The role of CD44 in fetal and adult hematopoietic stem cell regulation

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ABSTRACT

Throughout development, hematopoietic stem cells migrate to specific microenvironments, where their fate is, in part, extrinsically controlled. CD44 standard as a member of the cell adhesion molecule family is extensively expressed within adult bone marrow and has been previously reported to play important roles in adult hematopoietic regulation *via* CD44 standard-ligand interactions. In this manuscript, CD44 expression and function are further assessed and characterized on both fetal and adult hematopoietic stem cells. Using a CD44$^{-/-}$ mouse model, conserved functional roles of CD44 are revealed throughout development. CD44 is critical in the maintenance of hematopoietic stem and progenitor pools, as well as in hematopoietic stem cell migration. CD44 expression on hematopoietic stem cells as well as other hematopoietic cells within the bone marrow microenvironment is important in the homing and lodgment of adult hematopoietic stem cells isolated from the bone/bone marrow interface. CD44 is also involved in fetal hematopoietic stem cell migration out of the liver, *via* a process involving stromal cell-derived factor-1α. The absence of CD44 in neonatal bone marrow has no impact on the size of the long-term reconstituting hematopoietic stem cell pool, but results in an enhanced long-term engraftment potential of hematopoietic stem cells.

Introduction

CD44, a cell adhesion molecule (CAM) encoded by one gene, but with more than 20 isoforms, is produced by alternative mRNA splicing and/or post-translational modifications.[1] CD44 standard (CD44s) is the most abundant isoform, expressed by most mammalian cells[2] and involved in hyaluronan uptake and degradation,[3] angiogenesis,[4] wound healing,[5] tissue formation and patterning.[2] CD44s is also a critical signaling receptor.[6] In contrast, CD44 variants (CD44v) are up-regulated in neoplasia,[7] and involved in tumor metastasis[8] and aggression.[9]

Via different binding domains and regulated by post-translational modifications, CD44s binds the extracellular matrix (ECM) components hyaluronan,[10] collagen,[11] fibronectin,[12] as well as transmembrane receptors such as E-selectin.[13] Among these, hyaluronan is the most common ligand. Although many cells express high levels of CD44s, they do not constitutively bind hyaluronan, with N-glycosylation[14] and carbohydrate-sulfation[15] each independently modulating binding capacity. Hyaluronan is synthesized by three hyaluronan synthases: HAS1, HAS2 and HAS3 at the inner face of the plasma membrane and extruded to the outer surface, where it is secreted. CD44 is the most common receptor for hyaluronan, and hyaluronan uptake and degradation occurs in a CD44-dependent manner.[16,17] Hyaluronan is an important component of the hematopoietic stem cell (HSC) niche,[18,19] participating in HSC lodgment in the endosteal region as well as functioning in HSC proliferation and differentiation.

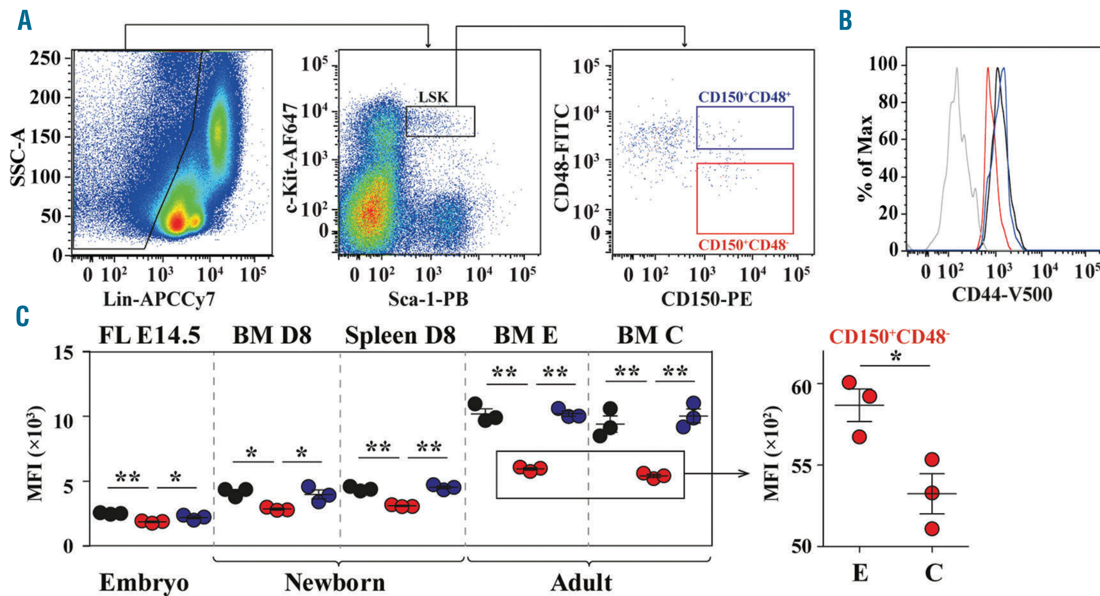

Figure 1. CD44 expression by stem and progenitor cells during development. (A) Flow cytometric analysis of LSK (black), LSKCD150+CD48- (red) and LSKCD150+CD48+ (blue) cells. (B) Representative histograms of CD44 expression on LSK, LSKCD150+CD48- and LSKCD150+CD48+ cells from (A) and unstained (gray) control. (C) The level of CD44 expression on LSK, LSKCD150+CD48- and LSKCD150+CD48+ cells during ontogeny. Each dot is an individual adult animal or a pool of 2 or more embryos or newborn pups. Data are representative from 2 biological repeats. MFI: mean fluorescence intensity; E: endosteal; C: central. *$P<0.05$, **$P<0.01$. Data show mean±SEM.

Furthermore, it has been reported that CD44, hyaluronan and stromal cell-derived factor-1 (SDF-1) interaction affects HSC and progenitor cell trafficking.[20] Hence, CD44s is anticipated to be involved in hematopoietic regulation.

Previously, important roles for CD44s in adult hematopoiesis had been identified. In mice, blocking CD44s inhibits lymphopoiesis in long-term bone marrow (BM) cultures,[21] T-precursor trafficking to the thymus and lymph nodes,[22] and memory cell activation.[23] Furthermore, CD44s expression is down-regulated during myeloid and erythroid development[24] and involved in the retention of hematopoietic progenitors in BM and spleen.[25] Similarly, CD44s plays a role in human hematopoietic regulation, including lymphocyte migration[22,26,27] and activation,[28-30] as well as progenitor cell proliferation and homing to BM.[31] CD44 has been shown to regulate HSC and their BM microenvironment by influencing: 1) matrix assembly; 2) cytokine/chemokine capture and/or release; 3) cytoskeletal linker protein binding (eg. ankyrin, ezrin, radixin and moesin) and signal transduction; and 4) matrix degradation *via* protease production to influence HSC adhesion, homing, migration, quiescence, resistance to oxidative stress as well as mobilization (reviewed by Zoller[32]). These networks are extremely complex and depend on CD44 post-translational modifications.

In this manuscript, we further investigated the role of CD44 on highly enriched HSC (Lineage⁻Sca-1⁺c-Kit⁺CD150⁺CD48⁻ cells) and extended our studies to embryonic hematopoiesis, as CD44s is known to be extensively expressed in fetal hematopoietic organs[33,34] but their roles are poorly understood.

Methods

Mice

CD44$^{-/-}$ mice, devoid of all CD44 isoforms,[35] (Tak Mak, Amgen Institute, Canada), red fluorescent (RFP)[36] mice and wild-type (WT) controls were bred on the most common genetic mouse strain (C57BL/6J, CD45.2) at Monash Animal Research Platform (MARP) (Monash University, Australia). Protein tyrosine phosphatase receptor type C (PTPRCA, CD45.1) mice were purchased from the Animal Resources Centre (Perth, Australia). C57BL/6J and PTPRCA mice are congenic strains, genetically identical except for the CD45 locus. Therefore, when transplanted together, specific CD45.1 and CD45.2 antibodies allow the specific contribution of each donor population to be determined. Adult mice were 6-8 weeks old and sex-matched. Non-ablated recipients and WT BM carrier cells were used in homing and spatial distribution assays, while irradiated recipients and BM carrier cells were used for long-term transplants. Recipients were irradiated using a split dose (4.5Gy each), 4-5 h apart, 24 h prior to transplant and carrier cells exposed to a single dose of 15Gy on the day of transplant. The MARP ethics committee approved all experiments and institutional and national guidelines for the care and use of laboratory animals were followed.

Timed pregnancies were set up late afternoon and separated early the following morning with vaginal plugs designated as 0.5 days (E0.5). Pups were harvested from embryonic day 14.5 (E14.5) until newborn day 9 (d9), as previously described.[37]

Hematopoietic cell isolation

Endosteally and centrally located adult BM HSC and fetal HSC (Lineage⁻Sca-1⁺c-Kit⁺ (LSK) CD150⁺CD48⁻) were isolated and analyzed as previously described.[37,38] Cell counts were performed on a Sysmex KX-21N automated cell counter (Sysmex, Japan).

Flow cytometry

Flow cytometric analysis used an LSRII and cell sorting used an Influx (Becton Dickinson, NJ, USA) equipped as previously described.[39] HSC cells were sorted at approximately 20,000 cells per second and re-analyzed to confirm purity (>95%). CD44 expression was analyzed on hematopoietic stem and progenitor cells using anti-CD44-biotin-streptavidin-V500.

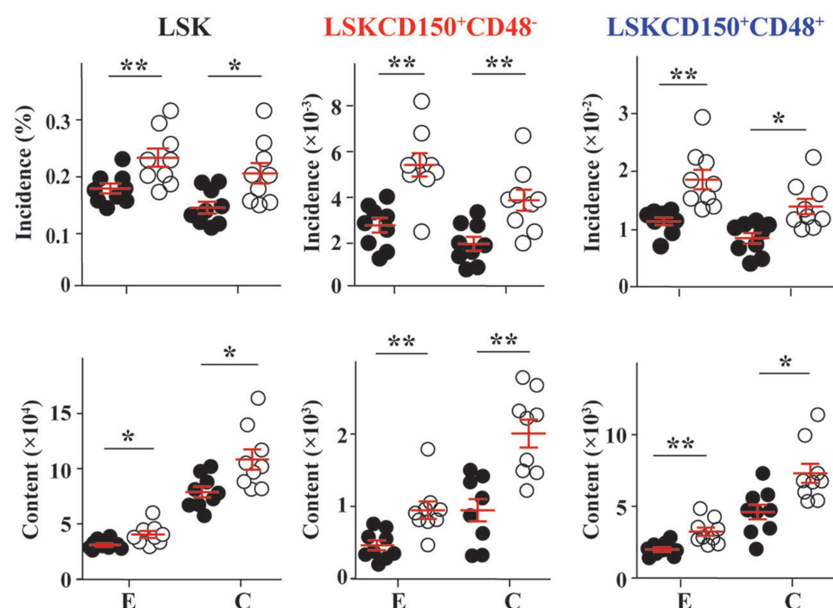

Figure 2. The incidence and content of HSC and progenitors in adult bone marrow. Data represent the cell content after normalization using individual mouse weights. Each dot represents an individual animal. WT: closed circle; CD44$^{-/-}$: open circle; E: endosteal, C: central. *P<0.05, **P<0.01. Data show mean±SEM, n=9 from 3 independent experiments.

Homing and spatial distribution assays

Sorted fetal liver HSC or adult BM HSC were stained with CFDA-SE (CFSE when incorporated) or SNARF at 0.5 μM and 1 μM, respectively, as previously described.[40] The homing efficiency of donor cells was determined by co-transplanting 1-3x10^3 CFSE$^+$ CD44$^{-/-}$ HSC and SNARF$^+$ WT HSC with 2x10^5 unlabeled carrier cells into non-ablated recipients as previously described.[39] Homing efficiency was calculated as the number of donor HSC homed to the BM divided by the number of donor HSC transplanted, assuming one iliac crest, femur and tibia contain 15% of total BM cells.[41] A homing assay in a non-ablated mouse model[42,43] allows for assessment in steady-state hematopoiesis, with ablation disrupting BM integrity[44,45] and causing increased cytokine activity[46] that drives donor cells to cycle.[47]

Sequential transplant spatial distribution assays were performed as previously described.[40] Specifically, irradiated WT and CD44$^{-/-}$ mice were initially transplanted with either WT or CD44$^{-/-}$ BM cells and allowed to reconstitute for 20 weeks (>95% chimerism). WT or CD44$^{-/-}$ CFSE$^+$ LSK cells with 2x10^5 unlabeled carrier cells were then transplanted into non-ablated pre-reconstituted mice to assess their lodgment 15 h post-transplant both *via* flow cytometry as well as histologically in paraffin embedded femur sections.[48] The number of cells located in the endosteal (within 12-cell diameters from the bone/BM interface in the diaphysis or in the metaphysis) and central BM regions were counted manually.[40]

Cell lysis and SDF-1α quantification via *ELISA*

Whole embryonic livers were dissociated in PBS and separated into supernatant and pellet fractions by centrifugation and the pellet was lysed as previously described.[39] E16.5 fetal bones were dissected and cut with fine scissors before lysis, as for the method above. SDF-1α expression was assayed in duplicates from 3 biological repeats at each time point using an ELISA kit (R&D) according to the manufacturer's instructions and normalized for total protein.

Limiting dilution reconstitution assay

The frequency of long-term multi-lineage reconstituting cells in d8 CD44$^{-/-}$ and WT BM HSC or adult CD44$^{-/-}$ and WT BM stem and progenitor was assessed using limiting dilution transplant analysis in RFP recipients with 2x10^5 irradiated RFP carrier cells (carrier cells were irradiated to provide short-term radioprotection before donor HSC reconstituted the recipient BM). The number of mice failing to reconstitute at each dilution (10, 30, 100 or 300 HSC) was measured and the frequency of long-term multi-lineage reconstituting cells calculated using L-Calc (Stem Cell Technologies, Vancouver, Canada).[38,49,50] Multi-lineage reconstitution was detected by staining with Gr-1, Mac-1 (myeloid), B220 and CD3 (lymphoid) antibodies as previously described.[38] Mice with more than 1% donor multi-lineage reconstitution were designated positive.

Competitive limiting dilution reconstitution assay

The multi-lineage reconstitution potential of CD44$^{-/-}$ d8 BM HSC was assessed using competitive limiting dilution transplant analysis, which compares the hematopoietic potential of one population with another.[38,51,52] A serial dilution of 10, 30, 100, 300 CD44$^{-/-}$ (CD45.2) LSKCD150$^+$CD48$^-$ cells were competed with 100 PTPRCA (CD45.1) WT LSKCD150$^+$CD48$^-$ cells and 2x10^5 irradiated RFP (red fluorescent CD45.2) carrier cells in irradiated RFP recipients were used to compare the hematopoietic potential of CD44$^{-/-}$ HSC in relation to WT HSC. Analysis was assessed as described above, except with the addition of anti-CD45.2 and anti-CD45.1 to discriminate donor populations.

Statistical analysis and data presentation

Flow cytometric analysis was performed using FlowJo and Sigma Plot for data comparison. Student's *t*-test, Mann-Whitney rank sum test, one-way ANOVA or one-way ANOVA on ranks was used to determine statistical significance as appropriate. A Kaplan-Meier and log rank test were used to assess survival.

Results

HSC express CD44 throughout development, but at lower levels than progenitors

Fetal and adult BM LSK cells, HSC (LSKCD150$^+$CD48$^-$) and restricted progenitors (LSKCD150$^+$CD48$^+$) (Figure 1A)

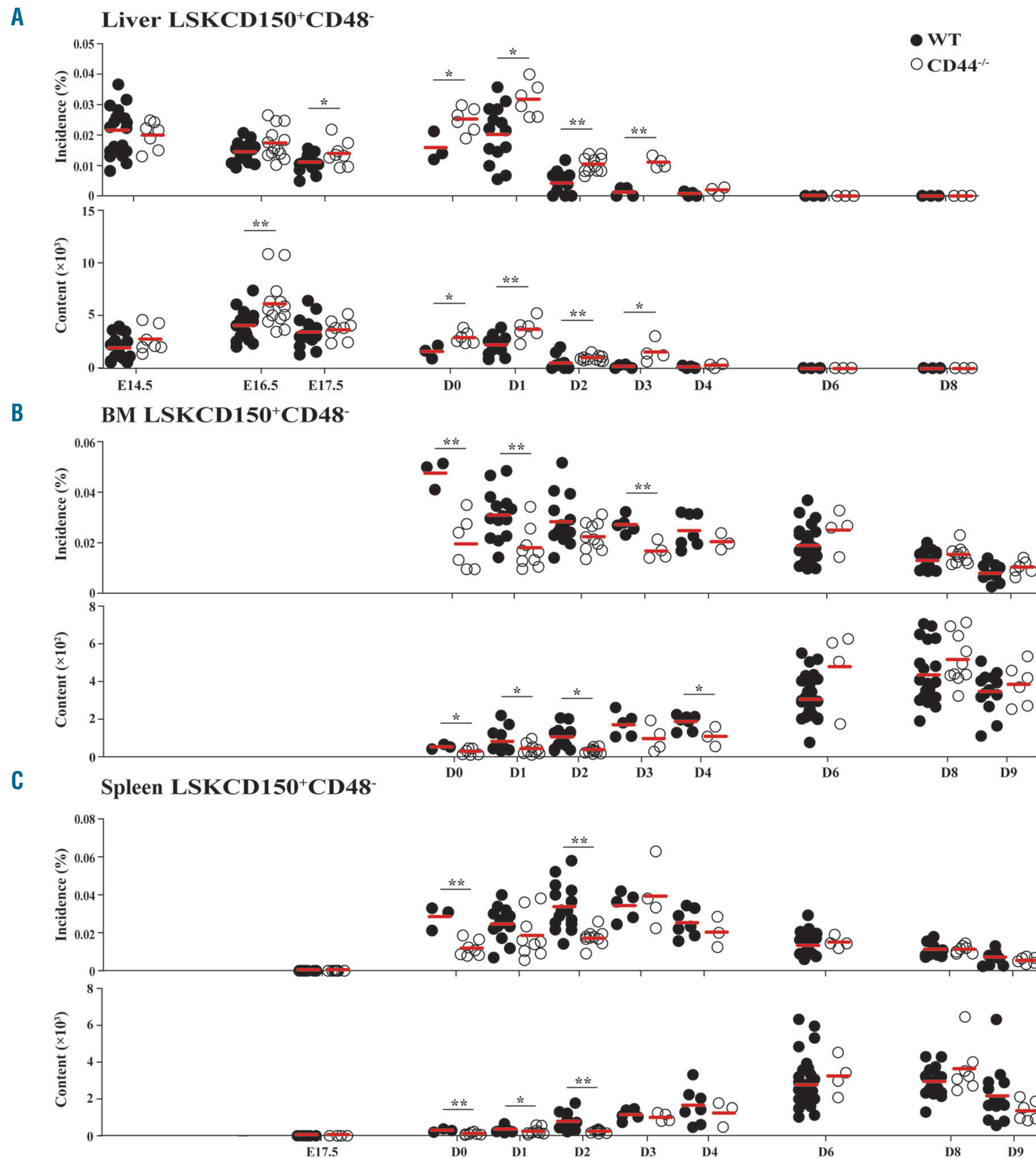

Figure 3. The incidence and content of HSC in fetal and newborn hematopoietic organs. Each dot represents a pool of 2 or more embryos or newborn pups for each time point different. **P*<0.05, ***P*<0.01. Line shows mean value, n≥3.

were analyzed for the presence of CD44 (Figure 1B). CD44 expression was significantly reduced on HSC (1.5-2-fold) compared to LSK and LSKCD150+CD48+ cells (*P*<0.05) (Figure 1C). Furthermore, CD44 expression on adult BM HSC was significantly higher when they were located in the endosteal region compared to those in the central BM region (*P*<0.01) (Figure 1C, inset). Previously we have shown that these two populations are identical in their LSKCD150+CD48– phenotype, but those isolated from the endosteal region have a higher hematopoietic potential.[38]

CD44-/- mice are consistently smaller than WT, but the absence of CD44 does not alter their lineage composition

From E16.5 onwards, including during adulthood, CD44–/– mice consistently weighed significantly less than age- and sex-matched WT controls (*Online Supplementary Figure S1A*). After normalizing for weight, CD44–/– fetal liver (FL) cellularity was significantly higher at E14.5 and E16.5 (*Online Supplementary Figure S1B*). In contrast, normalized fetal and adult BM as well as fetal spleen cellularities were comparable between CD44–/– and WT mice

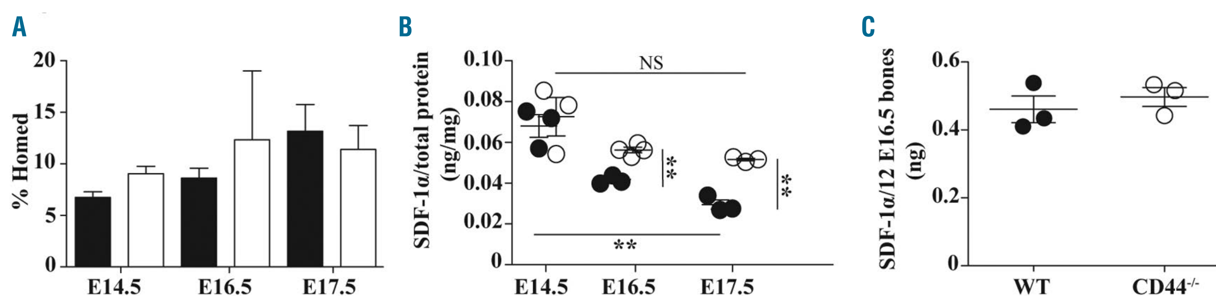

Figure 4. The role of CD44 in FL HSC homing and SDF-1α expression in fetal tissue. (A) Homing efficiency of FL HSC to adult BM. WT: black bar; CD44$^{-/-}$: white bar. (B) SDF-1α in FL and (C) E16.5 fetal bones. WT: closed circle; CD44$^{-/-}$: open circle. Data are the mean±SEM, n=3. ***P*<0.01. NS: not significant.

(*Online Supplementary Figure S1B*). Normalized values were used for all subsequent calculations of cell content. Furthermore, lineage analysis of fetal and adult hematopoietic organs revealed no significant differences in the proportions of granulocytes (Gr-1$^+$ in fetal[53] and Gr-1$^+$/Mac-1$^+$ in adult), T cells (CD3$^+$) or B cells (B220$^+$) in CD44$^{-/-}$ and WT mice (*Online Supplementary Figure S1C*).

The absence of CD44 significantly alters HSC and progenitor pools in fetal and adult hematopoietic organs in a developmental age dependent manner

The incidence and number of HSC and progenitors were assessed in the absence of CD44, providing important but different information; with a number of examples occurring where one is altered but the other is not affected or is altered in the opposite direction due to changes in total cellularity.[54,55] In adult BM, CD44 acts as a negative regulator with a significant increase in both the proportion and number of LSK, LSKCD150$^+$CD48$^-$ and LSKCD150$^+$CD48$^+$ cells in CD44$^{-/-}$ mice compared to WT controls (*P*<0.05) (Figure 2). Furthermore, cell cycle analysis of adult CD44$^{-/-}$ BM LSK cells revealed no changes in cell cycle (n=3) (*data not shown*).

In FL, the absence of CD44 resulted in increased frequencies and numbers of HSC (Figure 3A), LSK (*Online Supplementary Figure S2A*) and LSKCD150$^+$CD48$^+$ cells (*Online Supplementary Figure S3A*) at E16.5 up until d3 after birth. Furthermore, the increased CD44$^{-/-}$ FL HSC pool was accompanied by a delayed depletion of HSC from the liver, with a HSC content of zero being detected at d2 in WT pups, in contrast to d4 in CD44$^{-/-}$ pups (Figure 3A). Similarly to that evident in the adult, cell-cycle analysis revealed no changes in E16.5 CD44$^{-/-}$ FL LSK cells compared to their WT counterparts (n=3) (*data not shown*).

In CD44$^{-/-}$ pups, fetal BM had a significantly lower incidence and number of HSC from d0-d4 (Figure 3B). In contrast, the absence of CD44 had limited impact on BM progenitors (LSK and LSKCD150$^+$CD48$^+$), with a significant difference in their content only detected at d2 (*Online Supplementary Figures S2B and S3B*). The absence of CD44 in the spleen resulted in a significantly lower incidence and number of HSC at d0 and d2 (Figure 3C). Collectively, the absence of CD44 significantly altered the HSC and progenitor pools in fetal and adult hematopoietic organs in a developmental age dependent manner.

CD44 is implicated in the migration of fetal HSC from liver to BM via *changes in SDF-1α expression*

With no evident changes to cell cycle, a proliferative defect of CD44$^{-/-}$ FL HSC is unlikely to explain the observed augmented HSC pools or the delayed HSC depletion in FL (Figure 3A). However, the accompanying lower numbers of HSC in the BM and spleen at the same developmental ages (Figure 3B and C) suggest CD44 is involved in fetal HSC migration. As a consequence, the absence of CD44 on the homing efficiency of fetal FL HSC was assessed. No differences were observed between CD44$^{-/-}$ and WT FL HSC homing to adult BM (Figure 4A), suggesting the absence of CD44 does not result in an intrinsically altered homing ability.

Alternatively, the impact on HSC migration in the absence of CD44 could be the consequence of changes in the FL microenvironment. As the CXCR4/SDF-1α pathway has been demonstrated to be important in HSC migration during development,[56] CXCR4 and SDF-1α expression was examined. Analysis of the expression of CXCR4 on CD44$^{-/-}$ and WT FL HSC revealed no differences at any of the time points assessed (*data not shown*). In FL, SDF-1α was exclusively detected in the cellular fraction. In WT FL, the concentration of SDF-1α significantly decreased from E14.5 to E17.5. In contrast, no significant changes in FL SDF-1α concentration was evident over this period in the absence of CD44, resulting in a significantly greater amount of SDF-1α in CD44$^{-/-}$ FL at E16.5 and E17.5 (Figure 4B). However, analysis of the concentration of SDF-1α in E16.5 fetal bones found no difference between CD44$^{-/-}$ and WT animals (Figure 4C). This organ and developmental age specific change in SDF-1α levels directly correlated with the delayed HSC migration from FL.

CD44 is important for adult HSC homing and lodgment

As the presence of CD44 was important for the migration of FL HSC, a potential role in the homing of adult BM HSC was also assessed (Figure 5A). The presence of CD44 on both donor endosteal HSC and in the recipient microenvironment was critical for homing of endosteal HSC, with its absence from either resulting in a significant decrease in homing efficiency (Figure 5B). However, the presence of CD44 on donor HSC or in the microenvironment had no impact on the ability of central HSC to home to the BM (Figure 5B). Importantly, significantly increased homing of WT endosteal HSC into a WT animal was observed compared to the homing of HSC isolated from the central BM region (Figure 5B), which is in agreement with that previously published.[38]

After homing to the BM, HSC and progenitors lodge and reside in specific BM microenvironments. The role of CD44 on hematopoietic progenitors lodging in the

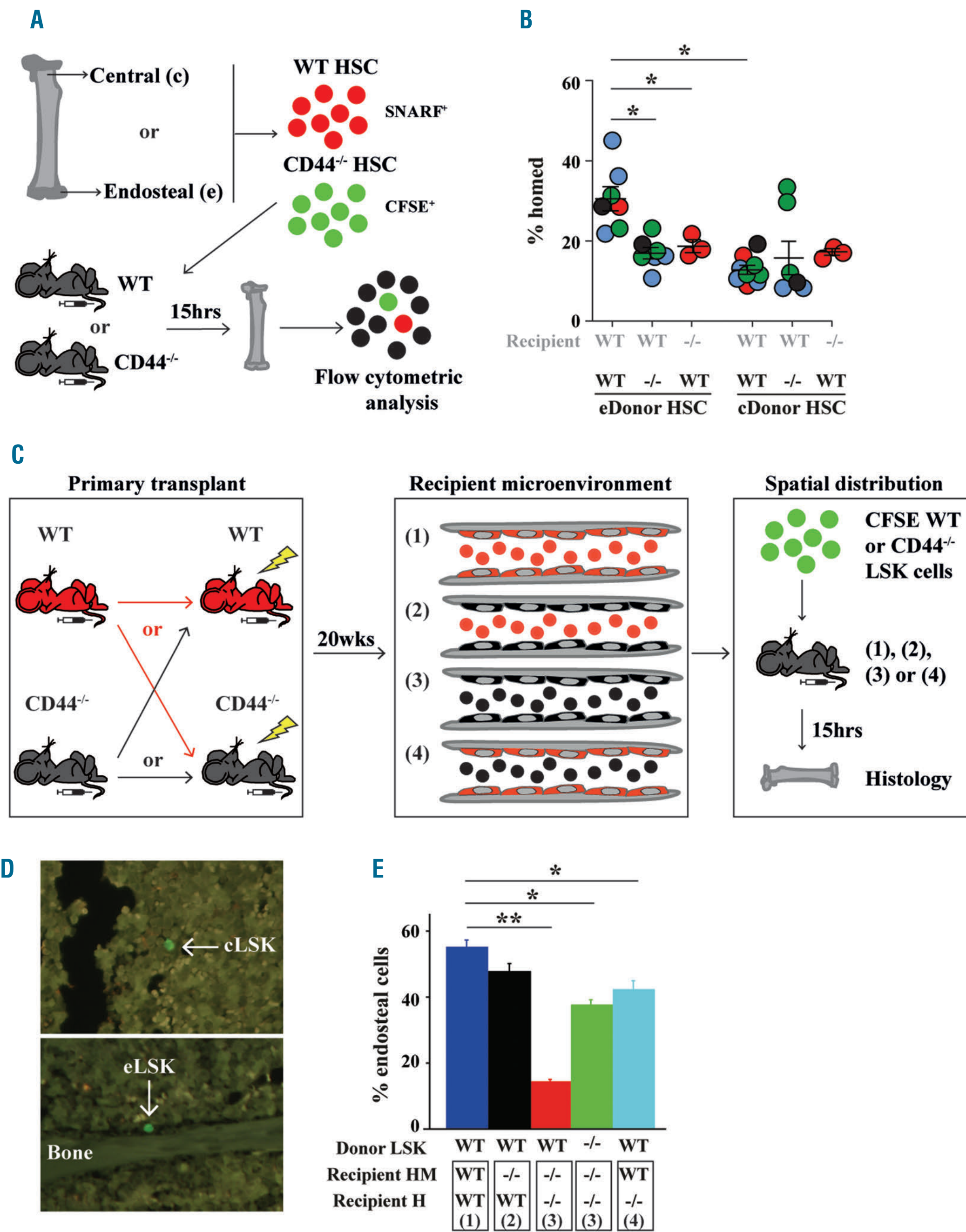

Figure 5. CD44 is critical in adult HSC homing to BM and lodgment in the endosteal region. (A) Schematic presentation of homing assay. (B) Homing efficiency. Each dot represents an individual recipient and different colors represent independent experiments. $*P<0.05$. Data show mean±SEM, n≥3. (C) Schematic presentation of spatial distribution assay into primary reconstituted recipients with different microenvironments (HM) and hematopoietic cells (H). The spatial distribution of total BM LSK cells was assessed. (D) Centrally and endosteally located CFSE+ donor cells in recipient BM sections. (E) Proportion of donor cells located in the endosteal BM region. $*P<0.05$, $**P<0.01$. Data show mean±SEM, n=5.

endosteal BM region was assessed using a sequential transplant spatial distribution assay which allows the evaluation of the role of CD44, either expressed in the microenvironment (HM) or on hematopoietic cells (H) (Figure 5C and D). Consistent with our previous findings,[40] approximately 55% of WT donor cells lodged in the endosteal region 15 h post transplant (Figure 5E). The absence of CD44 only in the recipient HM did not significantly change the spatial distribution of donor WT cells. When WT or $CD44^{-/-}$ LSK cells were transplanted into WT or $CD44^{-/-}$ mice reconstituted with $CD44^{-/-}$ BM (H), the proportion of cells homing to the endosteal region was significantly reduced (≥25% of WT cells into WT mice) (Figure 5E). Furthermore, the absence of CD44 on both the recipient BM and recipient HM resulted in an even greater reduction (approx. 70%) of WT cells lodging in the endosteal region (Figure 5E). Together the data suggest that CD44 expressed by adult HSC and recipient BM cells, rather than in the recipient HM, is more critical in the lodgment of transplanted hematopoietic progenitors.

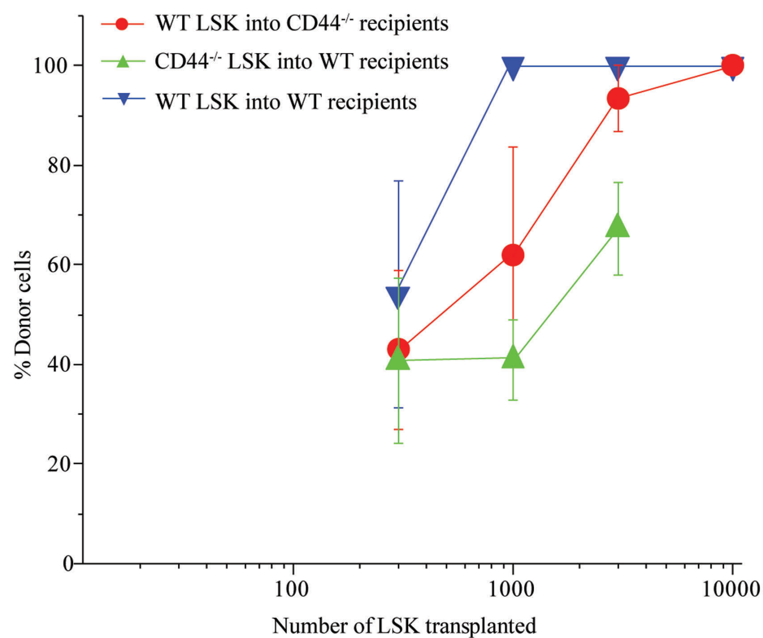

Figure 6. CD44 on adult BM LSK cells has a functional role in supporting stem and progenitor engraftment. A serial dilution of 300, 1000, 3000 and 10000 WT or CD44$^{-/-}$ LSK cells isolated from total BM were transplanted into WT or CD44$^{-/-}$ recipients and assessed 20 weeks post transplant for multi-lineage reconstitution. Data show mean±SEM, n=5.

CD44 in adult BM has a functional role in supporting stem and progenitor engraftment but does not change the frequency of long-term multi-lineage reconstituting HSC

Analysis of adult stem and progenitor potential in a limiting dilution assay *in vivo* demonstrated a critical requirement for CD44 on both the donor LSK cells as well as in the recipient microenvironment (Figure 6). When CD44 was absent from the donor cells, less than 50% chimerism was obtained 20 weeks following a transplant of 1000 cells compared to 100% donor reconstitution following a transplant of equivalent numbers of WT cells. Furthermore, when WT LSK cells were transplanted into a CD44$^{-/-}$ recipient, significantly more donor cells were required to obtain 100% donor reconstitution 20 weeks post transplant compared to WT cells transplanted into a WT recipient.

Although there was a functional role for CD44 in the regulation of HSC potential, the absence of CD44 did not change the frequency of long-term multi-lineage reconstituting HSC in adult LSK cells. Following a transplant of CD44$^{-/-}$ LSK cells into a WT recipient, the frequency of long-term multi-lineage reconstituting HSC was 1:181 (95% confidence interval 1 in 108 to 1 in 304, n=5), equivalent to transplants of WT LSK cells into a WT recipient (1:277, 95% confidence interval 1 in 176 to 1 in 437, n=5) or WT cells into a CD44$^{-/-}$ recipient (1:391, 95% confidence interval 1 in 249 to 1 in 615, n=5).

The absence of CD44 in neonatal BM also has no impact on the number of the long-term multi-lineage reconstituting HSC, but results in an enhanced long-term engraftment potential

We examined the impact of the absence of CD44 on the number of HSC with long-term reconstitution capability *in vivo* (Figure 7). To assess the frequency of long-term multi-lineage reconstituting HSC, a limiting dilution long-term transplant was performed using CD44$^{-/-}$ and WT d8 newborn BM HSC (Figure 7A). No differences in the survival (Figure 7B), or donor chimerism (Figure 7C) were evident between mice transplanted with limiting numbers of WT or CD44$^{-/-}$ HSC. In addition, an equivalent frequency of long-term multi-lineage reconstituting HSC was detected in CD44$^{-/-}$ (1:17, 95% confidence interval 1 in 8 to 1 in 38) and WT (1:17, 95% confidence interval 1 in 7 to 1 in 39) newborn d8 HSC, which was also equivalent to adult endosteal HSC (1:12, 95% confidence interval 1 in 7 to 1 in 20) (Figure 7D). Together with the observed equivalent number of HSC observed in CD44$^{-/-}$ and WT d8 BM (Figure 3B), the data suggest the absence of CD44 does not alter the long-term multi-lineage reconstituting HSC pool in newborn BM.

The role of CD44 in long-term multi-lineage reconstituting HSC potential was then investigated by competing CD44$^{-/-}$ HSC with WT HSC in a competitive long-term limiting dilution transplant (Figure 7E). Analysis of recipient peripheral blood (PB) at 6 and 12 weeks post transplant revealed equivalent hematopoietic potential, with the expected proportions of donor contribution (Figure 7F). However, at 20 weeks, the proportion of donor CD44$^{-/-}$ cells was significantly higher than expected when equivalent numbers of CD44$^{-/-}$ and WT cells were transplanted (61±5 and 39±5%, respectively) (Figure 7F). Importantly, analysis of BM also revealed an increased contribution from CD44$^{-/-}$ cells (Figure 7F). Furthermore, lineage commitment analysis demonstrated that when equivalent numbers of CD44$^{-/-}$ and WT cells were transplanted, CD44$^{-/-}$ donor cells resulted in a significantly higher proportion of B cells at all time points and more myeloid cells at 20 weeks in both PB and BM (Figure 7G). Together, the data suggest CD44$^{-/-}$ HSC have higher long-term engraftment potential than their WT counterparts.

Discussion

Previous studies had shown CD44 is highly expressed on adult HSC[57] and early erythroid progenitors, but down-regulated during lineage commitment.[24] In addition, adult BM cells with colony-forming-unit granulocytes-macrophage and burst-forming unit-erythroid are

predominantly found in the $CD44^{bright}$ population.[58] The current analysis extended these findings to reveal CD44 expression on HSC is significantly reduced compared to that detected on progenitor cells throughout development. Furthermore, in adult BM, CD44 is expressed at a higher level on HSC isolated from the endosteal BM region compared to those isolated from the central BM. Although there is no difference in the incidence of HSC between endosteal and central BM, due to the higher total cellularity in central BM, the total number of HSC in this region is significantly higher than in the endosteal region.[38]

$CD44^{-/-}$ mice are born healthy and fertile with no apparent developmental disorders.[35] Previous studies have reported $CD44^{-/-}$ mice develop normal organ morphology with normal nucleated cell counts in hematopoi-

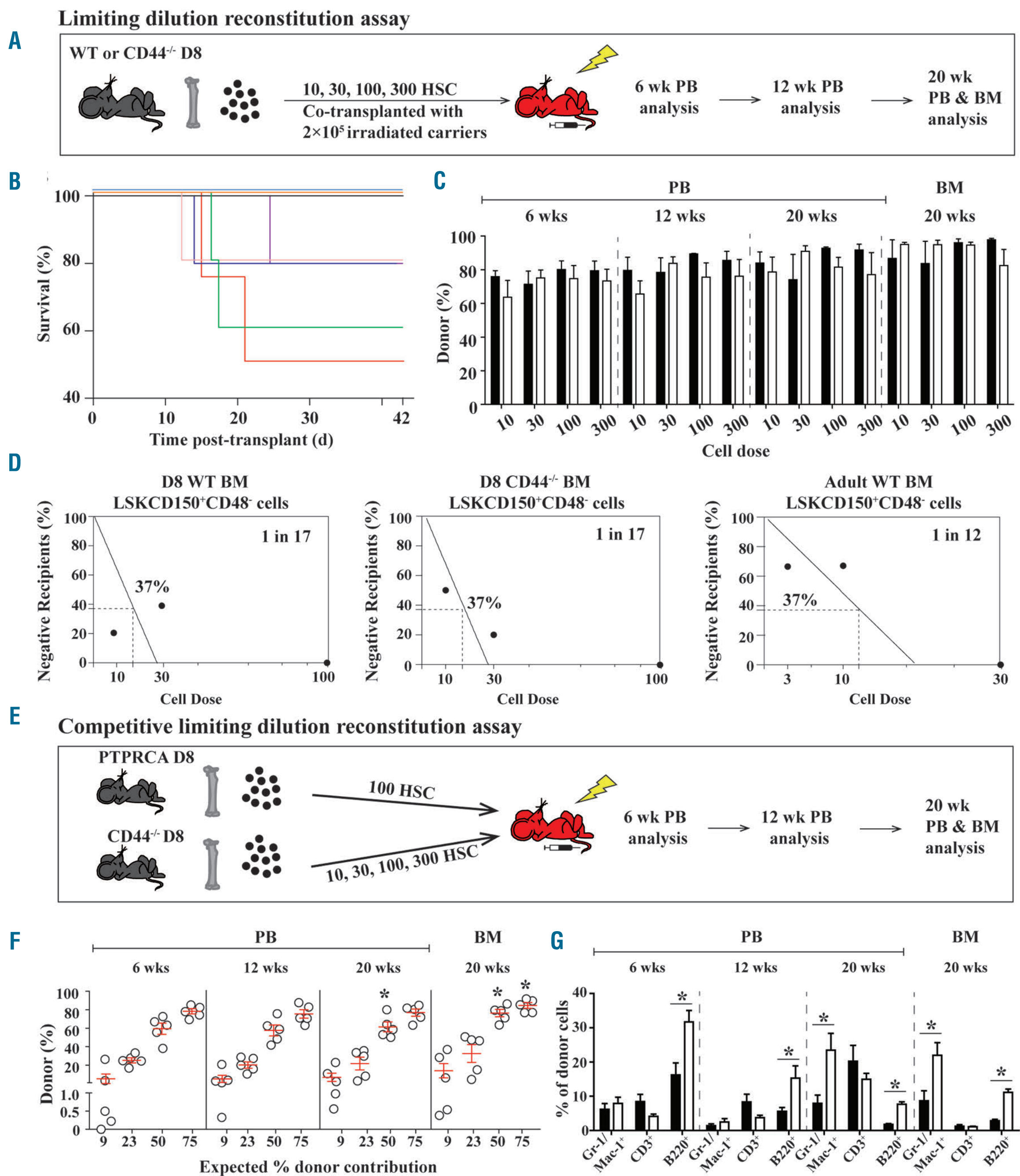

Figure 7. Neonatal $CD44^{-/-}$ stem cell pool has equivalent numbers of long-term multi-lineage reconstituting HSC but higher long-term engraftment potential compared to WT counterparts. (A) Limiting dilution transplant analysis of D8 BM $LSKCD150^{+}CD48^{-}$ cells. (B) Survival analysis: WT 10 (red), $CD44^{-/-}$ 10 (pink), WT 30 (purple), $CD44^{-/-}$ 30 (green), WT 100 (dark blue), $CD44^{-/-}$ 100 (light blue), WT 300 (black), and $CD44^{-/-}$ 300 (orange). (C) Peripheral blood (PB) and BM reconstitution from $CD44^{-/-}$ (white bars) and WT (black bars) donor HSC. (D) Frequency of long-term multi-lineage reconstituting HSC. Each symbol represents a group of 5 mice. (E) Competitive limiting dilution transplant analysis of $LSKCD150^{+}CD48^{-}$ cells. (F) PB and BM reconstitution from $CD44^{-/-}$ donor HSC. (G) $CD44^{-/-}$ (white bars) and WT (black bars) donor lymphoid and myeloid reconstitution following a transplant of 100 $CD44^{-/-}$ and 100 WT donor cells. Data are mean±SEM, n=5. *P<0.05.

etic tissues.[35,59] However, our CD44$^{-/-}$ mice are consistently smaller than their WT counterparts. These differences may be due to differences in mouse strains, with Schmits *et al.* using heterozygous CD44$^{+/-}$ mice as controls[35] and Oostendorp *et al.* using CD44$^{-/-}$ mice on a PTPRCA background,[59] whilst our CD44$^{-/-}$ mice were bred on a C57/Bl6 background. However, despite the reduced body weight of CD44$^{-/-}$ mice, normalization for body weight revealed equivalent organ cellularities from E16.5. Furthermore, analysis of lineage commitment in CD44$^{-/-}$ mice did not show any evidence of lineage skewing in fetal or adult hematopoietic organs.

However, the data demonstrate CD44 functions in maintaining the HSC and progenitor pools in a developmental age and location dependent manner. In adulthood, CD44$^{-/-}$ BM had significantly increased stem and progenitor cell pools. Together the data suggest CD44 acts as a negative regulator of HSC proliferation, which is potentially regulated through the CD44/hyaluronan interaction. Previously, hyaluronan had been shown to be highly

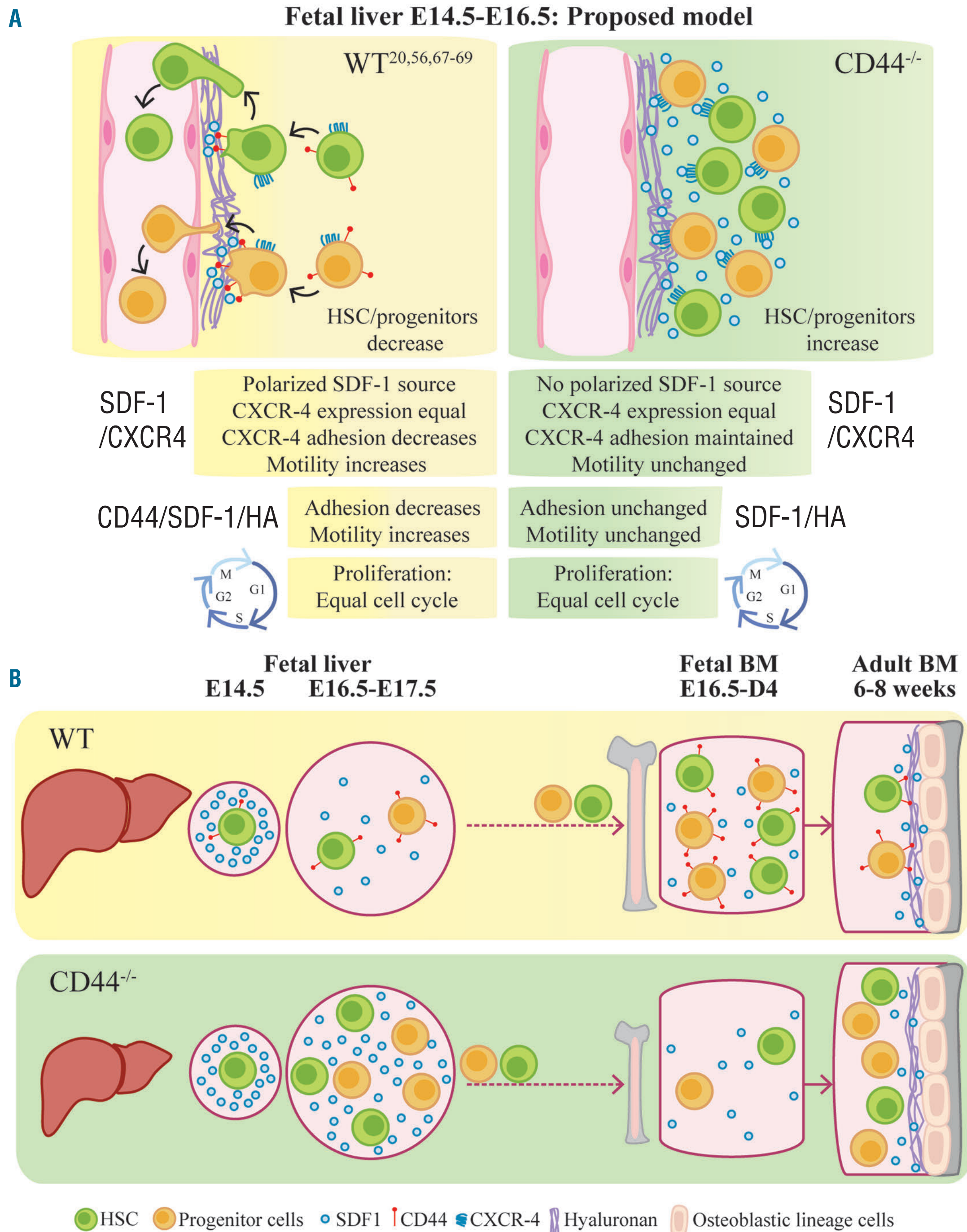

Figure 8. Schematic presentation summarizing the role of CD44 in fetal and adult hematopoiesis. (A) Proposed model for the mechanism of the role of CD44 in the migration of HSC and progenitors out of the FL. (B) Observed changes in fetal and adult hematopoiesis in the absence of CD44.

expressed at the endosteal BM surface as well as acting as a negative regulator of HSC proliferation.[18,48] This could also explain why endosteal HSC have significantly higher CD44 expression compared to centrally isolated HSC.

Previous analysis of HSC devoid of CD44 revealed no functional changes, with CD44$^{-/-}$ adult BM and FL cells having equivalent capacity to initiate long-term marrow cultures *in vitro*[59] and form d12 spleen colonies *in vivo*.[35] These findings are consistent with our data, which showed an equivalent frequency of long-term multi-lineage reconstituting HSC in CD44$^{-/-}$ and WT newborn BM HSC as well as CD44$^{-/-}$ and WT adult LSK cells. However, the use of a HSC competitive limiting dilution transplant assay revealed CD44$^{-/-}$ neonatal (d8) HSC have higher engraftment potential than WT controls. This finding is in contrast to that previously published by Oostendorp *et al.*[59] who showed equivalent frequency of competitive repopulating units in adult CD44$^{-/-}$ BM. The difference may reflect the roles of CD44 in fetal and adult HSC regulation, although it is known that while fetal HSC expand from E14.5[60] they have acquired adult characteristics by one week after birth (d8).[61] Alternatively, these differences may be due to the assay conditions used. In this study, purified HSC were assessed, while Oostendorp *et al.*[59] used total BM. In addition, Waterstrat *et al.*[62] have suggested that, due to superior homing ability, CD45.2 cells naturally have increased engraftment potential compared to CD45.1 cells; a possible contributing explanation to our results. However, this difference was not evident until 30 weeks post transplant, whereas we detected a significant difference in engraftment potential by 20 weeks post transplant, making this unlikely to be the sole explanation.

Furthermore, we demonstrate CD44 is an important molecule in adult HSC homing. The reduced homing was evident when CD44 was absent on endosteal HSC or in the recipient microenvironment and could be due to the interaction between CD44, hyaluronan and SDF-1 in anchoring homed hematopoietic progenitor cells.[20] We have previously demonstrated that HSC isolated from the endosteal region home to BM better than their central counterparts as well as preferentially home back to the endosteal region,[38] and that hyaluronan is highly expressed in the endosteal BM region compared to central BM.[18,48] Therefore, without CD44, HSC and progenitors may have impaired interactions with their microenvironment during the homing process. Furthermore, hyaluronan has been shown to act as a chemoattractant *via* CD44[63] and we did not find any differences in the expression of HAS3, critical in the generation of hyaluronan, in CD44$^{-/-}$ mouse BM (*data not shown*). Together, these data suggest an extrinsic effect on HSC in the absence of CD44.

While Khaldoyanidi *et al.*[31] had previously demonstrated a key role for CD44 in the homing of progenitor cells, Oostendorp *et al.*[59] did not. The discrepancy between results could be due to assay conditions: Oostendorp *et al.*[59] analyzed the homing of whole BM cells transplanted into ablated recipients, with altered cytokine release,[46] cell cycling,[47] and vascular permeability post irradiation,[44,45] while we used purified HSC separated based on their anatomical location and demonstrated a significant reduced homing of both endosteal CD44$^{-/-}$ HSC into WT steady-state, non-ablated recipients as well as WT HSC into CD44$^{-/-}$ steady-state, non-ablated recipients. Because only one-quarter to one-third of HSC are endosteal in origin,[38,64] simply assaying total BM HSC, let alone total BM, could miss the effect we identified. In further support of a role for CD44 in progenitor cell homing and migration, Protin and colleagues[65] found CD44$^{-/-}$ T-cell progenitors showed a homing defect to the thymus and lymph nodes as well as a dysregulation of progenitor cell mobilization with a significant decrease in the number of CFU-GM in the spleen following G-CSF administration.[35]

The current data extend our understanding of fetal hematopoiesis and highlight a role for CD44 in fetal HSC anchoring and migration, with a proposed model of the underpinning mechanisms described in Figure 8A. During embryogenesis, HSC initially migrate to FL before colonizing the spleen and BM later in development. Migration is essential for fetal hematopoiesis as multiple sites allow different inductive signals from the microenvironments to support hematopoietic development.[66] We show that, in WT mice, FL SDF-1α concentration progressively decreased from E14.5, with HSC migrating from the FL to the BM after E16.5. The accumulated data in other *in vivo* and *in vitro* contexts, including the homing of HSC and progenitors to BM,[20] suggest this occurs due to a co-ordinated sequence of events including: 1) a polarized source of SDF-1 developing; 2) the cells producing projections extending towards the SDF-1, with cell-associated CD44 migrating into the projections; and 3) CD44 binding to an external source of hyaluronan and the cells undergoing trans-endothelial migration. However, in the absence of CD44, the concentration of FL SDF-1α remained unchanged from E14.5 to E17.5 and HSC and progenitors were not released from the FL microenvironment to migrate to the BM (Figure 8A). These data are supported by Petit *et al.*[67] who demonstrated that decreased SDF-1 levels result in HSC detachment and migration from their microenvironment. The major source of SDF-1 in E15.5 mouse FL is hepatic stem/progenitor (stromal) cells[68] and it has been shown that SDF-1$^{-/-}$ HSC are not retained in the FL but migrate into the PB.[56] Furthermore, genetic silencing of CD44 expressed by endothelial cells increased SDF-1 mRNA expression *via* NFκB.[69] This suggests the accumulation of SDF-1α in CD44$^{-/-}$ FL is the direct result of the absence of CD44, with a resulting increase in HSC and progenitors that remain firmly attached to the FL microenvironment, delaying their migration to the BM. Our data demonstrate that this increase in HSC and progenitors was not due to changes in cell cycle or due to an intrinsic homing defect. In addition, examination of CXCR4 on WT and CD44$^{-/-}$ FL HSC and progenitors at E16.5 revealed no differences. This finding is also in agreement with previous studies reporting that while human HSC and progenitors pre-incubated with CD44 blocking antibody had impaired homing ability *in vivo* as well as decreased migration towards a SDF-1 gradient *in vitro*, this was not due to the downregulation of CXCR4 expression.[20]

In summary, the current study demonstrates a number of roles of CD44 in hematopoiesis throughout development (Figure 8B). CD44 is highly expressed on HSC and progenitors and regulates their pools *in situ*. Furthermore, CD44 participates in HSC anchoring and migration *via* hyaluronan and SDF-1α, with roles in adult HSC homing and lodgment in the BM post transplant as well as in fetal HSC migration out of the liver. As an in depth study of the roles of key molecular interactions during hematopoietic

development, the current study is critical for both our understanding and the treatment of hematologic disorders, as well as the translation of this to the derivation of HSC from human embryonic stem cells and/or induced pluripotent stem cells.

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