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Hemangioblast Commitment in the Avian Allantois: Cellular and Molecular Aspects

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We recently identified the allantois as a site producing hemopoietic and endothelial cells capable of colonizing the bone marrow of an engrafted host. Here, we report a detailed investigation of some early cytological and molecular processes occurring in the allantoic bud, which are probably involved in the production of angioblasts and hemopoietic cells. We show that the allantois undergoes a program characterized by the prominent expression of several "hemangioblastic" genes in the mesoderm accompanied by other gene patterns in the associated endoderm. VEGF-R2, at least from stage HH17 onward, is expressed and is shortly followed by transcription factors GATA-2, SCL/tal-1, and GATA-1. Blood island-like structures differentiate that contain both CD45$^+$ cells and cells accumulating hemoglobin; these structures look exactly like blood islands in the yolk sac. This hemopoietic process takes place before the establishment of a vascular network connecting the allantois to the embryo. As far as the endoderm is concerned, GATA-3 mRNA is found in the region where allantois will differentiate before the posterior instestinal portal becomes anatomically distinct. Shortly before the bud grows out, GATA-2 was expressed in the endoderm and, at the same time, the hemangioblastic program became initiated in the mesoderm. GATA-3 is detected at least until E8 and GATA-2 until E3 the latest stage examined for this factor. Using *in vitro* cultures, we show that allantoic buds, dissected out before the establishment of circulation between the bud and the rest of the embryo, produced erythrocytes of the definitive lineage. Moreover, using heterospecific grafts between chick and quail embryos, we demonstrate that the allantoic vascular network develops from intrinsic progenitors. Taken together, these results extend our earlier findings about the commitment of mesoderm to the endothelial and hemopoietic lineages in the allantois. The detection of a prominent GATA-3 expression restricted to the endoderm of the preallantoic region and allantoic bud, followed by that of GATA-2, is an interesting and novel information, in the context of organ formation and endoderm specification in the emergence of hemopoietic cells. © 2001 Academic Press

Key Words: **hemangioblasts; hemopoiesis; allantois; avian embryo; GATA factors; VEGF-R2; SCL/tal-1.**

INTRODUCTION

Hemopoiesis in the embryo exhibits dramatic versatility, one obvious aspect being that distinct organs provide sequential microenvironments wherein hemopoietic stem cells (HSC) receive appropriate growth and differentiation signals. These HSC are extrinsic, i.e., they colonize the organs, with the exception of the yolk sac, which produces some progenitors *in situ* (Moore and Owen, 1965; Le Douarin *et al.*, 1984). For some time, indeed, the only site known to produce progenitors was the yolk sac, which was consequently held to have a unique role in the building of the hemopoietic system (Moore and Owen, 1967). Then, the aortic region within the embryo proper was shown to have a hemogenic capacity in the avian (Dieterlen-Lièvre, 1975), amphibian (Turpen *et al.*, 1981), and mammalian embryo (Garcia-Porrero *et al.*, 1995; Cumano *et al.*, 1996; Medvinsky and Dzierzak, 1996; Tavian *et al.*, 1999). We recently

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reported that a commitment event also directs mesodermal cells into the hemopoietic pathway in the allantois of avian embryos (Caprioli *et al.,* 1998). Evidence for the latter occurrence was obtained by transplanting early quail allantoic buds into chick hosts; the bone marrow of hosts became significantly populated not only by hemopoietic progenitors but also by angioblasts originating from the graft. This dual blood-borne colonization suggested the involvement of a common progenitor, the hemangioblast.

Several lines of evidence support the existence of such progenitors with dual potential. The first argument is the shared expression by hemopoietic progenitors, angioblasts, and endothelial cells of a number of molecules, belonging to the classes of transcription factors and surface antigens, in mammals, birds, amphibians, and fishes (for reviews see Orkin and Zon, 1997; Evans, 1997; Dieterlen-Lièvre *et al.,* 2000). Examples are transcription factors SCL/tal-1 (Kallianpur *et al.,* 1994; Sanchez *et al.,* 1999), GATA-2 (Yamamoto *et al.,* 1990; Dorfman *et al.,* 1992), tyrosine kinase receptors VEGF-R2 and Tie-2 (Shalaby *et al.,* 1997; Hamaguchi *et al.,* 1999), cell surface protein CD34 (Young *et al.,* 1995; Tavian *et al.,* 1996; Wood *et al.,* 1997), etc. The second argument is the impaired development of both lineages in mouse, fish, and amphibian embryos bearing a mutation or a dominant negative form of one of the relevant genes (for review, see Orkin and Zon, 1997 and references therein). The third argument is the recent demonstration that a single ES cell in culture can give rise to both endothelial and hemopoietic cells (Choi *et al.,* 1998). Moreover, the *in vivo* production of HC from ECs in the aortic floor was demonstrated by some of us in the avian model (Jaffredo *et al.,* 1998, 2000). Angioblasts, i.e., progenitors with potential restricted to the endothelial lineage, have also been detected in the avian embryo, two modes of contribution to the blood-forming system being demonstrated, each proceeding from distinct subdivisions of the mesoderm. Hemangioblasts, responsible for the production of hemopoietic progenitors and of the visceral organ endothelial network, derive from splanchnopleural mesoderm (i.e., the mesoderm, associated to endoderm) both in the yolk sac and embryo proper, while angioblasts, destined to colonize the body wall and limbs of the embryo, derive from the somites (Pardanaud and Dieterlen-Lièvre, 1993, 1999; Pardanaud *et al.,* 1996).

When we began investigating the avian allantois potential, it was on the premise that it might produce hemopoietic and endothelial progenitors, since the mesoderm in this appendage is associated with endoderm. A critical role of endodermal–mesodermal interactions in the emergence of the blood system has indeed been assumed for many years (Miura and Wilt, 1969; Wilt, 1965). In the mouse, visceral endoderm has been demonstrated to induce endothelial cells and hemopoietic progenitors in the mesoderm (Belaoussoff *et al.,* 1998). In birds, a transient association of endoderm to somatopleural mesoderm or somites also confers a hemangioblastic capacity to these tissues (Pardanaud and Dieterlen-Lièvre, 1999). However, the molecular mechanisms specifying hemangioblastic progenitors still remain elusive. So far, few endoderm-produced molecules which might be involved in the emergence of the blood system have been identified. Most of the studies focused on VEGF; this molecule is produced by the splanchnopleural endoderm during the early steps of higher vertebrate development (Dumont *et al.,* 1995; Flamme *et al.,* 1995; Wilting *et al.,* 1997; Miquerol *et al.,* 1999) and the inactivation of only one allele results in complete loss of both endothelial and hemopoietic cells by the yolk sac (Carmeliet *et al.,* 1996; Ferrara *et al.,* 1996). Recently, transcription factors GATA-3 and Lmo2 were also reported to be expressed by the endoderm during a precise window consistent with an instructive event determining aorta-associated hemopoiesis (Manaia *et al.,* 2000). GATA-3 deletion is known to cause anemia in E12 mice embryo at the time when definitive erythropoiesis sets in (Pandolfi *et al.,* 1995). However GATA-3$^{-/-}$ ES cells integrated in chimeras are able to contribute to all hemopoietic lineages except the T lymphoid lineage (Ting *et al.,* 1996). Thus, GATA-3 appears to be required in cells interacting with hemopoietic progenitors in other lineages than the T lymphopoietic lineage, but this role has remained elusive and the expression pattern at the cellular level largely unexplored.

The purpose of the present work was to investigate in more detail the early cytological and molecular processes involved in the production of allantoic angioblasts and hemopoietic progenitors demonstrated earlier (Caprioli *et al.,* 1998). The development of the chicken allantoic vascular network has been investigated mainly at a histological level (Sethi and Brookes, l97l; Ausprunk *et al.,* l974; Shumko *et al.,* l988; Yang and Moses, l990; Rizzo *et al.,* l995). Using RT-PCR analysis, we already showed that both allantois and yolk sac expressed GATA-1 and GATA-2 but only allantois expressed GATA-3 (Caprioli *et al.,* 1998). Gene inactivations in mice have pinpointed the respective roles of GATA-1 and -2 during hemopoietic development. GATA-1 deletion causes a block late in erythropoiesis while GATA-2 is involved in the multiplication of multipotential progenitors (see Orkin, 2000 for a review).

We report *in situ* expression patterns of hemangioblastic genes by the allantoic mesoderm and the presence of two of the GATA factors studied (2 and 3) in the associated endoderm. GATA-3 was detected in the posterior intestinal portal (PIP) long before outgrowth of the bud, thus representing to date one of the earliest molecular signs of allantoic formation. GATA-2 followed shortly after, while at the same time the associated mesoderm expressed VEGF-R2, SCL/tal-1, GATA-2, and GATA-1 and erythropoiesis became initiated. These expressions are followed, before the allantois became vascularized, by the differentiation of groups of cells, in which a few express CD45. Inner cells of these groups then rapidly accumulate hemoglobin. An endothelial network thereafter connects these groups, similar in appearance to yolk sac blood islands. The intrinsic origin of this network determined experimentally, its ca-

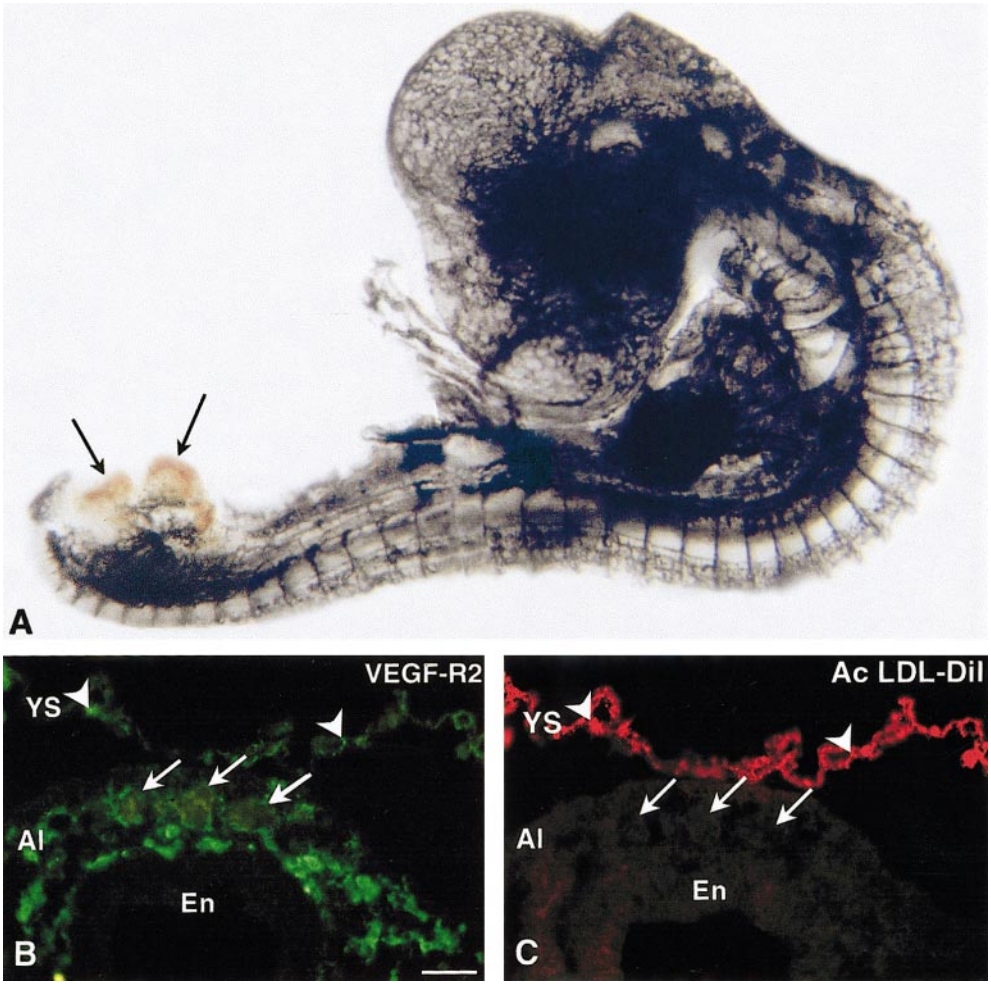


FIG. 1. Microangiographies with India ink or AcLDLs-DiI reveal the vascular network. (A) HH20 chick embryo. India ink inoculation followed by methyl salicylate clearing. The whole vascular network including plexuses in the brain, neural tube, and posterior intestinal portal are filled with ink. Only the base of the allantois is vascularized, while the large aggregates of red blood cells at the apex remain free of ink (arrows). (B, C) HH19 embryo, double-stained for VEGF-R2 in green (B) and AcLDL-DiI in red (C), sectioned through allantois (Al) and yolk sac (YS). VEGF-R2$^+$ cells delineate blood island-like structures filled with erythrocytes (arrows in B). Endothelial cells of the yolk sac are VEGF-R2$^+$ (arrowheads in B) and endocytose AcLDLs$^+$ vigorously (C). In contrast, no LDL signal is found at the level of the allantoic mesoderm. En, endoderm. Bar = 15 μm.

pacity when grafted to sprout into host tissue and to produce blood cells *in vitro*, are also reported.

MATERIALS AND METHODS

Embryos

Quail (Coturnix coturnix japonica) and chicken (JA57 strain) eggs were incubated at 38 ± 1°C. Stages were determined according to Hamburger and Hamilton (1951).

Histochemistry and Immunocytology

Benzidine staining. Erythroid cells were revealed with benzidine. Benzidine reacts with hemoglobin, an endogenous peroxidase, to form an intracellular precipitate (Glick, 1949; Pearce, 1960; Davenport, 1960; Martoja and Martoja, 1967). The staining was performed according to Palis *et al.* (1995) in PBS or culture medium. In these conditions, the precipitate turns brown in erythrocytes and blue in erythroblasts. For the blue color to be preserved, the medium must be acid, otherwise the color of the precipitate in erythroblasts changes from blue to brown.

Stained allantoises and yolk sacs were fixed for 2 h in 100% ethanol and 1% acetic acid at −20°C, dehydrated, embedded in paraplast (Prolabo), and serially cut at 5 μm.

QH1 staining. Chimeric embryos with grafted allantoic buds were fixed in Bouin's solution, dehydrated, embedded in Paraplast (Prolabo), serially cut at 5 μm, and stained with the QH1 mAb (Pardanaud *et al.*, 1987) specific for quail hemopoietic and endothelial cells. QH1 expression was revealed by an anti-IgM fluores-

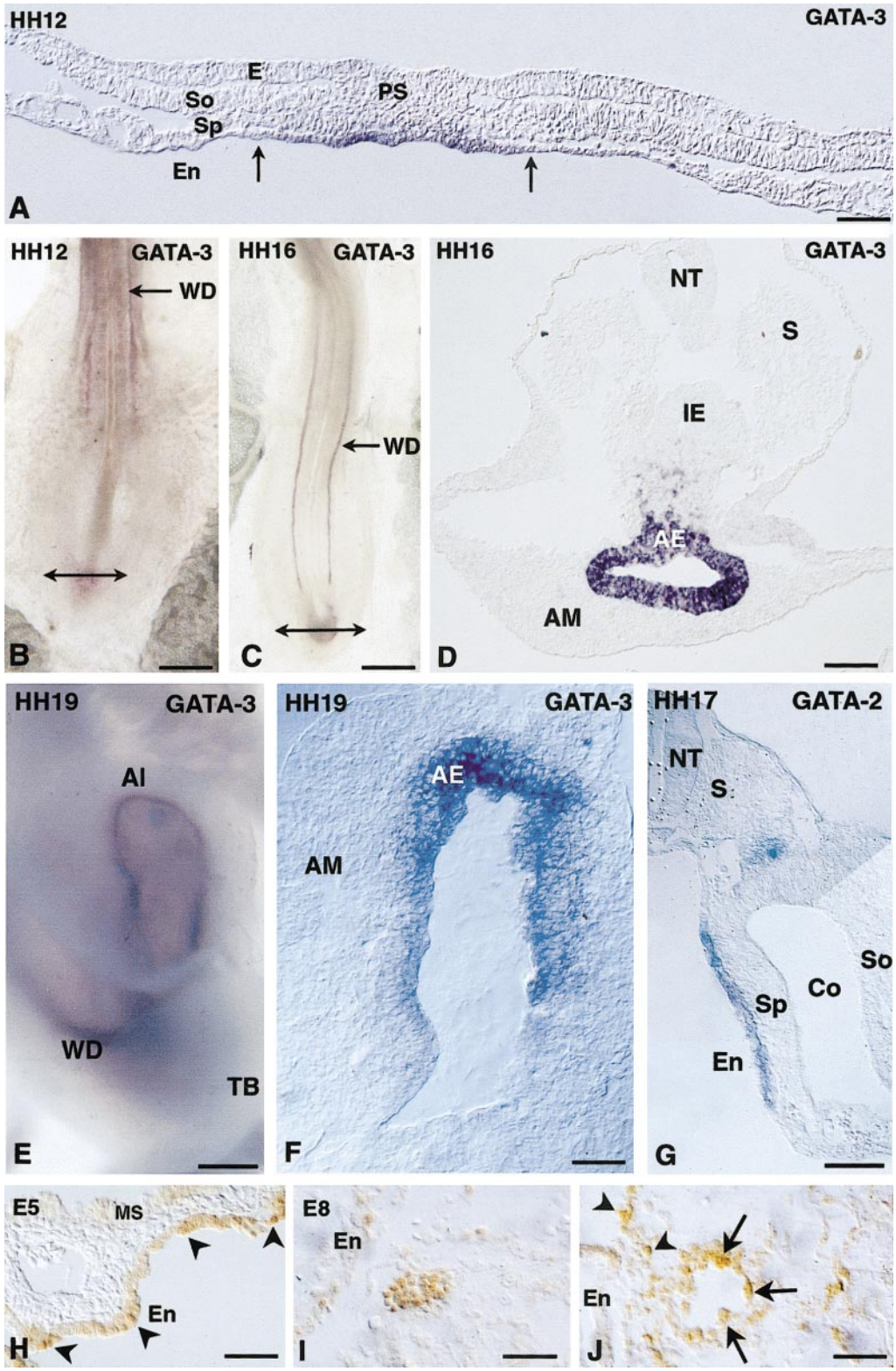


FIG. 2. GATA-3 (A–F) and -2 (G) expression in the allantoic endoderm. *In situ* hybridizations. (H–J): GATA-3 immunocytology. (A) Cross section through the level indicated in (B) by the double-headed arrow. GATA-3 is restricted to an axial stretch of endoderm (delineated by arrows). Bar = 200 μm. (B) Whole mount from the same embryo. GATA-3 mRNA is found in the caudal region at the level of the tail bud

cent antibody (Southern Biotechnology Associates). Sections were observed with a Nikon microscope equipped for epifluorescence with appropriate filters.

CD45/VEGFR-2 double staining. Immunostaining was performed on frozen sections of chicken allantois embedded into gelatin. Anti-CD45 antibody (HISC-7) was obtained from ID-DLO (The Netherlands). Anti-VEGF-R2 antibody (anti-Quek-1) was obtained following immunization of mice with the extracellular portion of the quail VEGF-R2 protein (Eichmann *et al.,* 1997). Staining was performed according to the tyramide amplification method (DuPont NEN, Europe), as modified by Jaffredo *et al.* (1998, 2000). Sections were observed on a Nikon microscope equipped for epifluorescence with appropriate filters.

Anti-GATA-3 immunohistochemistry. Anti-GATA-3 staining was performed on E2.5–E3 whole embryos (HH16–HH19) and on E4, -5, -6, and -8 isolated allantois. Since standard immunocytological protocols appeared unsuccessful to reveal the GATA-3 protein, we adapted a protocol previously described for cell cultures (Carmo-Fonseca *et al.,* 1992). In order to make the GATA-3 protein accessible to the antibody, tissues and embryos were first immersed in CSK buffer (10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 2 mM EGTA) supplemented with 0.5% Triton (Carmo-Fonseca *et al.,* 1992) on ice between 20 min to 1 h, depending on tissue thickness. Tissues were then washed in CSK, fixed overnight at 4°C in 4% paraformaldehyde and CSK, frozen, and cut at 5 or 7 μm. Sections were incubated overnight at 4°C with a monoclonal anti-GATA-3 antibody (Santa Cruz Biotechnology, ref. no. sc 268) diluted 1/50 followed by a 1-h incubation at room temperature with a secondary biotinylated anti-mouse IgG1 antibody (Amersham) diluted 1/50 in TNB. Diaminobenzidine staining was performed after tyramide amplification (DuPont NEN, Europe). Sections were observed with a Nikon microscope.

In situ hybridization. GATA-2 and GATA-3 plasmids were a gift from Dr. Engel (Northwestern University, Evanston) (Yamamoto *et al.,* 1990; Leonard *et al.,* 1993) and SCL/tal-1 was from Dr. Green (University of Cambridge, U.K.) (Goodwin *et al.,* 1992). GATA-1 was provided by Dr. Evans (Albert Einstein College of Medicine, NY). Whole-mount *in situ* hybridizations were performed following the method modified by Henrique *et al.* (1995). *In situ* hybridization on sections were performed according to Wilting *et al.* (1997).

Whole-mount stained embryos were photographed, then frozen in 0.12 M phosphate buffer, 15% saccharose, and 7.5% gelatin, prior to sectioning at 25–30 μm. Sections were deposited on Superfrost plus slides (Menzel Gläser, Germany) and stored at −80°C until use. Gelatin was removed by using a bath of PBS at 37°C for 10 min and sections were mounted with Glycergel (DAKO GmbH).

Organ Cultures of Quail and Chick Allantoises

Buds were taken out from HH18–HH19 embryos and cultured *in toto* in 24-well dishes in the presence of DMEM supplemented with 10% fetal calf serum, 1% chicken serum, 1% penicillin—streptomycin, and 1% glutamine (Gibco BRL, Europe) at 37°C in an atmosphere containing 5% CO_2. After 2–5 days in culture, they were fixed and stained with benzidine.

Grafting

Two distinct series of grafts were performed. In the first, eggs were incubated up to stage HH18. Quail allantois was grafted either in the umbilical region or inside the brain vesicles of a chicken host. The reverse combination with chicken allantois in the brain vesicles of quail embryos was also performed. The engrafted embryos were sacrificed at embryonic day 6 (E6). Grafts were dissected out together with the surrounding tissues; in the case of grafts to the brain, the whole head was fixed. In the second series, younger recipient embryos (HH16–HH17) were used. The allantoic bud or the margin of the caudal intestinal portal from quail embryos was grafted in the chicken coelom. Engrafted embryos were sacrificed after 1–3 days (E4–E6): the grafted allantois alone or the whole embryo was fixed in Bouin's fluid and processed for QH1 staining.

In Ovo Ac LDL-DiI and India Ink Injections

Chick embryos between stages HH16 and HH19 were inoculated with 3 μl of AcLDL-DiI solution (Molecular probes) into the heart according to Jaffredo *et al.* (1998). Care was taken not to injure the extraembryonic vascular network when inoculating the embryos, which were incubated for an additional 2–3 h before sacrifice. In some cases, embryos were injected in the heart with India ink (Pelikan) according to Caprioli *et al.* (1998). AcLDL-DiI- and India ink-inoculated embryos were fixed overnight in 4% formaldehyde in phosphate buffer (0.12 M), rinsed out, and embedded in gelatin as described in "*In Situ* Hybridization." Cryostat sections (7- to 10-μm thick) from AcLDL-inoculated embryos were stained with anti-VEGFR-2 by using the tyramide amplification method (Du-

in a triangle shaped area and in the nascent Wolffian duct (WD). Bar = 700 μm. (C) GATA-3 mRNA expression now clearly delineates Wolffian duct (arrow) and the future allantoic region (double-headed arrow). Bar = 1.2 mm. (D) Cross section through the level indicated in (C) by the double-headed arrow. GATA-3 is restricted to the allantoic part of the endoderm and is not expressed by the intestinal part. Bar = 150 μm. (E) Allantoic region. GATA-3 mRNA is restricted to the allantois and Wolffian duct. The allantoic signal located in the endoderm is visible through the mesoderm. Al, allantois; TB, tail bud. Bar = 3 mm. (F) Transverse section through the allantoic region in (E). GATA-3 is prominent in the allantoic endoderm but progressively disappears to become undetectable in the intestinal endoderm, both being contiguous at that stage. Bar = 500 μm. (G) Cross section at PIP level. The GATA-2 signal is restricted to the lateral aspect of the endoderm. Also note expression at the level of the intermediate mesoderm. Bar = 100 μm. (H) The GATA-3 protein in the endoderm is especially strong in nuclei (arrowheads). Bar = 30 μm. (I, J). E8 embryo. The GATA-3 protein is now detected in groups of cells located in the mesoderm (probably hemopoietic islands). (I) Blood island-like structure. Bar = 25 μm. (J) GATA-3^+ mesodermal cells (arrowheads) and GATA-3^+ cells protruding in a vessel lumen (arrows). Bar = 30 μm. AM, allantoic mesoderm; AE, allantoic endoderm; Co, coelom; E, epiblast; En, endoderm; IE, intestinal endoderm; MS, mesoderm, NT, neural tube; PS, primitive streak; S, somite; So, somatopleural mesoderm; Sp, splanchnopleural mesoderm; WD, Wolffian duct.

Pont NEN). India ink-inoculated embryos were cleared in methyl salicylate to visualize the vascular network in whole mounts.

RESULTS

Onset of Circulation between the Allantois and the Embryo

In the avian embryo, the allantois is an embryonic appendage that develops as a swelling of the PIP at stage HH17 (29–32 somite pairs). At HH18 (30–36 somite pairs), when the intestine has closed, the allantoic bud has formed as a small vesicle lined by mesoderm outside and endoderm inside. A few red cell clusters are visible at that stage at the apex of the bud. They increase in size and number, filling the whole allantoic mesoderm at stages HH19–HH20 (37–43 somite pairs, Fig. 1A). Around E5, the allantois is destined to fuse with the chorion, an extraembryonic membrane composed of ectoderm and mesoderm.

In our earlier experiments (Caprioli *et al.*, 1998), we used India ink as a probe to monitor the circulation between the allantois and the embryo. Here, we have refined the visibility of the vascular arborisation by clearing the inoculated embryos in methyl salicylate. As a complementary approach, we implemented AcLDLs-DiI inoculations (Brand-Saberi *et al.*, 1995; Jaffredo *et al.*, 1998) to precisely determine the onset of vascularization. Five embryos between stages HH18 and HH19 (30–36 and 37–40 somite pairs, respectively) were inoculated with India ink. The heart, aorta, segmental arteries, and the delicate vascular plexuses in the brain, neural tube, and tail bud became infiltrated (Fig. 1A), indicating that even the smallest vessels do not become clogged (size of ink particles 2 μm^3 according to Pelikan GmbH consumer's office). The allantois remained clear in all embryos leaving aggregates of red blood cells conspicuous at the apex of the bud (Fig. 1A). This confirms our previous reports (Caprioli *et al.*, 1998).

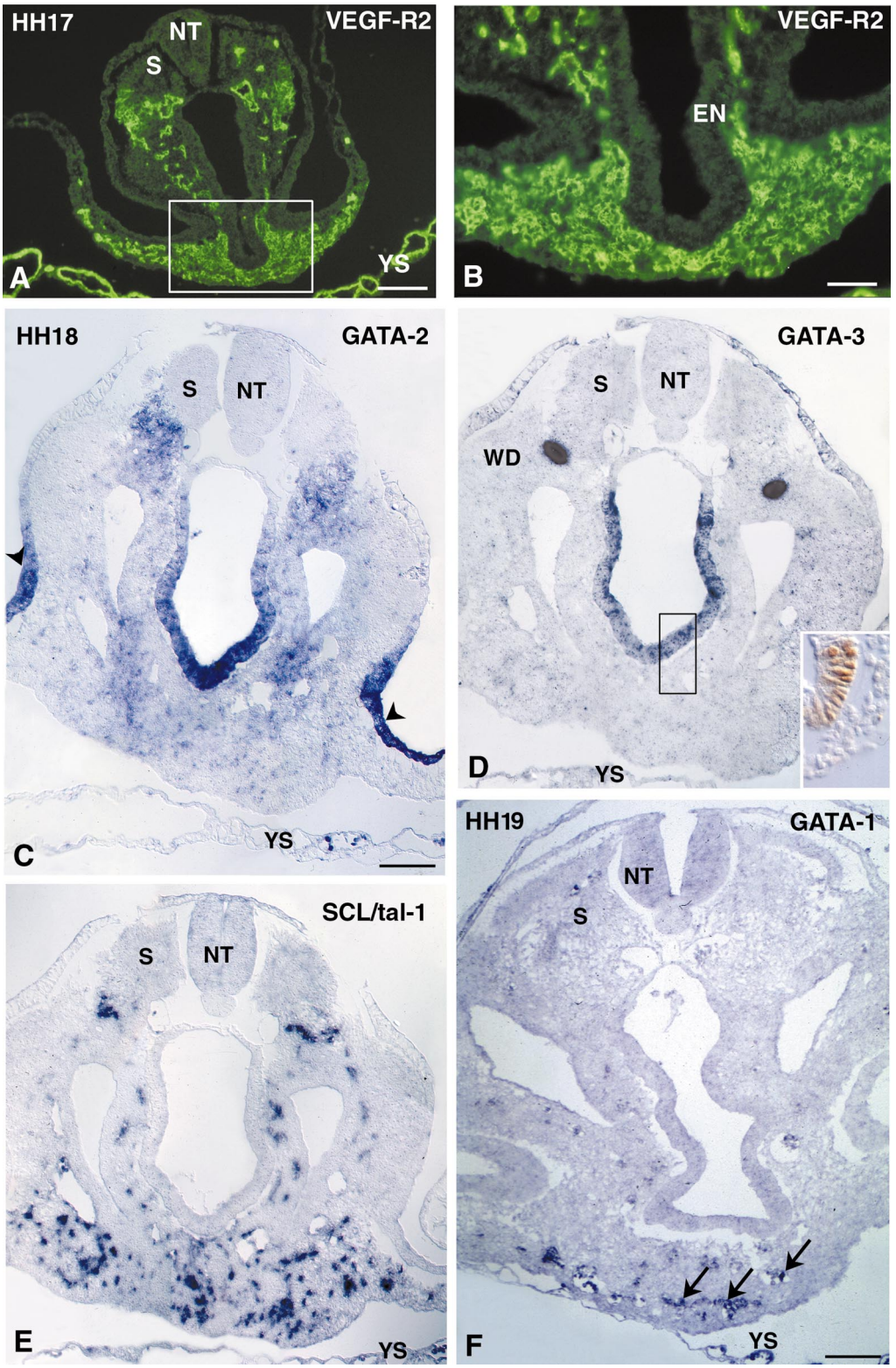
AcLDL uptake is slightly more sensitive than India ink microangiography to reveal the vascular network. AcLDLs readily distribute into the intravascular compartment and are rapidly receptor-endocytosed following inoculation. Contrary to AcLDLs, India ink never penetrates into ECs, and part of the charcoal particles may be washed out from sections by PBS rinces. Embryos inoculated with AcLDLs were left to develop for an additional 2–3 h. Endothelial cells and macrophages can be easily distinguished, since ECs exhibit a delicate punctate pattern while macrophages contain large positive vacuoles.

Upon injection at HH16-HH18 (respectively, 26–28 and 30–36 somite pairs, 11 embryos), no AcLDL uptake was detected in the posterior intestinal portal, where the allantoic bud was going to appear, although the whole vascular tree in the embryo and yolk sac were intensely labeled. The first signs of vascular connections between the allantois and the embryo were detected when HH19 embryos (37–40 somite pairs, 5 samples) were inoculated. A weak punctate fluorescence was visible on sections of 5 out of 11 embryos at the base of the allantois, indicating that the circulation progressively became established while the apex of the structure remained unlabeled (Figs. 1B and 1C). In contrast, all the blood vessels and capillaries in the body wall were labeled (not shown). Thus, the onset of circulation throughout the bud occurred around HH19 with slight variations between individuals. At HH20 (40–43 somite pairs), the whole allantois displayed a LDL^+ vascular network.

GATA Factors Expressed in the Endoderm

In our previous paper, we detected by RT-PCR an allantoic-specific expression of GATA-3 as well as GATA-1 and -2 at day 2.5 (≈HH17–HH18) and we put forward the hypothesis that the presence of GATA-3 mRNA might be linked to the emergence of definitive hematopoiesis. Here, the *in situ* distributions of GATA-2 and -3 mRNA were studied from HH11 to HH21 (respectively, 13 to 40–43 somite pairs). The analysis of GATA-3 expression was complemented by detection of the protein with a monoclonal antibody from HH17 to E8. GATA-3 mRNA first appeared at HH11–HH12 (respectively, 13 and 16 somite pairs) in the caudal-most endoderm before any sign of allantoic swelling (Figs. 2A and 2B). Judging on the localization, timing, and evolution, this expression probably corresponds to the future allantoic region. Our data are in agreement with a recent fate map of the posterior region of

FIG. 3. Hemangioblastic markers in the outgrowing allantoic bud. (A–F) Transverse sections. (A, B) VEGF-R2 detection. The future allantoic bud is framed in (A). The same area from an adjacent section is enlarged in (B). Strong VEGF-R2 reactivity of the bud and PIP mesoderm as well as ventrolateral somite region. Bar = 0.1 mm. (B) The allantoic and PIP mesoderm are strongly $VEGF\text{-}R2^+$. Bar = 20 μm. (C–E) HH18 (30–36 somite pairs). Adjacent sections through the allantoic region before outgrowth of the bud. (F) HH19 (37–40 somite pairs). *In situ* hybridization for GATA-2 (C), GATA-3 (D), SCL/tal-1 (E), and GATA-1 (F). (C) A strong GATA-2 expression, polarized to the ventral aspect, is detected in the allantoic endoderm. A diffuse GATA-2 signal is present in the allantoic mesoderm. GATA-2 is also expressed in the intermediate mesoderm region, in a few circulating cells in blood vessels of the yolk sac (YS) and in the ectoderm at the embryonic–extraembryonic junction (arrowheads). (D) GATA-3 mRNA is strongly expressed in the allantoic region endoderm (compare to C) and the Wolffian duct. The frame indicates the region enlarged from another embryo at the same stage in which the GATA-3 protein was revealed. (E) SCL/tal-1 is highly expressed by single cells or small groups of cells in the mesoderm. Note SCL/tal-1 reactivity in the intermediate mesoderm region as well as in free cells in the yolk sac blood vessels. (F) A few $GATA\text{-}1^+$ cells are localized at the apex of the bud in blood island-like structures (arrows).



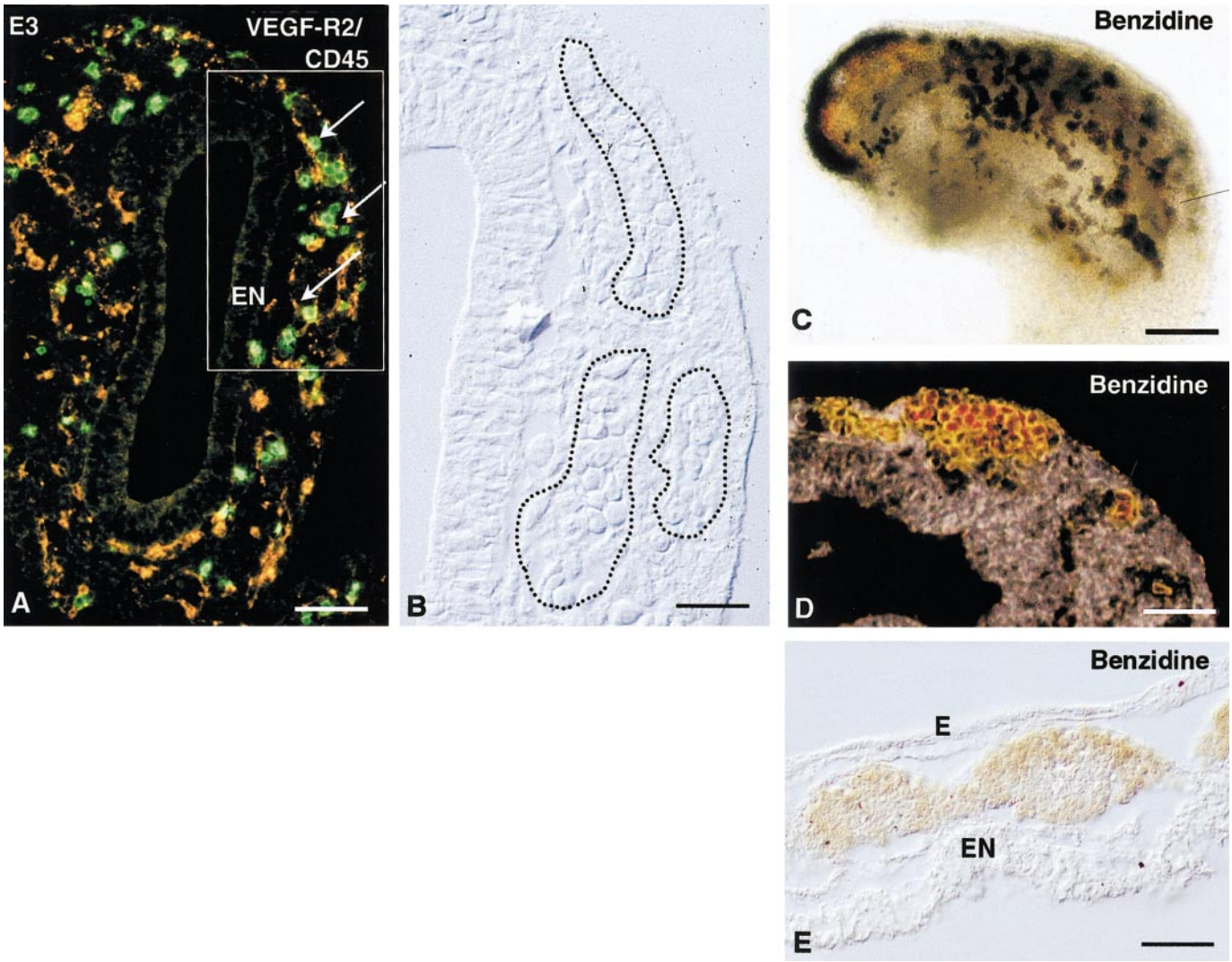


FIG. 4. Cytological evolution of the allantois during the third day. (A) Double staining for VEGF-R2 (red) and CD45 (green). VEGF-R2$^+$ endothelial profiles enclose CD45$^+$ cells (arrows). Bar = 30 μm. (B) Nomarski's interference view of the frame indicated in (A). Groups of round, hemopoietic cells looking like yolk sac blood islands, delineated by dotted lines are clearly visible (see also Fig. 4E). (C) *In toto* benzidine staining of the allantoic bud. Erythroblasts appear dark purple and erythrocytes reddish brown. Bar = 300 μm. (D) Benzidine staining + phase contrast, cross section. Bar = 20 μm. (E) Benzidine staining + Nomarski's interference contrast of yolk sac blood islands from an E2 chicken embryo. Allantoic and yolk sac blood islands are very similar. Compare (D) and (E). Bar = 15 μm. E, ectoderm; En, allantoic endoderm.

the 1.5-day chick embryo (Matsushita, 1999). Neither GATA-1 nor GATA-2 displayed a similar distribution at this stage (data not shown). From HH16, the GATA-3 signal was present in the endoderm of the future allantoic bud (Figs. 2C and 2D). From HH17 onward, GATA-2 was also detected in the allantoic endoderm which is not yet completely closed at that time (Fig. 2G), persisting at least until HH20, the latest stage examined. The endodermal expression of GATA-3 continued at HH18 and 19 (Figs. 2E, 2F, and 3D), E5 (Fig. 2H) and until E7. At E8, the signal disappeared from some regions in the endoderm, while some cell groups in the mesoderm became positive (Figs. 2I and 2J) as well as some cells inside vessel lumina (Fig. 2J).

Gene Expressions in Mesoderm

The cell constitution of the early allantois was characterized by means of a combination of endothelial/hemopoietic-specific genes. VEGF-R2 is one of the first genes expressed by the hemangioblastic mesoderm, as it

differentiates into yolk sac blood islands at the gastrula stage; VEGF-R2 later becomes restricted to ECs (Eichmann *et al.*, 1993, 1997; Millauer *et al.*, 1993; Dumont *et al.*, 1995; Nishikawa *et al.*, 1998; Jaffredo *et al.*, 1998). SCL/tal-1 is present in hemopoietic cells and is essential for the development of all hemopoietic lineages (Kallianpur *et al.*, 1994; Porcher *et al.*, 1996; Robb *et al.*, 1996). A central role of SCL/tal-1 in hemangioblast commitment has recently been demonstrated (Gering *et al.*, 1998; Robertson *et al.*, 2000). GATA-2 and GATA-1 are respectively expressed in multipotent hemopoietic stem cells and committed erythroblasts. Gene inactivation has revealed a respective role of these factors in the multiplication of multipotent progenitors and progression through erythroid differentiation (see Begley and Green, 1999; Orkin, 2000 for reviews). CD45 is a pan-leukocyte antigen expressed by hemopoietic progenitors and leukocytes (Trowbridge and Thomas, 1994).

VEGF-R2 was detected very early in the future allantoic mesoderm (ventral) which, at HH16–HH17, appeared entirely positive in contrast to the mesoderm present in sides and roof of the endodermal pouch, which contained sparser VEGF-R2$^+$ endothelial profiles (Figs. 3A and 3B). From HH17 onward, VEGF-R2 expression decreased and became restricted either to groups of cells or to VEGF-R2$^+$ cells delineating structures strikingly similar to yolk sac blood islands (see Fig. 1B) and contained erythroblasts. At HH18–H19, GATA-2 and SCL/tal-1 were expressed in this area. GATA-2 expression in the mesoderm was diffuse (Fig. 3C), while a strong signal, polarized to the ventral aspect, was detected in the endoderm. GATA-2 was also detected in the ectoderm at the embryonic–extraembryonic junction. Abundant SCL/tal-1 mRNA widely distributed in the mesoderm of the whole region (Fig. 3E). GATA-1 was detected slightly later (HH19, 37–40 somite pairs) in the mesoderm in a few cells at the apex of the allantois consistent with its role in erythropoiesis (Fig. 3F). At stages, HH18–HH19, blood island-like structures appear in the mesoderm. The more mature islands were located at the base of the bud. They were delineated by VEGF-R2$^+$ cells and contained free hemopoietic cells, a few of them CD45$^+$ (Figs. 4A and 4B). Less mature structures, found at the apex of the bud, were characterized by a VEGF-R2$^+$ EC envelope completely filled with blood cells. Some of these were CD45$^+$ while some others were CD45$^-$, probably already hemoglobinized (Figs. 4A and 4B). These features, typical of a vascular network differentiating between blood islands, are present before the allantoic bud becomes connected to the embryo's vascular network indicating an autonomous hemangioblastic program.

Indeed, numerous benzidine-positive cells were already present at HH19 prior to vascularization. In this regard, chick and quail allantois displayed similar patterns between stages HH17 and HH20. Erythroid islands composed of immature (dark blue) and mature (brown) cells were readily visible at HH19 in both species (Figs. 4C and 4D). Benzidine-positive cells were clearly enriched at the apex where they formed large pools (Fig. 4D). These islands are very similar to those present in the yolk sac (compare Figs. 4D and 4E).

Since we grafted quail allantois into chick embryos, we determined whether the allantois had a similar developmental program in the two species. QH1 immunoreactivity in E3–E9 quail allantois revealed profiles very similar to those shown for quail yolk sac with QH1/MB1 (Péault *et al.*, 1988) or CD34$^+$ cells in the human yolk sac (Tavian *et al.*, 1999) or demonstrated with combined VEGF-R2 and CD45 antibodies in the chicken. On the basis of these results, we concluded that, in the two species, the timing of allantoic development is similar (data not shown).

Experimental Evidence for Vasculogenesis and Blood Progenitor Production

The capacity of the allantoic mesoderm to differentiate into endothelial or hemopoietic cells was probed through organ culture or grafts from the quail into chicken hosts, both approaches involving prevascular buds. Quail or chick prevascular allantoic buds and associated PIPs were dissected and cultured whole in liquid culture medium for 2–5 days in 24-well dishes. As a control, some allantoic buds were fixed immediately and stained with benzidine. No benzidine-positive cell was observed, indicating that committed erythrocytes were not present at the onset of culture. When put in culture, the buds rapidly spread onto the substrate. In 100% of the samples, abundant erythroblasts (blue after benzidine staining) organized in small groups (Fig. 5A) or remained isolated in the mesoderm. In some cases, erythrocytes, belonging to the definitive lineage judging from their ovoid shape, were clearly visible (Fig. 5B), in agreement with our earlier RT-PCR analysis in which globin mRNA make-up characteristic for the definitive lineage was detected (Caprioli *et al.*, 1998).

In the case of grafts, three distinct sites were elected for grafting. The first site was the vicinity of, or through, the host allantois, with the aims of replacing the host appendage and of obtaining a development of the graft comparable to normal. This proved impossible, because, when cut out, the host allantois always regenerated, precluding orthotopic replacement of the appendage. Inserted close to the host umbilical stalk in three cases out of eight, the grafted allantois developed as a large intensely red pouch linked to the host body by a fine stalk (Figs. 5C and 5D). On sections, these pouches appeared filled with red cells. The walls of the pouches were extensively vascularized (Fig. 5D). In the remaining five cases, the allantois was floating in the amniotic cavity, where it survived without growing. In this situation, no red blood cells matured, while abundant QH1$^+$ cells persisted (Fig. 5E). Inserted into a slit of the host allantois (nine cases), the grafts became pyramid-shaped red appendixes, filled with red blood cells and extensively vascularized (data not shown). These structures were surrounded by a QH1$^+$ layer of cells (Fig. 5F). Many vessels emanated from these structures and penetrated into the host linking with host vessels.

The second site was into the coelom (seven cases), the allantois usually developed as a small vesicle linked to the host liver or lung through vascular connections. $QH1^+$ endothelial and hemopoietic cells from the grafts always strayed into the host liver, sometimes in the mesoderm of the limb bud and, in one case, in the floor of the aorta (data not shown), thus quite a distance away from the graft.

The third site was the host brain at the level of the mesencephalon, in view of testing the angiogenic capacity of the allantois. In the 10 cases, the allantois developed in the ventricle or protruded outside the brain forming a sparsely vascularised ball with no red blood cell accumulation. In this situation, allantoic vessels anastomosed with the host blood system, and gave rise to chimeric vessels in the host brain tissue. In this situation, sparse quail blood island-like formations were always detected in the graft, and sometimes in host tissues (data not shown).

In the reverse combination, namely chicken allantoic bud in quail brain, host $QH1^+$ cells never invaded the graft, on the contrary chimeric vessels, identified by the presence of $QH1^+$ and $QH1^-$ EC, developed in the host brain all around the graft.

DISCUSSION

The cytological evolution, the molecular patterns detected in the early avian allantoic bud, and the experimental approaches undertaken lead us to conclude that both angioblasts and hemopoietic progenitors derive *in situ* from the allantoic mesoderm: strikingly, all cells in the mesoderm display receptor 2 for VEGF and many express transcription factors SCL/tal-1, GATA-2, and GATA-1; the CD45 antigen then appears on some cells and hemoglobinization of cell groups shortly follows. All these signs of commitment and differentiation, which pertain to a process we have earlier designated as "hemangiopoiesis" (Pardanaud and Dieterlen-Lièvre, 1999), occur prior to the establishment of vascular connections between the allantois and the embryo. In our earlier work (Caprioli *et al.*, 1998), the allantoic rudiment was dissected out for grafting between HH17 and HH18, about 6 h before vascular connection; the timing of this event was herein determined very precisely by the injection of AcLDLs; it occurs between HH18 and HH19 (30–36 to 37–40 somite pairs).

Actually, prior to vascularization, the allantoic bud exhibits prominent blood island-like structures. On the molecular level, there is, like in the yolk sac, a hemopoietic program, characterized by the expression of GATA-2, GATA-1, SCL/tal-1, and globin genes, which appears predominantly erythroid. On the basis of the ellipsoid shape of red cells recognizable in cultures and of RT-PCR identification of the globin types (Caprioli *et al.*, 1998), the erythrocytes produced by the allantois can be allocated to the "fetal" rather than the embryonic lineage.

In the 1998 paper, we reported that the host bone marrow became invaded by hemopoietic and endothelial cells emitted by the grafted allantoic rudiment; thus, we considered the possibility that hemangioblasts, rather than distinct progenitors for the two lineages, colonized the host bone marrow. The presence of VEGF-R2 and SCL/tal-1, reported here in the allantoic mesoderm supports this hypothesis, since several pieces of evidence such as developmental expression, deletion and ectopic expression effects, indicate that these two genes are involved in the commitment of hemangioblasts. (1) Both molecules are expressed by mesodermal cells that give rise to endothelial and hemopoietic ligneages during gastrulation (Millauer *et al.*, 1993; Dumont *et al.*, 1995; Eichmann *et al.*, 1993, 1997; Gering *et al.*, 1998; Mead *et al.*, 1998), and yolk sac formation (Kallianpur *et al.*, 1994; Elefanty *et al.*, 1999). Single VEGF-R2-positive cells from the caudal mesoderm of avian gastrulating blastodisc (i.e., the region that gives rise to yolk sac blood islands) develop into either hemopoietic or endothelial colonies *in vitro* (Eichmann *et al.*, 1997). In the aortic region, which is the site of a secondary step of hemopoietic progenitor production, these genes are also expressed (Labastie *et al.*, 1998; Jaffredo *et al.*, 1998; Marshall *et al.*, 1999; Delassus *et al.*, 1999; Sinclair *et al.*, 1999; Manaïa *et al.*, 2000; Minko and Jaffredo, unpublished). (2) Mice lacking VEGF-R2 fail to generate both vasculature and hemopoietic cells (Shalaby *et al.*, 1995, 1997). (3) Ectopic expression of SCL/tal-1 specifies hemopoietic mesoderm in *Xenopus* animal cap explants (Mead *et al.*, 1998) and results in excessive production of blood and endothelial cells at the expense of somitic and pronephric duct cells in the zebrafish embryos (Gering *et al.*, 1998).

In culture, isolated allantoic buds produce erythrocytes. In the quail–chick grafting experiments, both EC and HC were detected when the bud was introduced into the coelom or close to the umbilical stalk. In contrast, when grafted into the host brain, a site known to promote blood vessel formation but not hemopoiesis, the graft produced EC participating to the formation of the host blood vessels but failed to produce detectable amounts of HC. In the mouse, the allantoic rudiment has also been shown to express a number of molecules related to vasculogenesis and/or hemopoiesis (Yamaguchi *et al.*, 1993; Shalaby *et al.*, 1995; Sanchez *et al.*, 1999; Gory-Fauré *et al.*, 1999; Manaia *et al.*, 2000; Drake and Flemming, 2000). It also develops its own vascular network (Downs and Harmann, 1997), but appears not to produce erythrocytes when explanted prior to fusion with the ectoplacental cone and cultured for 2 days (Downs *et al.*, 1998). An important difference distinguishes the mouse and chicken allantois: the endoderm is anatomically marginal in the mouse rudiment, i.e., it remains at the base of the appendage, staying in contact with the endoderm for only a short period of time, never entering the stalk. In the case of the avian embryo, mesoderm and endoderm remain in close contact during the major part of the budding process at least until E8. This time-limited tissue interaction for the mouse species may cause a restriction to the angiopoietic potential of the allantoic mesoderm. However, a specific contribution from the mouse

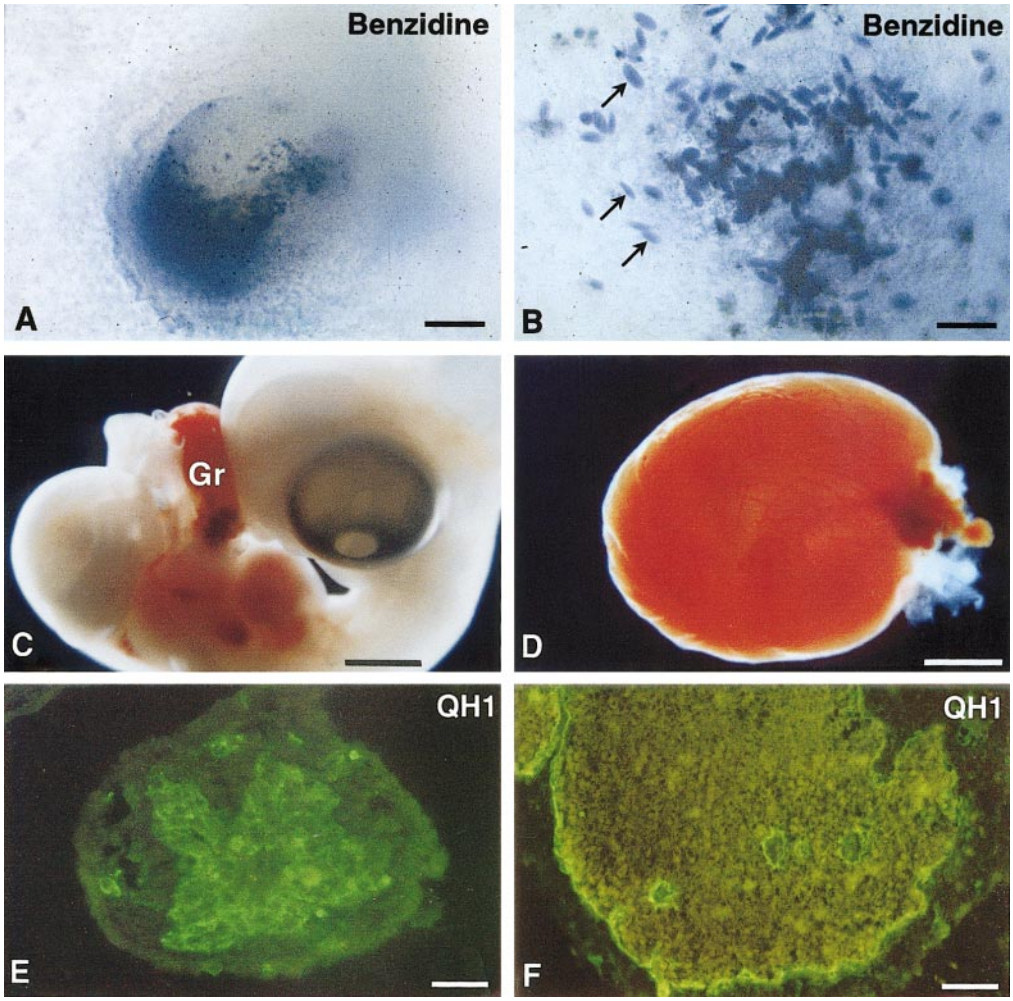


FIG. 5. Vasculogenesis and blood cell production by the allantois. (A, B) Allantoic bud, 2-day *in vitro* culture of nondissociated buds; benzidine staining. (A) A large aggregate of erythroid cells (stained blue) is visible inside the organ. Bar = 150 μm. (B) Definitive erythrocytes with a characteristic elongated shape are present in the culture. Bar = 20 μm. (C–F) Quail allantois grafts dissected out at HH18 and grafted into HH19–HH20 chick hosts. (C) E6 host engrafted in the umbilical region. Gr, graft. Bar = 2 mm. (D) The same graft after dissection. Bar = 1 mm. (E, F) Sections of quail allantoic buds engrafted for 3 days in chick hosts and stained for QH1 expression. (E) A poorly developed graft harbors a large aggregate of $QH1^+$ cells. Bar = 50 μm. (F) Section through a well-developed graft seen in (D): a large pool of erythrocytes is surrounded by $QH1^+$ endothelium and contains several smaller $QH1^+$ endothelial profiles. Bar = 25 μm.

allantois to the hemopoietic system would be difficult to identify, because the rudiment develops during gastrulation at the same time as, and in close contact with, the yolk sac and very soon fuses to the ectoplacental cone. We feel that other strategies are needed to verify whether or not the mouse allantoic mesoderm is a site where commitment of HSC may occur.

Many experimental data have pointed out the critical role of endoderm as a source for hemopoiesis-supporting factors in the chick embryo, (Wilt, 1965; Miura and Wilt, 1969; Kessel and Fabian, 1987; Gordon-Thomson and Fabian, 1994; Pardanaud and Dieterlen-Lièvre, 1999) and in the mouse embryo (Belaoussoff *et al.*, 1998; Dyer *et al.*, 2001). The nature of the molecular signal is only partially understood. Expression and knock-in studies have shown that VEGF is expressed by the endoderm (Flamme *et al.*, 1995; Dumont *et al.*, 1995; Miquerol *et al.*, 1999) and is required in full dosage for yolk sac hemopoiesis and vasculogenesis to proceed (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). Recently, Dyer *et al.* (2001) demonstrated that Indian hedgehog was present in the YS endoderm and was also necessary for the specification of EC and HC from the mesoderm. In the splanchopleural mesoderm of the embryo proper, where definitive hemopoiesis takes place, commitment to the hemopoietic lineage also appears to be mediated by the endoderm; moreover a short term contact with

endoderm induces hemangiopoiesis in the somatopleural mesoderm and this effect is mimicked by growth factors such as VEGF, TGFβ, or bFGF (Pardanaud and Dieterlen-Lièvre, 1999). The experiments reported here reveal an interesting molecular feature of the allantoic endoderm. We had previously detected by RT-PCR that GATA-3 is expressed in the allantois but not in the yolk sac (Caprioli *et al.*, 1998). Here, we determined that this expression, both at the mRNA and protein levels, is localized in the endoderm. GATA-3 appears at HH11–HH12 at least 6 h before allantoic outgrowth. Judged on territory of expression and progressive restriction, this expression delineates the future allantoic region, in agreement with a recent fate map of the caudal part of the 1.5-day chick embryo (Matsushita, 1999). In addition, we found that GATA-2 is also unexpectedly expressed in the endoderm, shortly following GATA-3. Previously the activity of this gene in hemopoiesis was thought to be restricted to hemopoietic cells proper (see Orkin, 2000 for review). Strikingly, neither GATA-2 nor GATA-3 were detected in the endoderm of nonhemogenic intestinal segments contiguous to the allantoic endoderm. When GATA-2 became conspicuous in the endoderm, its expression also switched on in the associated mesoderm, in parallel with that of VEGF-R2, SCL/tal-1, and GATA-1. This schedule of expression may be consistent with an inductive signal from endoderm to mesoderm. Similar expression profiles were recently reported in the mouse embryo aortic region. In this species, GATA-3 is absent from the yolk sac. It is expressed by the caudal-most endoderm, transitorily associated with Lmo2, another transcription factor, previously reported in hemopoietic progenitors themselves and critical for their commitment (Manaïa *et al.*, 2000). GATA-3 and Lmo2 expression only overlap in the endoderm during the 5-somite stage, prior to the delamination of the aortic endothelium into hemopoietic clusters described by several groups (Wood *et al.*, 1997; Garcia-Porrero *et al.*, 1998; Tavian *et al.*, 1996; Debacker *et al.*, 1999; Godin *et al.*, 1999; Manaïa *et al.*, 2000). It thus appears that several genes, previously known to be present in hemopoietic cells and thought to be implicated in the specification of blood cell lineages, are also unexpectedly found in the endoderm associated to the mesoderm of definitive hemogenic sites. This observation suggests that, in addition to a cell-autonomous role for several of these gene products in the initiation of hemopoiesis, non-cell-autonomous mechanisms can be considered which might explain the hereto elusive mechanism of the GATA-3 knock-out effect on definitive erythropoiesis. We propose that the endodermal expressions of GATA-2 and -3 (and maybe others), alone or in combination, depending on the hemogenic site considered, may be required to switch on an endoderm signal necessary for mesoderm commitment to the hemangioblastic lineage, at the time when definitive hemopoiesis sets in.

A number of recent studies indicate that other GATA factors play a role in the patterning of primordial germ layers or in different induction processes. Several members of the gene family are critical for the specification of both mesoderm and endoderm in vertebrates and in invertebrates. In mouse, *Xenopus*, and Zebrafish, GATA-4 and -5 play a central role patterning the cardiac area and specifying the digestive endoderm (Weber *et al.*, 2000; Soichet *et al.*, 2000; Yasuo *et al.*, 2000 and references therein). In invertebrates, the *Caenorhabditis elegans* end-1 gene was proposed to be a master gene for endodermal development (Zhu *et al.*, 1997, 1998). In *Drosophila*, the GATA-related *serpent* gene, expressed by subsets of endodermal and mesodermal cells, is essential for both midgut and hemopoietic cell specifications (Reuter, 1994; Rehorn *et al.*, 1996; Lebetsky *et al.*, 2000).

In conclusion, the combination of genes expressed in the allantoic bud is in agreement with an ongoing hemopoietic or, more probably, hemangiopoietic program. Interestingly the present study uncovers a striking endodermal expression of GATA-3 and GATA-2. The avian allantoic bud appears as a favorable model system to study the combinatorial effects of gene activities mediating the endoderm/mesoderm interactions involved in the specification of definitive erythropoiesis.

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