

Dual role of Mpl receptor during the establishment of definitive hematopoiesis

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Cytokine signaling pathways are important in promoting hematopoietic stem cell (HSC) self-renewal, proliferation and differentiation. Mpl receptor and its ligand, TPO, have been shown to play an essential role in the early steps of adult hematopoiesis. We previously demonstrated that the cytoplasmic domain of Mpl promotes hematopoietic commitment of embryonic stem cells in vitro, and postulated that Mpl could be important in the establishment of definitive hematopoiesis. To answer this question, we investigated the temporal expression of *Mpl* during mouse development by in situ hybridization. We found *Mpl* expression in the HSCs clusters emerging in the AGM region, and in the fetal liver (FL) as early as E10.5. Using *Mpl*^{−/−} mice, the functional relevance of *Mpl* expression was tested by comparing the hematopoietic progenitor (HP) content, long-term hematopoietic reconstitution (LTR) abilities and HSC content of control and *Mpl*^{−/−} embryos at different times of development. In the AGM, we observed delayed production of HSCs endowed with normal LTR but presenting a self-renewal defect. During FL development, we detected a decrease in HP and HSC potential associated with a defect in amplification and self-renewal/survival of the lin[−] AA4.1⁺ Sca1⁺ population of HSCs. These results underline the dual role of Mpl in the generation and expansion of HSCs during establishment of definitive hematopoiesis.

KEY WORDS: Cytokine receptor, AGM, Fetal liver, HSC, Hematopoiesis, Development

INTRODUCTION

Hematopoietic stem cells (HSCs) are defined by their ability to supply all the various types of blood cells throughout life and to self-renew (Weissman, 2000). The most stringent experimental procedure to underline the existence of HSCs is to show their ability to provide long-term multilineage hematopoietic reconstitution (LTR) when transplanted into a lethally irradiated adult animal (Morrison et al., 1995), and to further repopulate secondary lethally conditioned recipient animals. It is now accepted that the first definitive HSCs endowed with LTR ability are generated in the AGM (aorta-gonad-mesonephros) region at mid-gestation [embryonic day (E) 10.5], and localize to the dorsal aorta and the vitelline and umbilical arteries (Cumano et al., 1996; Medvinsky and Dzierzak, 1996; Muller et al., 1994). By E11, LTR-HSCs are found in the yolk sac (YS) and the fetal liver (FL) rudiment. From E11 onwards, the number of HSCs increases dramatically in the FL, which is, with the thymus, the major lymphohematopoietic organ during mouse embryonic development (Ema and Nakauchi, 2000; Morrison et al., 1995). This very rapid increase of the FL HSC pool has raised the question of whether the HSCs from the AGM alone could account for this expansion. Indeed, it has recently been shown that E12 YS also contributes to this process (Kumaravelu et al., 2002), and that an HSC pool expands in the placenta from E10.5–11 until E12.5–13.5 (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). Finally, HSCs colonize the thymus, the spleen and the bone marrow, which is the primary site of adult hematopoiesis.

Among the various factors that contribute to the maintenance of the HSC compartment, the cytokine signaling pathways are important in promoting HSC self-renewal, proliferation and differentiation in the adult. Yet little is known about the role of cytokines in embryonic hematopoiesis. Recent work on the earliest identified cytokine interleukin 3 (IL3), has shown that although this cytokine shares functional redundancy with other cytokines for HSC growth in the adult, it is an important embryonic HSC regulator as a proliferation and survival factor for the earliest HSCs in the embryo (Robin et al., 2006). The Mpl receptor and its ligand, thrombopoietin (TPO; also known as Thpo – Mouse Genome Informatics), the primary regulator of megakaryopoiesis and platelet production (Eaton and de Sauvage, 1997; Kaushansky, 1995), have been shown to play an essential role in the early steps of adult hematopoiesis. Long-term self-renewing stem cell activity segregates with Mpl expression (Solar et al., 1998) and, conversely, bone marrow cells from mice lacking Mpl (*Mpl*^{−/−} mice) are unable to compete with normal marrow for long-term hematopoietic repopulation in irradiated mice (Kimura et al., 1998). High Mpl expression is found in marrow cell populations enriched for HSCs (Terskikh et al., 2001; Terskikh et al., 2003). TPO is a potent early-acting cytokine that promotes the survival and proliferation of primitive hematopoietic cells in vitro in synergy with other early-acting cytokines such as Flt3 ligand and SCF (also known as Kitl – Mouse Genome Informatics) (Borge et al., 1997; Luens et al., 1998; Murray et al., 1999; Ramsfjell et al., 1996). TPO has also been shown to be important for the expansion of HSCs in vivo (Fox et al., 2002).

Although the role of Mpl/TPO signaling in adult HSCs is well-documented, little is known about its importance during the establishment of definitive hematopoiesis in the mouse embryo. Analysis of hematopoiesis during development in *Mpl*^{−/−} mice indicated that whereas production of megakaryocytes was compromised during fetal hematopoiesis, E12 FL contained normal numbers of hematopoietic progenitors (HPs), including multipotent progenitors and progenitors of blast cell colonies

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(Alexander et al., 1996). On the other hand, comparison of the *in vivo* hematopoietic reconstituting activity of AA4.1⁺ Sca1⁺ Mpl⁺ and AA4.1⁺ Sca1⁺ Mpl⁻ cells sorted from E14.5 FL showed that all the long-term repopulating activity of the AA4.1⁺ Sca1⁺ population is confined within the Mpl⁺ subset (Solar et al., 1998). Interestingly, passage of HSCs through the FL seems to be an important step for HSCs to acquire their definitive potential, and liver also appears to be the main site of TPO production in fetuses, neonates and adults (Wolber et al., 1999). In the mouse, *TPO* mRNA is detected in the FL as early as E12.5 (Nomura et al., 1997). At this stage, a high level of *Mpl* expression is also detected in this organ (Souyri et al., 1990). Our own results have shown that in the E12.5 FL, the cytoplasmic domain of Mpl is active only in immature hematopoietic cells. This result, added to the observation that the Mpl cytoplasmic domain promotes hematopoietic commitment of embryonic stem (ES) cells led us to hypothesize that Mpl could be important in the establishment of definitive hematopoiesis (Challier et al., 2002). In order to answer this question, we first investigated the temporal expression of *Mpl* by *in situ* hybridization in the main sites of hematopoiesis during mouse C57Bl6 development. We next tested the functional relevance of Mpl expression *in vitro* and *in vivo* using *Mpl*^{-/-} mice: we compared the colony-forming cell (CFC) content, LTR abilities and HSC content of control C57Bl6 and *Mpl*^{-/-} embryos at different times of development. We show that Mpl is expressed by clusters of emerging HSCs in the AGM region and as early as E10.5 in the FL. *In vivo* LTR assays indicated that *Mpl*^{-/-} embryos indeed present a delayed production of HSCs by the AGM region, accompanied by delayed seeding of the liver by HSCs at E11.5. During FL development, a decrease in HP and HSC potential is observed, associated with a defect in amplification and self-renewal/survival of the lin⁻ AA4.1⁺ Sca1⁺ population of HSCs. Our results indicate that Mpl plays a dual role in the establishment of definitive hematopoiesis: first, in the process of emergence/production of the first HSCs in the AGM region, and then in the amplification and survival/self-renewal of HSCs during their passage in the FL.

MATERIALS AND METHODS

Mice

C57Bl6 mice (B6-Ly5.2) and their congenic strain (B6-Ly5.1) were bred and maintained in our animal facility. Heterozygous *Mpl*^{+/-} mouse couples (B6-Ly5.2 background), kindly provided by Dr Fred de Sauvage (Genentech), were interbred to generate homozygous *Mpl*^{-/-} animals as previously described (Levin et al., 2001).

Embryo generation and processing

Embryos were produced by natural mating of B6-Ly5.2 or *Mpl*^{-/-} mice. Vaginal plugs were checked in the morning, marking E0.5. Pregnant females were killed by cervical dislocation at different times of gestation. Uteri were taken and placed in phosphate-buffered saline (PBS) (Invitrogen). The stages of the embryos were confirmed by somite counting and/or morphological analysis. Thereafter, embryos were either dissected further to recover AGM, liver and spleen and thymus at later stages of development, or embedded in paraffin for *in situ* hybridization (ISH).

For ISH, embryos were fixed overnight in 4% paraformaldehyde (PAF) (Sigma) at 4°C, and dehydrated by successive baths in 70, 90 and 100% ethanol (Prolabo, VWR International), and toluene (Carlo Erba). Embryos were then infiltrated with paraffin (Thermo Shandon-Biosciences Technologies) at 56°C and embedded at room temperature until solidification of paraffin. Blocks of paraffin were either directly sliced with a microtome (5 µm sections) or stored at 4°C.

In situ hybridization

A full-length murine *Mpl* probe cloned in the pSPT18 vector was used. Sense and antisense ³⁵S-UTP *Mpl* riboprobes were produced using the Riboprobe In Vitro Transcription System (Promega), following the manufacturer's instructions. After hydrolysis and purification on a G-50 Sephadex column (Amersham Biosciences), RNA probes were precipitated with ethanol. The RNA pellet was resuspended in 100 µl of 100 mM dithiothreitol (Invitrogen). An aliquot was counted in a radioactivity counter (LS 1800, Beckman Coulter).

For ISH, slides were dewaxed in toluene and treated with proteinase K (Roche Diagnostics) as previously described (Labastie et al., 1998). Hybridization of ³⁵S-labeled riboprobes was performed as described by Labastie et al. (Labastie et al., 1995). Slides were in contact with NTB2 autoradiographic emulsion (Kodak) for 5-6 weeks. After development, sections were counterstained with Gill's Hematoxylin (Sigma), dehydrated and mounted in Entellan medium (Merck) before observation.

RT-PCR analysis of total cellular RNA

Dissected embryos were lysed with Trizol (Invitrogen) and total RNA was extracted as recommended by the manufacturer. Reverse transcription was performed on 1 µg or less of RNA, as previously described (Challier et al., 2002). Samples of cDNAs were submitted to 35 cycles of amplification with Taq polymerase (New England Biolabs). A sample (5 µl) was used with primers specific for β-actin (*Actb*) and 10 µl with primers specific for the sequence encoding the intracellular region of the Mpl receptor. Sequences for β-actin primers have been described (Cocault et al., 1996). For *Mpl*, the 5' primer was 5'-TACAG-CTTGACGCTGCGTGCCAG-3', and the 3' primer was 5'-TGT-GTGGTGCAGCAGGACCCCTC-3'.

Relative quantitative PCR (q-PCR) was performed using the QuantiTect SYBR Green PCR Kit (Qiagen) according to the manufacturer's guidelines with gene-specific PCR primers on a LightCycler Instrument (Roche). After one step at 95°C for 15 minutes to activate the

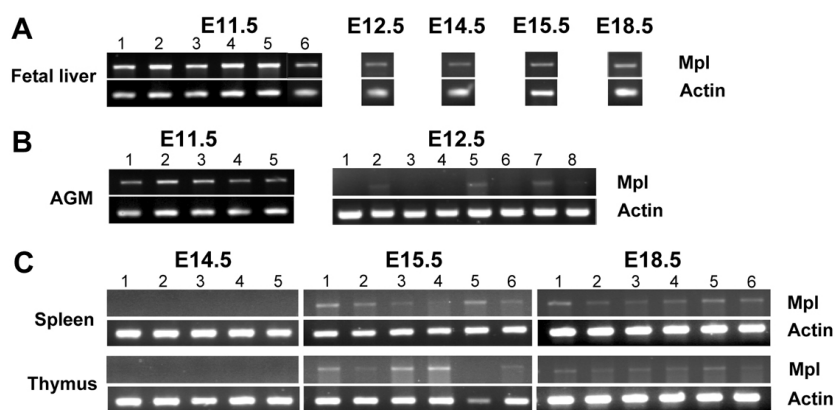


Fig. 1. *Mpl* expression in hematopoietic organs of control C57Bl6 embryos. *Mpl* expression was assessed by RT-PCR analysis in the mouse fetal liver (A), AGM region (B), spleen and thymus (C) from individual embryos. PCR of *Actb* (Actin) and *Mpl* were performed in parallel, in order to normalize *Mpl* signal after 35 cycles of amplification.

HotStarTaq DNA polymerase, the samples were cycled 40 times (denaturation at 95°C for 15 seconds, annealing at 60°C for 25 seconds, and extension at 72°C for 15 seconds). Crossing point (Cp) values of the sequences of interest (*Mpl* and *TPO*) were measured using the LightCycler software (automated calculation was by the second derivative maximum method). Relative expression was calculated according to the $E^{-\Delta C_p}$ formula. Data were normalized to *Gapdh*. The following primer sequences (5' to 3') were used: *Mpl* forward primer, CATCCTTGTAGAGGTGACCACAG; *Mpl* reverse primer, TCCAGCCTTCCACTTGAGAC; *TPO* forward primer, TGTGACCCAGACTCTAAATAAA; *TPO* reverse primer, GGCAGCAGAACAGGGATAGA; *Gapdh* forward primer, ATGGTGAAGGTCGGTGTGAA; *Gapdh* reverse primer, AATGAAGGGGTCGTTGATGG.

Cell preparations

Single-cell suspensions of YS, AGM and FL were prepared in IMDM medium (Invitrogen) with 2% fetal calf serum (FCS) (Hyclone) by repeated flushing through 18- to 26-gauge needles. At E12.5 and E14.5, FL sorted cells were isolated as follows: nucleated cells were depleted of lineage-positive cells using MACS Micro Beads (Miltenyi Biotec) with a mixture of Ter119 (also known as Ly76 – Mouse Genome Informatics), Gr1 (Ly6g), B220 (Ptpcr), CD11b (Itgam), CD4 and CD8a monoclonal antibodies (BD Biosciences-Pharmingen) (20 µg/ml), and CD34⁺ c-kit⁺ (c-kit is also known as Kit) (E12.5) and Sca1⁺ AA4.1⁺ (also known as Ly6a and Cd93, respectively) (E12.5 and E14.5) cells were subsequently sorted on a FACS-Diva cell sorter (BD Biosciences). Placentas were dissected (free of umbilical cord and maternal decidua), and treated for 1.5 hours at 37°C with 0.125% type 1 collagenase (Sigma) in PBS containing 10% FCS. Cells were dispersed gently by successive flushing through 18- to 26-gauge needles, washed, filtered through 70 µm nylon mesh (Bio-technofix), and resuspended in IMDM supplemented with 2% FCS. For the total number of cells per organ, see Table S1 in the supplementary material.

Clonogenic assays

Clonogenic assays were performed as previously described (Gurney et al., 1994). Briefly, 0.5 embryo-equivalent (ee) AGM or 5×10^4 FL cells were plated in 1 ml methylcellulose in IMDM supplemented with 30% FCS, 1% crystallized BSA (Sigma), and 10^{-4} M β -mercaptoethanol (Sigma), in the presence of 2 U/ml erythropoietin, 10 ng/ml recombinant mouse IL3 (Promocell) and 20 ng/ml recombinant human TPO (kindly provided by Kirin Brewery Co., Japan). Samples were plated in duplicate 35-mm Petri dishes and incubated for 7 days at 37°C.

Assay for long-term culture-initiating cells (CAFCs)

MS5 stromal feeder cells (Itoh et al., 1989) were seeded at 3×10^3 cells/100 µl LTC-IC medium in 96-well flat-bottom microplates (Dutscher). LTC-IC medium consisted of IMDM supplemented with 12.5% FCS, 12.5% horse serum, 0.5 mg/l ascorbic acid, 37 mg/l myo-inositol, 10 mg/l folic acid, 5×10^{-5} M β -mercaptoethanol and 10^{-6} M hydrocortisone hemisuccinate (Sigma). 24 hours later, serial half-dilutions of test cells in 100 µl LTC-IC medium were seeded over feeder cells. Cultures were maintained at 37°C with weekly half-medium changes. The input number of cells seeded per well was plotted against the log percentage of negative wells on day 28 of culture. The frequency of CAFCs was extrapolated from the linear regression curves at the log percentage of -0.37.

Analysis of long-term repopulating (LTR) ability

Adult B6-Ly5.1 mice were exposed to a single dose of 9.25 Gy (0.3 Gy/minute) from a ^{137}Cs source (IBL637, CIS Bio International); at this dose, mice died within 2 weeks. Pooled YS, AGM, blood and FL cells were co-injected intravenously into the retro-orbital venous plexus with 7×10^5 normal B6-Ly5.1 spleen cells for short-term radioprotection. Peripheral blood was collected at different times after injection and nucleated cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-Ly5.1 and biotinylated anti-Ly5.2, followed by addition of streptavidin-phycoerythrin (PE) (BD Biosciences-Pharmingen), and analyzed by flow cytometry (FACSCalibur cytometer, BD Biosciences).

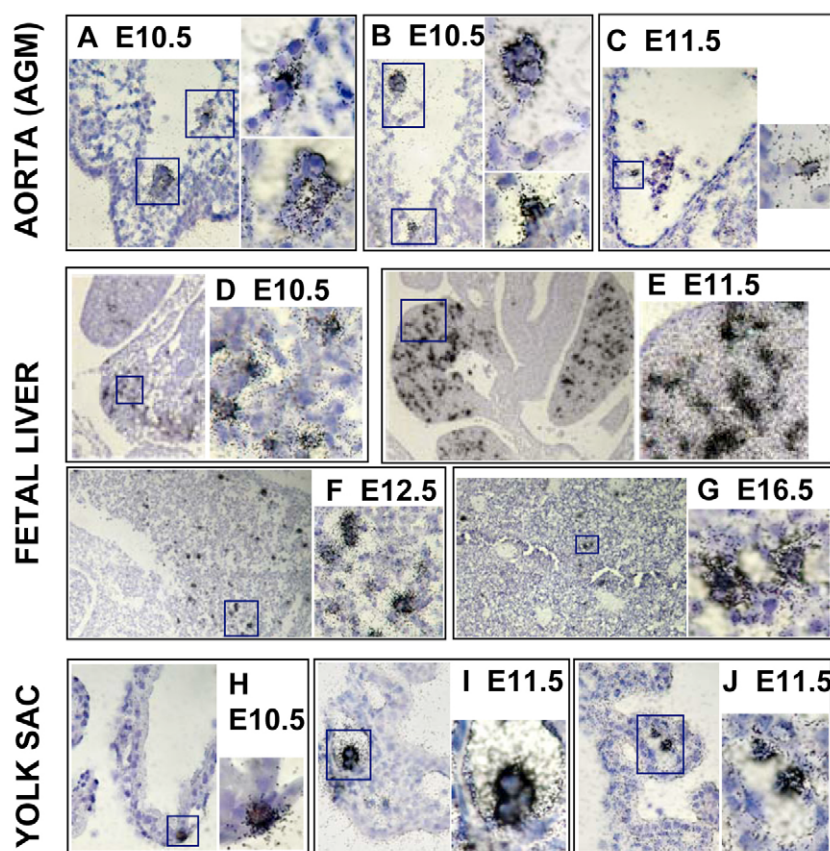


Fig. 2. Detection of *Mpl* expression in the AGM and FL by in situ hybridization. Transverse sections of E10.5 (A,B,D,H), E11.5 (C,E,I,J), E12.5 (F) and E16.5 (G) mouse embryos were hybridized with *Mpl* antisense riboprobes. (A,B,C) *Mpl* expression in the hematopoietic cells emerging from the ventral wall of the aorta in the AGM region. (D,E,F,G) *Mpl* expression in the liver at various times of development. (H,I,J) *Mpl* expression in the yolk sac. The boxed areas are shown at high magnification to the right of each panel.

for the presence of donor-derived (Ly5.2⁺) cells. The percentage of reconstitution was determined by the following formula: $(\% \text{Ly5.2}/\% \text{Ly5.1} + \% \text{Ly5.2}) \times 100$. A recipient mouse was considered positive when the percentage of reconstitution was above 5%.

Multilineage analysis and secondary transplantation

For LTR studies, primary recipient mice were sacrificed 20 weeks after primary transplant, and bone marrow, spleen and thymus were analyzed by flow cytometry. Bone marrow and spleen cells were co-incubated with Ly5.2 and CD34, Sca1, Thy1, Ter119, Gr1 or B220; thymus cells were co-incubated with Ly5.2 and CD4 or CD8. Cells were then analyzed on a FACSCalibur cytometer, and the percentage of coexpression of Ly5.2 and lineage markers was determined using WinMDI2.8 software.

At the same time, 5×10^5 bone marrow cells from *Mpl*^{+/+} and control C57Bl6 mice with equivalent percentage of Ly5.2 chimerism were injected into new B6-Ly5.1 irradiated mice for secondary reconstitution. Mice were bled between 7 and 16 weeks after injection to determine the percentage of reconstitution.

Statistical analysis

Statistical analysis was performed using the Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

Mpl is expressed by the first clusters of hematopoietic cells associated with the ventral endothelium of the embryonic aorta

In adult mice, *Mpl* has been shown to be expressed in hematopoietic organs such as the spleen and bone marrow (Souyri et al., 1990). We therefore started to investigate the temporal expression of *Mpl* by RT-PCR in the main sites of intraembryonic hematopoiesis: the AGM region, FL, spleen and thymus. As shown in Fig. 1A, strong *Mpl* expression was found in the E11.5 FL, which persisted until the neonatal stage of development (not shown). At this stage, *Mpl* signal was also found in the AGM region, but this decreased strongly as early as E12.5 (Fig. 1B). In the spleen and thymus, *Mpl* expression was first detected around E15.5, and persisted until E18.5 (Fig. 1C).

We performed in situ hybridization (ISH) to determine the spatial and temporal expression of *Mpl* during mouse development. For each ISH experiment, one slide was dedicated to hybridization with the sense *Mpl* riboprobe in order to check the specificity and the level of background. Analysis of serial sections of C57Bl6 embryos at various times of development with a full-length ³⁵S-labeled *Mpl* antisense riboprobe revealed that, interestingly, a high level of *Mpl* expression is detected at E10.5–11 in the clusters of hematopoietic cells that emerge from the ventral wall of the aorta within the AGM and represent the first intraembryonic HSCs (Fig. 2A–C). No clear *Mpl* expression above the background level was observed in the endothelial floor of the aorta. High *Mpl* expression was detected in

the FL as early as E10.5, and persisted throughout development (Fig. 2D–G). The number of *Mpl*-positive spots in the FL reached a peak at E11.5. *Mpl*-positive cells were also detected in the lung (where they are likely to correspond to megakaryocytes) and brain (data not shown). Sections of E10.5 and E11.5 YS were also hybridized, and sparse clusters of *Mpl*-positive cells were seen at these stages (Fig. 2H–J). We did not detect *Mpl* expression in the E9.5 embryo by ISH. In order to check whether this negative result could be attributed to the detection threshold of ISH, rather than to a true absence of *Mpl* expression before E10, we used relative q-PCR to test whether *Mpl* expression could be detected earlier than E10.5. As shown in Fig. 3A, low-level *Mpl* expression was found in the para-aortic splanchnopleura (PSP) region at E9.5 (this region will develop into aorta, gonads and mesonephros, and is called AGM after E10). With this method, *Mpl* could also be detected in the whole embryo and in the YS at E8, although at a very low level (Fig. 3A). We also checked *TPO* expression in the AGM region and FL. As expected, *TPO* was detected in the FL as early as E10.5 (Fig. 3B). Interestingly, we also found *TPO* expression in the AGM region at E10.5 (Fig. 3B).

Delayed production of HSCs with an impaired activity in the AGM of *Mpl*^{+/+} embryos

We tested the functional relevance of *Mpl* expression in the AGM clusters of hematopoietic cells using *Mpl*^{+/+} mice. We compared the CFC content (which corresponds to the HP content), the LTR ability and the HSC content of the AGM and liver from control C57Bl6 and *Mpl*^{+/+} E11.5 embryos (expressing the Ly5.2 allele of CD45) (Fig. 4). In order to make this comparison as rigorous as possible, embryos were staged precisely by counting somites and only embryos with 38 to 45 somites (E11–11.5) were used in all experiments. As shown in Fig. 5A, *Mpl*^{+/+} AGM presented a 1.4-fold defect in total CFCs (47.4 ± 13.4 total CFCs in *Mpl*^{+/+} versus 65.6 ± 22.6 in C56Bl6 AGMs), mainly owing to a deficiency in BFU-E and in mixed colonies containing megakaryocytic progenitors (CFU with MK). At this stage, no difference in CFC content was observed in the YS or placenta for *Mpl*^{+/+} and C57Bl6 embryos (data not shown). A 2-fold defect in LTR ability was also observed [14% (1 reconstituted mouse for 7 transplanted) and 33% (2 reconstituted mice for 6 transplanted) of reconstituted mice grafted with 1 and 3 ee *Mpl*^{+/+} AGM, respectively, versus 30% and 57% when the mice were grafted with 1 and 3 ee control C57Bl6 AGM] (Table 1). However, FACS analysis of the bone marrow, spleen, thymus and blood of reconstituted recipient mice for coexpression of CD45.2 and specific lineage markers indicated that HSCs present in the AGM of *Mpl*^{+/+} mice were able to colonize all hematopoietic organs and to give rise to all hematopoietic lineages in adult recipients (Fig. 6). Solid lymphoid reconstitution is indicative of the presence of

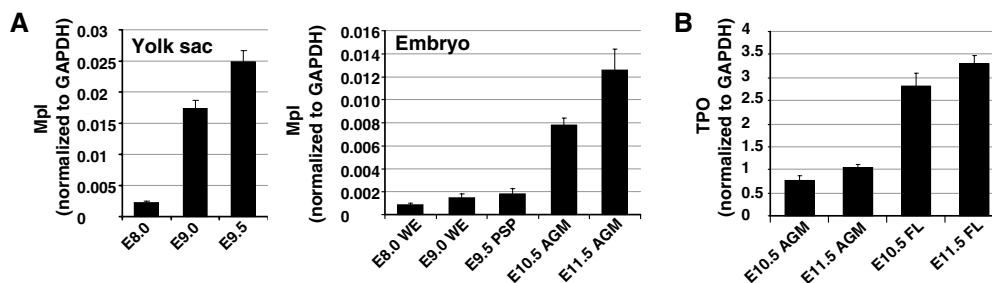


Fig. 3. *Mpl* and *TPO* levels assessed by q-PCR. (A) *Mpl* expression was assessed in mouse early yolk sacs (E8.0, E9.0, E9.5) and embryos (E8.0, E9.0, PSP E9.5, AGM E10.5 and E11.5) by relative q-PCR. (B) *TPO* expression was also evaluated by relative q-PCR in E10.5 and E11.5 AGMs and fetal livers. Each column represents an average of *Mpl* or *TPO* level normalized to the *Gapdh* internal control \pm s.d.

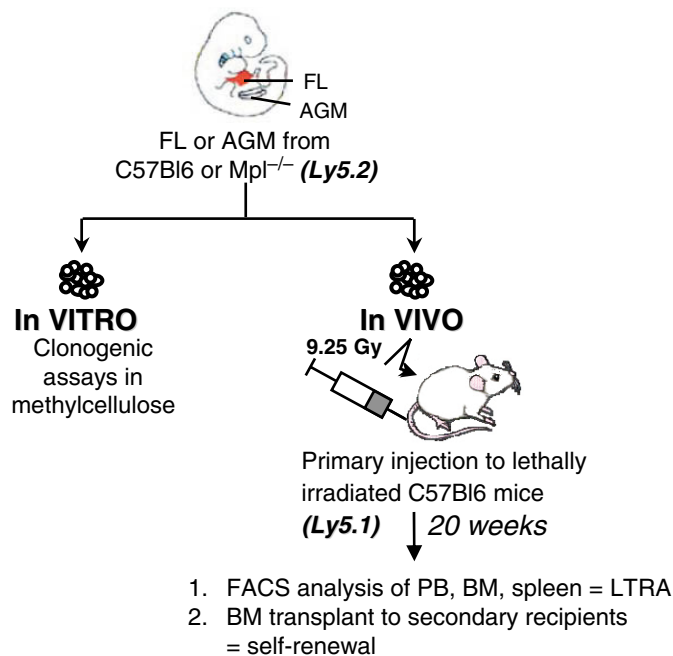


Fig. 4. Experimental design of in vitro clonogenic and in vivo long-term reconstitution assays. Single-cell suspensions were prepared from the AGM region (E11.5 and E12.5) and liver (E11.5, E12.5 and E14.5) of control C57Bl6 and *Mpl*^{-/-} embryos (expressing the *Ly5.2* allele of CD45), and analyzed in vitro in clonogenic assays, or in vivo for long-term reconstitution (LTR) of lethally irradiated C57Bl6 mice expressing the *Ly5.1* allele of CD45. At E12.5, single-cell suspensions of yolk sac or peripheral blood were also tested. The engraftment and repopulating activity of the injected cells were assessed at different times after injection (5, 10 and 20 weeks) by FACS analysis on the basis of the detection of *Ly5.2* in the peripheral blood (minimal level of reconstitution considered for positive reconstitution was 5%). Twenty weeks after injection, LTR ability (LTRA) was evaluated (FACS analysis) in positive mice by the percentage of *Ly5.2*⁺ cells present in the bone marrow (BM). The HSC content was thereafter determined by secondary transplantation of bone marrow cell suspensions from primary recipients into lethally irradiated B6-*Ly5.1* mice.

HSCs in *Mpl*^{-/-} AGM. Self-renewal being a key property of HSCs, bone marrow cells from primary recipients were used for secondary reconstitutions 20 weeks post-transplant. As illustrated in Table 1, although both *Mpl*^{-/-} and C57Bl6 bone marrow cells were able to reconstitute secondary irradiated mice with the same efficiency, the median of participation of grafted cells to hematopoietic reconstitution was much lower with bone marrow issued from *Mpl*^{-/-} AGM injection than with bone marrow injected with control AGM (28% versus 63%). This is indicative of a lower number of HSCs in the AGM of *Mpl*^{-/-} mice, and could reflect a delay in the production of these HSCs. In order to test this hypothesis, we studied the LTR potential of E12.5 AGM. We showed that it was higher than that of E11.5 for *Mpl*^{-/-} AGM, whereas, as described, it decreased for control AGM. In addition, E12.5 *Mpl*^{-/-} AGM was found to have a better LTR potential than C57Bl6 AGM (Table 1). Secondary reconstitutions indicated that the LTR ability detected in the E12.5 *Mpl*^{-/-} AGM is linked to the presence of HSCs (Table 1). However, as for E11.5 *Mpl*^{-/-} AGM, these secondary transplantations underscored an HSC defect (median of participation of grafted cells to hematopoietic reconstitution in mice reconstituted with primary

bone marrow of only 6%) compared with control E12.5 AGM (Table 1). This indicates that in addition to their delayed production, HSCs generated by *Mpl*^{-/-} AGM present an impaired activity.

Defect in HP content during development of *Mpl*^{-/-} FL

We detected *Mpl* expression as early as E10.5 in FL. We therefore analyzed the hematopoietic content of *Mpl*^{-/-} FL (Fig. 5B). At E11.5, whereas the total cellularity of *Mpl*^{-/-} FL was not significantly different to that of C57Bl6, a 1.7-fold defect in CFC content was observed (982±122 versus 1686±226 for C57Bl6 controls). This defect mainly concerned CFU-GEMM (5±2 for *Mpl*^{-/-} FL versus 25±12 for C57Bl6) and CFU with MK (44±5 versus 288±67) and to a lesser extent CFU-GM (451±62 versus 733±208). At E12.5, total cellularity and total CFC content in *Mpl*^{-/-} livers were very similar to those of C57Bl6 livers, except for CFU with MK and CFU-GEMM, which were found to be less abundant (691±63 CFU with MK versus 1357.5±165 for C57Bl6 controls, and 148.5 CFU-GEMM versus 212.5 for C57Bl6). However, 2 days later, at E14.5, *Mpl*^{-/-} FL was found to be profoundly defective in all classes of HP (76% deficiency in total CFC, with 73, 66, 63 and 95% defects in CFU-GM, BFU-E, CFU-GEMM and CFU with MK, respectively). Statistical analysis revealed that this defect is significant for total CFC, CFU-GM and for CFU with MK.

HSC potential decreases during *Mpl*^{-/-} FL development

We next analyzed the HSC potential of *Mpl*^{-/-} FL. No LTR activity was detected in the FL at E11.5, even when as many as 4 ee were used to reconstitute one adult irradiated recipient (Table 2). By contrast, LTR activity was observed in 70% of reconstituted mice injected with 1 ee control C57Bl6 FL at the same stage (Table 2), and this LTR activity was associated with the presence of HSCs (100% of secondary reconstituted mice injected with 5×10⁵ bone marrow cells from primary recipients, data not shown). These results indicate that at E11.5, *Mpl*^{-/-} FL already presents a profound HSC defect.

Surprisingly, not only did E12.5 *Mpl*^{-/-} FL not display any defect in primary transplants, but they even exhibited a better LTR ability than control E12.5 C57Bl6 FL (Table 2). However, as observed for AGM, in secondary transplantations the median of participation of grafted cells to hematopoietic reconstitution was much lower with bone marrow issued from *Mpl*^{-/-} FL injection (11% versus 35%) (Table 2), which indicates a defect of HSCs in E12.5 *Mpl*^{-/-} FL. This normal LTR ability observed in the E12.5 FL was rather surprising, given the fact that at E11.5 we observed an impaired HSC activity in the AGM associated with a profound HSC defect in the FL. The delayed production of HSCs by *Mpl*^{-/-} AGM can be taken into account to partly explain this, yet it cannot explain by itself the high LTR ability observed. Recently, placenta has been shown to be a source of definitive HSCs during mouse development (Gekas et al., 2005), with a peak production at E12-13 (Ottersbach and Dzierzak, 2005). We therefore investigated the LTR ability of *Mpl*^{-/-} placenta at E12.5. As shown in Table 2, E12.5 *Mpl*^{-/-} placentas contained more HPs endowed with LTR ability than C57Bl6 placentas. However, a defect in HSC content was detected, as illustrated by the low percentage of secondary reconstituted mice and the low median of reconstitution. Although to a lesser extent, an input of LTR cells from E12.5 *Mpl*^{-/-} YS was also detected (Table 2). This contribution of LTR cells from AGM, placenta and YS through the blood stream could play a significant

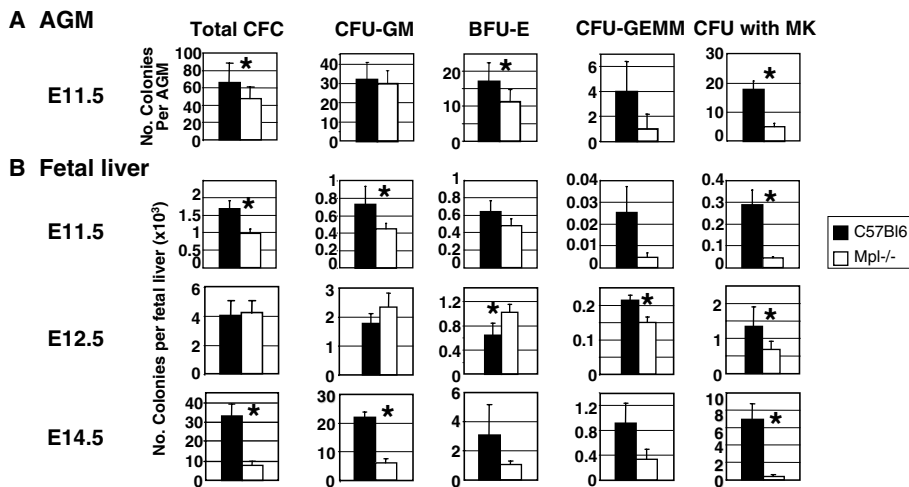


Fig. 5. Comparison of the CFC content of *Mpl*^{-/-} and C57Bl6 mouse embryos at E11.5, E12.5 and E14.5. Total CFCs and CFU-GM, BFU-E, CFU-GEMM and CFU with MK content of (A) E11.5 AGM and (B) E11.5, E12.5 and E14.5 FL are presented. Half an AGM suspension and 5 × 10⁴ FL cells were used in each Petri dish. The results of a single experiment representative of two other independent experiments are shown. For each experiment, three independent Petri dishes were prepared and scored, and the results shown represent the mean ± s.e. *, *P* < 0.05.

part in the high LTR ability of E12.5 *Mpl*^{-/-} FL. This was supported by the fact that LTR cells were slightly more abundant in blood from E12.5 *Mpl*^{-/-} embryos than in blood from control C57Bl6 embryos (data not shown).

As maximum expansion of HSCs takes place at E14.5 in the FL (Morrison et al., 1995), we analyzed E14.5 crude *Mpl*^{-/-} FL cells in reconstitution assays. No animals could be reconstituted with 1 × 10⁴ *Mpl*^{-/-} cells, and only 40% of the mice were reconstituted with 5 × 10⁴ cells (instead of 20 and 60% when 1 × 10⁴ and 5 × 10⁴ C57Bl6 FL cells were injected, respectively; see Table 2), which seems to indicate that the *Mpl*^{-/-} E14.5 FL presents a defect in LTR ability. Secondary transplantations showed that this deficiency is accompanied by a deficit in HSCs (40% reconstitution with bone marrow cells prepared from mice reconstituted with *Mpl*^{-/-} FL cells, with a maximum level of reconstitution of only 38%, versus 90% with bone marrow cells prepared from mice reconstituted with control C57Bl6 FL cells, with a maximum level of reconstitution of 80%) (Table 2). It appears, then, that E14.5 *Mpl*^{-/-} FL presents a general defect in HPs and HSCs. Taken together, these results indicate that HSC potential decreases during FL development in *Mpl*^{-/-} embryos.

Defect in amplification and self-renewal/survival of lin⁻ AA4.1⁺ Sca1⁺ HSCs in *Mpl*^{-/-} FL

In order to more accurately analyze the *Mpl*^{-/-} HSC content of the FL at E12.5, we sorted the lin⁻ CD34⁺ c-kit⁺ population (enriched in HPs and HSCs) and the lin⁻ AA4.1⁺ Sca1⁺ population (highly

enriched in HSCs) (Jordan et al., 1990), and compared their LTR ability to that of the same populations sorted from control C57Bl6 FL. Whereas no significant difference in the LTR ability was observed between *Mpl*^{-/-} and C57Bl6 FL lin⁻ CD34⁺ c-kit⁺ cells (Table 3), *Mpl*^{-/-} lin⁻ AA4.1⁺ Sca1⁺ cells presented a 4- to 5-fold defect in their ability to engraft irradiated recipients (only 12.5% engrafted recipients with 2000 *Mpl*^{-/-} cells, versus 57% with the same number of C57Bl6 cells). Likewise, long-term in vitro cultures on MS5 stroma revealed that *Mpl*^{-/-} lin⁻ AA4.1⁺ Sca1⁺ presented three times fewer day-28 (D28) CAFs than control lin⁻ AA4.1⁺ Sca1⁺ (1/1750 instead of 1/620). Interestingly, whereas E12.5 *Mpl*^{-/-} FL presented a total cellularity equivalent to that of C57Bl6 FL, it was found to contain three times more lin⁻ AA4.1⁺ Sca1⁺ cells than control FL (10,767 ± 4592 for *Mpl*^{-/-} FL, versus 3597 ± 1985 for control FL) (see Fig. S1 in the supplementary material).

As for E12.5, at E14.5 the total cellularity per FL was equivalent in *Mpl*^{-/-} and C57Bl6 embryos. Nevertheless, at this stage, *Mpl*^{-/-} FL contained half the number of lin⁻ AA4.1⁺ Sca1⁺ HSCs cells than control FL (4104 ± 1231 for *Mpl*^{-/-} FL, versus 8219 ± 4244 for control FL; see Fig. S1 in the supplementary material). Indeed, whereas the number of lin⁻ AA4.1⁺ Sca1⁺ cells per FL was found to have increased 2.3-fold in control embryos between E12.5 and E14.5 (3597 ± 1985 at E12.5, and 8219 ± 4244 at E14.5), it decreased 2.6-fold in *Mpl*^{-/-} animals (10,767 ± 4592 at E12.5, and 4104 ± 1231 at E14.5). A substantial defect in LTR activity was also observed in the *Mpl*^{-/-} sorted cells (Table 3), associated with a 3-fold defect in D28 CAFs (1/176 for *Mpl*^{-/-} lin⁻ AA4.1⁺ Sca1⁺ versus 1/60 for controls).

Table 1. In vivo LTR (primary transplantation) and HSC activity (secondary transplantation) of *Mpl*^{-/-} and C57Bl6 AGM

		Primary transplantation (LTR)			Secondary transplantation (HSC)		
		No. organs or cells injected	Engrafted mice/Total mice	Median of reconstitution (%)	No. cells injected	Engrafted mice/Total mice	Median of reconstitution (%)
AGM	E11.5	<i>Mpl</i> ^{-/-}	1	1/7	16	5 × 10 ⁵	28
			3	2/6	75		
		C57Bl6	1	3/10	78		
			3	4/7	89		
	E12.5	<i>Mpl</i> ^{-/-}	1	6/10	62	5 × 10 ⁵	6
		C57Bl6	1	1/10	81	5 × 10 ⁵	38

Single-cell suspensions of AGM were injected into irradiated adult recipients. For LTR activity, each result represents the number of engrafted mice at ≥ 20 weeks post-transplantation (showing more than 5% donor CD45.2 cells in peripheral blood) out of the total number of mice transplanted. Two or three independent experiments were performed. For secondary transplantations, 5 × 10⁵ bone marrow cells isolated from primary recipients with high chimerism were injected, and mice were analyzed 7 weeks post-transplantation (two independent experiments).

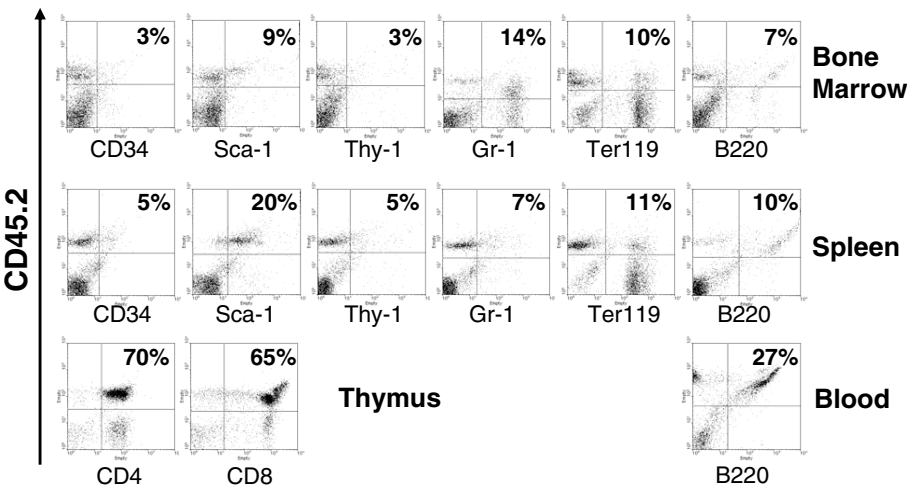


Fig. 6. Flow cytometric analysis of bone marrow, spleen, thymus and blood from mice reconstituted with *Mpl*^{-/-} E11.5 AGM. Twenty weeks after injection, primary reconstituted mice were sacrificed and cell suspensions prepared from femoral marrow, spleen and thymus. A peripheral blood sample was also taken and treated as described in Materials and methods. Bone marrow and spleen cells were co-incubated with Ly5.2 antibody coupled to phycoerythrin (PE) and CD34, Sca1, Thy1, Ter119, Gr1 or B220 antibodies directly or indirectly coupled to FITC. Thymus cells were co-incubated with Ly5.2-PE and CD4-FITC or CD8-FITC. Blood cells were co-incubated with Ly5.2 and B220. Percentages of double-positive cells are indicated in the upper right quadrant.

DISCUSSION

A decade ago, it was shown that in the mouse embryo, adult-type HSCs are generated autonomously in an intraembryonic region surrounding the dorsal aorta known as the aorta-gonad-mesonephros (AGM) region (Durand and Dzierzak, 2005). This finding was subsequently extended to the human embryo (Tavian and Peault, 2005). These observations have since led to many investigations into the generation of definitive HSCs during development, and among the various approaches used, gene-targeted mice have proven helpful in drawing up a map of regulatory molecules that direct blood cell development (Teitell and Mikkola, 2006). In the present report, we show that *Mpl*, the receptor of the TPO cytokine known to be an important regulator of adult HSC self-renewal and proliferation, is also an important embryonic HSC regulator.

We determined the expression of *Mpl* in the embryo by ISH at various times of development. *Mpl* was found to be expressed from E10.5 onwards, in the YS, FL rudiment and in the first intraembryonic HSCs that emerge from the floor of the dorsal aorta in the AGM region (see Fig. 2). In this latter region, no *Mpl* signal was detected in the endothelial floor of the aorta. Notably, *TPO* transcripts could also be detected in the AGM region by q-PCR (Fig. 3B). We did not detect any *Mpl* expression before E10.5 by ISH. In a transgenic mouse model in which the human placental alkaline phosphatase gene was under the control of the promoter and regulatory elements of the mouse *Mpl* gene, *Mpl*-positive cells were found in the YS and embryo proper as early as E8.5 (Ziegler et al., 2002). This discrepancy may be attributed to the fact that very few cells express *Mpl* at this stage, and/or that *Mpl* expression is too faint

Table 2. In vivo LTR (primary transplantation) and HSC activity (secondary transplantation) of *Mpl*^{-/-} and C57Bl6 FL, placenta and yolk sac

		Primary transplantation (LTR)			Secondary transplantation (HSC)		
		No. organs or cells injected	Engrafted mice/Total mice	Median of reconstitution (%)	No. cells injected	Engrafted mice/Total mice	Median of reconstitution (%)
Fetal liver	<i>Mpl</i> ^{-/-}	1	0/5				
		2	0/8				
		3	0/6				
		4	0/3				
	C57Bl6	0.5	1/10	91			
		1	7/10	72			
Fetal liver	<i>Mpl</i> ^{-/-}	5×10 ⁴	10/15	90	5×10 ⁵	4/10	11
		1×10 ⁵	11/15	89			
		5×10 ⁴	2/15	78	5×10 ⁵	4/10	35
		1×10 ⁵	7/15	83			
	<i>Mpl</i> ^{-/-}	1×10 ⁴	0/5				
		5×10 ⁴	2/5	91	5×10 ⁵	4/10	25
Fetal liver	C57Bl6	1×10 ⁴	1/5	81	5×10 ⁵	9/10	51
		5×10 ⁴	3/5	80			
Placenta	<i>Mpl</i> ^{-/-}	0×1	3/15	79	5×10 ⁵	5/15	21
		0×3	10/20	70			
		0×1	2/15	49	5×10 ⁵	10/10	49
		0×3	8/20	88			
	C57Bl6	1	3/12	85			
		1	0/10				

Single-cell suspensions of FL, placenta and yolk sac were injected into irradiated adult recipients. For LTR activity, each result represents the number of engrafted mice at ≥20 weeks post-transplantation out of the total number of mice transplanted. Two or three independent experiments were performed. For secondary transplantations, 5×10⁵ bone marrow cells isolated from primary recipients with high chimerism were injected, and mice were analyzed 7 weeks post-transplantation (two independent experiments). ND, not determined.

Table 3. In vivo LTR and HSC activity of *Mpl*^{-/-} and C57Bl6 lin⁻ CD34⁺ c-kit⁺ and lin⁻ Sca1⁺ AA4.1⁺ cells sorted from E12.5 and E14.5 FL

	Primary transplantation (LTR)		
	No. cells injected	Engrafted mice/ Total mice	Median of reconstitution (%)
lin⁻ CD34⁺ c-kit⁺			
E12.5 <i>Mpl</i> ^{-/-}	5000	9/10	61
	20,000	7/10	81
C57Bl6	5000	9/10	60
	20,000	10/10	79
lin⁻ Sca1⁺ AA4.1⁺			
E12.5 <i>Mpl</i> ^{-/-}	500	0/5	56
	2000	1/8	
C57Bl6	500	3/5	74
	2000	4/7	90
E14.5 <i>Mpl</i> ^{-/-}	100	0/3	40
	500	0/8	
	2000	2/5	
C57Bl6	100	6/14	30
	500	6/9	84

lin⁻ CD34⁺ c-kit⁺ and lin⁻ AA4.1⁺ Sca1⁺ cells were sorted from *Mpl*^{-/-} and C57Bl6 FL at E12.5 and E14.5 and injected into irradiated adult recipients.

to be detected by ISH. Indeed, using relative q-PCR analysis, we were able to detect a low level of *Mpl* in the PSP region at E9.5 (Fig. 3A), indicating that this latter possibility is likely to be the case. In the FL, the number of *Mpl*-positive spots reaches a peak at E11.5, and although it decreases sharply after E14.5, expression persists in this organ until birth (Fig. 2). Therefore, our results indicate that *Mpl* expression is associated with the main phases of hematopoiesis in the embryo and is linked to the emergence (AGM), expansion and maturation (FL) of HSCs.

The overall pattern of *Mpl* expression parallels that of the platelet glycoprotein IIb (GpIIb; also known as CD41 and αIIb integrin), classically viewed as a megakaryocyte- and platelet-specific marker (Ginsberg et al., 1995), which has recently been detected on immature hematopoietic cells in the mouse embryo (including sites of HSC emergence) and on multipotential myeloid progenitors (Corbel and Salaun, 2002; Berridge et al., 1985; Debili et al., 1992; Tronik-Le Roux et al., 1995). A functional role for GpIIb in the regulation of progenitor cell number in the YS, FL and bone marrow was demonstrated in *GpIIb*-null mice (Emambokus and Frampton, 2003).

Our analysis of the hematopoietic potential of *Mpl*^{-/-} embryonic and fetal sites of hematopoiesis allowed us to underline the functional role of *Mpl* receptor during the various stages of the establishment of definitive hematopoiesis in the mouse (Fig. 7).

The AGM is a site of emergence of definitive HSCs that are able to colonize the FL. In the AGM, induction and generation of hematopoietic cells coincide with that of endothelial cells, and HSCs are produced and proliferate without differentiating. In the AGM, the absence of *Mpl* signal transduction leads to a delayed production of HSCs, as shown by: (1) the 2-fold lower LTR ability of AGM, associated with the lack of LTR potential of the FL at E11.5 (indicating that this organ has not yet been colonized by HSCs); and (2) the fact that LTR potential increases in E12.5 *Mpl*^{-/-} AGM (Table 1). Importantly, not only did the lack of *Mpl* lead to delayed production of clusters of definitive hematopoietic cells in the AGM region, but it also resulted in the production of HSCs with an impaired activity, as demonstrated by secondary transplants.

The importance of *Mpl* signaling in the hematopoietic commitment of mesodermal cells has already been suggested by our previous data based on in vitro differentiation of ES cells (Challier et al., 2002). This has been strengthened by the study of Perlingeiro et al., who showed that TPO stimulates hemangioblast formation during ES cell differentiation in vitro, indicating a role for TPO at the earliest stages of hematopoietic cell commitment (Perlingeiro et al., 2003). Interestingly, it has recently been shown that the expression of VEGF-A (also known as Vegfa), a key factor in the process of induction of the hemangioblastic and hematopoietic

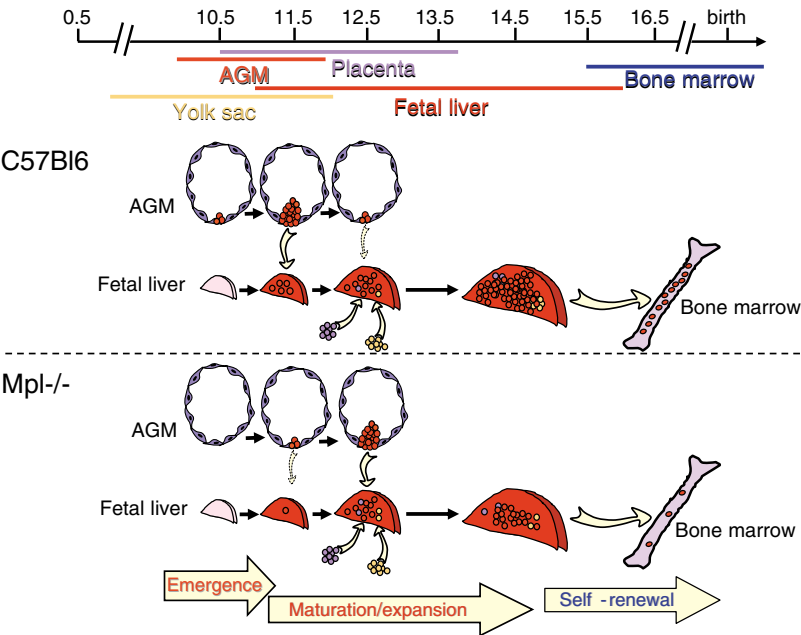


Fig. 7. Different impacts of the absence of *Mpl* signaling during establishment of definitive hematopoiesis in the mouse embryo. The anatomical locations involved in the generation of the pool of HSCs are shown, together with the timing of their activity on a timescale of embryonic days. AGM and placenta are the sites of production of the first HSCs (Emergence). These cells (red) migrate to the FL, where they mature further and expand (Maturation/expansion). The FL is also colonized by HSCs coming from the YS (yellow) and placenta (purple). Ultimately, HSCs establish steady-state levels in the bone marrow, where balance between self-renewal, quiescence and differentiation is tightly regulated. The absence of *Mpl* leads to a delayed and defective production of HSCs by the AGM region, and to a defect in amplification and self-renewal/survival of HSCs in the FL, which in turn results in the HSC defect described for the adult bone marrow.

development of ES cells (Choi et al., 1998), is enhanced by TPO in primitive hematopoietic cells (Kirito et al., 2005). In the AGM, TPO could act synergistically with VEGF-A, therefore enhancing the commitment and induction of hematopoietic cells, which would explain the delayed production of HSCs in the absence of Mpl signaling. The defect in the activity of HSCs produced by *Mpl*^{-/-} AGM underlines an additional specific role of Mpl signaling in the self-renewal/survival of HSCs at the earliest stages of their emergence in the embryo. Of interest, Mpl has possible interactions with *Runx1*, one of the genes involved in the emergence of definitive HSCs in the AGM region (Teitell and Mikkola, 2006): on the one hand, TPO/Mpl signaling can regulate the activity of Runx1 through the ERK pathway (Hamelin et al., 2006); on the other hand, Runx1 can putatively regulate Mpl expression, as three Runx1 binding sites are present in the promoter region of *Mpl* (Heller et al., 2005).

The FL is the main site of HSC expansion and differentiation; the first HSCs appear in this organ at E11.5 and are likely to come from the AGM and placenta (Mikkola and Orkin, 2006). Indeed, the delayed production of HSCs by *Mpl*^{-/-} AGM led to a delayed colonization of FL by HSCs, as no LTR activity could be detected in the E11.5 *Mpl*^{-/-} FL (Table 2). Interestingly, 1 day later, normal amounts of both HPs and LTR cells were detected in this organ (Fig. 5B and Table 2). Our data showed that this can be explained by an influx of LTR cells derived not only from the AGM at E12.5, but also from the placenta and YS through blood circulation (Table 2). These results are in agreement with recent studies indicating that besides AGM, inputs of HSCs come from E12 YS (Kumaravelu et al., 2002) and placenta (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). However, HSCs present in E12.5 *Mpl*^{-/-} FL had a defect in their ability to self-renew. This is at least in part the result of the colonization of FL by the HSCs produced by the *Mpl*^{-/-} AGM, which already presented an impaired activity. Moreover, our data clearly indicate that HP, LTR activity and HSC potential decrease during *Mpl*^{-/-} FL development (Fig. 5B and Table 2), and underline a defect in amplification and self-renewal/survival of the lin⁻ AA4.1⁺ Sca1⁺ population of HSCs. This step in HSC maturation in the FL also corresponds to the timing of TPO production by this organ (Nomura et al., 1997). HSCs lacking Mpl are not exposed to the signals mediated through TPO, which would normally help them to become functional adult-type HSCs and to retain self-renewal and differentiation properties. The role of TPO in the self-renewal capacity of adult HSCs in vivo has indeed been demonstrated in a hematopoietic reconstitution model in which wild-type HSCs were grafted in TPO^{-/-} mice (Fox et al., 2002; Kaushansky, 2003). The genes involved in the TPO-mediated steps of amplification and self-renewal of HSCs during their passage in the FL remain to be determined.

The differential role of Mpl that we underline in this work might be related to the properties of the various hematopoietic sites employed during development, and to the inductions/restrictions that are imposed on HSCs in these various territories. At any rate, our findings indicate that Mpl expression is important for HSCs at the earliest stages of their emergence in the mouse embryo, and that it could have different impacts on the generation and the expansion of HSCs during the establishment of definitive hematopoiesis.

We thank Adeline Gein and Vincent Ravault for technical assistance; Marie Claude Labastie for introducing us to the ISH technique; and Thierry Jaffredo, Charles Durand and Laure Coulombel for critical reading of the manuscript and helpful discussions around it. This work was supported by the Institut National de la Recherche Médicale (Inserm), the Institut de Cancérologie et d'Immuno-Génétique (ICIG) and by grants from the Ligue Nationale contre le Cancer (LNC), the Association ANRB Vaincre le Cancer, and the Groupement

des Entreprises Françaises dans la Lutte contre le Cancer (GEFLUC). M.F. is supported by the Ministère de l'Éducation Nationale, de la Recherche et de la Technologie (MENRT).

Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/16/3031/DC1>

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Table S1. Total number of cells per fetal liver, placenta and yolk sac in *Mpl*^{-/-} and C57Bl6 embryos at the various times of development used for the different experiments described in the manuscript

		Total number of cells per organ (\pm s.d.) $\times 10^6$	
		C57Bl6	<i>Mpl</i> ^{-/-}
Fetal liver	E11.5 ($n=7$)	0.28 \pm 0.13	0.23 \pm 0.16
Fetal liver	E12.5 ($n=29$)	1.41 \pm 0.91	1.59 \pm 0.94
Fetal liver	E14.5 ($n=15$)	9.2 \pm 3.2	8.9 \pm 3.6
Placenta	E12.5 ($n=5$)	1.2 \pm 0.85	1.1 \pm 0.28
Yolk sac	E12.5 ($n=7$)	1.37 \pm 0.74	1.32 \pm 0.75