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# Immature hematopoietic stem cells undergo maturation in the fetal liver

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## SUMMARY

Hematopoietic stem cells (HSCs), which are defined by their capacity to reconstitute adult conventional mice, are first found in the dorsal aorta after 10.5 days post coitus (dpc) and in the fetal liver at 11 dpc. However, lympho-myeloid hematopoietic progenitors are detected in the dorsal aorta from 9 dpc, raising the issue of their role in establishing adult hematopoiesis. Here, we show that these progenitors are endowed with long-term reconstitution capacity, but only engraft natural killer (NK)-deficient *Rag2 $\gamma$ <sup>−/−</sup>* mice. This novel population, called here immature HSCs, evolves in culture with thrombopoietin and stromal cells, into HSCs, defined by acquisition of CD45 and MHC-1 expression and by the capacity to reconstitute NK-competent mice. This evolution occurs during ontogeny, as early colonization of fetal liver by immature HSCs precedes that of HSCs. Moreover, organ culture experiments show that immature HSCs acquire, in this environment, the features of HSCs.

**KEY WORDS:** MHC class I, Fetal liver, Hematopoietic reconstitution, Hematopoietic stem cells

## INTRODUCTION

In the mouse embryo, the first hematopoietic progenitors, found in the yolk sac (YS) starting at 7 days post-coitus (dpc), rapidly provide the embryo with erythro-myeloid cells. The first hematopoietic stem cells (HSCs) defined by their ability to display long-term reconstitution (LTR) activity in conventional adult irradiated mice, have been first identified after 10.5 dpc (precisely after the 34- to 35-somite stage), in the embryonic structure comprising the aorta, gonads and mesonephros (AGM) (Müller et al., 1994; Medvinsky and Dzierzak, 1996). These HSCs are detected in the fetal liver (FL) at later stages [11 dpc to 42 somites (S)] (Müller et al., 1994; Sánchez et al., 1996).

At earlier developmental stages (from 9 dpc, corresponding to 15–20S), multipotent hematopoietic progenitors, defined by single cell assays in vitro, are detected in the para-aortic splanchnopleura (P-Sp), an intra-embryonic structure that later evolves into the AGM region (Godin et al., 1995; Godin et al., 1999). These multipotent progenitors reach their maximum numbers in the AGM at the 35S stage. These progenitors, of intra-embryonic origin (Cumano et al., 1996; Cumano et al., 2001), were phenotypically characterized by the expression of high levels of Kit, medium levels of CD41, low to undetectable expression of CD45 and a combination of markers also present in endothelial cells, such as CD31, CD34 and AA4.1. Moreover, these progenitors, isolated from the P-Sp/AGM at 10 dpc (30–35S), are capable of LTR activity when transferred into natural killer (NK)-deficient *Rag2 $\gamma$ <sup>−/−</sup>* mice (Bertrand et al., 2005).

In contrast to the AGM, where hematopoietic progenitors are generated in situ, the FL relies on the colonization by exogenous progenitors to initiate hematopoiesis. The onset of FL colonization has been staged at 10 dpc, precisely at the 28–32S stages (Houssaint, 1981). The colonizing cells detected at this stage are considered as erythromyeloid cells from the YS, because, as mentioned previously, AGM-derived HSCs endowed with LTR activity in conventional recipient, are only detected in the FL after 11 dpc.

Experimental data suggest that the multipotent hematopoietic progenitors detected in the P-Sp/AGM prior to AGM-HSC production might contribute to definitive hematopoiesis in the adult: in vivo experiments, performed to trace the onset of HSC development, used the inducible Cre/lox system controlled by Runx1 or VE-Cadherin regulatory sequences to induce Cre recombinase, leading to a persisting expression of  $\beta$ -galactosidase ( $\beta$ -gal) (Samokhvalov et al., 2007; Zovein et al., 2008). Induction starting at 9.5 dpc and during the next few hours, resulted in  $\beta$ -gal expression in virtually all adult HSCs. Importantly, induction performed before 9.5 dpc resulted in less than 10% of  $\beta$ -gal expressing adult Lin<sup>−</sup>Sca-1<sup>+</sup>Kit<sup>+</sup> (LSK) cells. This observation indicates that all adult HSC progenitors derive from 9.5–10 dpc progenitors expressing high levels of Runx1 (or VE-Cadherin) and raises the issue of its relationship to multipotent hematopoietic progenitors found in the P-Sp at this stage. These data suggest that, before the appearance of the first HSC in the embryo, the multipotent progenitors detected at 9–9.5 dpc can contribute to the definitive hematopoiesis in the adult.

We thus tested the possibility that these multipotent progenitors also present a LTR ability that is restricted to *Rag2 $\gamma$ <sup>−/−</sup>* recipients, like P-Sp/AGM cells at 10 dpc, and that these progenitors further evolve into HSCs.

We show that the first progenitors endowed with LTR activity in NK-deficient mice, called here immature HSCs (imHSCs), are detected in the P-Sp as soon as 9 dpc. However, their transfer into irradiated recipients results in a hematopoietic chimerism lower than that of HSCs. This low hematopoietic chimerism neither results from an inability to home in the bone marrow nor from an

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inadequate response to the proliferative signals from the bone marrow environment, but rather from the low frequency of cells that efficiently integrate the HSC compartment. Using a culture in the presence of thrombopoietin (TPO), we demonstrate that imHSCs from 10 dpc P-Sp/AGM are capable of evolving into HSCs, as defined by phenotype and by the ability to reconstitute hematopoiesis of NK-competent mice. This effect results, in part, from the inhibition of host NK cell activity.

Our results also bring in a new kinetics of FL development. We provide evidence that FL colonization by YS-derived hematopoietic cells occurs as soon as 9 dpc (20S) and that multipotent progenitors/imHSCs colonize the FL starting at 10 dpc (30S), shortly after being generated in the P-Sp/AGM. We further show, using organ culture of 10 dpc FL rudiment, that imHSCs give rise to HSCs.

Taken together, the data presented here define a novel stage in HSC development, the imHSC stage, which is characterized by phenotype, by multipotent hematopoietic potential and by the capacity to reconstitute hematopoiesis in NK-deficient mice. Shortly after their emergence, they reach the FL through circulation where the maturation events that drive the acquisition of HSC properties occur.

## MATERIALS AND METHODS

### Dissection of embryonic structures

The two C57BL/6 congenic lines bearing the Ly5.2 and Ly5.1 alleles of the pan-hematopoietic marker CD45 and their F1 progeny were used in this study. Two strains of immunodeficient mice, *Rag2<sup>-/-</sup>* (Shinkai et al., 1992) and *Rag2<sup>γc</sup><sup>-/-</sup>* (Colucci et al., 1999), bearing the Ly5.2 or Ly5.1 alleles, respectively, were used as recipients in the reconstitution experiments.

The day of vaginal plug observation was designated as day 0.5. Each embryo was staged by somite counting or by development of the limb buds. Dissections of 9–11 dpc P-Sp/AGM and FL were carried out as previously described (Godin et al., 1999). The staging terminology used throughout this study was: P-Sp, 9–9.5 dpc, 15–30S; P-Sp/AGM, 10 dpc, 30–35S; AGM, over 10.5 dpc, 35S.

### Culture conditions

#### imHSC culture

One hundred to 450 CD31<sup>+</sup>Kit<sup>+</sup>CD45<sup>low</sup>Mac-1<sup>low</sup> cells purified from the P-Sp/AGM were cultured for 7 days on an OP9 monolayer in 96-well plates in OptiMEM (Invitrogen), 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 × 10<sup>-5</sup> M 2-mercaptoethanol without cytokine or supplemented with TPO (20 ng/ml), Ang-3 (200 ng/ml) (R&D Systems), IL3 (supernatant of a cell line transfected with the cDNA encoding IL3, a kind gift from F. Melchers, Basel, Switzerland) or Kit ligand. Cells were re-fed after 4 days with fresh medium. At the end of the culture, cells were re-suspended by gentle pipetting.

#### Organ culture

Whole FLs were individually transferred onto polycarbonate filters (0.8 µm; Millipore) floating on culture medium (OptiMEM, 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 × 10<sup>-5</sup> M 2-mercaptoethanol) in wells of six-well plates and cultured for 3 days. Single-cell suspensions were then obtained from five pooled explants by passage through a 26-gauge needle. P-Sp explants were similarly maintained in organ culture, except that they were placed directly in the culture medium instead of on top of a filter.

#### B lymphoid culture

Cells from individual FL were cultured for 14 days on OP9 stromal cells in OptiMEM, 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 × 10<sup>-5</sup> M 2-mercaptoethanol supplemented with IL7, Kit ligand and Flt3 ligand, provided by the supernatant of a stably transfected cell line (from F. Melchers). Under these conditions, myeloid differentiation also occurs.

### Flow cytometry analysis and cell sorting

Analyses were performed in a FACS Canto II running with the FACS Diva software (Becton Dickinson). Dead cells were excluded in all analysis using propidium iodide. The following antibodies, from BD Pharmingen except when mentioned otherwise, were used: anti-CD31-PE, anti-Kit-APC, anti-CD45-FITC, anti-Mac-1-FITC, anti-CD45.1-FITC, anti-CD45.2-FITC, anti-CD19-PE-Cy7, anti-Gr1-APC, anti-CD4-PE-Cy7 (eBioscience), anti-CD8-APC, anti-Sca-1-FITC, anti-Kit-APC-Alexa Fluor 750, anti-CD150-PE (Biolegend), anti-CD48-APC (eBioscience), anti-MHC-1-biotin and streptavidin-PE-Cy7. Lin<sup>-</sup> cells were stained with a biotinylated Lin<sup>+</sup> antibody cocktail (anti-CD4, anti-CD8, anti-NK1.1, anti-CD11c, anti-B220, anti-CD19, anti-Gr1 and anti-Ter119) followed by streptavidin-PE-Cy5. Intracellular Ki67 expression was analyzed using anti-Ki67 FITC (BD Pharmingen) after use the Foxp3 Staining Set (eBioscience). Cell sorting was performed in a MoFlo cell sorter running with the Summit software (Cytomation, Ft Collins, CO, USA).

### Reconstitution experiments

Cells from F1 embryos (C57BL/6 CD45.1xCD45.2) were injected intravenously in the retro-orbital sinus or directly in the left femur of sublethally irradiated (600–800 rad, Cesium source) *Rag2<sup>γc</sup><sup>-/-</sup>* recipient mice. Reconstitution of sublethally irradiated *Rag2<sup>-/-</sup>* or lethally irradiated C57BL/6 (Cesium source, 6 and 9.5 Gy, respectively) recipient was similarly performed. C57BL/6 recipients also received 10<sup>5</sup> total bone marrow (BM) cells. In long-term reconstitution assays, donor-derived contribution was followed in peripheral blood cells, and after 5 to 6 months, the recipient mice were sacrificed and the BM, spleen and thymus were analyzed by flow cytometry. Mice were considered reconstituted when the % donor-derived granulocytes in BM was at least 0.5.

### Gene expression analysis

Total RNA was isolated using the RNeasy Micro Kit (Qiagen). cDNA was prepared from total RNA using avian myeloblastosis virus reverse transcriptase (RT) under conditions recommended by the manufacturer (Invitrogen).

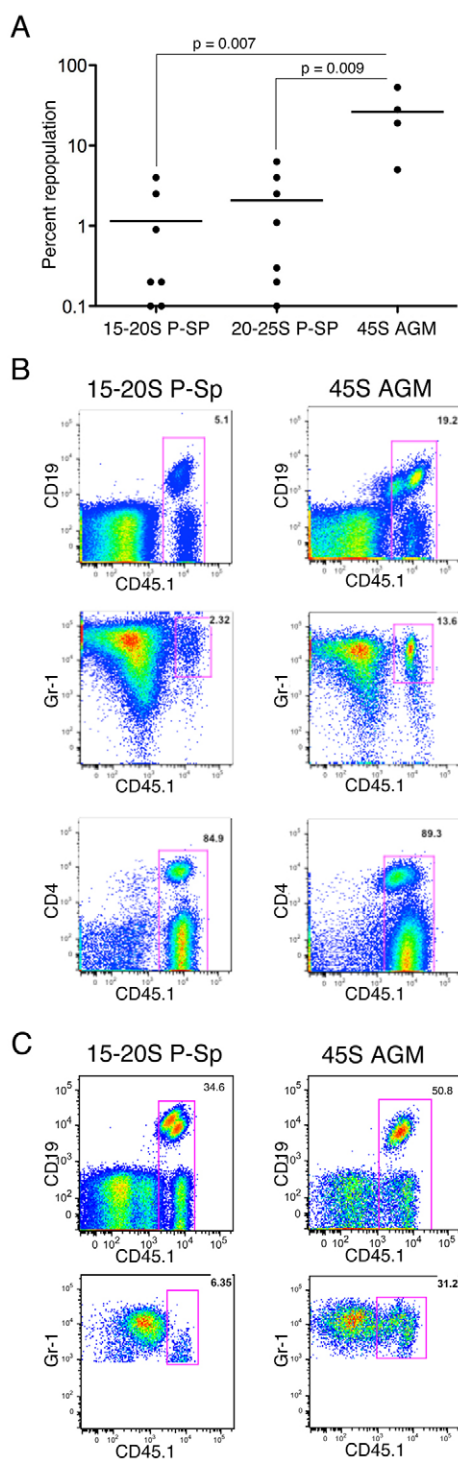
A quantitative real-time PCR (RT-PCR) was then performed on cDNAs samples to evaluate the mRNA levels of Mpl and Hprt. All samples were analyzed in triplicate using TaqMan gene expression assays (Applied Biosystems). Hprt was used as an endogenous control. Thermal cycler conditions were 50°C for 2 minutes, 95°C for 10 minutes, and 45 cycles of 95°C for 0.15 minutes and 60°C for 1 minute. Data were analyzed with the Sequence Detection System software version 1.6.3 (Applied Biosystems).

## RESULTS

### Onset of immature HSC activity in the dorsal aorta

Previous experiments established that HSCs, defined by LTR activity in conventional irradiated mice, are detected in the AGM, after 10.5 dpc (34–35S) (Müller et al., 1994; Medvinsky and Dzierzak, 1996). However, we previously observed (Bertrand et al., 2005) that the P-Sp/AGM isolated prior to this stage contains multipotent hematopoietic progenitors displaying LTR activity in *Rag2<sup>γc</sup><sup>-/-</sup>* but not *Rag2<sup>-/-</sup>* or conventional recipients. These hematopoietic progenitors are designated throughout this report as imHSCs, in contrast to HSCs, which are able to reconstitute hematopoiesis of recipient mice displaying NK activity.

We first determined in a reconstitution assay the stage of appearance of imHSCs in the P-Sp, the embryonic territory that later develops into the AGM. The LTR potential of cells isolated from 15–20S and 20–25S P-Sp was analyzed by intra-femoral transplantation into *Rag2<sup>γc</sup><sup>-/-</sup>* mice. At 9–9.5 dpc, the P-Sp contains a limited number of multipotent progenitors comprising most of the Kit<sup>+</sup>CD31<sup>+</sup> subset (Bertrand et al., 2005). We quantified Kit<sup>+</sup>CD31<sup>+</sup> cells at the three stages analyzed (supplementary material Fig. S1) and injected an equivalent number of multipotent progenitors from 9–9.5 dpc P-Sp and 11 dpc AGM. Three out of



seven and four out of nine *Rag2* $\gamma^{-/-}$  recipients injected with 9 (15-20S) or 9.5 dpc (20-25S) P-Sp, respectively, showed long-term multilineage hematopoietic reconstitution. The myeloid chimerism in the BM ranged from 0.9-6.2% in host reconstituted with 9-9.5 dpc P-Sp cells and 5-53% when AGM cells from 11 dpc (>45S) embryos were used for transplantation (Fig. 1A). Fig. 1B shows myeloid and lymphoid reconstitution in the BM of *Rag2* $\gamma^{-/-}$  mice that received P-Sp and AGM cells. All positive mice also displayed donor-derived B lineage (CD19 $^{+}$ ) cells in the BM, T lymphocytes in the spleen and BM, and myeloid cells in the blood 12 weeks

**Fig. 1. imHSC emergence in the P-Sp.** (A) CD45.1 $^{+}$  cells derived from 9 dpc (15-20S) P-Sp (12 ee/recipient), 9.5 dpc (20-25S) P-Sp (10 ee/recipient) or 11 dpc (45S) AGM (3 ee/recipient) were injected in the femur of CD45.2 $^{+}$  *Rag2* $\gamma^{-/-}$  recipients. Engraftment was analyzed 5-6 months after transplantation. Chimerism was expressed as the frequency of donor derived (CD45.1 $^{+}$ ) myeloid cells (Gr-1 $^{+}$ ) in the host BM. Data cumulate three and two independent experiments for P-Sp and AGM, respectively. (B) Flow cytometry analysis of donor-derived (CD45.1) B cells (top panels) and myeloid (middle panels) cells in the BM of *Rag2* $\gamma^{-/-}$  mice 5-6 months after transfer of 15-20S P-Sp (left profiles) and 45S AGM (right profiles). T cell chimerism in the spleen is shown in the bottom panels. (C) Flow cytometry analysis of B cells (upper panels) and myeloid cells (lower panels) in the blood of the two animals shown in B, 12 weeks after transplantation of 15-20S P-Sp (left profiles) and 45S AGM (right profiles).

after post-transplantation (Fig. 1C). The injection of an equivalent number of multipotent progenitors derived from 9-9.5 dpc P-Sp (15-25S) resulted in a chimerism significantly lower than that obtained from 11 dpc AGM (Fig. 1A).

We next determined whether imHSCs (30-35S P-Sp/AGM) and HSCs (45S AGM) could equally integrate the BM stem cell pool and proliferate after transplantation. We found a significantly higher frequency of donor LSK when mice were injected with 45S than with 30-35S P-Sp/AGM multipotent progenitors (supplementary material Fig. S2A), although they exhibited a similar frequency of cells in cycle, as shown by Ki67 staining (supplementary material Fig. S2B). As a control, we determined the profile of Ki67 expression in freshly isolated cells. imHSCs (30-35S P-Sp/AGM) and HSCs (45S AGM) showed a similar frequency to Ki67 $^{+}$  cells, which was consistently higher than that of BM HSC frequency (supplementary material Fig. S3A).

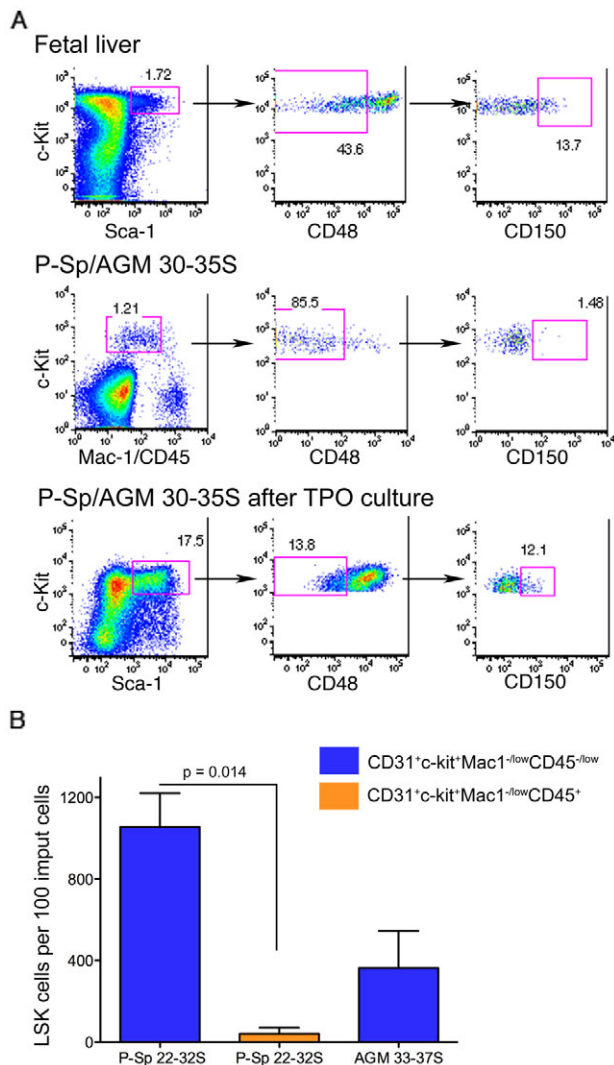
These results establish that imHSCs appear in the P-Sp at 9 dpc (15-20S), therefore at the same stage when multipotent progenitors are first detected. After transplantation, imHSCs (<35S P-Sp/AGM) are capable of long-term multilineage reconstitution, although they are less efficient than HSCs (after 45S AGM) at reconstituting the hematopoietic compartment of adult BM.

### Immature HSCs adopt a HSC phenotype and LTR activity in NK-competent recipients

HSCs appear in the AGM one and a half days later in development than do imHSCs. To test the possibility that imHSCs can give rise to HSCs, we defined culture conditions that allow the conversion of imHSCs into HSCs.

FL HSCs are defined as LSKCD150 $^{+}$ CD48 $^{-}$  (Kiel et al., 2005; Kim et al., 2006) (Fig. 2A, upper panel). By contrast, 20-35S P-Sp/AGM multipotent progenitors, identified by their CD31 $^{+}$ Kit $^{+}$ CD45 $^{-/low}$ Mac-1 $^{-/low}$  phenotype (Bertrand et al., 2005), express undetectable levels of CD150 while most cells are CD48 $^{-}$  (Fig. 2A, middle panel). We cultured 100 imHSCs (CD31 $^{+}$ Kit $^{+}$ CD45 $^{-/low}$ Mac-1 $^{-/low}$ ) from 30-35S P-Sp/AGM in the presence of OP9 stromal cells and various cytokine combinations. Whereas freshly isolated 20-35S Kit $^{+}$  cells have low frequency of Sca-1 $^{+}$  cells (supplementary material Fig. S4A), a well-defined compartment of CD45 $^{+}$ Kit $^{+}$ Sca-1 $^{+}$  cells was detected after culture (Fig. 2A, lower panel, supplementary material Fig. S4A,C; Fig. S5A). The highest yield of cells with the LSK CD150 $^{+}$  phenotype (Fig. 2A, lower panel) was obtained when thrombopoietin (TPO) was added to OP9 cells for 7 days. ImHSCs express similar levels





**Fig. 2. imHSCs acquire a mature phenotype after culture with TPO and stromal cells.** (A) (Top panel) Lin<sup>+</sup>Kit<sup>+</sup>Sca-1<sup>+</sup> profile of 14.5 dpc FL cells (left profile). The middle profile shows CD48 expression in LSK cells. The expression CD150 in LSK CD48<sup>+</sup> FL cells appears in the right profile. (Middle panel) CD150 and CD48 expression on CD31<sup>+</sup>Kit<sup>+</sup>CD45<sup>low</sup>Mac1<sup>low</sup> cells from 30-35S P-Sp/AGM. (Bottom panel) Profile of Lin<sup>+</sup> cells derived from imHSCs cultured on OP9 stroma with TPO. Lin<sup>+</sup>Kit<sup>+</sup>Sca-1<sup>+</sup> profile (left) of imHSCs after 7 days in culture. CD48 and CD150 expression is shown in the middle and right profiles, respectively. (B) Numbers of LSK cells recovered from 100 sorted P-Sp/AGM cells after culture on OP9 stroma with TPO. CD31<sup>+</sup>Kit<sup>+</sup>CD45<sup>low</sup>Mac1<sup>low</sup> (blue) and CD31<sup>+</sup>Kit<sup>+</sup>CD45<sup>+</sup>Mac1<sup>low</sup> (orange) cells were sorted from 22-32S P-Sp (n=4) and CD31<sup>+</sup>Kit<sup>+</sup>CD45<sup>low</sup>Mac1<sup>low</sup> (blue) cells from 33-37S AGM (n=2). Thirty wells for each population were analyzed.

of the TPO receptor *Mpl* as adult LSK (supplementary material Fig. S5B); in the absence of TPO (supplementary material Fig. S5A, middle panels), no Kit<sup>+</sup>Sca-1<sup>+</sup>CD48<sup>+</sup>CD150<sup>+</sup> cells were detected. With TPO and OP9 stroma, there was a 15-fold expansion of LSK (supplementary material Fig. S4C), over to the total input cells after 5 days and a 40-fold expansion after 7 days. Intermediate levels of expansion and maturation were obtained with Ang-1, IL3, Kit ligand or combinations of these cytokines.

The production of cells with a LSK CD150<sup>+</sup> phenotype upon TPO culture might result from the maturation of imHSCs and/or from the expansion of the first few HSCs present in the 34-35S AGM (Müller et al., 1994; Medvinsky and Dzierzak, 1996). We therefore assessed LSK CD150<sup>+</sup> cell production from either 22-32S or 33-37S P-Sp/AGM sorted cells. Fig. 2B shows that similar numbers and frequency of LSK CD48<sup>+</sup>CD150<sup>+</sup> progenitors were obtained at both stages. Moreover, CD31<sup>+</sup>Kit<sup>low</sup>CD45<sup>+</sup> hematopoietic cells, mostly myeloid progenitors, did not acquire the LSK CD150<sup>+</sup> phenotype during culture, indicating that only multipotent progenitors can do so (Fig. 2B). We also analyzed the ability of single CD31<sup>+</sup>Kit<sup>+</sup>CD45<sup>low</sup> P-Sp/AGM cells to give rise to LSK CD150<sup>+</sup> cells. The frequency of clones producing LSK CD150<sup>+</sup> cells (around 10% of LSK CD150 progenitors) obtained was comparable at the 22-32S or 33-37S stages (Fig. 3A). This result confirmed at the single cell level the similarity of potential displayed by multipotent progenitors at both stages. Interestingly, among these clones, a few could yield up to 100 LSK CD150<sup>+</sup> cells (Fig. 3A, lower panel).

As in vitro maturation of imHSCs might result from cytokine or stroma-driven inductive signals, we investigated whether this maturation steps occurs in situ. To that end, we implemented P-Sp organ culture and assessed the expression CD150 at day 3 and 5. Fig. 3B (top panel) shows that, after organ culture, 15-22S P-Sp cells acquire the expression of Sca-1 and CD150. Moreover, such analysis performed at different 9-10 dpc sub-stages (15-22S; 28-32S; 34-36S) showed that the phenotype evolution is similar at the different stages (Fig. 3B, lower panel). We also show MHC-1 expression in these cultures (see below).

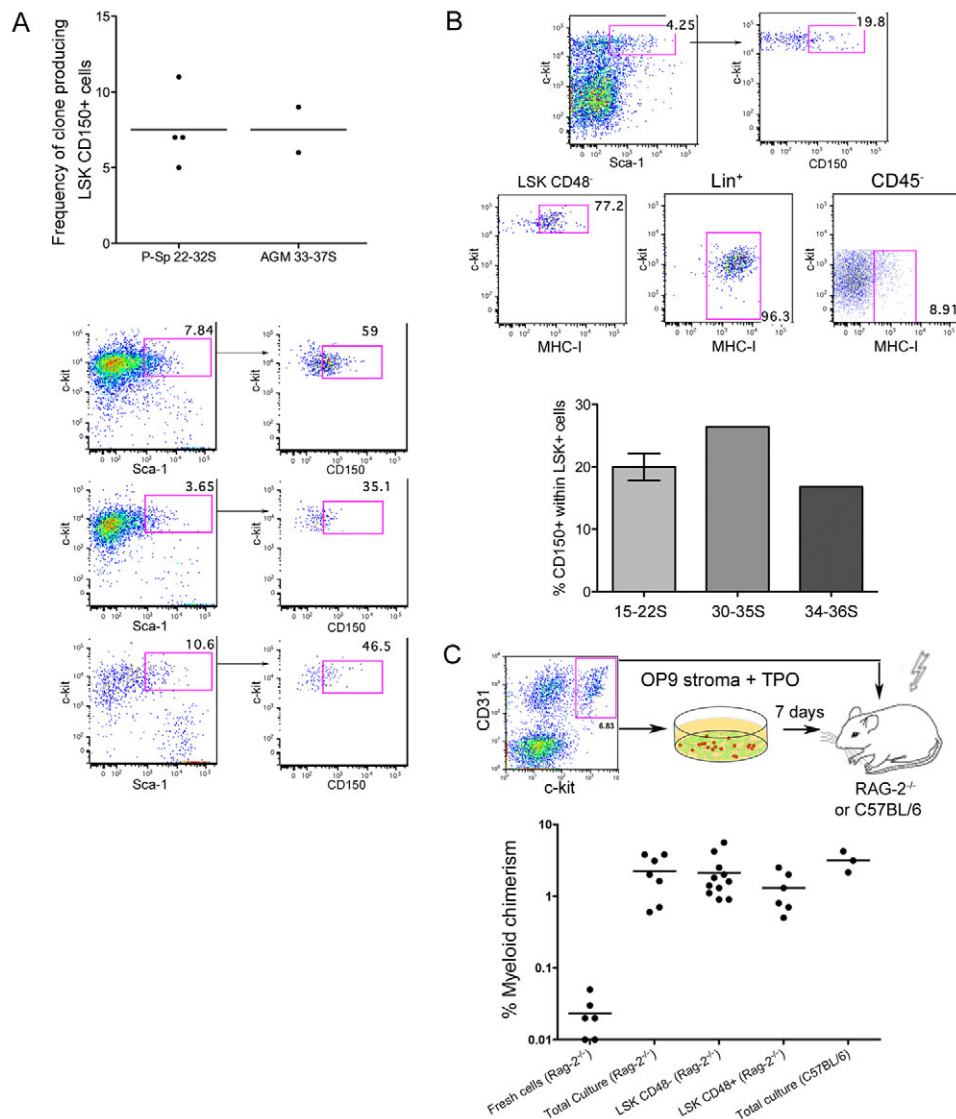
We next characterized the reconstitution potential of imHSC-derived cells. We injected 30-35S imHSCs or the progeny of an equivalent numbers of progenitors obtained after culture with TPO and stromal cells into *Rag2*<sup>-/-</sup> or into conventional (C57BL/6) recipient mice. No hematopoietic repopulation was obtained with CD31<sup>+</sup>Kit<sup>+</sup>CD45<sup>low</sup>Mac1<sup>low</sup> cells freshly isolated from 30-35S P-Sp/AGM (none positive out of six mice). When the same population was cultured with OP9 and TPO for 7 days, 100% (three positive mice out of three) of transplanted mice were reconstituted and displayed a BM myeloid chimerism ranging from 0.6 to 3.8%. Contrary to imHSCs, their progeny have acquired, upon culture, the ability to reconstitute hematopoiesis for more than 6 months into either *Rag2*<sup>-/-</sup> or in wild-type C57BL/6 recipient mice (Fig. 3C; see also supplementary material Fig. S6), demonstrating the conversion of imHSCs to HSCs.

To further define the phenotype of the cells that exhibit HSC properties in vivo, we injected LSKCD48<sup>-</sup> and LSK<sup>+</sup>CD48<sup>+</sup> sorted after culture of imHSCs with TPO and stromal cells (Fig. 3C). All populations containing LSK cells displayed LTR activity. As expected, the transplantation of LSKCD48<sup>-</sup> cells resulted in 100% positive recipient mice (11/11 mice) and a mean of 2.1% donor myeloid cells. Surprisingly, five out of six mice were also reconstituted with the LSK<sup>+</sup>CD48<sup>+</sup> population (mean chimerism: 1.3%). This result could suggest that the HSC phenotype is not completely established at the end of the culture.

Altogether, these data show the emergence of cells with phenotypic and functional properties similar to HSCs, in cultures seeded with imHSCs.

### Immature HSCs acquire, after culture, an inhibitory phenotype for NK cells

The expression of MHC class I (MHC-1) molecules is acquired in embryonic cells after 10.5 dpc (Jaffe et al., 1991). The absence of



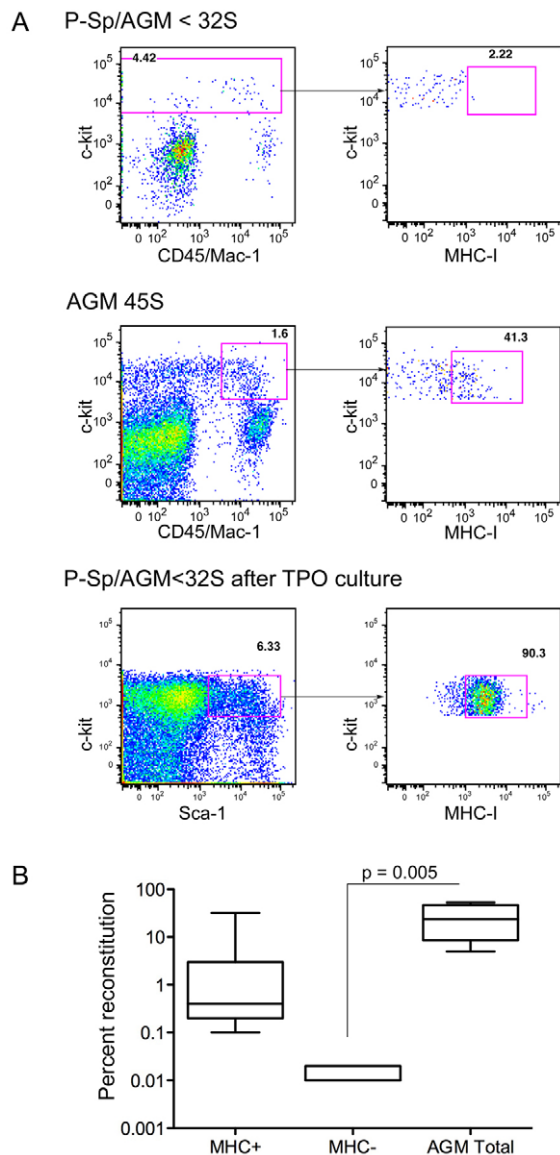
**Fig. 3. imHSCs can evolve into HSCs in vitro and in organ culture.** (A) Single cell analysis. (Top panel) Frequency of single CD31<sup>+</sup>Kit<sup>+</sup> CD45<sup>-low</sup> P-Sp/AGM cells capable of giving rise to LSK CD150<sup>+</sup> cells. The progeny of 300 single cells was analyzed for each developmental stage (22-32S,  $n=4$ ; 33-37S,  $n=2$ ). (Bottom panels) Three representative profiles of the progeny of single CD31<sup>+</sup>Kit<sup>+</sup> CD45<sup>-low</sup> cells from 22-32S P-Sp that gave rise to LSK cells (left profiles) containing CD150<sup>+</sup> cells (right profiles). (B) Organ culture analysis. (Top panels) After 5 days in organ culture, 15-22S P-Sp gave rise to CD150<sup>+</sup> cells (upper profiles). MHC-1 is expressed by LSK CD48<sup>-</sup> and Lin<sup>+</sup> cells produced by 15-22S P-Sp maintained for 5 days in organ culture, while its expression is lower in CD45<sup>-</sup> cells (lower profiles). (Bottom panel) A similar percentage of CD150<sup>+</sup> cells within LSK cells is obtained after organ culture of 15-22S P-Sp, 30-35S P-Sp/AGM and 34-36S AGM. (C) LTR activity. CD45.2<sup>+</sup> Rag2<sup>-/-</sup> recipient mice were injected with either 200 sorted (CD31<sup>+</sup>Kit<sup>+</sup>CD45.1<sup>-low</sup>Mac-1<sup>-low</sup> cells) 30-35S P-Sp/AGM cells or with their progeny after culture on OP9 stroma with TPO. At day 7 of culture, cells were either injected directly (total culture) or stained and sorted as LSKCD48<sup>-</sup> or CD48<sup>+</sup> cells. Five to 6 months after transplantation, the reconstitution level obtained with the three samples of cultured cells was significantly higher (total culture,  $P=0.0013$ ; LSKCD48<sup>-</sup>,  $P=0.004$ ; LSKCD48<sup>+</sup>,  $P=0.003$ ), compared with fresh cells. Data cumulate two independent experiments. The reconstitution level obtained when total culture is transplanted is similar in Rag2<sup>-/-</sup> and C57BL/6 recipients ( $P=0.401$ ).

MHC-1 expression on hematopoietic progenitors isolated before 35S could activate NK cells from recipient mice, leading to their elimination. The differential engraftment capacity of imHSCs could thus be explained by the absence of NK cell activity in Rag2<sup>-/-</sup> compared to Rag2<sup>-/-</sup> or wild-type mice.

We analyzed MHC-1 expression on P-Sp cells before the 32S stage, on 30-35S P-Sp/AGM cells and on 45S AGM cells. MHC-1 expression was virtually undetectable in P-Sp cells below the 32S stage (Fig. 4A) and in 30-35S P-Sp/AGM multipotent progenitors (CD31<sup>+</sup>Kit<sup>+</sup> CD45<sup>-</sup>Mac-1<sup>-</sup>), whereas in 45S AGM, 40% of

CD31<sup>+</sup>Kit<sup>+</sup> (CD45/Mac-1<sup>+</sup>) cells were MHC-1 positive (supplementary material Fig. S7A). The expression of MHC-1 and CD45 thus occurs in hematopoietic progenitors between the 30S and the 45S stages (supplementary material Fig. S7). We next followed the evolution of MHC-1 upon TPO culture (Fig. 4A). After culture with TPO, 99% of LSK cells generated from P-Sp cells below the 32S stage now expressed MHC-1 (Fig. 4A).

To obtain a functional link between MHC-1 expression and the capacity to repopulate the hematopoietic system of NK<sup>+</sup> mice, we injected Rag2<sup>-/-</sup> mice with MHC-1-positive and -negative cells



**Fig. 4. MHC-1 expression is required for the engraftment of HSCs in NK competent mice. (A)** Flow cytometry analysis of MHC-1 expression (H-2D<sup>b</sup>) in P-Sp/AGM cells. Top profiles, Kit<sup>+</sup>CD45<sup>low/-</sup>/Mac-1<sup>low</sup> cells from P-Sp/AGM below 32S; middle profiles, Kit<sup>+</sup>CD45<sup>low/-</sup>/Mac-1<sup>low</sup> cells from 45S AGM; bottom profiles, the LSK progeny of Kit<sup>+</sup>CD45<sup>low/-</sup>/Mac-1<sup>low</sup> cells from P-Sp/AGM below 32S after 7 days in culture on OP9 stroma with TPO. See supplementary material Fig. S7A. **(B)** Chimerism in sublethally irradiated *Rag2*<sup>-/-</sup> mice injected with total 45S AGM cells, with Kit<sup>+</sup>CD31<sup>+</sup>MHC-1<sup>+</sup> or with double the number of Kit<sup>+</sup>CD31<sup>+</sup>MHC-1<sup>-</sup> cells. The chimerism level, shown as the frequency of Gr-1<sup>+</sup> donor (CD45.1) cells in the BM of recipient mice 6 months after transplantation, was not statistically different when reconstitution was achieved with Kit<sup>+</sup>CD31<sup>+</sup>MHC-1<sup>+</sup> or total AGM cells ( $P=0.055$ ). By contrast, no reconstitution was obtained using Kit<sup>+</sup>CD31<sup>+</sup>MHC-1<sup>-</sup> cells.

from 45S AGM, the first stage of development where both populations co-exist in the same organ (Fig. 4A; supplementary material Fig. S7). None of the mice injected with twice as many MHC-1<sup>-</sup> as MHC-1<sup>+</sup> cells showed hematopoietic chimerism, whereas there was no significant difference when the long-term

chimerism of total and MHC-1<sup>+</sup> AGM cells was compared ( $P=0.055$ ) (Fig. 4B).

However, three out of seven mice reconstituted with sorted MHC-1<sup>+</sup> cells showed chimerism levels below 0.5%. We considered the possibility that the antibody recognizing H-2D<sup>b</sup> used to mark MHC-1<sup>+</sup> cells may interfere with the reconstitution capacity of labeled cells. To test this possibility, the same numbers of total 45S AGM cells were incubated with either anti-H-2D<sup>b</sup> or anti-Kit as a control, prior to injection into lethally irradiated wild-type mice. None of the three mice injected with AGM cells incubated with anti-H2D<sup>b</sup> antibody were reconstituted, in contrast to two out of three mice injected with cells incubated with anti-Kit (supplementary material Fig. S8). This result indicates that the presence of anti-H2D<sup>b</sup> antibody on AGM cells impairs their reconstitution capacity. Consequently, the reconstitution frequency obtained with MHC-1<sup>+</sup> cells is largely underestimated. Particularly significant was the consistent inability of H2D<sup>b</sup>-negative cells to engraft recipient mice, even when double numbers of H2D<sup>b</sup>-compared with H2D<sup>b</sup><sup>+</sup> cells were transplanted (supplementary material Fig. S8).

These results implicate MHC-1 expression that occurs concomitantly with CD45 upregulation, as a major event in the transition from imHSCs to HSCs. The acquisition of an inhibitory phenotype for NK cells explains the capacity of cultured imHSCs to repopulate *Rag2*<sup>-/-</sup> and wild-type recipients.

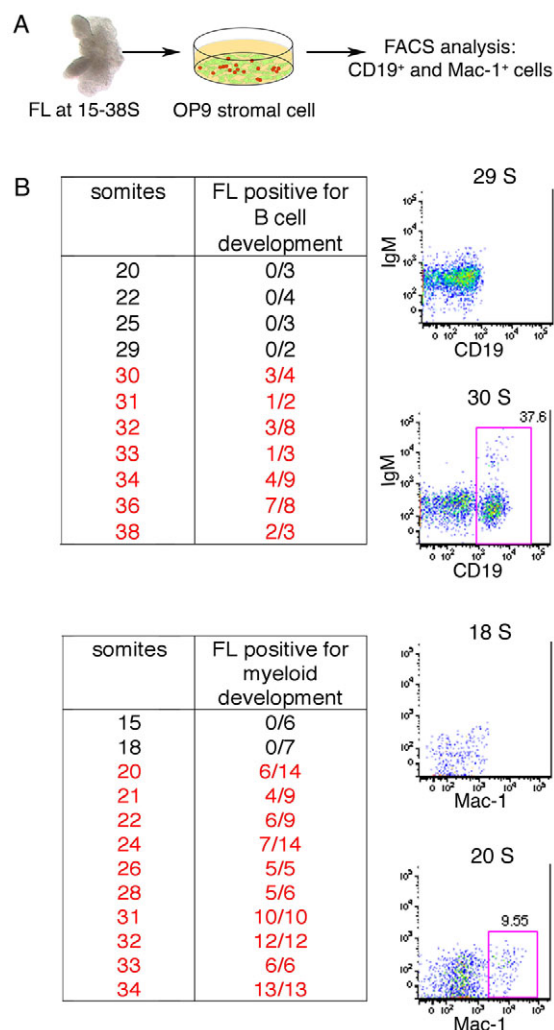
### Immature HSCs acquire HSC properties in the FL

FL hematopoiesis relies on the colonization by hematopoietic progenitors, a process previously shown to be initiated at 10 dpc (28-32S) (Houssaint, 1981). HSCs were first detected in the FL at 11 dpc (42S), shortly after they are produced in the AGM. We tested whether imHSCs, which may rapidly circulate and colonize the FL, can give rise to HSCs in situ.

To define precisely the stage of FL colonization, we analyzed the differentiation potential of hematopoietic progenitors recovered from 20-38S FL, through culture on OP9 stroma with cytokines (Fig. 5A). Although restricted myeloid progenitors could be identified from 9 dpc (20S), CD19<sup>+</sup> B-lineage cells were detected after the 30S stage (Fig. 5B). This kinetic analysis shows that the first FL colonization occurs 1 day earlier than previously thought. The FL receives a first wave of erythro-myeloid restricted progenitors from 9 dpc, before the colonization by imHSCs. The second wave of FL colonization, which occurs at 10 dpc (from the 32S-stage), is performed by imHSCs, shortly after they emerge in the P-Sp/AGM and long before the detection of HSCs.

Similar to P-Sp/AGM cells (Fig. 4A; supplementary material Fig. S4A, Fig. S7A), 30-35S fetal liver hematopoietic progenitors did not express sca-1 or MHC-1 (Fig. 6A; supplementary material Fig. S4B, Fig. S7B). One day later (45S), 25% of Kit<sup>+</sup>CD31<sup>+</sup>CD45<sup>+</sup> FL cells expressed MHC-1 (Fig. 6A; supplementary material Fig. S7B). A 4-day organ culture of 30-35S FL resulted in 98% of MHC-1 expression in LSK cells (Fig. 6A). To confirm the presence of imHSCs in the FL, we transplanted FL cells from 10 dpc embryos (30-35S) into *Rag2*<sup>-/-</sup> recipient mice (Fig. 6B). Five out of seven mice displayed long-term reconstitution and showed donor-derived myeloid cells. The absence of HSCs in the FL at this stage was confirmed by the lack of LTR activity in *Rag2*<sup>-/-</sup> recipients (0/3) (Fig. 6B). We isolated FL at the 30-35S stages, performed a 3-day organ culture and injected the cells obtained into *Rag2*<sup>-/-</sup> mice. In contrast to freshly isolated FL cells, cells derived from 30-35S FL after organ culture displayed LTR activity in 4/4 of *Rag2*<sup>-/-</sup> recipient mice (Fig. 6B).





**Fig. 5. Colonization of FL by imHSCs and myeloid progenitors.**

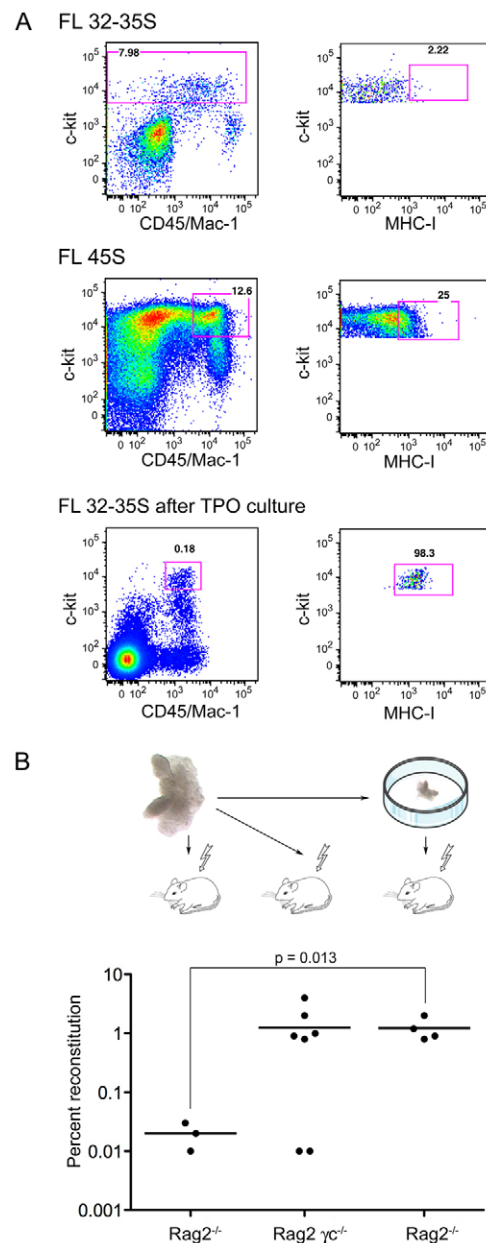
(A) Scheme of cultures of total cells isolated from FL at different stages, in B lymphoid and myeloid conditions. B and myeloid cells were identified as CD19<sup>+</sup> and Mac-1<sup>+</sup> cells, respectively. (B) Frequency of FL positive for B lymphoid (top) or myeloid (bottom) cell production. Flow cytometry profiles on the right show the presence of B lymphoid cells in culture derived from a 30S FL, compared with a 29S FL (top panels). Myeloid cells are present in cultures derived from FL after the 19S stage (bottom panels).

These data demonstrate that after 3 days in the FL environment, the imHSCs that colonized the FL after the 30S stage evolved into HSCs. The conversion of imHSCs to HSCs thus occurs in situ.

## DISCUSSION

A better characterization of the events that precede the detection of the first HSC in the embryo is crucial for the understanding of the maturation steps leading to the development of adult type HSCs.

We focused on the potential and the outcome of multipotent hematopoietic progenitors that are present in the P-Sp/AGM before 10.5 dpc, as hematopoietic activity at this stage remains poorly characterized. The appearance of these multipotent progenitors precedes by nearly 2 days the detection of the first HSC defined by its ability to perform long-term multilineage reconstitution of irradiated adult recipients. HSCs have first been identified in the



**Fig. 6. imHSCs from 10 dpc FL evolve into HSCs after organ culture.** (A) MHC-1 expression (H-2D<sup>b</sup>) by FL cells. (Top profile) 32-35S FL. (Middle profile) 45S FL. (Bottom profile) Progeny of 32-35S FL cells after 4 days in organ culture. See supplementary material Fig. S7B. (B) The experimental protocol (top panel). 30-35S FL were injected directly into CD45.2<sup>+</sup> Rag2<sup>-/-</sup> and Rag2<sup>-/-</sup> recipients, or after a 3-day organ culture into Rag2<sup>-/-</sup> recipient mice. See supplementary material Fig. S5. (Bottom panel) Engraftment (% Gr-1<sup>+</sup> cells in the BM) 5-6 months after transplantation in mice that received freshly isolated cells (left and middle) or cells obtained from organ culture. Data cumulate two independent experiments.

10.5 dpc AGM, after the 34-35S-stage, and slightly later in the FL (Müller et al., 1994; Sánchez et al., 1996; Medvinsky and Dzierzak, 1998). However, hematopoietic activity starts in the P-Sp at 9 dpc (after the 15S stage) with the emergence of clonally defined multipotent progenitors (Godin et al., 1995; Yokota et al., 2006). When specifically tested for their reconstitution activity, 9-9.5 dpc



hematopoietic progenitors were found to be unable to reconstitute conventional recipients.

Here, we tested the possibility that 9-9.5 dpc multipotent progenitors constitute the progenitors of HSCs. Our phenotypic and functional analyses led to the identification of a population that lays immediately upstream of HSCs in the hierarchical progression from mesoderm to HSCs during ontogeny. This population, referred to as imHSCs, is detected in the P-Sp from 9 dpc, at the same stage when multipotent progenitors, defined *in vitro*, were previously identified (Godin et al., 1995). Attempts to find imHSCs in other locations before the establishment of circulation, which would unambiguously assign their origin, were unsuccessful. We therefore concentrated our study on the P-Sp/AGM. It is possible that imHSCs are also present and/or generated in other sites [YS, placenta or the umbilical and omphalomesenteric (or vitelline) arteries].

Immature HSCs are defined by their ability to reconstitute in the long-term the hematopoiesis of *Rag2 $\gamma$ <sup>-/-</sup>* recipient mice together with the absence of LTR activity in either *Rag2 $\gamma$ <sup>-/-</sup>* or conventional mice. More importantly, these cells that also differ from HSCs by their phenotype, can progress, in monolayer or in organ cultures, to a stage when they now show the functional and phenotypic properties of HSCs. Indeed, our results demonstrate that sorted CD31<sup>+</sup>Kit<sup>+</sup>CD45<sup>-</sup> imHSCs cultured on OP9 stromal cell line in the presence of TPO can give rise to HSCs defined by (1) an adult HSC phenotype (Kiel et al., 2005), namely LSKCD150<sup>+</sup>CD48<sup>-</sup>; and (2) a LTR activity in *Rag2 $\gamma$ <sup>-/-</sup>* or conventional (C57BL/6) recipient mice. Upon culture with TPO, HSCs that display a LTR activity are recovered from both the LSKCD48<sup>-</sup> and Lin<sup>-</sup>CD48<sup>+</sup> populations. The phenotype of the cells isolated from culture that generate long-term chimerism is identical to that obtained with sorted freshly isolated 14 dpc FL cells. The LSKCD48<sup>-</sup> population, which is derived from the culture with TPO, is composed of CD150<sup>+</sup> and CD150<sup>-</sup> populations, both of which display LTR activity in *Rag2 $\gamma$ <sup>-/-</sup>* recipient mice. It has been documented that FL and BM HSCs share an LSKCD150<sup>+</sup>CD48<sup>-</sup> phenotype. However, it was recently shown that HSCs are also present within the CD150<sup>-</sup> population (Weksberg et al., 2008; Kent et al., 2009).

Two lines of evidence indicate that imHSCs from the P-Sp below the 32S stage and HSCs from 33-37S AGM are highly similar, although they differ in the type of recipient required to reveal their LTR ability: (1) single CD31<sup>+</sup>Kit<sup>+</sup>CD45<sup>-</sup> cells from both stages gave rise to similar numbers and frequencies of CD45<sup>+</sup>MHC-1<sup>+</sup> cells and of CD150<sup>+</sup> cells; (2) at both stages, P-Sp or AGM explants maintained in organ culture displayed a similar phenotypic evolution characterized by the progressive acquisition of Sca-1, CD45 and MHC-1 expression. Although hematopoietic cells derived from organ culture were not tested here for their reconstitution capacity, they are likely to behave *in vivo* as HSCs. This conclusion is strengthened by the fact that 36-39S AGM cells maintained in organ culture gave rise to LTR-HSCs (Medvinsky and Dzierzak, 1996).

Using various experimental set-ups, others authors have attempted to characterize intermediate steps in HSC development. Ema et al. (Matsumoto et al., 2009) identified pre-HSCs in an embryonic stem cell (ES) differentiation assay combined with an inducible HOXB4 enforced expression. These ES derived progenitors display the same phenotype as the imHSCs described here, namely CD41<sup>+</sup>Kit<sup>+</sup>CD45<sup>-</sup>, and are also unable to engraft recipient mice displaying NK activity.

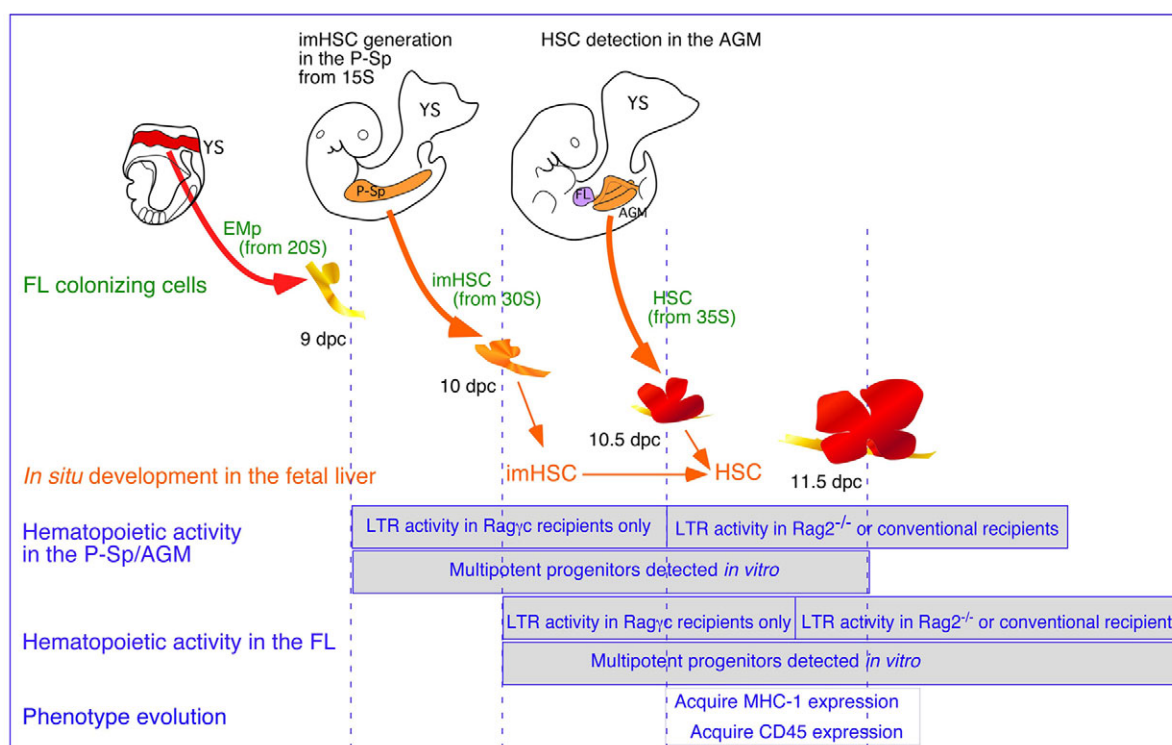
Medvinsky's group used organ cultures of re-aggregated AGM to reveal the presence of VE-Cadherin<sup>+</sup> CD45<sup>+</sup> or CD45<sup>-</sup> cells after

11 dpc (45S) that they qualify as 'pre-HSC type II' (Taoudi et al., 2008; Rybtsov et al., 2011). These cells, which mature during the organ culture, are responsible for the increased HSC number recovered from the culture. Our study refers to a developmental stage that precedes by 2 days the appearance of VE-cadherin<sup>+</sup>CD45<sup>+</sup> pre-HSC type II, which express MHC-1 molecules. The maturation steps described by Taoudi et al., which are subsequent to the ones described here, might further confer robust engraftment capacity to imHSCs. Recently, although focusing on earlier development stages, these authors showed that VE-cadherin<sup>+</sup>CD45<sup>-</sup> cells sorted from 10.5 dpc embryo (36-39S stage), which poorly reconstitute conventional recipients, are able to efficiently reconstitute these recipients when they are maintained for 4 days in a 3D-reaggregation culture with OP9 cells (Rybtsov et al., 2011). The 11 dpc 'pre-HSC type II' are considered as deriving from VE-cadherin<sup>+</sup>CD45<sup>-</sup> cells, termed 'pre-HSC type I', through maturation steps that occur during the 3D-reaggregation culture. Using similar approaches (culture with TPO or P-Sp/AGM organ culture) at an earlier stage, we identify an earliest maturation stage, and further define the acquisition of MHC-1 expression as an important step in this process.

As stated above, HSCs may be distinguished from imHSCs by the ability to long-term reconstitute hematopoiesis in *Rag2 $\gamma$ <sup>-/-</sup>* mice. This distinct *in vivo* behavior results in part from the difference in the expression of MHC-1, an inhibitory ligand of NK activation. From our results, it appears that the inhibitory phenotype of NK activation is acquired during the transition to HSCs. Yoder et al. previously evidenced that CD34<sup>+</sup>Kit<sup>+</sup> cells from 9 dpc P-Sp or YS could display LTR activity when transferred into the fetal liver of myelo-ablated newborn mice (Yoder et al., 1997). As NK activity is not developed in newborns (Dussault and Miller, 1995), the reconstituting cells described in this study probably correspond to imHSCs.

Although both imHSCs and HSCs are capable of long-term reconstitution when engrafted into sublethally irradiated *Rag2 $\gamma$ <sup>-/-</sup>* mice, the hematopoietic chimerism obtained with P-Sp/AGM-derived imHSCs is lower than the level achieved by HSCs. In the same way, HSCs derived from imHSCs during the culture with TPO or the organ culture of FL resulted in a low hematopoietic chimerism in *Rag2 $\gamma$ <sup>-/-</sup>* mice. This difference in hematopoietic chimerism does not result from an impaired homing to the BM nor from a decreased proliferation capacity. One possible explanation would be that maturation events, other than acquisition of MHC-1 (and CD45 and Sca-1), are required. Nevertheless, the few imHSCs that successfully integrated the recipient bone marrow compartment behave as HSCs as they maintain, in the long term, their contribution to hematopoiesis.

We also demonstrate here that imHSCs readily contribute to further hematopoietic development, as we detected the presence of imHSCs in the FL as soon as the 30S stage, 1 day earlier than the FL colonization by HSCs (Müller et al., 1994; Sánchez et al., 1996). These imHSCs are able to give rise to HSCs in the FL. The establishment of the HSC pool in the FL may thus result from both *in situ* maturation of imHSCs and further contribution of AGM-derived HSCs at later stages. FL colonization by imHSCs occurs at the same stage when Houssaint et al. timed FL colonization by YS-derived erythro-myeloid progenitors (Houssaint, 1981). However, in these experiments, hematopoietic cells were identified in the liver rudiment after culture using histological and morphological techniques that did not allow the characterization of hematopoietic lineages. Our present results suggest that the hematopoietic cells they detected were derived from imHSCs. This lead us to re-



**Fig. 7. Schematic representation of HSC development.** Erythromyeloid progenitors generated in the YS between 7 and 9.5 dpc (Palis et al., 1999; Lux et al., 2008) colonize the FL starting at the 20S stage. imHSCs produced in the P-Sp/AGM starting at 9 dpc, develop into HSC detected in the AGM at 10.5 dpc. Rapidly after their generation, imHSCs colonize the FL at 30S and give rise in situ to HSCs. AGM-derived HSCs may also contribute to the HSC pool present in the FL at 11 dpc.

investigate the timing of FL colonization by YS-derived cells, defined by their inability to give rise to lymphoid progeny (Cumano et al., 1996; Cumano et al., 2001). Our kinetic analysis shows that the first FL colonization, seeded by YS-derived progenitors, occurs 1 day earlier than previously thought, as progenitors restricted to the erythro-myeloid lineage were detected in the FL from 9 dpc (20S).

The FL is the main site that produces TPO (Cardier and Dempsey, 1998), a cytokine involved in the amplification and quiescence of HSCs (Kimura et al., 1998; Qian et al., 2007; Yoshihara et al., 2007). The production of TPO in the FL begins at around 10.5 dpc at the same stage when we detected imHSCs in the FL (Petit-Cocault et al., 2007) and at that stage imHSCs express similar levels of Mpl than BM HSCs, suggesting a role for TPO in imHSC development. Indeed, a function of TPO in the maintenance/amplification of imHSCs is suggested by the non-exhaustion of HSCs after 7 days of culture. A pending issue is whether TPO also plays a direct role in the transition from imHSC to HSC. It has been shown that HSCs from the FL of *Mpl*<sup>-/-</sup> mice have a impaired self-renewal ability (Petit-Cocault et al., 2007). Moreover, FL colonization by HSCs is delayed in *Mpl*<sup>-/-</sup> embryos, leading to the absence of LTR activity at 11 dpc. Taking into account the data presented here, the absence of HSCs in *Mpl*<sup>-/-</sup> FL at 11 dpc might result from a defective development of imHSCs. However, HSCs could be recovered from *Mpl*<sup>-/-</sup> FL (Qian et al., 2007) at 12 dpc, indicating that other components of microenvironment can compensate for the function of TPO, during HSC maturation.

Our data functionally define for the first time the existence of imHSCs, in the P-Sp/AGM, that lie immediately upstream of

HSCs during development (Fig. 7). Moreover, we redefine the kinetics of FL colonization as comprising a first wave involving erythro-myeloid progenitors from the YS followed by a second wave composed of imHSCs from P-Sp/AGM, which are able to give rise in situ to HSCs (Fig. 7). This latter population is also found in the AGM and could correspond to a third wave of FL colonization.

This revised sequence of events in developmental hematopoiesis has consequences in the interpretation of previous observations regarding early FL hematopoiesis. It is also relevant to studies aimed at identifying and elucidating the functions of factors that regulate the maturation of imHSCs into HSCs and further expansion of these cells.

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#### Competing interests statement

The authors declare no competing financial interests.

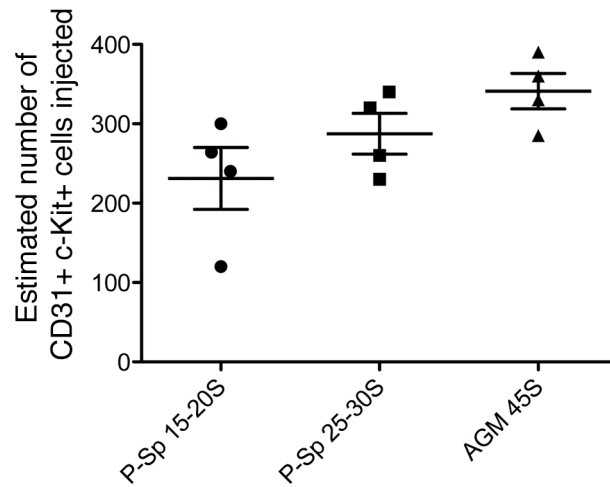
#### Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.079210/-/DC1>

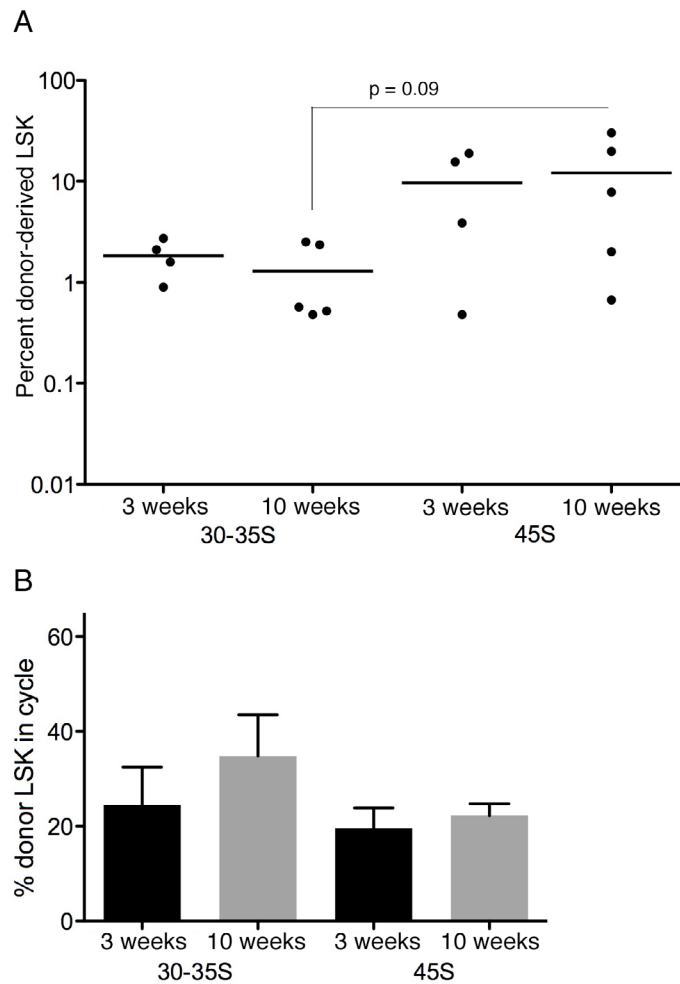
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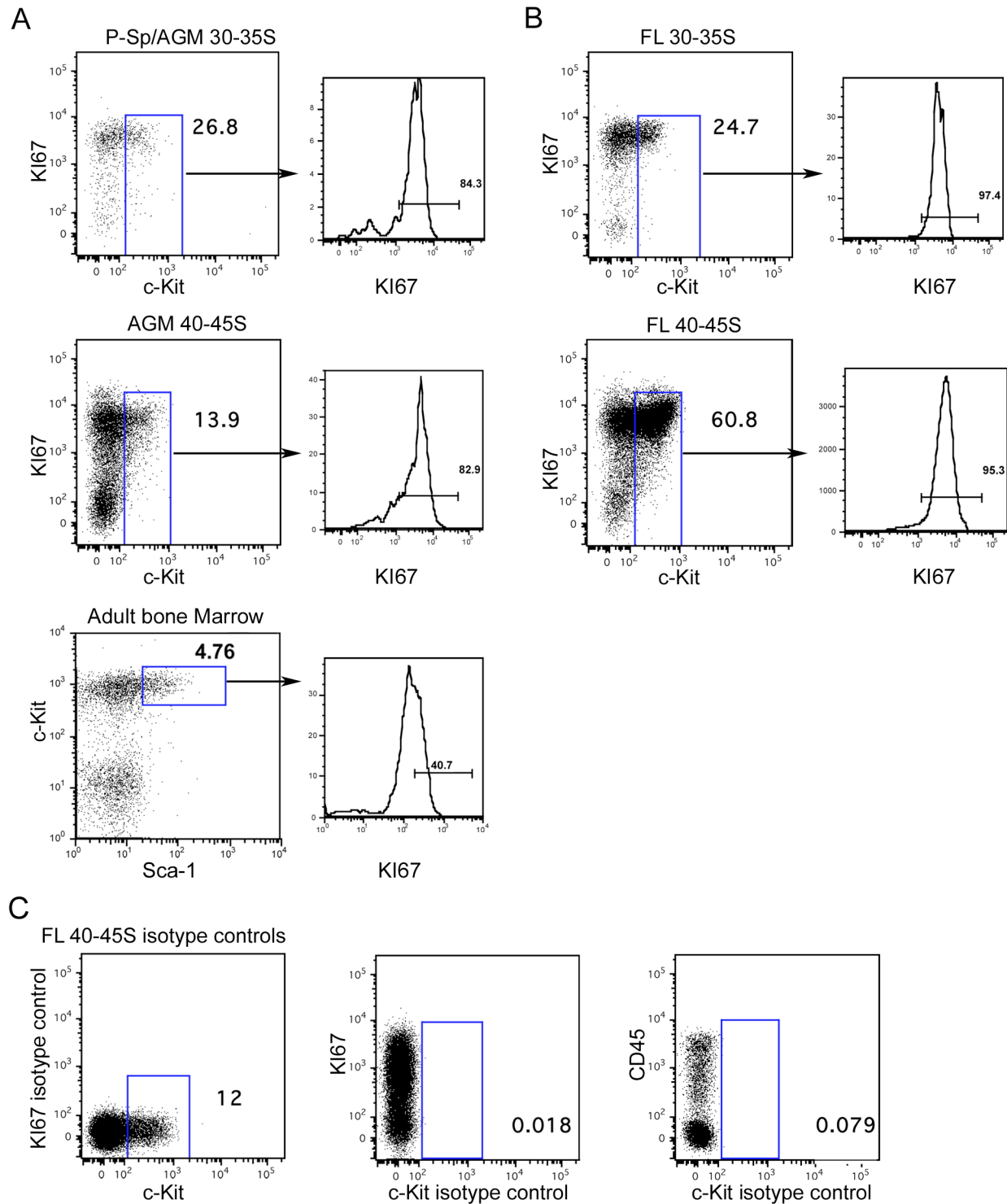




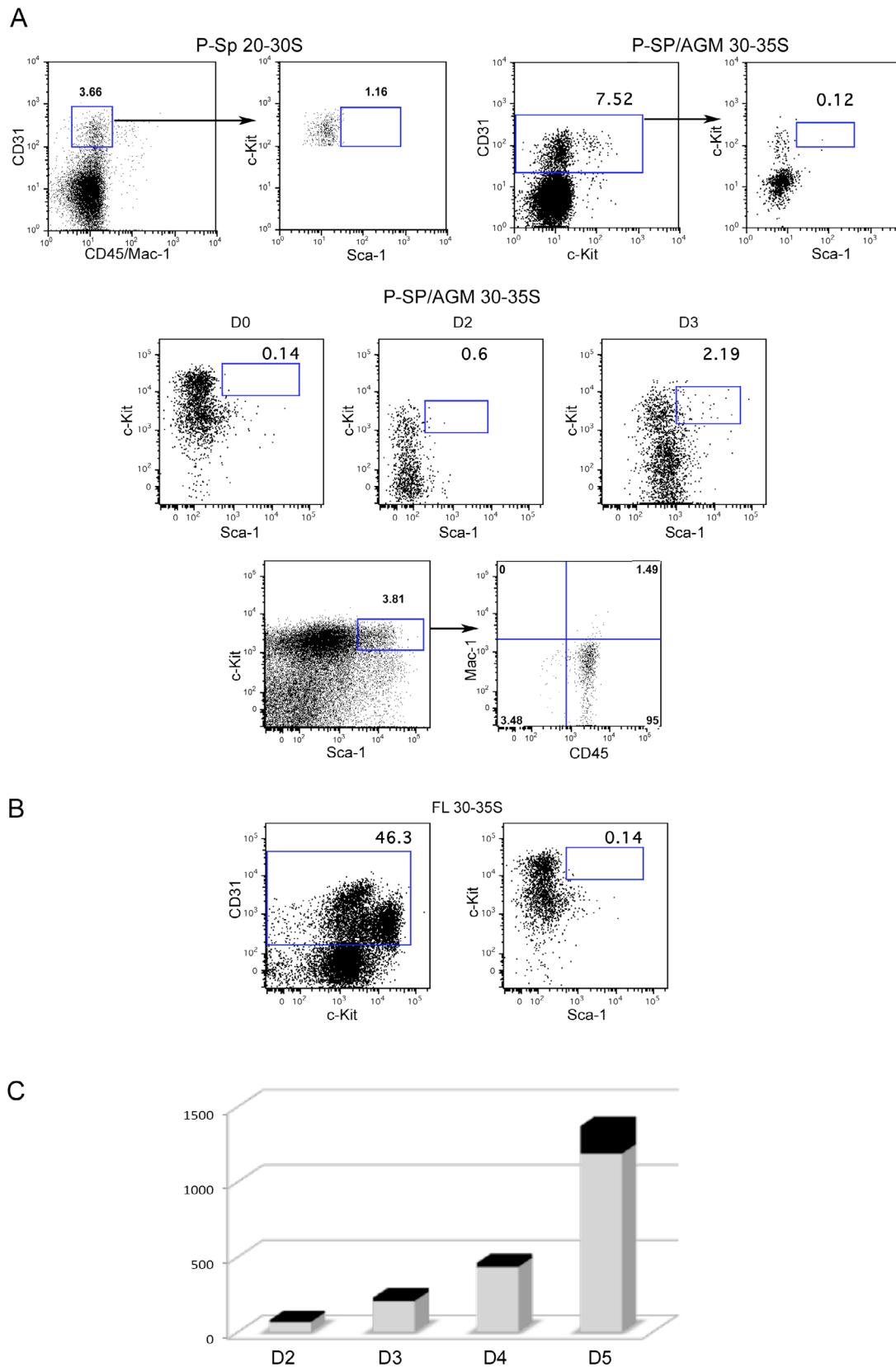
**Fig. S1. Estimated number of multipotent progenitors transplanted into *Rag2*<sup>γc</sup><sup>-/-</sup> recipients.** CD31<sup>+</sup> Kit<sup>+</sup> multipotent progenitors were quantified by flow cytometry (top panel). To inject similar number of progenitors at the three stages analyzed (lower panel), the recipients were transplanted with 12 P-Sp per mouse at 9 dpc (15-20S), 10 P-Sp per mouse at 9.5 dpc (20-25S) or 3 AGM per mouse at 11 dpc (45S).



**Fig. S2. Frequency of LSK cells in cycle after reconstitution.** Sublethally irradiated *Rag2*<sup>γc</sup><sup>-/-</sup> recipients were intravenously transplanted with cells isolated from 30-35S P-Sp/AGM (4 e.e.) or 45S AGM (2 e.e.). Donor-derived LSK cells in the BM were analyzed using the intracellular Ki67/DAPI staining 3 and 10 weeks after transplantation. The frequency of cells expressing high levels of Ki67/DAPI is shown.

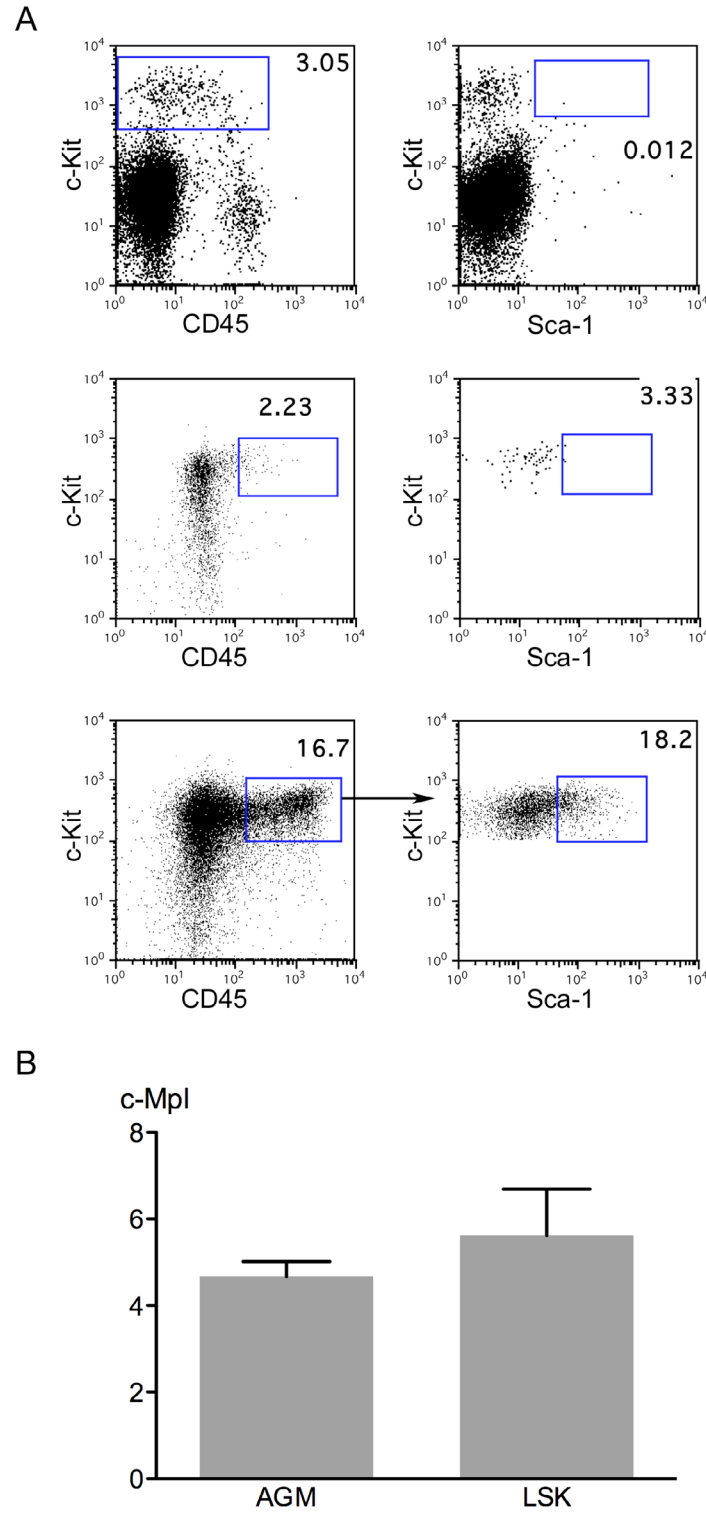


**Fig. S3. Proliferative activity of AGM and FL cells.** (A) Flow cytometry profiles of KI67 expression in CD31<sup>+</sup>Kit<sup>+</sup>CD45<sup>low</sup> imHSC from 30-35S P-Sp/AGM (top panel) and in CD31<sup>+</sup>Kit<sup>+</sup> CD45<sup>low/+</sup> HSC from 40-45S AGM (middle panel). (Lower panel) KI67 expression in LSK cells from adult BM. (B) Flow cytometry profiles of KI67 expression in CD31<sup>+</sup>Kit<sup>+</sup>CD45<sup>+</sup> cells from 30-35S FL (top panel), from 40-45S FL (lower panel). (C) Staining of 40-45S FL cells with isotype controls.

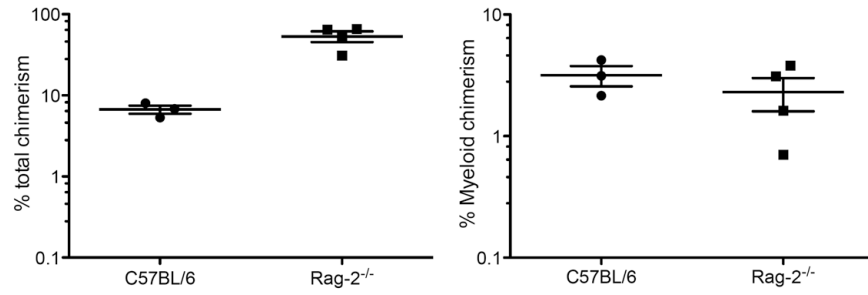


**Fig. S4. Flow cytometry profile of imHSC from the P-Sp/AGM, freshly isolated or after culture.** (A) ImHSC from 20-30S P-Sp or from 30-35S P-Sp/AGM do not overtly express Sca-1 (upper panel). After culture with TPO on OP9 stromal cells, the expression of Sca-1 is progressively acquired (middle panel). LSK cells derived from the culture of Kit<sup>+</sup> CD45<sup>-/low</sup> Mac-1<sup>-/low</sup> imHSC (30-35S P-Sp/AGM) express CD45 and Mac-1 (lower panel). (B) Similar to imHSC from the 30-35S P-Sp/AGM, imHSC from the 30-35S FL do not express Sca-1. (C) Number of LSK (grey bars) and LSK CD48<sup>-</sup> CD150<sup>+</sup> (black bars) per 100 CD31<sup>+</sup> Kit<sup>+</sup> CD45<sup>-</sup> sorted P-Sp/AGM at days 2, 3 4 and 5 of culture with OP9 stroma and TPO.

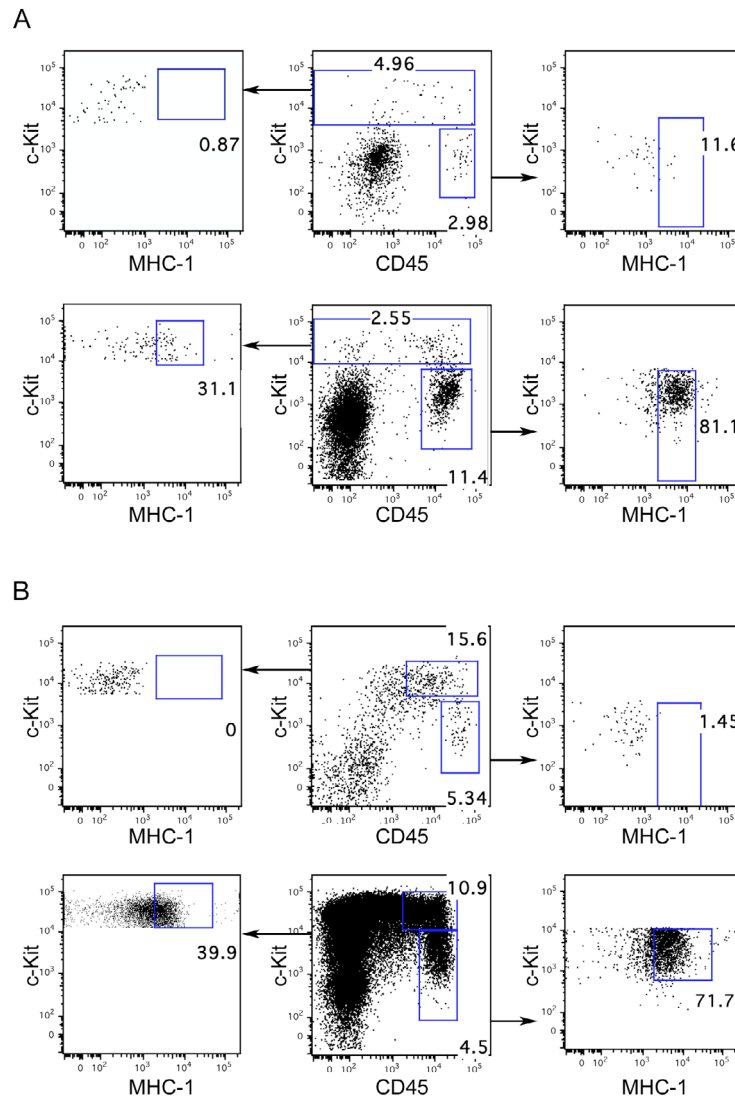




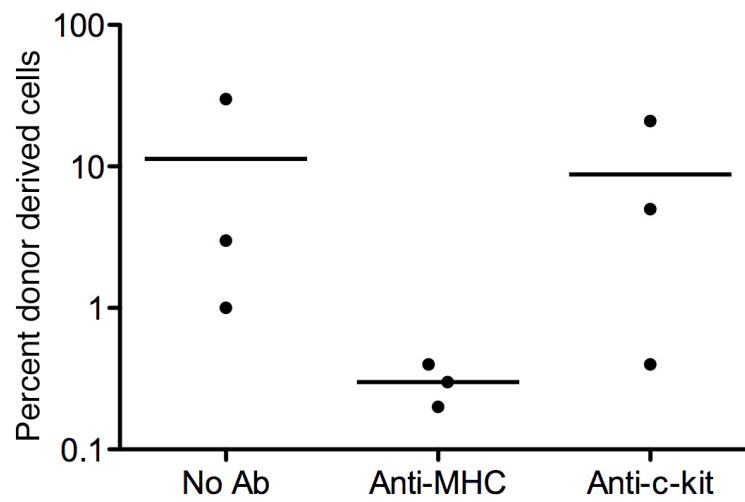
**Fig. S5. TPO promotes in vitro maturation of 30-35S P-Sp/AGM multipotent progenitors.** (A) Freshly isolated CD31<sup>+</sup>Kit<sup>+</sup> cells from 30-35S P-Sp/AGM initially lack the expression of CD45 and Sca-1 (top panel). After culture on OP9 stroma, in the absence of TPO, these 30-35S P-Sp/AGM cells give rise to a limited number of CD45<sup>+</sup>Sca-1<sup>+</sup> cells (middle panel), whereas when TPO is added to the culture medium, a well-defined population of CD45<sup>+</sup>Sca-1<sup>+</sup> cells appears (lower panel). (B) Sorted 30-35S Kit<sup>+</sup>CD31<sup>+</sup> P-Sp/AGM cells and adult bone marrow LSK were subjected to Q-RT-PCR for the detection of Mpl and HPRT mRNA (the receptor of TPO). Results are expressed as arbitrary units (ratio of Mpl to HPRT).



**Fig. S6. Comparison of total and myeloid chimerism levels in C57BL/6 and *Rag2*<sup>-/-</sup> recipients.** This figure relates to Fig. 3C. Analysis of the chimerism level in *Rag2*<sup>-/-</sup> and C57BL/6 recipients 6 months after engraftment of total cells recovered from 30-35S P-Sp/AGM cells cultured on OP9 stroma with TPO. The total chimerism level in the BM of *Rag2*<sup>-/-</sup> recipients often reaches levels close to 90%, owing to the 100% donor-derived contribution to the lymphoid compartment, when it reaches about 10% in similarly injected C57BL/6 recipients (left panel). The corresponding myeloid chimerism in the two types of recipients (right panel) is similar ( $P=0.4148$ ) and ranges on average from 2% to 4%.



**Fig. S7. Acquisition of MHC class I correlates with CD45 expression.** This figure relates to Figs 4 and 6. (A) CD45 against MHC class I (H2D<sup>b</sup>) profile (middle panels) of Kit<sup>+</sup> progenitors from 30-35S P-Sp/AGM (top panel) and 45S AGM (bottom panel). Within the hematopoietic progenitor compartment, MHC class I expression is restricted to CD45<sup>+</sup> cells (right panels). At earlier stages, Kit<sup>+</sup> cells express low levels of CD45, and MHC class I is virtually undetectable (Fig. 4A). (B) CD45 against MHC class I (H2D<sup>b</sup>) profile (middle panels) of Kit<sup>+</sup> progenitors from 30-35S (top panel) and 45S (bottom panel).



**Fig. S8. Incubation of AGM with Anti-H2D<sup>b</sup> antibody interferes with reconstitution capacity.** CD45.2<sup>+</sup> recipient mice were injected with 3 e.e. AGM cells from 45S embryos. Prior to injection, cells were incubated with anti-Kit or anti-H2D<sup>b</sup> antibodies. Results show percentage donor-derived cells in the blood 24 weeks after transfer.





