

Integrin α_{IIb} (CD41) plays a role in the maintenance of hematopoietic stem cell activity in the mouse embryonic aorta

Jean-Charles Boisset*, Thomas Clapes*, Reinier van der Linden, Elaine Dzierzak and Catherine Robin*[‡]

Erasmus University Medical Center, Department of Cell Biology, Erasmus MC Stem Cell Institute, PO Box 2040, 3000 CA Rotterdam, The Netherlands

*Present address: Hubrecht Institute, Royal Netherlands Academy of Arts and Sciences and University Medical Center Utrecht, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands
[‡]Author for correspondence (c.robin@hubrecht.eu)

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Summary

Integrins are transmembrane receptors that play important roles as modulators of cell behaviour through their adhesion properties and the initiation of signaling cascades. The α_{IIb} integrin subunit (CD41) is one of the first cell surface markers indicative of hematopoietic commitment. α_{IIb} pairs exclusively with β_3 to form the $\alpha_{IIb}\beta_3$ integrin. β_3 (CD61) also pairs with α_v (CD51) to form the $\alpha_v\beta_3$ integrin. The expression and putative role of these integrins during mouse hematopoietic development is as yet unknown. We show here that hematopoietic stem cells (HSCs) differentially express $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins throughout development. Whereas the first HSCs generated in the aorta at mid-gestation express both integrins, HSCs from the placenta only

express $\alpha_v\beta_3$, and most fetal liver HSCs do not express either integrin. By using α_{IIb} deficient embryos, we show that α_{IIb} is not only a reliable HSC marker but it also plays an important and specific function in maintaining the HSC activity in the mouse embryonic aorta.

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Key words: Integrin, Hematopoietic stem cells, Aorta, Mouse development, Placenta, Fetal liver, CD41

Introduction

Hematopoietic stem cells (HSCs) are at the foundation of the blood system and are the key cell type in transplantation protocols for blood-related disorders. The number of HSCs available for clinical applications and fundamental research is limited. The efficient expansion and/or generation *in vitro* is thus far not possible because our knowledge of the mechanisms underlying HSC growth, including the specific *in vivo* interactions between HSCs and the surrounding microenvironment are poorly understood.

Adult HSCs are generated only during a short window of developmental time (Boisset and Robin, 2012). They are first detected at embryonic day (E)10.5 of mouse development in the Aorta–Gonad–Mesonephros (AGM) region (Medvinsky and Dzierzak, 1996; Müller et al., 1994). Starting at E11, HSCs are also found in the yolk sac (YS), placenta (PL) and fetal liver (FL). The pool of HSCs expands in the PL and FL before colonizing the bone marrow (BM) from E17 onward (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). Studies performed in the zebrafish, chicken and mouse models have clearly demonstrated that HSCs originate from specialized endothelial cells referred as hemogenic (Boisset and Robin, 2012). HSCs most likely reside in clusters of cells (Intra-Aortic Hematopoietic Clusters, IAHCs) that are tightly attached to the endothelium of the aorta, the vitelline and umbilical arteries, and the vascular labyrinth of the placenta (Rhodes et al., 2008; Yokomizo and Dzierzak, 2010). In adult BM, HSCs localize in specialized

niches that maintain the balance between HSC self-renewal, quiescence and differentiation. Adhesion molecules (including integrins) are important for the binding of HSCs to the BM niches (Grassinger et al., 2009; Notta et al., 2011; Potocnik et al., 2000; Qian et al., 2006; Umamoto et al., 2006; Wagers and Weissman, 2006). In contrast to adult, the specific interactions and cell adhesion properties of HSCs in the aorta and the successive developmental niches are as yet poorly described.

Integrins are transmembrane glycoproteins (gp) that play an important role in cell adhesion, survival, proliferation, differentiation, migration, gene regulation, and cytoskeletal arrangement. They are a family of 24 heterodimeric receptors composed of α (18 types) and β (8 types) subunits (Prowse et al., 2011). While some integrins are ubiquitously expressed, others are tissue- or cell lineage-specific (Bouvard et al., 2001). Adult HSCs express several integrins important for homing and migration (e.g. $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$) (Bonig et al., 2009a; Bonig et al., 2009b; Grassinger et al., 2009; Lapidot et al., 2005). In the embryo, α_{IIb} (platelet (gp)IIb or CD41) is one of the earliest surface markers of hematopoietic commitment (Emambok and Frampton, 2003; Ferkowicz et al., 2003; McKinney-Freeman et al., 2009; Mikkola et al., 2003; Mitjavila-Garcia et al., 2002; Robin et al., 2011) and its expression is developmentally regulated. E11 AGM HSCs express α_{IIb} , whereas HSCs in the E12 AGM, E12 PL or E14 FL are α_{IIb} negative (Matsubara et al., 2005; McKinney-Freeman et al., 2009; Robin et al., 2011). By

performing time-lapse confocal imaging on live mouse embryo slices, we have shown that the onset of α_{IIb} expression coincides with the formation of hematopoietic stem/progenitor cells (HSPCs) from the hemogenic aortic endothelium (Boisset et al., 2011; Boisset et al., 2010) and that α_{IIb} protein localizes at the point of contact between the cells in IAHCs.

To date little is known about integrin function and expression in HSCs throughout development. The BM of α_{IIb} deficient mice show no hematopoietic lineage commitment problems (Tronik-Le Roux et al., 2000) but possess defective platelets and display bleeding disorders similar to those seen in β_3 deficient mice (Hodivala-Dilke et al., 1999) and humans with Glanzmann thrombasthenia disease. Here we examine the expression of α_{IIb} , β_3 and α_v integrin subunits on HSCs in the main hematopoietic sites (AGM, YS, PL, and FL) and the function of α_{IIb} in these cells. α_{IIb} exclusively associates with β_3 to form the major platelet integrin $\alpha_{IIb}\beta_3$ (gpIIb/IIIa or CD41/CD61). β_3 also associates with α_v to form the $\alpha_v\beta_3$ integrin (CD51/CD61). We show by performing *in vivo* transplantation assays of sorted populations that newly generated E11 AGM HSCs express $\alpha_{IIb}\beta_3$ integrins whereas HSCs from E12 PL and E14 FL do not. $\alpha_v\beta_3$ is expressed on all AGM and PL HSCs but on only a few E14 FL HSCs. All three types of HSCs were found in the E12 YS. Interestingly, α_{IIb} deficient embryos show an HSC defect in the AGM but not in the YS and FL, indicating that α_{IIb} plays a fundamental and precise spatio-temporal role to maintain the HSC activity in the aorta of the mouse embryo.

Materials and Methods

Mice and embryo generation

Embryos were generated from crosses of *h β -globin* *ln72* mice and wild-type (C57BL/10 \times CBA)F1 females; Ly5.1 males and females; $\alpha_{IIb}^{+/tk}$ males and $\alpha_{IIb}^{+/+$ or $\alpha_{IIb}^{+/tk}$ females; $\alpha_{IIb}^{tk/tk}$ males and $\alpha_{IIb}^{+/+$ or $\alpha_{IIb}^{+/tk}$ females; wild-type C57BL/6 females and males. The day of vaginal plug observation is embryonic day (E)0. *ln72*, *YMT* and α_{IIb} genotypes were determined by DNA PCR. Mice were housed according to institutional guidelines and all animal procedures were carried out in compliance with the Standards for human care and use of laboratory animals.

Dissections and cell preparation

E11–E14 embryos were isolated. Tissues (AGM, FL, YS, PL) were dissected and dissociated as previously described (Robin and Dzierzak, 2005). Viable cells were counted (using trypan blue) and kept in phosphate-buffered saline (PBS), 10% fetal calf serum, and penicillin/streptomycin (PBS/FCS/PS) at 4°C for further analysis.

Explant culture

Whole AGMs were cultured as explants at 37°C for 3 days as previously described (Medvinsky and Dzierzak, 1996). Explant cultures of α_{IIb} mutant AGMs were performed with no added cytokines. To test the number of CD41^{int}CD61^{int}CD45^{int}c-kit⁺ cells per wild-type AGM, the medium was supplemented or not with 200 ng/ml of recombinant murine IL-3 (Robin et al., 2006) or 250 ng/ml of gremlin (Durand et al., 2007).

Hematopoietic progenitor assay

In vitro hematopoietic progenitor analysis was performed on dilutions of sorted cells plated in triplicates in methylcellulose medium (StemCell Technologies). Plates were incubated at 37°C in a humidified chamber under 5% CO₂. Hematopoietic colonies were counted with an inverted microscope at day 12 of the culture.

In vivo transplantation assay and transplanted mice analysis

Intravenous injection of total or sorted cells into irradiated adult wild-type recipients was as previously described (Robin et al., 2006). Recipient female mice ((129/SV \times C57BL/6), C57BL/6 or (CBA \times C57BL/10)) were exposed to a split dose of 9 Gy of γ -irradiation (¹³⁷Cs source) and injected with several cell doses (or embryo equivalent, ee). 2 \times 10⁵ spleen cells (recipient background) were co-injected to promote short-term survival.

Blood was obtained at 1 and 4 months post-transplantation and DNA was analyzed for donor cell markers by semiquantitative PCR (*h β -globin*, α_{IIb} , *Ymt*).

The percentage of donor contribution was calculated from a standard curve of DNA control dilutions (0 to 100% donor marker). Recipients were considered repopulated when the chimerism was greater than 10%. For multilineage repopulation analysis, thymus, lymph nodes, bone marrow and spleen were dissected from the repopulated recipients. T, B, erythroid, and myeloid cells were sorted from recipient BM and spleens after antibody staining (see below). Primary recipient BM cells were injected into secondary irradiated recipients to assess self-renewal capacity (0.5 \times 10⁶ or 2 \times 10⁶ cells injected per recipient; supplementary material Fig. S4D). Chimerism level of the Ly5.2 recipients (C57BL/6) injected with Ly5.1 cells was determined by flow cytometry (see below). Recipients were considered repopulated when the chimerism was greater than 5%.

For statistical analysis, student's *t* test (Mann–Whitney) was used to determined statistical significance.

Flow cytometric analysis and sorting

Flow cytometric analysis and sorting were performed on a FACScan and/or AriaIII (BD Bioscience) with CellQuest and FACSDiva. Stainings were performed in PBS/FCS/PS for 30 min at 4°C. Cells were washed and resuspended in PBS/FCS/PS and 7-AAD (Molecular Probes, Leiden, NL) or Hoechst 33258 (1 μ g/ml, Molecular Probes) for dead cell exclusion. The positive gates were defined from staining with isotype-matched control antibodies. Monoclonal antibodies (BD Pharmingen, eBioscience, Invitrogen, Santa Cruz, Biolegends) used: FITC-anti-Ter119, FITC-anti-CD42c (Gp1b β); PE-anti-CD41; APC-anti-CD61; FITC, APC-Cy7 or APC-AlexaFluor750-anti-c-kit; FITC, PE or PE-Cy7-anti-CD31; PerCP-Cy5.5-anti-CD45; APC or Pacific Blue-anti-CD34; Alexa-Fluor488-anti-CD51; Lin cocktail (PE-anti-Ter119, PE-anti-B220, PE-anti-Mac1, PE-anti-Gr1, PE-anti-CD3 ϵ); PE-anti-CD48; APC-anti-CD150 and PE-Cy7-anti-Sca-1.

Ly5.2/Ly5.1 cell chimerism was determined by flow cytometry on blood after erythrocyte lysis (Beckman Coulter) and staining with FITC-anti-Ly5.2, PE-anti-Ly5.1 and 7-AAD. Flow cytometry data were analyzed with FlowJo.

For multilineage repopulation analysis, spleen cells were stained with FITC-anti-B220, PE-anti-CD8a and PE-anti-CD4; bone marrow cells were stained with FITC-anti-Ly6C and PE-anti-CD31.

Cell cycle analysis

Cells from dissociated E11 AGM were stained with PE-anti-c-kit antibody. Cells were washed prior to fixation with 2% PFA for 30 min. Cells were then permeabilized for 1 h in 0.2% Triton X-100. After washing, cells were incubated during 1 h with FITC-anti-Ki-67 antibody or IgG isotype control. Cells were washed and incubated with Hoechst 33342 (1 μ g/ml, Molecular Probes) prior to analysis by flow cytometry.

Pre-apoptosis analysis

Cells from dissociated E11 AGM were stained with PE-anti-c-kit antibody. Cells were washed in cold PBS and resuspended in 1 \times binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) and stained for 20 min with Annexin-V-FITC and Hoechst 33258 (1 μ g/ml, Molecular Probes). Analyses were performed within 1 h by flow cytometry.

Whole-mount immunostaining

The whole-mount immunostaining was performed as previously described (Yokomizo and Dzierzak, 2010). Rat anti-mouse primary antibodies for c-Kit (2B8) and biotinylated anti-CD31 (MEC13.3) were used (BD Biosciences). Secondary antibodies were goat anti-rat IgG-Alexa647 (Invitrogen) and streptavidin-Alexa594 (Invitrogen). Embryo caudal halves were imaged using a Leica SP5 confocal microscope.

Staining and confocal microscopy of non-fixed embryo and placenta slices

Non-fixed wild-type embryos were cut into thick transversal slices (200 μ m) and stained with directly conjugated monoclonal antibodies as previously described (Boisset et al., 2011; Boisset et al., 2010). The antibodies include: PE-anti-CD41 (MWreg30), Alexa488-anti-CD51 (RMV-7), APC-anti-CD61. Similarly, placentas isolated from E12 non-fixed wild-type embryos were cut into slices (200 μ m) and were incubated with PE-anti-Tie-2, Alexa488-anti-CD51 (RMV-7) and APC-anti-CD61. Slices were immobilized into agarose gel and imaged by using a Leica SP5 confocal microscope.

Results

Hematopoietic stem cells differentially express $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins throughout development

HSCs are restricted to the CD41^{intermediate} (CD41^{int}) fraction in E11 AGM (McKinney-Freeman et al., 2009; Robin et al., 2011). However, HSCs in E12 AGM and PL, and E14 FL are exclusively in the CD41⁻ fraction. In YS, HSCs are in both

populations (Robin et al., 2011). To further investigate integrin subunit expression on HSCs, we performed flow cytometric analyses for α_v (CD51) and β_3 (CD61), in addition to α_{IIb} (CD41) expression. Cells were analysed at the time points corresponding to organ-specific peaks of HSC activity and subunit expression was found to differ between tissues (Fig. 1). CD41^{int}CD61⁻ cells were found only in E11 AGM (Fig. 1A), and not in E12 YS (Fig. 1C), E12 PL (Fig. 1E) or E14 FL (Fig. 1G). CD41⁻CD61^{high} cells were found mainly in E12 PL (Fig. 1E) and to a lesser extent in E11 AGM (Fig. 1A). Three distinct cell populations were present in all tissues: CD41⁻CD61⁻, CD41⁻CD61^{int} and CD41^{int}CD61^{int}.

To determine whether HSCs in E11 AGM expressed both α_{IIb} and β_3 subunits, CD41^{int}CD61⁻ and CD41^{int}CD61^{int} fractions were sorted and injected into adult wild-type irradiated recipients ($n=2$ (n =independent experiments when not specified otherwise)). Four months post-transplantation, the mice injected

with CD41^{int}CD61^{int} cells were reconstituted (5 mice reconstituted out of 6 mice transplanted, 5/6). No mice (0/8) injected with CD41^{int}CD61⁻ cells were reconstituted, even with a high cell dose (3 ee per mouse) (Fig. 1B). High-level multilineage engraftment of blood, BM, spleen, lymph nodes and thymus was found in the recipients receiving CD41^{int}CD61^{int} cells (supplementary material Fig. S1, top panel) and secondary recipients were successfully engrafted with BM from these primary recipients, thus demonstrating that the CD41^{int}CD61^{int} population contains bona fide HSCs (supplementary material Fig. S1, bottom panel). 95% of the CD41^{int}CD61^{int} cells also expressed CD51 (Table 1). At E12, AGM HSCs were CD41⁻ (Robin et al., 2011) but still expressed CD51 (Table 1). Thus, all HSCs in E11 AGM express both $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins.

YS HSCs are both CD41⁻ and CD41^{int} at E11 and E12 (Robin et al., 2011). In combination with CD61, three cell fractions were sorted from E12 YS (CD41⁻CD61⁻, CD41⁻CD61^{int} and

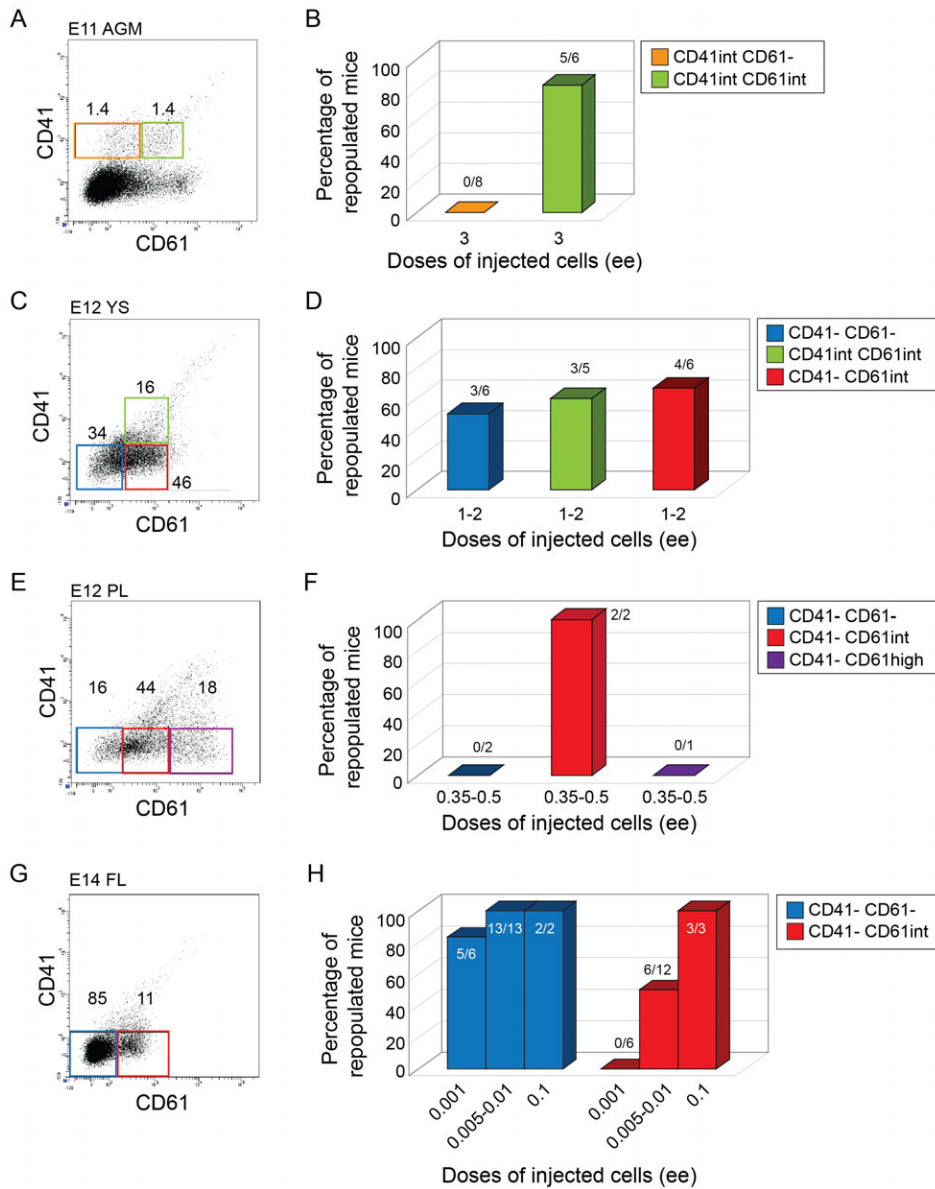


Fig. 1. Phenotypic and functional analyses of integrin-based sorted cell fractions. (A,C,E,G) Flow cytometric analyses of E11 Aorta-Gonad-Mesonephros (AGM), E12 yolk sac (YS), E12 placenta (PL) and E14 fetal liver (FL). Representative sorting gates of each population are indicated. (B,D,F,H) Hematopoietic repopulation analyses after injection of integrin-based sorted fractions of AGM ($n=2$), YS ($n=4$), PL ($n=2$) and FL ($n=3$). Numbers above columns indicate number of mice repopulated/number of mice injected. Dose of injected cells is indicated as embryo equivalent (ee).

Table 1. Percentage of CD51⁺ cells in the cell fractions enriched in hematopoietic stem cells.

Tissue	Embryonic day	Cell fraction	Percentage of CD51 ⁺ cells in the cell fraction
AGM	E11	CD41 ^{int} CD61 ^{int}	95
	E12	CD41 ⁻	96
Placenta	E12	CD41 ⁻ CD61 ^{int}	86
YS	E12	CD41 ^{int} CD61 ^{int}	97
		CD41 ⁻ CD61 ^{int}	72
		CD41 ⁻ CD61 ⁻	34
FL	E14	CD41 ⁻ CD61 ^{int}	98
		CD41 ⁻ CD61 ⁻	8

CD41^{int}CD61^{int}) (Fig. 1C) and transplanted ($n=4$). Multilineage engraftment was obtained with all fractions (Fig. 1D). Similar to E11 AGM HSCs, YS cells in the CD41^{int}CD61^{int} and CD41⁻CD61^{int} fractions expressed CD51 (97% and 72% respectively) (Table 1). On the other hand, the majority of CD41⁻CD61⁻ cells did not express CD51 (Table 1). Thus, YS and AGM contain HSCs that express both $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins, whereas some YS HSCs express solely $\alpha_v\beta_3$ integrin or none of these integrins.

HSCs in PL and FL are CD41⁻ (Robin et al., 2011). CD41⁻CD61⁻, CD41⁻CD61^{int} and CD41⁻CD61^{high} (only present in PL) fractions were sorted (Fig. 1E,G) and transplanted (PL: $n=2$, FL: $n=3$). The PL CD41⁻CD61^{int} fraction (but not the CD41⁻CD61⁻ fraction) contained HSCs (Fig. 1F). Most of CD41⁻CD61^{int} cells expressed CD51 (86%) (Table 1). Thus, CD41⁻CD61^{int} HSCs in PL express only $\alpha_v\beta_3$ integrin and therefore resemble CD41⁻CD61^{int} YS HSCs. In E14 FL, HSCs were in both CD41⁻CD61⁻ and CD41⁻CD61^{int} fractions (Fig. 1H). Limiting cell dilution transplantations (0.001 to 0.1 ee) showed that HSCs were enriched in the CD41⁻CD61⁻ fraction (Fig. 1H). Similar to E12 PL and YS, CD41⁻CD61^{int} FL cells also expressed CD51 (98%) (Table 1). In contrast, CD41⁻CD61⁻ FL cells did not express CD51 (Table 1). Thus, all HSCs in the PL express $\alpha_v\beta_3$ but not $\alpha_{IIb}\beta_3$ integrins, whereas most FL HSCs do not express these integrins.

E11 AGM hematopoietic stem cells are enriched in the CD41^{int}CD61^{int}CD45^{int}c-kit⁺ subpopulation

Further enrichment of the E11 AGM CD41^{int}CD61^{int} population was attempted with the pan-hematopoietic marker CD45 and the HSC marker c-kit (Sánchez et al., 1996). All committed cells from erythroid (Ter119⁺) and megakaryocytic (Gp1b β ⁺) lineages were first excluded (supplementary material Fig. S2A, left panel) and the CD41^{int}CD61^{int} HSC containing fraction (Fig. 1A,B) was sorted into four subfractions: CD45⁻c-kit⁻, CD45⁻c-kit⁺, CD45^{int}c-kit⁺ and CD45^{high}c-kit⁺ (supplementary material Fig. S2A, middle and right panels). Cells were injected into irradiated adult recipients and engraftment was measured at 1 month (short-term repopulation, STR) and 4 months (long-term repopulation, LTR) post-transplantation ($n=8$). As expected, the CD45⁻c-kit⁻ and CD45⁻c-kit⁺ subfractions yielded no repopulation, even with high doses of injected cells (5–10 ee) (supplementary material Fig. S2C). The CD45^{int}c-kit⁺ and CD45^{high}c-kit⁺ subfractions yielded short-term repopulation (supplementary material Fig. S2C, left panel), but only the CD45^{int}c-kit⁺ subfraction was capable of long-term repopulation (supplementary material Fig. S2C, right panel). These cells provided high-level multilineage

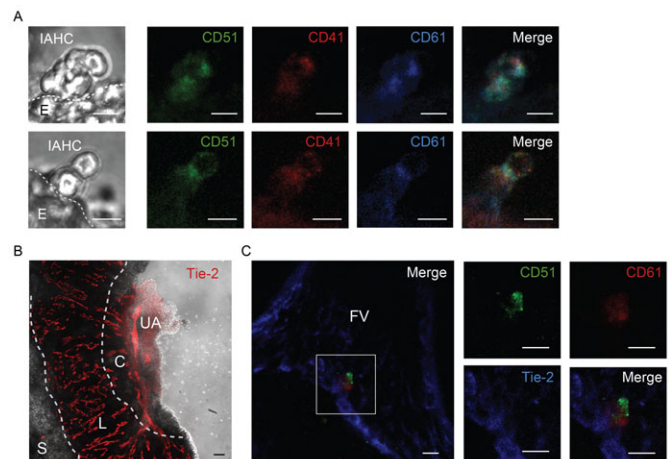
reconstitution of the primary and secondary transplanted recipients (supplementary material Fig. S2B). Thus, the CD41^{int}CD61^{int} population contains both STR-HSCs and LTR-HSCs that both express CD45 and c-kit. However, STR-HSCs and LTR-HSCs can be discriminated based on the level of CD45 expression: STR-HSCs are CD45^{high} while LTR-HSCs are CD45^{int}. There is as few as 96 ± 31 CD41^{int}CD61^{int}CD45^{int}c-kit⁺ cells per E11 AGM ($n=6$). Thus, the combination of integrin expression with other hematopoietic markers such as CD45 and c-kit can be used to discriminate cell populations highly enriched in STR-HSCs and LTR-HSCs in E11 AGM.

Interestingly, the number of CD41^{int}CD61^{int}CD45^{int}c-kit⁺ cells per AGM was increased when E11 AGM explants were cultured in the presence of IL-3 and decreased in the presence of Gremlin, as compared to non-supplemented controls (supplementary material Fig. S2D,E). This is consistent with our previous findings that IL-3 is a powerful amplifying factor (Robin et al., 2006) and Gremlin (a BMP antagonist) is an inhibitory factor for HSC activity in E11 AGM (Durand et al., 2007; Robin and Durand, 2010). Therefore, the refined CD41^{int}CD61^{int}CD45^{int}c-kit⁺ HSC phenotype provides a rapid readout for testing the effects of specific molecules/reagents in AGM and perhaps other embryonic culture systems.

Localization of α_{IIb} , α_v and/or β_3 integrin subunits expressing cells in the AGM and placenta

To determine the precise location of cells expressing α_{IIb} , α_v and/or β_3 , multicolour stainings were performed using the technique that we previously developed to stain non-fixed embryo slices (Boisset et al., 2011). We observed CD41^{int}CD51^{int}CD61^{int} cells in the IAHCs of E10.5 embryos (Fig. 2A). Interestingly, the integrin subunits were concentrated at the junction between the IAHC cells.

The embryo slice staining technique was adapted to non-fixed PL. E12 PL slices stained with directly labelled

**Fig. 2. Location of phenotypically defined HSCs in AGM and placenta.**

(A) Intra-aortic hematopoietic clusters (IAHCs) of E10.5 wild-type embryos. Non-fixed embryo slices were stained with antibodies against CD51 (α_v), CD41 (α_{IIb}) and CD61 (β_3). Scale bars: 10 μ m. (B) Non-fixed placenta slices (E12) were stained with antibodies against Tie-2. Transmitted light and fluorescent images are merged. Scale bar: 100 μ m. (C) Confocal stack image of an E12 non-fixed placenta slice stained with antibodies against CD51, CD61 and Tie-2. Close up of the boxed area (left panel) shows a group of labelled cells (single and merged fluorescent channels are shown). Scale bars: 10 μ m. UA: umbilical artery, C: chorionic plate, L: labyrinth, S: spongiotrophoblast layer, FV: fetal vessel.

anti-Tie-2 antibodies (Fig. 2B) showed specific staining of both placental vessels and umbilical artery (UA). This allowed discrimination of the chorionic plate (C), the vascular labyrinth (L) and the spongiotrophoblast layer (S) (Fig. 2B). To localize phenotypically defined HSCs in the Tie-2⁺ placental vessels (FV), multicolour staining was performed with anti-CD51 and CD61 antibodies (Fig. 2C). Groups of CD51^{int}CD61^{int} cells were observed in the vasculature of the chorionic plate and similar to the aorta, the integrin subunits were concentrated at the junctions between cells (Fig. 2C). Thus, integrin expressing HSCs are localized in the IAHCs as well as in the vasculature of the placental chorionic plate.

α_{IIb} deficient embryos have no qualitative or quantitative defects in AGM hematopoietic progenitors or IAHCs

All hematopoietic progenitors are CD41^{int} in the E11 AGM (Robin et al., 2011). To test whether α_{IIb} plays a role in the production of hematopoietic progenitors, AGM cells from E11 wild-type ($\alpha_{IIb}^{+/+}$) and α_{IIb} mutant ($\alpha_{IIb}^{+/tk}$ and $\alpha_{IIb}^{tk/tk}$) embryos (Tronik-Le Roux et al., 2000) were isolated and tested in the colony forming unit-culture assay (CFU-C; $n=3$). As shown in Fig. 3A, AGMs of E11 mutant embryos contained erythroid and myeloid progenitors. The numbers of total CFU-C and of each type of progenitor were similar in the mutants as compared to wild-type AGMs, demonstrating that α_{IIb} does not influence the production or function of AGM progenitors.

Whole embryo imaging of $\alpha_{IIb}^{+/+}$, $\alpha_{IIb}^{+/tk}$ and $\alpha_{IIb}^{tk/tk}$ E11 embryos immunostained for CD31 (to visualize endothelial and IAHC cells) and c-kit (to visualize IAHC cells) revealed no differences in IAHC cell number (Fig. 3B), organization or shape (Fig. 3C,D). To investigate whether the lack of α_{IIb} affects the expression of α_v (CD51) and β_3 (CD61), we performed flow cytometric analysis on $\alpha_{IIb}^{+/+}$, $\alpha_{IIb}^{+/tk}$ and $\alpha_{IIb}^{tk/tk}$ E11 AGMs (supplementary material Fig. S3). CD51 and CD61 expression levels were similar between the different phenotypes, although

the CD51⁺CD61^{high} population was absent in the $\alpha_{IIb}^{tk/tk}$ AGMs. Thus, the absence of CD41 does not influence expression of CD51 and CD61. Together, these results show that α_{IIb} is not needed for the formation, organization and anchorage of IAHCs, or there is functional redundancy with other integrins/adhesion molecules (e.g. $\alpha_v\beta_3$).

α_{IIb} deficient embryos have a hematopoietic stem cell defect in the AGM

To test whether α_{IIb} plays a role in the function of AGM HSCs, transplantations were performed with cells isolated from E11 wild-type ($\alpha_{IIb}^{+/+}$) and α_{IIb} mutant ($\alpha_{IIb}^{+/tk}$ and $\alpha_{IIb}^{tk/tk}$) AGMs ($n=3$). Whereas 72% of the mice injected with $\alpha_{IIb}^{+/+}$ cells were reconstituted, only 29% and 38% of mice were reconstituted with $\alpha_{IIb}^{+/tk}$ or $\alpha_{IIb}^{tk/tk}$ cells, respectively, at four months post-transplantation (Fig. 4A). The percentage of donor cell chimerism was significantly lower in the few mice repopulated with $\alpha_{IIb}^{+/tk}$ or $\alpha_{IIb}^{tk/tk}$ cells (10% average) as compared to the mice injected with $\alpha_{IIb}^{+/+}$ cells (60% average) (Fig. 4A, red bars). The reconstitution with $\alpha_{IIb}^{tk/tk}$ cells resulted in multilineage engraftment (supplementary material Fig. S4B,C) similar to that found in mice reconstituted with $\alpha_{IIb}^{+/+}$ cells (supplementary material Fig. S4A). Secondary transplantations of BM cells (two cell doses, supplementary material Fig. S4D,E) isolated from two primary recipients repopulated with $\alpha_{IIb}^{tk/tk}$ AGM HSCs and one recipient repopulated with $\alpha_{IIb}^{+/+}$ cells (Fig. 4A, red lozenges; supplementary material Fig. S4A,B,C) showed that $\alpha_{IIb}^{tk/tk}$ AGM HSCs were self-renewing. $\alpha_{IIb}^{tk/tk}$ AGM-derived cells successfully repopulated secondary recipients with similar chimerism as $\alpha_{IIb}^{+/+}$ AGM-derived cells (supplementary material Fig. S4D,E). Thus, although $\alpha_{IIb}^{+/tk}$ and $\alpha_{IIb}^{tk/tk}$ embryos have fewer HSCs in the AGM as compared to $\alpha_{IIb}^{+/+}$ embryos, these are fully functional HSCs.

It was previously shown that the number of HSCs increases when AGMs are cultured as explants for 3 days (Medvinsky and

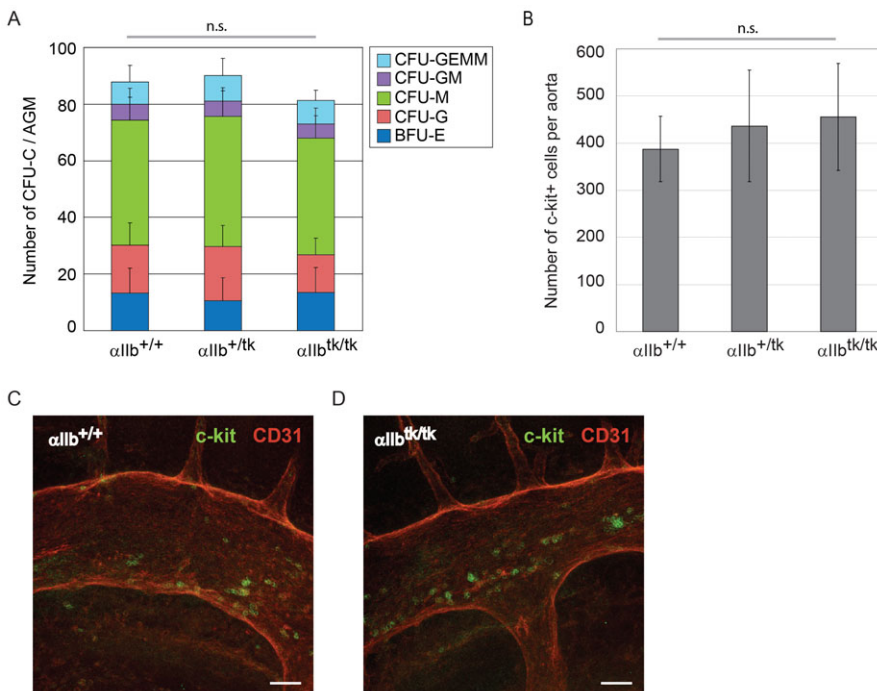


Fig. 3. Intra-aortic hematopoietic clusters and *in vitro* clonogenic progenitor activity of AGM cells isolated from E11 CD41 (α_{IIb}) deficient embryos. (A) *In vitro* clonogenic analyses. AGMs were isolated from E11 wild-type ($\alpha_{IIb}^{+/+}$), heterozygous ($\alpha_{IIb}^{+/tk}$) or homozygous deficient embryos ($\alpha_{IIb}^{tk/tk}$). Error bars: standard deviations for $n=3$ independent experiments. CFU-GEMM: CFU-Granulocyte–Erythroid–Macrophage–Megakaryocyte; CFU-GM: CFU-Granulocyte–Macrophage; CFU-M: CFU-Macrophage; CFU-G: CFU-Granulocyte; BFU-E: Burst-Forming Unit-Erythroid. (B) Number of c-kit⁺ cells per E11 $\alpha_{IIb}^{+/+}$ ($n=5$ embryos), $\alpha_{IIb}^{+/tk}$ ($n=5$ embryos) and $\alpha_{IIb}^{tk/tk}$ ($n=3$ embryos) aorta. Error bars: standard deviations. Confocal stack images of the mouse aorta region of E11 $\alpha_{IIb}^{+/+}$ (C) and $\alpha_{IIb}^{tk/tk}$ (D) embryos after whole mount staining for c-kit and CD31. Scale bars: 50 μ m. ns: not statistically significant. Error bars: standard deviations.

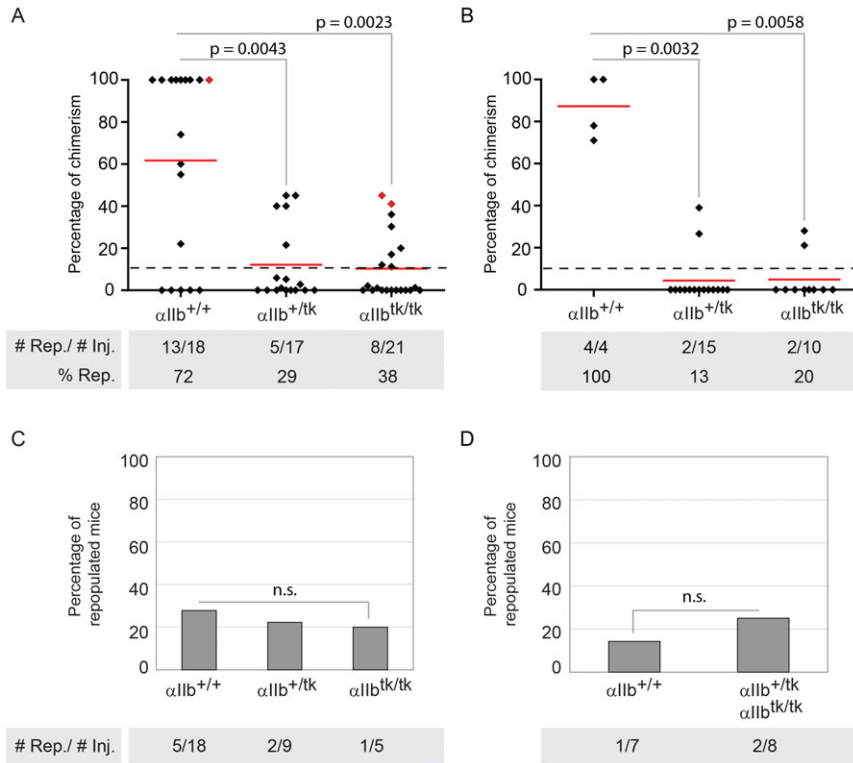


Fig. 4. Functional analyses of AGM, yolk sac and fetal liver cells isolated from E11 CD41 (α_{IIb}) deficient embryos. Hematopoietic repopulation analysis of mice after injection of (A,B) AGM, (C) yolk sac or (D) fetal liver cells isolated from E11 wild-type ($\alpha_{IIb}^{+/+}$), heterozygous ($\alpha_{IIb}^{+/tk}$) or homozygous deficient embryos ($\alpha_{IIb}^{tk/tk}$). (A,B) Percentage of chimerism in peripheral blood for each injected mice. Lozenge: one transplanted mouse. Red lozenge: mice used to perform secondary transplantations. Dashed line: limit of positivity (>10% of chimerism). Red line: chimerism average. (A) AGM cells were injected directly ($n=3$) or (B) after explant culture ($n=3$). The genotype of embryos, the number of mice repopulated/number of mice transplanted (# Rep./# Inj.) and the percentage of repopulated mice (% Rep.) are indicated below the graphs. 1 embryo equivalent (ee) of AGM cells was injected per recipient ($n=3$). p-values are indicated. (C,D) Percentage of repopulated mice. Numbers below columns: number of mice repopulated/number of mice transplanted. (C) 1 ee of yolk sac cells ($n=3$) and (D) 3 to 4.5 ee of fetal liver cells ($n=4$) were injected per recipient. Due to the very low number of HSCs in E11 FL, $\alpha_{IIb}^{+/tk}$ and $\alpha_{IIb}^{tk/tk}$ cells were pooled. ns: not statistically significant.

Dzierzak, 1996). We tested whether α_{IIb} deficient HSCs can be maintained and expanded in AGM explant cultures ($n=3$). Cells from $\alpha_{IIb}^{+/tk}$ or $\alpha_{IIb}^{tk/tk}$ AGM explants were able to reconstitute 13% and 20% of the transplanted recipients, as compared to 100% of recipients transplanted with cells from $\alpha_{IIb}^{+/+}$ AGM explants (Fig. 4B). As expected the HSC repopulation ability of the $\alpha_{IIb}^{+/+}$ AGM cells was higher after explant culture, as compared to transplantations performed without pre-culture (Fig. 4A). In contrast the HSC repopulation ability of the α_{IIb} mutant AGM cells was significantly lower. This was not due to abnormal cycling or increased apoptosis of c-kit⁺ cells in the α_{IIb} mutant AGM (supplementary material Fig. S5A,B, respectively). Altogether, the results support a role for α_{IIb} in the maintenance of AGM HSC repopulating activity.

α_{IIb} deficient embryos have no major hematopoietic stem cell defect in the YS or FL

HSC activity of α_{IIb} mutant YSs was also tested (only some YS HSCs express α_{IIb}). Transplantations with YS cells isolated from E11 $\alpha_{IIb}^{+/+}$, $\alpha_{IIb}^{+/tk}$ and $\alpha_{IIb}^{tk/tk}$ embryos ($n=3$) showed similar percentages of mice reconstituted (28%, 22% and 20%, respectively) at four months post-transplantation (Fig. 4C). Thus, while there is a strong HSC defect in the AGM of the α_{IIb} mutant embryos, it is not the case in the YS.

Similarly, we performed transplantations with FL cells isolated from E11 $\alpha_{IIb}^{+/+}$, $\alpha_{IIb}^{+/tk}$ and $\alpha_{IIb}^{tk/tk}$ embryos ($n=4$), at the time when HSCs start to colonize the FL. The percentage of reconstituted mice after the injection of $\alpha_{IIb}^{+/tk}$ or $\alpha_{IIb}^{tk/tk}$ cells (25%) was similar to the percentage of reconstituted mice after the injection of $\alpha_{IIb}^{+/+}$ cells (14%) (Fig. 4D) thus showing that α_{IIb} mutant HSCs are able to migrate and colonize the FL *in vivo*.

We further tested the HSC activity in the FL of α_{IIb} mutant embryos at E14, a time point when HSCs extensively expand.

Flow cytometry for Lin⁻Sca-1⁺c-kit⁺CD48⁻CD150⁺ cells showed that the absolute numbers of these phenotypically defined HSCs were similar in α_{IIb} mutant embryos as compared to wild type (supplementary material Fig. S6). Limiting dilution transplantation of FL cells (0.001 [$n=3$] and 0.005 [$n=3$] ee per recipient) (Fig. 5) showed that α_{IIb} mutant FL cells were as competent as $\alpha_{IIb}^{+/+}$ FL cells in recipient reconstitution. Thus, HSCs have no proliferative defect in the FL of the α_{IIb} mutant embryos.

Discussion

The α_{IIb} integrin subunit (CD41) is one of the first surface markers indicative of hematopoietic commitment (Ferkowicz et al., 2003; McKinney-Freeman et al., 2009; Mikkola et al., 2003; Robin et al., 2011). It is notably expressed by the first hematopoietic cells emerging from the hemogenic endothelium in the aorta (Boisset et al., 2010). We found here that α_{IIb} is not only a reliable HSC marker, but it also plays an important role in maintaining HSC activity in the aorta.

We have also found that HSCs differentially express α_{IIb} , β_3 and α_v integrin subunits during ontogeny. Since HSCs in embryos are difficult to identify *in situ*, integrin expression can be used in combination with other markers (as CD45 and c-kit) to enrich and localize HSCs throughout development. Surprisingly, a substantial fraction of HSCs in the E11/E12 YS do not express α_{IIb} and/or β_3 integrin subunits. Such HSCs are not detectable in the AGM at the same time point, the YS might generate such a subset of HSCs. The results of *in vivo* CD41-Cre-mediated genetic tagging suggest that all/most HSCs go through an α_{IIb} expressing phase, as is reflected by a high percentage of labelled hematopoietic cells in the adult animals (35–65%) (Rybtsov et al., 2011). Thus, the expression of integrins on the surface of HSCs appears to not only be

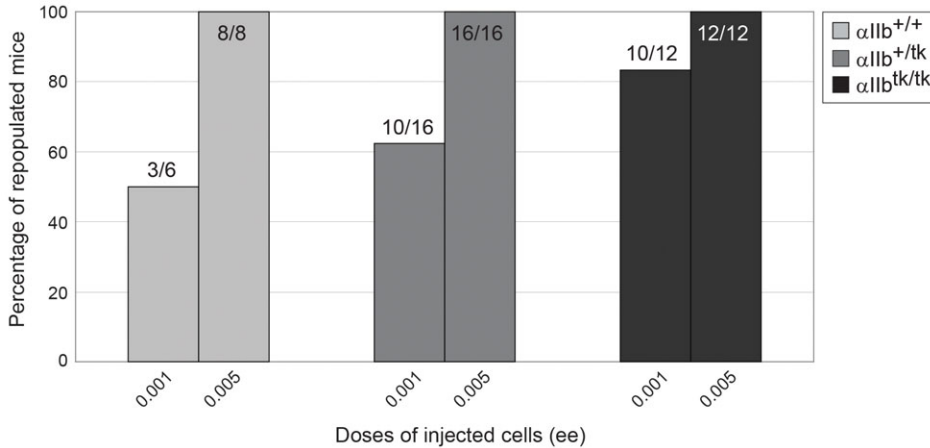


Fig. 5. Functional analyses of E14 fetal liver cells isolated from CD41 (α_{IIb}) deficient embryos. Hematopoietic repopulation analysis of mice after the injection of fetal liver cells isolated from E14 wild-type ($\alpha_{IIb}^{+/+}$; grey bars), heterozygous ($\alpha_{IIb}^{+/tk}$; dark grey bars), or homozygous deficient ($\alpha_{IIb}^{tk/tk}$; black bars) embryos. Numbers above columns: number of mice repopulated/number of mice transplanted.

regulated developmentally, but also by the surrounding cells that compose the HSC niches.

IAHC cells express α_{IIb} (Corbel and Salaün, 2002; Yokomizo and Dzierzak, 2010). Due to low expression levels, the visualization of CD41 by immunostaining embryo cryosections is rather difficult and often is in the context of high background. However, our results show that immunostaining of non-fixed embryo slices with directly conjugated antibodies allows the visualization of low level of integrin expression in the aorta with good resolution (Boisset et al., 2011; Boisset et al., 2010). We observed that IAHC cells co-express α_{IIb} , β_3 and α_v . Interestingly, α_{IIb} , β_3 and α_v were mainly localized at the junction between the cells that form the IAHCs. Using this improved technique to immunostain viable PL slices, we were also able to observe the placental vasculature and groups of HSPCs expressing both α_v and β_3 in the vasculature of the chorionic plate. Similar to the IAHCs, α_v and β_3 were mainly at the junctions between the cells.

We have previously shown that hematopoietic progenitors in the E11 AGM and YS are CD41^{int}, whereas they are in both CD41⁻ and CD41^{int} fractions in E12 PL and E14 FL (Robin et al., 2011). To test whether α_{IIb} plays a role in the progenitor activity in the AGM, we examined a mouse line ($\alpha_{IIb}^{tk/tk}$) in which the α_{IIb} locus was disrupted by the integration of a *tk* gene, resulting in the lack of α_{IIb} protein expression (Tronik-Le Roux et al., 2000). We observed no differences in the total number or types of progenitors in the AGM of E11 $\alpha_{IIb}^{+/+}$, $\alpha_{IIb}^{+/tk}$, or $\alpha_{IIb}^{tk/tk}$ embryos. Such results indicate that α_{IIb} does not play a role in the regulation of the hematopoietic progenitors in the AGM region. This is in contrast to a study in which the disruption of α_{IIb} results in an increased number of hematopoietic progenitors (CFU-Myeloid, BFU-E and CFU-Mk) in E9.5 YS, and in E12.5, E13.5 and E15.5 FL (Emambokus and Frampton, 2003). This difference in the requirement for α_{IIb} on progenitors might depend on the resident microenvironment. Thus, α_{IIb} does not play a functional role on the progenitors despite the fact that all progenitors express this marker in the AGM (Robin et al., 2011).

We observed fewer HSCs and a decrease in the HSC activity in the AGMs of α_{IIb} mutant embryos. The few remaining HSCs were found to be functional in transplantation experiments, ruling out the possibility that α_{IIb} mutant AGM HSCs are defective in homing to the adult BM niche. Interestingly, we did not find differences in the HSC activity in α_{IIb} mutant YS and FL at the same time point of development (E11). The HSC activity was

also normal at a later stage (E14), when HSC expansion occurs in the FL, indicating that α_{IIb} mutant HSCs can undergo normal expansion. The HSC defect is thus restricted to the AGM region. The HSC activity was also lower after explant culture of the α_{IIb} mutant AGMs showing that HSC activity is not maintained in the AGM when α_{IIb} is absent. The defect seems to be cell intrinsic since only IAHC cells (where HSCs reside) express α_{IIb} in the aorta at this stage of development. The observation that the HSC defect is similar in the AGMs in both $\alpha_{IIb}^{+/tk}$ and $\alpha_{IIb}^{tk/tk}$ embryos suggests that a certain threshold of α_{IIb} on the surface of HSCs might therefore be necessary to maintain the HSC activity in the AGM.

We found in α_{IIb} mutant embryos that the number of c-kit⁺ IAHC cells and the shape of the IAHCs are similar to that in wild-type embryos. Thus, either α_{IIb} does not play a role in IAHC cell anchorage, or other adhesion molecules compensate for the absence of α_{IIb} (e.g. $\alpha_v\beta_3$). The normal number of IAHCs also shows that α_{IIb} , which is expressed by the emerging IAHCs and HSCs, is not required for the endothelial to hematopoietic transition (EHT).

Integrin binding to extra-cellular matrix compounds induces outside-in signaling through clustering of integrin heterodimers at focal adhesion sites (Gong et al., 2010; Hynes, 2002). This will recruit other cell surface receptors and many proteins that will activate intracellular signaling pathways. Therefore, the absence of α_{IIb} might have direct or indirect consequences, resulting for example in the lack of recruitment of important receptors to the focal adhesion points (e.g. cytokine receptors) or the lack of signaling downstream of $\alpha_{IIb}\beta_3$ integrins. It was shown that platelet clot formation is mediated through phosphorylation of the c-Src kinase (regulating downstream effectors such as RhoA) after binding of $\alpha_{IIb}\beta_3$ to the $G\alpha_{13}$ subunit (Gong et al., 2010). It was also recently shown that outside-in signaling via pY747 of β_3 (β_3 Y747) following activation of $\alpha_v\beta_3$ integrin by TPO-mediated inside-out signaling is indispensable for TPO-mediated maintenance of HSC activity *in vitro* and *in vivo* within the BM niche (Umamoto et al., 2012). Whether such mechanisms are also active on E11 AGM HSCs will be the focus of further investigations.

Altogether our study shows that β_3 and α_v integrin subunits, in addition to α_{IIb} , are reliable markers of the first HSCs found in the AGM. Such subunits are then differentially expressed by HSCs throughout embryonic development and can be used to isolate and localize HSCs. It has been shown that some integrin subunits are important for the retention of HSPCs in the adult BM

niches (e.g. α_4 (Scott et al., 2003)) and/or for the colonization of HSPCs into the FL and BM (e.g. β_1 (Hirsch et al., 1996; Potocnik et al., 2000)). Here we highlight a new role for integrin subunits and provide evidence that α_{IIb} is important to maintain the HSC activity in the aorta.

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Competing Interests

The authors have no competing interests to declare.

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