MOLECULAR ASPECTS OF HAEMATOPOIETIC STEM CELL EMERGENCE IN THE AVIAN EMBRYO

Co-directed thesis for PhD

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FREQUENTLY USED ABBREVIATIONS

ac-LDL-DiI: acetylated-low-density-lipoprotein-3,3'dioctadecylindocarbocyanine-iodide

AGM: aorta-gonad-mesonephros

AO: area opaca

AOE: area opaca endoderm

AP: area pellucida

APE: area pellucida endoderm

bFGF: basic fibroblast growth factor

BL-CFC: blast colony-forming cell

BMP: bone morphogenetic protein

CD: cluster of differentiation

CFU-S: colony forming unit in the spleen

DLP: dorsal lateral plate

dpc: day post coitum

E: embryonic day

EB: embryoid bodies

Ep: epiblast

ES cell: embryonic stem cell

Hb: haemoglobin

HH: stage of Hamburger and Hamilton

HSC: haematopoietic stem cell

ICM: intermediate cell mass

LTRA: long-term repopulating ability

M: mesoderm

mAb: monoclonal antibody

PIP: posterior intestinal portal

PS: primitive streak

P-Sp: paraaortic splanchnopleura

SCID: severe combined immuno-deficiency

SCL/tal-1: stem cell leukaemia/T-ALL, acute T-cell lymphoblastic leukaemia-1

TF: transcription factor

VBI: ventral blood island

VEGF: vascular endothelial growth factor

VEGFR-2/flk-1: receptor 2 for the vascular endothelial growth factor/ fetal liver kinase-1

PREFACE

Haematopoietic stem cells (HSC) are routinely used today in clinical haematology to treat leukaemia and recent findings offer further possibilities of using them in other fields of medicine such as genetic disorders or cell and tissue replacement. Before of this type of application, however, it is very important to precisely reveal their characteristics, origin and genetic programmes. To understand the properties of the adult HSCs it is important to know from where they come from in the embryo and what are the regulatory mechanisms that specify them.

One of the useful models to investigate these processes is the avian embryo which has contributed much to our knowledge of how the haematopoietic system develops during the embryogenesis of higher vertebrates. Pioneer experiments on bird embryos demonstrated that haematopoietic stem cells, which ensure the needs of the adult blood system in the bone marrow, are not derived from the yolk sac but have an intraembryonic source, namely the dorsal aorta region. The relatively large size and accessibility of avian embryos to manipulation provide the possibility to study cellular migrations and interactions that occur during development. In addition, the growing number of chicken genetic databases raises the possibility of revealing the molecular regulatory mechanisms of these processes.

INTRODUCTION

THE HAEMATOPOIETIC STEM CELL

What are stem cells?

Defining a stem cell today is not an easy task following the findings of the past years and with our changing understanding about them. However most scientists are in agreement on at least two criteria: self-renewal and the capability for terminal differentiation; in other words stem cells are capable to give rise to two types of cell, one for keeping the stem cell pool, and one for giving rise more differentiated cells. During embryogenesis stem cells are rather the units of the tissue differentiation and organogenesis while in the adult they are responsible for the maintenance of rapidly differentiating tissues (like epidermis, gut epithelia and blood) and also for tissue repair (in the case of the liver and muscles). Surprisingly, in the last decade, multipotent stem cells were also isolated in the central nervous system, where previously none of this type of cells was described (1). Thus, the results of the last decade confirmed the earliest suggestion that stem cells are present in all type of tissues and organs in the adult.

Stem cells have "come into fashion" in the past few years because of their potential medical importance. The unexpected findings that stem cells of different tissues can substitute for each other - and thus have wider differentiation potential - (for review see Weissman (2)) placed stem cells at the center of scientific and social interest. However the recent claims that both neural and haematopoietic stem cells can contribute to a number of mesodermal cell types, needs to be validated by future studies regarding the purity of the input stem cell population.

Stem cells could be used in tissue repair or replacement and provide a tool for gene therapy. Before the general use of these cells however, there are

some important problems that need to be resolved. How do these cells develop during embryogenesis and once formed, how they are regulated? What are the commitment events from stem cells toward the more differentiated progenitors and terminally differentiated cells? And the most important question from the medical point of view, how the balance between self-renewal and commitment becomes wrong and leads to disease.

The haematopoietic system provides a complete hierarchical tree from the single pluripotent stem cell, which gives rise to numerous cell types and is relatively accessible for examination. In addition, it is one of the best-characterised developmental systems, therefore the investigation of this system can provide further data both on the development of the haematopoietic system, and on the differentiation or cell fate decisions in general.

Haematopoietic stem cells in the adult

Adult haematopoietic stem cells (HSC) (that originate from embryonic precursors) are pluripotent, self-renewing cells (3; 4) and produce the progenitors of all blood cell lineages. In adult mammals, they occur in the bone marrow, and represent only the 0.01 % of mononucleated cells (5). Because they do not show characteristic morphological properties, HSCs were characterised mainly by functional tests. Development of the monoclonal techniques made possible to identify them also by their surface phenotype. In the following chapters several methods that were developed for the investigation of the adult HSC will be summarised because they are also used for studying the embryonic haematopoietic progenitors.

Functional tests for HSC

Functional studies from the 1950's demonstrated HSCs as cells to be capable of reconstituting the haematopoietic system of the host animal, after lethal irradiation (6). Classical experiments of Till and McCulloch (7), characterising the properties of HSCs, identified macroscopic colonies in the

spleen of irradiated mice after injection of normal bone marrow cells. Founder cells of these clonal aggregates were called later CFU-S (Colony Forming Unit in the Spleen) and were believed to be equivalents of the HSCs for several years (8; 9). Actually, the CFU-S cells are considered to be short-term repopulating cells (10) and only a subpopulation of these cells possess the property of long-term reconstitution (11).

Other *in vivo* tests were developed for the identification of the HSC. *In vivo* tests include radioprotection assays to distinguish between short-term and long-term repopulating ability (LTRA) of haematopoietic progenitors (10; 12; 13; 14). The SCID (severe combined immuno-deficiency) mouse is used in experiments to study the human HSC *in vivo*. Homozygote animals bearing the *scid* mutation do not produce T and B cells which makes them deficient for both the cellular and humoral response (15) therefore offers the possibility to populate them with xenogenic human haematopoietic cells.

In vitro tests are also used to investigate the haematopoietic progenitors. Two approaches were developed: clonal cultures in semi-solid medium (agar, methylcellulose) with added growth factors, or culture on stromal feeder layer. The advantage of these *in vitro* methods is the identification of the haematopoietic precursor, before it displays the morphological properties of the differentiating haematopoietic cell. However, for just this reason, these approaches suggest only the developmental potentialities of the cells rather than the real *in vivo* capabilities. Table 1. summarises the most frequently used methods.

Table 1. Functional tests to characterise the HSC and haematopoietic progenitors				
Functional assay		**HSC/ Haematopoietic progenitor type or test**	**description**	**references**
in vivo		CFU-S	colony-forming unit-spleen	7
		radioprotection ST-HSC	short-term repopulating ability	10; 12
		LT-HSC	long-term repopulating ability	11
in vitro	semi-solid medium	mix-CFC	mixed-lineage colony -forming cell (committed precursors for erythro-myeloid lineages)	16
		HPP-CFC	high-proliferative potential colony-forming cell	17
	semi-solid medium and stromal feeder layer	BI-CFC	blast colony-forming cell	18
	stromal feeder layer	CAFC	cobblestone area-forming cell	19
		bone marrow cultures LTC and LTC-IC	long-term culture and long-term culture initiating cell	20; 21
	organ culture	FTOC	fetal thymic organ cultures	22

Selection methods based on the quiescent state of the HSCs

HSCs, which are capable for long term reconstitution, are in the G_0/G_1 phase of the cell cycle, thus are resistant for some chemical agent acting only on dividing cells (17; 23; 24). This feature can be used to enrich the HSC population.

Rhodamin 123 (Rho) is a vital colorant which is capable to fix on the mitochondrial membrane. Quiescent HSCs, having few mitochondria than active, dividing cells, fix thus small amount of Rho. Studies on mouse and human haematopoietic progenitors showed that, in correlation with the data cited above, the Rho^{low} population of progenitors contains the most primitive

HSCs (11; 25), which are in G_0/G_1, and in the mouse, reconstitute lethally irradiated animals.

Another dye to select hematopoietic progenitors is the fluorochrome Hoechst 33342 (Hst). This molecule reversibly binds to DNA and makes possible to distinguish the G_0/G_1 phase from the S/G_2+M, when the DNA content of the cells is doubled (26). The RNA quantity of the cells in G_1 is higher than in G_0, so further selection can be achieved on the G_0/G_1 progenitor population. Pyronine Y (PY) is used for this selection, which detects the RNA contents of the cells. Goodell et al. (27) identified a subpopulation (called side-population, SP) of the Hst positive cells. These cells are capable to reconstitute the bone marrow of irradiated mice in competition assays.

The 5-fluoruracil (5-FU), is an antimitotic agent, toxic to dividing cells. While HSCs are in dormant state, the treatment of 5-FU eliminates the mature progenitors from the haematopoietic populations but spares the HSC (28).

Phenotype of the adult HSC

In the absence of unique markers, investigators use more than one surface marker for subsequent positive or negative selection of HSC populations in experimental or clinical practice. Some of these markers are described in table 2.

Table 2. Some surface marker of haematopoietic progenitors		
Marker gene	**Description**	**Reference**
CD34	glycoprotein, adhesion molecule	29
CD45 (also called Ly-5 in the mouse)	pan leukocyte antigen, membrane-associated phosphatase	30
Sca-1/Ly-6^E/TAP	stem cell antigen, surface glycoprotein	13
c-kit	receptor tyrosine kinase	31
Thy-1	glycoprotein	32
AA4.1	cell surface protein	33
flk-1/VEGFR-2	receptor tyrosine kinase for VEGF	34
tie (tie1)	receptor tyrosine kinase	35
tek (tie2)	receptor tyrosine kinase for angiopoetins	35
CD43	cell surface molecule	36

Expression of CD34, the sialomucin type cell surface glycoprotein, is thought to be a good marker for HSC, the exact function of this molecule, however, has remained elusive until recently. It is expressed by haematopoietic progenitors, endothelial cells, embryonic fibroblasts and bone marrow stromal cells (37; 38; 39; 40). In the haematopoietic population the most primitive cells strongly express this antigen, and the expression is progressively downregulated during differentiation (41).

Patients transplanted with bone marrow or blood enriched for $CD34^+$ cells engraft rapidly and seem to have fewer transplant-related complications (42). The idea of using this marker in clinical applications came from the observation that CD34 is present only in a small fraction of human bone marrow cells (43). Until recently it seemed that the CD34+ population contains the true stem cells of the haematopoietic system. Further selection indicated that stem cells are in the $CD34^+$ population which express Thy-1 (surface glycoprotein) and c-kit receptor, and that do not express neither the CD38 antigen nor any blood cell lineage markers (such as CD3, CD4, CD5, CD8, CD13, CD15, CD19, CD20, CD33, CD66, CD71, Mac-1, B220, NK-1.1, they are Lin^-).

Using the colorants Hst and PY, the G_0 and G_1 subpopulations of the $CD34^+$ haematopoietic progenitors can be selected. The $G_0/CD34^+$ cells are

more effective in *in vitro* assays and in terms of reconstitution than the $G_1/CD34^+$ cells (44;45).

While CD34 is a useful marker in clinical research, recent data by Osawa, Goodell and co-workers suggest that at least some primitive progenitors of human and mouse bone marrow lack CD34 on their surface but are capable of generating CD34+ stem cells (46; 47). These authors suggest thus the existence of some kind of $CD34^-$ pre- HSC, which could be the precursor of the $CD34^+$ HSCs.

Most recent studies examine the specific gene profiles and the genetic program of the stem cell populations which characterise the stem cell state, rather than some particular surface markers (48).

All of these *in vivo* and *in vitro* approaches to characterise the HSC contributed to the construction of the hierarchical pyramid of adult haematopoietic populations. According to the current conception, the population of haematopoietic cells can be subdivided into sequential compartments. The most primitive, long-term repopulating stem cells (long-term HSC: LT-HSC) compose the top of the pyramid. They are not numerous and divide slowly. The next progenitor population can be divided into three subpopulations: the short-term HSCs (ST-HSC), the preprogenitor and lineage-committed progenitor cells. Finally the dividing and maturing cells make up the base of the pyramid. The cells in each compartment are the amplified progeny of cells in the preceding compartment. The hierarchical pyramid of these populations is presented on Figure 1.

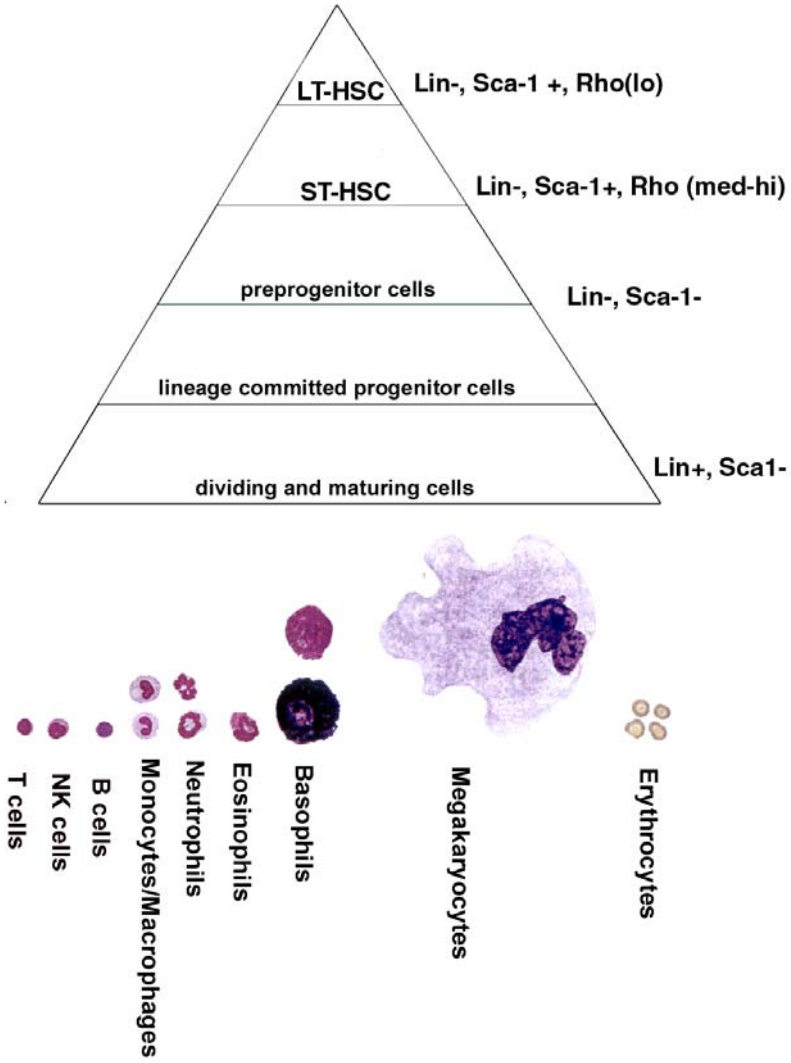


Figure 1. Haematopoietic subpopulations. Cells can be separated using mature lineage-specific markers (Lin), stem cell antigen-1 (Sca-1), or by staining with Rhodamine 123 (Rh) (Adapted from Metcalf (49)).

FORMATION OF HAEMATOPOIETIC STEM AND PROGENITOR POPULATIONS DURING EMBRYOGENESIS

Developmental processes of haematopoiesis have been described in different vertebrate model organisms (Zebrafish, Xenopus, chicken and mouse). These data are equally important to understand the main characteristics of this process. In this chapter, the main steps of vertebrate embryonic haematopoiesis will be summarised, focusing on the avian embryo model, which I used in my experiments.

Embryonic haematopoiesis, a historical overview

The first theories explaining embryonic haematopoiesis were based mainly on histological observations of the mouse embryo. During embryogenesis, the first blood cells are detected in the extraembryonic yolk sac. Subsequently, haematopoiesis is detected in the fetal liver, thymus, spleen and finally in the bone marrow. Two models were constructed to explain these observations; one of them supposed the *in situ* emergence of haematopoietic progenitors from the mesenchyme of each of these organs (stromal origin); the other postulated that the fetal and adult progenitors were derived from the original embryonic blood precursors emerging in the yolk sac (extrinsic origin). For a long time the second theory was accepted as a dogma.

Haematopoietic organs are colonised by haematopoietic progenitors of extrinsic origin

The extrinsic origin of stem cells in haematopoietic organs has been demonstrated for the first time in birds, and has been shown only later to be true for all vertebrates. Moore and Owen originally showed, that stromal haematopoietic organ rudiments are colonised by extrinsic, blood-borne stem cells in order to become functional (50;51). They obtained chimeric haematopoietic organs by parabiosis of chick embryos, and analysed the organs of the partners using the pairs of sexual chromosomes as markers.

These experiments demonstrated that haematopoietic cells colonising the haematopoietic organs come from elsewhere and do not develop *in situ* from the mesenchyme.

Moore and Owen thought that the yolk sac, an embryonic appendage was the only source of the seeding cells. In this classical model, the progenitors, developing in the yolk sac migrate to a fetal haematopoietic site, such as the liver, where they differentiate into fetal liver stem cells. In a second wave, stem cells migrate to the bone marrow to ensure the constant existence of blood cells during lifetime. Scientists accepted this idea for a long time, since other experiments, also undertaken on the avian model, changed this view.

Definitive haematopoietic cells have an intraembryonic source

The quail/chick chimera system, developed by Nicole Le Douarin offered a powerful experimental method to trace cell fates in the avian embryo (52). When pieces of the quail embryo are grafted into a similar region of the chick, the cells become integrated into the host and participate to the construction of the appropriate organs or tissues. This grafting can be done in ovo and the chick that hatches is a "chimera", having a part of its body composed of quail cells. Quail-derived cells can be distinguished from that of the chicken by their nuclei. In quail cells the heterochromatin is a large, deeply stained mass, which is concentrated around the nucleoli. In chicken nuclei the heterochromatin is diffuse, and is distributed between fine chromocenters. In the last 15 years, some monoclonal antibodies have been developed against antigens that are species-specific (such as QCPN to detect quail nuclei, or QH1 against quail haematopoietic and endothelial cells) further refining the chimerism detection.

The chimera method further extended the theory of Moore and Owen that progenitors of extrinsic origin colonise the haematopoietic organs. In particular, this method revealed that the thymus is colonised by successive waves of haematopoietic cells, and colonisation periods are interrupted by refractory stages (53).

The quail/chick chimera method also revealed the existence of an intraembryonic source of haematopoietic stem cells. In order to determine the contributions of the extraembryonic yolk sac and the embryo proper in the haematopoietic progenitor production, Françoise Dieterlen-Lièvre and their colleagues developed the use of the yolk sac chimera (54). This technique associated a quail embryo onto an extraembryonic area of a chick embryo. Analysis of the haematopoietic organs of chimeras showed that colonising haematopoietic cells were exclusively of quail origin. In addition, a large amount of circulating blood cells had the nuclear characteristics of quail cells (55). Similar experiments were undertaken by associating different chicken strains (differing by various markers) and these homospecific chimera experiments reinforced that the data of the quail-chick combinations were correct (56).

Thus, these experiments refuted the long-held notion that the yolk sac was the site of origin of the adult haematopoietic system, and turned attention to the intraembryonic source of the HSC.

Diffuse haematopoietic foci were first described by Miller, in 1913 in the avian embryo (57). Dieterlen and co-workers reinvestigated the emergence of these cells by the yolk-sac chimera method (56). The experiments demonstrated that the cells developing in these foci were formed *in situ*, in the mesenchyme, and did not originate from the yolk sac, suggesting a possible source for definitive haematopoietic progenitors.

Origin and characteristics of the primitive and definitive haematopoietic progenitors

According to the current knowledge, formation of vertebrate blood cells takes place in successive waves at different anatomical locations during development. The molecular program and the basic morphological aspects of these events appear to be conserved between vertebrate species. Two types of haematopoietic stem cell activity exist in the developing vertebrate embryo: primitive and definitive. Primitive haematopoiesis (or "ventral" type, according to the frog nomenclature, see later) takes place in the yolk sac analogue compartments and only gives rise to a transient wave of blood cells, endowed with a limited developmental potential that is probably restricted to the erythro-myeloid lineage. The definitive wave of haematopoiesis (or "dorsal" type) occurs slightly later in the embryo proper, associated to the aorta. Progenitors developing in this location have broader developmental potential and are capable of producing the complete set of haematopoietic lineages. Thus a second and stable population of definitive HSC replaces the initial haematopoietic progenitors. The primitive progenitors and definitive stem cells apparently originate independently from different embryonic sites. Sites of haematopoietic progenitor emergence in vertebrates are summarised in Table 3.

Table 3. Comparison of the haematopoietic sites and organs in vertebrates

Species	**ventral site**	**dorsal site**	**main haematopoietic organs**
Fish (Danio rerio)	ICM	aorta	pronephros anterior mesonephros thymus
Frog (Xenopus laevis)	VBI	DLP and aorta	thymus bone marrow
Bird (Gallus gallus)	yolk sac	aorta intraaortic clusters paraaortic foci	thymus bursa of Fabricius spleen bone marrow
Mammals Mus musculus Homo sapiens	yolk sac	P/Sp; AGM	liver thymus spleen bone marrow

ICM: intermediate cell mass; VBI: ventral blood island; DLP: dorsal lateral plate; P-Sp: paraaortic splanchnopleura; AGM: aorta-gonad mesonephros; see references in the text

Origin of the primitive precursors and haematopoiesis in the yolk sac analogue compartments

As previously mentioned, the yolk sac was believed for a long time to be the source of haematopoietic progenitors colonising the fetal haematopoietic organs. Experiments on different vertebrate species have shown that this embryonic appendage has a limited contribution to this process. In contrast, blood progenitors developing in this extraembryonic location have important roles at the beginning of development. They produce the first wave of erythrocytes to fill the gap between passive oxygen diffusion and the production of fetal and adult erythrocytes by later progenitors.

In higher vertebrates, the first haematopoietic cells develop in the yolk sac. The origin of these cells is the extraembryonic mesoderm, which is the earliest tissue formed from the epiblast during gastrulation. In birds, fate mapping studies have demonstrated that extraembryonic mesoderm is derived from the posterior part of the primitive streak (58;59). Mesodermal cells leaving the primitive streak migrate laterally and anteriorly and invade a horse-shoe shaped area between the yolk-rich extraembryonic endoderm and extraembryonic ectoderm. As gastrulation proceeds and the primitive streak regresses cranio-caudally, extraembryonic mesodermal cells remain in more central positions in the blastoderm.

In the yolk sac, haematopoietic cells differentiate in close association with endothelial cells in specific cell aggregates, called blood islands. During the initial formation of these structures, mesenchymal cells become packed together, close to the endoderm, in the postero-lateralmost aspect of the blastoderm, in the avian embryo. These aggregates progressively mature into blood islands as peripheral cells flatten into endothelial cells and internal cells become rounded, haematopoietic cells. During the somitic stages, blood islands begin to differentiate into blood vessel and intravascular blood cell groups, which remain attached to the vessel wall. Thereafter, haematopoietic cells are progressively released into the circulation. Steps of this morphological differentiation process have been described in detail (60).

The problem of the hemangioblast

Observations made in the early 20th century that haematopoietic cells differentiate in close association with endothelial cells lead to the hypothesis that both cell types originate from a common precursor, the hemangioblast (61; 62). Recent molecular and functional studies also support this idea. For example, increasing numbers of molecules is expressed by both haematopoietic and endothelial lineages (see Table 4.). Two of them, the receptor 2 for the vascular endothelial growth factor (VEGFR-2), and the transcription factor SCL (from stem cell leukaemia); were shown to be critical for the development of the hemangioblast population. Loss- and gain-of-function experiments performed on higher and lower vertebrates (details will be reported later), showed that both are important for the development of the haematopoietic and endothelial lineages (63;64; 65; 66; 67; 68; 69).

Table 4. Common markers for hematopoietic and endothelial cells

marker	description	reference
CD34	adhesion molecule	70 38
VEGFR-2/flk-1	receptor tyrosine kinase	71; 72 73
Tie	receptor tyrosine kinase	35
Tie2/Tek	receptor tyrosine kinase	74
SCL/tal-1	bHLH transcription factor	75
PECAM-1/ CD31	adhesion molecule	76;77; 78
flt-1	receptor tyrosine kinase	79
VE-cadherin	adhesion molecule	80
GATA-2	Zn finger transcription factor	81; 82
EpoR	cytokin type receptor for erythropoietin	83

Functional assays also suggest the existence of this common precursor. The group of Gordon Keller developed the in vitro embryonic stem (ES) cell system to study the first steps of embryonic haematopoiesis. ES cells from the mouse embryo spontaneously differentiate and form embryoid bodies (EB) in culture (84). These EBs, differentiated for 2.5-3.5 days, contain a unique cell

population the blast colony-forming cell (BL-CFC). The particularity of these cells is that they form blast colonies in response to VEGF in methylcellulose cultures. These colonies express a number of genes common for endothelial and haematopoietic lineages, i. e. CD34, SCL, VEGFR-2 (85) and give rise to primitive, definitive haematopoietic and endothelial cells when replated in suitable culture conditions (86; 85). Therefore, these blast colonies are thought to be the *in vitro* equivalents of hemangioblasts.

Other groups have attempted to isolate hemangioblasts from early chick and mouse embryos (72; 73; 80). Because the VEGFR-2 receptor is expressed very early in the extraembryonic mesoderm, the posterior part of the avian blastoderm was dissected from gastrulating embryos, and sorted for VEGFR-2 expression. Cells expressing the receptor were plated in clonal culture, where they differentiated to either haematopoietic or endothelial cell colonies. Experiments performed on the mouse showed that a considerable fraction of VEGFR-2 positive cells were able give rise to both haematopoietic and endothelial cells. This fact however, does not make clear the question whether the receptor-expressing cells represent bipotent hemangioblasts or earlier progenitors.

A recent fate map study on the mouse embryo brings also into question the existence of the hemangioblasts. Kinder et al. (87) investigated the fate of the cells in the early, mid, and late primitive streak of the mouse gastrula and found that distribution of both the erythroid and endothelial populations in the same region of the yolk sac was rarely observed simultaneously. This indicates that mesodermal cells generating erythrocytes emerge earlier than those that give rise to endothelial ones, and that these lineages may arise from different progenitors already existing within the primitive streak. Thus further work should be directed at examining the existence of the common precursor *in vivo*.

Functional characteristics of the progenitors, developing in the yolk sac analogue compartments

In ***amphibian embryos***, the ventral region of the developing neurula, /called ventral blood island (VBI)/, represents the homologue of the yolk sac blood islands of higher vertebrates. Ablation and transplantation studies indicated that this region is responsible for the generation of primitive erythrocytes, the definitive wave of haematopoiesis take place in a different, dorsal compartment of the embryo (88; 89). These results were later confirmed on the Xenopus, and the contribution of VBI derived cells in the thymus and spleen was also shown (90; 91). The VBI cells contribute thus to both primitive and definitive lineages (92).

The yolk sac chimera experiments on the ***bird model*** showed that primitive haematopoiesis in this class of vertebrates is only transitory. Yolk sac derived blood cells decrease in number during embryogenesis to become absent at hatching. These experiments also demonstrated however, that the avian yolk sac contributes to two different generations of erythrocytes: primitive red blood cells (containing embryonic haemoglobins) derived entirely from the yolk sac, while definitive ones (containing fetal haemoglobins) are derived from both the yolk sac and the embryo proper (55; 93). In addition to these two lineages, yolk sac-derived primitive macrophages were also described (94). These studies thus indicate that the early yolk sac is only capable of producing progenitors for the erythro-myeloid lineages. This restricted capacity probably has environmental causes, because under experimental conditions, when the thymus rudiment of an E6 chick is grafted onto a quail yolk sac, lymphoid differentiation occurs in the rudiment, and lymphoid cells are of quail origin (95).

Several groups investigated the developmental potential of the yolk sac progenitors in the ***mouse***. While the *in utero* development of mammals excludes grafting experiments, functional assays developed for the investigations of the adult HSC were used for the characterisation of the embryonic haematopoietic populations. Therefore, the main approaches of the

investigation of mammalian haematopoiesis are indirect, and often allow more than one possible interpretation of the results.

Early yolk sac cells are unable to repopulate adult irradiated recipients upon direct transplantation. However, when blood island cells dissected from E8 and E10 embryos are transplanted *in utero* into another embryo of the same stage, they contribute to the colonisation of bone marrow and the thymus (96; 97). Similar results have been obtained by transplantation of yolk sac cells into newborn recipients (98; 99). In addition, by E8.5, the yolk sac contains cells that can mature into definitive HSCs if they are co-cultured with an AGM-derived stromal cell line (100). According to these findings, the yolk sac of the mouse embryo contains pre-definitive HSCs, which are capable of developing into definitive HSC upon maturation in an embryonic or newborn microenvironment, at least from E8.5 (101).

Recently, the developmental potential of the yolk sac progenitors was investigated as well during ***human*** embryonic development in co-cultures with M5 stromal cells, and by subsequent FACS analysis (102). While T and B lymphoid potentials were never detected under these conditions, cultured yolk sac cells were capable of differentiating into myeloid (detected by the CD15 antigen) and NK (natural killer, detected by CD56) cells.

In summary, according to these experiments on different species, it seems that haematopoietic progenitors developing in the yolk sac analogue compartments of vertebrates differentiate mainly into erythro-myeloid cells at the beginning of development; most probably to satisfy the immediate demands of the embryo. However, as data on different species indicate, the yolk sac can also be a source of pre-definitive HSCs during later development. At this time, it remains unclear if during normal embryo development, these cells mature *in situ* into definitive HSC or if they have to migrate to an inductive, intraembryonic microenvironment, in order to do so.

The definitive haematopoiesis

Origin of the definitive precursors

According to the current understanding, haematopoietic stem cells developing in the embryo proper give rise to the source of blood cells during adult life. Compared to the primitive wave of haematopoiesis, the definitive progenitors can be detected slightly later during development.

However, detailed fate mapping studies have not been performed on the avian embryo to reveal the earliest mesodermal precursors of the definitive blood cell lineages, some data indicate the independent origin of these cells from the primitive lineages. The fate map of the early gastrulating avian embryo shows that mesodermal cells invading the embryonic area leave the primitive streak anteriorly compared to the mesodermal cells invading the extraembryonic area (58). As the mesoderm differentiates in the embryonic region, a cavity opens inside, which is called the coelomic cavity. The mesodermal layer, which remains in contact with the endoderm, is the splanchnopleural mesoderm, the other layer composed by the ectoderm and the dorsal part of the mesoderm is called somatopleure. In between the splanchnopleural mesoderm and endoderm, cell aggregates are established, which are very similar to the yolk sac blood islands but never give rise to haematopoietic cells. Later in development they unite with each other to give rise the first embryonic blood vessels and the aorta.

The avian aorta however has a dual origin. Orthotopic grafting experiments have shown that when the latest-formed somites are transplanted from quail to chick hosts, endothelial cells of somitic origin colonised only "dorsal" structures, such as the body wall, periphery of neural tube and kidney, and with respect to the aorta, were found only on the roof and sides of it. By contrast, when splanchnopleural mesoderm (with the vascular aggregates mentioned above) was placed in the place of the latest-formed somites, it did give rise to the endothelium of "dorsal" structures while also contributed to the floor of the aorta (103). These experiments show that the aortic endothelium has a mixed origin, the roof and sides coming from the

paraxial mesoderm (somites) and the floor is derived from the splanchnopleural mesoderm.

On the 3rd day of development, clusters of haematopoietic cells appear on the ventral aspect of the dorsal aorta. These aggregates, budding into the lumen, develop in one stripe on each side of the ventrolateral aortic floor. In birds, these intraaortic clusters represent the first wave of intraembyonic haematopoiesis. In a second phase, between E6-E8, large cell groups develop in the mesenchyme, ventral to the aorta, called paraaortic foci (Fig. 2) (104).

The origin and fate of these clusters were investigated in the avian embryo by vital labelling of the endothelia with acetylated Low Density Lipoprotein (ac-LDL) coupled to a fluorescent lipophilic tracer (DiI), which was specifically endocytosed by endothelial cells. Injection of the tracer was performed on the 2nd day of development into the heart, before the appearance of CD45 (pan leukocyte marker) expressing cluster cells (105). Cells were traced thereafter, and subsequently LDL-DiI and CD45 double positive cells appeared on the ventral wall of the aorta, indicating that the clusters were generated from the endothelial layer. In the same experiment, labelled cells expressing the haematopoietic marker CD45 were also found not only in the clusters, but also in the mesenchyme, ventral to the aorta, probably the progeny of the paraaortic foci. In order to investigate the relationships between the aortic clusters and the paraaortic foci, further tracing experiments were performed. Because the LDL-DiI is not appropriate for long-time tracing, the labelling was repeated with a non-replicative Lac-Z-bearing retroviral vector, which marker made possible a clonal labelling of cells for a longer period of time. The virus was inoculated in the same conditions into the heart of the embryos and labelled clones were detected either in the endothelia or haematopoietic cells, or in some cases, in haematopoietic cells, ingressing into the dorsal mesentery. By E7, numerous LacZ+ cells were detected in the paraaortic foci suggesting a direct progeny in between the cluster bearing aortic endothelia and the paraaortic foci (106).

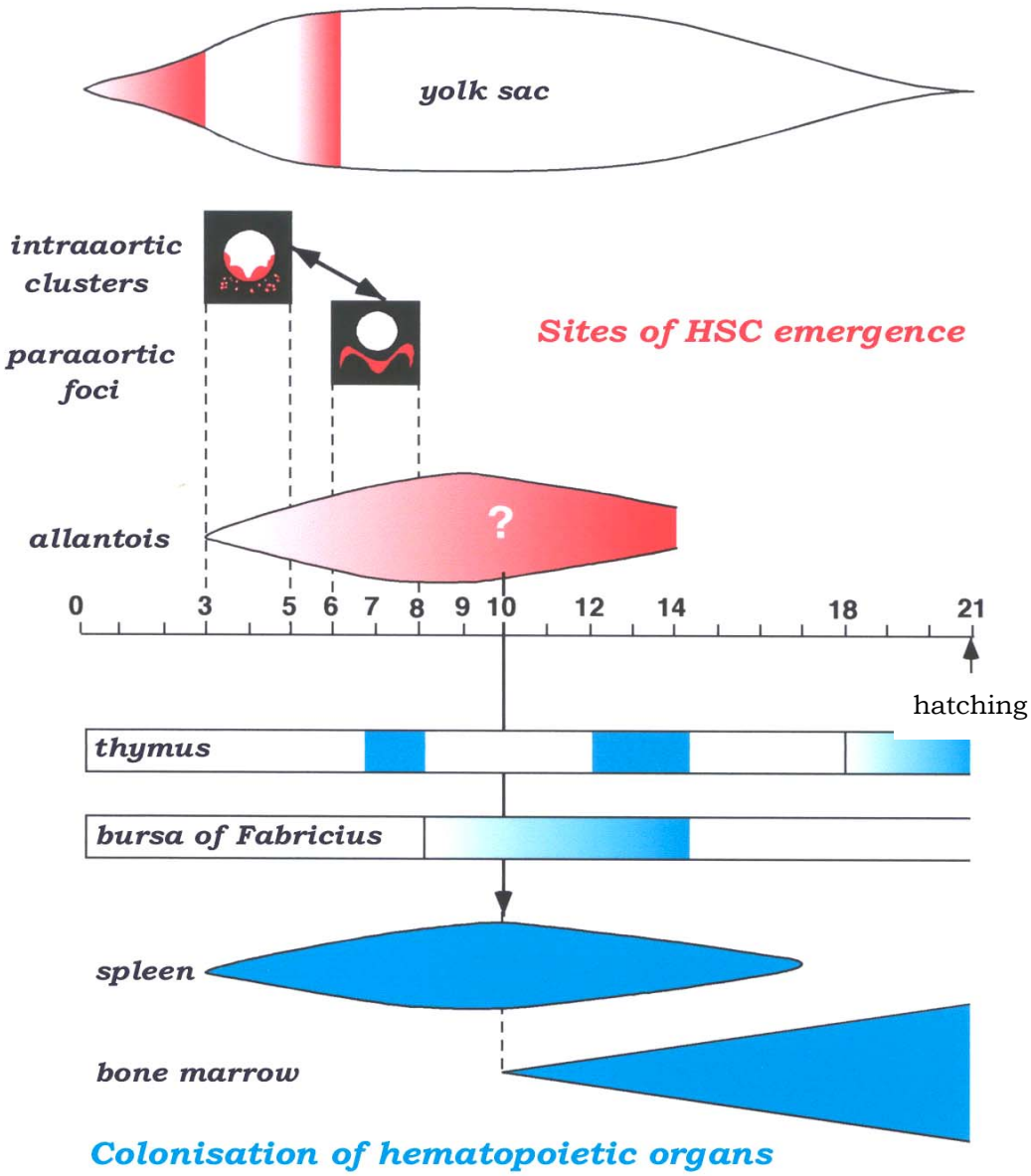


Figure 2. Development of the hematopoietic system in the avian embryo (adapted from Dieterlen-Lièvre).

Characteristics of the definitive progenitors, developing in the embryo proper, associated to the aorta

The developmental potential of the precursors of definitive haematopoietic cells was analysed by *in vitro* and *in vivo* studies using different models.

In ***amphibians***, the second wave of haematopoietic progenitors emerges in a dorsal location at the neurula stage, called dorsal lateral plate (DLP). When the DLP region was transplanted from a diploid embryo to a triploid host, graft derived cells were detected in the thymus and mesonephros of the host animal (90). Recent lineage mapping of single blastomeres suggested direct relationships between DLP cells and haematopoietic cells associated with the floor of the dorsal aorta (107). Thus it seems that haematopoietic progenitors emerging in the DLP region migrate to the aorta to give rise to the progeny of definitive haematopoietic cells.

Experiments on the ***avian model*** showed that multipotent haematopoietic progenitors emerge in the aorta region. Cell suspensions prepared from E3, E4 aorta, seeded in appropriate semi-solid cultures, yielded macrophage, granulocyte or erythrocyte clones. In addition, when they were associated with a thymic rudiment, cells left the quail aorta, entered this rudiment and underwent lymphopoiesis (108–110). When cell suspensions from E7 chick intraembryonic mesenchyme were obtained and introduced into lethally irradiated E14 recipients, T and B cells, derived from the graft, were detected in the thymus, spleen and bone marrow and were capable of long term reconstitution (111; 112).

By the early 1990's, experiments on the ***mouse embryo*** provided also additional data on the potentials of intraembryonic haematopoietic progenitors. Two independent groups showed first the presence of an intraembryonic site of haematopoiesis in the mouse embryo. Isabelle Godin and co-workers investigated the region corresponding to one that was found to be haematopoietic in the avian embryo. The paraaortic splanchnopleure (P-Sp), comprising endoderm, mesoderm and blood vessels, was dissected from

embryos with 10 to 20 pairs of somites, and grafted under the kidney capsule of SCID mouse. The splanchnopleure reconstituted a specific compartment of CD5+ B lymphocytes, the B1a cells (113). During embryogenesis, part of the P-Sp transforms into a morphologically distinct, composite axial structure, named AGM, consisting of the dorsal aorta, genital ridges and mesonephros. Dzierzak and colleagues demonstrated CFU-S activity, and in later experiments also long-term reconstitution activity in this region (114; 115; 116). Thereafter de Bruijn et al. (117) identified the dorsal aorta and surrounding mesodermal cells, as the origin of the definitive haematopoietic cells. In addition, it was demonstrated that the E8.5 P-Sp contains cells that can mature into definitive HSCs by co-culturing with an AGM-derived stromal cell line (100). Although at this stage the P-Sp is incapable of reconstituting adult recipients, it contains multipotent lymphomyeloid progenitors as was shown by a multistep culture system (118; 119).

In summary, experiments on the mouse showed that the embryonic haematopoietic hierarchy is in reverse orientation compared to the adult. This means that the system first yields short-term repopulating stem cells in the P/Sp-AGM region, but long-term repopulating potential (LTR-HSC activity) appears only during the later stages (120).

Intraembryonic haematopoiesis was also investigated in ***human embryos***. Haematopoietic clusters emerge in the human aorta early in development, in the pre-liver embryo, and process the characteristics of primitive haematopoietic progenitors (121). In vitro analysis of the lineage potential of these progenitors showed that they were capable to give rise B (CD19+), T (CD4+), NK (CD56+) and myeloid (CD15+) cells (102).

All of this data, obtained on different model organisms, suggest that haematopoiesis during development is a multistep process. It seems that short-lived, rapidly differentiating haematopoietic progenitors develop first, in the yolk sac analogue compartments, with restricted developmental potential to ensure the immediate needs of the growing embryo. Slightly later, an intraembryonic source of haematopoietic progenitors emerges. Haematopoietic progenitors developing in this location give rise to the progeny of the adult

blood system. However, as some data indicate, haematopoietic precursors with broader developmental potentials also appear later in the yolk sac analogue compartments. It is interesting to note, that emergence of the early haematopoietic precursors is always linked with the development of the vascular system. A summary of these events is shown in Fig. 3.

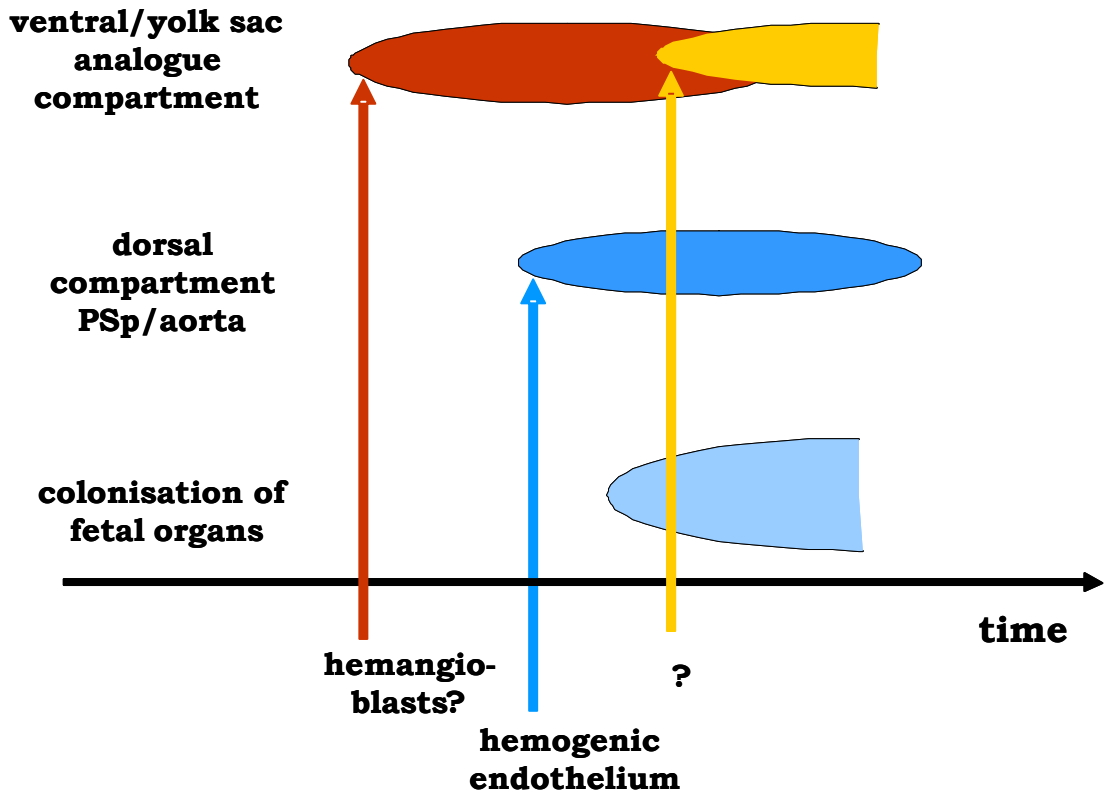


Figure 3. Events of the vertebrate embryonic haematopoiesis

A new site of haematopoiesis in birds: the allantois

Data obtained from developmental studies on avian haematopoiesis are summarised in Figure 2. As it is shown in the diagram, the activation of the intraaortic clusters and paraaortic foci is compatible with the colonisation of the thymus and bursa of Fabricius, but not with the seeding of the bone marrow. This process only begins at E10.5, 2-3 days after the activity of the paraaortic foci. Therefore, it seemed possible that there exist another source of haematopoietic progenitors in the avian embryo. In addition, previous results obtained on yolk sac chimeras indicated that the allantois may have haematopoietic function (55).

The avian allantois is the last to appear of the extraembryonic appendages, at around 2.5 days of incubation (20-28 pairs of somites, HH17). It develops from a bud, which grows out into the extraembyonic coelom from the posterior intestinal portal (PIP). Slightly later (HH18), when the posterior intestine has closed, the allantoic bud has formed as a small vesicle lined by mesoderm outside and endoderm inside. Until recently it was thought that the allantois has two main functions during embryonic life. It serves as respiratory organ and a reserve of metabolic wastes of the kidneys. To fulfil its respiratory functions it fuses with the chorion around E5.5, forming the chorioallantois membrane, which will cover the entire embryo and contacts the shell membrane.

When the allantois bud was grafted from a quail into the coelom of a chicken host at E3, the host bone marrow was colonized by QH1 positive endothelial and haematopoietic cells later in development (122). These results suggest that this extraembryonic organ also release progenitors for the adult haematopoiesis.

REGULATION OF HAEMATOPOIESIS

Studies on vertebrate embryonic haematopoiesis examined first the morphological and functional aspects. During the last decades, the major breakthroughs of the molecular biology offered the possibility to understand better these events at molecular level.

This chapter discuss two important issues in the regulation of haematopoiesis. First is that how the emergence of haematopoietic cells is regulated during ontogeny; and the second one demonstrates how the resulting pluripotent HSCs undergo the commitment processes which give rise to the multiple blood cell lineages. Two main processes regulate these commitment events: the intrinsic, cell-autonomous program of each lineage, and the extrinsic microenvironment acting through the signal transduction pathways.

Tools of the molecular studies

A wide variety of experimental approaches, such as embryonic stem cell technology and homologous recombination in the mouse, make it possible to study genes suspected of playing a role in haematopoietic development. Genes that were first biochemically analysed have now been characterised *in vivo* by using mouse knockouts. Investigation of the contribution of mutant ES cells to haematopoietic lineages in chimeric mice, and estimation of the developmental potential of *in vitro* differentiated ES cells permit evaluation of the consequences of gene knockouts on haematopoietic potential, independent of the viability of the germline defects. Mouse genetic offered thus a great tool permitting to reveal molecular networks working in the specification of blood cells, but our understanding is still incomplete on the steps involved in the commitment of haematopoietic cells.

A knockout phenotype shows only the earliest stage at which a gene product is required in development. In addition, the small mammalian embryos develop in safety, in the uterus of their mothers, which makes them fairly accessible for studying. The way of revealing these processes is based on

the use of lower vertebrate species. Zebrafish and the African clawed toad become popular to study developmental processes because of the high number of embryos with good access to them. These vertebrate models extended our knowledge on the molecular regulation of the haematopoiesis and provide further possibilities to understand these processes.

Early patterning events regulating the induction and specification of the ventral mesoderm and embryonic blood

Cell fate determination during ontogenesis is governed by signalling molecules emitted and captured between populations of different cells during a process called embryonic induction. Prior to primitive haematopoiesis, which gives rise to the first generation of blood cells in the embryo, the establishment of the haematopoietic program begins with the induction and specification of the ventral mesoderm to a haematopoietic fate. The specification of blood from mesoderm is regulated by many of the same positional cues that pattern dorso-ventrally the mesoderm and generate other mesodermal derivatives, such as muscle or vasculature. Because this process has been most extensively studied in the frog Xenopus laevis, I will summarise these data in the following chapter.

The BMP pathway and its targets

To understand the mesoderm induction and blood specification it is important to note some general aspects of the development of the Xenopus. The early embryo is composed of two types of cells: the animal pole cells which are on the upper portion of the embryo, and the vegetal cells which compose the bottom, yolk rich part.

The mesodermal germ layer, which is the origin of blood cells, takes shape during the gastrulation, when cells migrate through the blastopore, inside the blastula, and the rapidly dividing animal pole cells cover the vegetal

cells by the process of epiboly. In Xenopus, Nieuwkoop showed first that mesoderm arises through an inductive interaction, in which cells of the vegetal hemisphere induce overlying equatorial cells to form mesoderm (reviewed in Smith (123)). This induction results in the formation of mesoderm that is patterned along the future dorso-ventral axis, which is established at fertilisation. This means that dorsal vegetal pole cells induce animal pole cells to form dorsal mesodermal cell types (notochord and muscle), while ventral vegetal cells induce ventral mesodermal cells, such as erythrocytes (124). The further investigations revealed the molecular background of these induction processes. Ligands and receptors which belong to the TGF-β family have been identified as mesoderm inducing factors. Receptors of this family are serine/threonine transmembrane kinases, which become activated by binding peptide ligands. One of them, the BMP (bone morphogenetic protein) pathway appears to be the primary inducer in the specification of ventral, blood-forming mesoderm. Arguments supporting this idea are as follows. It was shown that injection of BMP-4 mRNA into fertilised Xenopus eggs ventralizes embryos (125; 126) (they do not contain muscle tissue or notochord (dorsal type mesoderm) but mesenchyme and blood, expressing globin). In addition, a dominant negative truncated form of a type I BMP receptor causes cultured ventral marginal zone (VMZ) cells to change their fates and become dorsal mesoderm (127; 128) and further results in a decrease of globin expression (129). The expression pattern studies on different vertebrate species and gene disruption experiments on the mouse embryo also justify the role of the BMP pathway in the specification of the ventral mesoderm (reviewed by Evans (130)).

What are the targets of the BMP pathway in the specification of the ventral, blood-forming mesoderm? Binding the ligand to the BMP receptors initiates a signal transduction pathway via phosphorylation of the Smad proteins. Activated Smads enter the nucleus and form complexes with other Smads and with gene-specific transcription factors to regulate gene expression. Further studies are required to understand which ligands, receptors and Smad molecules play a key role in the BMP pathway to induce blood specification. In contrast, there are some data concerning downstream genes of the BMP pathway, which are probably activated by Smad-associated

complexes in the ventral mesoderm. Mix.1, Xvent-1 and 2 homeobox genes were identified as BMP signalling activated genes (131; 132; 133; 134) but their role in the mesoderm pattern specification needs further investigation.

More direct targets of BMPs, required for haematopoiesis were also identified in Xenopus animal cap assay, which is the equivalent method of the ES cell culture applied in mammals. Induction of transcription factors acting in early haematopoiesis, such as SCL/tal-1 and GATA-2 (see below) were detected when ectopic BMP-4 was added to cultured animal cap cells (135; 136). In addition, GATA-1 (a Zn finger transcription factor acting in erythropoiesis, see below) expression was also activated. According to the current understanding, BMPs could lead to a positive feedback loop between SCL and GATA-1 because GATA-1 is capable of activating an SCL promoter (137; 138) to promote haematopoiesis in the early embryo. The schematic diagram in Figure 4 summarises the role of BMP-4 in early haematopoiesis.

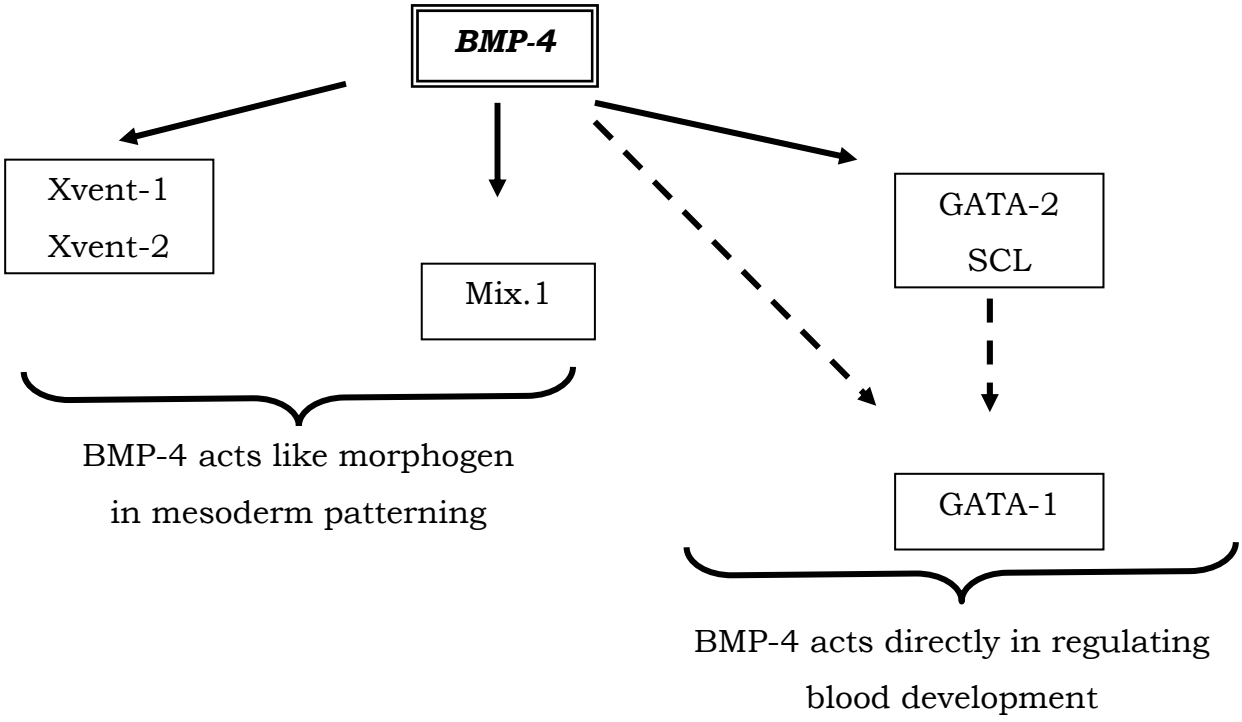


Figure 4. Dual role for BMP-4 in early haematopoiesis

Endoderm delivered signals for early haematopoiesis identified in higher vertebrates

Tissue recombination studies on chick embryos have indicated that blood formation in the yolk sac requires diffusible signals from the extraembryonic endoderm (139; 140; 141). Gordon-Thompson and Fabian (142) later showed that at least a bFGF (basic fibroblast growth factor)-like signal is responsible for this induction. Studies on mouse embryos revised this question, and pointed out the importance of the secreted signals by primitive (visceral) endoderm which is required for the initiation of haematopoiesis and vasculogenesis. Recently, in the laboratory of Margaret Baron, a transgenic explant culture system has been developed and demonstrates that cellular interactions are needed between primitive, visceral endoderm (VE) and adjacent embryonic ectoderm or nascent mesoderm to initiate haematopoiesis and vasculogenesis (143). Moreover the primitive endoderm could respecify the prospective neural ectoderm to a posterior mesodermal fate. To understand the nature of the signal delivered by the endoderm, the explant culture system was used again. Gene expression studies indicated that a hedgehog family member, Indian hedgehog (Ihh) is expressed specifically in the VE and mature yolk sac of mouse embryos (144). In the explants, Ihh was sufficient alone to induce the formation of haematopoiesis and vasculogenesis and could respecify the prospective neuroectoderm to gives rise to haematopoietic and endothelial cells (145). In addition, downstream genes of the hedgehog pathway (patched, the receptor for Hh, smoothened and Gli1) were upregulated by the prospective neuroectoderm in culture, as well as BMP-4.

A hypothesis explaining the possible interactions of the BMP and Hedgehog pathways in the primitive haematopoiesis

It is known that BMPs and Hedgehog proteins are involved in many processes during embryogenesis. Often they are expressed in neighbouring tissues and Hedgehog proteins modulate BMPs (146). The group of Baron demonstrated the same effect in explant cultures. When the anterior epiblast of the mouse embryo was cultured in the presence of recombinant Indian Hedgehog (Ihh-N), the BMP-4 gene was upregulated as detected by RT-PCR (145). They propose therefore a model pathway working in the yolk sac regulating haematopoiesis and vasculogenesis. According to this hypothesis Ihh is secreted from visceral endoderm to target extraembyonic mesoderm, where it activates expression of BMP-4. BMP-4 protein in turn feeds back to extraembyonic mesoderm, activating VEGFR-2, CD34, and SCL and formation of haematopoietic/vascular stem/progenitor cells (147).

The VEGF pathway

Vascular endothelial growth factors (VEGFs) are involved in the adult and embryonic angiogenesis. Several members of this subfamily of PDGF-like (platelet derived growth factor) signal transduction pathway have been identified at the level of the dimeric glycoprotein ligands and tyrosine kinase type receptors. Among them, the VEGF-VEGFR-2 pathway seems to be the most important during early development, in the specification of hemangioblasts.

One argument supporting the role of this pathway in early development is that similar gene expression profiles characterise all early vertebrate embryos, with the presence of VEGF in the endoderm, underlying the extraembryonic mesoderm, and VEGFR-2 in the extraembryonic mesoderm proper (71; 72; 148; 149; 150). Other data comes from the genetic studies on mouse. Loss of function studies provided some idea about the roles of these molecules during early development. Targeted gene inactivation of the receptor

made the embryos unable to generate yolk sac blood islands and caused death between E8.5-9.5 (63). The defect in these embryos is thought to result from the inability of VEGFR-2-/- mesodermal cells to normally migrate to the appropriate regions of the yolk sac and differentiate there, rather than from a specific block in the developmental pathway of these lineages (64). Other evidence suggesting the importance of gene dosage is that disruption of a single allele of VEGF was sufficient to impair the development of the hemangioblastic population (151; 152). This data strongly suggest a role for these signalling molecules in the process of the early hemangiopoiesis. However, natural inducers of the VEGF/VEGFR-2 expression in the early haematopoiesis are not yet known, it was shown that the *vegf* gene acts downstream of the sonic hedgehog pathway in the control of arterial fate (153).

Are the same factors driving the primitive and definitive haematopoiesis?

Data cited above concerned the first, primitive wave of haematopoiesis, taking place in the yolk sac analogue compartments in vertebrates. In the following, pathways involved in the definitive haematopoiesis will be summarised, and compared with factors acting in the primitive wave, because data from several mouse mutants suggest developmental differences between primitive and definitive haematopoietic cells.

BMPs appear to have a role not only in primitive but also in definitive haematopoiesis. For example, BMP receptor and Smad signalling components are present and functional in CD34+ CD38- stem cells isolated from human haematopoietic tissue (154). In addition, BMP-4 is expressed in the AGM region of human embryos when haematopoietic clusters emerge in the aorta (155). In the adult bone, marrow BMP-4 collaborates with cytokines and Sonic hedgehog to regulate the haematopoietic microenvironment that maintains the homeostasis of the stem cells (156).

It was also suggested that erythropoietin (Epo) and its cytokine type receptor (EpoR) are involved in the survival, proliferation and differentiation of the definitive erythroid cells but are not required for primitive erythropoiesis.

The independence of primitive erythropoiesis from this cytokine pathway was certified by knockout experiments as well (157; 158).

Another signal transduction pathway appears to be specific for the definitive wave of haematopoiesis: the ligand (KL) coded by the Steel locus (also called Steel factor or stem cell factor), a growth factor for multipotential and mast cells, and its receptor c-kit, the membrane tyrosine kinase of the PDGF (platelet-derived GF) family. This receptor is one of the genes, which are localised on the 5th chromosome in the mouse, distributed in three clusters. This type of physical organisation of the genes generally refers to functional consequences. One of the neighbours of c-kit in its cluster is VEGFR-2, which receptor is important for the development of the very early progenitors in hemangiopoiesis as seen above. It is likely that these genes are switched on sequentially when cells differentiate toward haematopoiesis during development. Another interesting observation is that the KL/c-kit pathway cross-talk with the Epo/EpoR pathway. The two receptors form a complex on the cell surface and after binding to the ligand, EpoR is phosphorylated which drives to the proliferation and maturation of erythroid progenitors (158).

Cell autonomous regulators of haematopoiesis

It has became evident in the last decades, that growth factors are not required for the lineage commitment in the development of the blood system but exert their effects by supporting the survival, proliferation and maturation of committed blood cells (159). In contrast, recent studies indicate that the transcriptional regulation and the changes of the chromatin structure play primary roles in these processes. Transcription factors are cell-autonomous regulators, functioning downstream of the growth factor network to control proliferation and differentiation. They can bind to cis-acting regulatory elements of lineage specific genes and regulate gene expression. Each blood cell type can be distinguished by a distinctive, well-defined set of effector genes. In the following chapter, models explaining lineage decisions will be described and important transcription factors acting in haematopoiesis which were in focus in my experiments will be reported.

Combinatorial action of the transcription factors or the "cocktail party" model of Sieweke and Graf

In the commitment of cell lineages, lineage-specific transcription factors (TF), working with general TFs play important roles. In addition, lineage-specific gene expression appears to be regulated not by single master regulators but by the combinations of transcription factors (160). Expression of a given transcription factor may have different consequences in different cell types, in part because of the different transcriptional environment. The combinatorial action of transcription factors is more important in the control of gene expression, which occurs by many physically interacting factors, forming large, multiprotein complexes. During differentiation, lineage specific complexes are selected, and define the direction of commitment. Sieweke and Graf suggested in their model that these complexes obtain new functions during haematopoietic differentiation through successive changes in composition, such as the topic of discussion changes and take other directions as new people join and others leave a cocktail party. The changes in the composition drive to sequential gene expression activations and inhibitions responsible for specific determination and differentiation (161).

Models for molecular processes underlying self-renewal versus differentiation

It has been recently shown that multipotent haematopoietic progenitors co-express several lineage-restricted genes prior to commitment (162; 163). These observations suggested that, in these uncommitted cells, several gene expression profiles exist at the same time from which distinct patterns emerge during lineage commitment. In other words, these progenitors are 'primed' for commitment. During differentiation one gene expression profile is upregulated, and others are inactivated selectively (164).

The mechanism, by which gene expression profiles become upregulated or inactivated, can be described by the cross-antagonistic model. In their model, Cantor and Orkin (165) explain why the level of the TFs in the uncommitted, multigene expressing cells is important.

Earlier experiments suggested that the lineage-specific TFs simultaneously exert inhibitory effects on alternate lineage gene programs in addition to playing a role in activating others. According to the cross-antagonistic model, TFs that are co-expressed at equivalent levels in the uncommitted cell hinder each other in activating the target gene regulating elements. When the concentration level of one TF increases relative to the other -due to external mechanisms- the unopposed molecules start to activate their targets and establish the cellular phenotype. Afterwards this decision on the fate of the cell is probably confirmed by feedback mechanisms. An example for factors working by this way is GATA-1 and FOG (see below).

Transcription factors required for formation/maintenance of the earliest haematopoietic progenitors

SCL/tal-1 and Lmo2

Two factors, SCL/tal-1 and Lmo2, are essential for haematopoietic cells to emerge, either at the primitive or definitive stage of haematopoiesis (65; 66; 166; 167). Moreover, both are required for the proper establishment of the primary capillary plexus in the yolk sac (67; 168). SCL/tal-1 is a member of the basic helix-loop-helix (bHLH) family, which includes cell-specific regulators (e. g. MyoD, Mash-1) as well as ubiquitous proteins (e. g. E2A products) with established known roles in development. Lmo2, a LIM-only polypeptide, does not bind to DNA, but acts as a modulator in transcriptional complexes.

Both factors were identified through chromosomal translocations in acute T-cell lymphoblastic leukaemia (T-ALL) (169). The expression pattern of SCL and Lmo2 is very similar during early development, namely in the extraembryonic mesoderm and fetal liver erythroid cells, however, SCL is also expressed in the central nervous system(75, 170, 171, 172; 173; 174). In addition, knock-out mouse embryos for SCL and Lmo2 have similar

phenotypes (171). Mutant embryos are "bloodless", consequently pale and growth-retarded. Non-haematopoietic tissues however do not show any abnormality.

The co-expression and similar phenotypes obtained after loss-of-function of these genes can be explained by their characteristics, i. e. they participate to the same transcriptional complex during early development. Lmo2 protein is specifically associated with SCL/tal-1 as well as with LIM-interacting polypeptide, Ldb1 in erythroid cell nuclei (175; 176). Moreover, a transcriptional complex containing SCL, Lmo2, Ldb1, E12/47, and GATA-1 has been observed in erythroid and progenitor cells (177). Whereas SCL (as a heterodimer with E2A products, E12 and E47), recognizes canonical E-box motifs (CAN-NTG) on DNA target sites, Lmo2 appears to provide a bridging function in this complex.

Functional data suggests that these factors are required for the specification of the haematopoietic fate from mesoderm. Gain-of-function experiments in zebrafish established SCL as an inducer of the expansion of cell populations expressing haematopoietic and endothelial markers (69) whereas in Xenopus, SCL could orient the bFGF-induced mesoderm towards haematopoiesis (68). Overexpression of SCL was able to rescue the haematopoietic and endothelial defects in zebrafish mutant *cloche* embryos (178). As a working model, SCL and Lmo2 may be considered as counterparts of a transcriptional complex acting in erythroid cells and haematopoietic progenitors.

The target genes regulated by SCL or Lmo2 and required for specifying haematopoiesis is largely unknown. Possible genes acting upon by SCL are c-kit, the receptor of SCF (179), and a new gene identified by chromatin immunoselection (180).

How SCL and Lmo2 function to promote early haematopoietic development is an unanswered question. Defining the roles of SCL in transcription is complicated because of the recent findings of Orkin and co-workers on the DNA-binding independent functions of SCL in development (181). It was shown in this study, that an intact HLH domain that directs heterodimerization is needed for formation of primitive erythroid cells and definitive haematopoietic progenitors but this process occurs without site-

specific DNA binding of SCL. These findings show that protein-protein interactions of SCL, presumably with Lmo2, are critical for HSC generation.

In summary, the temporal gene expression patterns, the downregulation of SCL and Lmo2 during erythroid development, and the complete lack of haematopoiesis in the KO mouse, placed these two factors near the top of the haematopoietic hierarchy.

GATA-2

GATA-2 is highly expressed in haematopoietic progenitor cells but probably does not play a pivotal role in the specification of the HSC. GATA factors are members of the zinc-finger family of transcription factors. All of them contain a highly homologous domain with two C2H2 zinc-fingers which has been shown to be capable of binding to the DNA sequence WGATAR (W=A or T, and R=A or G) and related sequences, such as CGATGG and AGATTA (182; 183). There is minimal sequence similarity among GATA family members outside of the Zn-finger domain, suggesting distinct functions for each GATA protein. GATA-2 itself was originally cloned from a chicken embryo cDNA library (184). When its expression is enhanced, GATA-2 inhibits transition from a multipotential progenitor to a committed erythroid precursor (185). In accordance with this observation its expression is down-regulated in many, but not all, haematopoietic lineages.

Mice with targeted mutation in the GATA-2 locus is embryonic lethal at E11 due to marked anaemia, however the loss of the gene does not eliminate completely the generation of haematopoietic progenitors (186). In contrast, the absence of functional GATA-2 significantly impairs the expansion and/or maintenance of the stem or progenitor pools. The numbers of progenitors in the yolk sac are moderately reduced in knockout embryos; definitive progenitors are ~100-fold less than the wild type. The presence of HSCs, albeit at a greatly reduced number in the absence of GATA-2, is consistent with the hypothesis according to which GATA-2 is required somewhat later in the haematopoietic pathway, perhaps to sustain the viability or proliferative capacity of immature progenitors. It was also shown, that GATA-2 functions

within transcriptional complexes, perhaps including SCL and Lmo2 (187), involved in the establishment of the haematopoietic program.

Transcription factors characteristics for particular lineages

GATA-1

The founding member of the GATA subfamily of zinc-finger transcription factors, GATA-1 (also called as NF-E1), was originally identified on the basis of its binding to the promoter or enhancer regions of chicken and mammalian globin genes. It was shown to be essential for their erythroid-specific pattern of gene expression, and deletion or mutation of GATA-1 binding sites resulted in the abolition of such expression (188; 189). Within the haematopoietic system, GATA-1 is expressed by erythroid, megakaryocytic, eosinophilic, mast and multipotential progenitor cells (190; 191; 192) but not by myeloid or lymphoid cells. During terminal differentiation of the erythroid and megakaryocyte lineages in culture, GATA-1 expression level has been reported to diminish (193, 194).

Mice with targeted disruption in the GATA-1 gene led to a failure of red blood cell formation, although all other cell types were still produced (195). Maturation arrest occurred at the proerythroblast stage, and cells underwent apoptosis (196; 197). This suggests a role for GATA-1 in cell survival as well as maturation. It has recently been shown that GATA-1 is also essential for megakaryocytic differentiation, indicating that it plays a key role in the generation of more than one haematopoietic cell type (198).

GATA-1 is not only needed for the development of particular lineages but its enhanced expression is sufficient to reprogram cultured myeloid cells to megakaryocytes, and progenitors along the erythroid, eosinophilic, and megakaryocytic differentiation pathway (199; 200).

Targets of the GATA-1 protein are the previously mentioned globin genes and it has also been implicated in the erythroid-specific gene expression of the porphobillinogen deaminase gene (201).

Recently, a cofactor of this protein was identified through a yeast two-hybrid screen, named Friend of GATA-1 (FOG-1) which was also found to be important in erythro- and megakaryopoiesis (202; 203). Other factors interacting physically with GATA-1 include EKLF/Sp1 (204), PU.1 (205), p300/CBP (206) and the previously mentioned Lmo2 and SCL (176, 177).

In summary, the presence of GATA-1 has important effects in lineage selection, in particular in the development of the erythroid and megakaryocyte lineages.

GATA-3

Increasing numbers of data suggests a key role for GATA-3 in T cell development (rewieved in 207). GATA-3 was initially cloned as a TF that binds to the TCRα gene enhancer (208), and multiple T cell specific genes have GATA-3 binding sites in their regulatory regions (209). GATA-3 appears to be a regulator of the earliest committed T cells (CD4/CD8 double negative) and also has an important role in the development of the T helper 2 cells.

In contrast, effects of GATA-3 on the early haematopoiesis remained elusive until recently. Mice lacking GATA-3 display haemorrhage and defective fetal liver haematopoiesis, and embryos die by day 11 dpc (day post coitum) (210). Recent data indicate however, that the embryonic lethality is due to the noradrenalin deficiency of the sympathetic nervous system rather than the defect in embryonic haematopoiesis (211). Interestingly, the co-expression of Lmo2 and GATA-3 was revealed in the caudal endoderm of the mouse embryo, associated with the aorta haematopoiesis, suggesting a combined function during specific steps of definitive haematopoietic development (212). In addition, GATA-3 expression was also revealed in the rudiments of haematopoietic organs (fetal liver and thymus) before the entry of the colonising haematopoietic cells. This presence is interpreted as a "stromal" function of GATA-3 in these organs.

Other factors acting in haematopoiesis

Genetic studies in the mouse indicate that haematopoiesis can be perturbed at different levels when transcription factor functions are impaired. Two factors, ***c-myb and Runx-1*** have been demonstrated to be required for definitive haematopoietic cells. In c-myb-/- knockout mice yolk sac haematopoiesis occurs normally, thus early development is not affected by the absence of the gene. In contrast, fetal liver haematopoiesis is severely impaired and embryos die by E15 (213). Loss of the other protein Runx-1 causes embryonic death at mid-gestation and the absence of definitive haematopoietic cells (214; 215). In addition, *lacZ* knock-in mutant at the Runx-1 locus lack haematopoietic clusters in the floor of the aorta, at the site of definitive haematopoietic cell formation (216).

Many transcription factors have been demonstrated to have important roles in the determination of particular blood cell lineages. Table 5 summarises the results of several knockout experiments.

Table 5. Key transcription factors (TF) involved in the regulation of haematopoiesis

TF	Lineage affected in loss-of-function studies	References
SCL/ tal-1	all blood cell lineages	217; 166; 65; 66
Lmo2	all blood cell lineages	167
GATA-2	decreased progenitor numbers	186
c-myb	definitive haematopoiesis	213
CBF Cbfa2/AML 1/Runx1 Cbfb	definitive haematopoiesis	214, 215; 218; 219
TEL	adult haematopoiesis in BM	220
PU.1	both myeloid and lymphoid	221
Ikaros	lymphoid	222
GATA-3	T cells	223
Pax-5	B cells	224
c-abl	B cells	225
RB-1	erythroid	226
ELFK-1	erythroid	227
FOG	erythroid and megakaryocyte	203
GATA-1	erythroid and megakaryocyte	195; 196; 198
NF-E2	megakaryocyte	228

Is there a hierarchical pathway of transcription factors?

According to the knockout and chimera experiments on the mouse a provisional hierarchical pathway of transcription factors was proposed (169). In this scheme SCL and Lmo2 were placed on the top of the hierarchy followed by GATA-2 and GATA-1 which is in accordance with their expression dynamics revealed on the mouse embryo (170). However, the authors indicate that the exact position of GATA-2 in the cascade is uncertain. In addition, expression pattern studies on lower vertebrates revealed that GATA-2 mRNAs are present very early in the embryo, prior to SCL and Lmo2 (229; 230 ; 231; 232; 68; 233) suggesting an earlier role in the haematopoietic hierarchy.

AIMS OF THE STUDY

I. ***To study the combined spatio-temporal expression pattern of some key haematopoietic transcription factors that work in multimeric complexes (SCL, Lmo2, GATA-1, 2, 3) in distinct phases of avian embryonic haematopoiesis (in the yolk sac; allantois, and the aorta region).***

II. ***Complete cloning of the chicken pat.pk0056.h5.F EST (expressed sequence tag) which is likely to be the chicken ortholog of Lmo2.***

III. ***To further trace the fate of the haematopoietic progenitors emerging in the dorsal aorta region of the 3 day-old embryos.***

MATERIALS AND METHODS

Embryos

Fertilized outbreed chick (Gallus gallus, JA57, Institut de Sélection Animale, Lyon, France) and quail (Coturnix coturnix japonica) eggs obtained from commercial sources were incubated at 38±1°C until the time when the embryos had reached the appropriate stages. Stages were determined according to Hamburger and Hamilton (HH) (234).

Identification of the chicken Lmo2 ortholog

The Chick EST database from the University of Delaware (http://www.chickest.udel.edu/) was screened for the presence of avian orthologs of the Lmo2 gene. One clone with significant homology to human, mouse, zebrafish and Xenopus Lmo2 cDNAs was identified. The size of the cDNA was evaluated by digesting pcDNA3-containing cDNA with EcoRI and NotI (Biolabs). cDNA was sequenced on both strands using Sp6 and T7 primers.

Sequence analysis

GenBank searches, cDNA translation and sequence comparisons were performed with the Mac Vector 7.0 program (Oxford Molecular Group).

RNA isolation and 5' RACE-PCR

The 5' end of the Lmo2 gene was obtained using the 5' RACE system Version 2.0 (GIBCO Life Technologies). Total RNA were isolated from HH8 chicken embryos using the RNAble kit (Eurobio). RNAs were reverse transcribed using a primer 5'-CGATACGCCTTTGTTAGC-3'. Two successive nested PCRs were then performed using the primers GSP2 5'-TGCTTCAGTCTCCCTTCTTC-3' and GSP3 5'- CCAAAGAGCCTGAGATAGTC-3'

respectively. Primers were designed using the MacVector 7.0 program. After PCR, cDNAs were subcloned into the pCR II plasmid using the TA cloning method (TA cloning kit dual promoter version F; InVitrogen) and sequenced.

Probes for in situ hybridisation

The chick GATA-1 probe was provided by Dr. Todd Evans, Albert Einstein College of Medicine, N.Y. GATA-2 and GATA-3 plasmids were kind gifts from Dr. Douglas Engel, Northwestern University, Evanston (184; 235); SCL/tal-1 from Dr. Anthony Green, University of Cambridge, UK (236). ChT was provided by Dr. Susan Mackem, NIH, Bethesda (237).

Table 6. Plasmids used to obtain probes for hybridisation

gene	plasmid name and antibiotic resistance	enzyme for linearization to obtain AS probe and polymerase for in vitro transcription	enzyme for linearization to obtain S probe and polymerase for in vitro transcription
GATA-1	pBluescriptII- Ampicilin	XbaI T7	HindIII T3
GATA-2	pGEM3Zf Ampicilin	StuI Sp6	XbaI T7
GATA-3	pGEM4Z Ampicilin	SacI T7	ClaI Sp6
SCL/tal-1	SKII+ Ampicilin	BamHI T7	HindIII Sp6
ChT	BS I/ SK- Ampicilin	Xba I T7	XhoI T3
Lmo2	pcDNA3 Ampicilin	EcoRI Sp6	XbaI T7

Digoxigenin labelled antisense and sense probes were synthetised from linearized plasmids with the Riboprobe System (Promega) supplemented with digoxigenin-UTP (Boehringer Mannheim).

In situ hybridisation on paraffin sections

The expression patterns of different genes were compared on serial sections from single embryos.

Dissected blastoderms were washed in PBS to remove the yolk and then fixed in modified Carnoy's solution (60% abs. ethanol, 30% formaldehyde 37%, 10% glacial acetic acid). After fixation, the tissues were dehydrated in ethanol

(70%, 90%, 100%) and toluol then embedded in paraffin. Sections were cut under RNAse-free conditions. For hybridisation, sections had been deparaffinated, rehydrated, digested with 10µg/ml proteinase K (20 mg/ml, Boehringer Manheim) in PBS for 7 minutes, postfixed in 4% paraformaldehyde, rinsed in PBS and washed two times in 2X SSC (3M NaCl, 0.3M sodium citrate, monohydrate). After hybridisation with digoxigenin-UTP-labeled probes (diluted in hybridisation solution: 50% formamide, 10% dextran sulfate, yeast tRNA 1mg/ml, 1X « salt » :/NaCl, Tris-HCl, Tris-Base, NaH_2PO_4, Na_2HPO_4, EDTA/, 1X Denhardts solution) overnight at 70°C under coverslips, slides were rinsed in 3 baths of 50% formamide, 2X SSC, 0,1% Tween at 70°C then in one change of MABT (0.1 M maleic acid, 0.15 M NaCl, pH:7.5 and 0.1% Tween 20) for 30 minutes at room temperature. Non-specific antibody binding was blocked by incubation in MABT containing 20% goat serum and 2% blocking reagent (Boehringer Mannheim) for 1 hour. AP-conjugated anti-digoxigenin antibody (Boehringer Mannheim) was added to this solution for overnight incubation, slides were covered with coverslips. The following day, slides were rinsed 4 times 30 minutes in MABT before equilibration in NTM (0.1 M Tris HCl pH:9.5, 0.1M NaCl, 0.05 M $MgCl_2$). The antibody was detected by a mixture of NBT (nitroblue tetrazolium, 75mg/ml)/BCIP (5-bromo-4 chloro-3-indolylphosphate, 50 mg/ml) in NTM (350 µl NBT and 45µl BCIP in 100 ml NTM), pH 9,5 at 37°C. The reaction was stopped by washing the slides in PTW (PBS containing 0.1% Tween 20) once the required signal intensity was achieved.

Whole-mount in situ hybridisation

All the whole mount in situ hybridization presented in the results has been obtained from at least four individual embryos.

Dissected blastoderms were washed in PBS to remove the yolk and then fixed in 3.8% formaldehyde (MERCK) overnight. Fixed embryos were dehydrated in PBS/methanol mixture (75/25%, 50/50% and 25/75%) and stored in 100% methanol at -20°C.

For hybridisation, embryos were rehydrated and permeabilised in methanol/PTW mixture (75/25%, 50/50% and 25/75%) then in PTW for 10

minutes in each solution. After treatment by proteinase K (20 mg/ml, Boehringer Manheim), embryos were postfixed (3.8% formaldehyde and 0.1% glutaraldehyde) for 30 minutes. After several washes in PTW to remove the fixative, embryos were incubated in PTW/hybridisation solution (50/50 %) for 5 minutes and in hybridisation solution (50% formamide, 1.3X SSC, 5mM EDTA, 50 μg/ml yeast tRNA, 0.2% Tween, 0.5% CHAPS, 100 μg/ml heparin) for 1 hour at 70 °C before the addition of appropriate probe, and overnight incubation at 70°C. After hybridisation, embryos were washed 2 times for 30 minutes with hybridisation solution at 70°C and then cooled to room temperature in MABT (0.1 M maleic acid, 0.15 M NaCl, pH:7.5 and 0.1% Tween 20) and washed 3 times for 1 hour.

For immunostaining, non-specific binding was blocked by incubating the embryos in MABT containing 2% blocking reagent (Boehringer Mannheim), 20% goat serum (GIBCO) for 2 hours. AP-conjugated anti-digoxigenin antibody was added to this solution, and incubated overnight at room temperature with rocking. The embryos were washed five times for 30 minutes in MABT at room temperature, followed by two times for 10 min in NTM, and developed for AP color reaction as described above. Once the required signal intensity was achieved, the reaction was stopped by washing the embryos in PTW, and then embryos were photographed, and conserved in PBS containing 0.1% sodium-azide, and eventually treated for gelatin embedding (see below).

Gelatin embedding of embryos

This method was applied for immunohistochemical staining of embryos or after the whole-mount in situ hybridisation procedure.

Before immunohistochemistry, embryos were washed in phosphate buffer (0,12M) and then fixed in paraformaldehyde (4%) overnight at 4°C. After removal of the fixative with washes in 0,12M phosphate buffer, embryos were transferred into 0,12M phosphate buffer containing 15% saccharose and were incubated for 24 hours at 4°C for cryoprotection. This step was repeated once again. After cryoprotection, tissues were impregnate in 0,12M phosphate buffer, 15%saccharose, 7,5% gelatine for 1 hour at 37°C and were embedded

into the same solution, chilled at 4°C, and then frizzed in cold isopentane (-65°C). Blocks were conserved at -80°C before sectioning.

Immunohistochemical procedures

CD45/VEGFR-2 double staining

Immunostaining was performed on gelatin-embedded frozen sections. Anti-CD45 antibody (HISC-7, mouse IgG2a) was obtained from ID-DLO (The Netherlands), anti-VEGF-R2 (Quek-1) antibody was provided by Dr. Anne Eichmann. Staining was performed according to the modified Tyramide Signal Amplification system (TSA, NEN Life Science) as described by Jaffredo et al. (1998). Briefly, gelatine was removed from the sections by washing in warm PBS (37°C). After inhibition of the endogenous peroxidases, with incubation for 30 min in 3% H_2O_2, non-specific binding sites were blocked by blocking buffer. Primary antibodies were applied overnight at 4°C in humified chamber alone or together, followed by secondary Abs: HRP coupled sheep anti-mouse IgG2a for CD45 or biotinylated sheep anti-mouse IgG1 for VEGFR-2 specific monoclonal Ab (Southern Biotechnology). For double fluorescent detection, after the two primary Abs, non-specific interactions were blocked for 30 minutes with TNB (Tris-buffered saline containing 0.5% NEN Life Science Blocking reagent). Secondary Ab against VEGFR-2 was then added first in TNB for 1 hour. After 3 rinses in TNT (Tris-HCl 0.1M pH: 7.5, NaCl 0.15M, Tween 20 0.05%), streptavidine-HRP in TNT was applied for 30 minutes followed by biotinilated tyramide in amplification buffer for 10 minutes and Streptavidin Cy3 (red, Amersham) in TNT for 30 minutes. Excess HRP was inactivated by 3.3% H_2O_2 (Merck) in PBS for 45 minutes between the two amplification steps. CD45 was revealed in the same manner with a sheep anti-mouse IgG2A-HRP in TNB for 1 hour, followed by biotinylated tyramide for 10 minutes and then Streptavidin Cy2 (green, Amersham) in TNT for 30 minutes. The slides were mounted in Glycergel (DAKO). Sections were observed on a Nikon microscope equipped for epifluorescence with appropriate filters.

QH1 staining of chimeras

Haematopoietic organs of chimeric embryos and in some cases whole chimeric embryos with grafted aortic region were fixed in Bouin's solution dehydrated and embedded in Paraplast (Prolabo). Organs or embryos were serially cut at 5μm and sections were dehydrated. Before incubation with the QH1 mAb (specific for quail haematopoietic and endothelial cells, 238) non-specific antigenic sites were blocked by incubation with FCS. QH1 was detected by anti-mouse IgM antibody coupled with alkaline-phosphatase (Southern Biotechnology Associate Inc., USA) revealed by NBT-BCIP. Sections were mounted by Aquatex.

Benzidine staining

Erythroid cells were revealed using benzidine staining according to the modified method of Palis et al. (239). Erythroblasts were stained in blue, whereas erythrocytes turned brown. Whole embryos were stained immediately after dissection in 0.2M sodium acetate buffered saline/benzidine dihydrochloride (Sigma) 50/50%, supplemented with 5‰ hydrogen peroxide. Embryos were immediately photographed prior to fading. Stained embryos were washed in PBS and fixed in acetate buffer containing 3.7% formaldehyde/0.1% glutaraldehyde.

In ovo AcLDL-DiI and India ink injections

Chick embryos between stages HH16 to 19 were inoculated with 3μl of AcLDL-DiI (acetil-low-density-lipoprotein-3,3'-dioctadecylindocarbocyanine-iodide) solution (Molecular Probes) into the heart according to Jaffredo et al. (105). Special care was taken not to injure the extraembryonic vascular network when inoculating the embryos. The inoculated embryos were incubated for two to three hours before sacrifice. In some cases, embryos were injected into the heart with India ink (Pelican) according to Caprioli et al. (122). AcLDL-DiI-and India ink-injected embryos were fixed overnight in 4% formaldehyde in 0.12M phosphate buffer and rinsed out. In other cases,

AcLDL-DiI-inoculated embryos were observed as whole-mounts under UV illumination. India ink-inoculated embryos were cleared in methyl salicylate in order to visualize the vascular network. AcLDL-DiI-inoculated embryos were embedded in gelatine as described above. 7 to 10 μm cryostat sections from AcLDL-inoculated embryos were stained with anti-VEGFR-2 mAb using the tyramide amplification method as described above.

Grafting experiments

Chicken and quail eggs were incubated up to embryonic day 3. Thoracic aorta regions were retrieved according to Cormier et al. (110) from quail donors ranging from HH17-21. Briefly, quail embryos were pinned down on its left side and the body wall was removed above the aorta. The thoracic part of the aorta was then dissected from the aortic arches to the mesonephros. Erythrocytes and other cells were removed by gently streaming of the buffer around the aorta. Control grafts (wing buds and allantoic rudiments) were also obtained from quail donors. Quail organ rudiments were then washed in PBS and grafted into the coeloms of chicken hosts ranging from stage HH17-20. Chimeras were incubated for different stages of embryonic development and sacrificed for immunohistological or flow cytometry analysis.

Flow cytometry analysis

Chimeras and control chick or quail embryos were sacrificed on appropriate days of incubation. Thymus, bursa of Fabricius, spleen and bone marrow were taken out and dissected. Thymi and bursas were then treated with collagenase type V. (Sigma, 1mg/ml) at 37°C for 30 minutes. Bone marrow cells were collected from the tibias and femurs by flushing with culture medium (DMEM supplemented with 3% fetal calf serum, 1% chicken serum, 1% penicillin-streptomycin, and 1% glutamine) and passing the suspension through a 22-gauge needle. Cells of the different organs from each chimera were pooled and suspensions were incubated with QH1 mAb for 30 min followed by anti-mouse IgG-FITC conjugated Ab (Biosys) in PBS. The cells were washed three times for 10 min with culture medium between

incubations. Before analysis cells were resuspended in PBS, and propodium-iodide was added to each suspension to reveal the living cells. The analysis was carried out on FacScan (Becton Dickinson).

RESULTS

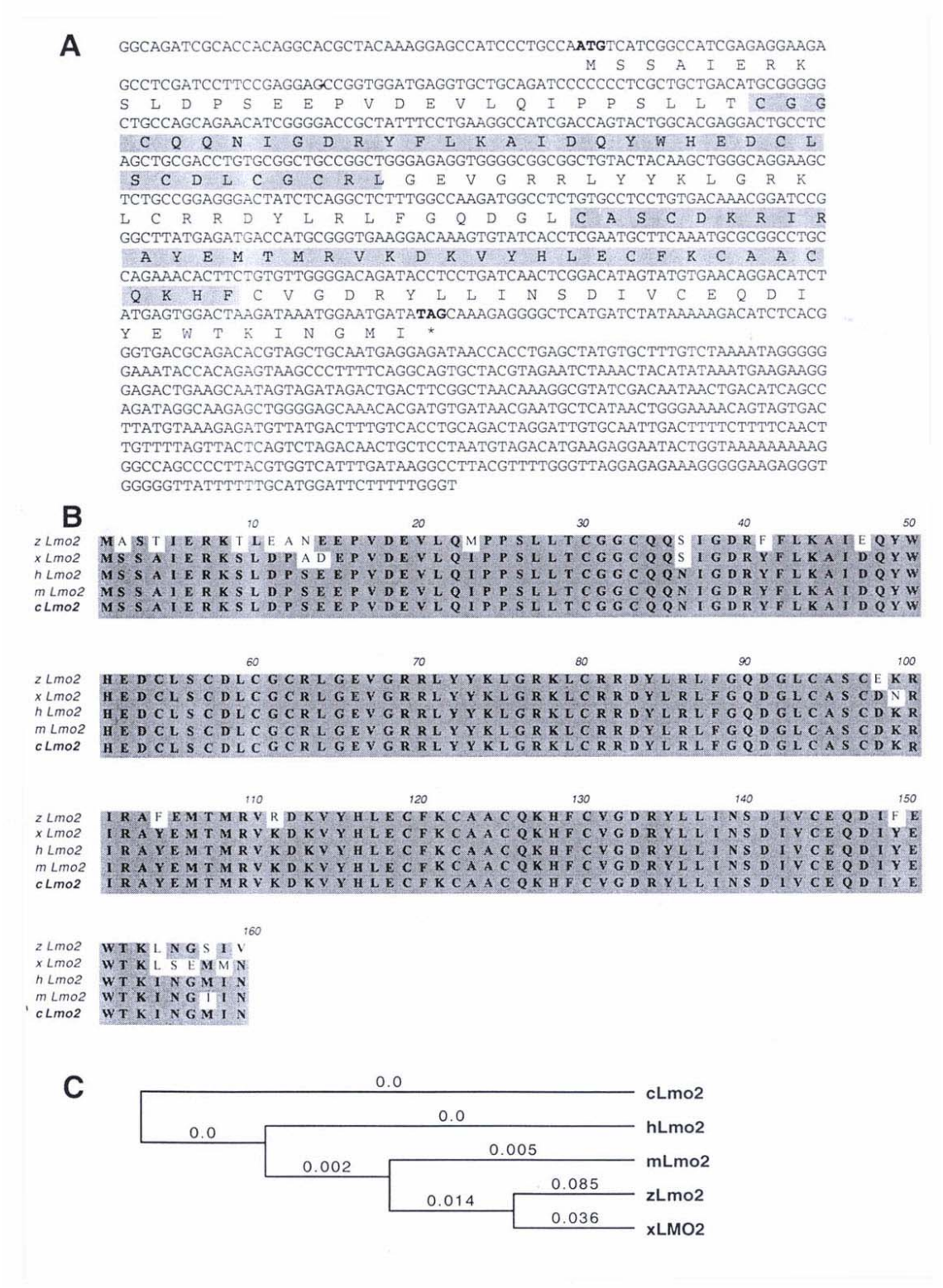
ISOLATION AND CHARACTERISATION OF THE CHICKEN LMO2 ORTHOLOG

The chicken EST database maintained at Delaware Biotechnology Institute (http://www.chickest.udel.edu/) was screened for the presence of sequences homologous to Lmo2. One clone displayed significant homologies to human (240), mouse (241), zebrafish (230) and the recently isolated Xenopus (242) Lmo2 cDNAs. This clone, identified as pat.pk0056.h5.F, originated from a cDNA library prepared from ConA activated chicken splenic T cells. Its sequenced 396 bp strech displayed more than 80% homology with the already cloned Lmo2 cDNAs and thus was likely to be the avian ortholog for Lmo2. Both strands were further sequenced; however the Sp6 3' end sequencing was ineffective. The T7 5' end sequencing yielded 719 bp covering the whole putative coding sequence except the 5' end. An internal oligonucleotide (5' GGTGACGCAGACACGTAGCT 3'; position 525-544) was designed to sequence the 3' end of the cDNA. The sequence yielded an additional 485 bp fragment. Taken together, the two sequences cover 1047 bp encompassing the whole putative coding sequence, except the 5' end, and 560 bp of the 3' untranslated region. Since the Sp6 end contained sequences that may preclude synthesis of single strand RNA probes, part of the 3' end of the cDNA was removed. A single XbaI digestion was performed at position 891 and a fragment of approximately 250 bp was removed. After ligation, the plasmid contained a cDNA of 855 bp that was used to synthesize sense and antisense probes for in situ hybridization.

A 5' RACE PCR was performed to isolate the 5' end of Lmo2, recovering an additional 80 bp sequence containing the initiation codon and 46 bp of 5' untranslated sequence. The full-length chick Lmo2 cDNA encompasses 1083 bp (Fig. 5/A) with an open reading frame (ORF) of 476 bp, the start codon located at position 48 and the stop codon at position 520 (Fig. 5/A). The ORF encodes a predicted protein of 158 amino acids that shares greater than 90% sequence identity with the human, mouse, Xenopus and zebrafish genes

(Fig.5/A, B). Protein domain analysis (Pro CITE) revealed the presence of two Lim 1 domains shared with human, mouse, zebrafish and Xenopus Lmo2, localized at nucleotides 134 to 238 and 326 to 433 (Fig. 5/A). The phylogenetic tree demonstrates close evolutionary conservation of the Lmo2 proteins from fish to human (Fig. 5/C). The cLmo2 sequence is available at GenBank under the accession number AF468789.

Figure 5. DNA sequence and amino acid sequence homology between chicken Lmo2 and the mouse, human, zebrafish and Xenopus orthologs. (A) DNA sequence and protein translation. Start and stop codons are indicated in bold characters. Shaded boxes localize the two LIM domains. (B) Pile-up analysis of the Lmo2 protein sequences reveals extensive sequence identity between vertebrate Lmo2 proteins. Accession numbers for sequences used in this comparison: human (hLmo2, NM-005574; 241), mouse (mLmo-2, NM-008505; 241), zebrafish (zLmo-2, AF191560; 230) and Xenopus xLmo2, AF374473, 242). (C) A phylogenetic tree of the LIM-only proteins shows the close relationship of the vertebrate Lmo2 proteins.


A
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M S S A I E R K
GCCTCGATCCTTCCGAGGAGCCGGTGGATGAGGTGCTGCAGATCCCCCCCTCGCTGCTGACATGCGGGGG
S L D P S E E P V D E V L Q I P P S L L T C G G
CTGCCAGCAGAACATCGGGGACCGCTATTTCCTGAAGGCCATCGACCAGTACTGGCACGAGGACTGCCTC
C Q Q N I G D R Y F L K A I D Q Y W H E D C L
AGCTGCGACCTGTGCGGCTGCCGGCTGGGAGAGGTGGGGCGGCGGCTGTACTACAAGCTGGGCAGGAAGC
S C D L C G C R L G E V G R R L Y Y K L G R K
TCTGCCGGAGGGACTATCTCAGGCTCTTTGGCCAAGATGGCCTCTGTGCCTCCTGTGACAAACGGATCCG
L C R R D Y L R L F G Q D G L C A S C D K R I R
GGCTTATGAGATGACCATGCGGGTGAAGGACAAAGTGTATCACCTCGAATGCTTCAAATGCGCGGCCTGC
A Y E M T M R V K D K V Y H L E C F K C A A C
CAGAAACACTTCTGTGTTGGGGACAGATACCTCCTGATCAACTCGGACATAGTATGTGAACAGGACATCT
Q K H F C V G D R Y L L I N S D I V C E Q D I
ATGAGTGGACTAAGATAAATGGAATGATATAGCAAAGAGGGGCTCATGATCTATAAAAAGACATCTCACG
Y E W T K I N G M I *
GGTGACGCAGACACGTAGCTGCAATGAGGAGATAACCACCTGAGCTATGTGCTTTGTCTAAAATAGGGGG
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GAGACTGAAGCAATAGTAGATAGACTGACTTCGGCTAACAAAGGCGTATCGACAATAACTGACATCAGCC
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TTATGTAAAGAGATGTTATGACTTTGTCACCTGCAGACTAGGATTGTGCAATTGACTTTTCTTTTCAACT
TGTTTTAGTTACTCAGTCTAGACAACTGCTCCTAATGTAGACATGAAGAGGAATACTGGTAAAAAAAAAG
GGCCAGCCCCTTACGTGGTCATTTGATAAGGCCTTACGTTTTGGGTTAGGAGAGAAAGGGGGAAGAGGGT
GGGGGTTATTTTTTGCATGGATTCTTTTTGGGT
B
10 20 30 40 50
z Lmo2 MASTIERKTLEANEEPVDEVLQMPPSLLTCGGCQQSIGDRFFLKAIEQYW
x Lmo2 MSSAIERKSLDPADEPVDEVLQIPPSLLTCGGCQQSIGDRYFLKAIDQYW
h Lmo2 MSSAIERKSLDPSEEPVDEVLQIPPSLLTCGGCQQNIGDRYFLKAIDQYW
m Lmo2 MSSAIERKSLDPSEEPVDEVLQIPPSLLTCGGCQQNIGDRYFLKAIDQYW
c Lmo2 MSSAIERKSLDPSEEPVDEVLQIPPSLLTCGGCQQNIGDRYFLKAIDQYW
60 70 80 90 100
z Lmo2 HEDCLSCDLCGCRLGEVGRRLYYKLGRKLCRRDYLRLFGQDGLCASCEKR
x Lmo2 HEDCLSCDLCGCRLGEVGRRLYYKLGRKLCRRDYLRLFGQDGLCASCDNR
h Lmo2 HEDCLSCDLCGCRLGEVGRRLYYKLGRKLCRRDYLRLFGQDGLCASCDKR
m Lmo2 HEDCLSCDLCGCRLGEVGRRLYYKLGRKLCRRDYLRLFGQDGLCASCDKR
c Lmo2 HEDCLSCDLCGCRLGEVGRRLYYKLGRKLCRRDYLRLFGQDGLCASCDKR
110 120 130 140 150
z Lmo2 IRAFEMTMRVRDKVYHLECFKCAACQKHFCVGDRYLLINSDIVCEQDIFE
x Lmo2 IRAYEMTMRVKDKVYHLECFKCAACQKHFCVGDRYLLINSDIVCEQDIYE
h Lmo2 IRAYEMTMRVKDKVYHLECFKCAACQKHFCVGDRYLLINSDIVCEQDIYE
m Lmo2 IRAYEMTMRVKDKVYHLECFKCAACQKHFCVGDRYLLINSDIVCEQDIYE
c Lmo2 IRAYEMTMRVKDKVYHLECFKCAACQKHFCVGDRYLLINSDIVCEQDIYE
160
z Lmo2 WTKLNGSIV
x Lmo2 WTKLSEMMN
h Lmo2 WTKINGMIN
m Lmo2 WTKINGIIN
c Lmo2 WTKINGMIN
C
0.0
cLmo2
0.0
hLmo2
0.0
0.005
mLmo2
0.002
0.085
zLmo2
0.014
0.036
xLMO2

Figure 5.

GENE EXPRESSION DURING YOLK SAC HAEMATOPOIESIS

Deposition of the early extraembyonic mesoderm

Prospective extraembryonic mesoderm is laid down at the beginning of the gastrulation, by the ingression of cells from the superficial epiblast into the primitive streak. Fate maps of different vertebrate species are very similar and show that the yolk sac mesoderm is derived from the posterior part of the streak (58; 59; 87; 243). In the avian embryo, cells deposited first (HH2-3) undergo extensive lateral and anterior migrations and reach lateral extraembryonic regions, while cells passing through the streak immediately thereafter occupy more medial positions. Thus, earlier deposited cells have a longer life history than their immediate neighbours and undergo differentiation earlier. These migrating cells will form the mesoderm of the Area Opaca (AO), the peripheral region of the blastoderm where blood islands form. No obvious mesodermal cell clumps are visible at the initiation of this process, mesodermal cells show mesenchymal morphology. The precise timing of events may vary somewhat from embryo to embryo.

At the intermediate streak stage (12-13h, HH3) and HH3+, the earliest stages examined, GATA-2 was detected in the whole blastoderm except in an inverted U-shaped area surrounding the primitive streak (Fig. 6/A, B). The whole mesoderm appeared positive although expression levels varied between individual cells. Some intensively positive cells located next to the endoderm (Fig. 6/C, D; arrows). Epiblast cells also expressed the transcripts. SCL, Lmo2, and GATA-1 were not detected at HH3. SCL and Lmo2 transcripts appeared somewhat later, in few mesodermal cells scattered in the posterior region of the blastoderm (at early definitive streak stage, 18-19h, HH4; not shown).

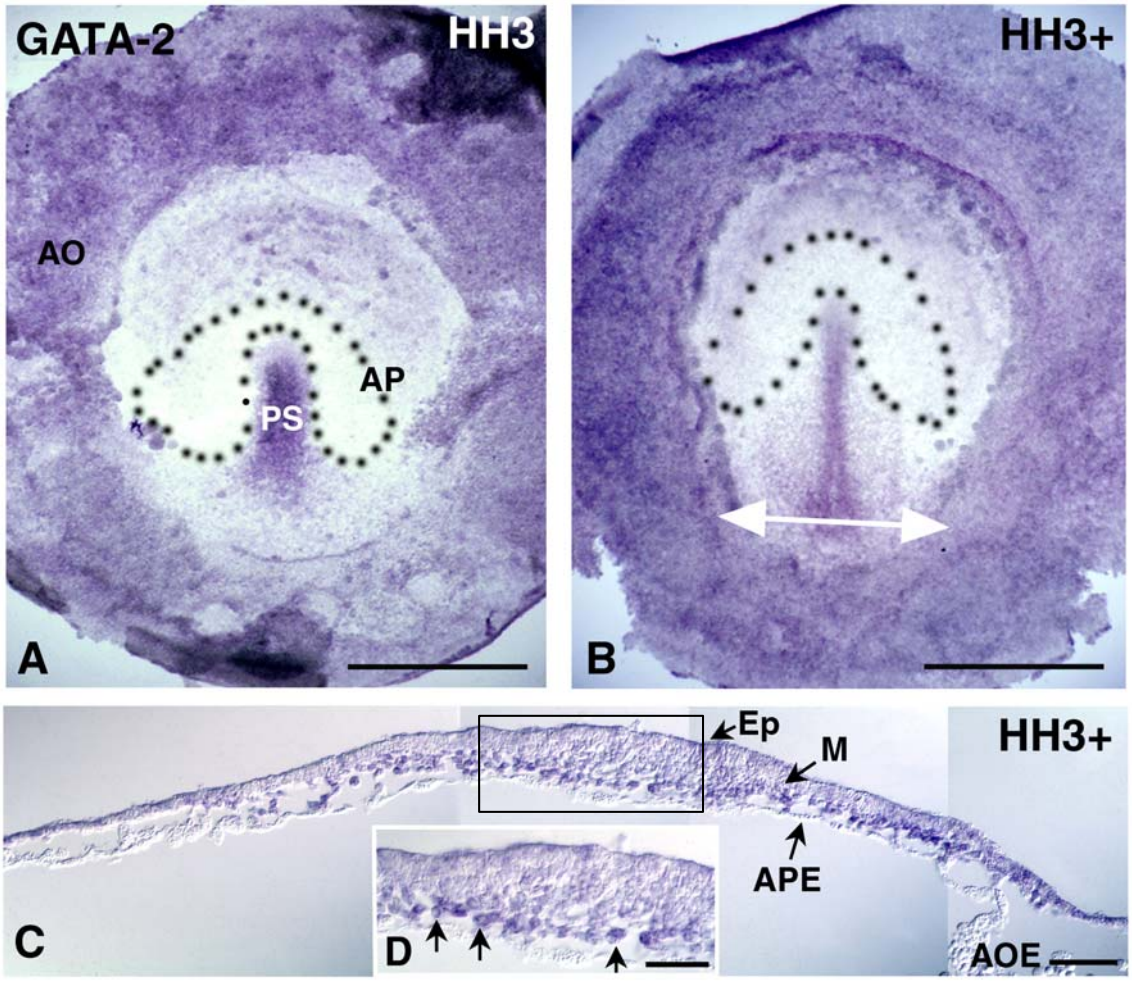


Figure 6. GATA-2 expression during early and late intermediate streak stages (HH3 and HH3+). (A, B) Whole-mount in situ hybridization; dorsal views, anterior side up. (C, D) Sections at the level indicated in B (Nomarski optics).

(A) Elongating primitive streak. GATA-2 transcripts are present in the primitive streak and the epiblast except in an inverted U-shaped area surrounding the streak (dotted area). Bar=1mm. (B) Just prior to the definitive streak stage, the GATA-2 negative territory has expanded (dotted area). In the area pellucida, GATA-2 expressing cells are restricted to the posterior part of the blastodisc. Bar=1mm. (C) Cross-section at the level indicated in B. GATA-2 mRNA is present both in the epiblast and the mesoderm but is absent from the endoderm. The frame shows the position of the insert (D). Bar=200µm. (D) Higher magnification of the region indicated in C. A layer of strongly positive cells is found in the mesoderm close to the endoderm (arrows). Bar=100µm (AO: Area Opaca; AOE: Area Opaca Endoderm; AP: Area Pellucida; APE: Area Pellucida Endoderm; Ep: Epiblast; M: Mesoderm; PS: Primitive Streak).

Hemangioblasts and the first blood islands

Definitive streak stage (HH4)

When the primitive streak reached its maximal extension (18-19h of incubation, HH4) GATA-2 continued to be expressed at varying levels by all mesodermal cells in the posterior part of the primitive streak (Fig. 7/A, D). SCL and Lmo2 positive cells rapidly increased in number to form a horseshoe-shaped area, characteristic of the developing blood-forming system (Fig 7/B, C). At this stage morphologically distinct blood island-like structures were not yet visible in the mesoderm. The transcripts were located in single cells or groups of cells scattered in the mesoderm (Fig. 7/E, F). Neither GATA-1 nor hemoglobin was detected at this stage (data not shown).

VEGFR-2 mRNA has been found in the caudal region of the blastoderm (148); however its protein distribution had not previously been investigated in detail. Anti-Quek-1 antibody was used to reveal VEGFR-2 protein distribution and compared with the other markers. The receptor protein was distributed throughout the nascent mesoderm (Fig. 7/G, H).

Figure 7. Definitive streak stage (HH4). (A, B, C) GATA-2, SCL and Lmo2 whole mount in situ hybridizations. Dorsal views, future head region is above. Bar=1mm. (D, E, F) GATA-2, SCL and Lmo2 expressions at the level indicated in A, parallel transverse sections from a single embryo. Bar=100μm (G, H) Anti-VEGFR-2 immunohistochemistry at the same, posterior level. Bar=100μm. (A) GATA-2 transcripts are present in the entire blastoderm except in the anterior part of the area pellucida. GATA-2 mRNA is also present in the posterior primitive streak. SCL (B) and Lmo2 (C) positive groups of cells are found in the postero-lateral region of the blastoderm. (D) GATA-2 transcripts are present throughout the entire epiblast and mesoderm. At the level of the streak, scattered mesodermal cells express the transcript at a higher level. SCL (E) and Lmo2 (F) expressions are detected in isolated cells or groups of cells in the GATA-2-expressing mesoderm (compare D, E and F). (G) The VEGFR-2 protein (green) is widely distributed throughout the mesoderm. (H) Computer superimposition of the VEGFR-2 signal (red) and Nomarski optics view. (AOE: Area Opaca Endoderm; APE: Area Pellucida Endoderm; PS: Primitive Streak, Ep: Epiblast; M: Mesoderm).

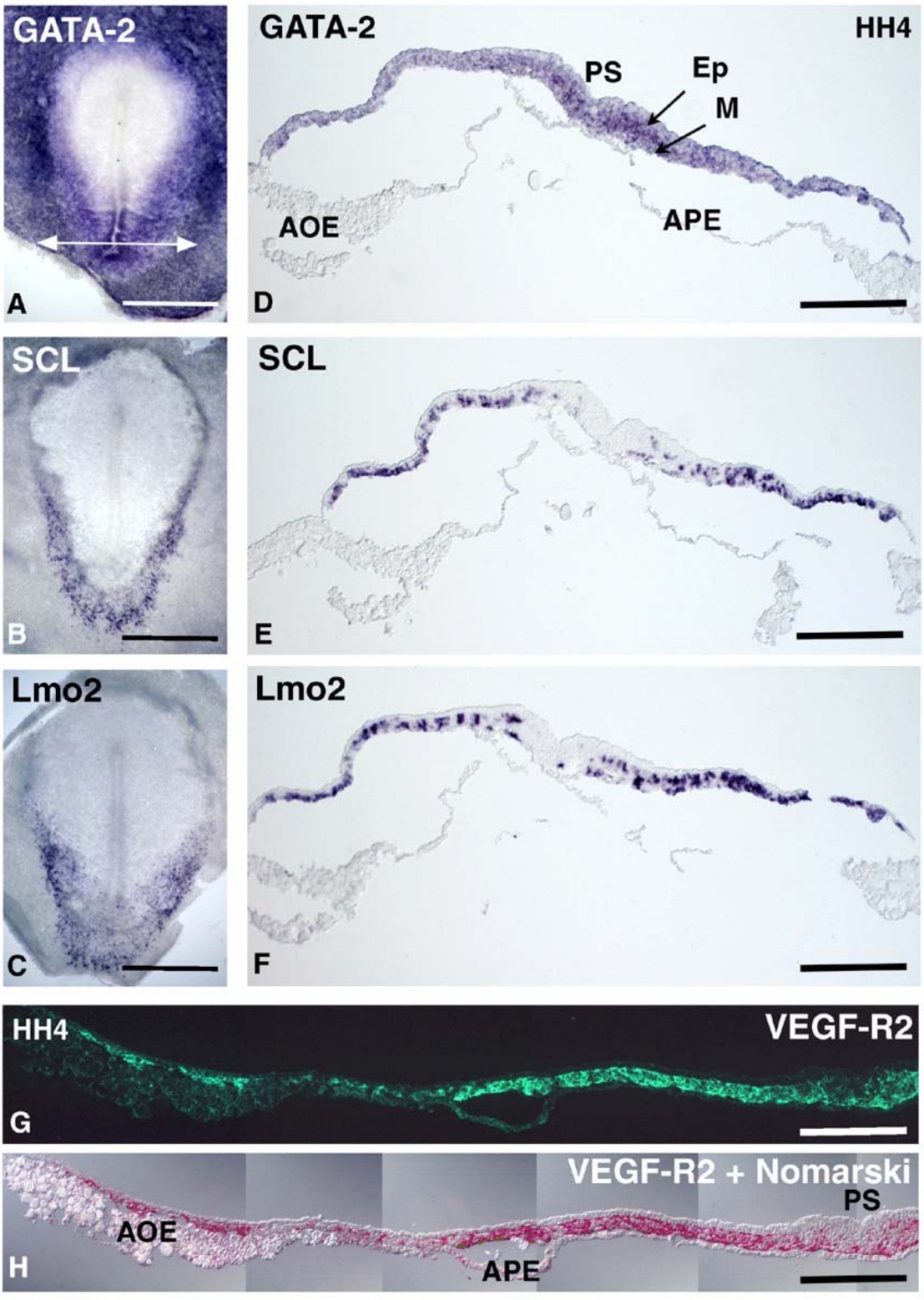

GATA-2
A
GATA-2
HH4
PS
Ep
M
AOE
APE
D
SCL
B
SCL
E
Lmo2
C
Lmo2
F
HH4
VEGF-R2
G
VEGF-R2 + Nomarski
PS
AOE
APE
H

Figure 7.

Head process and head fold stages (HH5 and 6)

Patterning of the blood-forming system became conspicuous at the head process stage (19-22 h of incubation, HH5), characterised by the histological emergence of cell aggregates in the peripheral area of the blastoderm. Some mesenchymal cells became packed together close to the endoderm and formed blood islands in the postero-lateralmost aspect of the AO. These aggregates became characteristic and more numerous at the head fold stage (23-25 h. of incubation; HH6) (60).

By the head process stage and more obviously by the head fold stage, the U-shaped GATA-2/SCL/Lmo2^{+} territories spread in lateral and anterior directions, in accordance with the centrifugal migration of extraembryonic mesoderm (Fig. 8/A, B, C). GATA-2 expression was upregulated in the future blood islands (arrows in Fig. 8/A), which became visible in the posterolateral aspect of the blastoderm by head fold stage (Fig. 8/E, F). SCL and Lmo2 expression levels were high in blood islands (Fig. 8/G, I; arrows). Furthermore in the GATA-2 positive region, single cells or small groups of cells scattered between blood islands also expressed SCL and Lmo2 (Fig. 8/G, I, arrowheads). GATA-1 mRNA, first detected by HH5, was also expressed in prospective blood islands (Fig. 8/D), although at very low levels (Fig. 8/H).

In summary, all posterior mesodermal cells expressed GATA-2 immediately after ingression through the primitive streak. SCL and Lmo2 labelled clusters of cells in the GATA-2 positive region which upregulated GATA-2 and become blood island. GATA-1 expression was detectable in the GATA-2 upregulating peripheralmost islands. The combined patterns for the head process stage are represented in Figure 9.

Figure 8. Emergence of blood islands, head process and head fold stages. (A, B, C, D) Whole-mount in situ hybridization, head process stage (HH5). (E-I) head fold stage (HH6). (E-I) Serial sections at the level indicated on the insert of E.

(A) GATA-2 is expressed throughout the blastoderm, except in the anterior part of the AP. Arrows indicate cell aggregates expressing high levels of GATA-2 at the periphery of AO. (B, C) SCL and Lmo2 expression. Transcripts of both of these genes are restricted to cell groups in the posterolateral domain. (D) GATA-1 mRNA expression is high in the most peripheral cell aggregates of the AO (arrows), compare with A. The positive area around the PS is due to non-specific staining. Bar=1mm.

(E) GATA-2 mRNA is present throughout the mesoderm but the expression is higher in lateral forming blood islands. Bar=200µm. (F, G) Neighbouring transverse sections from the posterior region, indicated on E. F is a higher magnification of the area framed in E. The same cell aggregates are indicated by arrows. SCL (G) is present in the same peripheral forming blood islands as GATA-2 (F) but is also found in single cells or groups of cells (arrowheads) that do not display high GATA-2 expression. (H, I) Neighbouring sections, arrows indicate the same forming blood islands. Lmo2 (I) distribution is similar to that of SCL (G). Arrowheads indicate single cells not organized into multicellular structures. GATA-1 (H) is only faintly expressed at this level (arrows). Bar=100µm.

APE: area pellucida endoderm, AOE: area opaca endoderm, E: ectoderm, PS: primitive streak.

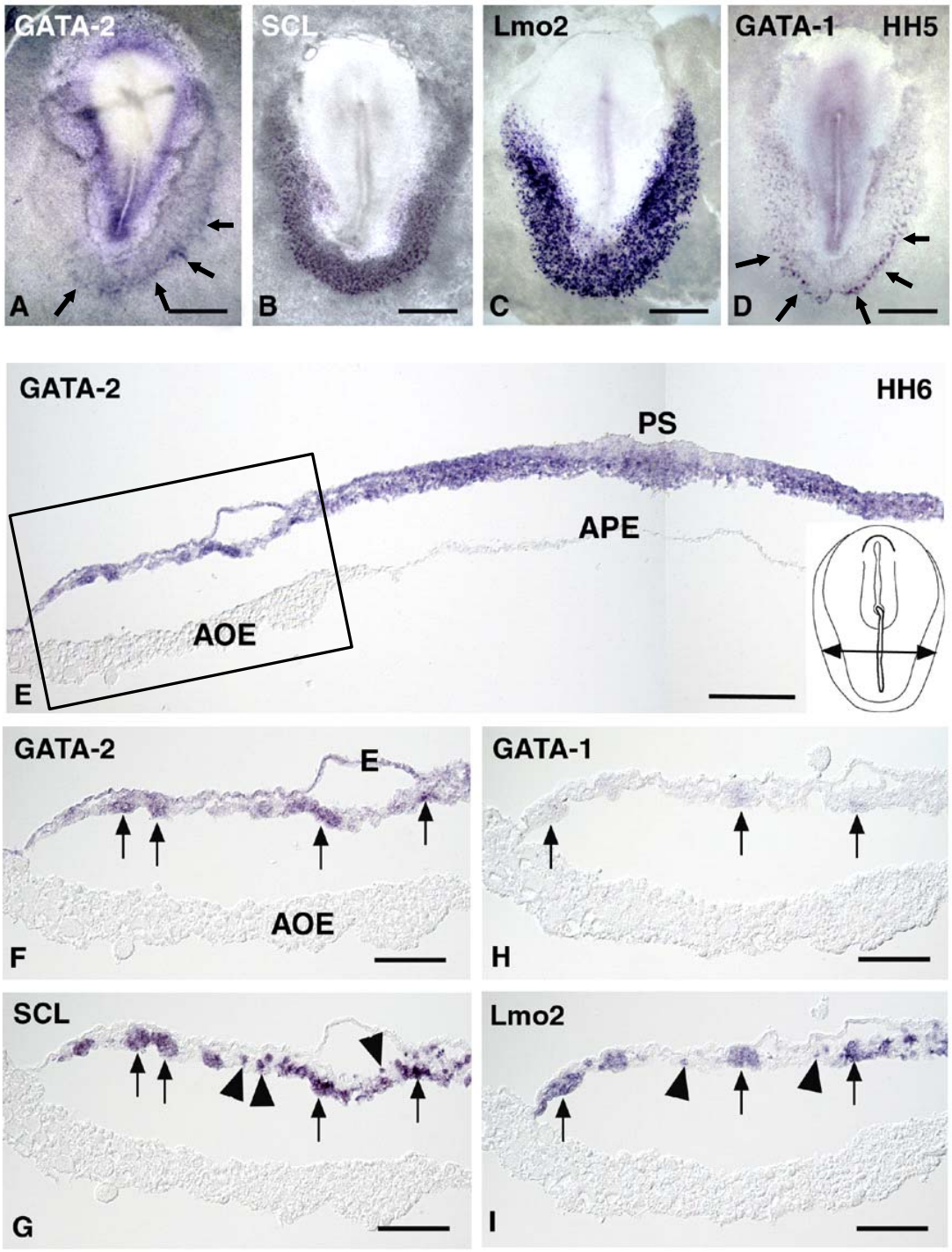

GATA-2
SCL
Lmo2
GATA-1
HH5
A
B
C
D
GATA-2
HH6
PS
APE
AOE
E
GATA-2
E
AOE
F
GATA-1
H
SCL
G
Lmo2
I

Figure 8.

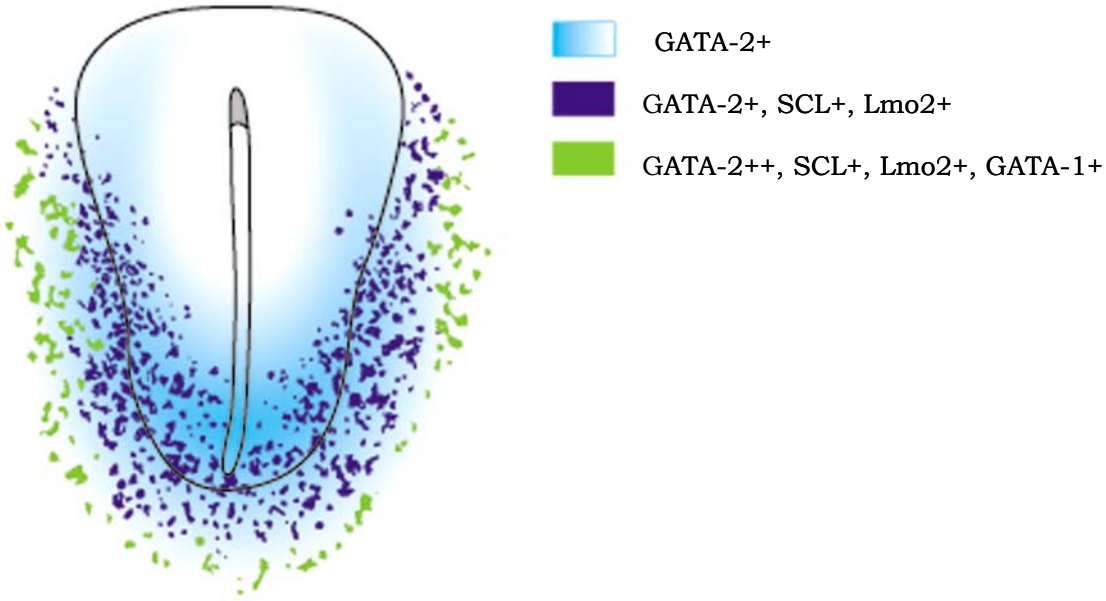


Figure. 9.

Differentiation of blood islands (somitic stages)

When segmentation occurs in the embryo, peripheral blood islands begin to differentiate in the yolk sac: peripheral cells flatten into endothelial cells at the edges of the aggregates while cells of the interior region became rounded and progress toward overt haematopoiesis.

At the 4- to 5 pairs of somite stage (26-29 h of incubation, HH8); GATA-2 continued to be expressed in the extraembryonic and lateral plate mesoderm and non-neural ectoderm. The expression was upregulated in the blood islands according to a centrifugal gradient (Fig. 10/A, F). In the caudal area pellucida (AP, transparent area around the embryo) a strong homogeneous signal was also detected around the primitive streak (Fig. 10/A). SCL and Lmo2 transcripts were distributed in clusters of cells (Fig. 10/B, C). GATA-1 expression was very similar to that of GATA-2 in the AO (compare Fig. 10/A

with 10/D). GATA-1 transcripts, present mainly in lateral blood islands, were also detected at very low levels in more medial cell groups with high SCL and GATA-2 expression (compare Fig. 10/H to Fig. 10/F, G). The very first hemoglobin-containing cells were detected at this stage at the periphery of the AO (Fig. 10/E, arrows). The combined patterns constructed from pictures of whole mount stained embryos are shown in Figure 10/I.

Figure 10. Differentiation of blood islands, 4 pairs of somite. (A-E) Whole mount in situ hybridization and haemoglobin staining. (F-H) Serial sections from the posterior part of the primitive streak at the level indicated in A. (I) Summary of the patterns at HH8. (A) GATA-2 transcripts are present throughout the extraembryonic area and the posterior part of the AP. A strong signal is detected in peripheral and lateral blood islands. (B, C) SCL and Lmo2 mRNAs are found in the whole extraembryonic area and the embryo proper. (D) GATA-1 transcripts are present in peripheral blood islands where GATA-2 is upregulated. (E) Hemoglobin, as revealed by benzidine staining, marks the more mature islands in the AO (arrows). Bar=1mm. (F) GATA-2 transcripts are distributed in the whole mesoderm and ectoderm. Developing blood islands in the AO show high GATA-2 expression (arrowheads). (G) SCL mRNA is restricted to single cells or groups of cells in the AP and is strongly expressed in blood islands of the AO. (H) GATA-1 transcripts are detected in blood islands at the periphery of the AO but are also present at very low levels in more medial structures. Arrow indicates the same level in the three sections, characterized by increased GATA-2, high SCL and low GATA-1 expressions. Small arrowheads indicate the same blood islands in G and H. Bar=100μm. (I) Summary of the patterns at HH8.
AOE: area opaca endoderm, APE: area pellucida endoderm, PS: primitive streak.

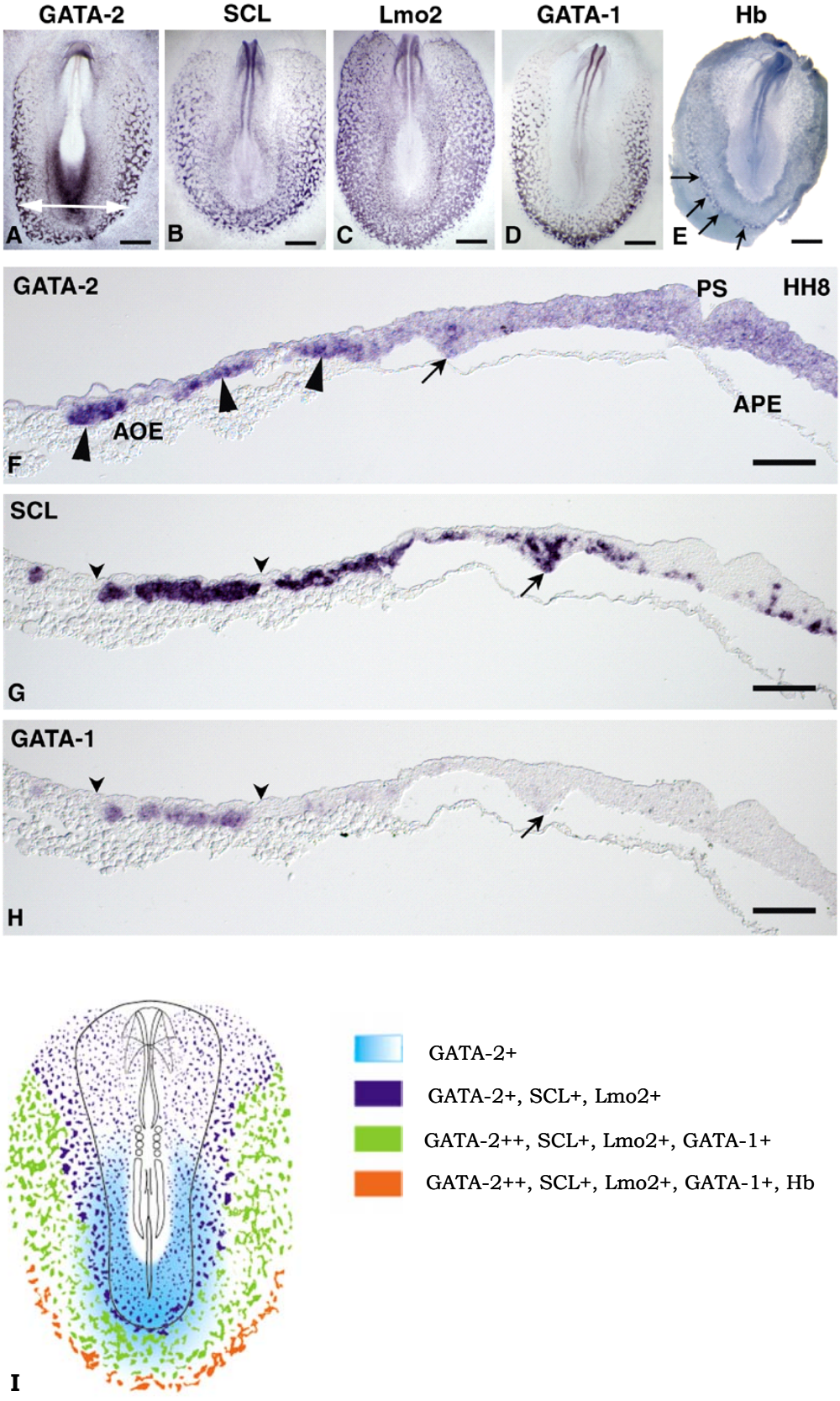

GATA-2
SCL
Lmo2
GATA-1
Hb
A
B
C
D
E
GATA-2
PS
HH8
APE
AOE
F
SCL
G
GATA-1
H
GATA-2+
GATA-2+, SCL+, Lmo2+
GATA-2++, SCL+, Lmo2+, GATA-1+
GATA-2++, SCL+, Lmo2+, GATA-1+, Hb
I

Figure 10.

Maturation of blood islands (somitic stages)

During the somitic stages, blood islands of the lateral AO begin differentiating into blood vessel and intravascular blood cell groups which remain attached to the dorsal aspect of the vessel wall. Maturation of blood islands progresses towards the AO/AP borderline while gastrulation proceeds in the regressing primitive streak. The 9-somite stage was selected for comparative study since it displays all steps from the commitment of the mesoderm to the full differentiation of blood islands in a single transverse section, as evidenced by morphological signs.

Blood islands expressing GATA-2, SCL, Lmo2 and GATA-1 extended throughout the whole extraembryonic mesoderm (Fig. 11/A-D). Haemoglobin synthesis became conspicuous in the posterior and lateral blood islands (Fig. 11/E). ChT expression, the chick Brachyury orthologue (237), used as a marker for nascent mesoderm, was restricted to mesodermal cells in the streak proper, at very low levels (Fig. 11/F). SCL and Lmo2 were detected in single cells or groups of cells in the GATA-2^{+} mesoderm, close to the endoderm (Fig. 11/H, I). GATA-2 expression increased in the forming blood islands at the border between the AP and the AO. GATA-1 was expressed in the same structures (compare Figs. 11/G with J and 12/H). As blood islands differentiated, GATA-2 expression persisted in interior haematopoietic cells whereas it weakened in exterior endothelial cells (Fig. 11/G, 12/E). At the same time, SCL decreased in externalmost cells of the island although some interior cell retained high SCL levels (Fig. 11/H, 12/F). Lmo2 was enhanced in external cells, forming a continuous layer surrounding haematopoietic cells (compare Figs. 11/H, I, and 12/F, G). In the mature, most lateral intravascular blood islands, GATA-2 was decreased (Fig. 11/A, G, 12/I), whereas GATA-1 was enhanced (Fig. 11/D, J, 12/L). SCL and Lmo2 expressions decreased in haematopoietic cells, whereas Lmo2 persisted in endothelial cells (Fig. 12/J, K). The combined expressions are represented in Figure 11/K.

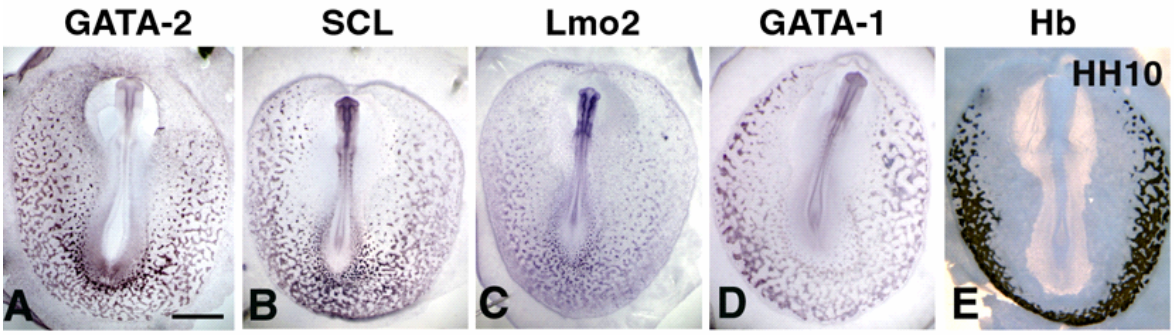
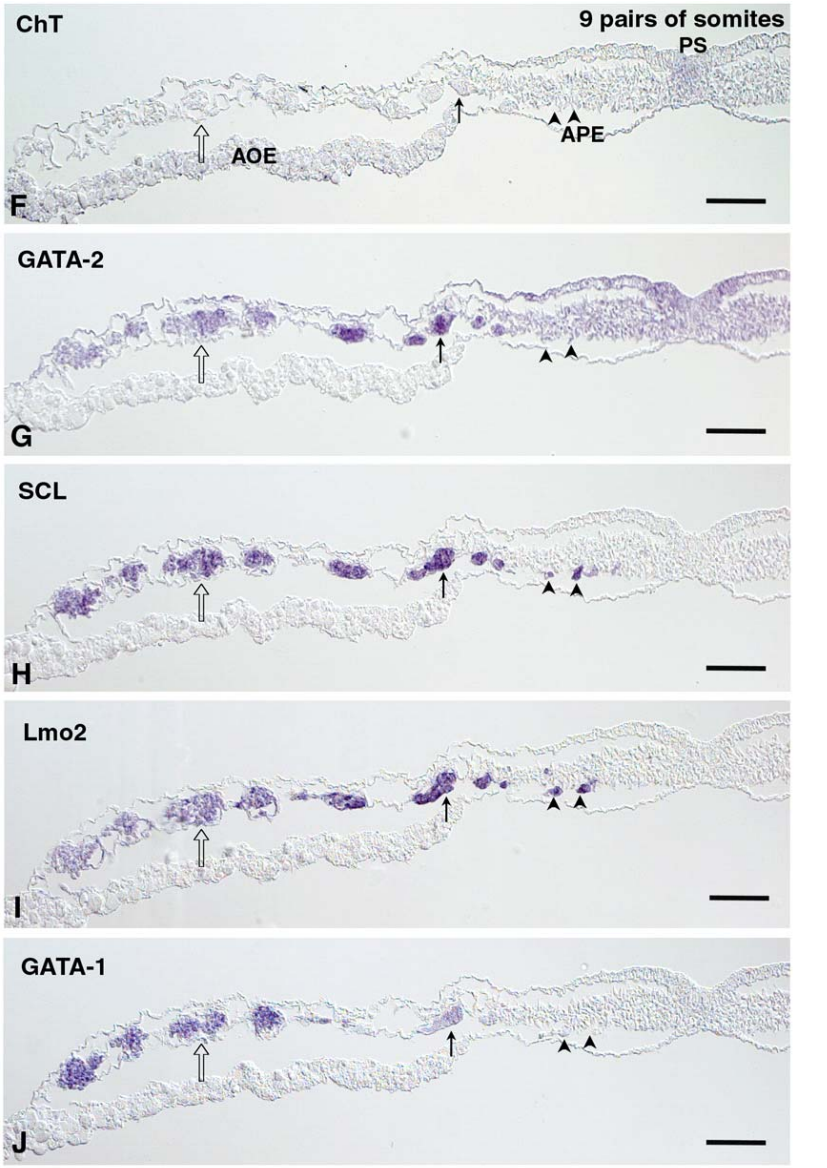


Figure 11. HH10: from mesoderm to intravascular blood cell groups. In situ hybridisation on whole mounts, HH10 (33-38 h of incubation) (A-E), and parallel sections from the same embryo (F-J, see next page), 9 pairs of somite (HH10). (A) GATA-2 expression has progressed throughout the AO. (B) SCL is strongly expressed by lateral blood islands as well as around the posterior part of the embryo. (C) Lmo2 displays a similar pattern, but designates the valls of the vessels as well. (D) GATA-1 is enhanced in the lateral blood islands. (E) Haemoglobin is now very strong in lateral islands. Bar= 2mm. (F) Brachyury (ChT) is restricted to the mesoderm at the level of the streak proper, at very low level. (G) GATA-2 is expressed by the mesoderm starting from its ingression through the streak. The signal is enhanced in nascent blood islands at the AP/AO border (solid arrow) but decreased in lateral, mature blood cells (empty arrow). (H) SCL is expressed by single cells or groups of cells, close to the endoderm, adjacent from the streak (arrowheads). Expression persists in nascent (solid arrow), and in mature (empty arrow) islands although expression levels vary from cell to cell. (I) Lmo2 expression resembles that of SCL and varies as soon as Lmo2 becomes detectable in the blood islands. (J) GATA-1 transcripts are found first in nascent blood islands where GATA-2 is upregulated (compare G to J). The GATA-1 signal increases as the islands mature, the highest expression level being found in the most lateral ones (empty arrow). Arrowheads, solid arrows and empty arrows point out the same structures on the different sections. Bar=100µm. (K, see next page) Summary of the patterns at HH10.

AOE: area opaca endoderm, APE: area pellucida endoderm, PS: primitive streak.


ChT
9 pairs of somites
PS
AOE
APE
F
GATA-2
G
SCL
H
Lmo2
I
GATA-1
J

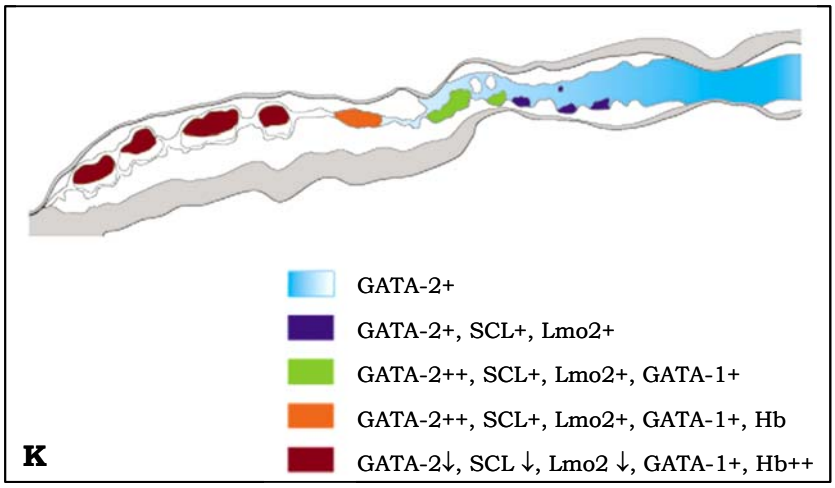

GATA-2+
GATA-2+, SCL+, Lmo2+
GATA-2++, SCL+, Lmo2+, GATA-1+
GATA-2++, SCL+, Lmo2+, GATA-1+, Hb
GATA-2↓, SCL ↓, Lmo2 ↓, GATA-1+, Hb++
K

Figure 11.

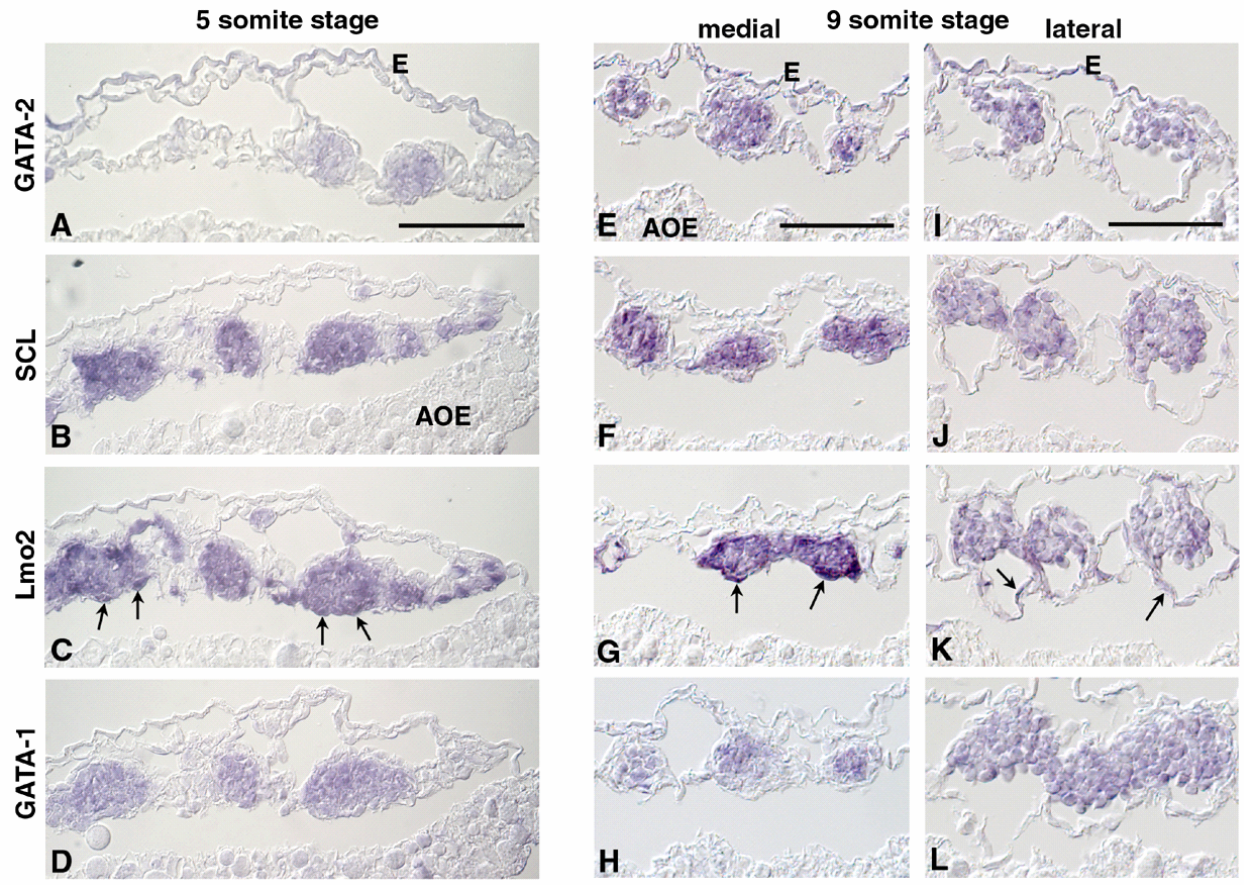


Figure 12. Comparison of GATA-2, SCL, Lmo2 and GATA-1 expression during blood island differentiation. (A-D) 5 pairs of somite, peripheral blood islands. (A) GATA-2 expression is obvious in blood islands. External cells of the islands display a weak or no signal. The ectoderm displays a weak GATA-2 signal. (B) SCL is strongly expressed by cells of the islands. Some internal cell upregulate the expression. A few single cells expressing the transcript are also detected between the islands. (C) Lmo2 is expressed by cells of the blood islands and appears upregulated in some of the external most cells (arrows). Like SCL, Lmo2 is also detected in isolated cells close to the islands. (D) GATA-1 is restricted to the internal cells of the islands. (E-H) 9 pairs of somite, maturing blood islands in the middle of the AO. (E) GATA-2 continues to be preferentially expressed by internal cells of the islands and by the ectoderm. (F) SCL expression is maintained in internal cells of the islands but decreases in external cells. (G) Lmo2 expression is maintained in internal cells of the island but increases in external cells (arrows). (H) GATA-1 is still expressed by internal cells. (I-L) 9 pairs of somite, intravascular islands at the edges of the blastoderm. (I) Blood cells and ectoderm express GATA-2. No signal is detected in endothelia. (J) SCL expression is maintained by blood cells. (K) Lmo2 expression is maintained in blood cells and endothelia (arrows). (L) GATA-1 is expressed only by blood cells. Bar (A-D): 100 µm, Bar (E-L): 150 µm. AOE: area opaca endoderm, E: ectoderm.

The VEGFR-2 protein signal was seen in a few scattered cells and in small clusters of cells close to the endoderm in the AP region, contrasting the wide expression at the definitive streak stage. In maturing blood islands, the receptor persisted only in endothelia (Fig. 13/A-F).

Figure 13. VEGFR-2 protein in maturing and mature blood islands. (A-F) Immunfluorescent staining of a representative embryo with Quek-1 mAb (for VEGFR-2), 10 pairs of somite. (A, C, E) fluorescence signal, (B, D, F) computer superimposition of the fluorescent labelling (red) and Nomarski optics views, the receptor expression appears in false colour.

(A, B) Cross-section through the posterior part of the primitive streak. Single cells or small groups of VEGFR-2+ cells are found scattered in the mesoderm. Most positive cell groups are close to the endoderm. A nascent blood island is clearly visible (arrowhead). A maturing island is detected at the borderline between AP and AO (arrow) with the external but not internal cells displaying VEGFR-2 protein. Bar =100μm. (C, D) Maturing islands representative of the AP/AO region (arrows). External cells express VEGFR-2 at high level while internal cells are negative. (E, F) Mature blood islands from the lateral part of the AO. VEGFR-2 shows mosaic expression levels in endothelia. Arrow indicates a positive endothelial cell. Bar=50 μm.

AOE: area opaca endoderm, APE: area pellucida endoderm, E: ectoderm, PS: primitive streak.

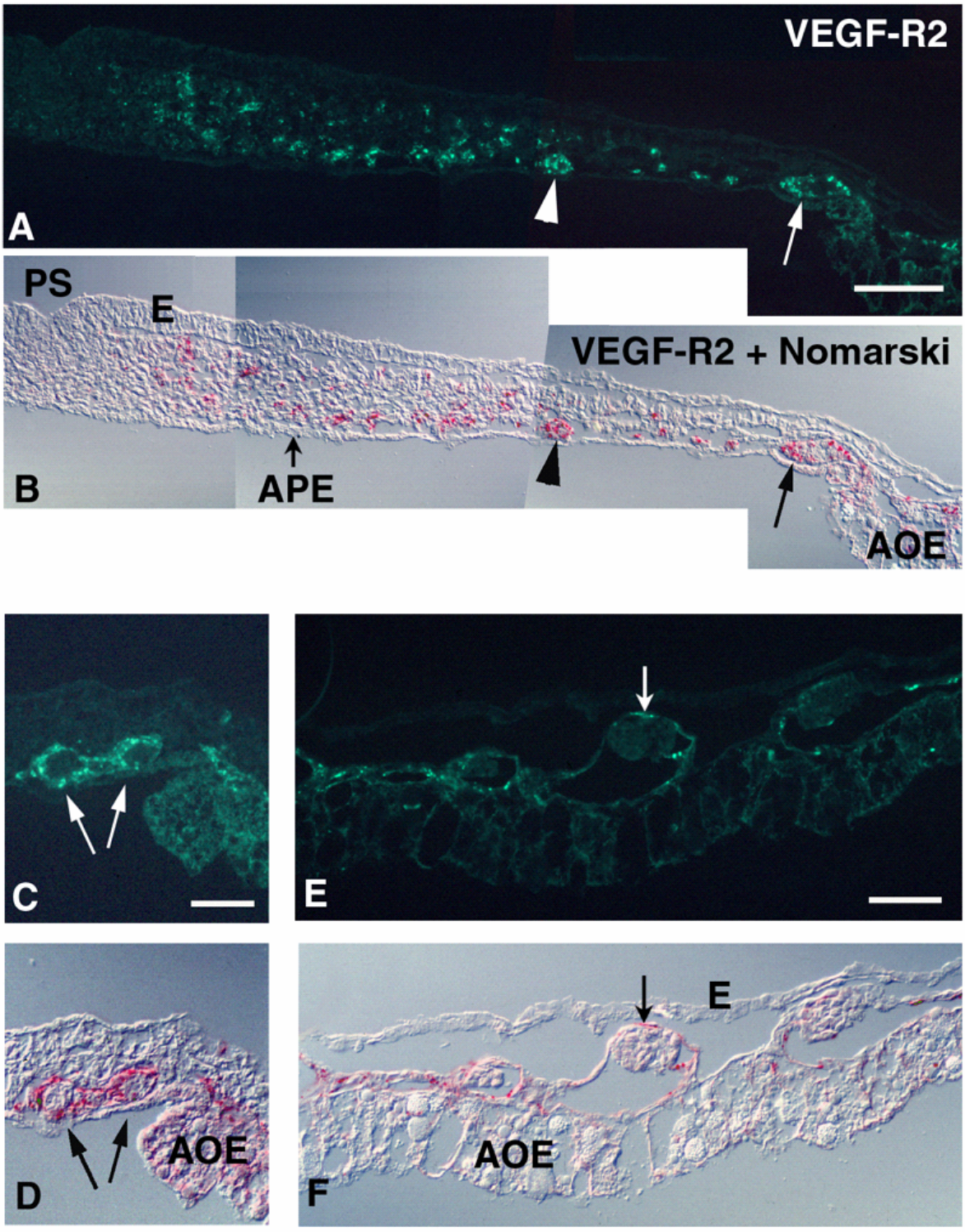


Figure 13.

Establishment of the circulation and vascular remodelling

The expression patterns of the above-mentioned factors were followed on the second day of incubation, when the onset of circulation between the embryo and the yolk sac is established (around HH12-13, 16 to 19 somite pairs) and when vascular remodelling occurs (by HH15, 24-28 somite pairs).

At HH12-13, GATA-2 expression became restricted to a caudal area where the lastly formed, less mature blood islands are located (Fig. 14/A, B). A similar centripetal decrease characterized the SCL, Lmo2 and, to a less extent, GATA-1 expressions (Fig. 14/C-H) while concurrently, haemoglobin expression extended throughout the whole yolk sac (Fig. 14/I, J).

At HH14-15, GATA-2 strongly decreased in the posterior AP blood islands and became barely detectable in yolk sac blood vessels (Fig. 14/K-M). SCL and Lmo2 expression areas also regressed, localizing in the posterior part of the AP as a few, small blood islands (Fig. 14/N, O, Q, R). Lmo2 was strongly positive in the whole vascular network of the yolk sac and the embryo proper (Fig. 14/Q-S). Few GATA-1 positive blood islands corresponding to the lastly developed islands were still visible in the posterior AP (Fig. 14/T, U). GATA-1 transcripts were also scarce in small clusters around blood vessels (Fig. 14/V). Erythrocytes occupied the entire yolk sac within blood islands and within a clear vascular network (Fig. 14/W, X).

Figure 14. Onset of circulation and vascular remodelling. (A-J) HH13, (K-X) HH15 whole mounts, dorsal views, anterior side up. (A, B) The GATA-2 expression area has medially regressed in the yolk sac. Posterior, less mature blood islands in the AP, still display a high GATA-2 signal. (C, D) SCL followed a similar pattern. Highly expressing cells are visible inside the posterior most islands. (E, F) The Lmo2 pattern is very similar to that of SCL, but blood vessels are better designated. (G, H) The GATA-1 expression area is larger than that the former ones. The signal is restricted to the blood islands. Note that GATA-1 fades in the postero-lateralmost aspect of the blastoderm where mature islands are found. (I, J) Haemoglobin distribution has now extended throughout the whole AO. Fusion between adjacent islands is occurring. (A, C, E, G, I) Bar=2mm, (B, D, F, H, J) Bar =1mm. (K, L, M) GATA-2 expression is strongly reduced in the AO and has nearly disappeared from the lastly formed blood islands.

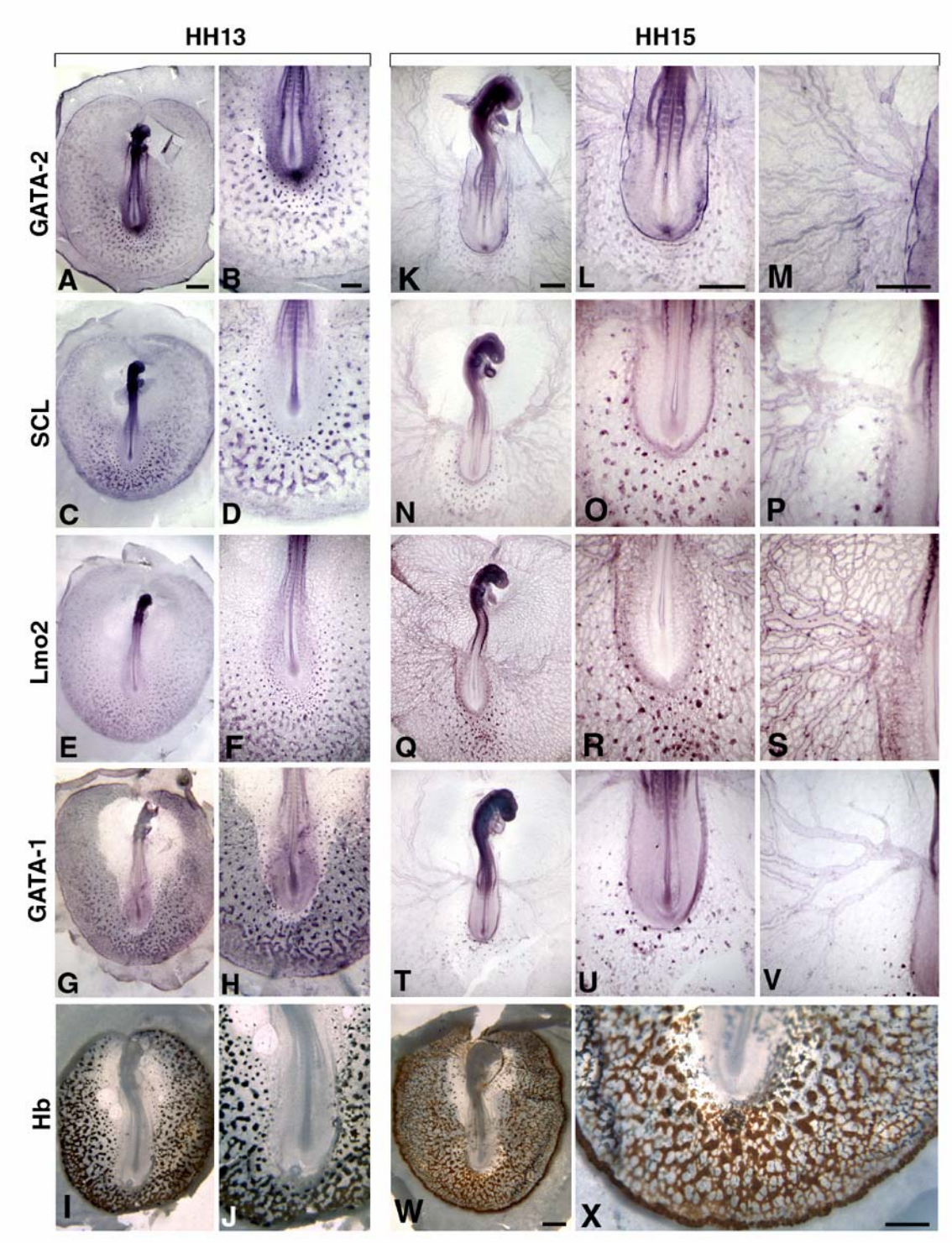


Figure 14.

(N, O, P) SCL expression becomes restricted to the posterior most aspect of the blastoderm where the last formed blood islands are localized. (Q, R, S) Lmo2 is expressed by the last formed blood islands, but is also present in the whole vascular system. (T, U, V) GATA-1 expression is restricted to the last formed islands in the AP. (W, X) Haemoglobin is now detectable in the whole blastoderm. Fusion between blood islands forming the vascular network is apparent from the staining. (K, N, Q, T, W) Bar=2 mm. (L, O, R, U) Bar=2 mm. (M, P, S, V) Bar=1mm, (X)=2 mm.

Expression changes in the 3-day-old yolk sac

On the third day of incubation the investigated transcription factors were detectable in 3 characteristic cell types in the yolk sac: circulating blood cells, endothelia and in clumps of cells. Circulating blood cells expressed mainly GATA-1 and SCL (Fig. 15/B, F and D, H) some of them were GATA-2, GATA-3 or Lmo2 positive (Fig. 15/A, E; J and C, G). Lmo2 expression outlined the endothelia of the whole vascular tree (Fig. 15/C, G) even on whole mount stained embryos (not shown).

GATA-2 was continuously expressed in the extraembryonic ectoderm (Fig. 15/A, E) and interestingly small clumps of cells in the mesoderm were positive as well (Fig. 15/E outlined). These aggregates were also positive for GATA-3 (Fig. 15/J outlined), which factor was not detected at earlier stages in the yolk sac.

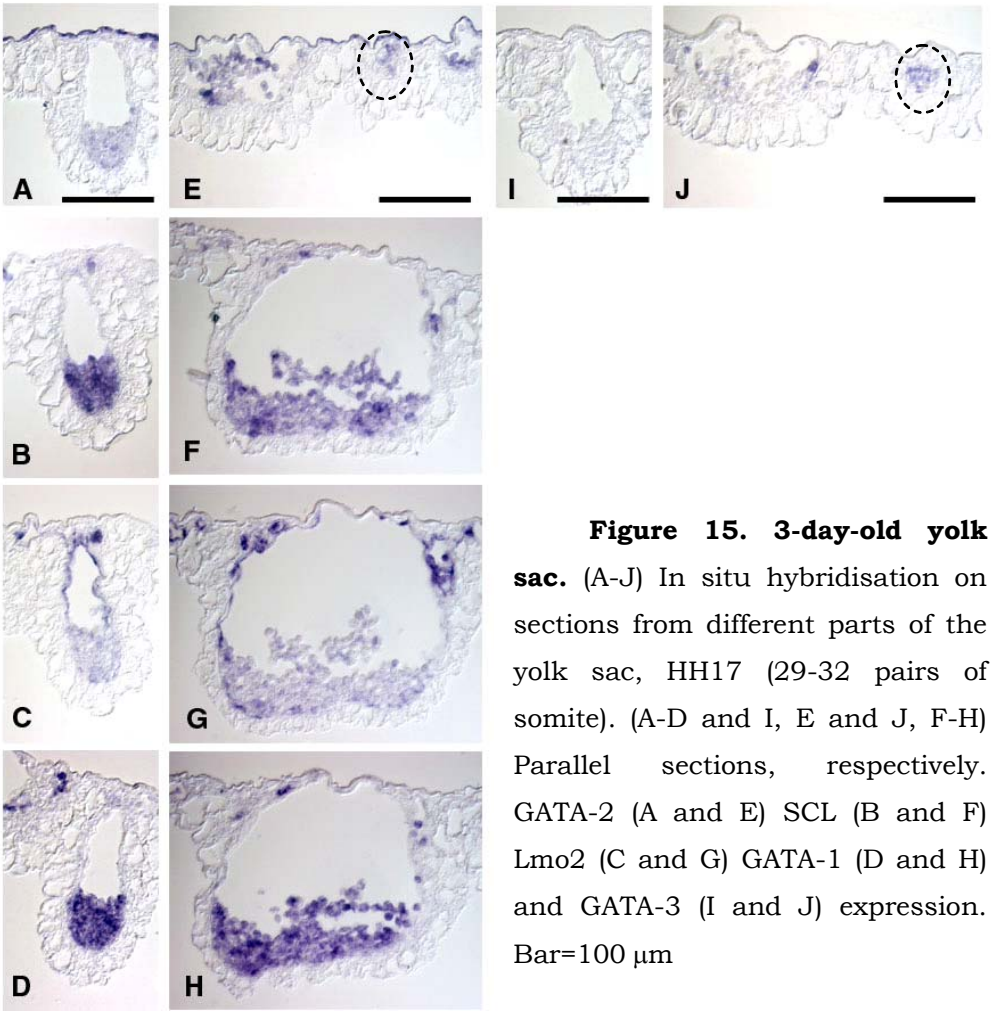


Figure 15. 3-day-old yolk sac. (A-J) In situ hybridisation on sections from different parts of the yolk sac, HH17 (29-32 pairs of somite). (A-D and I, E and J, F-H) Parallel sections, respectively. GATA-2 (A and E) SCL (B and F) Lmo2 (C and G) GATA-1 (D and H) and GATA-3 (I and J) expression. Bar=100 μm

GENE EXPRESSION PATTERNS IN THE ALLANTOIS

Onset of circulation between the allantois and the embryo

Red blood cell clusters in the allantois are visible very early at the apex of the bud. Mesodermal cells of the allantois form aggregates at HH17 surrounding the endodermal epithelia (Fig. 16/A, B). In later stages (HH19), they differentiate to form blood islands, and contain darkly stained erythroblasts (Fig. 16/C, D).

In order to pinpoint the time when the connection between the allantois and the embryonic circulation take place, India ink was used as a probe in the first series of experiments (Fig. 17.).

Figure 16. Blood island formation in the allantois. (A-D) Semithin sections from HH17 (A, B) and HH19 chick embryos (C, D). (A) Transverse section at the level of the allantoic bud. (B) Parallel section of A, higher magnification of the area framed on A. Red arrows indicate the mesodermal aggregates close to the endoderm. (C) Transverse section at the level of the posterior limb buds, containing the allantoic stalk and vesicle. (D) Parallel section of C, higher magnification of the area framed on C. The arrow shows a blood island with darkly stained erythroblasts. NT: neural tube, N: notochord, G: gut, A: lumen of the allantois, YS: yolk sac. Bar=0.4 mm

Figure 17. Microangiographies with India ink in order to reveal the vascular network of the embryo and its extraembryonic membranes. (A, B) HH17 (29-32 pairs of somite) chick embryo, posterior part. (A) The injected ink marks some vessels at the base of the allantoic bud (arrow). (B) Higher magnification of the detail framed on A. (C) HH19 embryo, the allantois is outlined. Only the base of the allantois is vascularized (white arrowhead), while the large aggregates of red blood cells at the apex remain free of ink. All: allantois, arrow: leg bud; TB: tail bud. Bar=1mm

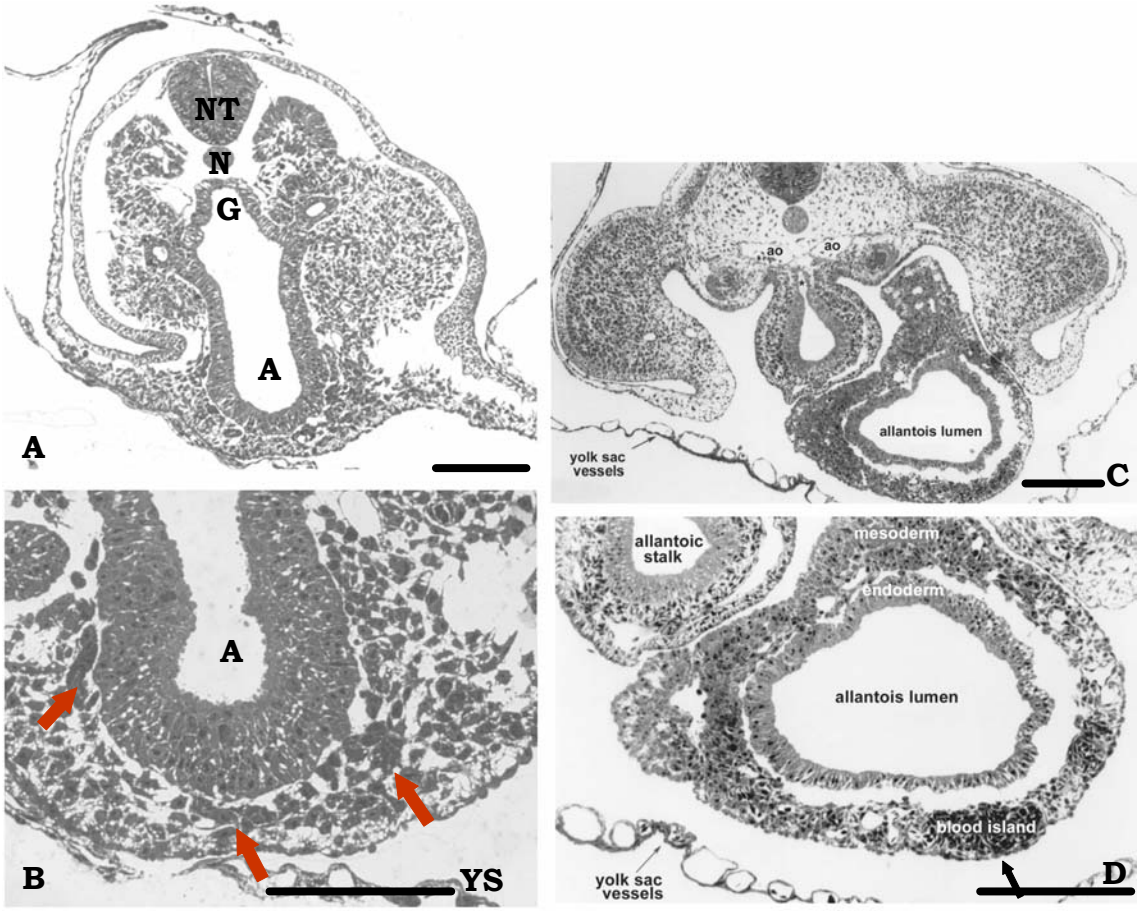


Figure 16.

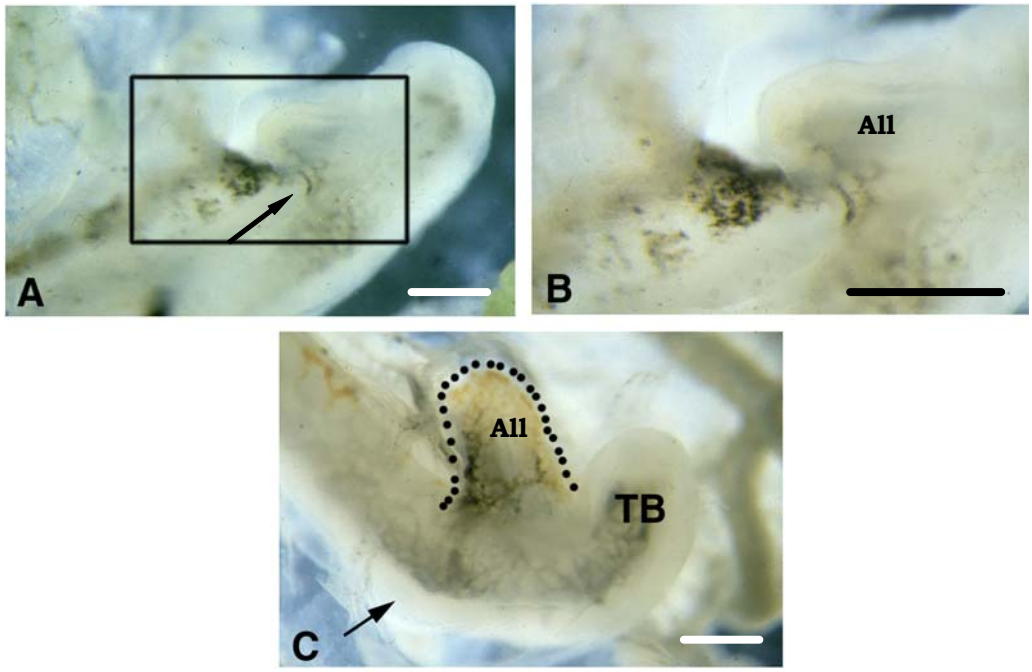


Figure 17.

Clearing the inoculated embryos in methyl salicylate refined the visibility of the vascular arborisation. Five embryos, between stages HH18 and 19 (30 to 36 and 37 to 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. 1/A of article 1) indicating that even the smallest vessels do not become clogged. The allantois except its base remained free of ink in all embryos, leaving aggregates of red blood cells at the apex of the bud (Fig. 1/A of article 1) that confirms the previous data (122).

As a complementary approach, AcLDLs-DiI inoculations were implemented (105; 244) to determine precisely the onset of vascularization. Upon injection of 11 embryos at HH16 to 18 (26-28 somite pairs to 30-36 somite pairs, respectively), no AcLDL uptake was detected in the developing allantoic bud, although the whole vascular tree in the embryo and yolk sac was intensely labelled (data not shown). The first sign of vascular connections between the allantois and the embryo was detected when HH19 embryos (37-40 somite pairs, 5 samples) were inoculated. A weak punctuate fluorescence was visible on 5 out of 11 embryo's sections at the base of the allantois indicating that the circulation progressively became established while the apex of the structure remained unlabelled (Fig. 1/B, C of article 1). In contrast, all the blood vessels and capillaries in the body walls were labelled (not shown). Thus the onset of circulation throughout the bud occurred around HH19 with slight variations between individuals. At HH20, the whole allantois displayed a LDL+ vascular network.

GATA factors are expressed in the allantoic endoderm very early

GATA factors, SCL and Lmo2 expressions were also revealed during the development of the allantois. The expressions of GATA-3 as well as GATA-1 and 2 at day 2.5 were detected by RT-PCR in chicken allantois in previous experiments (122). Loss-of-function experiments in mice and previous expression studies on different vertebrates indicated that GATA-3 has a role only in definitive haematopoiesis. Our in situ hybridization study is in agreement with these results since this factor was not detected in the yolk sac, during the first two days of development. We put forward the hypothesis that the presence of GATA-3 mRNA in the allantois might be linked to the emergence of definitive haematopoiesis and therefore decided to investigate the in situ distributions of different GATA factor mRNAs during early development of the allantois. GATA-3 mRNA first appeared at HH11-12 (13 and 16 somite pairs, respectively) in the caudalmost endoderm before any sign of allantoic swelling (Fig. 18/A-C). The positive area enlarged at HH12 (Fig. 18/A, C). Neither GATA-1 nor GATA-2 displayed a similar distribution at this stage (data not shown). From HH16-17, the GATA-3 signal was present in the allantoic bud endoderm (Fig. 18/D and 19/D). From HH17 onwards, GATA-2 was also detected in the allantoic endoderm (Fig. 3/D of article 1 and Fig. 19/C), and persisted at least until HH20, the latest stage examined (not shown). The endodermal expression of GATA-3 continued at HH18 and 19 (Figs. 2/E, 2F and 3/D of article 1), E5 (Fig. 2/H of article 1) and until E7. At E8, the signal disappeared from some regions in the endoderm, while some cell groups in the mesoderm (Figs. 2/I and 2/J of article 1) as well as some cells inside vessel lumina became positive (Fig. 2/J of article 1).

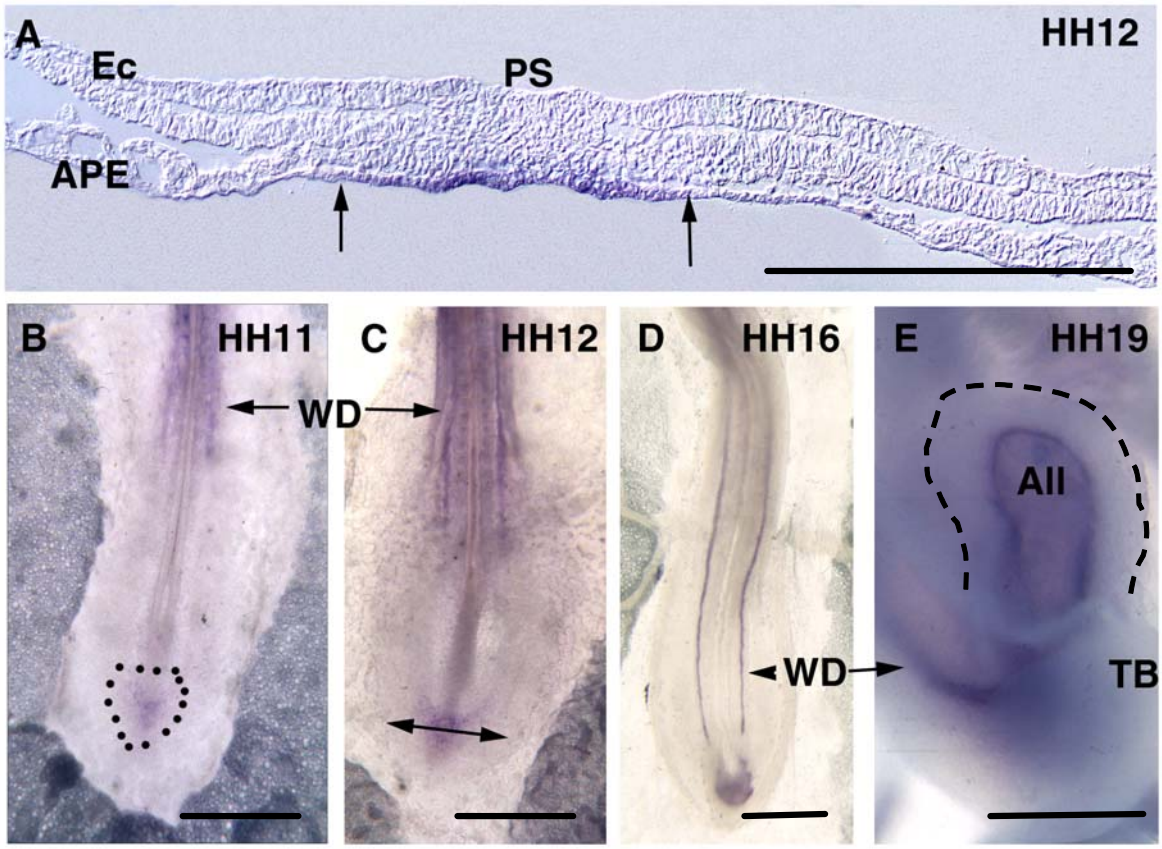


Figure 18. GATA-3 expression in the endoderm of the allantois. Whole-mount in situ hybridizations from HH11 to HH19. (A) Cross section through the level indicated in C by the double-headed arrow. GATA-3 is restricted to an axial stretch of endoderm (delineated by arrows). (B) First appearance of GATA-3 expression in the caudal endoderm (dotted area) at HH11. (C) Whole-mount of the same embryo represented on A, posterior region, HH12. GATA-3 mRNA is found in the caudal region in an extended triangle shaped area and in the nascent Wolffian duct. (D) GATA-3 mRNA expression now clearly delineates the Wolffian ducts (arrow) and the future allantoic region (arrowhead). (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. All: allantois bud, APE: area opaca endoderm, Ec: ectoderm, TB: tail bud, PS: primitive streak, WD: Wolffian duct. Bar=1mm.

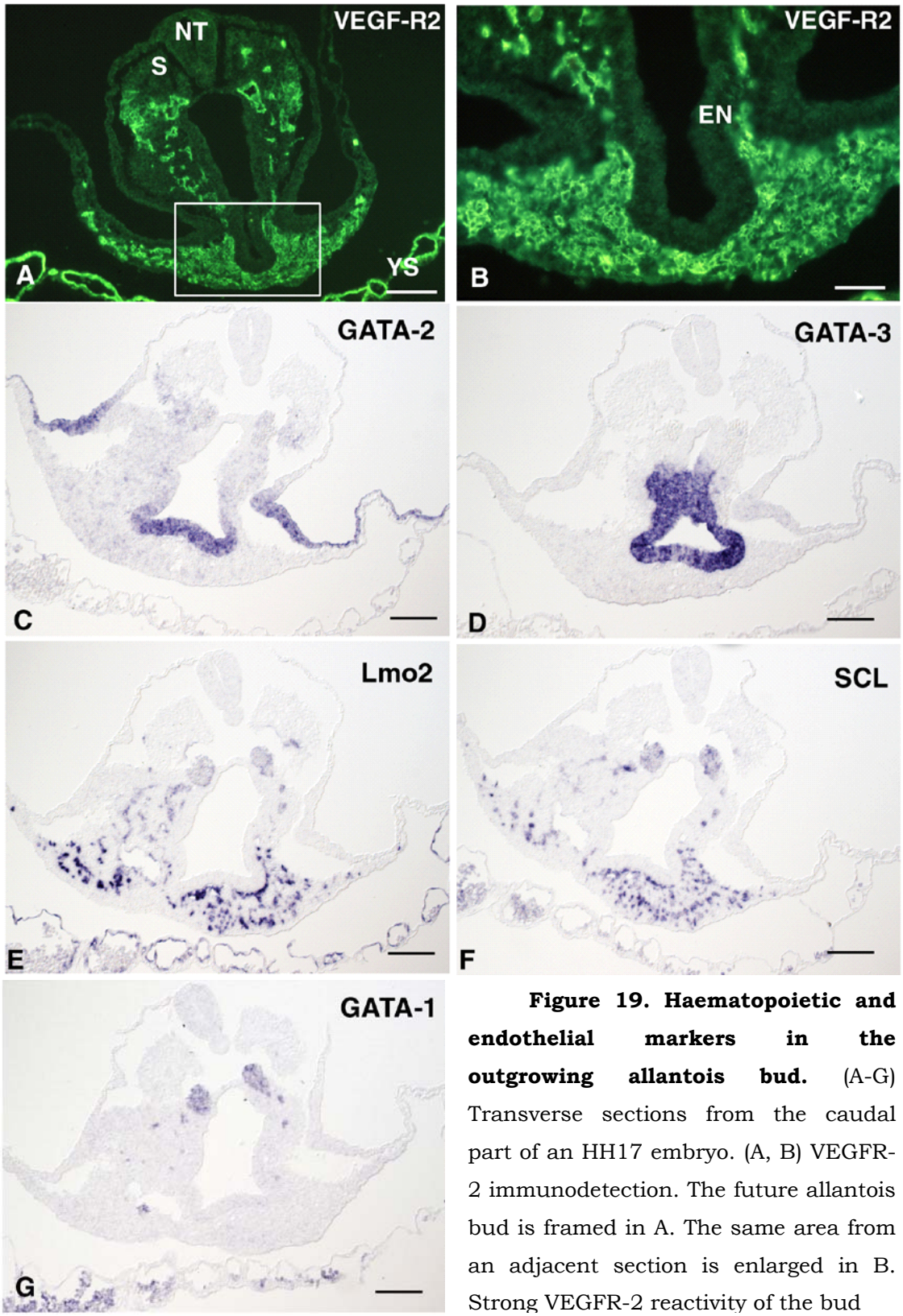


Figure 19. Haematopoietic and endothelial markers in the outgrowing allantois bud. (A-G) Transverse sections from the caudal part of an HH17 embryo. (A, B) VEGFR-2 immunodetection. The future allantois bud is framed in A. The same area from an adjacent section is enlarged in B. Strong VEGFR-2 reactivity of the bud and posterior intestinal portal (PIP) mesoderm as well as ventrolateral somite region. Bar=0.1mm. (B) The allantois and PIP mesoderm are strongly VEGFR-2+. Bar=20μm. (C-G) In situ hybridisation for GATA-2 (C), GATA-3 (D), Lmo2 (E), SCL (F), and GATA-1 (G) on adjacent sections through the allantoic region before outgrowth of the bud. Bar=0.1mm.

An interesting finding is that associated GATA factor expression (GATA-2 and 3) was also revealed not only in the allantois endoderm but also in the foregut of the 4 pairs of somite embryo (Fig. 20).

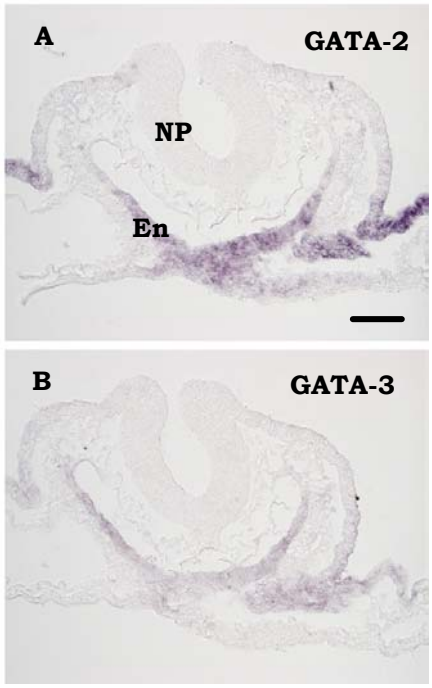


Figure 20. Associated GATA factor expression in the foregut. (A, B) In situ hybridisation on neighbouring sections from the anterior part of the same embryo, 4 pairs of somite. (A) GATA-2 is present in the endoderm, and in the extraembryonic ectoderm. (B) GATA-3 is present in almost the same locations; however the expression level is weaker. Bar=100mm En: endoderm, NP: neural plate

Gene expressions in the mesoderm of the allantois

The cell constitution of the early allantois was characterized by means of a combination of endothelial/haematopoietic-specific genes. VEGFR-2 was detected very early in the future allantoic mesoderm, which, at HH16-17, appeared entirely positive in contrast to the mesoderm surrounding the hindgut endoderm, which contained sparser VEGFR-2$^+$ cell profiles (Fig. 19/A, B). From HH17 onwards, VEGFR-2 expression decreased and became restricted either to groups of cells or to structures delineated by VEGFR-2$^+$ cells. The latter were strikingly similar to yolk sac blood islands (see Fig. 1/B of article 1). SCL and Lmo2 were also detected from this stage in clumps of cells in the allantoic mesoderm (Fig. 19/E, F). GATA-2 expression in the mesoderm was very weak and diffuse in HH17, while conspicuous in the endoderm and ectoderm (Fig. 19/C, Fig. 3/C of article 1). GATA-1 was absent from the allantois mesoderm (Fig. 19/G) until HH19 when it was expressed in few cells at the apex of the allantois (Fig. 3/F of article 1).

At stages HH18-19, blood island-like structures appear in the mesoderm of the allantois. During later development, the more mature islands are located at the base of the bud. These islands were investigated for the expression of VEGFR-2 and CD45, a pan-leukocyte antigen expressed by haematopoietic progenitors and leukocytes (245). The blood islands were delineated by VEGFR-2 positive cells and contained free haematopoietic cells, a few of them were CD45 positive (Figs. 4/A and B of article 1). Maturing blood islands, in the apex of the bud, were characterized by a VEGFR-2 positive endothelial envelope, completely filled with blood cells. Some of these were CD45 positive while some others were negative, probably already hemoglobinised (Figs. 4/A and B of article 1). 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.

THE EMBRYONIC AORTA-ASSOCIATED HAEMATOPOIESIS: GENE EXPRESSION PATTERNS AND EXPERIMENTAL DATA

Gene expression in the developing aortic region

Gene expression patterns in the aortic region were investigated from the HH17 stage (52-64 hours of development) after the two aortic anlagen had fused and the first rounded haematopoietic cells appeared on the floor of the aorta at the anterior levels of the embryo (105). SCL, Lmo2 and GATA-3 expression was detected in the aortic region. Their expression pattern was similar: among the endothelial cells, flattened positive cells were detected mainly on the ventral wall of the aorta, arranged in two ventrolateral groups (Fig. 21). Some positive cells located in the dorsal aspect of the aorta, and in the case of Lmo2, they were characteristic for sprouting vessels from the aorta (Fig. 21/A). On the other hand, round shaped cells on the ventral aspect of the aorta were infrequently detected (Fig. 21/B). Circulating blood cells expressed mainly SCL, while Lmo2 and GATA-3 was present only sparsely in these cells. A subset of cells, ventral to the aorta, also expressed SCL and Lmo2 (Fig. 21/A, B). GATA-2 and GATA-1 was not investigated in detail at this stage, but our preliminary data indicate the presence of GATA-2 in the ventral wall of the aorta (data not shown).

Between HH18 and 20 (68-72 hours of development), clusters of rounded haematopoietic cells develop in the ventral wall of the dorsal aorta (Jaffredo et al. 1998). The HH19 stage was selected for comparative gene expression study of these clusters. SCL and Lmo2 were strongly expressed by cluster-forming cells while GATA factors were barely (GATA-2 and 3) or not detected (GATA-1) (Fig. 22/A-E). Interestingly, different cells of the clusters showed a mosaic pattern of expression regarding the SCL and Lmo2 transcription factors (Fig. 22/A, B). GATA-2 and GATA-3 expression restricted mainly to the basal cells of the clusters (Fig. 22/C, D). GATA-1 positive cells were only detected in the lumen of the aorta, not associated with cluster-forming cells (Fig. 22/E). SCL, Lmo2, GATA-2 and GATA-3 expression was not restricted to the ventral aspect of the aortic endothelia, but was also revealed on the dorsal side. Circulating blood cells expressed mainly SCL and GATA-1 (Fig. 22/A, E),

barely GATA-2 (Fig. 22/C) and only some of them was positive for Lmo2 or GATA-3. Cells positive for Lmo2 and SCL were also detected in the dorsal mesentery (data not shown).

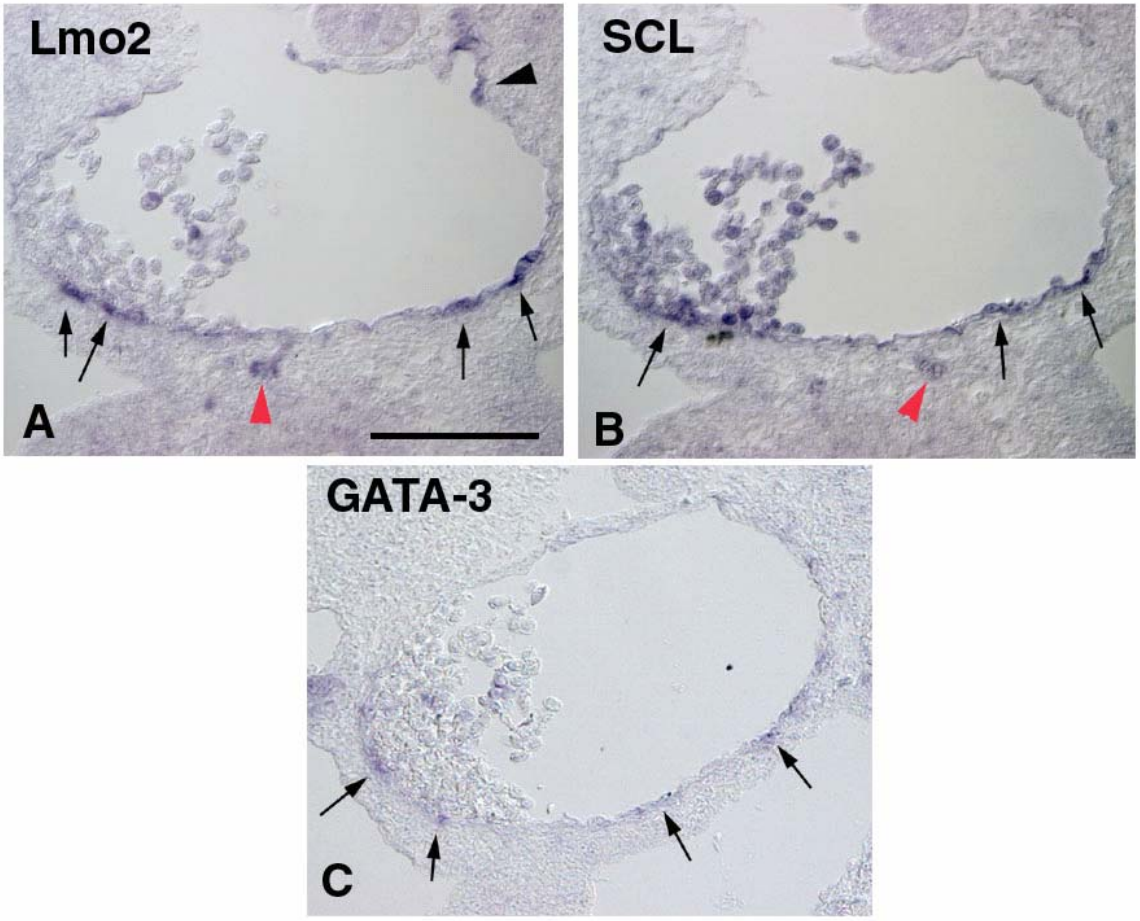


Figure 21. SCL, Lmo2 and GATA-3 expression in the dorsal aorta. (A-C) HH17, in situ hybridization on serial sections at thoracic levels. (A) Cells of the ventral aspect of the dorsal aorta (arrows) express Lmo2. Positive cells are also detected ventral to the aorta (red arrowhead) and in outgrowing blood vessels (arrowhead). (B) SCL is also expressed by ventral, flattened cells of the aorta (arrows) and by cells ventral to the aorta (red arrowhead). Most of the circulating cells also contain the transcripts. (C) GATA-3 expression is detected on the two ventrolateral sides of the aorta; however the cells display only a weak signal (arrows). Bar=100μm.

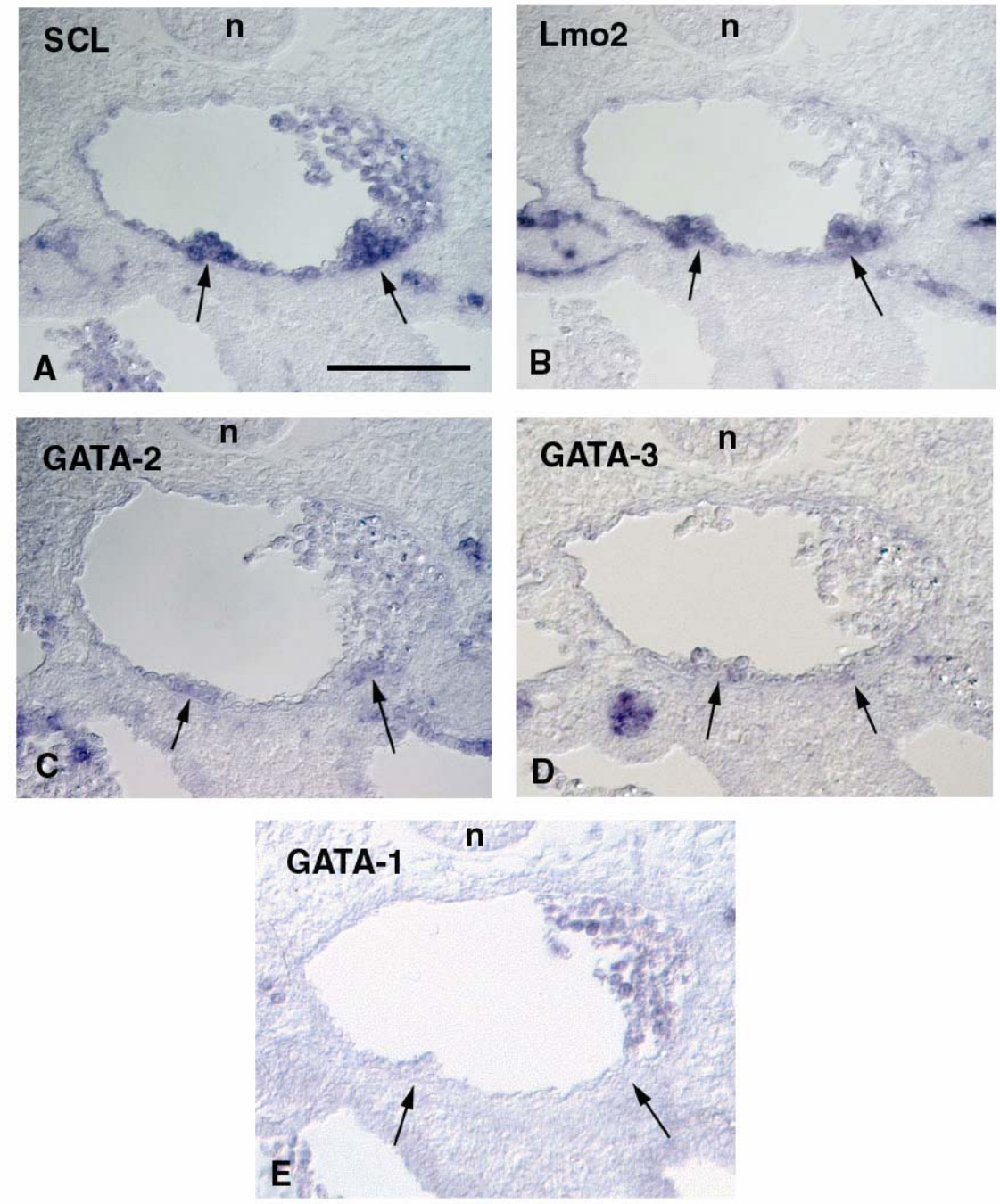


Figure 22. SCL, Lmo2 and GATA factor expression in the dorsal aorta region. (A-E) HH19, in situ hybridization on serial sections at thoracic levels. (A, B) Lmo2 and SCL are strongly expressed by the cells of the haematopoietic clusters on the ventral aspect of the dorsal aorta (arrows). (C, D) GATA-2 and 3 expressions are also detected in the clusters (arrows), however the cells display weak signals. Positive cells are also detected in the kidney. (E) GATA-1 transcripts are not present in the clusters (arrows), but are detected in circulating cells. Bar=100μm. n: notochord

Experimental analysis of the aorta-associated haematopoiesis

In order to determine the developmental potential of the haematopoietic cells emerging on the ventral aspect of the dorsal aorta in vivo, isochronic grafting experiments were performed on the third day of development. The thoracic part of the dorsal aorta region of quail embryos was grafted into the coelom of chicken hosts, and chimeras were analyzed by flow cytometry and immunocytology with the quail haematopoietic and endothelial cell specific antibody (QH1).

Grafting experiments

Two kinds of control grafts were performed to determine the colonisation capacity of the haematopoietic cells emerging in the aorta region. Wing bud, containing endothelial but not haematopoietic precursors at this stage, or allantois bud, which is capable of colonizing the bone marrow by in situ emerging endothelial and haematopoietic cells (122), were taken off from 3-day-old quail embryos and grafted into the coelom of 3-day-old chick host. Four haematopoietic organs of each chimeras (thymus, bursa of Fabricius, spleen and bone marrow) were then analyzed before hatching (on embryonic day 16-18) by flow cytometry with QH1 antibody, in order to follow the fate of the grafted cells (Fig. 23.).

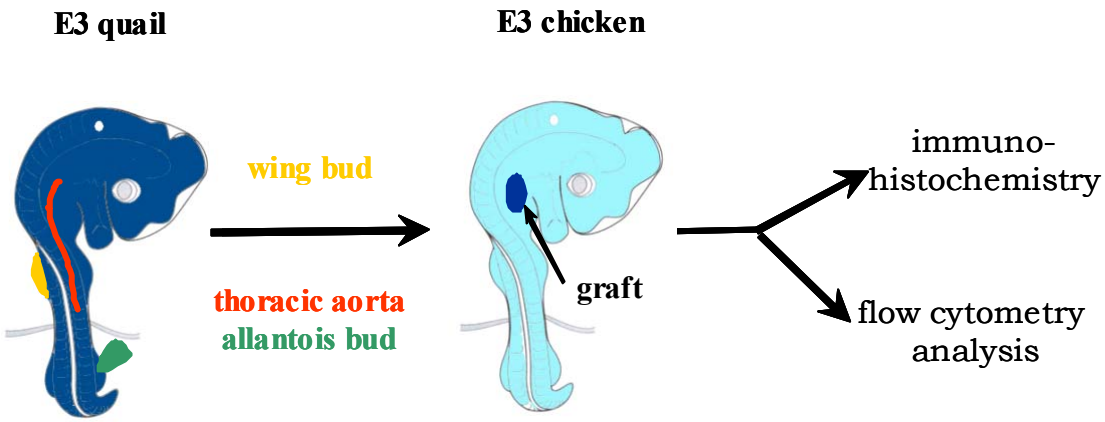


Figure 23. Scheme of the chimera experiments

When wing buds were grafted into chicken hosts, the ratio of QH1 positive cells to total cells in the haematopoietic organs was around 0.1% or below, as analysed by flow cytometry. Only 3 organs of 5 chimeras contained more than 0.1% QH1 positive cells among 20 analyzed organs (one thymus had 0.34% positive cells, and 2 spleen contained 0.34% and 0.42%). A ratio of positive cells less than 0.5% was therefore considered as the minimal background in the case of allantois or aorta implantations. Table 7 and 8 shows the summary of these results.

Table 7. Summary of the grafting experiments

Implanted tissue	Number of chimeras	Number of positive organs/all tested organs
wing bud	5	0/20 (0%)
allantois	10	8/40 (20%)
aorta	18	21/72 (29.17%)

Table 8. Percentage (number) of positive organs/ all tested organs

Grafted tissue	Analysed organs			
	Thymus	Bursa	Spleen	Bone Marrow
Allantois	10% (1/10)	30% (3/10)	30% (3/10)	20% (2/10)
Aorta	44% (8/18)	38% (7/18)	16% (3/18)	16% (3/18)

Figure 24. shows the organs from chimeras colonised by QH1 positive cells when allantois buds or thoracic aorta regions were grafted. Each column refers to an organ having more than 0.5% QH1 positive cells. The percentage of the positive cells indicates that the most successful colonisation was achieved with the thymi of chimeras grafted with the thoracic aorta. In contrast, cells derived from the implanted allantois bud did not show such preferences in the colonisation of the investigated organs. Figure 25. show FACS profiles of representative chimera organs, grafted with thoracic aorta colonised by QH1 positive cells.

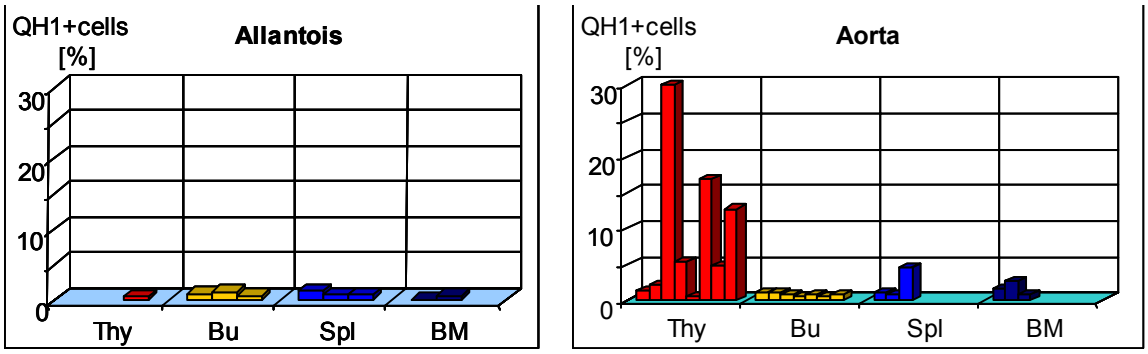


Figure 24.

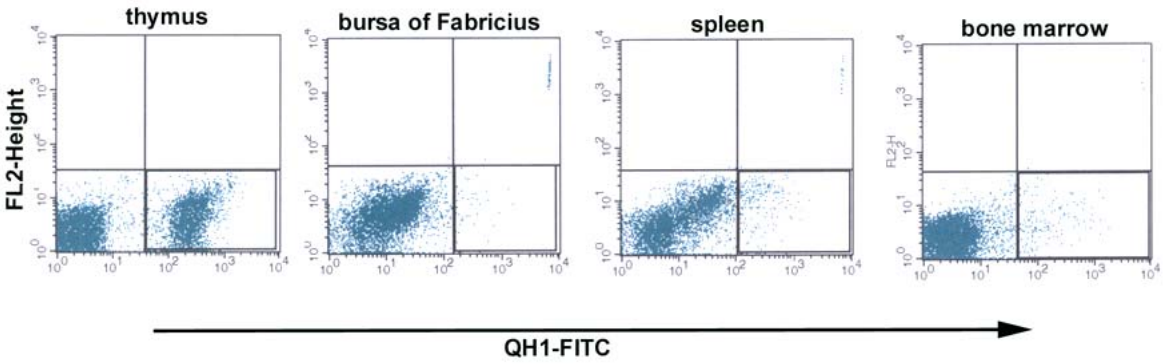


Figure 25.

Immunohistochemical analysis of the chimeras grafted with thoracic aorta

Haematopoietic organs of chimeras grafted with the thoracic aorta region were also analysed in some cases by immunohistochemistry. QH1 positive cells were detected in all the 4 investigated organs (thymus, bursa of Fabricius, spleen and bone marrow). Thymic lobules often were well colonised by QH1 positive cells. The numerous rounded cells are thought to be thymocytes (Fig. 26/A). Sometimes positive endothelial cells were also detected in the adipose tissue surrounding the thymic lobes (Fig. 26/B). QH1 positive round shaped cells colonised the bursas of Fabricius, they were detected in the interfollicular spaces as well as in the folliculi. Sometimes the whole folliculus was filled with positive cells (Fig. 26/C). In the spleen, rounded haematopoietic cells were sparced, and chimeric vessels containing QH1

negative and positive cells were sometimes revealed (Fig. 26/E). Bone marrows of chimeras were also positive, having QH1 positive round shaped cells dispersely (Fig. 26/F).

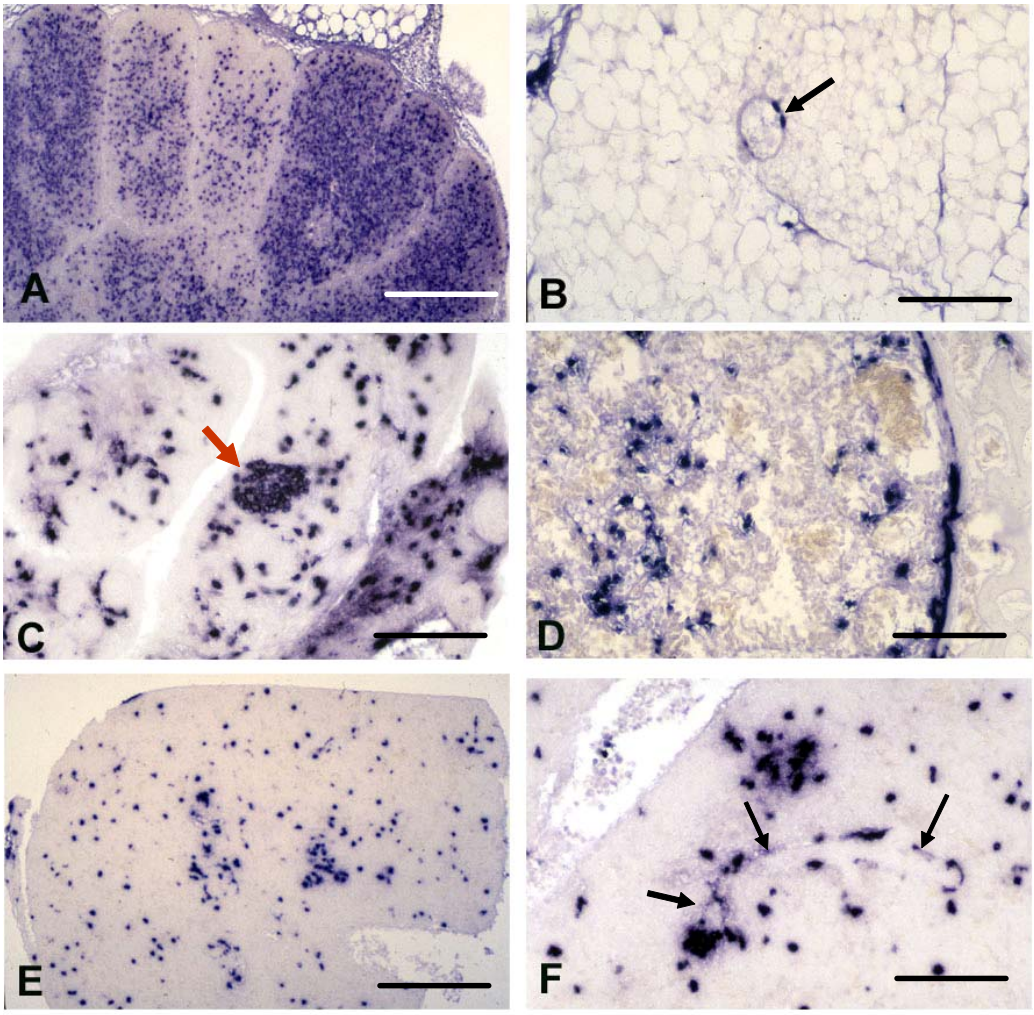


Figure 26. Immunohistochemical analysis of haematopoietic chimera organs with QH1. (A) Thymus of E17 chimera grafted with thoracic aorta. Each lobule contains QH1 positive cells some of them are filled with positive cells. (B) Higher magnification of the adipose tissue surrounding the thymus lobes with chimeric vessels (arrow). (C) Bursa of Fabricius from E18 chimera grafted with thoracic aorta. Rounded cells are dispersely detected; one follicule is filled with QH1 positive cells (red arrow). (D) Cross section of the tibia of an E17 chimera, containing rounded, QH1 positive cells in the bone marrow. (E) Spleen of an E18 chimera, with clusters of QH1 positive cells. (F) Parallel section of E with higher magnification showing clusters of positive cells and a chimeric vessel (arrows). Bars=400μm (A,E); 200μm (B,C,D,F)

SUMMARY OF THE RESULTS

1. The chicken ortholog of the Lmo2 gene has been isolated and characterised. Its detailed expression pattern was also revealed.

2. The detailed comparative expression patterns of key transcription factors in haematopoiesis, namely SCL, GATA-1, 2, 3 and Lmo2 has been established in different anatomical locations of haematopoiesis (such as the yolk sac, allantois bud and dorsal aorta) on the chicken embryo. Some important new findings of this work are summarised below:

I./ Two distinct expression levels of GATA-2 characterise the yolk sac primitive erythropoiesis, depending on the mesodermal compartment and stage of blood island maturation. Such a bimodal GATA-2 expression was not reported before on other vertebrate models, but reconciles the two roles postulated for GATA-2 during haematopoiesis related to ventral mesoderm commitment and to the maintenance of the haematopoietic progenitor pool.

II./ The pivotal role of SCL and Lmo2 genes during development of the hemangioblast population in the set off of the blood program was confirmed in this study but was also revealed that different levels of expression are required for haematopoietic or endothelial differentiation.

III./ SCL, GATA-2, Lmo2 expression followed by GATA-1 expression in the allantois mesoderm suggests the presence of an erythroid differentiation program. GATA factor expression was also demonstrated during allantois development in the endoderm. GATA-3 mRNA appears first in the presumptive allantois endoderm before any sign of allantoic swelling.

IV./ The expression patterns in the yolk sac and allantois mesoderm indicate that predominantly the erythro-myeloid lineages develop in the wall of these extraembryonic membranes. In contrast, the transcription factor expression pattern in the intraaortic clusters at E3 refers to progenitor emergence, with the

presence of early haematopoietic marker genes -such as SCL, GATA-2, 3 and Lmo2- and the absence of the lineage specific GATA-1. Interestingly, cluster cells show different levels of transcription factor expression.

3. The *in vivo* haematopoietic potential of the E3 cluster-containing dorsal aorta has been demonstrated in quail-chick chimera experiments. The implanted aorta derived cells colonised the haematopoietic organs (thymus, bursa, bone marrow and spleen) with haematopoietic and vascular marker (QH1) expressing cells. Among these organs the thymus was preferentially colonised by aorta derived cells.

DISCUSSION

To unravel the regulatory networks operating in haematopoietic progenitor cells and committed precursors is one of the major challenges in the field of haematopoiesis today. In this study the expression patterns of a combination of genes, which plays key roles in the specification of the blood forming system, was examined during the primitive and definitive phases of avian embryonic haematopoiesis. These processes occur in different anatomical locations, namely in the wall of extraembryonic membranes (the yolk sac and allantois) and in the embryo proper (associated with the dorsal aorta).

MOLECULAR SPECIFICATION OF THE HEMOGENIC MESODERM IN THE EARLY EMBRYO

The earliest marker is GATA-2 in the ventral/posterior mesoderm

In the earliest stage examined, GATA-2 expression as the only marker was detected in the mesoderm, which exits from the primitive streak to give rise to the extraembryonic mesoderm. The early presence of this factor in the mesoderm was also described on lower vertebrates (229; 230; 232; 233). Functional studies on the Xenopus showed that this early GATA-2 expression in the gastrulating mesoderm is regulated by BMP-4, because BMP-4 was sufficient to induce animal cap cells to express abundant GATA-2 (135). In addition its antagonist, noggin (a dorsalising signal) has the opposite effect (233).

The expression data presented below indicate that this signal transduction pathway seems to be conserved between lower and higher vertebrates because of its similar gene expression profiles. In Zebrafish, the ventral region of the early embryo is marked by the expression of BMP-2/4 (246; 247) and GATA-2. In the Xenopus and avian embryo, BMP-4 and its receptors are expressed at the right time and place to induce GATA-2 expression (231; 233; 248). This early GATA-2 expression probably has an

important role in the ventralising program according to the expression and functional data, but not sufficient to specify the blood program.

Molecular patterns defining hemangioblasts

Previous gene expression studies showed that VEGFR-2 is expressed very early in the posterolateral region of the chicken blastoderm (148). We observed the presence of this protein throughout the whole GATA-2 positive domain. In addition, expression of VEGFR-2 in mesodermal cells preceded, and was more widely distributed than that of SCL and Lmo2. In agreement to this observation, several reports indicate that, in the mouse embryo, VEGFR-2 is present in the newly deposited mesoderm likely before hemangioblastic commitment (71; 149; 150; 249).

SCL and Lmo2 were first observed in our study, in single cells or cell groups, scattered throughout the GATA-2 low expression domain and otherwise undistinguishable from adjacent cells. The early expression of SCL supports its suspected role as “master gene” in hemangioblast specification which was deduced from experimental analyses in zebrafish, Xenopus and mouse embryos (see introduction).

The early expression of GATA-2, which antedates that of SCL, as revealed by our study, suggests that this factor can regulate the expression of SCL. In accordance with this, previous biochemical analysis demonstrated that the promoter region of the SCL gene contains GATA site (137; 138; 250). More recent studies showed that binding of GATA-2 is needed for the activation of the enhancer region of the SCL gene, working in progenitor cells (251).

In summary, the expression data presented here raise the possibility that a GATA-2/SCL/Lmo2 containing transcriptional complex can act in hemangioblasts in accordance with the suggestion of Wadman et al. (177) and the model of Sieweke and Graf (161). GATA-2 mRNAs are already present in the ventral mesoderm when the hemangioblastic program is sets in by inductive effects (see Figure 27.).

Expression profiles accompanying the commitment to the haematopoietic lineages

GATA-1 expression was detected first in morphologically distinct blood islands. In the same time, GATA-2 expression was upregulated and was followed by the appearance of haemoglobin protein in the first erythroblasts, evidenced by the dark blue benzidine staining, on the lateral rim of the yolk sac. Such expression of GATA-1 is consistent with its role acting as a transcriptional globin activator. As GATA-1 and haemoglobin expressions progressed centripetally through the yolk sac, GATA-2 expression regressed and become confined to the caudal-most blood islands of the area pellucida. This pattern recalls the downregulation of GATA-2 during terminal erythroid differentiation (235). GATA-1 itself may also repress GATA-2 expression in a negative feedback loop (252). Haemoglobinisation of blood islands was accompanied by the down-regulation of Lmo2, GATA-2, SCL and, noticeably, of GATA-1 in erythrocytes (brown with benzidine staining). The downregulations are consistent with the inactivation of gene transcription following terminal erythroid differentiation in nucleated red blood cells. In vitro models showed that downregulation of GATA-1 and 2 during erythropoiesis is necessary for differentiation (185; 253; 254). It is also known, that GATA-2 is required for proliferation, survival of haematopoietic cells, but not for erythroid and myeloid terminal differentiation (81). SCL and GATA-1 expression have been reported to decline during terminal differentiation of adult human erythrocytes and megakaryocytes (193) and mouse erythrocytes (194). Such downregulations that had not been previously reported during embryogenesis indicate that gene transcription is silenced in mature blood islands or when red blood cells are released into the circulation.

A late SCL, Lmo2, GATA-2 and GATA-1 activity were still detected on the second day of incubation, in a few blood island in the posterior part of the AP, close to the tail bud region. This reflects the presence of a small number of maturing islands even after day two and half of development and consistent with the extended deposition of extraembryonic mesoderm in the avian embryo (58). While SCL, Lmo2 and GATA-1 expression restricted mainly to the circulating blood cells in the yolk sac on the third day of development, GATA-3

appeared and was co-localised with GATA-2 in newly formed yolk sac cell aggregates, probably as a second generation of blood islands.

Commitment to the endothelial lineage during the maturation of the blood islands

Our data indicate that SCL and Lmo2 are differentially regulated depending on commitment to the endothelial or haematopoietic lineages. SCL expression was downregulated in maturing endothelial cells, which is consistent with the results obtained in zebrafish (69) and mouse embryos (255). In contrast, Lmo2 expression was enhanced in endothelial cells which has not been reported previously, but is consistent with the recently reported role for Lmo2 in the formation of the vasculature (168).

As blood islands differentiate, VEGFR-2 becomes restricted to the endothelial layer displaying a mosaic expression. Recent evidence indicates that VEGFR-2 has additional roles in later stages of hemangioblast specification. It is displayed on the most primitive haematopoietic cells (256) and is required for their differentiation (73; 257). In contrast, endothelial commitment can occur independently from VEGFR-2 although progression to a differentiated state is blocked (258; 259). Recently, two SCL motifs were found to be critical for the endothelial-specific expression of VEGFR-2 (260) indicating that additional cross controls may occur at later stages. Lmo2 whose expression often parallels with that of SCL is also required in these processes (167; 168; 171).

Our study confirmed the pivotal role of SCL and Lmo2 genes during development of the hemangioblast population in the set off of the blood program but also revealed that different levels of expression may be required for haematopoietic or endothelial differentiation. Since vascular cells are present in the absence of SCL in mouse knock out embryos and gene expression is downregulated in endothelia, SCL would seem to be required after the hemangioblast stage, perhaps upon commitment to a haematopoietic fate. In contrast, the upregulation of Lmo2 in the angioblasts of the yolk sac blood islands suggests that this factor is equally important in the development of both endothelial and haematopoietic lineages (see Fig. 27.).

The tentative pathway of the primitive erythropoiesis

The present study completes and extends the hierarchy of gene activities that has emerged from genetic studies on haematopoietic specification on the mouse and zebrafish species. Figure 27. depicts a tentative pathway for primitive erythropoiesis in the yolk sac synthesizing our own data with that of others.

Our expression data are also consistent with that of other groups, working on embryonic bodies of cultured mouse embryonic stem cells as a model for primitive erythropoiesis. In this experimental system, the expression of the Brachyury gene defines pre-committed mesoderm (86; 261). In the mouse and avian embryo, Brachyury is expressed along the entire streak delineating the ingressing mesoderm compartment (262; 263; 264; 265). Indeed, VEGFR-2 expression immediately follows that of Brachyury during differentiation of embryoid bodies (86; 261; 266; 267). Thus VEGFR-2+ mesoderm appears to harbour hemangioblasts. In our study, Brachyury was detected at the level of the primitive streak, and in the adjacent nascent mesoderm, in cells that also expressed VEGFR-2 and GATA-2. These latter genes were widely expressed in the newly deposited posterior mesoderm but, differently from Brachyury, remained expressed in the lateral mesoderm. The early expression of GATA-2 antedating the expression of SCL, in accordance with our findings, was also reported during the haematopoietic differentiation of embryoid bodies (261). In addition, SCL-/- ES cells express Brachyury, VEGFR-2 and GATA-2 though they fail to undergo haematopoiesis (261; 266; 267) which is also consistent with the gene activities reported in the present study.

The scheme represented on figure 27, should be considered as a working model and provides a basis for further experimental analysis. The conservation of these gene activities among different vertebrate species is consistent with strikingly similar, if not identical, requirements of molecular pathways for haematopoietic differentiation in phylogeny.

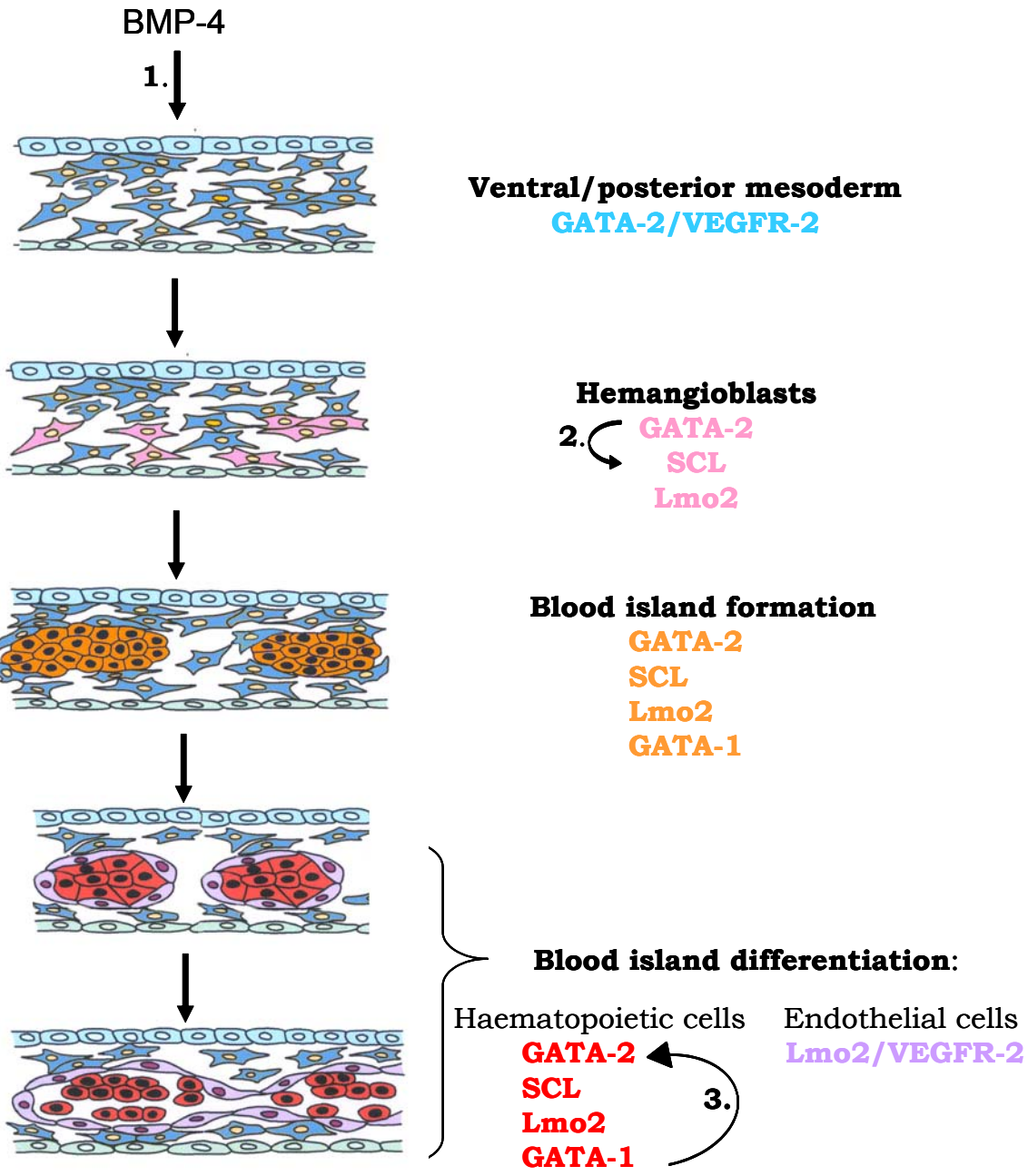


Figure 27. Schematic representation of the commitment of haematopoietic and endothelial lineages in the yolk sac. Synthesis of our expression data and that of functional studies. Same colours indicate the stage of commitment and the combination of transcription factors and VEGFR-2 expressed in the given compartment. Numbered arrows indicate the possible interactions suggested by functional studies. 1: BMP-4 is an important factor in the initiation of these processes. GATA-1 and SCL are activated depending on BMP-4 signalling (136) and all of the haematopoietic TFs need the BMP-4 signalling for initial activation (135).; 2: GATA-2 can positively act on the expression of SCL (137; 250; 138; 251); 3: GATA-1 negatively regulates expression of GATA-2 during erythroid development (252).

MOLECULAR PATTERNS IN THE EARLY ALLANTOIS

The cytological evolution, the molecular patterns detected in the early avian allantois bud and the experimental approaches undertaken lead us to conclude that both angioblasts and haematopoietic progenitors are derived *in situ* from the mesoderm. Strikingly, most if not all cells in the mesoderm display VEGFR-2 and many express the transcription factors SCL, GATA-2 and GATA-1; the CD45 antigen then appears on some cells and haemoglobinization of cell groups shortly follows. All these signs of commitment and differentiation, which pertain to a process, designated earlier as "hemangiopoiesis" (268), occur prior to the establishment of vascular connections between the allantois and the embryo. In our earlier work (122), the allantois rudiment was dissected out for grafting between HH17 and 18, about 6 hours before vascular connection; the timing of this event was herein determined very precisely by the injection of AcLDLs; it occurs between HH18 and 19 (about 72 hours of incubation). AcLDL uptake is slightly more sensitive than India ink microangiography to reveal the vascular network. AcLDLs are readily distributed into the intravascular compartment and is rapidly receptor-endocytosed following inoculation. India ink never penetrates into endothelial cells, and part of the charcoal particles may be washed out from sections by PBS rinses.

Actually, prior to vascularization, the allantois bud exhibits prominent blood island-like structures. On the molecular level, there is, like in the yolk sac, a haematopoietic program, characterized by the expression of GATA-2, GATA-1 and globin genes, which appears predominantly erythroid. On the basis of the ellipsoid shape of red blood cells recognizable in cultures and of RT-PCR identification of a primitive globin type (122), the erythrocytes produced by the allantois can be allocated to the "fetal" rather than the embryonic lineage.

In the previous paper (122), it was reported that the host bone marrow became invaded by haematopoietic and endothelial cells emitted by the grafted allantois rudiment; thus it was considered that hemangioblasts, rather than distinct progenitors for the two lineages, colonized the host bone marrow. The

presence of VEGFR-2, SCL and Lmo2 reported here in the allantoic mesoderm supports this hypothesis.

In the mouse, the allantoic rudiment has also been shown to express a number of molecules related to vasculogenesis and/or haematopoiesis (63; 149; 212; 255; 269; 270). It also develops its own vascular network (271), but appears not to produce erythrocytes when explanted prior to fusion with the ectoplacental cone (13-15 pairs of somite) and cultured for 2 days (272). 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, never entering the stalk; this may cause a restriction to the angiopoietic potential of the allantoic mesoderm. However a specific contribution from the mouse allantois to the haematopoietic system would be difficult to identify, because the rudiment develops during gastrulation at the same time as 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 allantois mesoderm is a site where commitment of HSC may occur.

The experiments reported here reveal an interesting molecular feature of the allantois endoderm. It was previously detected by RT-PCR that GATA-3 is expressed in the allantois (122). Here, we determined that this expression is localized in the endoderm. GATA-3 appears at HH11-12 at least six hours before allantoic outgrowth. Judged on the 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 (273). 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 haematopoiesis was thought to be restricted to haematopoietic cells proper (see review for Orkin (274)). When GATA-2 became conspicuous in the endoderm, its expression also switched on in the associated mesoderm in parallel with that of VEGFR-2, SCL, 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 (212). GATA-3 and Lmo2 expression only overlap in the endoderm during the 5-somite stage, prior to the delamination of the aortic endothelium into haematopoietic clusters described by several groups (212; 275; 276; 277; 278; 279). It thus appears that several genes, previously known to be present in haematopoietic 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 haematopoiesis, 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 haematopoiesis 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 (280; 281; 282 and references therein). In invertebrates, the Caenorhabditis elegans end-1 gene was proposed to be a master gene for endodermal development (283; 284). In Drosophila, the GATA-related *serpent* gene, expressed by subsets of endodermal and mesodermal cells, is essential for both midgut and haematopoietic cell specifications (285; 286; 287).

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

THE HAEMATOPOETIC PROGRAM OF THE EMBRYONIC DORSAL AORTA

The early haematopoietic key factors are expressed in the dorsal aorta

Previous studies showed that the first morphological transformations on the aortic floor, where haematopoietic clusters emerge, are also accompanied by gene expression changes (105). In the avian embryo, the entire aortic endothelium is positive for VEGFR-2 at E3, before the haematopoietic clusters emerge. At the time of cluster differentiation, VEGFR-2 is downgraded and CD45, the pan-leukocyte marker, is upgraded in ventral endothelial cells. As rounded haematopoietic cells bulge into the lumen of the aorta, they become all CD45 positives.

In the recent study, we further dissected these commitment events and followed the gene expression changes at the level of transcription factors. Even at the stage, when no morphological sign of cluster differentiation is seen, SCL, Lmo2 and GATA-3 co-expression was revealed on aortic endothelial cells. The expression levels were highest on the ventral wall of the aorta and indicated that haematopoietic progenitors emerging in this location are committed even before the morphological transformation. These data are consistent with the supposed role of SCL and Lmo2 in the initiation of haematopoietic progenitor emergence.

Co-expression of GATA-3 with SCL and Lmo2, which was not revealed in the early yolk sac, suggests that transcriptional complexes working in the primitive yolk sac haematopoiesis are different from that of the haematopoietic precursors emerging in the aorta.

In the intraaortic clusters SCL and Lmo2 were upregulated, while GATA-2 and GATA-3 showed modest expression levels. Interestingly, the expression of these different factors was not restricted to the clusters, but was also revealed on the dorsal wall of the aorta. This observation suggests that the cluster cells are not specified by the expression of the investigated factors but

by the presence of other factors such as c-myb or Runx-1 (see in 288). Within the clusters, a mosaic expression pattern of TFs was revealed, suggesting that haematopoietic progenitors emerging in this location represent a heterogenic population. At this stage GATA-1 was only detected in circulating blood cells, but not in the clusters, which indicates that these progenitors are not committed to blood cell lineages specified by this factor.

Colonisation potential of the haematopietic progenitors emerging in the dorsal aorta

As seen in the introduction, the developmental potential of the haematopoietic progenitors emerging in the aortic region was investigated on different vertebrate species by *in vitro* as well as *in vivo* methods. The most important result of these experiments is that the aorta region is the first site in the embryo where long-term repopulating haematopoietic stem cells emerge (120). Previous experiments made on the avian model showed that the origin of these progenitors is the aortic endothelium itself (105) and direct filiation was also proved between the intraaortic clusters (from E3) and paraaortic foci (from E6) (106). It was also shown that isolated E4 chick or quail aorta grafted onto a reverse host of the same age, produced haematopoietic progenitors capable of seeding the fetal haematopoietic organs and acquired surface antigens characteristics of T or B lymphocytes and granulocytes (289).

In our study, we micro-dissected various tissues from E3 (HH17-19) quail embryos, and grafted into the coelom of the same developmental stage chick embryos. This experimental system allows studying the developmental potential of the aortic region one day earlier than Cooper and co-workers, when the haematopoietic transcription factors are already present in the aortic wall (see above). When the aortic region was implanted, the graft-derived cells widely colonised the haematopoietic organs, and colonisations were comparable to that of the allantoic bud. These data clearly indicate that the implanted aorta already has the haematopoietic colonisation capacity. In addition, the unexpected finding of these experiments is that the implanted cells of the aorta region were found preferentially in the thymus of the host embryos. Such a significant difference between the analysed organs (thymus,

bursa of Fabricius, spleen, bone marrow) was not detected in the case of the allantois grafts. The different colonisation potentials can be explained by the distinct genetic program of the haematopoietic progenitors emerging in the aorta or allantois. The presence of GATA-3 (see above) on the cluster cells, which was demonstrated to be essential for T cell development from the earliest recognizable stage (223; 290), suggests that direct precursors of this lineage can occur in the aortic clusters.

CONCLUSIONS AND PERSPECTIVES

Assembling data presented in this work it appears that different genetic programs regulate the haematopoiesis during the embryonic life depending on the site and time. At the level of transcriptional regulation it is accompanied by the expression of different transcriptional complexes. Haematopoiesis taking place in the early chicken yolk sac or allantois is characterised by the co-expression of GATA-2, SCL, Lmo2 and GATA-1. In contrast, intraaortic clusters, appearing later in development, express SCL, Lmo2, GATA-2, and GATA-3 and never GATA-1. Such similarities and differences between the molecular programs of the various locations of the haematopoietic progenitor emergence are probably due to the origin of progenitors. All of mesodermal ancestor of the haematopoietic progenitors developing in the yolk sac, allantois and aorta derive from the slpanchnopleure but at distinct levels of the antero-posterior axis. The posterior part gives rise to the mesoderm of the yolk sac and allantois while hemogenic endothelial cells of the aorta come from more anterior positions. The positional information might be established at gastrulation when the mesodermal precursors leave the primitive streak. Afterward specifications probably occur when cells migrate into different microenvironments receiving different inductive signals before differentiation.

The established normal gene expression patterns revelaled by this study offers further possibilities to investigate the effects of different morphogens and growth factors on the process of embryonic haematopoiesis. The defined developmental stages, characterised by different TF gene expressions, are also important from the clinical point of view, because they raise the possibility of further dissecting the adult progenitor populations in order to obtain more efficient precursors for therapeutic goals.

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SUMMARY

The detailed comparative expression patterns of key transcription factors in haematopoiesis (such as SCL, Lmo2 and GATA factors) have been established during avian embryonic haematopoiesis. These factors were chosen because molecular studies have established they work together in multimeric transcription factor complexes. In order to study the gene expression pattern of Lmo2, its chicken ortholog has been isolated and characterised.

Two distinct expression patterns for GATA-2 were found in the yolk sac. One low, associated to the lateral-posterior mesoderm, and a second high, blood island-specific. This latter is contemporary with GATA-1 expression and post-dates SCL and Lmo2 in the hemangioblast population. Such a bimodal GATA-2 expression has not been reported before on other vertebrate models. According to the expression patterns, it seems that Lmo2 is equally important in the development of both the endothelial and the haematopoietic lineages, in contrast to SCL.

The molecular patterns revealed in the developing allantois mesoderm lead us to conclude that both angioblasts and haematopoietic progenitors, or hemangioblasts are derived *in situ* from the mesoderm prior to the establishment of vascular connections between the allantois and the embryo. Additionally, two GATA factors were detected in the endodermal layer. GATA-3 expression indicated the presumptive area of allantoic endoderm, and was followed by the switching on of GATA-2.

The established expression patterns in the yolk sac and allantois mesoderm refers predominantly to the development of the erythroid lineage. In contrast, the expression patterns in the intraaortic haematopietic clusters indicate progenitor emergence, with the presence of early haematopoietic marker genes -such as SCL, GATA-2, 3 and Lmo2- and the absence of the lineage specific GATA-1.

The haematopoietic potential of the E3 haematopoietic cluster-containing dorsal aorta has been demonstrated *in vivo*, in quail-chick chimera experiments. Grafting was performed one day earlier than that was previously reported. The unexpected finding of these experiments is that the implanted cells of the aorta region were found preferentially in the thymus of the host embryos.

ÖSSZEFOGLALÁS

A vérképzés korai szakaszában az SCL, Lmo2 és GATA faktorok egymással alkotnak transzkripciós komplexeket. Vizsgálataimban ezeknek a faktoroknak a részletes összehasonlító expressziós mintázatát határoztam meg a csirke embrió vérképző helyein a korai embrionális fejlődés során. Az Lmo2 faktor csirke homológját klónoztuk az expressziós mintázatának meghatározásához.

A korai vérképzésben fontos szerepet játszó szikhólyagban a GATA-2 faktor kettős mintázatot mutat. Egy alacsony szintű GATA-2 expresszió jellemzi az összes posterolateralis mezoderma sejtet, közvetlenül a primitív csíkból való kilépés után. Ezt követően a morfológiailag egységes mezodermában egyes sejtcsoportokban megjelenik az SCL és az Lmo2 faktorok mRNSe. Az SCL és Lmo2 expresszió által kijelölt leendő vérszigetekben a GATA-1 expresszió megjelenésével párhuzamosan felerősödik a GATA-2 expresszió. Korábbi vizsgálatok a GATA-2 expresszió ilyen változását nem fedték fel más gerinces modellállaton, de eredményeink jól összeegyeztethetők a GATA-2-nek az irodalomból ismert kettős szerepével. Az Lmo2 expressziójának felerősödése a vérszigetek angioblastjaiban arra utal, hogy ez a faktor nemcsak a vérsejtek, hanem az endothelium differenciációjában is szerepet játszik.

Az allantoison végzett vizsgálataink azt mutatják, hogy a szikhólyaghoz hasonlóan itt is *in situ* vérképzés történik. A hemopoetikus differenciáció már az embrióval való keringési kapcsolat kialakulása előtt elkezdődik. Az allantois korai fejlődése során kimutattam a GATA-3 faktor jelenlétét a caudalis endodermában. A mintázat és az irodalmi sorstérképezési adatok alapján a GATA-3 expresszió kijelöli a leendő allantois endodermáját, majd későbbi stádiumokban megjelenik ugyanitt a GATA-2 faktor is.

A szikhólyag és az allantois transzkripciós faktor mintázata az mutatja, hogy az itt képződő sejtek főleg erythroid irányba differenciálódnak. Ezzel ellentétben, az aortában kialakuló vérsejtképző aggregátumokban a korai vérsejt markerek (SCL, Lmo2, GATA-2 és 3) jelenléte, illetve a GATA-1 hiánya progenitorok kialakulására utal.

A hemopoetikus sejtcsoportokat tartalmazó embrionális aorta *in vivo* kolonizáló képességét bizonyítottuk csirke-fürj kiméra kísérletekkel. Az aorta eredetű sejteket kimutattuk a hemopoetikus szervekben. További vizsgálatokra ösztönző megfigyelésünk, hogy az aortából származó sejtek a thymus felé mutatnak preferenciát.

RESUME

J'ai analysé chez le jeune embryon de Poulet l'expression combinée de facteurs de transcription (SCL/tal-1, Lmo2 et GATA) dans les sites de l'hématopoiese embryonnaire. Ces facteurs ont été choisis car ils sont connus pour jouer des rôles importants dans l'hématopoïèse en participant à des complexes multiprotéiques. Pour obtenir le patron d'expression de Lmo2, son orthologue aviaire a été identifié et caractérisé.

Deux niveaux d'expression de GATA-2 ont été détectés dans le sac vitellin. Toutes les cellules mésodermiques dans la région postéro-latérale quittant la ligne primitive expriment ce facteur à niveau faible. Par la suite l'expression de GATA-2 remonte dans les futurs îlots sanguins marqués par l'expression de SCL et Lmo2, en même temps que GATA-1 commence à être exprimé aussi. Jusqu'à maintenant cette expression bimodale de GATA-2 n'a pas été détectée chez les autres Vertébrés. Selon les patrons d'expression, Lmo2 jouerait un rôle important dans le développement des cellules endothéliales et hématopoïétiques. En revanche SCL est dérégulé très vite dans les cellules endothéliales du sac vitellin.

Il a été démontré que l'allantoïde d'Oiseau produit des progéniteurs hématopoïétiques *in situ*. Notre analyse moléculaire a renforcé maintenant que l'émergence de ces précurseurs précède la vascularisation de l'allantoïde. En outre, on a détecté l'expression endodermique de GATA-3 dans le territoire présomptif de l'allantoïde. Cette expression est suivie par celle de GATA-2.

Les patrons d'expressions détectés dans le sac vitellin et l'allantoïde suggèrent l'émergence de cellules érythroïdes. Au contraire, la présence des marqueurs précoce de l'hématopoïèse (SCL, Lmo2, GATA-2) et l'absence de lignage spécifique GATA-1 dans les agrégats intra-aortiques suggère l'apparition des progéniteurs. Il semble que la détermination des cellules sur le plancher de l'aorte a lieu avant la transformation morphologique car la co-expression de SCL, Lmo2 et GATA-3 est détecté déjà dans les cellules endothéliales.

La capacité colonisatrise de la région aortique de trois jours de développement a été démontrée à l'aide des chimères caille/poule et comparée à celle de l'allantoïde. Les greffons ont été implantés à E3, un jour plus tôt que ce qui avait été rapporté jusqu'alors. Les cellules du greffon ont été détectées dans les organes hématopoïétiques à l'aide de l'anticorps monoclonal QH1, spécifique des lignées endothéliale et hématopoïétique. Le pourcentage de cellules $QH1^+$, issues de l'aorte greffée, atteint jusqu'à 30% des cellules du thymus.