Lmo2 and GATA-3 associated expression in intraembryonic hemogenic sites

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SUMMARY

It is now widely accepted that hemopoietic cells born intraembryonically are the best candidates for the seeding of definitive hemopoietic organs. To further understand the mechanisms involved in the generation of definitive hemopoietic stem cells, we analysed the expression of the hemopoietic-related transcription factors Lmo2 and GATA-3 during the early stages of mouse development (7-12 dpc), with a particular emphasis on intraembryonic hemogenic sites. We show here that both Lmo2 and GATA-3 are present in the intraembryonic regions known to give rise to hemopoietic precursors in vitro and in vivo, suggesting that they act together at key points of hemopoietic development. (1) Lmo2 and GATA-3 are expressed in the caudal mesoderm during the phase of intraembryonic precursors determination. (2) A highly transient concomitant expression is observed in the caudal intraembryonic definitive endoderm, suggesting that these factors are involved in the specification of intraembryonic hemopoietic precursors. (3) Lmo2 and GATA-3 are expressed within the hemopoietic clusters located in the aortic floor during fetal liver colonisation. Furthermore, a strong GATA-3 signal allowed us to uncover previously unreported mesodermal aggregates beneath the aorta. A combined in situ and immunocytological analysis strongly suggests that ventral mesodermal GATA-3 patches are involved in the process of intraembryonic stem cell generation.

Abbreviations: AGM, aorta-gonad-mesonephros; Ao, aorta; dpc, day postcoitus; BA, branchial arch; BP, branchial pouch; EC, endothelial cells; HSC, hemopoietic stem cell; LTR, long-term reconstitution; MoAB, monoclonal antibody; PGC, primordial germ cell; P-Sp, para-aortic splanchnopleura; Sp, splanchnopleura; YS, yolk sac.

Key words: Transcription factor, Hemopoiesis, Mouse, Lmo2, GATA-3

INTRODUCTION

In the mouse embryo, the yolk sac (YS) and the caudal intraembryonic splanchnopleura (Sp) independently generate hemopoietic precursors. In these two sites, hemopoietic precursors arise in situ, contrasting with later hemopoietic organs, such as the fetal thymus and liver, spleen and bone marrow that depend on colonisation to achieve hemopoiesis. In both extraembryonic and intraembryonic hemogenic sites, mesoderm is associated to endoderm, a combination thought to promote blood precursor formation (Miura and Wilt, 1969). However, YS and Sp precursors strikingly differ in their differentiation and maintenance potential (Cumano et al., 1996), as only intraembryonic precursors are endowed with lymphoid potential. They also persist longer in culture than YS precursors. These two features constitute a first evidence in favour of an intraembryonic origin of definitive hemopoiesis.

After the 15-somite stage, the tissues derived from the Sp, the para-aortic splanchnopleura (P-Sp; 8.5-10 dpc (Godin et al., 1995)), then the aorta-gonad-mesonephros (AGM) region (Godin et al., 1999) harbours multipotent hemopoietic precursors. AGM precursors are able to provide the long-term, multilneage reconstitution (LTR) of lethally irradiated adult recipients that characterize hemopoietic stem cells (HSC) (Medvinsky and Dzierzak, 1996; Müller et al., 1994). LTR activity of P-Sp precursors is revealed only when they are transplanted into the newborn environment (Yoder et al., 1997). In the fetal liver (FL), LTR activity appears one day later than in the AGM (Medvinsky and Dzierzak, 1996), providing further evidence that definitive HSC would originate from Sp-derived intraembryonic site.

During pre-liver stages (7-10 dpc), differentiated cells originate primarily in the YS and are qualified as primitive. The switch to definitive hemopoietic activity occurs after the seeding of the FL by HSC. Several phenotypes provided by targeted disruption of hemopoiesis-regulating genes indicate that primitive and definitive hemopoiesis do not depend on the same gene regulation. Besides common signaling pathways involving, for example, Tal-1/SCL, Lmo2, GATA-1 and GATA-2, a number of specific genes (including GATA-3, c-myc, AML-1; Shivdasani and Orkin, 1996) are only required for the development of definitive hemopoiesis.

The LIM-zinc finger proteins Lmo (Boehm et al., 1991) are thought to promote self-renewal of precursors and their
maintenance in an immature state (Visvader et al., 1997). Lmo2 null mutants die after 10.5 dpc due to failure of YS erythropoiesis (Warren et al., 1994). Lmo2 is also required for the development of definitive hemopoiesis, as null ES cells do not contribute to hemopoietic cells in chimeras (Yamada et al., 1998). This factor is detected in all the tissues involved in active hemopoiesis, both primitive (Silver and Palis, 1997) and definitive (Foroni et al., 1992). Lmo2 is considered as a bridging molecule that links combinations of transcription factors. The first described complex, involving Lmo2, Tal-1/SCL and GATA-1, was characterised in erythroid cells (Wadman et al., 1997). As Lmo2 can also bind GATA-2, the existence of complexes involving other members of the GATA family was postulated (Wadman et al., 1994; Yamada et al., 1998). More recently, Lmo2 was shown to interact with GATA-3 (Ono et al., 1998).

Targeted disruption experiments indicate that GATA-3 is not essential for primitive hemopoiesis (Pandolfi et al., 1995). GATA-3−/− ES cells normally contribute to the definitive hemopoietic compartments of chimeras, except the T-lymphoid lineage (Ting et al., 1996), indicating that GATA-3 function during the establishment of definitive hemopoiesis is non-cell autonomous. However, up to now, GATA-3 expression in the hemopoietic microenvironment has not been documented. Moreover, expression in hemopoietic cells has only been analysed after 10 dpc (George et al., 1994; Oosterwegel et al., 1992).

We focused here on Lmo2 and GATA-3 expression in intraembryonic hemogenic sites, to gather more information on the trans-acting factors involved in definitive HSC generation. In vitro functional studies clearly established the presence of

**Fig. 1.** Evolution of Lmo2 and GATA-3 expressions from gastrulation to 10 dpc.
(A-C) Neural plate stages. (A) Lmo2 transcripts delineate extraembryonic mesoderm (arrowheads); (B) Lmo2 expression restricts to immature blood islands (BI, arrowheads); (C) slightly later, BI development leads to the formation of a Lmo2-positive ring (arrowheads). (D) Late head-fold stage. Lmo2-positive cells are present at the boundary between intraembryonic and extraembryonic compartments and in the cephalic mesoderm (white arrowheads), in YS-BI (arrowheads) and in the allantois (Al). (E, top) At late head-fold stage, GATA-3 is expressed in the developing gut and lateral ectoderm (arrowheads). The area where hemopoietic cluster-bearing vessels develop (Al and caudal Sp) is labelled (white arrow). (E, bottom) At 8 dpc (3 somites), GATA-3 is expressed in the Al and intraembryonic Sp (white arrow); YS-BI (arrowhead) lack GATA-3 signal. (F) At late head-fold stage, beside a strong expression in YS-BI, Lmo2-positive cells are detected in the caudal Sp (arrow), in continuity with Al angioblasts (arrowheads). (G) At 8.5 dpc (15 somites), transcripts are seen in the BA (white arrowhead); signal in the paired Ao remains high caudally (arrows) and appears in the trunk (arrowhead). The posterior ventral mesoderm (pvm) is strongly stained. Expression is downregulated in the YS. (H) At 9-9.5 dpc (20-25 somites), expression is maintained in the BA (white arrowheads), truncal Ao (arrow) and pvm. Signal appears in cranial capillaries (arrowheads) and intersomitic vessels (white arrow). Lmo2 downregulation in circulating hemopoietic cells is clear in the YS due the lack of extraembryonic EC-signal. (I) At 9.5-10 dpc (25-30 somites), GATA-3 is expressed in BA2-4 (white arrowheads), the aortic region (arrows), mesonephros (white arrow) and Pvm. Ab, allantoic bud; fg, foregut; H, heart; NF, neural folds. Bar: 100 μm.
multipotent precursors in the P-Sp (Godin et al., 1995). However, no cytological evidence suggesting a hemopoietic-related process has been found before the AGM stage (10.5-11.5 dpc), when hemopoietic cell clusters are detectable in the floor of the main arteries (Garcia-Porrero et al., 1995; Wood et al., 1997). Previous attempts to trace back these precursors at earlier development stages confirmed the hemopoietic nature of the intra-arterial clusters, but failed to disclose precursors at the pre–AGM stage (Garcia-Porrero et al., 1998).

Several previously unreported expression patterns of these two factors bearing on vascular and hemopoietic development, are described here. Lmo2 is shown to be expressed by precursors of the vascular system and to persist only in intraembryonic endothelial cells. Moreover, Lmo2 mRNA is observed in all the sites endowed with a hemopoietic potential, where its expression is tightly regulated spatiotemporally. The rapid modifications of Lmo2 expression patterns suggest that it allocates specific combinations of transcription factors during key points of
development. The overlapping expressions of Lmo2 and GATA-3 suggest combined functions during specific steps of definitive hematopoietic development, namely: (1) endodermal induction leading to the emergence of hematopoietic precursors in the mesoderm, (2) determination of these cells from the mesoderm, and (3) their production in the aortic region from 9 to 12 dpc as well as their release into the blood stream.

Interestingly, a environmental role of GATA-3 is uncovered by an expression in the rudiments of hematopoietic organs (FL, thymus) before the entry of colonising hematopoietic cells. Moreover, the floor of the dorsal aorta, which harbours hematopoietic clusters during the AGM stage, constantly overlays a GATA-3-positive domain that appears involved in intraembryonic stem cell generation.

MATERIALS AND METHODS

Embryos
Embryos were derived from BALB/C mice (Iffa-Credo). The day of vaginal plug observation was considered as 0.5 day postcoitum (dpc). Embryos were staged by somite counting or, during presomite stages, according to the criteria of Downs and Davies (1993).

In situ hybridisation
DIG-labelled (Boehringer) Lmo2 and GATA-3 riboprobes were obtained from PCR fragments (Lmo2: 500 bp CDR fragment subcloned at the Bluescript EcoRI site; GATA-3: 367 bp fragment subcloned into a pCR™II vector). Whole-mount in situ hybridisation was performed according to Henrique et al. (1995). After an overnight incubation in alkaline phosphatase-coupled anti-DIG antibody (Boehringer; dilution 1/2000), hybridised embryos were revealed at 37°C with NBT/BCIP (Boehringer). To obtain sections, embryos were incubated in alkaline phosphatase-coupled anti-DIG antibody was performed according to Henrique et al. (1995). After an overnight incubation in 4% paraformaldehyde, 4% sucrose, 0.12 mM CaCl₂, washed

RESULTS

In the YS, Lmo2 expression progressively restricts to hematopoietic cells while GATA-3 is not expressed
Lmo2 expression, which appears in the whole extraembryonic mesoderm from the early streak stage, outlines mesoderm progression towards the anterior region of the gastrula, building up a ring after the mid-streak stage. Through early neural plate stages, individual patterns differ. In earlier embryos (7/17), Lmo2-positive cells still form a ring (Fig. 1A) while, in older (10/17) embryos, they are isolated or form small clusters scattered along the whole circumference of the extraembryonic mesoderm (Fig. 1B). The analysis of sectioned embryos shows that Lmo2 is downregulated in most mesodermal cells while it restricts to early blood islands. During blood island evolution, Lmo2 domain regains a ring form (Fig. 1C), which will acquire a network structure after head fold stages (Fig. 1D,F). At 8 dpc, when endothelial (EC) and hematopoietic cells become clearly distinct, it appears that Lmo2 is not expressed by extraembryonic EC (Fig. 2D), a feature that persists throughout development. From 8.5 dpc, Lmo2 signal in circulating hematopoietic cells gradually decreases and is no longer detected by 10 dpc. This downregulation is seen more clearly in the YS than in the embryo proper, due to the lack of Lmo2 expression in extraembryonic EC (Fig. 1G,H).

GATA-3 is not expressed in the YS, either in the blood islands or in YS-derived hematopoietic or endothelial cells (Fig. 1E). This observation was confirmed by RT-PCR analysis of YS explanted before the establishment of vascular connections with the embryonic vessels (Data not shown).

GATA-3-positive mesodermal cells lay close to Lmo2-positive hematopoietic cluster-bearing vessels
Intraembryonic Lmo2 transcripts appear in the basis of the allantois at the early head fold stage. By the late head fold (LHF) stage, numerous scattered Lmo2-positive cells flank the embryo proper, including the cephalic mesoderm (Fig. 1D). Both Lmo2 and GATA-3 are expressed in the allantois and caudal Sp where the hematopoietic cluster-bearing vessels (aorta, omphalomesenteric and umbilical arteries) develop. Whereas Lmo2-positive cells distribution (two parallel arrays: Figs 1F, 2A) correlates with that previously described for angioblasts (Coffin et al., 1991), GATA-3 expression (Figs 1E, 2E) is diffuse. When angioblasts assemble into the rudiments of the vascular system during the early somite stages (Coffin et al., 1991), Lmo2 remains expressed in intraembryonic EC. In aortic EC, Lmo2 expression, restricted initially to the caudal region, transiently extends to the trunk (8.5-10 dpc: Fig. 1G,H). The initial observation of whole-mount embryos suggested a GATA-3 expression in intraembryonic cluster-bearing vessels (Figs 1E, 2E). However, in sections, it appeared that EC did not express GATA-3. The vessels seemed labelled in toto because they lay close to GATA-3-positive cells (Figs 2F,G, 3A). In addition to the endothelial expression, a weak and uniform Lmo2 signal is present in the caudal extraembryonic mesoderm (Fig. 2C,D). The posterior ventral mesoderm of the tail bud

Immunostaining
To obtain multiple stainings, alternate sections were processed in parallel for immunostaining and in situ hybridisation. Rat monoclonal antibodies (MoAB) were used at the following dilutions: biotinylated anti-mouse CD44 (Caltag), 1/40; biotinylated anti-mouse CD45 (DAKO, M851) was used at a 1/50 dilution. Rabbit polyclonal anti-AA4.1 hybridoma line, 1/25. Mouse MoAB anti-smooth muscle actin (Pharmingen), 1/25; AA4.1, purified from the supernatant of the AA4.1 hybridoma line, 1/25. Mouse MoAB anti-smooth muscle actin (DAKO M851) was used at a 1/50 dilution. Rabbit polyclonal anti-bovine tyrosine hydroxylase (Eugene Tech International) was used at a 1/200 dilution. Goat anti-rat (DAKO), anti-mouse or anti-rabbit biotinylated antibodies (Vector) were used at a 1/100 dilution as secondary steps. Tyramide signal amplification (NEN Dupont) was used to detect antigens expressed at low levels (CD45 and AA4.1). Immunostainings were performed as previously described (Garcia-Porredo et al., 1998; Petrenko et al., 1999).

Histochemical identification of primordial germ cells (PGCs)
PGCs were identified through their alkaline phosphatase activity. Sections were incubated for 15 minutes in 25 mM Tris-maleate buffer containing 1 mg/ml Fast Red TR salt (Sigma), 0.4 mg/ml α-naphtyl MX phosphate (Sigma) and 4 mM MgCl₂. Enzyme activity was localised by a bright pink reaction product.

Computer-assisted overlap of alternate staining
Photographs of alternate sections were acquired with a Umax 1200CS scanner and superimposed using the Photoshop software after processing the signal in false colours.
maintains high Lmo2 (Fig. 1G,H) and GATA-3 (Fig. 1I) expression levels up to 10 dpc.

**During the 5-somite stage, the endoderm underlaying the aortae expresses Lmo2 and GATA-3**

Endodermal Lmo2 expression is restricted to the intraembryonic compartment (Fig. 2A-D). Moreover, only the caudalmost endoderm expresses Lmo2 (Fig. 2B-D). Finally, this signal, extremely transient, occurs only during the 5-somite stage (8 dpc). Even amongst the eight embryos analysed at this stage, only six displayed an endodermal signal. The timing of Lmo2 expression in the intraembryonic endoderm thus seems extremely tightly controlled. The intraembryonic endoderm also expresses GATA-3 (Fig. 2F-G). However, this signal is conspicuously absent from the dorsal aspect of the developing gut, between the paired dorsal aortae. Overlapping endodermal expression of the two factors thus only occurs during a few hours, precisely in the region shown to give rise to intraembryonic precursors (Cumano et al., 1996).

Lmo2 is transiently expressed in the first somites

A highly transient Lmo2 expression is also observed in the lateral part of the three most anterior somites (Fig. 2A,B) during the 5-somite stage. These new data concur with a possible participation of Lmo2 to somite development, already suggested by a highly reduced number of somites in homozygous mutants (Warren et al., 1994).

**GATA-3 is expressed beneath the Ao during the whole period of intraembryonic HSC generation**

From 8 to 11.5 dpc, the tissue underlying the aortic floor expresses GATA-3; until 8.5 dpc, this layer is the endoderm (Fig. 2F-G). As Sp evolves to P-Sp/AGM, the mesodermal cells accumulating underneath the Ao yield a homogeneous GATA-3 signal. At the beginning of the AGM stage (10 dpc), a few cells, lateral or ventral, display a stronger signal (Fig. 3A). From 10.5-11 dpc (38-45 somites: Fig. 3B), GATA-3 transcripts define previously unidentified subaortic patches. Numerous small aggregates (3-10 cells) or large bilateral groups (10-20 cells) are present beneath the aortic clusters. These patches extend along 800-900 μm, immediately caudal to the FL (Fig. 3C). They disappear at 12 dpc, when hemopoietic cell generation in the AGM decreases (Godin et al., 1999). The location of GATA-3 patches below the aortic clusters during the peak of intraembryonic hemogenic activity suggests a relationship between the two structures during hemopoietic cell generation.

**Characterisation of GATA-3-positive patches in the subaortic crossroad**

Between 10.5 and 11.5 dpc, the region ventrolateral to the Ao is the site of convergence of various cell populations, such as primordial germ cells (PGC), neural crest-derived cells and cells building up the aortic wall. We used computer-generated superposition of alternate sections, hybridised with GATA-3 or stained with markers specific for these cell types to precise the nature of the GATA-3-positive patches.

**Primordial germ cells**

Hemopoietic cells have been reported to be obtained from 7.5-8.5 dpc PGCs (Rich, 1995). The culture conditions used in this study are known to promote the formation of embryonic germ (EG) cells from early PGC. EG cells mostly behave as ES cells that can give rise to various cell types including hemopoietic cells (Buehr, 1997), so that the evidence that hemopoietic cells directly derive from “non-transformed” PGC is still lacking. However, we analysed whether PGCs were present in these patches. PGCs, characterised by their alkaline phosphatase activity, were never found within the GATA-3-positive population as the only PGC that had not already reached the genital ridges were localised more laterally (Fig. 3D).

**Neural crest cells**

Truncal neural crest cells, which will give rise to the aortic plexus, dorsal root and sympathetic ganglia, and adrenal medulla, progress through the subaortic region on their ventral migration pathway (Serbedzija et al., 1990). GATA-3 expression in dorsal root and sympathetic ganglia, as well as in the adrenal medulla, has already been reported (George et al., 1994; Lieuw et al., 1997; Oosterwegel et al., 1992). We used an antibody directed against the catecholamine-synthesising enzyme tyrosine hydroxylase to identify neural crest-derived cells amongst GATA-3 patches. Tyrosine-hydroxylase-positive clusters, likely sympathetic ganglia, were observed dorsolateral to the Ao, a location where clusters of GATA-3-positive cells were also detected on alternate sections (Fig. 3E).

**Vascular smooth muscle cells**

Smooth muscle cells, detected nearby the aortic wall from 9.5 dpc, already constitute a layer around this vessel at 10-11 dpc (Takahashi et al., 1996). Smooth muscle cells accumulation thus occurs concomitantly to hemopoietic cluster appearance. In chick embryos, when aortic clusters arise, the smooth muscle cell layer below the aortic floor is thicker than dorsally (Hungerford et al., 1996), suggesting a link between smooth muscle cell distribution and the development of a hemogenic activity in this region. To test whether some subaortic GATA-3-positive cells corresponded to these cells, we immunolabelled smooth muscle actin, which is expressed from the beginning of smooth muscle cell differentiation. At 11 dpc, smooth muscle cells lay close to aortic EC, and thus do not overlap with the GATA-3-positive cells, located two to three cell layers away (Fig. 3F).

**Hemopoiesis-related markers**

We concluded from these data that, amongst the GATA-3-positive patches, those ventral to the Ao constitute a new cell type, possibly involved in intraembryonic HSC generation. We therefore analysed its relationship with two hemopoietic markers, AA4.1 and CD45.

AA4.1 antigen defines, in the 14 dpc FL, a population that includes precursors endowed with LTR activity (Jordan et al., 1990). Moreover, we previously established that single AA4.1-positive cells from the P-Sp/AGM were multipotent (Godin et al., 1995, 1999). At 10-11.5 dpc, EC and aortic hemopoietic clusters express AA4.1 (Petrenko et al., 1999). At 11 dpc, AA4.1 signal appears in cells groups ventral to the Ao in the same location as GATA-3-positive patches. We combined GATA-3 detection with AA4.1 immunostaining. The analysis of adjacent sections (10 embryos) showed that AA4.1-positive cells underlying the aorta never coalesce into tubular structures with an open lumen, nor do they contain blood cells, indicating that they do not belong to the endothelial lineage. GATA-3-
positive patches form a core nested into AA4.1-positive cells, overlap only occurring in the outer limit. These structures are often symmetric and located below the aortic clusters (Fig. 3G).

We characterised non-erythroid hemopoietic cells through their CD45 expression. CD45-positive cells are considerably more concentrated within the area separating the GA TA-3 patches from the aortic floor than near the dorsal part of the vessel. However, overlap with GA TA-3 is highly unfrequent. CD45-positive cells are often located close to the ventral EC or in the clusters (Fig. 3H).

Aortic clusters
At 10.5-11.5 dpc, a few GATA-3-positive cells are located at the basis of the clusters rather than amongst the cluster cells protruding in the lumen (Fig. 3B). Lmo2 is strongly expressed by the whole aortic cluster or by a few cells in the aortic floor (Figs 3I, 4A).

Lmo-2 and GATA-3 expression in the liver and thymic rudiments
GATA-3 is expressed in the liver rudiment before its colonisation
Lmo2-positive cells, probably hemopoietic, appear in the FL only at 10.5 dpc, after its colonisation (Fig. 4A). In contrast, a homogeneous GATA-3 signal is detected from 9.5-10 dpc, in the septum tranversum that will contribute to FL development (Fig. 4B). At 10.5-11.5 dpc, hemopoietic aggregates expressing high GATA-3 levels are present in the colonised FL (Fig. 4C).

The thymic rudiment expresses GATA-3 until its colonisation
From 8.5 dpc, the developing branchial arches (BA) express Lmo2 (8.5-9.5 dpc: 15-25 somites; Fig. 1G,H) and GATA-3 (9.5-10 dpc: 25-30 somites; Fig. 1I). When it first becomes morphologically identifiable from BA3-4 (Metcalf and Moore, 1971), the thymic rudiment clearly expresses Lmo-2 and GATA-3 (10-10.5 dpc: 30-35 somites; Fig. 4D,E,G). Sections of the labelled heads showed Lmo2 signal to be located in mesodermal cells. The thymic epithelium (endoderm of the 3rd and 4th branchial pouches (BP)) is clearly unlabelled (Fig. 4F). In contrast, besides being expressed by the mesodermal cells of the BA, GATA-3 signal is also present from 10 dpc in the thymic epithelium with a restriction to its distal aspect, opposite the foregut (30-35 somites: Fig. 4H). GATA-3 expression is downregulated in both the mesodermal and endodermal components of the thymic rudiment at 11 dpc (40-45 somites). This disappearance could correlate with the onset of thymic rudiment colonisation, roughly timed at 10.5-11 dpc in the mouse (Metcalf and Moore, 1971). To more precisely
date this event, we combined GATA-3 in situ hybridisation with the immunohistological detection of the hyaluronate receptor CD44 (or Pgp-1), which is present on the earliest thymocyte precursors (Ruiz et al., 1995), in addition to the pan-hemopoietic marker CD45.

From 9.5-10.5 dpc (25-35 somites), CD44 is expressed by the EC of the aortic arches. Interestingly, the staining is always asymmetric, being stronger in the part of the vessel close to the foregut. The endoderm of all BP, including the thymic epithelium is also labelled until 10.5 dpc (35 somites: Fig. 4I). CD44 epithelial signal also is asymmetric, being higher in the distal side of the BP, opposite to EC staining, so that epithelial expression occurs in the area of BP3-4 that displays GATA-3 signal. At 11 dpc, CD44 signal disappears from the thymic epithelium, concomitantly to GATA-3 downregulation (Fig. 4K), while the asymmetric CD44 expression is maintained in the thymic rudiment (arrowhead). Isolated CD44-positive cells, some of which also express CD45 (arrowheads), are numerous close to the side of the AA4 that highly expresses CD44. As we used an antibody directed against the standard (hemopoietic) CD44 form that recognises also other variants, different isoforms may account for the expression in the EC, thymic epithelium and hemopoietic cells.

At 10-10.5 dpc (30-35 somites), CD45-positive cells, although present within the mesenchyme, are never observed in the thymic primordium (Fig. 4J), indicating that the thymus is not yet colonised. At 11 dpc (40-45 somites), CD45-positive cells are concentrated in the area spanning the aortic arches and the developing thymus. Some of these cells also express CD44 (Fig. 4K). Others are located in CD44-positive capillaries tightened to the thymic rudiment or grouped within the nearby mesenchyme. Moreover, isolated CD45-positive cells, likely the first thymocyte precursors, are found entering the thymic epithelium (Fig. 4K), opposite to the side that previously expressed both GATA-3 and CD44. These observations,
together with the asymmetric location of GATA-3 and CD44, and their disappearance at the time of the thymus colonisation, suggest that these factors may oppose the entry of thymocyte precursors.

**DISCUSSION**

**Lmo2 displays a highly dynamic expression pattern**

An important feature uncovered here is that Lmo2 switches on and off at precise time points of development. A highly transient Lmo2 expression is observed in the lateral aspect of the first three somites and the caudal endoderm during the 5-somite stage, two sites where expression had not been reported before. An “oscillating” expression is also clear during YS development (Fig. 5). First observed in the whole invaginating mesoderm, Lmo2 expression is switched off in most mesodermal cells, persisting only in a few cells, possibly hemangioblasts. Expression endures during blood island development to finally restrict to hemopoietic cells as segregation between extraembryonic EC and hemopoietic cells occurs. Lmo2 disappears from YS hemopoietic cells after 8.5 dpc, a feature consistent with experimental results indicating that the YS does not sustain the production of hemopoietic precursors (Cumano et al., 1996; Dieterlen-Lièvre, 1975). In contrast, Lmo2 expression is maintained in intraembryonic hemogenic sites (Sp/P-Sp/AGM) during the whole phase of HSC generation. Lmo2 associates various transcription factors into a protein complex. The combinations so far described comprise: (1) Tal-1/SCL, GATA-1, E47 and the LIM-binding protein Ldb1/NLI (Wadman et al., 1997), and (2) Tal-1/SCL and GATA-3 (Ono et al., 1998), but the existence of others complexes is postulated. The highly dynamic Lmo2 expression pattern uncovered here suggests that partners in the Lmo2-anchored complex may change to produce various effects.

**Lmo2 and GATA-3 expression in the sites of intraembryonic angioblasts development**

Caudal intraembryonic mesoderm concomitantly expresses GATA-3 and Lmo2 in the area where hemopoietic cluster-bearing vessels (Ao, omphalomesenteric and umbilical arteries) develop. Whereas Lmo2 is expressed by mesodermal cells, including angioblasts that subsequently differentiate into Lmo2-positive EC, the evolution of GATA-3 expression pattern does not favour an expression by angioblasts: intraembryonic angioblasts, that do not express GATA-3, are initially surrounded by GATA-3-positive aggregates. Later, they are lined ventrally by mesodermal GATA-3-positive cells during the whole phase of intraembryonic hemopoietic precursors generation. A possible function achieved by mesodermal Lmo2/GATA-3 could be to set apart, in the Sp, a subset of undetermined mesodermal cells that will later give rise to definitive HSC amongst other cell types, or pre-hemopoietic cells determined to generate only HSC. The few cells expressing both Lmo2 and GATA-3 observed at the basis of hemopoietic clusters may represent such a cell type. These later interpretations receives some support from the combined functions of the two factors, as Lmo2 maintains cells in an immature state and promotes self-renewal (Visvader et al., 1997), while GATA-3 seems to specify cell fate (Lieuw et al., 1997; Neave et al., 1995).

The distinct features of intraembryonic and extraembryonic hemopoietic precursors may result from different inductive signals

Extraembryonic visceral endoderm influences the development of hemopoietic cells from the mesoderm (Fig. 5). YS mesoderm autonomously produces erythroid cells when isolated from the endodermal layer after gastrulation (Belaoussoff et al., 1998; Kessel and Fabian, 1987; Palis et al., 1995). Nevertheless, a normal level of blood cell formation requires an inductive signal from the extraembryonic endoderm (Belaoussoff et al., 1998; Miura and Wilt, 1969). In the embryo proper, the absence of hemopoietic cell production concomitant to blood vessel development seems to result from an ectodermal inhibition of the endodermal induction (Kessel and Fabian, 1987; Pardanaud and Dieterlen-Lièvre, 1999). When aortic clusters were shown to be involved in the establishment of definitive hemopoiesis in birds, an endodermal influence on intraembryonic precursors generation was considered (Pardanaud et al., 1989), since the clusters are restricted to the aortic floor, the only part of the vessel that has been in contact with the endoderm (Fig. 5). In the mouse, we considered that the distinct features of Sp and YS hemopoietic precursors disclosed in vitro (Cumano et al., 1996) could result from different inductive signals. This possibility is supported by the fact that YS endoderm is of a primitive type whereas intraembryonic endoderm derives from the epiblast during gastrulation (Rossant, 1995).

We show here that endodermal Lmo2 expression is restricted to: (1) the intraembryonic compartment, (2) the caudal Sp, the only embryonic region shown to generate definitive hemopoietic precursors, and (3) the 5-somite stage, this highly transient expression thus occurring during HSC determination from the mesoderm, as determined in vitro (Cumano et al., 1996). In contrast, GATA-3 is expressed longer and more widely in the intraembryonic endoderm. However, endodermal GATA-3 being conspicuously absent between the paired aortae, Lmo2 and GATA-3 only overlap during the 5-somite stage, in the caudal intraembryonic Sp and specifically under the developing aortae (Fig. 6). This tight spatiotemporal window of Lmo2/GATA-3 coexpression is consistent with an inductive process. Whereas endodermal Lmo2/GATA-3-associated expression seems unrelated to EC development as it occurs too late, a function during the determination of intraembryonic hemopoietic precursors may be involved. The nature of the target cell in the mesoderm (undetermined mesodermal cell, hemangioblast or pre-hemopoietic cell), as well as the function of endodermal Lmo2/GATA-3, remains unclear. The viability of both Lmo2 and GATA-3 null mutants during early stages of intraembryonic hemopoietic development should allow an experimental approach of these questions.

**GATA-3 expression is restricted to sites involved in definitive hemopoietic development**

In mutants with disrupted GATA-3 gene (Pandolfi et al., 1995), YS hemopoiesis occurs normally, death occurring concomitantly with the onset of definitive hemopoiesis in the fetal liver. Observations collected here are in perfect agreement, as GATA-3 is never observed in the YS. In contrast GATA-3 is constantly expressed in the sites of intraembryonic hemopoietic cell generation (Sp, P-Sp/AGM).
In zebrafish (Neave et al., 1995), avian (Leonard et al., 1993) and human (Labastie et al., 1998) embryos, GATA-3 is also restricted to sites linked to the development of definitive hemopoiesis. In *Xenopus*, however, GATA-3 transcripts are present in both the ventral blood island (VBI) and dorsal lateral plate, regions respectively equivalent to YS and P-Sp/AGM (Bertwistle et al., 1996; Turpen et al., 1997). This may appear in contradiction with our claim that GATA-3 is necessary only for the establishment of definitive hemopoiesis, however, in *Xenopus*, the VBI also provides cells that contribute to definitive hemopoietic development (Turpen et al., 1997).

**Fig. 6.** Lmo2- and GATA-3-associated expression during the development of intraembryonic hemogenic sites.

**Fig. 5.** Steps involved in endothelial and hemopoietic development in the extraembryonic and intraembryonic splanchnopleura. Combinations of genes expressed in the two compartments. 1Silver and Palis 1997; 2Kallianpur et al., 1994; *present data. Each row displays equivalent stages of YS and Sp endothelial development. Note that, compared to the YS, intraembryonic events are delayed.

**GATA-3 is expressed in hemopoietic environment**

An extrinsic function of GATA-3 during the development of definitive hemopoiesis has been inferred from the respective phenotypes of the null mutants (Pandolfi et al., 1995) and chimeras (Ting et al., 1996), but this function is still elusive. Up to now, GATA-3 expression in the hemopoietic environment has never been documented. Our findings indicate that GATA-3 is expressed in the environment in which intraembryonic precursors emerge (Sp/P-Sp/AGM), but also in the developing hemopoietic rudiments before their colonisation, in the septum transversum from which the FL develops and in the thymic rudiment until the arrival of the first migrants. Strikingly, no CD45-positive hemopoietic cells are found within the GATA-3 domain, which is our main argument to attribute environmental roles to this transcription factor.

**Intraembryonic hemogenic sites**

Lmo2 and GATA-3 co-expression in mesodermal cells becomes lost during early somite stages, except in the caudalmost ventral mesoderm until tail formation. When...
expressions segregate, a close contact is maintained between the Lmo2-expressing aorta and a GATA-3-positive underlying domain during the whole duration of intraembryonic precursor generation (Fig. 6). Up to 8.5 dpc, this layer is endodermal. From the 15-somite stage, when the first intraembryonic hemopoietic precursors becomes detectable in vitro without a preliminary organotypic culture step (Godin et al., 1995), GATA-3-positive cells in contact with the aortic floor are part of the splanchnic mesoderm. We would like to suggest that GATA-3 endodermal expression might correlate with precursors determination from the mesoderm whereas GATA-3 mesodermal expression might be linked to their generation.

The size and frequency of occurrence of aortic hemopoietic clusters culminate when the number of precursors peaks in the AGM (Godin et al., 1999), while they become detectable in significant numbers in the blood stream (Delassus and Cumano, 1996). We thus interpret the hemopoietic clusters as reflecting the translocation of newly born stem cells into the blood stream through the ventral aspect of the aorta. This process most probably begins at 9 dpc (15 somites), when the first precursors are born in the P-Sp, but cannot be disclosed morphologically due to the minute number of precursors concerned. A direct relationship between GATA-3 expression and HSC generation is suggested by the fact that its expression domain: (1) is restricted to the ventral aspect of the aorta, that bears the clusters, (2) is better defined at 10.5-11.5 dpc when intra-aortic hemopoietic clusters are detected and the number of generated precursors is at its highest, (3) is preferentially located under the intra-aortic hemopoietic clusters, and (4) disappears after 12.5 dpc, when intraembryonic precursors are no longer generated (Godin et al., 1999). The lack of CD45 expression within the GATA-3 patches suggests that this previously unknown structure is of a “stromal” nature. Interestingly, CD45-positive cells are highly concentrated in the area spanning the patches and the intra-aortic clusters, suggesting that they may be produced in the GATA-3-positive subaortic region. We propose that CD45-positive cells migrate towards the aortic floor, building up the clusters, a hypothesis that we intend to check by a cytological and in vitro analysis of the GATA-3 null mutant.

P-Sp/AGM multipotent precursors may be isolated based on AA4.1 antigen expression (Godin et al., 1995, 1999). The external layer of GATA-3 patches that expresses AA4.1 may represent stromal cells that participate in the process of generation and/or release of the hemopoietic precursors or, alternatively mesoderm-derived “pre-hemopoietic” precursors. Further away from the GATA-3 patches, hemopoietic precursors expressing AA4.1 and/or CD45, as well as a few GATA-3/Lmo-2-positive cells are often seen close to the basis of the intra-aortic clusters.

**GATA-3 may play a role during thymus colonisation**

Information regarding the initial stages of thymus development are scarce. Thymus colonisation, for example, is roughly dated at 11-12 dpc (Fontaine-Perrus et al., 1981). We show here that besides being expressed widely in the BA mesoderm, GATA-3 and CD44 are expressed in the thymic epithelium. Interestingly, epithelial expression: (1) disappears shortly before the appearance of the first CD45-positive cell in the thymic rudiment at 11-11.5 dpc (40-45 somites), and (2) is higher on the side of BP3-4 opposite the translocation site of colonising cells, the aortic arches. In addition, aortic arch-EC also expresses CD44 asymmetrically with a higher level opposite the thymic rudiment. As CD44 is involved in cell migration (Ruiz et al., 1995), it is tempting to interpret the opposite expressions in aortic arch-EC and thymic epithelium as local cues influencing the seeding of the thymic rudiment. These expression patterns suggest that CD44 and GATA-3 may oppose thyromyocyte precursors entry before 11 dpc, a hypothesis strengthened by the fact that multipotent precursors are present in blood vessels at earlier stages (Delassus and Cumano, 1996). It has clearly been established through chimera analysis that the avian thymus is refractory to precursor entry before E5, despite the fact that precursors are available in the circulation and that, when colonised, the full thymus prevents other precursor entry (Le Douarin and Jotereau, 1975). Although a lot of attention has been given to the factors involved in lymphoid progenitor homing (Dunon and Imhof, 1993), no data are available on the mechanisms responsible for the “refractory period”.

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