paraxial mesoderm (PM). In Experimental Set B, the allantois was subdivided into three regions, tip, middle third, and base. Each of these regions was then transplanted to three sites in the same experiment: the base of the allantois, the prospective LPM, and the prospective PM.

Abbreviations: embs, embryos; expt., experiment; neg., negative; no., number; s.e.m., standard error of the mean.

*One graft had only four cells incorporated into the allantoic endothelium and was scored as negative.
†One graft had only one cell incorporated into the allantoic endothelium and was scored as negative.
environment during growth in the exocoelomic cavity and not to some intrinsic polarizing activity within the allantois itself. Benzidine staining revealed the presence of hemoglobin-producing cells in allantoises removed through both yolk sac sites (Table 2; Fig. 4D). Although there appeared to be fewer positive allantoises amongst those that had been removed through Site 1 (Fig. 2), the Student’s \(t\)-test revealed that this difference was not significant (\(P=0.069\), equal variances assumed, confidence interval, 95.0%, df=93).

This comparison suggests that the allantois may be intrinsically erythropoietic. However, from histological examination of a series of twelve headfold-stage conceptuses, it was concluded that the distance between the yolk sac blood islands and the site of removal of the allantois (al) for grafting. The upper arrow indicates a yolk sac blood island and the lower arrow points to the level of yolk sac puncture for removal of the allantois. Note that the distance between the arrows in E is greater than the 90 \(\mu\)m diameter of the capillary used to remove the donor allantois, whilst in F that distance is less than 90 \(\mu\)m. Thus, an allantois removed from a conceptus similar to that in F would have exited the yolk sac through broken blood islands. Scale Bar in C, for A-D: 25 \(\mu\)m (A); 20 \(\mu\)m (B); 21 \(\mu\)m (C); 35 \(\mu\)m (D). Scale Bar in F for E and F 100 \(\mu\)m.

**Table 2. Benzidine staining of allantoises cultured in isolation**

<table>
<thead>
<tr>
<th>Site of removal (YS)</th>
<th>Culture time (hours)</th>
<th>No. expts.</th>
<th>No. explants</th>
<th>No. positive explants (%)</th>
<th>Mean no. benzidine(^*) cells±s.e.m.</th>
<th>Median no. benzidine(^*) cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Non-blood islands</td>
<td>24</td>
<td>3</td>
<td>23</td>
<td>4 (17.4%)</td>
<td>5.8±2.9</td>
<td>3.5</td>
</tr>
<tr>
<td>40-43</td>
<td>3</td>
<td>35</td>
<td>4 (11.4%)</td>
<td>43.0±25.4</td>
<td>25.5</td>
<td></td>
</tr>
<tr>
<td>2. Blood islands</td>
<td>24</td>
<td>2</td>
<td>18</td>
<td>6 (27.3%)</td>
<td>24.5±11.1</td>
<td>18.5</td>
</tr>
<tr>
<td>40-43</td>
<td>2</td>
<td>22</td>
<td>6 (33.3%)</td>
<td>31.5±6.4</td>
<td>18.0</td>
<td></td>
</tr>
</tbody>
</table>

Donor allantoises (\(lacZ^+\)) were removed through two vitelline sites in the same experiments (shown in Fig. 2 as sites 1 and 2, and described in Materials and Methods). Isolated allantoises were then cultured for 24 or 40-43 hours, stained in benzidine and fixed briefly before examination in the compound microscope. Abbreviations: YS, yolk sac; s.e.m., standard error of the mean.

could not be ruled out. The question of whether the allantois is intrinsically erythropoietic awaits further analysis.

**Heterotopic transplantations of donor allantoic tissue**

**Prospective lateral plate mesoderm (LPM)**

To discover whether allantoic mesoderm would become incorporated into one or more of the control tissues formed at the level of the primitive streak that gives rise to LPM, the tip, middle third, and base of donor allantoises were transplanted into the prospective lateral plate mesoderm of host embryos (‘heterotopic’ transplantations; Fig. 3D). In contrast with the transplantations into the allantois, where most grafted cells contributed to the allantois, all three allantoic regions contributed descendants to the fetus when placed into prospective LPM (Fig. 6).

All three regions of the donor allantoises integrated into the endothelium of the dorsal aorta in all chimeras (Figs 6, 8C,D). Most grafts also colonized the mesenchyme adjacent to the

![Fig. 4. Benzidine staining in donor allantoises and location of yolk sac blood islands. (A) Double benzidine (and X-gal) staining of the yolk-sac of a non-transgenic host conceptus cultured for 23 hours. There is no background X-gal staining in the host. Arrow points to an example of a benzidine-positive red blood cell which, in this study, were brown. (B) Double benzidine and X-gal staining of transgenic donor conceptuses cultured for 23 hours. There is one red blood cell (arrow) in this area that appeared negative for staining with X-gal. (C) Two presumptive donor-derived benzidine-positive cells in the mid-allantoic region of a host conceptus cultured for 23 hours (arrows).](image-url)
Fig. 5. Grafts of donor allantoic tissue into the base of host allantoises, headfold stage. Results of transplants of the three donor allantoic regions, tip, middle third, and base, into the base of the allantois of host conceptuses are shown for every chimera scored after 23 hours in culture. For scoring, the host allantois was subdivided into the base, mid-region and tip. (A) Distribution of grafted basal allantoic cells. This figure combines the results of Table 1, in which the base of the allantois was transplanted orthotopically in two experimental sets, designated A and B. (B) Distribution of grafted donor allantoic cells whose origin was the mid-region. (C) Distribution of grafted donor allantoic cells whose origin was the distal third. (D) Summary of grafts of donor allantoic orthotopic and donor allantoic heterotopic transplantations into the base of the host allantois. HF, headfold stage; Omph Art, omphalomesenteric artery; Meso, mesoderm of the yolk sac; amn cav, amniotic cavity; ext. of ys, exterior of yolk sac. Ordinal numbers are the initial and final numbers of somite pairs. NS, not scored.
Fig. 6. Grafts into the primitive streak at the level of prospective lateral plate mesoderm, headfold stage. The number and location of donor transgenic cells are shown for every chimera 23 hours after orthotopic (same site) grafting of the region of prospective lateral plate mesoderm and heterotopic grafting of the three allantoic regions into the primitive streak at the level of prospective lateral plate mesoderm. The initial and final stages of each chimeric conceptus are shown. HF, headfold; ordinal numbers are pairs of somites.
dorsal aorta and a few exhibited donor descendants in the endothelium of the nearby intersegmental vessels. The mid-region of the allantois exhibited the highest rate of fetal chimerism whereas the base alone exhibited a wider colonization range than the other two allantoic regions, with basal cells found also in lateral plate mesoderm, surface ectoderm, and in endoderm of the future coelom (Figs 6, 8D).

Prospective paraxial mesoderm (PM)

None of the three allantoic regions colonized presomitic or paraxial (somitic) mesoderm (Table 1B; Figs 3E, 8E) but all three again colonized the endothelial lining of the dorsal aorta and adjacent mesenchyme (Fig. 7). The allantoic mid-region produced the highest average number of grafted cells, whereas the base and tip contributed relatively few. Interestingly, the base of the allantois did not exhibit the relative pluripotency in this region of the conceptus that it did when transplanted into prospective LPM.

**DISCUSSION**

We have investigated the developmental potential of the extraembryonic mesoderm of the headfold stage allantois. During the headfold stage, the primitive streak has fully elongated from its posteriormost site at the embryonic/extraembryonic junction of the conceptus to the distal tip of the egg cylinder; it is still an active source of mesodermal tissue at this stage (Tam and Beddington, 1987). The developmental potency of the headfold-stage allantois was challenged by placing it into two sites of the primitive streak: (1) the primitive streak where mesoderm emerges as lateral plate mesoderm, and (2) the primitive streak where paraxial mesoderm is formed, just posterior to the node (Tam and Beddington, 1987). In addition, placement of the distal two-thirds of the allantois into the base, originally intended to control for contribution of donor allantoic cells to the allantois in the second set of experiments (Table 1B), provided additional data on the state of differentiation of the headfold-stage allantois.

Ozdzenski (1967) hypothesized that a small population of alkaline phosphatase-positive cells that resides in the base of the headfold-stage allantois contributes either to the mature allantois, to the germ line or to both. It appears from our results that the basal portion of the allantois contributes only to the mature allantois. However, these results are complicated by the fact that there is no precise morphological feature that clearly defines where the base of the allantois begins. This was the...
reason that Copp et al. (1986) included the entire allantoic bud in orthotopic transplantations designed to discover the whereabouts of the primordial germ cells. Although we defined the base of the allantois according to Ozdzenski (Fig. 2), one of us had previously shown that allantoic tissue can be regenerated following aspiration of the headfold-stage allantois (Downs and Gardner, 1995). This argues that removal of the allantois via aspiration is either incomplete or, if it is complete, cells beneath the allantois retain the potential to regenerate an allantois (Downs and Gardner, 1995). Therefore, any future studies designed to analyze fate in the base of the allantois will need to define precisely the allantoic/posterior primitive streak region.

The allantois and embryonic mesoderm

All three regions of the donor allantois contributed predominantly to the endothelium of the dorsal aorta and adjacent mesenchyme when placed into the fetus. This mesenchyme is thought to be recruited into connective tissue and/or smooth muscle making up the surrounding tunicae of the mature aorta (Arey, 1965; reviewed by Noden, 1989).

Only the base of the allantois exhibited a greater developmental potential than the other two regions when placed into prospective lateral plate mesoderm, contributing descendants to the endoderm of the future coelom as well as to surface ectoderm and lateral plate mesoderm, similar to the orthotopic control grafts. Thus, the base of the allantois may contain a somewhat more developmentally labile population of cells than the two distal allantoic regions. The relative pluripotency of the base of the allantois is not surprising, given that cells in the base of the allantois are the most recent mesodermal cells to emerge from the posterior streak until the 3-somite stage (Tam and Beddington, 1987), and therefore, they may be the least differentiated.

The fetal lineages colonized by allantoic tissue following placement into the primitive streak at the level of prospective paraxial mesoderm revealed no overlap with the orthotopic control grafts. In particular, none of the regions of the allantois contributed to paraxial mesoderm. This suggests that the allantois shares few, if any, properties with pre-somatic, or paraxial, mesoderm. The base and the tip of the donor allantois exhibited the fewest number of grafted cells per embryo after placement into prospective paraxial mesoderm whereas the mid-region produced the most descendants.

This suggests that the environment at this level of the streak cannot support pluripotent basal cells and more specialized tip cells. It further suggests that those grafted cells that have become angioblasts may have the best chance of becoming incorporated into this less ‘allantoic-favorable’ region of the conceptus.

Grafts of allantoic tissue into the base of the allantois confirmed that there may be significant differences in the state of differentiation.
of differentiation along the proximodistal axis of the headfold-stage allantois (Fig. 5D). The majority of basal allantoic cells moved only as far as the allantoic mid-region. In contrast, the majority of donor cells whose origin was the allantoic tip moved further distally. The tip exhibited the largest fraction of cells in the chorioallantoic fusion junction (Fig. 9). This suggests that allantoic tip cells might be chorioadhesive as early as the headfold stage, although the full repertoire of gene expression required for chorioallantoic fusion is probably not yet in place, as previous studies have shown that the distal half of the allantois is not competent to fuse with the chorion until it has attained developmental maturity, typically at the equivalent of 4-6 somite pairs (Downs and Gardner, 1995). Alternatively, cells destined for the umbilical endothelial cell lineage at the fusion junction may be further differentiated and possess properties different from those in the mid-region, which may explain why tip cells did not avidly colonize the fetal vasculature.

**Umbilical vasculogenesis**

Previous studies have suggested that all mesoderm has the ability to undergo vasculogenesis, given the proper environment (Pardanaud et al., 1987; Coffin and Poole, 1988). The heart, large blood vessels and the vitelline vasculature are formed by vasculogenesis, a uniquely embryonic process in which pluripotent mesodermal cells differentiate into angioblasts that subsequently aggregate and assemble in situ into new blood vessels. Angiogenesis, which is the formation of blood vessels from pre-existing ones, occurs once the major vascular systems are in place. Angiogenesis occurs during both embryogenesis and throughout the life of the organism.

Very little is known about how the umbilical vasculature is formed. Electron microscopic analyses of the murine allantois have not been presented in enough detail to confirm the existence of angiogenic clusters (Tamarin and Boyle, 1976), but Ellington (1985) has described rudiments of the vascular system in the rat by 10 dpc (equivalent to approximately 4-somite pairs in the mouse). Expression studies have also shown that Vascular Endothelial Growth Factor (VEGF), its two receptors, VEGFR1 flt-1 and VEGFR2 flk-1 (Yamaguchi et al., 1993; Breier et al., 1995) and TEK (tie-2) (Schnurch and Risau, 1993) are expressed early in the allantois. These suggested that vasculogenesis was occurring in the prefusion allantois, but without examination of the developmental fate and potency of allantoic cells, the true significance of these expression patterns could not be verified.

Our transplantation experiments support the possibility that the umbilical vasculature is formed by vasculogenesis rather than by angiogenesis. This is because allantoic angioblasts were found incorporated in the large vessel endothelium of the dorsal aorta and intersegmental arteries rather than in small capillaries. Although it is possible that the nearby yolk sac contributes endothelial cells to the allantois, making angiogenesis the principal mechanism of formation of the umbilical vasculature rather than de novo vasculogenesis, we have some evidence that this is not the case. Yolk sac transplants demonstrated that the earliest time at which yolk sac cells move into the allantois is after fusion, at about 10-somite pairs (M. Blahnik, S. Gifford, and K. Downs, unpublished data). This is approximately 22 hours after the headfold stage, the time at which the donor allantoises used in this study were removed for transplantation. Also, benzidine staining and cell profiles demonstrated that all of the yolk sac-derived cells within the host allantois appear to be red blood cells.

If angioblasts are formed de novo within the allantois, then how do the extraembryonic mesodermal cells that constitute the allantoic bud differentiate into angioblasts? One possibility is that distance of pluripotent mesodermal cells from the posterior streak dictates angioblast differentiation. Extraembryonic mesoderm destined for the allantois emerges from the primitive streak in a relatively pluripotent state, but as it is pushed distally by sustained addition of nascent extraembryonic mesoderm into the allantoic base, it moves out of the sphere of influence of the primitive streak and differentiates into angioblasts. Movement farther into the tip entails further differentiation, either into chorioadhesive cells or possibly into specialized umbilical endothelial cells.

Alternatively, differentiation of allantoic cells may be temporally, rather than spatially regulated. The fate of allantoic cells may be dependent upon the amount of time they reside in different allantoic levels, the length of which may in turn be dependent upon the rate at which cells are recruited into the base of the allantois and/or the number of cell cycles completed by allantoic cells before translocating distally.

**Conclusions**

The main function of the allantois is to fuse with the chorion and vascularize, thereby forming the umbilical component of the chorioallantoic placenta. Allantoic vascularization is not dependent upon fusion with the chorion (Yamaguchi et al., 1993; Downs and Gardner, 1995). We have found that
angioblasts are formed almost as soon as the allantois emerges from the posterior primitive streak. Moreover, formation of the umbilical vasculature may not be dependent upon interaction with endoderm (Risau and Flamme, 1995) because, as far as is known, the murine allantois consists entirely of mesoderm. Our discovery of the whereabouts of pluripotent and differentiated cells within the allantois provides a developmental blueprint essential for understanding the role of genes expressed in the allantois during the headfold stage. The allantois, heretofore little studied, offers a promising and highly manipulable system to discover how angioblasts are formed from pluripotent mesoderm in the living mammalian conceptus.

This study would not have been possible without the generous support and tutelage of transplantation techniques from Dr Rosa Beddington. K. M. D. is also indebted to Professor Robert Auerbach for his gift of Leitz micromanipulation arms, an electrode puller, and the deFonbrune microforge. The authors are very grateful to Dr Karen Gould and Professor William Dove for the gift of backcrossed ROSA26 hemizygous mice; to Melanie Blahnik for expert animal care; to Kati Wesemeyl for expert assistance with benzidine staining; and to Professor Matthew Kaufman for a valuable discussion on the appellation of mesenchyme adjacent to the fetal aorta. K. M. D. is especially grateful to Professor Richard Gardner for thoughtful and valuable comments on the manuscript and always for his generosity of time and discussion, and to Dr Janet Rossant and the reviewers for their valuable criticisms. This study was supported by a grant to the University of Wisconsin Medical School under the Howard Hughes Medical Institute Research Resources Program for Medical Schools, The Graduate School of the University of Wisconsin, and by a University of Wisconsin Hilldale award to third year undergraduate students (C. H.).

REFERENCES


(Accepted 7 May 1997)