Syndecan-2 expression enriches for hematopoietic stem cells and regulates stem cell repopulating capacity

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Abstract:
The discovery of novel hematopoietic stem cell (HSC) surface markers can enhance understanding of HSC identity and function. We have discovered a population of primitive bone marrow (BM) HSCs distinguished by their expression of the heparan sulfate proteoglycan, Syndecan-2, which serves as both a marker and regulator of HSC function. Syndecan-2 expression was increased 10-fold in CD150^+CD48^-CD34^-c-Kit^-Sca-1^-Lineage^- cells (long-term – HSCs, LT-HSCs) compared to differentiated hematopoietic cells. Isolation of BM cells based solely on Syndecan-2 surface expression produced a 24-fold enrichment for LT-HSCs, 6-fold enrichment for alpha-catulin+c-kit+ HSCs, and yielded HSCs with superior in vivo repopulating capacity compared to CD150^+ cells. Competitive repopulation assays revealed the HSC frequency to be 17-fold higher in Syndecan-2^+CD34^-KSL cells compared to Syndecan-2^-CD34^-KSL cells and indistinguishable from CD150^-CD34^-KSL cells. Syndecan-2 expression also identified nearly all repopulating HSCs within the CD150^-CD34^-KSL population. Mechanistically, Syndecan-2 regulates HSC repopulating capacity through control of expression of Cdkn1c (p57) and HSC quiescence. Loss of Syndecan-2 expression caused increased HSC cell cycle entry, downregulation of Cdkn1c and loss of HSC long-term - repopulating capacity. Syndecan-2 is a novel marker of HSCs which regulates HSC repopulating capacity via control of HSC quiescence.

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- Syndecan-2 expression enriches for LT-HSCs
- Syndecan-2 regulates hematopoietic stem cell self-renewal via control of stem cell quiescence
Abstract

The discovery of novel hematopoietic stem cell (HSC) surface markers can enhance understanding of HSC identity and function. We have discovered a population of primitive bone marrow (BM) HSCs distinguished by their expression of the heparan sulfate proteoglycan, Syndecan-2, which serves as both a marker and regulator of HSC function. Syndecan-2 expression was increased 10-fold in CD150⁺CD48⁻CD34⁻c-Kit⁺Sca-1⁻Lineage⁻ cells (long-term – HSCs, LT-HSCs) compared to differentiated hematopoietic cells. Isolation of BM cells based solely on Syndecan-2 surface expression produced a 24-fold enrichment for LT-HSCs, 6-fold enrichment for alpha-catulin⁺c-kit⁺ HSCs, and yielded HSCs with superior in vivo repopulating capacity compared to CD150⁺ cells. Competitive repopulation assays revealed the HSC frequency to be 17-fold higher in Syndecan-2⁺CD34⁺KSL cells compared to Syndecan-2⁻CD34⁻KSL cells and indistinguishable from CD150⁺CD34⁺KSL cells. Syndecan-2 expression also identified nearly all repopulating HSCs within the CD150⁺CD34⁺KSL population. Mechanistically, Syndecan-2 regulates HSC repopulating capacity through control of expression of Cdkn1c (p57) and HSC quiescence. Loss of Syndecan-2 expression caused increased HSC cell cycle entry, downregulation of Cdkn1c and loss of HSC long-term - repopulating capacity. Syndecan-2 is a novel marker of HSCs which regulates HSC repopulating capacity via control of HSC quiescence.
Introduction

Hematopoietic stem cells (HSCs) comprise less than 0.01% of the total nucleated cells in the adult bone marrow (BM). Long-term - HSCs (LT-HSCs) possess self-renewal capacity and unrestricted hematopoietic differentiation potential. Characterization of the immunophenotype of HSCs is important both to understand the fundamental biology of HSCs and to facilitate the development of HSC-based regenerative therapies.

Murine HSCs display unique surface protein expression patterns, which enabled the purification of HSCs using antibody staining and fluorescence activated cell sorting (FACS). Seminal studies showed that Sca-1 and c-Kit expression enriched for hematopoietic stem and progenitor cells (HSPCs), while single c-Kit+ Sca-1 Lineage¬ (KSL) CD34low− cells were capable of long-term multilineage repopulation. Additional markers including Flk2/Flt3, CD201, and endothelial cell – selective adhesion molecule 1 (Esam1) have been used to further enrich for murine HSCs. Notably, the expression pattern of the signaling lymphocyte activation molecules (SLAM) proteins (CD150, CD48 and CD244) can purify distinct subpopulations of LT-HSCs, short-term HSCs (ST-HSCs) and hematopoietic progenitor cell populations.

HSC transcriptome studies have unveiled a molecular portrait of LT-HSCs. Expression of Sdc2, which encodes Syndecan-2, a heparan sulfate proteoglycan (HSPG), was shown to be increased in LT-HSCs compared to multipotent progenitor cells (MPPs). However, the function of Sdc2 in HSCs has not been defined. Syndecan-2 is a member of the Syndecan family of HSPGs and consists of a core protein decorated by heparan sulfate chains. Through their interactions with other proteins, Syndecans regulate cell proliferation, survival, and adhesion/migration. The role of Syndecans in stem cell regulation has been primarily confined to muscle stem cells and neural stem cells. However, inhibition of heparan sulfate synthesis by Mx1− stromal cells or pharmacological inhibition of heparan sulfate promoted HSPC egress from the BM into the peripheral blood (PB), while administration of
heparan sulfate mimetics promoted LT-HSC mobilization. However, the nature of HSPG expression on HSCs is poorly understood and the potential cell-autonomous functions of HSPGs on HSCs are unknown.

Here, we demonstrate that Syndecan-2 expression enriches for HSCs with enhanced self-renewal capacity and Syndecan-2 regulates HSC repopulating capacity via control of HSC quiescence.

**Methods**

For detailed methods, see Supplemental Methods.

**Mice**

All mouse procedures were performed using 8–12-week-old mixed gender mice in accordance with protocols approved by UCLA and Cedars Sinai Medical Center (PI, John Chute).

**Flow cytometry**

BM cells were isolated from murine long bones, lineage depleted, and stained using antibodies and/or 7-AAD/Annexin-V for cell death analysis. Stained cells were analyzed using a BD FACS Canto II or sorted using a BD FACS Aria.

**Competitive transplants**

HSCs were resuspended in 10% FBS/PBS supplemented with 2x10^5 competitor BM cells. Cells were transplanted into lethally irradiated (900 cGy) mice via tail vein injection. PB was analyzed every four weeks for donor chimerism using flow cytometry. For secondary transplants, BM was harvested from primary transplanted mice 16 weeks post-transplant and 3x10^6 BM cells were
transplanted along with 2x10^5 competitor BM cells. PB was analyzed every four weeks for donor chimerism.

**Homing assay**

BM HSPCs were resuspended in 10% FBS/PBS and transplanted intravenously into lethally irradiated mice. At +24 hours, BM cells were harvested and analyzed for donor cells by flow cytometry.

**HSC cultures, lentiviral transduction and colony assays**

BM HSCs were sorted using FACS, plated in TSF media and cultured in humidified, 5% CO_2 incubator. Cultured cells were analyzed after 3 or 7 days of culture. Sorted 34 KSL cells were pre-stimulated for 48 hours in StemSpan media supplemented with mouse SCF, IL-3, and IL-6. Cells were spin-occulted with shControl, shSdc2, shCdkn1c, MSCV Control or MSCV-Sdc2 viral supernatant and incubated for 48 hours. For colony forming cell assays, BM cells were plated in Methocult and incubated for 10 days prior to colony counts.

**Gene expression and RNA sequence analysis**

RNA was isolated using the RNeasy Micro Kit. For qRT-PCR analysis, RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit and gene expression was analyzed using an Applied Biosystems QuantStudio 6 PCR Machine. For RNA sequencing, RNA was sequenced using a HiSeq3000 Sequencing System and IPA was performed. RNA sequencing data are available at GEO under accession number GSE151733.

**Cell cycle analysis**

BM cells were stained for surface markers using antibodies and then fixed, permeabilized and stained for Ki-67 and 7AAD for flow cytometric analysis.
Confocal microscopy

Cells were plated on fibronectin-coated slides, fixed, permeabilized, and stained using antibodies to p57, p16, p21 and p27. Imaging was performed using the Leica Stellaris system and analyzed with ImageJ.

Results

Syndecan-2 is differentially expressed on LT-HSCs

We isolated murine LT-HSCs and hematopoietic progenitor populations to assess for Sdc gene expression in each population (Figure 1A). Using qRT-PCR, we quantified Sdc gene expression in adult BM LT-HSCs (CD150⁺CD48⁻CD34⁺c-Kit⁺Sca-1⁺Lineage⁻ cells, supplemental Table 1), CD34⁺c-Kit⁺Sca-1⁺Lineage⁻ (34⁺KSL) HSCs, short term – HSCs (ST-HSCs, CD150⁺/⁻CD48⁻34⁻KSL cells) and multipotent progenitor cells (MPPs, CD150⁺CD48⁻34⁺KSL cells). Sdc2 expression was significantly increased in LT-HSCs compared to 34⁺KSL HSCs, ST-HSCs and MPPs, while expression of Sdc1, Sdc3 and Sdc4 was not different between LT-HSCs and these other populations (Figure 1B). Surface expression of Syndecan-1, Syndecan-3 and Syndecan-4 protein was low on MPPs, ST-HSCs and LT-HSCs (supplemental Figure 1A-C). In contrast, the mean fluorescence intensity (MFI) of Syndecan-2 was increased on LT-HSCs and 34⁺KSL HSCs compared to MPPs (Figure 1C, D), and the percentages of LT-HSCs expressing Syndecan-2 were increased compared to ST-HSCs and MPPs (Figure 1E). Consistent with this, Syndecan-2⁺ BM cells were enriched for KSL, 34⁺KSL, and SLAM LT-HSCs compared to Syndecan-2 BM cells (Figure 1F, G). Syndecan-2⁺Lin⁻ cells also exhibited a 6-fold enrichment for α-catenin⁺c-kit⁺ HSCs compared to Syndecan-2⁺Lin⁻ cells (Figure 1H, I). Multiparameter bh-SNE analysis showed that Syndecan-2⁺ BM cells overlay with regions of
increased expression of c-Kit, Sca-1, CD150 and decreased CD48 and CD34 (supplemental Figure 1D). Syndecan-2 expression is also increased on human cord blood (CB) CD34⁺ HSPCs compared to CB mononuclear cells (MNCs) (supplemental Figure 1E, F). Syndecan-2 expression is also increased on human BM CD34⁺CD38⁺CD45RA⁺CD90⁺ HSCs compared to BM MNCs (supplemental Figure 1G).

We next evaluated whether Syndecan-2 as a sole marker could enrich for functional HSCs and HSPCs. Syndecan-2⁻Lin⁻ cells produced significantly more colony forming cells (CFCs) compared to CD150⁺Lin⁻ cells (Figure 1J).12,15 Furthermore, mice transplanted with Syndecan-2⁻Lin⁻ cells displayed increased total donor cell chimerism and increased donor B cell and myeloid cell chimerism in the BM at 16 weeks post-transplant compared to mice transplanted with CD150⁺Lin⁻ BM cells or Syndecan-2⁻Lin⁻ BM cells (Figure 1K, L). Therefore, Syndecan-2 surface expression alone enriches for HSCs with multilineage repopulating capacity.

Since terminally differentiated hematopoietic cells and HPCs comprise the majority of adult BM cells,¹³⁰ we analyzed Syndecan-2 expression on Lin⁻c-Kit⁺Sca-1⁻CD34⁻CD16/32⁻ megakaryocyte-erythocyte progenitors (MEPs), Lin⁻c-Kit⁺Sca-1⁻CD34⁻CD16/32⁺ common myeloid progenitors (CMPs), Lin c-Kit⁺Sca-1⁻CD34⁺CD16/32⁺ granulocyte-macrophage progenitors (GMPs) and Lin Flt3⁻Il7ra⁻c-Kitlow Sca-1low common lymphoid progenitors (CLPs). Syndecan-2 expression was minimally detected on CMP, MEP, GMP, and CLP cells and terminally differentiated hematopoietic cells (supplemental Figure 1H-I). Syndecan-2⁺ BM cells contained small percentages of hematopoietic progenitor cells and terminally differentiated cells compared to Syndecan-2⁻ BM cells (supplemental Figure 1J-L).

**Syndecan-2 expression enriches for HSCs with increased self-renewal capacity**

Since Syndecan-2 expression enriched for phenotypic LT-HSCs, we evaluated whether Syndecan-2⁺ expression marked HSCs with increased self-renewal capacity. We sorted donor
Syndecan-2^+34^KSL cells and Syndecan-2^34^KSL cells (hereafter referred to as Sdc2^+ HSCs and Sdc2^- HSCs, respectively) from the BM of B6.SJL (CD45.1^+) mice (Figure 2A) and transplanted 5x10^2 donor cells competitively with 2x10^5 BM cells (CD45.2^+) into lethally irradiated C57BL/6 (CD45.2^+) mice (Figure 2B). Through 16 weeks post-transplant, mice transplanted with Sdc2^+ HSCs displayed increased total and multilineage donor cell PB chimerism compared to mice transplanted with Sdc2^- or 34^KSL HSCs (Figure 2C, D). We detected increased multilineage donor chimerism in the BM at 16 weeks without lineage skewing (supplemental Figure 2A, B), and increased donor KSL cells and donor CD150^+CD48^- KSL cells in mice transplanted with Sdc2^+ HSCs compared to recipients of Sdc2^- or 34^KSL HSCs (Figure 2E, F). We detected no differences in homing of Sdc2^-Lin^- cells and Sdc2^-Lin^- cells to the BM at 24 hours post-transplant (supplemental Figure 2C). We also analyzed whether isolation of 34^KSL HSCs based on high (Sdc2^High) or medium Sdc2 expression (Sdc2^Med) could distinguish HSCs with distinct repopulating capacities (supplemental Figure 2D). Mice transplanted with Sdc2^High HSCs demonstrated significantly higher total and multilineage donor chimerism following competitive transplantation compared to Sdc2^Med or Sdc2^- HSCs (supplemental Figure 2E).

Secondary competitive repopulation assays to assess long-term HSC repopulating capacity revealed increased donor hematopoietic cell engraftment in the PB (Figure 2G) and BM (supplemental Figure 2F) of recipients of Sdc2^+ HSCs, without lineage skewing, compared to mice transplanted with Sdc2^- HSCs (supplemental Figure 2F, G). Secondary recipient mice also displayed increased donor BM CD45.1^-CD150^-CD48^-KSL HSCs compared to recipients of Sdc2^- HSCs (Figure 2H).

Surface expression of CD150 and absence of CD48 and CD41 expression select for HSCs with the highest demonstrated LT-HSC enrichment. Utilizing a limiting dilution assay and Poisson statistical analyses, we compared the HSC frequency within Sdc2^+34^KSL, Sdc2^-
34KSL and CD150+34KSL HSCs based upon donor hematopoietic engraftment in recipient mice at 16 weeks following transplantation of each population.31 We detected increased HSC frequency in both Sdc2+34KSL cells and CD150+34KSL cells compared to Sdc2+34KSL cells and no significant difference in the HSC frequency between Sdc2+34KSL cells and CD150+34 KSL cells (Figure 2I, J). Both Sdc2+34KSL cells and CD150+34KSL cells contained > 1 log increased HSC frequency compared to Sdc2− HSCs (Figure 2J).

We next evaluated whether Syndecan-2 expression could resolve heterogeneity within CD150+34KSL HSCs.32 We sorted donor BM Sdc2+CD150+34KSL cells and Sdc2−CD150−34KSL cells from B6.SJL mice (Figure 2K) and transplanted 3 x 10^2 donor cells, along with 2 x 10^5 BM competitor cells, into lethally irradiated C57BL/6 mice. Mice transplanted with Sdc2+CD150+34KSL HSCs exhibited 15-fold increased multilineage hematopoietic repopulation compared to mice transplanted with Sdc2−CD150+34KSL HSCs (Figure 2L), suggesting that Syndecan-2 expression resolves heterogeneity in self-renewal capacity within CD150+34KSL HSCs.

**Syndecan-2 expression delineates HSCs with enhanced quiescence**

Flow cytometric analysis revealed that Sdc2+ HSCs contain increased percentages of CD150+, CD48−, CD150+CD48−, α-catulin+ and Flt3− cells compared to Sdc2− HSCs or parent 34KSL HSCs (Figure 3A-C). Additionally, a subset of Lin− α-catulin+ and 34KSL Flt-3+ HSCs express Syndecan-2, suggesting partial overlap between Sdc2+ HSCs and HSCs defined by other markers (supplemental Figure 2H). We observed no differences in percentages of apoptotic Sdc2+ and Sdc2− HSCs at baseline (supplemental Figure 2I, J). In contrast, we detected significantly increased percentages of Sdc2+ HSCs in G0 and decreased percentages in G1 at baseline compared Sdc2− HSCs (Figure 3D).
We next examined whether Sdc2⁺ HSCs display an alteration in cell cycle capacity following competitive transplant (Figure 3E). We detected no difference in the cell cycle status of BM donor CD34⁺KSL cells in mice at 8 weeks following transplantation with 34⁺KSL HSCs, Sdc2⁺ HSCs or Sdc2⁻ HSCs (Figure 3F, G). However, as a percentage of total BM cells, mice transplanted with Sdc2⁺ HSCs displayed increased percentages of donor 34⁺KSL cells in G₀ at 8 weeks post-transplant compared to mice transplanted with Sdc2⁻ HSCs or parent 34⁺KSL cells (Figure 3H).

While quiescent HSCs are protected from exhaustion, HSCs must maintain the ability to enter the cell cycle to support hematopoietic demands. We isolated 34⁺KSL HSCs, Sdc2⁻ HSCs and Sdc2⁺ HSCs and plated these populations in complete IMDM supplemented with thrombopoietin, stem cell factor and Flt3 ligand (TSF media) to promote cell cycling (Figure 4A). Total cell expansion was observed in all populations after 7 days, but Sdc2⁺ HSCs produced significantly more 34⁺KSL HSCs in culture compared to Sdc2⁻ HSCs, which were nearly depleted of phenotypic HSCs (Figure 4B-E).

In order to determine if Syndecan-2 surface expression is interchangeable on HSCs, Sdc2⁺ HSCs or Sdc2⁻ HSCs were cultured with TSF media. By day +3 of culture, more than 80% of the 34⁺KSL cells in cultures initiated with Sdc2⁺ HSCs became Sdc2⁻, whereas more than 30% of the 34⁺KSL cells in cultures initiated with Sdc2⁻ HSCs became Sdc2⁺ (Fig. 4F, supplemental Figure 3A); these distributions persisted through day +7 (Fig 4G, supplemental Figure 3B). Cultures initiated with Sdc2⁺ HSCs produced significantly more Sdc2⁺ and Sdc2⁻ HSCs (Figure 4H, I), consistent with increased total cell expansion in the cultures initiated with Sdc2⁺ HSCs (Figure 4B, E). We also sorted Sdc2⁺34⁺KSL cells and Sdc2⁻34⁺KSL cells from day +7 cultures and re-plated each population in TSF media and quantified the percentages of Sdc2⁺34⁺KSL cells in each group after 14 total days of culture (supplemental Figure 3C). Both Sdc2⁺34⁺KSL cells and Sdc2⁻34⁺KSL cells derived from originating Sdc2⁻ HSCs continued to
produce a higher percentage of Sdc2⁺34⁺KSL cells in culture compared to the progeny of Sdc2⁺ HSCs (supplemental Figure 3D). These results suggest that a subset of Sdc2⁺ HSCs can convert to Sdc2⁺34⁺KSL cells in response to cytokine stimulation.

In vivo analysis of Syndecan-2 expression on BM donor 34⁺KSL cells at 8 weeks following competitive transplantation of Sdc2⁺ or Sdc2⁻ HSCs showed that donor Sdc2⁺ HSCs gave rise to significantly more Sdc2⁺ HSCs, whereas donor Sdc2⁻ HSCs rarely produced Sdc2⁺ HSCs (Fig. 4J, supplemental Figure 3E). We also quantified whether Syndecan-2 expression is retained upon hematopoietic differentiation following HSC transplantation. In the BM, < 30% of donor-derived B, T, and myeloid cells retained Sdc2 surface expression (supplemental Figure 3F). Conversely, 10-30% of donor T, B, and myeloid cells produced by transplanted Sdc2⁻ HSCs expressed surface Syndecan-2 (supplemental Figure 3F), suggesting that Sdc2 surface expression is acquired upon differentiation of Sdc2⁺ HSCs.

In cultures initiated with Sdc2⁺ HSCs, > 70% of 34⁺KSL cells were in G₀ at day 7, compared to 40% of the Scd2⁺ HSC cultures (Figure 4K). However, since the numbers of 34⁻ KSL cells significantly increased in Sdc2⁺ HSC cultures compared to cultures of Sdc2⁻ HSCs (Fig. 4E), the overall percentages of G₀ 34⁺KSL cells increased in the Sdc2⁺ HSC cultures compared to the progeny of Sdc2⁻ HSCs (Figure 4L). Mice transplanted with the progeny of cultured Sdc2⁺ HSCs exhibited increased donor multilineage hematopoietic cell repopulation compared to mice transplanted with the progeny of Sdc2⁻ HSCs or 34⁺KSL HSCs (Figure 4M). We also detected increased percentages of donor CD150⁺CD48⁺KSL HSCs in the BM of mice transplanted with the progeny of Sdc2⁺ HSC cultures compared to mice transplanted with the progeny of Sdc2⁻ HSCs or 34⁺KSL HSCs (Figure 4N). Secondary competitive transplantation assays utilizing BM cells isolated at 16 weeks post-transplant from primary recipient mice confirmed that long-term engraftment potential was increased in the cultured progeny of Sdc2⁺
HSC cultures compared to the progeny of Sdc2− HSCs or 34′KSL HSCs (supplemental Figure 3G).

**Syndecan-2 regulates HSC quiescence and repopulating capacity**

We next used a Sdc2 global knockout mouse (Sdc2<sup>−/−</sup>) to assess the requirement for Syndecan-2 in hematopoietic repopulation. Mice transplanted with BM cells from Sdc2<sup>−/−</sup> donor mice displayed modestly decreased total donor chimerism, T cell and B cell chimerism at 16 weeks compared to mice transplanted with BM cells from Sdc2<sup>+/+</sup> mice (supplemental Figure 4A-C).

However, utilization of this global knockout approach did not allow direct examination of the role of Syndecan-2 expressed by HSCs in mediating HSC function. We therefore utilized lentiviral shRNAs to silence Sdc2 expression in HSCs. Treatment with two unique shRNA clones targeting Sdc2 (shSdc2 Clone #1 and shSdc2 Clone #2) significantly decreased Sdc2 expression and Syndecan-2 surface expression in Sdc2<sup>−</sup> HSCs (supplemental Figure 5A-C).

We detected decreased percentages and numbers of CD150<sup>+</sup>CD48<sup>−</sup>34′KSL LT-HSCs in shSdc2-treated HSCs compared to shControl-treated Sdc2<sup>+</sup> HSCs and no change in percentages of CD150<sup>+</sup>CD48<sup>−</sup>34′KSL LT-HSCs in Sdc2<sup>−</sup> HSCs treated with shSdc2 (Figure 5A-C). HSCs treated with shSdc2 also contained decreased total CFCs and multilineage CFU-GEMMs compared to shControl-treated HSCs (Figure 5D, E).

Primary competitive repopulation assays performed using shSdc2-treated HSCs and shControl-treated HSCs demonstrated no differences in total donor cells, donor B cell or T cell chimerism in primary recipient mice (Figure 5F, supplemental Figure 5D). However, donor myeloid cell chimerism was decreased in mice transplanted with shSdc2-treated HSCs (Figure 5F). Donor cell engraftment within BM CD150<sup>−</sup>CD48<sup>−</sup>34′KSL HSCs was also decreased in mice
transplanted with shSdc2-treated HSCs compared to controls (Figure 5G). At 16 weeks, donor 34KSL cells in the BM of recipient mice transplanted with shSdc2-treated HSCs displayed decreased percentages of cells in G0 compared to donor 34KSL cells in mice transplanted with shControl-treated HSCs (Figure 5H, I). Secondary competitive repopulation assays utilizing BM cells from primary recipient mice transplanted with shSdc2-treated HSCs demonstrated significantly decreased donor multilineage chimerism in recipients compared to mice transplanted with BM cells from primary mice transplanted with shControl-treated HSCs (Figure 5J).

**Syndecan-2 regulates HSC quiescence through control of Cdkn1c**

Syndecans mediate context-specific effects on cell proliferation, whereas we have observed that Syndecan-2 expression is associated with HSC quiescence. We next measured the cell cycle status of BM 34KSL HSCs following treatment with lentiviral particles containing shControl or shSdc2. HSCs treated with shSdc2 displayed decreased percentages of cells in G0 compared to shControl-treated HSCs, indicating exit from quiescence in response to Syndecan-2 silencing (Figure 6A-C). Since the Cip/Kip family of cyclin dependent kinase (Cdk) inhibitors regulates HSC cell cycling, we measured the expression of Cdkn1c, which encodes the cell cycle inhibitor, p57, in shSdc2-treated HSCs and shControl-treated HSCs. At baseline, Sdc2+ HSCs expressed nearly 10-fold increased levels of Cdkn1c compared to Sdc2- HSCs (Figure 6D). Silencing of Sdc2 suppressed Cdkn1c expression in BM 34KSL cells (Figure 6D). Sdc2 knockdown in BM 34KSL cells also caused a moderate increase in the expression of Cdkn1a and p16 compared to control HSCs, but did not impact Cdkn1b expression (Figure 6E). Microscopic protein expression analyses revealed decreased p57 and increased p16 protein expression in shSdc2-treated HSCs compared to shControl-treated HSCs (supplemental Figure 5E - H).
To further analyze how Sdc2 regulates p57, we utilized a murine stem cell virus (MSCV) overexpression system to generate Sdc2-overexpression (Sdc2-OE) or GFP-control HSCs (supplemental Figure 6A, B). We detected increased percentages of Sdc2-OE HSCs in G_0 and decreased percentages in G_1 compared to GFP-control HSCs, suggesting that Sdc2 expression induces HSC quiescence (supplemental Figure 6C, D). Furthermore, Sdc2-OE HSCs exhibited increased expression of Cdkn1c, suggesting that Sdc2 promotes Cdkn1c expression in HSCs (supplemental Figure 6E).

Given the role of Sdc2 in regulating Cdkn1c expression, we next knocked down both Sdc2 and Cdkn1c in Sdc2^+ and Sdc2^- HSCs. Silencing of Cdkn1c, Sdc2, or both in Sdc2^- HSCs did not further decrease Cdkn1c levels, suggesting that Sdc2^- HSCs minimally express Cdkn1c (Figure 6D). Silencing of Cdkn1c, Sdc2, or both in Sdc2^- HSCs also did not impact the expression of Cdkn1a, Cdkn1b or p16 (Figure 6E). In Sdc2^+ HSCs, silencing of Cdkn1c and Sdc2 did not further decrease Cdkn1c expression or increase Cdkn1a expression compared to knockdown of Cdkn1c alone (Figure 6D, E). Double knockdown of Sdc2 and Cdkn1c in Sdc2^+ HSCs decreased CFU-GEMM colony production compared to shControl-treated HSCs, but this reduction was comparable to knockdown of either Sdc2 or Cdkn1c alone (Figure 6F). These results suggest that Sdc2 mediates molecular and hematopoietic effects through Cdkn1c.

The expression of Cdkn1c is regulated by TGFβ/SMAD signaling,^38^ so we treated Sdc2^+ HSCs with SIS3, a selective inhibitor of TGFβ-1-dependent SMAD3 phosphorylation, to determine if Sdc2 – mediated HSC quiescence was dependent on Cdkn1c. ^39,40^ Sdc2^+^ HSCs treated with SIS3 displayed a loss of quiescence compared to control Sdc2^+^ HSCs (Figure 6G). Silencing of Sdc2 expression decreased HSC quiescence in control Sdc2^+^ HSC cultures, but Sdc2 silencing had no significant effect on HSC quiescence in the presence of SIS3 treatment (Figure 6G). We next assessed the cell cycle status of CD150^-CD48^-34^KSL Sdc2^+^ HSCs upon knockdown of Sdc2, Cdkn1c or both. Silencing of Sdc2 alone, Cdkn1c alone or both Sdc2 and
Cdkn1c caused comparable reductions in the percentages of HSCs in G₀ and G₁, with concordant increases in percentages of HSCs in G₂/S/M phase (Figure 6H). These data suggest that Syndecan-2 regulates HSC cell cycle status through Cdkn1c.

To understand why Sdc2⁺ HSCs are susceptible to inhibition of TGFβ-1-dependent SMAD3 phosphorylation while Sdc2⁻ HSCs are not, we quantified phospho-SMAD3 expression in Sdc2⁺ and Sdc2⁻ HSCs. Sdc2⁺ HSCs exhibited elevated SMAD3 activation at baseline compared to Sdc2⁻ HSCs, but Sdc2⁺ and Sdc2⁻ HSCs were equally sensitive to TGFβ stimulation (Figure 6I), suggesting that Syndecan-2 expression marks HSCs with elevated basal SMAD3 signaling. We next treated HSCs with SIS3 to understand whether Sdc2⁺ or Sdc2⁻ HSCs are dependent on SMAD3 signaling for HSC maintenance. SIS3 treatment of Sdc2⁺ HSCs significantly decreased CFU-GEMM generation compared to control treated Sdc2⁺ HSCs (Figure 6J) and significantly decreased percentages of 34KSL cells in culture (Figure 6K). Since TGFβ/SMAD signaling regulates Cdkn1c expression⁴¹, these data are consistent with our genetic studies suggesting that Syndecan-2 regulates HSC quiescence and function through control of Cdkn1c.

To further evaluate the molecular basis through which Syndecan-2 regulates HSC quiescence and repopulating capacity, we performed RNA sequence analysis of BM 34KSL HSCs, Sdc2⁻ HSCs, and Sdc2⁺ HSCs (supplemental Figure 7A-C). Differentially expressed genes (Figure 6L) were analyzed using Ingenuity Pathway Analysis (IPA) to assess Diseases & Functions and Canonical Pathways modulated based on Syndecan-2 expression. Our data revealed alterations in the Hematopoietic System Development and Function Pathway in Sdc2⁺ HSCs compared to Sdc2⁻ HSCs (Figure 6M), and increased expression of genes associated with HSC function, including Hoxa10,⁴² Hoxb4,⁴³ Gata1 ⁴⁴, Gata3,⁴⁵ Esam,¹⁰ Tek,⁴⁶ and Slamf1¹⁵, Cttnal1,⁴⁷ Hoxb5,⁴⁷ Cd244, and Procr (CD201) ¹⁵ and Fgd5 ⁴⁸ (Figure 6N). Given the interplay between Syndecans and integrins, we also analyzed alterations in integrin gene
expression. We detected altered Integrin Signaling in Sdc2⁺ HSCs compared to Sdc2⁻ HSCs (supplemental Figure 7D) concordant with altered integrin protein expression (supplemental Figure 7E-F). Sdc2⁺ HSCs also displayed increased expression of Cdkn1c compared to Sdc2⁻ HSCs (Figure 6O).

**Discussion**

Syndecan-2 is a member of the family of HSPGs, which via their role as extracellular binding partners for secreted proteins,¹⁸,¹⁹ regulate morphogen gradients during development.²⁶,⁴⁹-⁵² Syndecans also modulate the differentiation, proliferation and regeneration of adult neural stem cells.²⁴,²⁵ In hematopoiesis, HSPGs produced by stromal cells contribute to cytokine-mediated regulation of HSPC growth⁵³-⁵⁵ and HSPC retention in the BM.²⁶ We discovered that BM HSCs are highly enriched for Syndecan-2 expression and isolation of BM cells based on Syndecan-2 surface expression yields a 24-fold enrichment for CD150⁺48 KSL LT-HSCs and approximately 6-fold enrichment for α-catenin⁺c-kit⁺ HSCs compared to Syndecan-2 negative BM cells. Furthermore, mice transplanted competitively with 300 Syndecan-2⁺ BM lin⁻ cells displayed enhanced BM engraftment of total cells, myeloid and B cells through 16 weeks post-transplant, whereas the identical dose of CD150⁺ BM lin⁻ cells or Syndecan-2⁻ BM lin⁻ cells failed to engraft in recipient mice. Kiel et al. reported the estimated HSC frequency in CD150⁺ BM cells to be 1 in 7,600 cells,¹³ and our data suggest that Syndecan-2 may provide unique advantages as a single marker to enrich for BM HSCs with in vivo repopulating capacity.

Previous landmark studies have demonstrated that CD150⁺48/41 KSL BM cells are the most highly enriched LT-HSC population, with an estimated HSC frequency of 1 in 3 to 4 cells based on competitive repopulation assays.¹² Subsequently, Acar et al. showed that approximately 30% of BM α-catenin-GFP⁺c-Kit⁺ BM cells in α-catenin-GFP mice were bona fide LT-HSCs.²⁹ Here, via limiting dilution analysis of competitive repopulation assays, we determined that the HSC frequency within Syndecan-2⁺34 KSL BM was indistinguishable from
CD150\(^{+}\)34\(^{-}\)KSL BM cells and more than 10-fold increased compared to Syndecan-2\(^{+}\)34\(^{-}\)KSL BM cells. Therefore, Syndecan-2 represents a novel cell surface marker that can be utilized to enrich and purify HSC populations. Prior studies have also shown that CD150\(^{+}\) HSC populations are heterogeneous with regard to their myeloid- or lymphoid-potential\(^{15,32}\), their rate of entry into cell cycle\(^{56}\) and their self-renewal potential in vivo\(^{57}\). Here, we demonstrated that CD150\(^{+}\)34\(^{-}\) KSL Sdc2\(^{+}\) cells contain nearly all of the in vivo repopulating capacity of the CD150\(^{+}\)34\(^{-}\)KSL HSCs. Therefore, Syndecan-2 surface expression may have utility toward resolving the heterogeneity in self-renewal capacity within the CD150\(^{+}\)34\(^{-}\)KSL HSC population. In keeping with this conclusion, we found that Sdc2\(^{+}\) HSCs are highly enriched for expression of numerous genes associated with enhanced hematopoietic cell stemness. Finally, our studies also suggest that Syndecan-2 surface expression on phenotypic HSCs changes in response to cytokine stimulation and following competitive transplantation in mice; further studies will address whether loss or gain of Syndecan-2 expression by phenotypic HSCs also reflects a change in self-renewal capacity.

Beyond its importance as a marker of LT-HSCs, Syndecan-2 uniquely regulates HSC function via control of HSC quiescence. Silencing of Syndecan-2 expression caused an increase in HSC cycling that persisted for several months following transplantation in mice. Commensurate with this, Syndecan-2 silencing depleted HSCs with long-term in vivo repopulating capacity. The functional role of other HSC markers such as CD150 and endothelial protein C receptor has not been defined\(^{6,12,15}\). Similarly, \(\alpha\text{-catulin}^{\text{GFP/GFP}}\) mice have been shown to have normal hematopoiesis and normal HSC content and function\(^{29}\). Surface expression of endothelial cell-specific adhesion molecule 1 (Esam1) and JAM-C have been shown to enrich for murine HSCs and deletion of Esam1 was associated with lineage skewing, whereas JAM-C deficient mice displayed increased myeloid cells\(^{10,58}\). In our studies, selection of BM lin\(^{-}\) cells or CD34\(^{-}\)KSL HSCs for Sdc2 expression caused significant gain of HSC repopulating function in vivo, whereas silencing of Sdc2 expression in HSCs increased HSC cycling and depleted long-
term repopulating HSCs. Syndecan-2 is uniquely modified by the addition of three heparan sulfate chains, whereas Syndecan-1 and Syndecan-3 are decorated by both chondroitin and heparan sulfate chains, which have been shown to have opposing physiological roles in other cell types. As such, our studies demonstrating the unique self-renewal capacity of Syndecan-2+ HSCs provides the basis for the development of strategies to increase heparan sulfate content on LT-HSCs.

Syndecan-2, expressed as a transmembrane HSPG, can serve as a co-receptor for TGF-β and either promote or inhibit TGF-β receptor–mediated signaling in a context–dependent manner. We have found that Syndecan-2 promotes HSC quiescence and enhanced repopulating capacity through induction of Cdkn1c expression, which can be suppressed by inhibition of TGF-β signaling. These data suggest that Syndecan-2 promotes HSC quiescence and enhanced repopulating capacity through activation of TGF-β receptor signaling and sustainment of Cdkn1c expression. TGF-β signaling and Smad pathway activation have also been shown to regulate p57 protein levels via control of proteolysis, so we will further explore this mechanism in regulating the quiescence of Syndecan-2+ HSCs. Since Syndecans can regulate the activity of other growth factors, integrins and extracellular matrix proteins that may act on HSCs, our findings also provide the basis to explore additional Syndecan-2–mediated pathways that regulate HSC quiescence and repopulating capacity. In summary, Syndecan-2 expression enriches for HSCs and Syndecan-2 regulates HSC repopulating capacity through control of HSC quiescence.
Figure Legends

Figure 1. Syndecan-2 is differentially expressed by LT-HSCs. (A) Gating strategy used to isolate adult BM MPPs, ST-HSCs, 34’KSL HSCs, and LT-HSCs. (B) qRT-PCR analysis of Sdc1, Sdc2, Sdc3, and Sdc4 in BM HSPCs (n=7 mice pooled/replicates/group). (C) Representative histograms of Syndecan-2 expression on HSPCs and quantification of (D) Syndecan-2 MFI and (E) percentages of Syndecan-2+ cells (n=7 mice/group). (F) Sequential gating of lineage depleted mouse BM cells based on Syndecan-2 expression and (G) quantification of the percent KSL of Lin−, 34’KSL of KSL, and LT-HSCs of 34’KSL (n=7 mice/group). (H) Representative analysis of α-catulinGFP-c-kit+ cells within lineage-depleted BM from adult α-catulinGFP mice based upon Syndecan-2 expression, and (I) quantification of % α-catulinGFP-c-kit+ LT-HSCs within each population (n=6 mice/group). (J) Quantification of CFCs from BM Syndecan-2’Lin− and Syndecan-2’Lin− cells and CD150+ Lin− cells (n=5 mice/pooled, n=4 replicates/group). (K) Schematic representation of competitive repopulation assay of CD45.1+ BM Syndecan-2’Lin− cells, Syndecan-2’Lin− cells or CD150+ Lin− cells transplanted into CD45.2+ mice. (L) Percentages of total donor CD45.1+ cells in the BM at 16 weeks following competitive transplantation of BM Syndecan-2’Lin− cells, Syndecan-2’Lin− cells and CD150+Lin− cells from CD45.1+ mice into CD45.2+ recipients (n=5 SJL mice/pooled for donor cells, n=3-5 recipient mice/group); Error bars = S.E.M.; statistics denote one- or two-way ANOVA followed by Holm-Sidak’s corrected unpaired t-test; * p< 0.05, *** p< 0.001, **** p< 0.0001; for competitive transplant.

Figure 2. Sdc2+ HSCs demonstrate increased self-renewal capacity. (A) Representative gating strategy to isolate 34’KSL cells (HSCs), Sdc2− HSCs or Sdc2+ HSCs. (B) Experimental design for primary competitive repopulation assay of 500 Scd2+ HSCs, Sdc2− HSCs and 34-KSL HSCs from CD45.1+ mice into CD45.2+ recipients. (C) Representative flow cytometric analysis of donor CD45.1+ engraftment in the PB of recipient CD45.2+ mice at 16 weeks post-transplant.
(D) Percentages of total donor CD45.1+ cells, B cells, T cells and myeloid cells in the PB over time in recipient CD45.2- mice in the groups shown (n=7 mice pooled for donor cell isolation; n=10-17 recipient mice/group). (E-F) Percentages of donor CD45.1+ KSL cells and CD45.1+CD150+CD48+KSL cells at 16 weeks in the BM of recipient CD45.2- mice (n=10-17 recipient mice/group). (G) Percentages of total donor CD45.1+ cells, B cells, T cells and myeloid cells in the PB over time after secondary competitive transplant in the treatment groups shown (n=10-17 recipient mice/group). (H) Percentages of donor CD45.1+ CD150+CD48+KSL cells in the BM of recipient mice at 16 weeks post-secondary transplant (n=10-17 recipient mice/group). (I) Graph of limiting dilution analysis of engraftment of Sdc2+ HSCs, Sdc2- HSCs, or CD150+ HSCs (CD45.1+) at 16 weeks following competitive transplant into lethally irradiated C57BL/6 (CD45.2-) mice. CD45.1+ donor engraftment > 0.1% was considered positive for engraftment (n=10 mice pooled for donor cell isolation; n=4-21 recipient mice/dose). (J) HSC frequency estimates in CD150+ HSCs, Sdc2+ HSCs and Sdc2- HSCs are shown based on Poisson statistical analysis of engraftment data shown. (K) Representative gating strategy used to isolate CD150-34KSL Sdc2+ cells and CD150-34KSL Sdc2- cells for competitive transplantation of 300 isolated cells. (L) Percentages of total donor CD45.1+ cells, B cells, T cells and myeloid cells in the PB over time after primary competitive transplant (n=10 mice pooled for donor cell isolation; n=8-10 recipient mice/condition). Error bars = S.E.M.; statistics denote one- or two-way ANOVA followed by Holm-Sidak’s corrected unpaired t-test; * p< 0.05, ** p< 0.01, *** p< 0.001, **** p< 0.0001 for comparison of Sdc2+ HSCs versus Sdc2- HSCs in (D) and (G) or comparison of CD150-34KSL Sdc2+ cells versus CD150-34KSL Sdc2- cells in (L); # p< 0.05, ## p< 0.01, ### p < 0.001 for comparison of Sdc2+ HSCs and 34KSL cells (HSCs) in (D) and (G).

Figure 3. Sdc2+ HSCs display increased quiescence and proliferative capacity. (A) At left, representative flow cytometric analysis of CD150-CD48- cells within BM 34KSL cells (HSCs),
Sdc2⁺ HSCs and Sdc2⁻ HSCs; at right, percentages of CD48⁺, CD150⁺ and CD150⁻CD48⁻ cells in each population are shown (n=8 mice/group). (B) At left, α-catenin^{GFP} expression in 34⁺KSL cells (HSCs), Sdc2⁺ HSCs and Sdc2⁻ HSCs; at right, percentages of α-catenin⁺ cells in each population (n=6 mice/group). (C) At left, Flt3 expression in 34⁺KSL cells (HSCs), Sdc2⁺ HSCs and Sdc2⁻ HSCs; at right, percentages of Flt3⁺ cells in each population (n=8 mice/group). (D) At left, representative flow cytometric analysis showing percentages of 34⁺KSL cells (HSCs), Sdc2⁺ HSCs and Sdc2⁻ HSCs in G₀ (Ki67⁻7AAD⁻), G₁ (Ki67⁺7AAD⁻) and G₂/S/M phase (Ki67⁺7AAD⁺); at right, percentages of cells in G₀, G₁ and G₂/S/M phase (n=3 mice/group). (E) Experimental design for primary competitive repopulation assay of Scd2⁻ HSCs, Sdc2⁻ HSCs and HSCs from CD45.1⁺ mice into CD45.2⁺ recipients for cell cycle analysis. (F) Representative flow cytometric analysis showing percentages of 34⁺KSL cells (HSCs), Sdc2⁺ HSCs and Sdc2⁻ HSCs in G₀, G₁ and G₂/S/M phase at 8 weeks post-transplant, and (G) quantification of the percentage of donor cells within each cell cycle phase (n=7-10 mice/group). (H) Quantification of the percent donor 34⁺KSL HSCs in G₀ relative to BM cells (n=7-10 mice/group). Error bars = S.E.M.; Statistics denote Holm-Sidak’s post-hoc unpaired t-test following one-way ANOVA; * p< 0.05, ** p< 0.01, *** p< 0.001, **** p< 0.0001.

Figure 4. Sdc2⁺ HSCs generate HSCs in culture with increased self-renewal ability. (A) Experimental design for ex vivo culture of 34⁺KSL HSCs, Sdc2⁺ HSCs or Sdc2⁻ HSCs in TSF media (n=4-5 replicates/group). (B) Mean numbers of total cells after 7 day culture (n=4-5 replicates/group). (C) Representative flow cytometric analysis of KSL cells and 34⁺KSL cells at day +7 in each group (n=4-5 replicates/group). (D) Percentages and (E) numbers of 34⁺KSL cells in each group at day +7 of culture (n=4-5 replicates/group). (F) Percentages of Syndecan-2⁺ cells of 34⁺KSL in each condition at day +3 or (G) day +7 after culture (n=4-5 replicates/group). Number of (H) Syndecan-2⁻ and (I) Syndecan-2⁺ cells of 34⁺KSL in each condition at day +7 after culture (n=4-5 replicates/group). (J) Percentages of Syndecan-2⁻ cells
of donor derived 34°KSL cells at 8 weeks following transplantation of 500 Sdc2° or Sdc2° HSCs (n=4-5 replicates/group). (K) At left, representative flow cytometric analysis showing percentages of 34°KSL cells (HSCs) derived from Sdc2° HSCs or Sdc2° HSCs in G0, G1 and G2/S/M phase at day +7; at right, percentages of cells in G0, G1 and G2/S/M phase (n=3 replicates/group). (L) Percentages of 34°KSL cells derived from Sdc2° HSCs or Sdc2° HSCs in G0 relative to total cell output (n=3 replicates/group). (M) Percentages of total donor CD45.1° cells, B cells, T cells and myeloid cells in the PB of recipient CD45.2° mice over time following transplantation of day +7 progeny of 34°KSL HSCs, Sdc2° HSCs or Sdc2° HSCs cultured with TSF media (n=5-6 recipients/group). (N) Percentages of donor CD45.1° CD150°CD48° KSL cells in the BM of recipient mice at 16 weeks post-transplant. Error bars = S.E.M.; Statistics denote Holm-Sidak’s post-hoc unpaired t-test following one- or two-way ANOVA; * p< 0.05, ** p< 0.01, *** p< 0.001, **** p< 0.0001 for Sdc2° HSCs versus Sdc2° HSCs; # p< 0.01, ## p< 0.01, ### p< 0.001 for comparison between Sdc2° HSCs and 34°KSL HSCs.

Figure 5. Syndecan-2 regulates HSC quiescence and self-renewal capacity. (A) Representative flow cytometric analysis of CD150°CD48° cells within the 34°KSL population treated for 48 hours with two Sdc2 shRNA clones (shSdc2 Clone #1 and shSdc2 Clone #2) or control shRNA (shCtrl, n=3-6 replicates/group). (B) Percentages of CD150°CD48° 34°KSL cells (LT-HSCs) in Sdc2° and Sdc2° cells treated with shSdc2 or shCtrl (n=3-6 replicates/group). (C) Numbers of phenotypic LT-HSCs following treatment with shSdc2 or shCtrl (n=3-6 replicates/group). (D) Numbers of total CFCs and (E) CFU-GEMMs in BM 34°KSL cells at 48 hours following treatment with shSdc2 or shCtrl (n=4 replicates/group). (F) Percentages of total donor CD45.1° cells in the PB of recipient CD45.2° mice over time and percentages of donor cells within the B cell, T cell, and myeloid cell populations over time following competitive transplantation of shSdc2-treated BM 34°KSL cells or shCtrl – treated BM 34°KSL cells (n=8-10 recipients/group). (G) Percentages of donor CD45.1°CD150°CD48° cells in the BM at 16 weeks
post-transplant in each group (n=8-10 recipients/group). (H) Representative cell cycle analysis of donor CD45.1+34 KSL cells in the BM of recipient mice at 16 weeks following transplantation of shSdc2-treated KSL cells or shCtrl-treated KSL cells, and (I) quantification of the percentages of donor cells in G0, G1 and G2/S/M phase in the groups shown (n=8-10 replicates/group). (J) Percentages of total donor CD45.1+ cells, B cells, T cells and myeloid cells in the PB over time after secondary competitive transplant in the treatment groups shown (n=8-10 replicates/group). Error bars = S.E.M.; Statistics denote Holm-Sidak’s post-hoc unpaired t-test following one- or two-way ANOVA; for secondary competitive transplants, statistics denote the overall effect of shSdc2 treatment, * p< 0.05, ** p< 0.01, *** p< 0.001, **** p< 0.0001.

Figure 6. Syndecan-2 regulates HSC quiescence through Cdkn1c. (A) Representative cell cycle analysis of shSdc2 Clone #1-treated and (B) shSdc2 Clone #2-treated and shCtrl-treated Sdc2+34 KSL cells. (C) Percentages of 34 KSL cells in G0, G1 and G2/S/M phase in each group (n=4-5 replicates/group). (D) qRT-PCR analysis for the expression of Cdkn1c in Sdc2+ and Sdc2- BM 34 KSL cells treated with shCtrl, shSdc2, shCdkn1c, or shSdc2 and shCdkn1c (n=3-12 replicates/group). (E) qRT-PCR analysis for the expression of Cdkn1a, Cdkn1b and p16 in Sdc2+ and Sdc2- BM 34 KSL cells treated with shCtrl, shSdc2, shCdkn1c, or shSdc2 and shCdkn1c (n=3-12 replicates/group). (F) Numbers of CFU-GEMMs at 10 days after treatment of 250 Sdc2+ HSCs with shCtrl, shSdc2, shCdkn1c, or shSdc2 and shCdkn1c shRNAs (n=4 replicates/group). (G) Percentages of Sdc2+ HSCs in G0 following treatment with shSdc2 or shCtrl and 5 μM SIS3 or Vehicle for 24 hours (n=3-6 replicates/group). (H) Percentages of CD150+CD48+34 KSL LT-HSCs in G0, G1 and G2/S/M phase from Ki-67/7-AAD analysis (n=3-4 replicates/group). (I) At left, representative flow cytometric analysis showing phospho-SMAD3 expression in 34 KSL, 34 KSL Sdc2+ and 34 KSL Sdc2- HSCs as baseline and after TGF-B stimulation for 30 minutes; at right, quantification of the percent phospho-SMAD3 cells within each population. (J) Quantification of CFU-GEMMs from 100 Sdc2+.
HSCs or Sdc2^- HSCs treated with 5 µM SIS3 or Vehicle (n=5 mice/pooled for sort, n=4 replicates/group). (K) Percent 34 KSL cells (percent of live cells) after SIS3 treatment of Sdc2^+ and Sdc2^- HSCs for 7 days in TSF media (n=3-6 replicates/group). (L) Volcano plots depicting differentially expressed genes in Sdc2^+ HSCs versus Sdc2^- HSCs. Differentially expressed genes were detected with parameters of p value < 0.05, and fold change > 2 or < -2. (M) Heat map depicting IPA comparing Sdc2^+ HSCs and Sdc2^- HSCs (n=10 mice pooled to sort each population; n=3 replicates/population; comparisons made between individual samples). (N) Heat map depicting selected differentially expressed genes within Hematological System Development, and (O) Cell Growth and Proliferation genes from IPA of 34-KSL HSCs, Sdc2^- HSCs and Sdc2^+ HSCs (n=10 mice pooled to sort each population; n=3 replicates/population).

Error bars = S.E.M.; statistics denote two-way ANOVA followed by unpaired t-test; * p< 0.05, ** p< 0.01, *** p< 0.001, **** p< 0.0001.

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Authorship
Project conceptualization was by JPC and CMT; experiments and analysis were performed by CMT, AP, ML, TF and VYC; CMT and AP made figures; CMT, AP and JPC wrote the paper with input from all authors.

**Conflict-of-interest disclosure**

The authors declare no competing financial interests.

**Data Sharing Statement**

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Figure 1

A. Flow cytometry plots showing the expression of Syndecan-2 and other markers across different hematopoietic populations.

B. Bar graph showing the expression (Normalized to MPP) of Syndecan-2, Sdc1, Sdc2, Sdc3, and Sdc4 in different populations.

C. histograms showing the MFI (Normalized to MPP) of Syndecan-2 in MPP, 34KSL, ST-HSC, and LT-HSC.

D. Bar graph showing the percentage of Syndecan-2+ cells in MPP, 34KSL, ST-HSC, and LT-HSC.

E. Bar graph showing the percentage of Syndecan-2+ cells in MPP, 34KSL, ST-HSC, and LT-HSC.

F. Flow cytometry plots showing the gating strategy for Lin- cells.

G. Bar graph showing the percentage of Lin- cells, Syndecan-2+ Lin- cells, and Syndecan-2+ BM cells.

H. Flow cytometry plots showing the gating strategy for Syndecan-2 Lin- cells.

I. Bar graph showing the percentage of α-catulin-GFP c-kit cells.

J. Bar graph showing the number of colonies per 300 cells for GEMM, E, and GM.

K. Flow cytometry plots showing the gating strategy for CD45.1, CD45.2, CD45.1+B cell, CD45.1+T cell, and CD45.2+Myeloid cells.

L. Bar graph showing the percentage of CD45.1, CD45.2, CD45.1+B cell, CD45.1+T cell, and CD45.2+Myeloid cells.
Figure 2

A Gated on KSL

34

SSC-A

CD34

Syndecan-2

Isotype

Alexa-647

Alexa-488

B

5x10^6 cells: 34 KSL

2x10^6 cells: WBM

CD45.1

CD45.2

C

34 KSL

Sdc2 HSCs

Sdc2 HSCs

D

Total

B cells

T cells

Myeloid cells

Weeks after transplant

% Donor KSL

% Donor CD150+488 KSL

G

Total

B cells

T cells

Myeloid cells

Weeks after transplant

% Donor CD150+488 KSL

H

% Donor CD150+488 KSL

I

% Negative

Cell Dose

J

Donor cell population

CD150-34 KSL

Sdc2+ HSCs

Sdc2 HSCs

K

Sheep IgG Alexa-488

Syndecan-2

L

Total

B cells

T cells

Myeloid cells

Weeks after transplant

% Donor CD150-34 KSL Sdc2

% Donor CD150-34 KSL Sdc2

% Donor CD150-34 KSL Sdc2

% Donor CD150-34 KSL Sdc2
Figure 4

A. CD45.1 FACS isolation of Sdc2- or Sdc2+ HSCs. 7 days. HSC analysis, Competitive repopulation.

B. 34 KSL
- Media: 20 ng/mL TPO, 150 ng/mL Scf, 50 ng/mL Flt3
- Gated on Lin-
- 7 days
- Total cells

C. 34 KSL: Sdc2+ HSCs, Sdc2- HSCs
- CD34-
- Sca-1
- SSC-A
- c-Kit
- CD45.1

D. % 34 KSL cells

E. # 34 KSL cells

F. % Syndecan-2+(of 34 KSL)

G. % Syndecan-2+(of 34 KSL)

H. # 34 KSL Syndecan-2 cells

I. # 34 KSL Syndecan-2 cells

J. % Syndecan-2+(of donor 34 KSL)

K. Ki-67

L. % G0 34 KSL of total

M. Overall, B cells, T cells, Myeloid cells

N. % Donor CD150+CD48+KSL

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Figure 5

A. Gated on 34 KSL:

|          | shCtrl | shSdc2 Clone #1 | shSdc2 Clone #2 |
|----------|--------|-----------------|-----------------|
| CD48     | 3.0%   | 1.3%            | 1.4%            |
| CD150    |        |                 |                 |

B. % LT-HSCs

|        | LT-HSCs |
|--------|---------|
| Sdc2+  |         |
| Sdc2-  |         |

C. # LT-HSCs

|        | LT-HSCs |
|--------|---------|
| Sdc2+  |         |
| Sdc2-  |         |

D. Total CFU-C

|        | CFU-C   |
|--------|---------|
| Sdc2+  |         |
| Sdc2-  |         |

E. CFU-GEMM

|        | CFU-GEMM|
|--------|---------|
| Sdc2+  |         |
| Sdc2-  |         |

F. % Donor

| Weeks after transplant | Total Donor |
|------------------------|-------------|
|                        |             |

G. % Donor CD150/CD48/KSL

|          | CD150/CD48/KSL |
|----------|----------------|
| shCtrl   |                |
| shSdc2   |                |

H. Gated on CD45.1 34 KSL:

|                | shCtrl | shSdc2 |
|----------------|--------|--------|
| KI-67          | 17%    | 26%    |
| 7AAD           | 78%    | 47%    |

I. % Donor cells in phase

|        | G0   | G1   | G2/S/M |
|--------|------|------|--------|
| shCtrl |      |      |        |
| shSdc2 |      |      |        |

J. % Donor

| Weeks after transplant | Total | B cells | T cells | Myeloid |
|------------------------|-------|---------|---------|---------|
|                        |       |         |         |         |
