# Commitment of hematopoietic stem cells in avian and mammalian embryos: an ongoing story

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**ABSTRACT** **During ontogeny, hematopoietic stem cells (HSC) become committed outside of hematopoietic organs. Once held to emerge from the yolk sac, they are currently thought to originate from the early aorta. However we now show that the allantois in the avian embryo and the placenta in the mouse embryo produce HSC in very large numbers. These sites thus appear to have a central role in the process of HSC emergence.**

KEY WORDS: *hematopoietic system, yolk sac, aorta, allantois, placenta.*

Several articles in the present Special Issue describe how the emergence of blood stem cells and their relationship to the endothelial network were analyzed using the cell tracing efficiency of the heterospecific quail/chicken model (Le Douarin, 1969). Concerning the blood forming system, this model was first applied to the investigation of T lymphopoiesis. During ontogeny, extensive cell migrations were known to occur in the hematopoietic system, due to the pioneering work of Moore and Owen (review in Metcalf and Moore, 1971). In 1965 these authors began investigating blood formation in the embryo, a few years after the advent of modern hematology devised for the adult system. Till and McCulloch (1961) had shown that the hematopoietic system of an irradiated mouse could be reconstituted by intravenous injection of bone marrow cells from a healthy mouse. This experiment uncovered the existence of pluripotent, self-renewable hematopoietic stem cells (HSC). These cells, which give rise to colonies *in vivo* and *in vitro*, are able to migrate and settle in various microenvironnements, in which they are instructed to enter specific differentiation pathways.

In order to investigate how the blood system forms during development, Moore and Owen chose the chicken embryo, already the subject of many descriptive studies since the end of the XIXth century, because it is directly visible after shell opening and develops independently from a maternal circulation. They applied an array of experimental approaches: irradiation/restoration, parabiosis, grafting of rudiments (thymus, spleen, bursa of Fabricius) on the chorioallantoic membrane (review in Metcalf and Moore, 1971). To follow cell origins in these different designs, Moore and Owen used the pair of sexual chromosomes as a cell marker. Despite several drawbacks inherent to this labelling system (the chromosomes can be observed only in 5-10% of cells arrested at the metaphase stage of mitosis after a colcemid

treatment which elicits high mortality of the embryos; furthermore the adequate opposite sex combination is left to chance), these investigators discovered an important trait which characterizes the development of the blood system: Hematopoietic Stem Cells (HSC) colonize all rudiments to the exception of the yolk sac. They also grafted mouse embryo rudiments on the chicken chorioallantois and observed that, in contrast with cell type-specific recognition capacities displayed by most other embryonic cell types across different classes of vertebrates, attraction or recognition did not work between mouse stromal rudiments and chicken HSC. Retrieved at an early stage and grafted on the chorioallantois, the mouse thymic rudiment remained alymphoid, allowing Moore and Owen to conclude in 1970 that, in mammalian developmental hematopoiesis also, HSC emerge in one location and differentiate in another (review in Metcalf and Moore, 1971).

The quail/chicken labelling system, applied to the development of the thymus and the bursa of Fabricius made it possible, through different grafting schemes, to identify the origin of every single cell in early rudiments (review in Le Douarin *et al.,* 1984). The thymus endomesodermal rudiment was shown to be colonized by cyclical waves of HSC according to a precisely recurring time schedule. The mesoderm, in particular non-endothelial cells of blood vessel walls, was shown to derive from the neural crest (i.e., ectomesoderm) (review in Etchevers *et al.,* 2001), while endothelial cells, in the region of the head and neck, are produced by a dramatic amplification of a minute quantity of cephalic paraxial mesoderm (Couly *et al.,* 1995).

The time when colonization begins in the mouse thymus was established, by associating the cultured early rudiment with an

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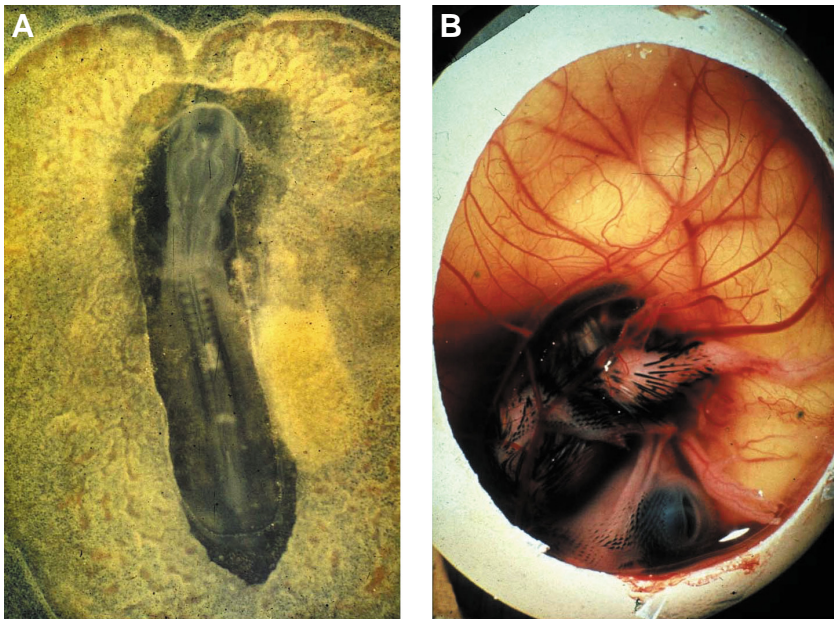
**Fig. 1. The yolk sac chimera. (A)** *Surgical construction of the chimera at E2. The suture line is visible between the quail embryonic body (neural tube and somites) and the chick extra-embryonic area.* **(B)** *Quail embryo growing with a chick yolk sac 12 days after the operation.*

HSC donor organ (fetal liver) (review in Dieterlen-Lièvre and Le Douarin, 1984). Sequential waves of colonization were uncovered in the mouse thymus but, due to overlap of these waves, the cycling was less clearly worked out (Jotereau *et al.,* 1987).

All in all, the quail-chicken model offered such experimental possibilities that it opened a variety of fields, allowing in particular to revisit the problem of HSC emergence. This problem benefited from the use of the monoclonal antibody MB1/QH1 (Péault *et al.,* 1983; Pardanaud *et al.,* 1989) which recognizes hematopoietic and endothelial cells in the quail species and not in the chicken. The rules thus uncovered were later extended to mammals. In the present volume, the articles by Jaffredo *et al.,* 2005; Pudlewski and Pardanaud, 2005 and Godin and Cumano, 2005 cover extensively the findings of my group about the commitment of HSC and their developmental relationship with the endothelial network in bird and mouse embryos. Here I shall put the history of these investigations in perspective, dwelling more lengthily on some issues not discussed in these articles and I will describe novel data about mouse embryo developmental hematopoiesis. These data are based on a hypothesis suggested by our recent finding in the avian embryo according to which the allantois is a hereto unrecognized site for HSC and angioblast production.

## Origin of HSC: contributions of the quail/chicken model

When Moore and Owen (1967) proposed that HSC must become committed from the yolk sac mesoderm early in development and once only for the whole life of the individual, this idea was enthusiastically endorsed by the hematologist and immunologist community. In 1975 I applied to this problem a surgical technique devised by Claude Martin (1972), in which the quail embryonic body is grafted on the chick yolk sac, before the onset of regular heart beat and circulation of blood cells. In these

chimeras (figure 1), cells belonging to blood lineages in the thymus, spleen, bursa and bone marrow were all chicken while stromal cells were quail (Dieterlen-Lièvre, 1975; Martin *et al.,* 1978). Circulating blood cells were chicken (derived from yolk sac progenitors) until day 5 of incubation, then became mixed, enriching with age in quail erythrocytes (Beaupain *et al.,* 1979). The results were confirmed in chimeras built between congenic strains of chickens, differing either in their immunoglobulin allotypes or their major histocompatibility antigens (Lassila *et al.,* 1978, 1982).

The demonstration of these intra-embryonic HSC and of their contribution to definitive hematopoiesis in birds was soon accepted. The experimental scheme was transposed to amphibian embryos, in which several species possess a so-called 'dorsal' compartment of hematopoietic progenitors (review in Turpen, 2001), which is actually the equivalent of the aortic region of birds and mammals. In the mouse model the problem was not approached until 1993, the consensus remaining until then that mammalian developmental hematopoiesis was 'different'.

Two distinct approaches then yielded results supporting and extending each other. Ours tackled the stages between 8 and 25 pairs of somites (E8.5-9.5 embryo), when the structures under study are very tiny and the number of cells available small, making the classical *in vitro* clonal culture technology appropriate (review by Godin and Cumano, 2005 in this issue). The region of the future caudal gut, widely open onto and continuous with the yolk sac, was tested; it included endoderm and mesoderm (splanchopleura) and the two aortic rudiments. We gave this region the name of 'Para-aortic Splanchnopleura' (P-Sp) (Figure 2). The tissues, dissected into single cells and seeded on a feeder layer of growth factor producing cells, in a semi-solid medium enriched with appropriate growth factors, gave rise to myeloid and lymphoid colonies between the stages of 15 and 25 pairs of somites. The yolk sac also yielded these colonies, while the rest of the embryo was non-productive. Blood circulation becoming established circa the stage of 6-7 pairs of somites in the mouse embryo, these experiments allowed no conclusion concerning the primary origin of progenitors present in each location. Thus the experimental scheme was applied to pre-7 somite embryos. Progenitors could detected in both the extra-embryonic and intra-embryonic compartments provided that one condition was fulfilled; the anatomically associated germ layers had to be cultured *in toto* for 2 days, prior to dissociation and clonal cultures. In these conditions, while vascular cell exchanges prior to tissue retrieval were excluded, cell types or cell layers could interplay, allowing the occurrence of commitment events. The P-Sp gave rise to myeloid and lymphoid colonies, while the yolk sac gave rise only to myeloid colonies, indicating that, like in birds, the embryo proper produces HSC with a wider potential than YS HSC.

The *in vitro* technology, well suited to detect progenitors present at early embryonic stages, has its limitations, since it identifies pluripotential colonies but not Long Term Reconstituting Hematopoietic Stem Cells (LTR-HSC) (see Godin and Cumano,

2005 in this issue for particulars). In order to find out whether such LTR-HSC are produced by the embryo proper, Elaine Dzierzak and her group applied the reconstitution technology: irradiated adult mice were injected with cells from the embryos. These investigators used older stages (30 to 42 pairs of somites, i.e., E10-11.5); by that time the two aortic rudiments have fused, the intestine has closed, new rudiments - gonads and kidney- have begun differentiating in this region (hence the designation 'Aorta-Gonad-Mesonephros' or AGM) (figure 2) and blood circulation is full blown (review in Ling and Dzierzak, 2001). The conclusions extended ours: the AGM yielded LTR-HSC, while the yolk sac did not.

## Dorsal (somitic) versus ventral mesoderm: different capacities regarding hematopoietic and endothelial differentiation

According to multifold evidence, endodermal contact is crucial for mesoderm to give rise to blood and blood vessels. A striking example is found when the yolk sac (mesoderm + endoderm), a powerful angioblastic and hemogenic appendage (Wilt, 1965; Kessel and Fabian, l987; Flamme, 1989; Flamme and Risau, 1992) is compared to the amnion. This latter appendage (mesoderm + ectoderm) never develops a vascular system nor blood cells.

We asked two questions: i) how and where do angioblasts, the precursors of endothelial cells (EC), become committed within the embryo proper? ii) what is the developmental relationship between angioblasts and HSCS? At the time we became concerned with this problem, somites were the only identified source of angioblasts in the embryonic body (Noden, 1991; Wilting *et al.,* 1995). Using MB1/QH1, we compared the development of the vascular network in the limb bud and in visceral organ rudiments (Pardanaud *et al.,* 1989). Limb bud and the bone marrow within the limb bud appeared colonized by angioblasts and HSC, both of extrinsic origin as earlier observed by Jotereau and Le Douarin (1978). Thus, somatopleural mesoderm does not have the potential for vasculogenesis (i.e., no cells in this mesoderm become committed to the angioblastic pathway). In contrast visceral rudiments, while they were also colonized by HSC, developed an intrinsic vascular network, derived from their mesodermal component. Original work by Pudlewski and Pardanaud, 2005 (this issue) confirm that these distinct modes of vascularisation are at work in mouse rudiments, namely that the angioblasts colonizing mouse limb buds grafted in quail embryos came from the host, while visceral rudiments developed a mouse, i.e., intrinsic, endothelial network.

To pinpoint the origin of these angioblasts and determine the territories they colonize, orthotopic grafts of somites or splanchnopleural mesoderm were performed from the quail into chick hosts (Pardanaud *et al.,* 1996; Pardanaud and Dieterlen-Lièvre, l999). Somite-derived angioblasts colonized the body wall and limb buds, but never penetrated into visceral derivatives; furthermore they gave rise to the roof and lateral endothelium of the aorta. Interestingly these angioblasts avoided the aortic floor. In contrast splanchnopleura-derived angioblasts, while they also colonized the body wall, proved capable of entering the viscera and contributed to the whole aortic endothelium, comprising the floor. The splanchnopleura- derived cells that integrated in the aortic floor, participated to 'intra-aortic clusters'. These cell groups, described in all species where they were looked for (Jordan, 1914; Emmel, 1916; Dieterlen-Lièvre and Martin, 1981; Garcia-Porrero *et al.,* 1995; Wood *et al.,* 1997; Tavian *et al.,* 1996) are tightly associated to the ventral endothelium of the aorta and are considered as the source of intra-embryonic HSC. Their blood progenitor nature is attested by the expression of CD41, an HSC-specific antigen, which is restricted to these cell clusters in the chicken and mouse (reviewed in Corbel *et al.,* 2005 in this issue).

Thus i) somites give rise to angioblasts, whose potential is restricted to vasculogenesis and which are responsible for vascularisation of the body wall; ii) splanchnopleural-derived 'hemangioblasts' (see the article by Eichmann *et al.,* 2005 in this issue) have a much wider potential and are capable of vascularizing the whole embryo. The splanchopleural cells that locate to the floor of the aorta display the dual potential, i.e., give rise to both endothelial and hematopoietic cells. Homing to the aortic floor is a requirement for the expression of this hematopoietic potential.

It is possible to manipulate the potential of these two types of precursors: somites pre-treated by contact with endoderm or culture on a medium containing VEGF, BFGF or TGFβ acquire the



**5-10 somites E 8.5**  **20-25 somites E 9.5**  **35-40 somites E 10.5**
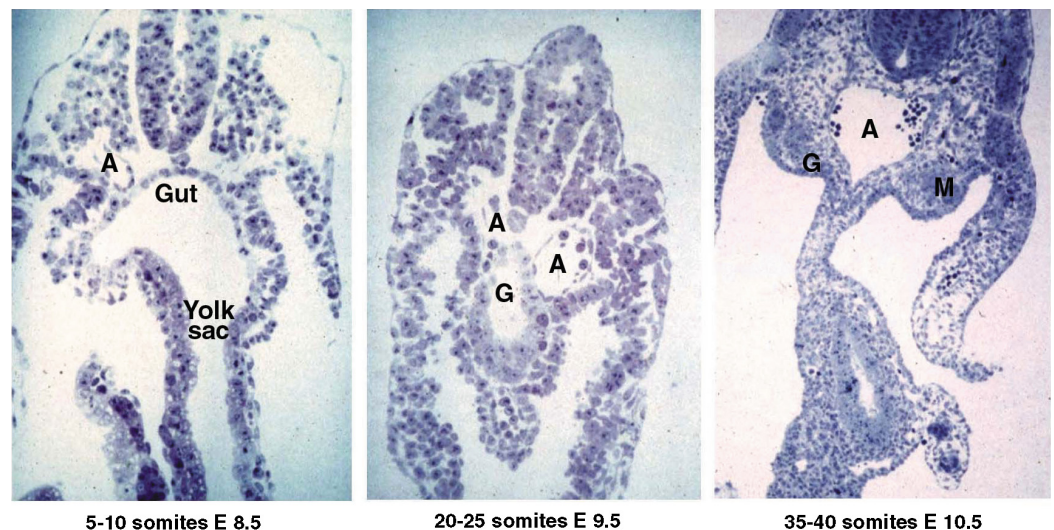
**Fig. 2. Developmental relationship between Para-aortic Splanchnopleura (P-Sp) and Aorta-Gonad-Mesonephros (AGM).** *Semi-thin transverse sections in the post-umbilical region, toluidine blue. At the 5-10 pairs of somites stage, the gut is wide open onto and continuous with the yolk sac, the mesoderm lining the endoderm contains the two aortic rudiments (A) but no organ rudiments; at 20-25 pairs of somites, the gut (G) has closed; at 35-40 pairs of somites, the dorsal aorta has formed by fusion of the two initial rudiments, the gonads and mesonephros rudiments have appeared.*

extended potential, while splanchnopleural mesoderm treated by ectodermal contact or cultured on TGF or EGF loose it (Pardanaud *et al.,* 1999).

Using the endothelium-specific endocytosis of low density lipoproteins, we followed *in situ* the evolution of the aortic endothelium and found that phenotypically characteristic endothelial cells, present in the aortic floor on E2, gave rise 24 hours later to hematopoietic clusters (Jaffredo *et al.,* 1998). A similar approach could be carried out in cultured mouse embryos and established the same derivation (Sugyiama *et al.,* 2003). Tavian, Péault and co-workers (see Tavian and Péault*,* 2005 in this issue), relying on antigenic specifications of different cell types in the human embryo, arrived at similar conclusions. These findings thus support the notion, formulated by the cytologists in the first half of the XXth century, of a 'hemogenic endothelium'.

## The avian allantois as a source of hematopoietic progenitors and angioblasts

In 1998, we showed that the allantoic bud retrieved from the quail at a prevascular stage and grafted in the chicken coelom produced progenitors that colonized the host bone marrow and gave rise there to both hemopoietic and endothelial cells (Caprioli *et al.,* 1998, 2001; see Jaffredo *et al.,* 2005 in this issue). This provocative double colonization may mean either that distinct precursors of the two lineages travel through the circulation or that a common progenitor, the hemangioblast, is the cell that circulates and differentiates into the bone marrow microenvironment along the two pathways. Experimental arguments for the existence of the hemangioblast are accumulating (see Jaffredo *et al.,* 2005 and Eichmann *et al.,* 2005, both in this issue), supporting the second interpretation. This finding in the avian model should be put together with the fact that endothelial progenitors bearing the HSC-specific antigen CD34 are known to circulate in the blood of the adult mouse during repair processes (Asahara *et al.,* 1997; Raffii, 2000).

Whatever the case, two interesting perspectives for future research open up: i) is the embryonic and fetal bone marrow vascularized by blood borne progenitors, in parallel with the process of HSC seeding? ii) the discovery of a new site of stem cell production raises the question as to how long during development this process may occur.

## The mouse placenta as a hematopoietic organ

The fetal liver, whose rudiment has to be seeded by HSC (Johnson and Jones, 1973; Houssaint, 1981) is considered as the main hematopoietic organ of the mammalian fetus. The placenta had not seriously been contemplated for such a function, until we undertook an investigation into a possible role of this appendage during hematopoietic development (Alvarez-Silva *et al.,* 2003). The idea was suggested by our finding that the avian allantois could give rise to HSCs *in situ* and that formation of the mammalian placenta entails fusion of the allantois to the ectoplacental cone.

Downs *et al.* (1998) had previously looked for a possible erythropoietic function of the prefusion mouse allantois. Their experiments dealt with allantoises from the head-fold stage which were explanted in organ culture until the 15 somite stage. They observed that vascularization developed *in situ* but did not find

any signs of red blood cell formation, but it is likely that the culture time and conditions were inadequate.

We first looked for colony-forming progenitors in the placenta between E10 and E17 (Alvarez-Silva *et al.,* 2003) using collagenase dissociation into single cells followed by culture in a semi-solid media containing a mixture of cytokines. To determine whether the colonies originated from the fetal or maternal component of the placenta, heterozygote fetuses, carrying one allele of a ubiquitously expressed GFP transgene, were obtained from crosses between GFP+ males and wt females. Very strikingly most colonies were GFP positive, they were 2-4 times more numerous than the ones obtained from the liver at all stages and the proportion of colonies derived from very early colonies (CFU-GEMM) was very high. Furthermore huge colonies that could be subcloned for at least 60 days were obtained. These data indicated that, indeed, the placenta is a site of active hematopoiesis. By comparison with the fetal liver, at all stages studied, the hematopoietic population in the placenta is richer in very early progenitors, in particular in High Proliferation Potential-HSC.

However, as mentioned above, the LTR-HSC is considered as the hallmark of a true HSC. Since our first publication, irradiation-reconstitution experiments have disclosed such cells (Gekas *et al.,* 2005; Ottersbach and Dzierzak, 2005). Interestingly the concentration in these LTR-HSC peaks very early, at about the same time as those which are present in the region of the AGM (E11 or around 42 somites) but they persist longer in the placenta, namely until E13. Their disappearance however antedates by several days the disappearance of *in vitro* colony-forming progenitors.

The question which persists concerns the origin of these stem cells and progenitors in the placenta. Do they become determined in this region or do they invade the placenta, for example after originating in the AGM? We are investigating this aspect using the approach that was efficient in the case of the P-Sp: prefusion allantoises, retrieved from GFP/+ embryos between the stages of 1 and 7 pairs of somites, are cultured *in toto* for 4-5 days and, only thereafter, dissociated into single cells and seeded in semi-solid media. Clearly, colony-forming progenitors are produced by the allantois (Salaün, Corbel, Dieterlen-Lièvre, submitted).

## Conclusion and perspectives

Why is it important to understand when and where and how stem cells that are at the origin of a tissue become committed? This endeavour has been going on, in the case of HSC, for nearly 40 years and is evidently not a closed case. While the molecular events are actively investigated using mouse embryonic stem cells differentiating *in vitro*, it remains important to establish what happens in the growing embryo, which clearly does not obey the same mechanisms. We have found that the emergence of HSC moves around from one site to another and is an event spread out on a long period of development. This is not such a surprise, considering the enormous amplification of the blood cell population that embryonic growth requires. How long in life the commitment of HSC may occur is still a black box. Seeing the vivid interest presently devoted to adult stem cells, because of their apparently extended plasticity and the therapeutic potential this plasticity may provide, it is crucial to understand precisely how these cells become specified during development.

YS cells should not be considered as HSC, but rather as progenitors. Indeed they do not display long term renewal potential and their differentiation capacities appear restricted to the myeloid lineage. This restriction may be due to environmental pressures but also to distinct molecular features of this first generation. For instance P-Sp progenitors were shown to lack LTR-HSC potential, because they do not display full MHC class I expression, which makes them prone to elimination by NK cells (see Godin and Cumano, 2005 in this issue).

The pendulum now swings back to an extraembryonic appendage, the allantois in birds and the placenta in mammals. Notably the sites, in which HSC become committed, are as a rule 'ventral' sites, in which the mesoderm is associated to endoderm. This is indeed the case in the allantois. The endoderm is known to produce ventralizing molecules such as BMPs and VEGF.

The developmental relationship between HSC and angioblasts appears complex with a population of purely angioblastic progenitors of dorsal origin and a population of hemangioblasts of ventral origin. The possible plasticity between the two populations even in the grown organism is evidently a crucial issue for therapeutic delivery of drugs and repair of tissues.

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