

Mpl receptor defect leads to earlier appearance of hematopoietic cells/hematopoietic stem cells in the Aorta-Gonad-Mesonephros region, with increased apoptosis

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ABSTRACT In a previous study, we underlined the functional role of the TPO receptor, *Mpl*, in the establishment of definitive mouse hematopoiesis, by demonstrating that the lack of *Mpl* led to a delayed production of definitive hematopoietic cells in the aorta-gonad-mesonephros (AGM) region, and resulted in the production of hematopoietic stem cells (HSCs) with an impaired activity at E11.5. In order to more accurately estimate the role of *Mpl* during generation of HSCs in the aorta, we performed an analysis of these AGMs at the time of the first HSC emergence (E10.5). Our results indicated that while *Mpl*^{-/-} AGMs were found to contain more hematopoietic cells (HC) than C57Bl6 AGMs at E10.5, a defect in the expansion process of the HC/HSCs was detected in explant cultures of these AGMs, likely due to an increased apoptosis of these cells. To determine the molecular mechanisms by which invalidation of *Mpl* receptor affects the temporal distribution and expansion of HC/HSCs in the AGM, a study of the transcription level of *Mpl* target genes was conducted. Expression of *Runx1*, a master transcription factor for the formation of hematopoietic progenitor (HP) cells and HSCs from the vasculature, as well as expression of *Meis1* and *HoxB4*, known to play a role in self-renewal and expansion of HSCs, were found to be down regulated in E10.5 *Mpl*^{-/-} AGMs. Our data indicate that *Mpl* is an active player during the first steps of definitive hematopoiesis establishment through direct regulation of the expression of transcription factors or genes important for the self-renewal, proliferation and apoptosis of HSCs.

KEY WORDS: *Mpl* receptor, HSC, AGM, apoptosis, *Runx1*

Introduction

In the mouse the first definitive intraembryonic Hematopoietic Stem Cells (HSCs) are generated in the aorta, in the Aorta-Gonad-Mesonephros (AGM) region, at embryonic day 10.5 (E10.5) (de Bruijn *et al.*, 2000; Godin *et al.*, 1993; Medvinsky and Dzierzak, 1996; Medvinsky *et al.*, 1993). The microenvironment provided by the AGM site plays a key role in this process, and extensive analysis of this microenvironment can reveal important regulatory molecules which direct blood cell development. One of the key molecules involved in cluster formation is the transcription factor *Runx1*. *Runx1* is the DNA-binding subunit of the heterodimeric transcription factor *Runx1*-*CBFβ* and it is a specific marker of hematopoietic clusters in many species (North *et al.*, 1999; Bollerot *et al.*, 2005). Embryos deficient for *Runx1* show no hematopoietic clusters, and do not display any detectable HSC (North *et al.*, 1999; Cai *et al.*, 2000; Yokomizo *et al.*, 2001; North

et al., 2002). Very recently, *Runx1* function has been demonstrated to be essential in endothelial cells for the formation of hematopoietic progenitors (HP) and HSCs from the vasculature (Chen *et al.*, 2009). It is reasonable to hypothesize that cytokines and their receptors, which are important in promoting HSC self-renewal, proliferation and differentiation in the adult, could also represent critical regulators in the establishment of definitive hematopoiesis. Indeed, a recent body of data support this assumption, since IL-3 was found to be an important embryonic HSC regulator as a proliferation and survival factor for the earliest HSCs in the embryo (Robin *et al.*, 2006), and IL-1 was shown to

Abbreviations used in this paper: AGM, Aorta-Gonad-Mesonephros region; FACS, fluorescence activated cell sorting; HC, hematopoietic cell; HSC, hematopoietic stem cell; *Mpl*, myeloproliferative leukemia; TPO, thrombopoietin.

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play a role in the normal regulation of HP/HSCs in the midgestation mouse AGM (Orelia *et al.*, 2008). Our own work underlined the functional role of the TPO receptor, *Mpl*, in the establishment of definitive mouse hematopoiesis: we demonstrated that the lack of *Mpl* led to a delayed production of definitive hematopoietic cells in the AGM region, and resulted in the production of HSCs with an impaired activity in E11.5 AGM (Petit-Cocault *et al.*, 2007).

In the present study, we tried to more accurately estimate the role of *Mpl* during the generation of HSCs in the aorta as well as the molecular mechanisms which are deficient in E10.5 *Mpl*^{-/-} AGM. By comparing the hematopoietic content of E10.5 *Mpl*^{-/-} and C57Bl6 AGMs, we could show that indeed *Mpl*^{-/-} AGMs contain more hematopoietic cells (HC) than C57Bl6 AGMs. However, explants cultures allowed to underline a defect in the expansion process of the HC/HSCs in these AGMs, likely due to an increased apoptosis of these cells. In addition, we show that expression of *Runx1*, as well as expression of *Meis1* and *HoxB4*, two target genes of *Mpl* known to play a role in self-renewal and expansion of HSCs (Sauvageau *et al.*, 1994; Thorsteinsdottir *et al.*, 1999; Antonchuk *et al.*, 2001; Antonchuk *et al.*, 2002; Hisa *et al.*, 2004; Azcoitia *et al.*, 2005), are down regulated in E10.5 *Mpl*^{-/-} AGMs. Taken all together, our data indicate that *Mpl* is an active player during the first steps of definitive hematopoiesis establishment through direct regulation of the expression of transcription factors or genes important for self-renewal, proliferation and apoptosis of HSCs.

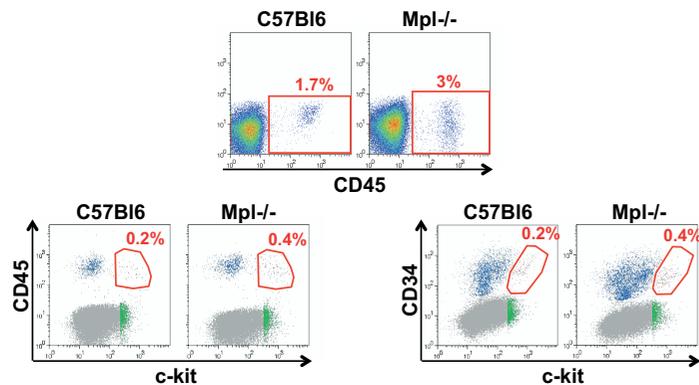
Results

High hematopoietic content of E11.5 and E10.5 *Mpl*^{-/-} AGMs

In a previous study, we showed that E11.5 *Mpl*^{-/-} AGMs presented an impaired HSC activity, and we postulated that lack of *Mpl* receptor led to a delayed production of clusters of definitive cells in the AGM region (Petit-Cocault *et al.*, 2007). In order to further investigate this hypothesis, we first compared the hematopoietic content of E11.5 AGMs from *Mpl*^{-/-} and C57Bl6 embryos. After dissection and dissociation by collagenase treatment, AGM cell suspensions were tested by FACS analysis for the presence of CD45⁺ HCs, CD45⁺*c-kit*^{hi} HC/HP cells, and CD34⁺*c-kit*^{hi} HP/HSC enriched populations. No defect in the percentage of HC/HSCs was detected in *Mpl*^{-/-} AGMs (sometimes a higher percentage was even detected in these later, as illustrated in Fig. 1A). When these percentages were related to the total number of cells per AGM, the number of CD45⁺*c-kit*^{hi} and CD34⁺*c-kit*^{hi} cells per AGM

was equivalent in *Mpl*^{-/-} and C57Bl6 AGMs (Fig. 1C). Then the HP/HSC content of *Mpl*^{-/-} AGMs at E11.5 does not corroborate the delayed production of these cells. Since the first clusters of HC emerge in the AGM region around E10.5, we next compared the CD41⁺ (early HCs), CD45⁺, CD45⁺*c-kit*^{hi}, CD144⁺CD45⁺ and CD34⁺*c-kit*^{hi} content in AGMs from *Mpl*^{-/-} and C57Bl6 embryos at this stage. The total number of cells per AGM was found to be equivalent in *Mpl*^{-/-} and C57Bl6 (144 707 ± 46 733 versus 127 863 ± 23 313, respectively). As illustrated in Fig. 1B, the *Mpl*^{-/-} AGMs did not show any defect in the percentage of HCs (CD41⁺ and

E11.5 AGM



E10.5 AGM

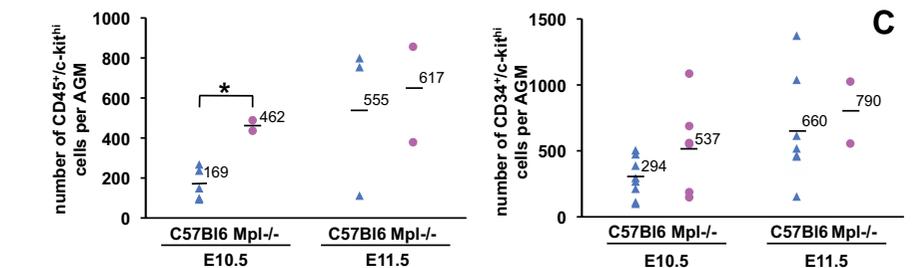
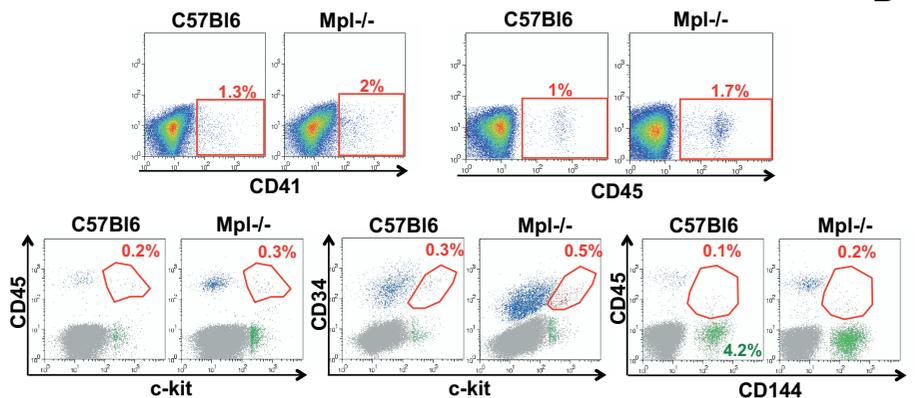


Fig. 1. Fluorescence-activated cell sorting (FACS) analysis of the hematopoietic content of *Mpl*^{-/-} AGMs. AGMs dissected from E11.5 (A) and E10.5 (B) C57Bl6 and *Mpl*^{-/-} embryos were dissociated by collagenase treatment, and tested for the presence of CD45⁺, CD45⁺*c-kit*^{hi} and CD34⁺*c-kit*^{hi} (E11.5) or CD41⁺, CD45⁺, CD45⁺*c-kit*^{hi}, CD34⁺*c-kit*^{hi} and CD144⁺CD45⁺ (E10.5) populations by FACS analysis. The majority of *c-kit*⁺ cells express *c-kit* as a continuum. *c-kit*^{hi} refers to the distinct minor population, which expresses high levels of *c-kit* and is indicated by a polygon on the slot dots. (C) Comparison of the total number of CD45⁺*c-kit*^{hi} and CD34⁺*c-kit*^{hi} per C57Bl6 and *Mpl*^{-/-} AGM. * *p* < 0.05.

CD45⁺), HPs (CD45⁺c-kit^{hi}) or HP/HSCs populations (CD144⁺CD45⁺ and CD34⁺c-kit^{hi}): on the contrary, most of the time the percentages of hematopoietic cells were higher in the Mpl^{-/-} AGMs. As for E11.5, when these percentages were related to the total number of cells per AGM, Mpl^{-/-} AGMs presented an equivalent or greater number of hematopoietic cells than C57Bl6 AGMs, as illustrated on Fig. 1C and 2A for CD34⁺c-kit^{hi} (537 ± 316 versus 294 ± 143) and CD45⁺c-kit^{hi} (462 ± 26 versus 169 ± 71) cells. The difference is statistically significant for the CD45⁺c-kit^{hi} population (p<0.05).

Taken all together, these data indicate that the LTR-HSCs defect that we described in the Mpl^{-/-} E11.5 AGMs does not result from a delayed production of hematopoiesis at E10.5.

E10.5 AGM explant cultures reveal a defect of CD34⁺c-kit^{hi} and CD45⁺c-kit^{hi} expansion in Mpl^{-/-} embryos

Cultured E10/E11 AGMs have been shown to autonomously generate and expand HC/HSCs (Medvinsky and Dzierzak, 1996). We therefore compared the expansion of the CD45⁺c-kit^{hi} and CD34⁺c-kit^{hi} populations after organ explant cultures of E10.5 Mpl^{-/-} or C57Bl6 AGMs. Both populations were amplified during Mpl^{-/-} AGMs explant cultures, and after 3 days of culture an equivalent number of CD45⁺c-kit^{hi} and CD34⁺c-kit^{hi} cells per AGM was generated in Mpl^{-/-} and C57Bl6 AGMs (Fig. 2A and Fig. 2B, left panel). However, the amplification of these populations was less important for Mpl^{-/-} AGMs than for C57Bl6 AGMs. While an average of 13.2 fold increase of the CD45⁺c-kit^{hi} population was observed with C57Bl6 AGMs explant cultures, Mpl^{-/-} AGMs only displayed a 6.3 fold increase. This difference of expansion was further emphasized for the CD34⁺c-kit^{hi} population, which was increased only 3 fold during the Mpl^{-/-} explant culture and 7.6 fold during C57Bl6 AGM culture (p<0.05)(Fig. 2 A,B, right panels). The most important defect in amplification was observed for the CD41⁺ population, which was not amplified at all in Mpl^{-/-} AGMs (3800 CD41⁺ cells at D0, and 3350 at day 3 of culture explants), while a 2.7 fold amplification could be observed in C57Bl6 AGMs (1900 CD41⁺ cells at D0, and 5190 at day 3 of culture explants).

Increased apoptosis in E10.5 Mpl^{-/-} AGM

The reduced amplification of the HC/HSC population during Mpl^{-/-} AGM explant cultures prompted us to analyze the expression of proapoptotic (*Bim*) and antiapoptotic (*Bcl-2* and *Bcl-X_L*) genes in E10.5 Mpl^{-/-} and C57Bl6 aortas. In fact, antiapoptotic and proapoptotic processes have been shown to be active in the earliest HSCs in the AGM region, suggesting that apoptosis was an important player in the regulation of these first HSCs expansion (Orelia *et al.*, 2004). The level of *Bim*, *Bcl-2* and *Bcl-X_L* expression was evaluated by q-PCR. A significant reduction of both *Bcl2* (1.6 fold, p<0.001) and *Bcl-X_L* (1.5 fold, p<0.05) antiapoptotic genes expression was observed in Mpl^{-/-} AGMs, while the level of expression of

the proapoptotic *Bim* gene was comparable in E10.5 Mpl^{-/-} and C57Bl6 AGMs (Fig. 3A).

The reduced expression of antiapoptotic genes in Mpl^{-/-} AGM prompted us to examine whether the decreased expansion of CD34⁺c-kit^{hi} and CD45⁺c-kit^{hi} populations in Mpl^{-/-} AGMs after 3 days of explant culture could be related to an increased apoptosis. Flow cytometric analysis with Annexin V, an early marker of apoptosis and 7AAD staining was therefore performed on gated CD45⁺c-kit^{hi} and CD34⁺c-kit^{hi} fractions (Fig. 3 B,C). As shown on Fig. 3D, the percentage of Annexin V⁺ 7AAD⁻ preapoptotic cells in the CD45⁺c-kit^{hi} and CD34⁺c-kit^{hi} fractions of Mpl^{-/-} AGM explants were increased 1.4 fold (p<0.05) and 1.9 fold respectively, as compared to wild-type AGM explants. In addition, a 1.2 fold reduced expression of *Bcl-2* (p<0.05) and *Bcl-X_L* (p<0.05) was still detected in Mpl^{-/-} AGM explants after 3 days of culture (Fig. 3E). This increased apoptosis can at least in part explain the reduced expansion of these populations during explant cultures of Mpl^{-/-} AGMs.

Molecular targets of Mpl are downregulated during emergence of HP/HSCs in E10.5 Mpl^{-/-} AGMs

We attempted to determine the molecular mechanisms by which invalidation of Mpl receptor affects the temporal distribution and the expansion of HC/HSCs in the AGM. TPO has been shown to enhance HoxB4 and Meis1 expression (Kirito *et al.*, 2003; Kirito *et al.*, 2004; Orelia *et al.*, 2004), two Hox genes known to play a role in self-renewal and expansion of adult HSCs. Expression of VEGF-A, a key factor in the process of induction of the hemangioblastic and hematopoietic development of ES cells (Choi *et al.*, 1998) and required for HSC survival (Gerber *et al.*,

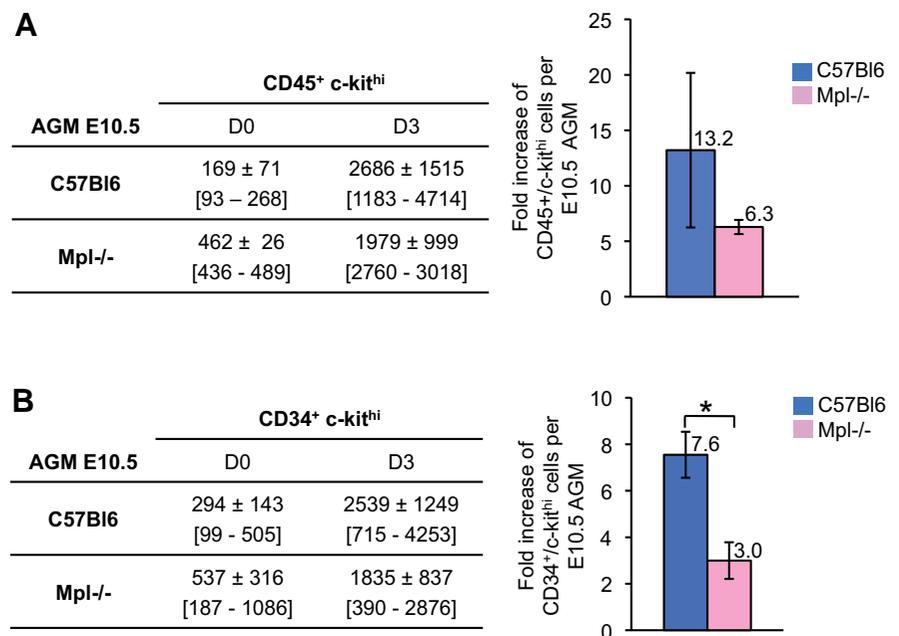


Fig. 2. Analysis of the hematopoietic content of Mpl^{-/-} aorta-gonad-mesonephros (AGMs) before and after culture explant. Total number of CD45⁺c-kit^{hi} (A) and CD34⁺c-kit^{hi} (B) per C57Bl6 or Mpl^{-/-} AGMs ± SD (n=3) determined after FACS analysis at D0 and D3 after culture explants are reported on the left tables. Range is indicated in the brackets [range]. Histograms on the right show the fold increase of CD45⁺c-kit^{hi} (A) and CD34⁺c-kit^{hi} (B) populations per E10.5 AGMs during the 3 days of culture explants. * p<0.05.

2002), is also induced by TPO (Kirito *et al.*, 2005). We therefore studied the level of transcription of these target genes by q-PCR in E10.5 AGMs from *Mpl*^{-/-} and control embryos. As shown on Fig. 4A, both *Meis1* and *HoxB4* expression were found to be decreased in *Mpl*^{-/-} AGM (1.5 fold, $p < 0.001$ and 1.2 fold respectively) compared to control C57Bl6 AGMs, while no difference could be observed for VEGF-A expression.

The high hematopoietic content of E10.5 *Mpl*^{-/-} AGMs is reminiscent of the change in the temporal distribution of HSCs observed with *Runx1* haploinsufficiency (Cai *et al.*, 2000). In addition, interactions between *Mpl* and *Runx1* have been recently demonstrated: 3 *Runx1* binding sites were found in the promoter region of *Mpl* (Heller *et al.*, 2005), and *Runx1* was described to work as a negative regulator of *Mpl* in adult HSC (Satoh *et al.*, 2008). Furthermore, Hamelin *et al.* (Hamelin *et al.*, 2006) have shown that TPO/*Mpl* signaling can regulate the activity of *Runx1* through the ERK pathway. As a matter of fact, when we explored

the level of *Runx1* expression in *Mpl*^{-/-} E10.5 AGMs by q-PCR, we found a 2.3 fold decrease compared to control E10.5 AGMs ($p < 0.01$) (Fig. 4B).

Discussion

In the present report, we investigated in more depth the HSCs defect that we described in E11.5 *Mpl*^{-/-} AGMs, by performing an analysis of the hematopoietic content of these AGMs at the time of the first HSCs emergence (E10.5). We showed that at E10.5, *Mpl*^{-/-} AGM contains twice more HC/HSCs than C57Bl6 AGM, as assessed by FACS analysis of the *CD41*⁺, *CD45*⁺, *CD45*⁺*c-kit*^{hi}, *CD34*⁺*c-kit*^{hi}, and *CD144*⁺*CD45*⁺ populations. Interestingly enough, we found a two-fold reduction of *Runx1* expression. This reduction of *Runx1* expression is not observed anymore at E11.5, and seems therefore to be related to the developmental stage of emergence of HC/HSCs in the aorta. Using a conditional deletion

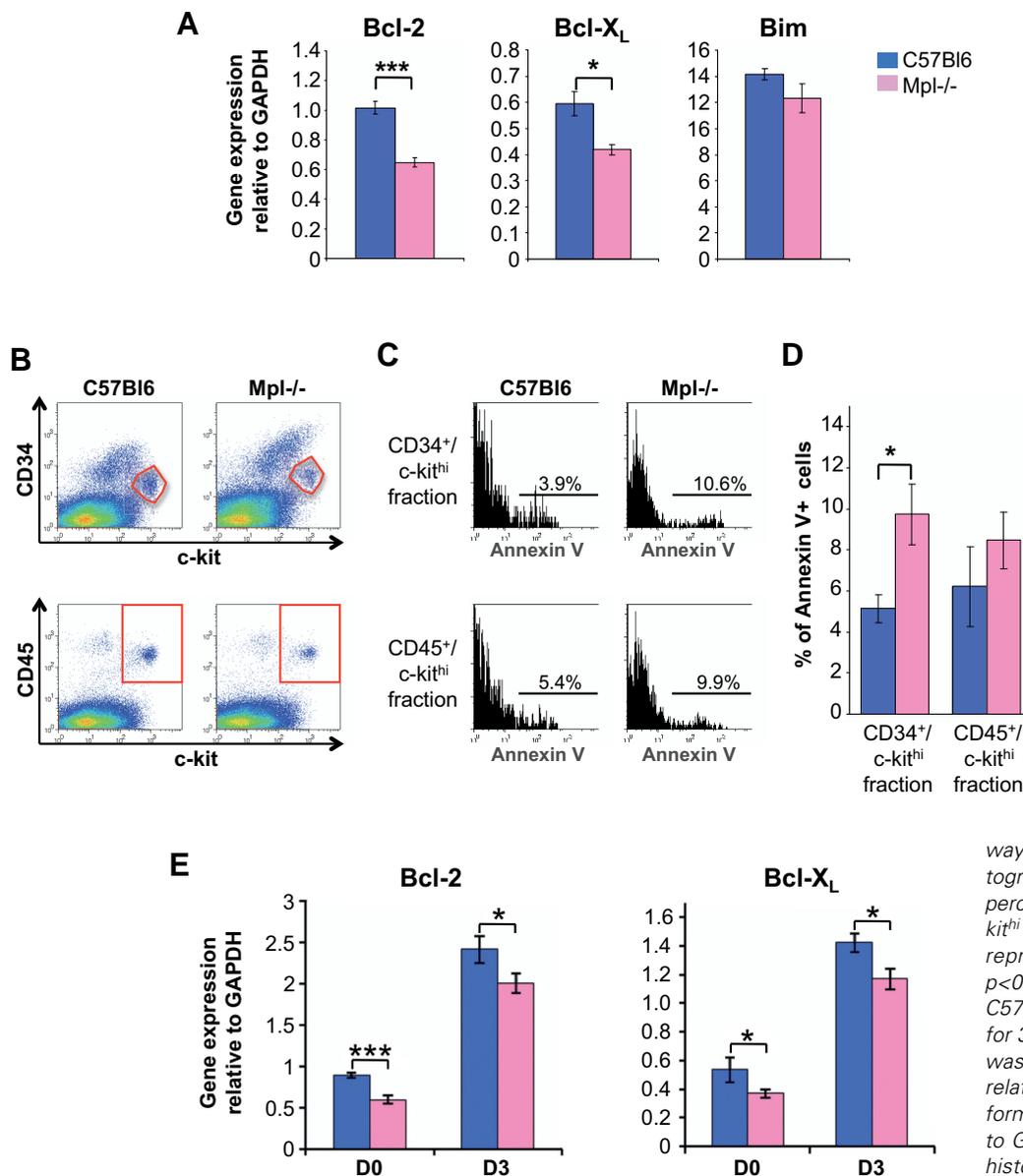


Fig. 3. Decreased expression of antiapoptotic genes in E10.5 *Mpl*^{-/-} AGM and increased apoptosis in the *CD45*⁺*c-kit*^{hi} and *CD34*⁺*c-kit*^{hi} populations after explants culture.

(A) Fresh E10.5 AGMs from *Mpl*^{-/-} and C57Bl6 embryos were lysed with Trizol and total RNA was extracted. After reverse transcription, relative quantitative PCR (q-PCR) was performed with gene-specific PCR primers on a LightCycler 480 Instrument. *Bcl-2*, *Bcl-XL*, and *Bim* expression relative to GAPDH expression are reported on the histograms. **(B,C,D)** E10.5 AGM from *Mpl*^{-/-} and C57Bl6 embryos were dissected and cultured as explants for 3 days. Single cells suspensions were prepared after collagenase treatment and stained with antibodies specific for *CD45* or *CD34* and *c-kit* and with Annexin V and 7AAD. **(B)** FACS plots for *CD45*⁺*c-kit*^{hi} and *CD34*⁺*c-kit*^{hi} populations for C57Bl6 and *Mpl*^{-/-} AGMs. **(C)** Within the *CD45*⁺*c-kit*^{hi} and *CD34*⁺*c-kit*^{hi} fractions of AGM, the percentage of Annexin V⁺ cells, which indicates the percentage of cells entering the apoptotic pathway, was determined. Representative histograms plots are shown. **(D)** The mean percentage of Annexin V⁺ cells in the *CD45*⁺*c-kit*^{hi} and *CD34*⁺*c-kit*^{hi} fractions of AGM is represented on the histogram ($n = 3$). * $p < 0.05$.

(E) E10.5 AGM from *Mpl*^{-/-} and C57Bl6 embryos were cultured as explants for 3 days, lysed with Trizol, and total RNA was extracted. After reverse transcription, relative quantitative PCR (q-PCR) was performed. *Bcl-XL*, and *Bim* expression relative to GAPDH expression are reported on the histograms. * $p < 0.05$; *** $p < 0.005$.

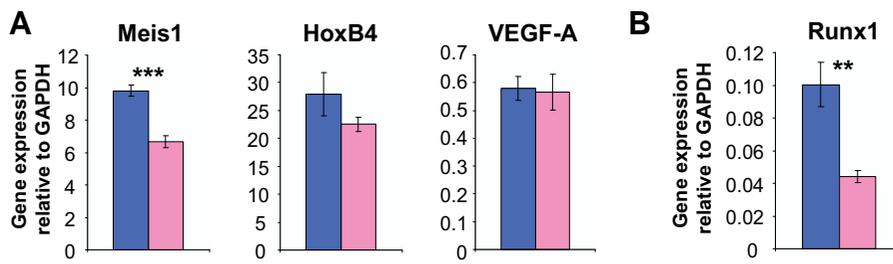


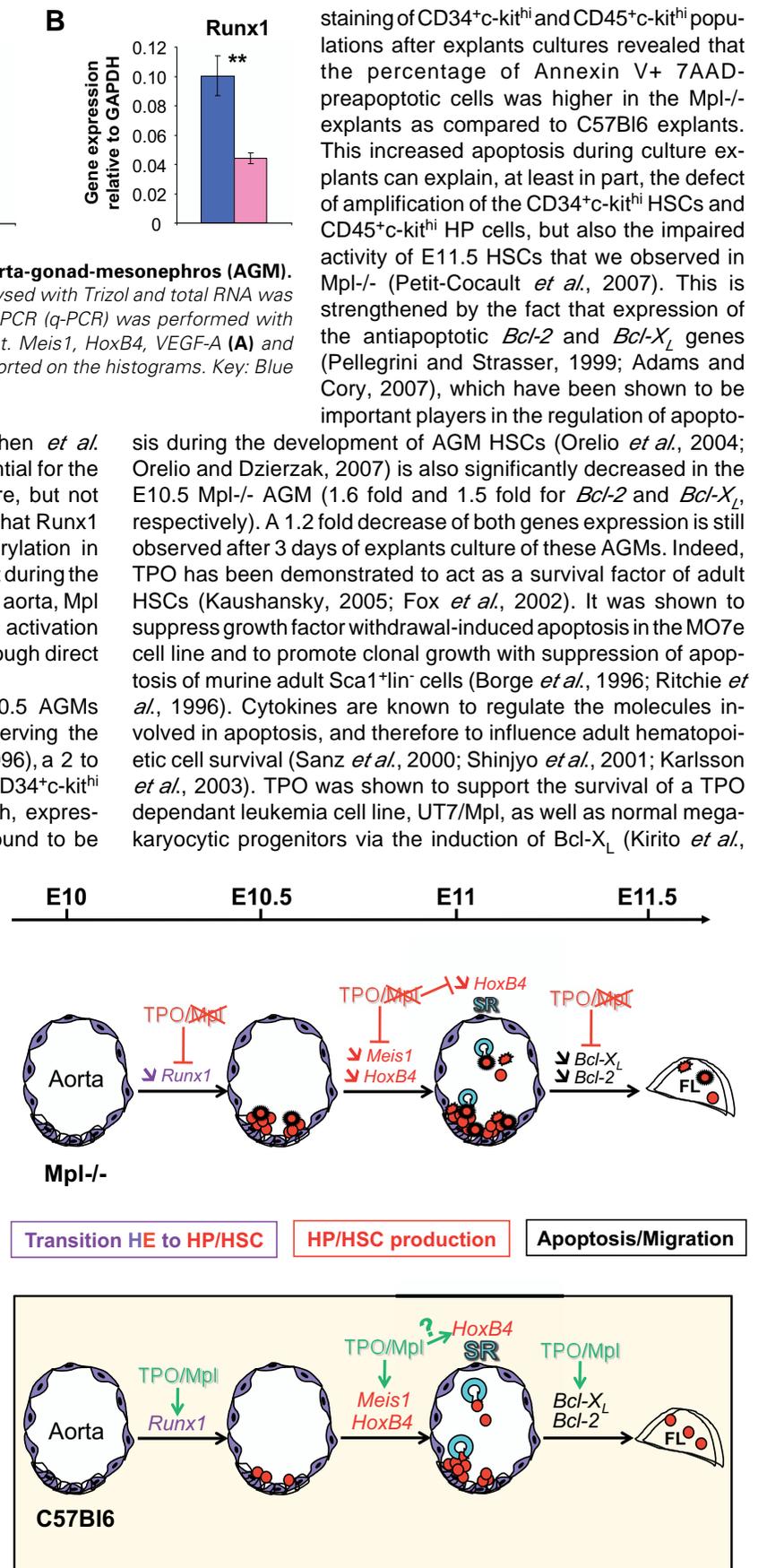
Fig. 4. Expression of Mpl target genes in E10.5 Mpl^{-/-} aorta-gonad-mesonephros (AGM). Fresh E10.5 AGMs from Mpl^{-/-} and C57Bl6 embryos were lysed with Trizol and total RNA was extracted. After reverse transcription, relative quantitative PCR (q-PCR) was performed with gene-specific PCR primers on a LightCycler 480 Instrument. Meis1, HoxB4, VEGF-A (A) and Runx1 (B) expression relative to GAPDH expression are reported on the histograms. Key: Blue C57Bl6; Pink, Mpl^{-/-}. ** $p < 0.01$; *** $p < 0.005$.

of Runx1 in the VE-cadherin⁺ endothelial cells, Chen *et al.* recently demonstrated that Runx1 expression is essential for the formation of HP cells and HSCs from the vasculature, but not thereafter (Chen *et al.*, 2009). This, added to the fact that Runx1 has been shown to be regulated by ERK phosphorylation in response to TPO (Hamelin *et al.*, 2006), let assume that during the transition from endothelial to hematopoietic cells in the aorta, Mpl receptor could play a role in the maintenance of Runx1 activation not only via TPO-mediated ERK pathway, but also through direct regulation of Runx1 transcription.

When culture explants were set up to allow E10.5 AGMs expanding and generating HSCs *in vitro* while preserving the cellular microenvironment (Medvinsky and Dzierzak, 1996), a 2 to 2.5 fold defect of amplification of CD45⁺c-kit^{hi} HP and CD34⁺c-kit^{hi} HSC populations was observed. Interestingly enough, expression of two homeogenes, HoxB4 and Meis1, was found to be reduced in E10.5 Mpl^{-/-} AGMs. HoxB4 has been shown to be important for HSCs expansion (Antonchuk *et al.*, 2002; Antonchuk *et al.*, 2002; Schiedlmeier *et al.*, 2003). A role of Meis1 in the amplification and self-renewal of HSCs was demonstrated in the Meis1 KO mice, which present a defect of fetal liver HSCs (Hisa *et al.*, 2004; Azcoitia *et al.*, 2005). Both HoxB4 and Meis1 were described as Mpl target genes and shown to likely account for part of the favorable effects of TPO on adult HSC self-renewal and expansion (Kaushansky, 2005). Their reduced level of expression in E10.5 Mpl^{-/-} AGMs which present a defect of HP/HSC amplification in culture explant, let make the assumption that Mpl/TPO signaling plays an important role also in HSCs self renewal and expansion during the establishment of definitive hematopoiesis.

Flow cytometric analysis with annexin V and 7AAD

Fig. 5. Mpl, a key "sensor" gene of the AGM microenvironment? The genes affected in the Mpl^{-/-} environment during the different steps of emergence and production of the first HP/HSCs in the AGM between E10 and E11.5 are represented on the top part of the sketch. The consequent control of these different target genes by Mpl during these precise steps is shown on the bottom part of the sketch. FL, fetal liver; HE, hemogenic endothelium; SR, self-renewal.



staining of CD34⁺c-kit^{hi} and CD45⁺c-kit^{hi} populations after explants cultures revealed that the percentage of Annexin V⁺ 7AAD⁺ preapoptotic cells was higher in the Mpl^{-/-} explants as compared to C57Bl6 explants. This increased apoptosis during culture explants can explain, at least in part, the defect of amplification of the CD34⁺c-kit^{hi} HSCs and CD45⁺c-kit^{hi} HP cells, but also the impaired activity of E11.5 HSCs that we observed in Mpl^{-/-} (Petit-Cocault *et al.*, 2007). This is strengthened by the fact that expression of the antiapoptotic *Bcl-2* and *Bcl-X_L* genes (Pellegrini and Strasser, 1999; Adams and Cory, 2007), which have been shown to be

important players in the regulation of apoptosis during the development of AGM HSCs (Orelia *et al.*, 2004; Orelia and Dzierzak, 2007) is also significantly decreased in the E10.5 Mpl^{-/-} AGM (1.6 fold and 1.5 fold for *Bcl-2* and *Bcl-X_L*, respectively). A 1.2 fold decrease of both genes expression is still observed after 3 days of explants culture of these AGMs. Indeed, TPO has been demonstrated to act as a survival factor of adult HSCs (Kaushansky, 2005; Fox *et al.*, 2002). It was shown to suppress growth factor withdrawal-induced apoptosis in the MO7e cell line and to promote clonal growth with suppression of apoptosis of murine adult Sca1⁺lin⁻ cells (Borge *et al.*, 1996; Ritchie *et al.*, 1996). Cytokines are known to regulate the molecules involved in apoptosis, and therefore to influence adult hematopoietic cell survival (Sanz *et al.*, 2000; Shinjyo *et al.*, 2001; Karlsson *et al.*, 2003). TPO was shown to support the survival of a TPO dependant leukemia cell line, UT7/Mpl, as well as normal megakaryocytic progenitors via the induction of *Bcl-X_L* (Kirito *et al.*,

2002). More recently, IL-3 and IL-1 were shown to play also a role in the survival and regulation of HP cells and HSCs in the midgestation mouse AGM (Robin *et al.*, 2006; Orelia *et al.*, 2008). Our results indicate that TPO/Mpl signaling could also regulate apoptosis of the first HSCs emerging in E10.5 AGM, during the stage of transition from endothelial to hematopoietic cells, and during the phase of HP/HSC production. Implication of Bcl-2 in this process differs from the Bcl-2 independent HSC survival and maintenance which has been described for adult HSCs (Qian *et al.*, 2007)

Transcription factors have always been considered as master genes for the regulation of developmental hematopoiesis (Teitell and Mikkola, 2006). Our present results with Mpl indicate that a cytokine receptor can also be an active player in this process through direct regulation of the expression of transcription factors or genes important for self-renewal, proliferation and apoptosis of HSCs. *Mpl* therefore looks like a key "sensor" gene of the AGM microenvironment, that can control different target genes depending on the precise steps of emergence of the first HSCs in the AGM region (Fig. 5).

Material and Methods

Mice

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

Dissections and tissue/cell preparation

Embryos were produced by natural mating of C57Bl6 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 morphological analysis.

Explant cultures

AGM were dissected and explanted AGMs were cultured onto Durapore 0.65 µm filters (Millipore) at the air-liquid interface as described by Medinsky and Dzierzak (Medvinsky and Dzierzak, 1996). Briefly, tissues were cultured for 3 days at 37°C in 5% CO₂ in myeloid long-term culture media (IMDM supplemented with 12.5% FCS (Dominique Dutscher), 12.5% horse serum (Fisher), 0.5 mg/l ascorbic acid, 37 mg/l myo-inositol, 10 mg/l folic acid, 5x10⁻⁵ M β-mercaptoethanol and 10⁻⁶ M hydrocortisone hemisuccinate (Sigma) in the presence of 20ng/ml TPO.

FACS analysis

Before staining, uncultured and cultured explants were treated for 30 minutes at 37°C with 0.125% type I collagenase (Sigma) in PBS with 10% FCS. Cells were dispersed by gentle pipetting, washed, filtrated through 70µm nylon mesh (Bio-technofix), resuspended in IMDM supplemented with 2% FCS and counted using Trypan Blue for exclusion of non viable cells. Cell staining was done in PBS with 0.5% bovine serum albumine (BSA) using the following antibodies: Allophycocyanin (APC)-CD45 (Biolegend), APC-CD34 (eBioscience), phycoerythrin (PE)-c-kit (Biolegend). CD144 antibody (BD Biosciences-Pharmingen) was biotinylated using Fluoreporter Mini-Biotin kit (Molecular Probes). biot-CD144 stained cells were detected by addition of PE-streptavidin (BD Biosciences-Pharmingen). For Annexin V analysis, immuno-stained cells were resuspended in Annexin V buffer and stained with fluorescein isothiocyanate (FITC)-Annexin V (Biolegend) according to the

manufacturer's guidelines. Dead cells were excluded by 7AAD (Beckman Coulter) staining, and FACS analysis was performed on a FACScalibur cytometer (BD Biosciences).

RNA isolation, cDNA synthesis and q-PCR analysis

Dissected AGM were lysed with Trizol (Invitrogen) and total RNA was extracted as recommended by the manufacturer. Reverse transcription was performed on 1 µg of total RNA, as previously described (Challier *et al.*, 2002). 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 480 Instrument (Roche). After one step at 95°C for 15 minutes to activate the HotStarTaq DNA polymerase (Qiagen), the samples were cycled 40 times (denaturation at 95°C for 20 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 20 seconds). Crossing point (Cp) values of the sequences of interest were measured using the LightCycler software (automated calculation by the second derivative maximum method). Relative expression was calculated according to the E^{-DCp} formula. Data were normalized to Gapdh. The following primer sequences (5' to 3') were used:

Bcl-2 forward primer (fw): TGTGGATGACTGAGTACCTGAACC

Bcl-2 reverse primer (rv): AGAGACAGCCAGGAGAAATCAAAC

Bcl-X fw GGAAAGCGTAGACAAGGAGATG

Bcl-X rv GTTCCCGTAGAGATCCACAAAA

Bim fw ACGAGTTCAACGAAACTTACACAA

Bim rv CAATGCCTTCTCCATACCAGA

Meis1 fw ACAGCAGTGAGCAAGGTGATG

Meis1 rv CGCTTTTTGTGACGCTTTTTG

HoxB4 fw GGAGTTTCACTACAATCGTACTCT

HoxB4 rv TGGGCAACTTGTGGTCTTTT

VEGF-A fw CAGGCTGCTGTAACGATGAA

VEGF-A rv CTCCTATGTGCTGGCTTTGG

Runx1 fw CTACTCGGCAGAACGAGAAATG

Runx1 rv ACGGTGATGGTCAGAGTGAAG

Gapdh fw ATGGTGAAGGTCGGTGTGAA

Gapdh rv AATGAAGGGGTCGTTGATGG

Statistical analysis

All experiments were analyzed using a 2-tailed Student t test. Data were expressed as mean plus or minus standard error of the mean (SEM).

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