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A population of hematopoietic stem cells derives from GATA4-expressing progenitors located in the placenta and lateral mesoderm of mice

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ABSTRACT

GATA transcription factors are expressed in the mesoderm and endoderm during development. GATA1-3, but not GATA4, are critically involved in hematopoiesis. An enhancer (G2) of the mouse *Gata4* gene directs its expression throughout the lateral mesoderm and the allantois, beginning at embryonic day 7.5, becoming restricted to the septum transversum by embryonic day 10.5, and disappearing by midgestation. We have studied the developmental fate of the G2-Gata4 cell lineage using a G2-Gata4^{Cre};R26R^{EYFP} mouse line. We found a substantial number of YFP$^+$ hematopoietic cells of lymphoid, myeloid and erythroid lineages in embryos. Fetal CD41$^+$/cKit$^+$/CD34$^+$ and Lin$^-$/cKit$^+$/CD31$^+$ YFP$^+$ hematopoietic progenitors were much more abundant in the placenta than in the aorta-gonad-mesonephros area. They were clonogenic in the MethoCult assay and fully reconstituted hematopoiesis in myeloablated mice. YFP$^+$ cells represented about 20% of the hematopoietic system of adult mice. Adult YFP$^+$ hematopoietic stem cells constituted a long-term repopulating, transplantable population. Thus, a lineage of adult hematopoietic stem cells is characterized by the expression of GATA4 in their embryonic progenitors and probably by its extraembryonic (placental) origin, although GATA4 appeared not to be required for hematopoietic stem cell differentiation. Both lineages basically showed similar physiological behavior in normal mice, but clinically relevant properties of this particular hematopoietic stem cell population should be checked in physiopathological conditions.

Introduction

The six transcription factors belonging to the GATA family in mammals play important roles in mesoderm and endoderm development. GATA1-3, but not GATA4-6, play critical roles in hematopoiesis.[1] Mice deficient for GATA4 show defects in the heart and intestine and die around embryonic day (E) 13.5.[2-4]

A mesodermal-specific enhancer of *Gata4*, called G2, drives Gata4 expression in the lateral mesoderm and allantois starting at E7.5. Later, this activity is restricted to the septum transversum and ceases by E12.5.[5] Our previous work using G2^{Cre};R26R^{EYFP} mice has shown that the cell lineage where *Gata4* is activated by G2 contributes to hepatic stellate cells. Inactivation of *Gata4* using this G2^{Cre} driver is lethal by midgestation. The anemia observed in the *G2^{Cre};Gata4$^{flox/flox}$* embryos was attributed to a failure in the expansion of the hematopoietic progenitors in the fetal liver. Interestingly, a small population of hepatic YFP$^+$ cells from G2^{Cre};R26R^{EYFP} embryos was positive for leukocyte and megakaryocyte markers.[6] We have performed a comprehensive analy-

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sis of hematopoietic G2-Gata4 lineage cells in mice. A significant fraction of the fetal and adult blood cells derives from this lineage, despite the short embryonic period in which the G2 enhancer is active. Thus, the adult hematopoietic stem cells (HSCs) would belong to two distinct mesodermal lineages, depending on whether they derive from progenitors expressing GATA4 under control of the G2 enhancer or not. This observation raises multiple questions about the embryonic origin of this lineage, the hypothetical role played by GATA4 in hematopoiesis or the existence of different properties of the adult HSCs depending on their embryonic lineage. Answers to the these questions have been the aims of the study herein, which has confirmed the existence of a long-term repopulating adult HSC population derived from the embryonic G2-*Gata4* lineage. The experimental evidence collected suggests that this hematopoietic lineage has a placental origin, but GATA4 appears dispensable for its differentiation.

Methods

Transgenic mouse lines

The animals used in our research program were handled in compliance with the institutional and European Union guidelines for animal care and welfare and housed in the animal facility of the University of Málaga under controlled standard conditions. The procedure was approved by the Committee on the Ethics of Animal Experiments of the University of Malaga (procedure code 2015-0028). Additional animals were maintained in the CABD animal care facility with the approval of the ethical committee of CSIC and the University of Pablo de Olavide. All embryos were staged from the time point of vaginal plug observation, which was designated as E0.5.

$G2^{Cre}$ and *Gata4* floxed mice were generated as previously described.[5,7] $G2^{Cre}$ mice were crossed with the reporter line $Rosa26R^{EYFP}$ (B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J). The resulting $G2^{Cre/+}$;$R26R^{EYFP}$ mice constitutively express YFP in all the lineage of the cells where the enhancer G2 has been activated. The tamoxifen-inducible *Scl*-Cre^{ERT} mouse line was generated by Dr. Joachim Göthert.[8]

Blood and tissue samples from embryos and adult mice were obtained as described in the *Online Supplementary Methods.*

RT-PCR

Bone marrow (BM) cells obtained from the femur were washed in phosphate buffered saline (PBS) and homogenized in 1mL of Tri Reagent (Sigma). Aorta-gonad-mesonephros (AGM) and fetal liver were used as positive controls. Details of reverse transcription-polymerase chain reaction (RT-PCR) and primer sequences are shown in the *Online Supplementary Methods.*

Flow cytometry

Cell suspensions were incubated with fluorochrome-conjugated antibodies for 30 min on ice. After washing, the labelled cells were analyzed in a FACSverse flow cytometer. For fluorescence activated cell sorting, the labelled cells were resuspended in 1 mL of working solution and sorted in a MoFlo cell sorter. Hoechst 33342 staining of bone marrow cells to identify the side population was performed according to standard protocols.[9] A list of the antibodies used is shown in the *Online Supplementary Table S1.*

Colony assay in MethoCult

Cells obtained from tissue homogenization were cultured in the semisolid medium MethoCult (GF M3434, Stem Cell Technologies Inc. Vancouver, Canada). Ectoplacental cones together with the allantois were obtained by fine dissection of E8.0 embryos and preincubated for 5 days in 24-well multidishes (Nunc). After 7-14 days the colonies were photographed. When the fluorescence of the cells had to be detected, the colonies were picked and adhered to a slide *via* cytospin. More details are provided in the *Online Supplementary Methods*.

Adult bone marrow cells transplant into irradiated adult mice

2,5 million bone marrow cells were suspended in 300 μL of Dulbecco's phosphate buffered saline (DPBS) +1% fetal calf serum (FCS) +1% penicillin/streptomycin (P/S) and injected in the tail vein of C57Bl/6 x CBA irradiated mice as described in the *Online Supplementary Methods.*

Adult bone marrow and embryonic cells transplant in newborn mice treated with Busulfan

Busulfan (1, 4-Butanediol dimethanesulfonate, Sigma-Aldrich) is a myeloablative agent used to improve the efficiency of a hematopoietic graft in newborn mice.[10,11] Pregnant females are injected by intraperitoneal (i.p.) injection on days 17 and 18 of gestation with a 15 mg busulfan/Kg dose. Newborns at stage P1 are injected through the facial vein with cells suspended in 50 μL of DPBS + 1% FCS + 1% P/S.[12]

Statistical analysis

Quantitative data were always expressed as mean±SEM. Statistical comparison of values was performed using the Student's *t*-test.

Results

A fraction of adult HSC derive from the G2-GATA4 embryonic lineage

We analyzed the peripheral blood (PB) and bone marrow of adult $G2^{Cre}$;$R26R^{EYFP}$ mice and investigated the presence of YFP^+ cells. After erythrocyte lysis, we found that about 20% of leukocytes ($CD45^+$), circulating monocytes ($CD11b^+$), T lymphocytes ($CD3^+$) and B lymphocytes ($B220^+$) in blood samples were YFP^+ (Figure 1A; Table 1).

In bone marrow samples, 20% of all the cells and 24% of all the $CD45^+$ cells were YFP^+. Similar percentages were found for $B220^+$, $CD11b^+$ and $CD41^+$ cells. Erythroblasts ($Ter119^+$) and lymphocytes ($CD3^+$, $CD4/CD8^+$) or lymphoid progenitors ($CD127^+$, *Online Supplementary Figure S1*) showed a lower and higher proportion of YFP^+ cells, respectively, but the differences were not significant. Similar proportions of YFP^+ cells were found in both the spleen and thymus (Table 1).

We then identified the bone marrow KSL population, i.e., cells expressing c-Kit and Sca1 but not lineage markers (Lin^-, defined as negative for CD3, Ly-6G/Ly-6C, CD11b, CD45R and Ter-119). The KSL population constitutes about 0.075±0.02% of all the bone marrow cells and includes hematopoietic stem and progenitor cells. 26,6±2,8% of the bone marrow KSL cells were YFP^+. The 1:4 proportion between YFP^+ and YFP^- cells was also observed between the $CD135/Flk2^-$ and $CD135/Flk2^+$ KSL cells, representing long-term and short-term HSCs, respectively (Table 1 and Figure 1B). Additionally we performed an experiment to estimate the percentage of YFP^+ cells within the bone marrow side population identified by Hoechst 33342 staining.[9] As shown in Figure 1C, 18.5% of this side population was YFP^+.

The presence of YFP^+ cells in the bone marrow was con-

firmed by confocal microscopy. We observed colocalization of YFP with c-Kit and also with CD44, a marker present in most hematopoietic cells (Figure 2A,B).

About 30% of the SCA1$^+$ cells from the bone marrow were YFP$^+$ (Table 1). Thus, we checked the colocalization of YFP with other mesenchymal stem cell markers. We found a relatively high percentage of YFP$^+$ cells within the CD73$^+$, CD90$^+$ and CD105$^+$ Lin negative bone marrow populations, reaching 30-40%. Furthermore, half of the Lin$^-$/Sca1$^+$/PDGFRα^+ population, enriched in mesenchymal stem cells,[13] was YFP$^+$ (*Online Supplementary Figure S2*). Thus, we cannot discard a contribution of the G2-*Gata4* cell lineage to the bone marrow mesenchymal stem cells.

Finally, we checked the expression of GATA4 in the adult bone marrow in order to disregard postnatal reactivation of the G2 enhancer. Adult bone marrow cells do not express GATA4 (Figure 1D).

G2^{Cre};R26R^{EYFP} bone marrow cells contain transplantable, long-term repopulating hematopoietic stem cells

2.5x10^6 bone marrow cells from G2^{Cre};R26R^{EYFP} mice were injected in irradiated adult recipient mice. About 20% of the injected cells were derived from the G2-*Gata4* lineage. Multilineage contribution from YFP$^+$ progenitors was determined at long-term (4 months posttransplantation) (Table 2). YFP$^+$ cells were identified in peripheral blood, bone marrow and the spleen. In lysed peripheral blood, 17.7±6.2% of all the cells were YFP$^+$. This percentage was higher in the cases of the T and B lymphocytes, reaching 25% for B220$^+$ and 28% for the CD3$^+$ population, respectively (Table 2). In bone marrow, the proportion of YFP$^+$ cells was well correlated with that found in peripheral blood, reaching 14.4±8.0% of the total cells, again with a higher proportion of CD3 lymphocytes (25%) and a lower fraction of erythroid cells (5.5%). The different contribution of YFP$^+$ cells to the CD3$^+$ and Ter119$^+$ populations was statistically significant (Student's *t*-test, *P* value=0.04).

Hematopoietic progenitors, including HSCs, have been shown to contribute to vascular endothelial cells in transplantation assays.[14] Therefore, we analyzed the distribution of YFP$^+$ cells in non-hematopoietic organs, such as the heart, kidneys, lungs and liver. Most of the YFP$^+$ cells found in these organs expressed CD45 and were found close to the walls of some vessels, probably indicating foci of extramedullary hematopoiesis. A number of endothelial (CD31$^+$/CD45$^-$) YFP$^+$ cells were localized in the heart and liver vessels, including the endocardium (Figure 2N-O). In samples of lysed peripheral blood 28.4±9.1% (n=2) of the CD45$^-$/CD31$^+$ cells were YFP$^+$ (*Online Supplementary Figure S3*). These cells could be putative circulating endothelial progenitors.

We also tested the long-term multilineage repopulation

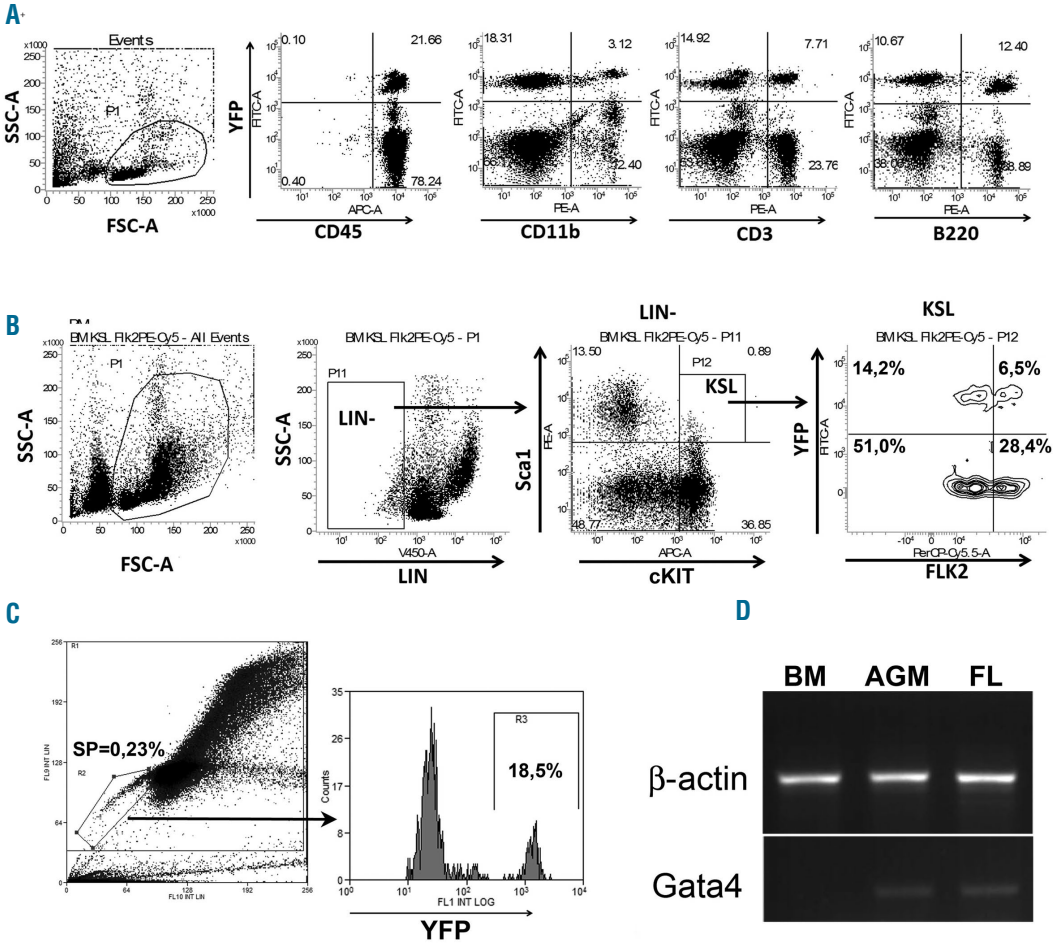

Figure 1. Analytical flow cytometry analysis of peripheral blood and bone marrow from G2^{Cre};R26R^{EYFP} mice. A: Gating strategy for the identification of different subpopulations of YFP$^+$ cells in lysed peripheral blood B: Gating strategy for the identification of c-Kit$^+$/SCA1$^+$/Lin$^-$ (KSL) hematopoietic cells in bone marrow (BM). A fraction of the KSL progenitors from bone marrow was YFP$^+$, and 80% of them were Flk2$^-$, representing long-term hematopoietic stem cells. Detailed data of these experiments are shown in Table 1. C: The side population of the bone marrow identified by Hoechst 33342 staining includes a fraction of YFP$^+$ cells. D: GATA4 is not expressed in bone marrow (BM) cells by RT-PCR. As a positive control, GATA4 expression was checked in fetal liver (FL) and aorta-gonad-mesonephros tissue (AGM). SSC-A: side scatter; FSC-A: forward scatter; FLK2: fetal liver kinase-2; SP: side population.

Table 1. Frequency of YFP$^+$ cells within different cell populations in G2^{Cre};R26R^{EYFP} mice.

Tissue		All cells	CD3	B220	CD45	CD11b	Ter119	CD127	CD41	Sca1	KSL-
Peripheral blood	Nº experiments	10	5	9	10	5					
	% total cells		19.3±3.5	52.5±1.2	83.3±5.5	21.5±2.8					
	% YFP$^+$	17.7±2.1	21.2±5.8	20.1±2.0	18.8±1.5	20.3±1.8					
Bone Marrow	Nº experiments	17	6	6	16	2	14	2	7	4	6
	% total		2.7±0.4	18.9±2.8	74.1±3.3	51,6±14,7	29.3±2.8	8.5±1.3	3.8±0.3	9.19±0.35	0.075±0.02
	% YFP$^+$	19.9±1.1	26.4±4.9	25.1±3.5	24.1±1.6	24,6±0,54	19.1±2.4	23.1±0.9	24.9±2.4	30.4±0.66	26.6±2.8
Spleen	Nº experiments	4	4	4			4	2			
	% total		26.3±0.6	52.5±8.6			24.3±9.9	15.2±1.2			
	% YFP$^+$	20.6±2.9	19.4±2.8	21.3±3.3			27.3±4.6	26.8±2.1			
Thymus	Nº experiments	4	4					2			
	% total		14.2±0.7					5.9±2.5			
	% YFP$^+$	18.9±2.9	19.2±1.9					19.8±0.8			

The first row of each tissue (% total cells) indicates the frequency of the population identified with each marker with respect to the total number of cells analyzed. The second row (% YFP$^+$) shows the proportion of YFP$^+$ cells within each population (mean±SEM). KSL: c-Kit$^+$/Sca1$^+$/Lin$^-$; Sca1: stem cell antigen 1.

Table 2. Frequency of YFP$^+$ cells after transplantation of bone marrow from G2^{Cre};R26R^{EYFP} mice into irradiated adult mice and into busulfan-treated newborns.

		Peripheral blood				Bone marrow						Thymus	Spleen
		Total	CD45	B220	T cells	Total	CD45	CD11b	T cells	Ter119	KSL	Total	Total
BM into irradiated	4 months	17.7±6,2		25.1±7.2	28.1±7.9 (CD3)	14.4±8.0	23.8±7.8	16.1±14.4	25.3±6.2[a] (CD3)	5.5±3,4[a]			23.1±6.5
BM into busulfan	1 month	17.7±1.0	17.3±1.1		7.6±1,6[b] (CD4+CD8)								
	4-9 months	16.8±3.3	17.2±3.4		14.4±2,8[b] (CD4+CD8)	14.6±2.7	15.3±2.9	17.1±3.6	14.2±2.8 (CD4+CD8)	13.4±2.6	18.6±4.5	16.0±5.6	16.2±2.2

The table shows the frequency (%) of YFP$^+$ cells in peripheral blood, bone marrow, the thymus and spleen after transplantation of bone marrow from G2^{Cre};R26R^{EYFP} mice into irradiated adults (N=4) and into busulfan-treated newborns (N=8). Data represent mean±SEM. [a]The difference found between the % of YFP$^+$ cells from the CD3 and the Ter119 populations was statistically significant (Student's *t*-test, *P* value=0.04). [b]The difference found between the % of YFP$^+$ cells in the CD4$^+$CD8 populations in the short- and long-term was statistically significant (Student's *t*-test, *P* value=0.03). BM: bone marrow; KSL: c-Kit$^+$/Sca1$^+$/Lin$^-$.

potential of bone marrow cells from G2^{Cre};R26R^{EYFP} mice by performing transplantation into myeloablated newborn mice, a model previously shown to allow hematopoietic reconstitution by early embryonic as well as adult HSC types.[15] Busulfan-treated newborn mice received 5x10^6 bone marrow cells (i.e., about 10^6 YFP$^+$ cells). Peripheral blood was analyzed one month (short-term) and 4-9 months (long-term) after the transplant (Table 2). Out of 14 mice transplanted, 8 showed YFP$^+$cells in their peripheral blood, with a mean percentage of YFP$^+$ nucleated cells of 17.7±1.0% a month after the transplantation. This proportion was similar in the long-term, and also in the bone marrow, thymus and spleen. No differences were found in the proportion of YFP$^+$/Ter119$^+$ cells as compared with other lineages. CD45$^+$ cells showed similar percentages in the long- and short-term, but YFP$^+$ T lymphocytes were less represented in the blood samples obtained in the short-term (7.6±1.6%) compared with the long-term (14.4±2.8%). This difference was statistically significant (Student's *t*-test, *P* value=0.03). In the long-term in busulfan-treated chimaeras injected with bone marrow, CD31$^+$/CD45$^-$/YFP$^+$ endothelial cells were also detected in the lungs and liver (*data not shown*).

Hematopoietic progenitors from the G2-*Gata4* lineage are mainly localized in the placenta

We performed a confocal microscopy study of the embryonic hematopoietic tissues (AGM, placenta, fetal liver) to study the distribution of the YFP$^+$ cells (Figure 2C-M). Most of the YFP$^+$ cells located close to the aorta by E10.5 are found in its dorsal part, around the notochord, and they mainly represent sclerotome cells. A smaller population of YFP$^+$ cells can also be seen in the mesentery and ventral part of the aorta, sometimes in the aortic endothelium (Figure 2C-E). Colocalization of YFP with CD31 demonstrated the endothelial nature of these cells (Figure 2C). YFP colocalizes with GATA4 protein in the mesothelium of the periaortic area, suggesting that the enhancer G2 is activating *Gata4* expression in these cells (Figure 2D). Colocalization with RUNX1 also suggests that these YFP$^+$ cells are hemogenic endothelium (Figure 2E).

In the placenta, YFP$^+$ cells are very abundant in the chorionic plate (Figure 2F-I), mainly around large vessels. Colocalization of YFP with CD41 and CD31 was frequent. Some YFP$^+$ cells were observed apparently detaching from the vascular endothelium (Figure 2G). As described for the aorta, YFP$^+$ cells also showed expression of GATA4 and RUNX1, mainly in the vascular walls (Figure 2H,I). However, YFP$^+$ cells were GATA2-negative in the placenta (Figure 2L).

Finally, colocalization of YFP and GATA4 was very frequent in the hepatic mesothelium and submesothelial areas. As previously described,[6] these cells represent mesothelial-derived cells invading the liver to contribute to the hepatic stroma (Figure 2J). Some of the YFP$^+$ cells in the fetal liver showed expression of endothelial and hematopoietic markers, including RUNX1 (Figure 2K) and GATA2 (Figure 2M).

We performed several experiments to establish the

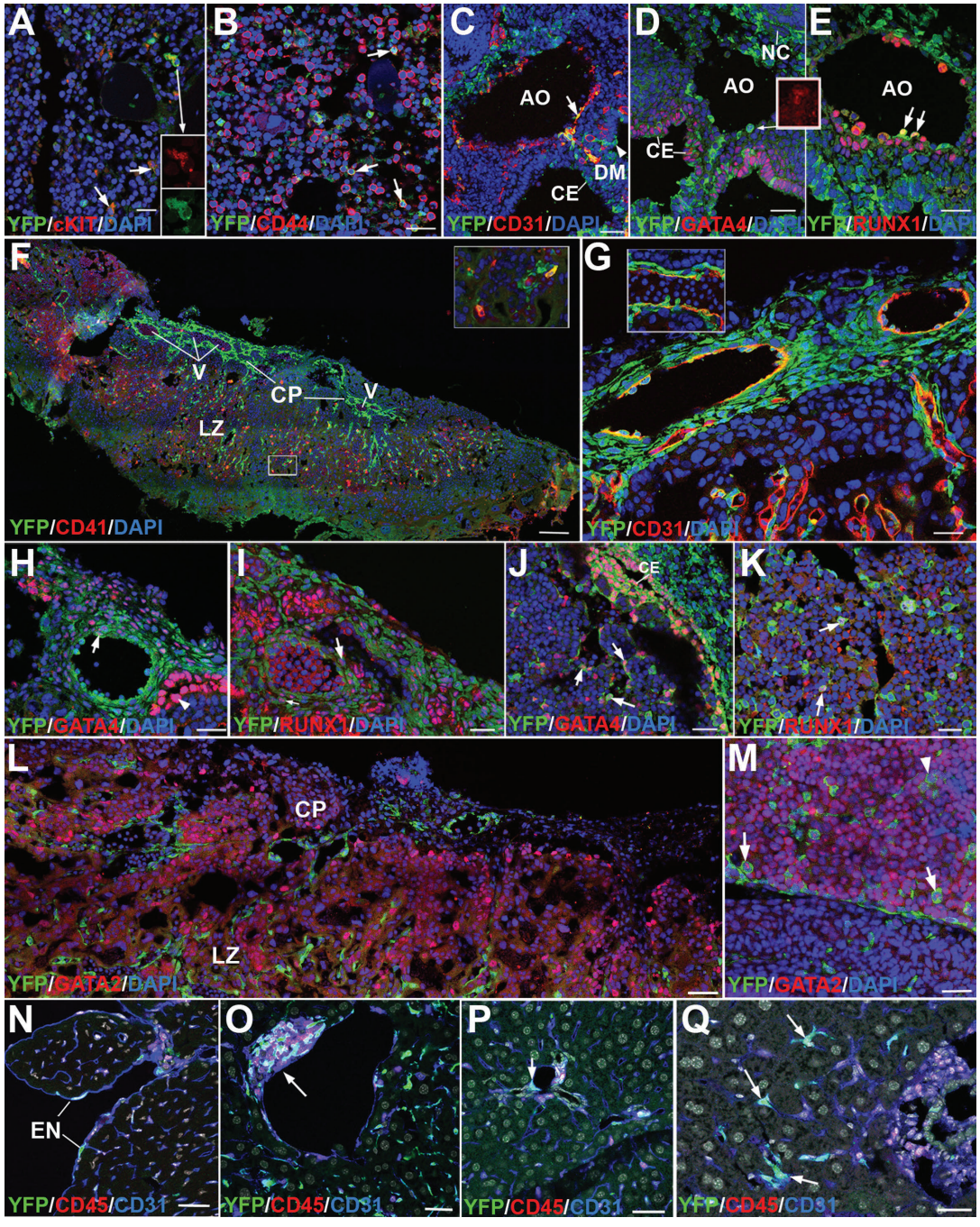

Figure 2. Localization of G2-GATA4 lineage cells (YFP$^+$) in adult and embryonic tissues by confocal microscopy. A,B: YFP$^+$ cells are localized in the bone marrow. Some of these YFP$^+$ cells express c-Kit (arrows in A, separate channels are shown in insert) and CD44 (arrows in B). C-E: YFP$^+$ cells are also localized close to the embryonic aorta (AO) by stage E10. YFP$^+$ cells are abundant around the notochord (NC) and they are also observed in the dorsal mesentery (DM) and in the adjacent coelomic epithelium (CE) (arrowhead in C). Some YFP$^+$ cells can be seen in the aorta, expressing the endothelial marker CD31 (arrow in C). Expression of GATA4 is prominent in the coelomic epithelium, suggesting activity of the enhancer G2 in specific areas of this tissue (D). Some YFP$^+$ cells of the aortic endothelium are GATA4 immunoreactive (arrow and insert in D). The hematopoietic marker RUNX1 is expressed in some YFP$^+$ cells of the aortic endothelium (arrows in E). F-I: YFP$^+$ cells are also abundant in the placenta at stage E12.5. A general view of the placenta (F) shows the distribution of the YFP$^+$ cells, more abundant in the chorionic plate (CP) and around the large vessels (V). The insert shows colocalization with the hematopoietic marker CD41 in the labyrinthine zone (LZ). YFP$^+$ cells are very abundant in the vascular walls, sometimes forming part of the CD31$^+$ endothelium of the large vessels and apparently detaching from it (insert in G). Colocalization with GATA4 and RUNX1 was observed in the vascular walls (arrows in H,I). Note the population of cells expressing GATA4 without activation of the G2 enhancer (arrowhead in H). J,K: YFP$^+$ cells were also found in the fetal liver at stages E11.5 (J) and 13.5 (K). GATA4 is expressed by coelomic epithelium (CE) and mesenchymal cells of the liver, and most of them are YFP$^+$ (arrows in J). Some YFP$^+$ cells also express the hematopoietic marker RUNX1 (arrows in K). L,M: Immunolocalization of GATA2 in the placenta (L) and liver (M) of E11.5 and E12.5 embryos, respectively. No colocalization of GATA2 and YFP is evident in the placenta, but some YFP$^+$ cells are GATA2$^+$ cells in the liver (arrows) and others are not expressing GATA2 (arrowhead). N,O: YFP$^+$ cells in the heart (N) and liver (O) of irradiated mice after four months of injections of bone marrow cells from G2^{Cre};R26R^{EYFP} mice. A number of these cells express the endothelial marker CD31 but they are negative for CD45. Some of these cells are integrated in the endocardium (EN). Clusters of hematopoietic cells appear in the wall of some hepatic sinusoids (arrow in O). P,Q: Busulfan-treated mice injected with cells obtained from G2^{Cre};R26R^{EYFP} embryos. After 4-7 months, putative endothelial CD31$^+$/CD45$^-$/YFP$^+$ cells can be seen in the liver after injection of aorta-gonad-mesonephros (P) and placental (Q) cells (arrows). Bars represent 33 μm except for F (200 μm), L (50 μm) and M (25 μm). The images were acquired as 3-channel images by a Leica SP5 II confocal microscope (Leica, Heidelberg, Germany) using LAS AF software and 40X and 63X oil immersion objectives (numerical apertures 1.35 and 1.40, respectively). Levels were adjusted for the entire images using Photoshop 8.0.1.

Table 3. Absolute and relative number of HSCs/YFP$^+$ cells found in hematopoietic embryonic tissues.

Tissue E11-5	Total number of cells/tissue (x10000) (n=5)	% of CD41$^+$ CD34$^+$cKit$^+$ cells within the P1 gate (n=4)		Estimated number of CD41$^+$ CD34$^+$ cells by organ (n=4)		% of LIN$^-$CD31$^+$cKit$^+$ cells within the P1 gate (n=3)		Estimated number of LIN$^-$CD31$^+$cKit$^+$ cells by organ (n=3)	
		YFP$^+$	YFP$^-$	YFP$^+$	YFP$^-$	YFP$^+$	YFP$^-$	YFP$^+$	YFP$^-$
Fetal liver	27.2±14.7	0.29±0.03 (n=3)	2.29±0.88 (n=3)	514±193 (n=3)	3813±996 (n=3)	0,45±0,17	12.56±1.96	1016±607	26689±4235
AGM	21±5,6	0.02±0.01	0.07±0.02	29±13	108±31	0.003±0.003	0.12±0.07	11±8	181±104
Placenta	379.2±83.2	0.90±0.21	2.55±0.64	2508±363	7151±1531	0.14±0.05	1.19±0.45	359±163	3322±2367
YS	100	0.32±0.12	0.27±0.07	1297±470	1099±197	0.13±0.07	0.83±0.47	479±208	3096±1316

The table shows the estimated absolute and relative number of YFP$^+$ and YFP$^-$ hematopoietic progenitors (defined by two criteria: cKit$^+$/CD41$^+$/CD34$^+$ and cKit$^+$/CD31$^+$/Lin$^-$) in different embryonic tissues (mean±SEM). The number of cells in the yolk sac is an estimation based on our previous work. HSCs: hematopoietic stem cells; AGM: aorta-gonad-mesonephros; YS: yolk sac.

embryonic origin of the HSCs of the G2-*Gata4* lineage. The cells used for these experiments were obtained from the fetal liver, AGM, placenta and yolk sac of E11.5 G2^{Cre};R26R^{EYFP} embryos, when definitive HSCs have already emerged.[16] We considered two criteria to identify the fetal hematopoietic progenitors: CD41$^+$/cKit$^+$/CD34$^+$ and Lin$^-$/cKit$^+$/CD31$^+$.[17,18] The four embryonic organs examined contained a population of YFP$^+$ cells in all the stages studied, but the highest absolute and relative numbers of CD41$^+$/cKit$^+$/CD34$^+$/YFP$^+$ cells were found in the placenta (Table 3 and *Online Supplementary Figure S4*). The Lin$^-$/cKit$^+$/CD31$^+$/YFP$^+$ cells were also relatively abundant in the placenta, while they were virtually absent from the AGM region, where Lin$^-$/cKit$^+$/CD31$^+$/YFP$^-$ cells were more frequent (Table 3 and *Online Supplementary Figure S4).*

We have assayed the potential of the G2^{Cre};R26R^{EYFP} cells isolated from hematopoietic tissues of E10-E11 embryos to form hematopoietic colonies in the MethoCult assay. In three independent experiments, and seeding five plates by organ (10000 cells in each experiment), the cells obtained from fetal liver exhibited the highest clonogenic ability (Table 4). 29.2% of them were YFP$^+$. Interestingly, the placenta showed a higher potential than the AGM to form colonies *in vitro*, and most (81.3%) of these placenta-derived colonies were YFP$^+$ *versus* only 20% of the AGM-derived colonies. Thus, YFP$^+$ colony forming cells are far more abundant in the placenta than in the AGM region. Furthermore, the culture of ten E8 ectoplacental cones in MethoCult also gave rise in one case to four YFP$^+$ colonies, suggesting that G2-*Gata4* hematopoietic progenitors are present in the early placenta, even before the onset of circulation.

In order to directly compare the hematopoietic potential of the G2-*Gata4* lineage cells obtained from different organs, we isolated the YFP$^+$ fraction from each organ through fluorescence-activated cell sorting (FACS, E11.5 and E12.5) and seeded the purified cells in MethoCult. The result of three experiments is shown in Table 4. Fetal liver contained the highest number of YFP$^+$ colony-forming cells, followed by the placenta. However, only a few colonies were obtained when the same numbers of YFP$^+$ cells isolated from the AGM region were cultured.

We next checked the potential of the embryonic G2-*Gata4* lineage cells to repopulate the adult hematopoietic system, using the busulfan-treated newborn mouse model. We transplanted AGM, fetal liver and placental cells from stages E11.5-E12.5, as well as purified YFP$^+$ cells sorted from the same tissues (Table 5). After four weeks we observed that the level of reconstitution of the hematopoietic system, as assessed by the percentage of YFP$^+$ cells in peripheral blood, was similar when AGM, fetal liver and placental cells were injected in the newborn mice, between 6.7% (placenta) and 11.0% (fetal liver). This percentage reached 19.2% in the AGM-injected chimaeras after 4-9 months, but the increase was small or absent in the chimaeras injected with placental or liver cells. The percentages of YFP$^+$ cells in the bone marrow, thymus and spleen were similar in all the experiments.

A significant difference between the short-term and long-term reconstitution of the T-lymphoid compartment was observed (Table 5), as described above for the busulfan-treated mice injected with adult bone marrow. The percentage of YFP$^+$ T lymphocytes increased between the short- and long-term independently of the origin of the cells. Considering all the experiments, the frequency of the YFP$^+$ lymphocytes increased three times, from 4% to 12% (Student's *t*-test, *P* value=0.015), while the increase of the CD45$^+$/YFP$^+$ cells was not significant.

When busulfan-treated newborns were injected with YFP$^+$ cells purified by FACS from the three embryonic tissues, the fetal liver showed the highest ability to reconstitute the hematopoietic system, followed again by the placental cells (Table 6). YFP$^+$ cells isolated from the AGM region showed a very weak ability to reconstitute the hematopoietic system in these mice, never reaching >1% of the nucleated blood cells. Thus, YFP$^+$ cells from the placenta showed a much higher short- and long-term reconstitution potential than YFP$^+$ cells isolated from the AGM region. In these mice, we again observed a statistically significant difference between short- and long-term percentages of lymphoid YFP$^+$ cells (Table 6).

We also analyzed the contribution of YFP$^+$ cells to non-hematopoietic organs in the long-term busulfan-treated chimaeras injected with embryonic tissues. Endothelial cells of the G2-Gata4 lineage can be seen in many organs, and they were more abundant in the liver sinusoids (Figure 2P,Q).

GATA4 is not directly involved in the differentiation of hematopoietic progenitors

To ascertain if GATA4 is required for embryonic hematopoiesis we have studied the conditional deletion of GATA4 in Scl-expressing cells (SCLCre^{ERT};Gata4$^{flox/flox}$). When GATA4 is deleted in the cells of the G2-*Gata4* lineage, mice develop anemia.[6] We inactivated GATA4 in cells of the SCL lineage through tamoxifen induction at different stages (E9.5-E11.5), but no differences were observed in the fetal hematopoiesis at different ages (E12.5-E14.5) (*Online*

Table 4. Number of colonies generated in MethoCult after seeding of embryonic cells from different origins.

Type of cells	Tissue	Stage	Number of colonies	Analyzed by cytospin	YFP+ colonies
Total	Fetal liver	E10-11	88.4±4.8	65	19 (29,2%)
Total	AGM	E10-11	1.7±0.89	10	2 (20%)
Total	Placenta	E10-11	3.4±1.0	16	13 (81,3%)
YFP$^+$ purified	Fetal liver	E11,5	55.9±7.3		
YFP$^+$ purified	Fetal liver	E12,5	44.3±6.7		
YFP$^+$ purified	AGM	E11,5	0.33±0.12		
YFP$^+$ purified	AGM	E12,5	0.1±0.058		
YFP$^+$ purified	Placenta	E11,5	5.7±0.64		
YFP$^+$ purified	Placenta	E12,5	1.0±0.38		

The number of colonies generated in MethoCult after seeding of cells obtained from three hematopoietic tissues is presented. 10000 total cells were obtained from the tissues (E10-11, N=5) or 2000-14000 YFP$^+$ cells were purified for each tissue (N=3, results are normalized to 1000 cells). In both cases the cells were seeded and the number of colonies counted. In the case of total cells seeded, the frequency of the YFP$^+$ colonies was analyzed by cytospin (mean±SEM). AGM: aorta-gonad-mesonephros.

Table 5. Frequency of YFP$^+$ cells after transplant of embryonic cells in busulfan-treated newborns.

Analyzed tissue	Donor tissue	Term	All cells	CD45	CD4+CD8[a]	Ter119	KSL
Peripheral blood	Fetal liver	ST	11.0±2.1 (2)	10.7±2.3 (2)	5.1±0.78 (2)		
	AGM	ST	8.4±0.57 (6)	10.1±1.4 (5)	1.8±0.61 (6)		
	Placenta	ST	6.7±0.45 (4)	6.5±0.6 (4)	5.3±2.9 (4)		
	Fetal liver	LT	10.7±4.5 (2)	10.8±4.5 (2)	11.6±4.7 (2)		
	AGM	LT	19.2±4.5 (6)	18.4±6.2 (6)	14.1±4.0 (6)		
	Placenta	LT	8.2±3.6 (4)	8.5±3.8 (4)	7.9±5.5 (4)		
Bone marrow	Fetal liver	LT	10.7±7.4 (2)	11.0±10.5 (2)	27.6 (1)[b]	10.5±11.3 (2)	11.7±9.7 (2)
	AGM	LT	16.7±6.4 (6)	22.8±24.6 (6)	17.2±18.9 (4)[b]	17.1±10.7 (6)	16.8±6.1 (6)
	Placenta	LT	18.5±17.1 (4)	18.6±33.9 (4)	24.3±30.4 (2)[b]	18.5±32.0 (4)	25.4±21.1 (4)
Thymus	Fetal liver	LT	26.6 (1)				
	AGM	LT	29.4±16.4 (4)				
	Placenta	LT	20.1±18.9 (2)				
Spleen	Fetal liver	LT	17.0 (1)				
	AGM	LT	16.3±7.4 (4)				
	Placenta	LT	17.5±8.3 (2)				

The table shows the frequency of YFP$^+$ cells (mean±SEM) in peripheral blood in the short- and long-term (1 and 4-9 months, respectively), bone marrow, the thymus and spleen (only long-term) after transplantation of cells from G2^{Cre};R26R^{EYFP} embryos into busulfan-treated newborns. The number of experiments is between parentheses. [a]The difference found between the % of YFP$^+$ cells in the CD4$^+$CD8 populations in the short- and long-term was statistically significant when the experiments were gathered (ST: 4.1±1.2%, LT: 11.8±2.6%, Student's t-test, *P* value=0.015). [b]CD3 was used instead of CD4$^+$CD8 in these samples. AGM: aorta-gonad-mesonephros; ST: short-term; LT: long-term; KSL: c-Kit$^+$/Sca1$^+$/Lin$^-$.

Supplementary Table S2).

Discussion

Early embryonic hematopoiesis occurs in three main territories; the yolk sac, AGM region and placenta. It has been well established that the hematopoietic progenitors originated in the yolk sac give rise to a transient wave of blood cells, mainly erythroid. Moreover, definitive HSCs are considered to derive from the AGM region, since the role played by the placenta in definitive hematopoiesis is more controversial, as discussed below. The lack of markers allowing one to distinguish between placental and intraembryonic HSCs has hampered the solving of this controversy.

We have identified two distinct lineages in the adult blood cells of mice, characterized by the activity, during embryonic life, of an enhancer of the *Gata4* gene (G2-*Gata4*) that drives its expression in the allantois/placenta and lateral mesoderm, starting at E7.5 and ceasing by midgestation. About 20% of the murine adult blood cells belong to this lineage, and this percentage shows a remarkable consistency between individuals. The G2-*Gata4* lineage of blood cells derive from bone marrow HSCs which can be transplanted and reconstitute, over the long-term, the hematopoietic system of both lethally irradiated and busulfan-treated mice. The HSCs belonging to the G2-*Gata4* lineage give rise to all kinds of blood cells and even to putative circulating endothelial progenitors and long-term engrafted vascular endothelial cells, and they show basically the same properties as the other HSCs. An exception was the lower percentages of YFP$^+$ T lymphocytes found in the short-term reconstitution of the hematopoietic system after myeloablation. This difference might be explained by the persistence of the host lymphocytes after the transplantation. T lymphocytes half-life in humans span for weeks,[19] thus, host lymphocytes of myeloablated mice are probably diluting the YFP$^+$ lymphocytes in the short-term and reducing their relative abundances. On the other hand, the smaller frequency of YFP$^+$ cells found among the Ter119$^+$ bone marrow population can be explained by downregulation of the expression of YFP as the erythroblasts differentiate.

The existence of heterogeneity among populations of

Table 6. Frequency of YFP⁺ cells after transplant of YFP⁺ cells purified from embryonic tissues in busulfan-treated newborns.

Donor tissue and number of YFP⁺ cells transplanted	Number of transplants	Survivors	Mice with >1% YFP⁺ ST	Mice with >1% YFP⁺ LT	Lysated PB								Bone marrow	Thymus	Spleen
					All cells		CD4+CD8[a]		CD45		B220	CD11[b]	All cells	All cells	All cells
					ST	LT	ST	LT	ST	LT	LT	LT	LT	LT	LT
Fetal liver (20000 cells)	4	4	2	2	26.0± 4.1	55,6± 12.7	19.6± 6.6	70.9± 4.1	31.7± 6.0	55.6± 5.0	68.1± 4.7	47.8± 9.1	60.3± 10.6	90.8± 0.78	76.4± 2.8
AGM (10000-25000 cells)	6	4[b]	0	0	-	-	-	-	-	-	-	-	-	-	-
Placenta (13000-25000 cells)	9	7	4	3	19.9± 10.3	43.5± 26.1	7.7± 4.7	40.9± 24.6	19.8± 10.6	42.9± 25.6	46.8± 28.8	54.3± 33.2	43.6± 23.5	62.6± 31.3	47.7± 20.9

The table shows the frequency of YFP⁺ cells (mean±SEM) in peripheral blood in the short- and long-term (ST and LT, 1 and 4-9 months, respectively), bone marrow, the thymus and spleen after transplantation into busulfan-treated newborns of YFP⁺ cells purified from tissues from G2^{Cre};R26R^{EYFP} embryos. [a]The difference found between the % of YFP⁺ cells in the CD4⁺CD8 populations in the short- and long-term was statistically significant when the experiments were gathered (ST: 12.4±4.1%, LT: 52.9±13.3%, Student's *t*-test, *P* value=0.036). [b]The percentages of YFP⁺ cells found in these mice were between 0.01% and 0.47%. AGM: aorta-gonad-mesonephros; ST: short-term; LT: long-term; PB: peripheral blood.

adult HSCs has been well established, but their causes and their potential relationships with different pathways of developmental hematopoiesis are unknown.[20-23] As acknowledged by Kaimakis *et al.*, the hematopoietic progenitors generated in the embryo result as being more diverse than previously appreciated.[24] These authors described a population of embryonic hematopoietic progenitors cells (HPCs) that did not express GATA2, and they suggested that GATA4 is expressed and might play some role in this specific population. This proposal might be related with our observation of a lineage of HSCs derived from GATA4 expressing progenitors. In fact, we found no expression of GATA2 in the YFP⁺ cells from the placenta, although part of the fetal liver YFP⁺ cells were GATA2⁺ (Figure 2L,M). Thus, we do not disregard that the G2-*Gata4* hematopoietic lineage can overlap totally or partially with the already described GATA2-independent population of HPCs.[24]

An important point revealed in our study is the embryonic origin of the G2-*Gata4* HSCs. The G2 enhancer activates GATA4 expression in hematopoietic cells from both the placenta and AGM, although YFP⁺ cells were far more abundant in the placenta. The yolk sac also showed a significant population of presumptive YFP⁺ hematopoietic progenitors, but they probably do not originate in the yolk sac, since the enhancer G2 is not driving GATA4 expression in that tissue.[5] In fact, conditional deletion of GATA4 in the G2 domain does not cause abnormalities in the yolk sac.[6] Thus, we compared the number of YFP⁺ hematopoietic progenitors identified under two different criteria, (CD41⁺/cKit⁺/CD34⁺ and Lin⁻/CD31⁺/cKit⁺),[17,18] in the placenta and in the AGM, and they were much more abundant in the former. In fact, Lin-/CD31⁺/cKit⁺/YFP⁺ cells were virtually lacking in the AGM region, whereas a significant number of Lin⁻/CD31⁺/cKit⁺/YFP⁻ cells were identified. YFP⁺ colonies were four times more frequent in MethoCult when we seeded placental cells than when AGM cells were seeded. When unfractionated AGM and placental cells were transplanted into busulfan-treated newborn mice, the contribution to the YFP⁺ blood cells was basically similar. Interestingly, we recorded a two-fold increase of the AGM contribution to the reconstituted YFP⁺ population in peripheral blood in the long-term as compared with the short-term, although this increase was not significant and it was not evident in bone marrow. Furthermore, YFP⁺ cells from the placenta, but not those isolated from AGM, reconstituted the hematopoietic system of busulfan-treated newborns. We cannot exclude that YFP⁺ cells from the AGM require accessory cells for engraftment and/or a process of maturation and expansion to acquire a potential of reconstitution similar to that exhibited by the placental YFP⁺ cells.[25] However, we think that our experimental evidence clearly supports a placental origin for most YFP⁺ hematopoietic progenitors, and presumably also for most of the adult HSCs derived from the G2-*Gata4* lineage.

Placental hematopoiesis has been well described,[16,26-27] although the primary origin of placental HSCs is controversial.[28] The allantois from embryos E8 pre-cultured *in toto* prior to seeding in a semisolid medium has demonstrated containing hematopoietic potential.[29] Using this experimental system, we performed the MethoCult assay with ectoplacental cones and allantois by E8, before the onset of the circulation, and have obtained YFP⁺ colonies in one case. This supports the ability of the placenta to generate definitive HSCs, as suggested by other reports.[30]

To date, there is no report of GATA4 being involved in hematopoiesis. We previously suggested that the anemia phenotype found in mouse embryos with conditional deletion of GATA4 in the lateral mesoderm domain was due to the failure of the expansion of hematopoietic progenitors in the liver, rather than from a direct impact of a lack of GATA4 in hematopoietic cells.[6] Our new results confirm this idea, as no hematopoietic defects were found when we inactivated *Gata4* either in the G2-GATA4 lineage, or in the hematopoietic progenitors using an inducible SclCreERT driver. However, redundancy among members of the GATA family have been previously described,[31-34] and we have discussed above how Kaimakis *et al.* do not disregard some redundancy between GATA4 and GATA2 in a subpopulation of embryonic hematopoietic progenitors cells.[24]

The embryonic origin of the different populations of bone marrow mesenchymal stem cells is still poorly known.[35,36] This was not an aim of our study, but we observed a contribution of YFP⁺ cells to the lineage-negative bone marrow cells within the CD90⁺, CD73⁺ and CD105⁺ subpopulations, classical markers of mesenchymal stem cells. This contribution was higher (30-40%) than that obtained for hematopoietic stem cells. Furthermore, 50% of the lineage-negative SCA1⁺/PDGFRα⁺ population, that is

enriched in mesenchymal stem cells,[13] was also YFP+. Further study would be necessary to establish the relevance of these observations, possibly related to the origin of a mesenchymal stem cell subpopulation from the lateral mesoderm.

In summary, we have identified a distinct lineage of adult HSCs characterized by its derivation of progenitors where Gata4 expression is activated by a specific enhancer. Most adult HSCs belonging to this lineage probably originate in the placenta. Despite the relatively normal behavior of this specific lineage, further experiments would be required to examine whether they exhibit differences in response to different physiopathological conditions.

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