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E3-ligase Skp2 regulates β-catenin expression and maintains hematopoietic stem cell homing

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ABSTRACT

The homing ability of hematopoietic stem cells (HSCs) was a critical step for transplantation and subsequent hematopoiesis. Although the HSC transplantation was widely used for many diseases, the mechanism by which HSC homing was regulated remained poorly understood. F-box protein S-phase kinase associated protein2 (Skp2), a component of the Skp2-SCF E3 ligase complex, was regarded as a cell cycle regulator by controlling the level of p21 and p27 through ubiquitination. We recently reported an important role of Skp2 in maintaining HSC pool size, quiescent stage and self-renewal ability. In this current study, we showed that Skp2 was a novel and critical regulator for maintaining the homing of HSCs as well as their residence in the endosteal niche. Microarray analysis together with biochemical validations revealed that *Skp2* deficiency profoundly reduced the expression of β-catenin and its target genes. Knockdown of β-catenin mimicked the decline of HSC homing upon *Skp2* deficiency, suggesting that Skp2 may regulate β-catenin and its target gene expression to orchestrate HSC homing. Our study not only identified Skp2 as a new regulator for maintaining β-catenin expression and HSC homing, but also suggested that Skp2 may serve as a predictive marker for monitoring the transplantation efficiency.

1. Introduction

Hematopoietic stem cells (HSCs) display a unique characteristic that enables them to home to bone marrow (BM) and spleen, which is a central process that not only maintains HSC pools important for lifetime hematopoiesis, but also determines the efficiency of HSC transplantation [1,2]. To date, HSC transplantation is widely used to cure many diseases, including autoimmune disease, genetic diseases, hematologic cancers and non-Hodgkin's lymphoma as well [3,4]. However, there are several aspects that need to be improved in HSCT; for example, the control of toxic graft-versus-host diseases, immunological reconstitution after transplantation, the risk reduction of relapse and so on. Therefore, understanding the intrinsic factors that critically control the HSC homing ability may provide novel therapeutic approaches for patients that are in need of HSC transplantation.

S-phase kinase associated protein2 (Skp2), an F-box protein, is a component of the Skp2-SCF E3 ligase complex that regulates cell cycle progression by targeting cell-cycle inhibitors such as $p21^{Cip/WAF1}$ and $p27^{Kip1}$ for degradation [5]. Previous studies from our group demonstrated that Skp2 accelerates the tumorigenesis, invasion, and metastasis [6], promotes the K63-linked ubiquitination of NBS1 (the component of MRN complex), which is important for its interaction upon the DNA double-strand breaks, thereby facilitating ATM recruitment to DNA foci for activation [7]. We also found that Skp2-SCF complex is a critical E3 ligase for ErbB-receptor-mediated Akt ubiquitination and membrane recruitment in response to EGF, therefore affect Akt activation, glycolysis as well as breast cancer

Abbreviations: Skp2, S-phase kinase associated protein2; HSC, hematopoietic stem cells; BM, bone marrow; WT, wild type; BSA, Bovine Serum Albumin; DAPI, 4′,6-diamidino-2-phenylindole; CFSE, carboxyfluorescein succinimidyl ester; LT-HSC, Lineage-Sca-1+c-Kit+Flk-CD34-; LSK, Lin-Sca-1+c-Kit+; shRNA, short hairpin RNA.

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progression [8]. Furthermore, we reported recently that Skp2 plays an important role in the regulation of HSC quiescence, pool size and self-renewal through affecting Cyclin D1 expression, which contributes to the HSC cycling [9]. Other group also found that Skp2 is involved in the basal homeostasis and stress-induced regeneration of HSC [10]. However, it is still unclear whether Skp2 also regulates HSC homing and its niche interaction.

In this study, we demonstrated that Skp2 maintains the HSC homing ability by mediating the binding between HSC and endosteal niches through the regulation of adhesion factor β-catenin in a cell cycle independent manner. Our findings not only reveal a novel regulating mechanism for HSC homing and its niche interaction but also underscore the critical function of Skp2 in the regulation of HSC transplantation efficiency.

2. Materials and methods

2.1. Mice and cells

Skp2$^{-/-}$ mice were maintained in 129 and C57/B6 mix background, and the primer sequences for genotyping were described previously [9]. *WT* and *Skp2*$^{-/-}$ MEFs were prepared from embryos 13.5 days by breeding *Skp2* heterozygous mice. All animal experiments were performed according to our Animal Care and Use Form animal protocol, approved by M.D. Anderson Cancer Center. Total BM cells or sorted HSCs were cultured in serum-free medium (StemSpan SFEM, Stemcell Technologies, Vancouver, Canada) containing 10% Bovine Serum Albumin (BSA, Stemcell Technologies, Vancouver, Canada) supplied with IL-3 and SCF (PeproTech, Rocky Hill, NJ, USA).

2.2. Homing assay

Homing assay was performed as described [11]. In brief, BM cells were stained with carboxyfluorescein succinimidyl ester (CFSE, Sigma Aldrich, St. Louis, MO, USA) (5 μM per million cells) for 10 min in 37 °C in dark. To quench the staining, five volumes of ice-cold culture media were added to the cells and incubated for 5 min on ice. Cell pellets were obtained by centrifugation, washed three times with fresh media, and re-suspended in PBS. Those fluorescence-labeled BM cells were injected into the irradiated recipient mice through the tail vein. 18 h after the injection, cells from BM and spleen of the recipient mice were collected for flow cytometry analysis. The homing ability was reflected by the frequency of fluorescence-positive cells. To assay the residence of HSCs to BM and spleen niche, we followed the protocols as described [12]. In brief, 18 h after the injection, tibia, femur and spleens from recipient mice were collected for frozen sections, and then the slides were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma Aldrich, St. Louis, MO, USA). The homing of HSCs was determined by the number of the fluorescence-labeled cells residing to BM and spleen.

2.3. Cell sorting and flow cytometry analysis

BM cells from 8 to 12 weeks old mice were stained with antibodies against various cell surface markers as described previously [9]. The antibodies (BD Bioscience, Franklin Lakes, NJ, USA) for surface markers included biotin-conjugated antibodies against 7 lineage markers (CD3, CD5, CD8, CD11b, Gr-1, B220, and Ter119), Sca-1 (PE-Cy5.5 conjugated), c-Kit (APC conjugated), CD34 (FITC conjugated), Flk-2 (PE conjugated), and secondary antibody for Lineage (PE-Cy7). Total BM cells were sorted by flow cytometry (BD Bioscience, Franklin Lakes, NJ, USA) to obtain LT-HSC (Lineage$^-$Sca-1$^+$c-Kit$^+$Flk$^-$CD34$^-$) or LSK (Lin$^-$Sca-1$^+$c-Kit$^+$) cells.

2.4. Adhesion assay

The adhesion assay was described previously [12]. In brief, equal number of LSK cells from *WT* and *Skp2*$^{-/-}$ mice were directly sorted into 96-well plate coated with fibronectin or collagen-I (BD Bioscience, Franklin Lakes, NJ, USA) for 3 h, after washed five times following the instructions, the attached cells were counted, and the ratio against the total number seeded were calculated.

2.5. Microarray analysis

We collected BM cells from *WT* and *Skp2*$^{-/-}$ mice (three mice from each group). We then sorted out the LT-HSCs and collected their total RNAs. Samples were delivered to the core facility of Department of Pathology of MDACC for gene expression analysis with Agilent chips. The data were analyzed and deposited to Gene Expression Omnibus (GEO), the record number is GSE23064 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=hfinxomsugyialo&acc=GSE23064).

2.6. Viral infection

For lentiviral short hairpin RNA (shRNA) infection, 293T cells were co-transfected with shRNAs for β-catenin and packaging plasmids (p-Helper and p-Envelope) following calcium-phosphate transfection methods. The lentiviral shRNA sequences for β-catenin were listed here: shRNA#3: (5′-GCTGATATTGACGGGCAGTAT-3′), shRNA#5: (5′-CCCAAGCCTTAGTAAACATAA-3′). Two days after transfection, viral particles were used to infect MEF or BM cells. The infected MEF cells were selected with the medium containing 2 μg/ml puromycin (Sigma, St. Louis, MO, USA). For BM cells infection, the medium of 293T cells were changed to mixture containing 50% of BM culture medium on 24 h after transfection. Then medium was used for infection on 48 h after the transfection, the ratio to fresh BM medium is 1:3 (0.5 ml into 1.5 ml, total 2 ml). The BM cells were subjected for G0/G1 analysis at 48 h after the infection.

2.7. Western blot analysis

LSK cells were harvested and lysed with RIPA buffer and then subjected for immunoblotting according to the standard protocols as previously described [13]. The following antibodies were used for Western blot analysis: Anti-β-catenin (BD Biosciences, Franklin Lakes, New Jersey) and anti-β-actin (Sigma, St. Louis, MO, USA).

2.8. Real-time quantitative PCR

Total RNA extracted from freshly sorted LT-HSC cells using the mirVana^{TM}miRNA Isolation Kit (Ambion, Grand Island, NY, USA) was treated with DNase I (Invitrogen, Grand Island, NY, USA), and cDNA was prepared with SuperScript II reverse transcriptase (Invitrogen, Grand Island, NY, USA) according to manufacturer's instruction. Real-time PCR were performed by using Applied Biosystems 7300/7500 Real Time PCR System together with SYBR Green PCR master mix (Applied Biosystems, Grand Island, NY, USA). The transcription level of β-catenin, MMP7, c-Myc, NF-kB, CD44, Axin2 and GAPDH was assessed with the primers listed in Supplemental Table S2. GAPDH was used as an internal control.

2.9. Cell cycle analysis

Cell cycle analysis was performed as described [9]. In brief, the infected BM cells were washed with PBS, fixed in 70% ethanol at 4 °C for 24 h. Cells were stained with propidium iodide and RNaseA (Sigma, St. Louis, MO, USA), incubated at 37 °C for

30 min in dark, then the cells were co-stained with Lineage, Sca-1 and c-Kit, and subjected for flow cytometry analysis. To dissect the G1 and G0 phase, infected BM cells were mixed with Hoechst 33342 (2 μg per million cells) at 37 °C for 45 min and then Pyronin Y were added to the final concentration of 1 μg/ml for another 45 min at 37 °C in dark. These stained cells were then incubated with antibodies against surface markers (Lineage, Sca-1, c-Kit, CD34 and Flk-2), and then subjected for the flow cytometry analysis.

2.10. Statistical analysis

Values were shown as mean ± s.d. The statistical analysis was performed with unpaired Student's *t*-test. $p < 0.05$ was considered as statistically significant.

3. Results and discussion

3.1. Skp2 orchestrates homing ability by maintaining the HSC binding to endosteal niche

As our previous report shows that HSC pool is expanded in the *Skp2* deficient mice [9], we next sought to determine whether the enhanced HSC number in *Skp2* deficient mice partly resulted from the increase in HSC homing ability. To test this possibility, we injected equal amounts of CFSE-labeled BM cells from wild type (*WT*) and *Skp2*$^{-/-}$ mice separately to *WT* recipient mice, and the HSCs homing ability and their residence to stem cell niche were examined. To our surprise, although *Skp2* deficiency significantly enhances HSC pools [9], it markedly reduced the homing ability of HSCs to both BM and spleen (Fig. 1A and B, $p < 0.05$, $n = 4$). In fur-

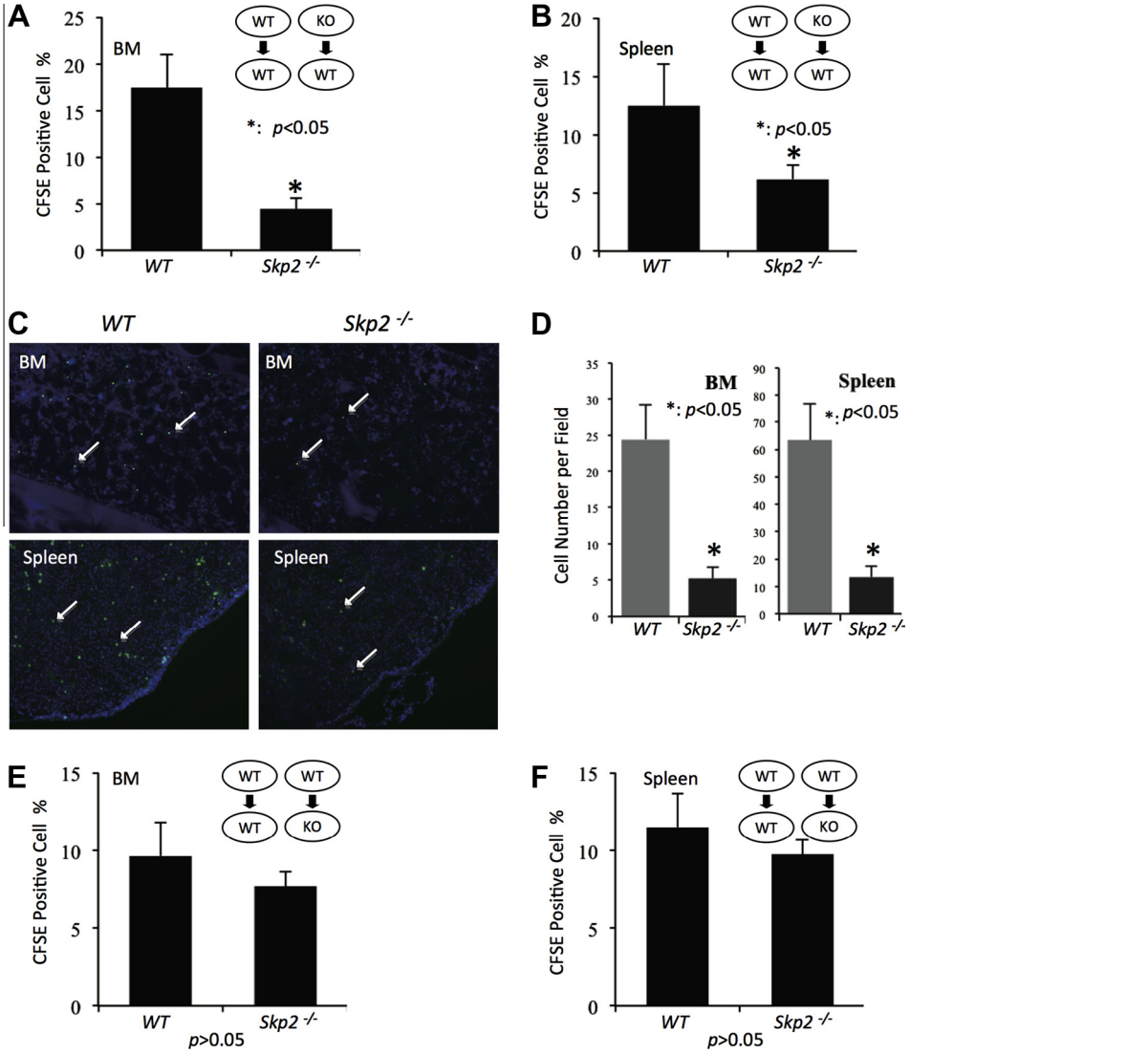

Fig. 1. Loss of *Skp2* decreases HSC homing ability but does not affect the stem cell niche. (A, B) BM cells from *WT* and *Skp2*$^{-/-}$ mice were stained with CFSE and injected to lethally irradiated *WT* recipients separately, the cells were then taken from recipient BM (A) and spleen (B) 18 h after injection and subjected for flow cytometry to detect the fluorescence labeled cells. $^{*}p < 0.05$, using unpaired Student's *t*-test, $n = 4$. (C, D) In homing assay, tibia, femur and spleens from recipient mice were collected for frozen sections 18 h after the injection. The slides were stained with 4′6-diamidino-2-phenylindole (DAPI) and then observed under fluorescence microscope (Green: HSC; Blue: the nucleic of the cells in the stem cell niche; donors BM cells were indicated by white arrow). The representative images from recipient *WT* BM and spleen (C) and the statistical results (mean ± s.d) by counting 8 random areas of slides (D) were shown. $^{*}p < 0.05$, using unpaired Student's *t*-test, $n = 3$. (E, F) BM cells from *WT* mice were stained with CFSE and injected to lethally irradiated *WT* and *Skp2*$^{-/-}$ recipients separately in order to define the differences of the stem cell niche function between *WT* and *Skp2*$^{-/-}$ mice. The cells were then taken from recipient BM (A) and spleen (B) 18 h after injection and subjected for flow cytometry to detect the fluorescence labeled cells. No significant difference was found, $p > 0.05$, $n = 4$.

ther support of this notion, we found that fluorescence-labeled *Skp2*$^{-/-}$ BM cells displayed marked reduction in their residence to BM and spleen niche *in vivo* compared to *WT* BM cells (Fig. 1C and D, $p < 0.05$, BM cells from donors were indicated by white arrow, $n = 3$). These results suggested that Skp2 is a critical factor for the maintenance of HSC homing.

Intrinsic HSC properties and endosteal niche are two important factors that may critically regulate HSC homing. We next determined whether Skp2 might regulate stem cell niche function and in turn orchestrate the homing ability of HSCs. We injected CFSE-labeled *WT* BM cells to *WT* and *Skp2*$^{-/-}$ recipients and found that the engraftment of *WT* HSCs to *WT* and *Skp2*$^{-/-}$ endosteal niche was comparable (Fig. 1E and F), suggesting that Skp2 does not alter endosteal niches to regulate HSC homing. Since the binding of HSCs to their niche is required for HSC homing, we determined whether Skp2 regulates HSC binding ability to stem cell niche related factors. We used a well-established *in vitro* adhesion assay as the readout for the HSC-endosteal niche interaction [12]. Collagen-I and fibronectin were applied as adhesion molecules for HSC binding, since they are two major extracellular matrix molecules that are present on the endosteal surface of the bone (osteoblast niche) and released by osteoblastic lineage cells [12]. To this end, LSK cells from *WT* and *Skp2*$^{-/-}$ mice were sorted into the 96-well plates coated with collagen-I or fibronectin, which mimicked the BM microenvironment, and the adherence of HSC to collagen-I or fibronectin were counted. Notably, we found that *Skp2* deficiency profoundly reduced the adhesion of HSCs to collagen-I and fibronectin (Fig. 2A and B), implying that Skp2 regulated HSC homing ability partly due to its impact on the residence of HSC to the osteoblast niche. Our current result underscored a critical role of Skp2 in the HSC homing and residence to endosteal niche in a cell autonomous manner.

3.2. β-Catenin is downregulated in *Skp2*$^{-/-}$ HSC

Our previous data [9] reveals that *Skp2* deficiency enhances HSC population and long-term reconstitution ability of HSCs, which appear to be counterintuitive to our current results that the homing ability of HSC from *Skp2*$^{-/-}$ mice was actually reduced. We reasoned that the pool size and repopulation ability of HSCs affected by Skp2 might be caused by the high expression of Cyclin D1 in *Skp2*$^{-/-}$ mice which rendered HSCs into the higher cycling and proliferation. However, the Skp2-mediated homing ability might possibly be modulated by other independent pathways. Our recent study along with others revealed a novel role of Skp2 in transcription regulation [6,14]. To gain further insight into how Skp2 may regulate the HSC homing ability, we performed the unbiased microarray analysis with total RNAs from *WT* and *Skp2*$^{-/-}$ LT-HSC cells (data were deposited to GEO as GSE23064). Among the genes regulated by Skp2, we found that β-catenin, a critical gene involved in the regulation of stem cell self-renewal and stem cells/niche interaction, was downregulated in *Skp2*$^{-/-}$ LT-HSCs (Supplemental Table S1).

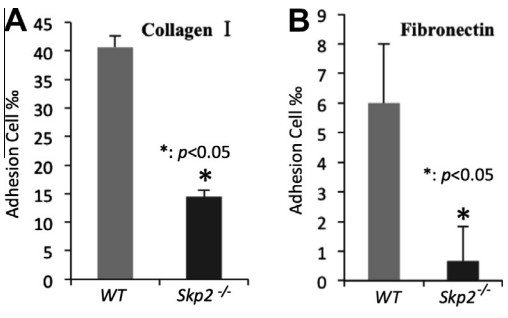

Fig. 2. Loss of *Skp2* decreases the *in vitro* binding ability of HSC. (A, B) LSK cells from *WT* and *Skp2*$^{-/-}$ mice were sorted into 96-well plates coated with either collagen-I or fibronection, the plates were washed several times 3 h after culture and the attached cells were counted under microscope. The *in vitro* adhesion ability was shown by the ratio between attached cells number and the total cell number seeded. $^{*}p < 0.05$, using unpaired Student's *t*-test, $n = 3$.

β-Catenin is an important factor that binds to cadherins at the plasma membrane to form a critical complex of adhesion junctions for cell–cell contact [15]. This process prevents β-catenin from translocating to the nucleus to turn on its downstream target gene transcription. However, upon Wnt signaling activation, β-catenin is stabilized and translocated to the nucleus to induce numerous target genes involved in a wide range of biological functions. Although β-catenin signaling pathway is important for tumorigenesis and stem cell functions, its role in HSCs remains controversial. One report shows that ectopic expression of a constitutively active β-catenin causes an increase of HSC population *in vitro* and enhanced repopulation ability *in vivo* [16]. Other report using β-catenin conditional knockout mice model reveals that β-catenin is dispensable for both HSC frequency and function [17]. Thus, the distinct models used in these studies may likely explain the discrepancy. Whether or not β-catenin is involved in HSC homing process, which represents an important feature that dictates the hematopoiesis and stem cell transplantation, remains currently unclear.

We verified the microarray data by real-time PCR analysis and further found that the mRNA level of β-catenin and its target genes including MMP7, c-Myc, CD44, NF-kB and Axin2 were profoundly reduced (Fig. 3A). Besides the transcriptional evaluation, we also detected the β-catenin protein expression in LSK cells and found that β-catenin protein levels in LSK cells from *Skp2* deficient mice were much lower than that from *WT* mice (Fig. 3B). Thus, our results demonstrated that Skp2 is a critical regulator for the expression of β-catenin and its target genes in HSCs.

3.3. Skp2 and β-catenin maintain the HSC homing ability in a cell cycle independent manner

We next determined whether the loss of β-catenin mimics the homing defect of *Skp2*$^{-/-}$ HSCs. We first used lentiviral short hairpin RNA (shRNA) to knockdown β-catenin in MEF cells to validate the shRNA knockdown efficiency and found that the shRNA #3 and shRNA #5 could efficiently knock down β-catenin expression (Supplemental Fig. S1A). We knocked down β-catenin in *WT* BM cells and assessed their homing ability. Consistent with the findings in *Skp2*$^{-/-}$ HSCs, the *WT* HSC homing ability was significantly reduced after β-catenin knockdown, indicating that β-catenin is likely a downstream executor of Skp2 to maintain the HSC homing ability (Fig. 3C and D).

Our previous report shows the loss of Skp2 promotes the cell cycle entry of HSC cells, which was partly caused by high Cyclin D1 expression [9]. We then knocked down β-catenin in BM cells isolated from *WT* mice and checked the cell cycle profile one week after cell culturing. Surprisingly, there was no significant difference in the cell cycle profile upon β-catenin knockdown (Supplemental Fig. S1B). We then further dissected the G0 and G1 phase of HSCs with Hoechst 33342 and Pyronin Y co-staining combined with HSC surface markers including Lineage, Sca-1, c-Kit, CD34 and Flk-3. Similarly, no difference was observed in the G0 and G1 phase cell populations upon β-catenin knockdown (Supplemental Fig. S2A-B). Taken together, these data suggest that the down-regulated β-catenin may contribute to the homing defect upon *Skp2* deficiency in a cell cycle independent manner.

HSC and osteoblast niche interaction are critical steps for the maintenance of HSC quiescence, pool size, self-renewal, and long-term reconstitution ability. However, how HSC homing to

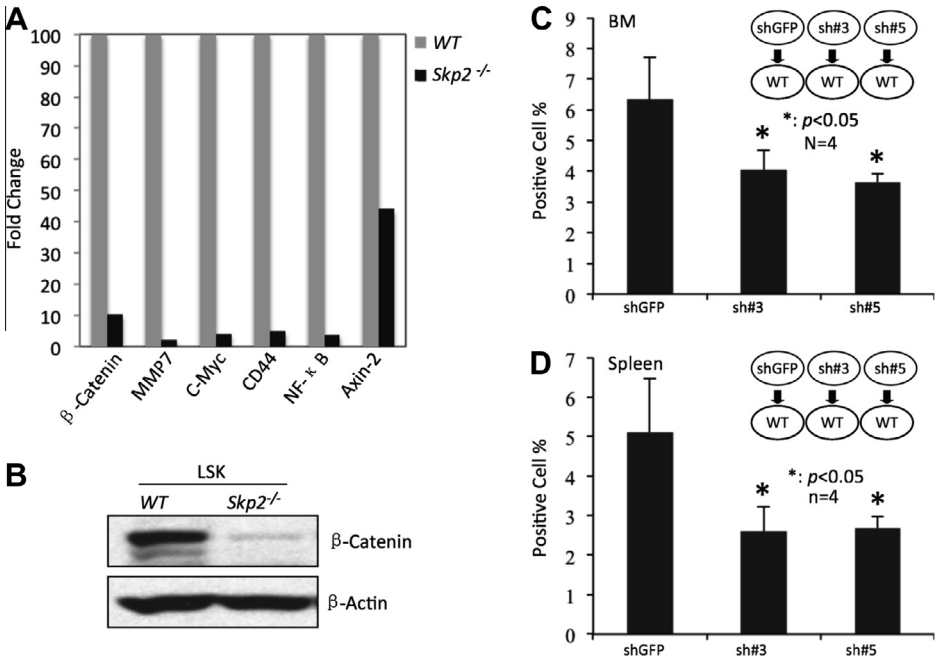

Fig. 3. Downregulated β-catenin mimics the BM homing defects in *Skp2*$^{-/-}$ mice. (A) LT-HSCs from *WT* and *Skp2*$^{-/-}$ mice were sorted and their total RNAs were subjected to real-time PCR to determine the transcription level of β-catenin and its target genes: MMP7, c-Myc, CD44, NF-kB, as well as Axin-2. (B) LSK cells from *WT* and *Skp2*$^{-/-}$ mice were sorted, lysed and subjected for Western blot analysis. β-catenin was detected and β-actin served as loading control. The expression level of β-catenin was reduced in *Skp2* deficient LSK cells compared to the *WT* LSK cells. (C, D) *WT* BM cells with vector control and β-Catenin knockdown were stained with CFSE and injected to lethally irradiated *WT* recipients. After 18 h, the cells were taken from recipient BM (C) and spleen (D), and the fluorescence labeled cells was then detected by flow cytometry. $^{*}p < 0.05$, using unpaired Student's *t*-test, $n = 5$.

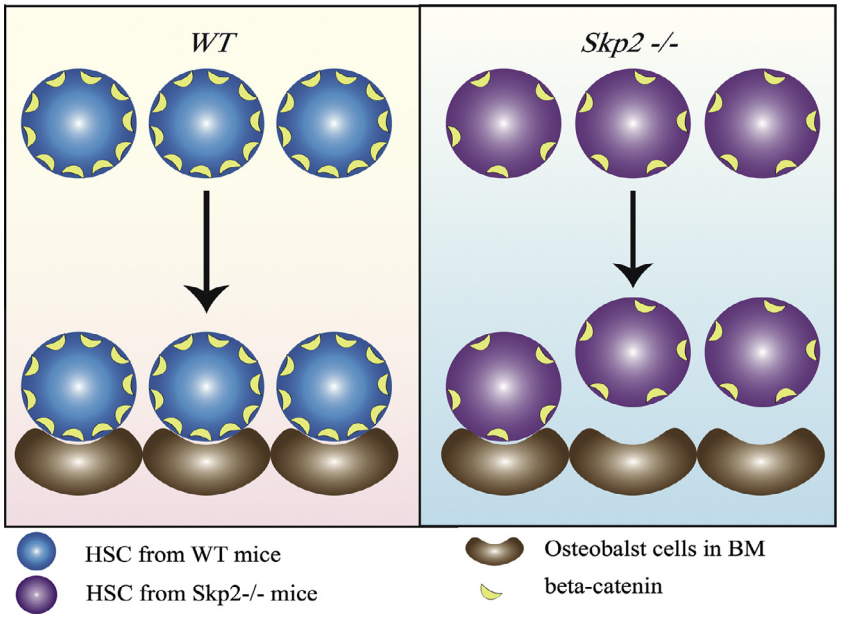

Fig. 4. Our working model on the role of Skp2 in HSC homing ability. HSCs bind to osteoblast niche when β-catenin expression is sufficient in steady state (left panel), while their binding to osteoblast niche is reduced likely due to the reduction of expression of β-catenin and its target genes upon Skp2 deficiency, which subsequently attenuates HSC homing ability of HSCs (right panel).

BM and subsequent residence to BM niche are regulated is not well understood. Osteoblast cells from mesenchymal stem cell (MSC) play critical roles in the interaction of HSCs with the microenvironments. The differentiation of MSC is dependent on multiple environmental, local, and hormonal factors including Wnt/β-catenin pathway [18,19]. Wnt/β-catenin stimulates a variety of responses in the osteoblast lineage cells, including progenitor cell proliferation and commitment to the osteoblast lineage and survival of mature osteoblast [20]. Furthermore, loss of β-catenin is expected to inhibit not only Wnt signaling activation, but also disrupts the architecture of adhesion junctions, which may affect cell–cell contact and adhesion of HSC to stem cell niche [21]. However, our study reveals for the first time that β-catenin plays an important role in HSC homing, as its knockdown displayed similar defect in HSC homing as *Skp2* deficiency does.

The previous report [9] from our group showing that *Skp2* deficiency increases pool size and long-term reconstitution of HSCs implies that Skp2 may likely promote HSC homing. However, in this study we surprisingly found that *Skp2* loss markedly reduces HSC homing and its niche interaction. This finding appeard to be counterintuitive to our previous observations. Although the detailed mechanism to explain this contradictory phenomenon remains unclear, we speculate that the *Skp2* deficient HSCs once entering the BM niches may undergo massive expansion to compensate for the loss of HSC pool size from the reduction in HSC homing, as we indeed found that *Skp2* deficiency promotes Cyclin D1 expression and facilitates cycling and proliferation of HSCs [9]. More studies are required to further resolve this puzzle.

4. Conclusions

In this study, we provided convincing evidence that Skp2 is a novel and critical regulator for HSC homing and subsequent engraftment to the BM niche. Mechanistically, Skp2 regulates β-catenin transcription and expression of its target genes, which in turn maintains HSC homing and its niche interaction (Fig. 4). Our study not only reveals a novel molecular insight into how HSC homing and engraftment to the BM niche is regulated, but also identifies Skp2 as a novel regulator in these processes. Importantly, we show that β-catenin plays an important role in HSC homing, although its role in HSC self-renewal has been so far controversial [17,21]. Understanding the mechanism by which Skp2 regulates the homing property of HSCs not only reveals novel insights into how HSC homing is regulated, but also provides novel paradigms for the treatment of stem cell-related diseases and improving stem cell transplantation efficiency.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.042.

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