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Regulation of mesenchymal stromal cells through fine tuning of canonical Wnt signaling

Jin-A Kim [a], Hyun-Kyung Choi [a], Tae-Min Kim [b], Sun-Hee Leem [c], Il-Hoan Oh [a,*]

[a] Catholic High-Performance Cell Therapy Center & Department of Medical Life Science, Republic of Korea
[b] Center for Cancer Evolution, Medical Research Center, The Catholic University of Korea, Republic of Korea
[c] Dept. of Biological Science, Dong-A University, Busan, Republic of Korea

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Abstract

Mesenchymal stromal cells (MSCs) have been extensively utilized for various cell therapeutic trials, but the signals regulating their stromal function remain largely unclear. Here, we show that canonical Wnt signals distinctively regulate MSCs in a biphasic manner depending on signal intensity, i.e., MSCs exhibit proliferation and progenitor self-renewal under low Wnt/β-catenin signaling, whereas they exhibit enhanced osteogenic differentiation with priming to osteoblast-like lineages under high Wnt/β-catenin signaling. Moreover, low or high levels of β-catenin in MSCs distinctly regulated the hematopoietic support of MSCs to promote proliferation or the undifferentiated state of hematopoietic progenitors, respectively. A gene expression study demonstrated that different intracellular levels of β-catenin in MSCs induce distinct transcriptomic changes in subsets of genes belonging to different gene function categories. Different β-catenin levels also induced differences in intracellular levels of the β-catenin co-factors, Tcf-1 and Lef-1. Moreover, nano-scale mass spectrometry of proteins that co-precipitated with β-catenin revealed distinctive spectra of proteins selectively interacting with β-catenin at specific expression levels. Together, these results show that Wnt/β-catenin signals can coax distinct transcription milieu to induce different transcription profiles in MSCs depending on the signal intensity and that fine-tuning of the canonical Wnt signaling intensity can regulate the phase-specific functionality of MSCs.

Introduction

Mesenchymal stromal cells (MSCs) are non-hematopoietic adherent cell populations derived from stroma of bone marrow (BM), adipose tissue, or placental tissue (Keating, 2006; Pittenger et al., 1999). These MSCs are being utilized for therapeutic trials, facilitating the establishment of a regenerative microenvironment for hematopoietic stem cells (HSCs), neuronal stem cells, and other tissue-specific stem cells (Frenette et al., 2013; Murphy et al., 2013; Satija et al., 2009). MSCs exert therapeutic effects via paracrine secretion of various growth factors that can stimulate regeneration of injured tissues (Caplan and Correa, 2011). In addition, MSCs exert niche effects on endogenous stem cells through cell–cell interactions, as typically exemplified by the BM stem cell niche in the endosteal and peri-vascular regions (Oh and Humphries, 2012; Oh and Kwon, 2010).

* Corresponding author at: Catholic High-Performance Cell Therapy Center, The Catholic University of Korea, 505, Banpo-Dong, Seocho-Ku, Seoul 137-701, Republic of Korea. Fax: +82 2 591 3994.
E-mail address: iho@catholic.ac.kr (I.-H. Oh).

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Accordingly, the signals controlling the biological properties of MSCs exert regulatory influences on tissue regenerative activity. For example, adipogenic differentiation of MSCs in BM suppresses the regeneration of HSCs in myeloablated hosts (Naveiras et al., 2009), whereas cells of the osteoblastic lineage support hematopoietic activity (Calvi et al., 2003; Zhang et al., 2003). In addition, the differentiation status of MSCs also influences the niche activity, i.e., recent studies have shown that among the heterogeneous subpopulation of MSCs in BM, primitive subsets of MSCs including prx-1(+) MSCs (Greenbaum et al., 2013) and nestin (+) MSCs (Mendez-Ferrer et al., 2010) play a major role in regulating HSC self-renewal in the perivascular niche of the BM. Accordingly, extensive studies are under way to identify the signals and molecular mechanisms that control the proliferation/self-renewal and differentiation of MSCs under various physiological and pathological conditions.

Wnts are secreted glycoproteins associated with the cell surface or extracellular matrix that influence diverse biological processes, including embryonic induction and cell fate specification. In the canonical Wnt signaling pathway, Wnt binds to seven-pass transmembrane Frizzled (Fz) family receptors and the single-pass co-receptors LRP 5, 6 (LDL-receptor-related protein 5,6), to induce β-catenin stabilization. Subsequently, the stabilized β-catenin translocates to the nucleus and forms a complex with the DNA-binding transcription factors TCF/LEF to activate a Wnt-controlled gene expression program (Kikuchi et al., 2006).

Several studies have shown that canonical wnt signaling plays a critical role in regulating cell fate decisions of MSCs, but variable biological effects on MSCs have been reported, i.e., up-regulation of canonical wnt signaling stimulated the proliferation of MSCs, inhibited their osteogenic differentiation, or promoted their osteogenic commitment (Boland et al., 2004; Cook et al., 2014; Gaur et al., 2005; Kawai et al., 2007; Takada et al., 2009), indicating that multiple, complex effects are mediated by canonical wnt signals.

In addition to the proliferation and differentiation of MSCs, canonical wnt signaling has been implicated in the regulation of the stromal activity of MSCs. For example, we and others recently showed that activation of Wnt/β-catenin signals in MSCs enhances the self-renewal of HSCs by triggering cross-talk of Wnt-Notch signals in the stem cell niche (Kim et al., 2009; Oh, 2010), and intra-femoral injection of β-catenin-stabilized MSCs promotes regeneration of HSCs (Ahn et al., 2010). Similarly, β-catenin expression in the BM stroma was shown to be necessary for maintenance of long-term hematopoietic cells (Kim et al., 2009; Nemeth et al., 2009). In contrast, myeloproliferative diseases developed in transgenic mice with constitutive activation of β-catenin in bone marrow osteoblastic cells (Kode et al., 2014), indicating that canonical Wnt signals in MSCs should be fine-tuned for their coordinated stromal function.

However, the regulatory mode of Wnt/β-catenin signals for MSCs, especially with regard to signaling intensity, remains unclear. Therefore, in this study, to precisely characterize the biological effects of canonical wnt signaling in MSCs, we investigated the possibility that MSCs are distinctively regulated by fine-tuning of canonical Wnt signaling. We show that β-catenin can cause distinct transactivation in MSCs depending on its level of accumulation, resulting in distinct behavior of MSCs. Our study reveals the fine-tuning of Wnt/β-catenin signaling for coordination of MSC function.

Materials and methods

Animals and cells

C57BL/6J-Ly 5.2 (BL6) from Jackson Laboratory (Bar Harbor, ME, USA) were used in the experiments under the approval from the Animal Experiment Board of the Catholic University of Korea. Enrichment of hematopoietic progenitors by 5-fluorouracil (5-FU BMCs) was performed as described (Kim et al., 2009). MSCs were isolated and passage cultured until become negative for CD45 as described (Kim et al., 2009) and characterized by surface phenotype (Supplemental Fig. 1).

Construction of expression plasmids, retrovirus production, and transduction of MSCs

A stable form of the β-catenin gene (S37A) (Kim et al., 2009) was cloned into two types of retroviral vector, MPG (MSCV-PGK-GFP) or QGCX2 (CMV-GFP-IRES). MSCs were transduced with each retroviral vector with multiplicity of infection (MOI) of 30, then sorted for transduced (GFP^+) cells as described (Hong et al., 2014).

Reporter assay

The TOPFLASH and FOPFLASH reporter constructs containing eight TCF/LEF binding sites or mutated binding sites (Biechele and Moon, 2008) were transfected into cells along with effector plasmids. Luciferase activity was measured by the Luciferase Assay System (Promega Corp., Madison, WI) according to the manufacturer's instructions. Transfection efficiency was normalized for β-galactosidase activity. To measure osteocalcin promoter activity, p6OSE2-luc containing six copies of the Runx2 binding site in the osteocalcin promoter region was used (Jung et al., 2009).

Cell proliferation and BrdU incorporation assay

Cell proliferation assay (4 days) for transduced (GFP^+) MSCs and their BrdU incorporation (12 h) were analyzed as previously described (Jung et al., 2009), visualized by secondary antibody conjugated with Alexa-593 (Molecular Probes, Eugene, OR), then mounted with DAPI (Molecular Probes) for analysis.

Colony formation and osteogenic and adipogenic differentiation of MSCs

Osteogenic and adipogenic differentiation of MSCs was performed as previously described (Jung et al., 2009), followed by Alizarin Red S (Sigma, St Louis, MO) or Oil Red O (Sigma) staining, respectively. The osteogenic mineralization or adipogenic lipid droplets were eluted and quantitatively measured by spectrophotometry at 570 and 520 nm, respectively. For colony formation, MSCs were maintained for 6 days under low (10 ng/ml) and high (100 ng/ml) concentrations of recombinant Wnt3a ligand and plated at

a density of 1000 cells per 100-mm dish. After incubation for 14 days, colonies (>50 cells) were fixed and stained with crystal violet (Sigma).

In-vivo ossification of MSCs in subrenal capsule

MSCs (1×10^5 each) were preconditioned (24 h), resuspended in Matrigel (with 100 ng/ml rhBMP-2) and inoculated underneath the renal capsule of 11 week old mice as previously described (Chan et al., 2009). After 2 weeks, the kidneys were dissected and fixed and paraffin sections were stained with either H&E or Alizarin Red stains. For immunohistochemistry staining of GFP, sections were stained with anti-GFP antibody (Abcam, Cambridge, UK), visualized with DAB substrate kit (BD Pharmingen), and followed by counterstaining with hematoxylin.

Measurement of β-catenin protein levels in the fresh bone marrow stromal cells

Fresh bone marrow cells were stained with antibodies against CD45, CD31, Ter119 (BD Pharmingen), and Leptin Receptor (Sigma). Stained cells were fixed and permeabilized with Fixation or permeabilization buffer (eBioscience), and then stained with anti-β-catenin antibody and anti-rabbit Cy5 (Bethyl Laboratories, Montgomery, USA) as described (Kim et al., 2009).

Nuclear extract and Western blot

For Western blot, MSCs were lysed in 2× Laemmli buffer, boiled for 5 min and cleared before electrophoresis. Nuclear extraction of MSCs was performed using NE-PER nuclear and cytoplasmic extraction reagents (Thermo). The membranes were probed with antibodies against HA (Sigma), active β-catenin (Merck Millipore, Billerica, MA), β-actin (Millipore), LEF1, TCF1 (Cell Signaling), TCF3, and TCF-4 (Santa Cruz).

Co-culture of hematopoietic cells and MSCs expressing β-catenin

MSCs transduced with each retroviral vector (GFP^+) were irradiated (1500 cGy) and co-cultured with 5-FU BMCs for 5 days in the presence of growth factors as described (Kim et al., 2009). The undifferentiated phenotype of co-cultured hematopoietic cells was analyzed by as previously described (Kim et al., 2009).

Microarray analysis

A gene expression study using microarrays was performed as previously described (Hong et al., 2014). Briefly, biotin-labeled cRNA samples were prepared using the Illumina TotalPrep RNA Amplification kit (Ambion, Austin, TX) and hybridized to a version 2 of the Illumina Mouse-6 BeadChip (48 K) according to the manufacturer's instructions (Illumina, San Diego, CA). Array data were analyzed using Illumina BeadStudio software. Average linkage hierarchical cluster analysis was performed with a 1-Pearson correlation as a similarity metric using the GeneCluster/TreeView program (http://rana.lbl.gov/EisenSoftware.htm). In total, 134 unique genes were selected by *t*-test ($p < .005$) with false discovery rate (FDR < 0.1) statistical confidence; the expression of these genes was increased by three-fold or more in MPG-β-catenin or QG-β-catenin compared with MPG or QG. The Gene Ontology program (http://david.abcc.ncifcrf.gov/) was then used to categorize genes in subgroups based on their biologic function.

Co-immunoprecipitation of β-catenin

Co-immunoprecipitation of proteins was performed as previously described (Antrobus and Borner, 2011). Briefly, cells were lysed and incubated with mouse anti-immunoglobulin G and anti-HA antibodies (Sigma), added with protein G plus/protein A-agarose beads (Calbiochem, Darmstadt, Germany), then precipitated, and subjected to proteomic analysis.

Nano-scale liquid chromatography/mass spectrometry of proteins co-precipitated with β-catenin

NanoLC–MS/MS analysis was performed on an Agilent 1100 Series Nano-LC and LTQ-mass spectrometer (Thermo Electron, San Jose, CA) as previously described (Gaspari and Cuda, 2011). Mass spectra were acquired using data-dependent acquisition with full mass scan (400–1800 m/z) followed by MS/MS scans. Each MS/MS scan acquired was an average of one microscan on the LTQ.

Database searching for proteomic analysis

The RAW files were converted and then searched using Mascot Server 2.2 (Matrix Science, Boston, MA). The data were searched against the NCBI human database (v.110704). Within the Mascot search, trypsin was selected as the enzyme, and one missed cleavage was allowed per peptide. All spectral count values were reported across samples, and only the proteins that included at least a top-ranked peptide and were reported in all samples were considered in the spectral counting analysis.

Statistical analysis

Data are expressed as the mean ± SEM. The significance of differences between groups was analyzed using Student's *t* test. $p < 0.05$ was considered to be statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Results

Establishment of bone marrow MSCs expressing high and low levels of active β-catenin protein

To establish MSCs that express high and low levels of β-catenin, we employed two types of retroviral vectors encoding the active form of β-catenin (S37A), where the expression of the coding gene was driven by the viral LTR (MPG-β-catenin) or CMV promoter (QG-β-catenin) (Figs. 1A, B). When each retroviral vector was transduced into 293T cells or primary BM-derived MSCS along with the TOPFLASH

reporter, the QG-β-catenin vector exhibited markedly higher transactivation of the target reporter gene than the MPG-β-catenin vector in both types of cells (108- and 38-fold higher in 293T cells and MSCs, respectively) (Fig. 1C). Moreover, BM-derived MSCs transduced with QG-β-catenin exhibited a much higher level of β-catenin protein expression compared to the levels in MSCs transduced with MGP-β-catenin (Fig. 1D). However, MSCs transduced with MPG-β-catenin exhibited only modestly increased levels of transactivation and protein levels compared to basal levels in control group MSCs (Fig. 1C right, Fig. 3D). These results show that these two types of MSCs can be employed as a model for MSCs expressing high (MSC-QG-β-catenin; H-BC/MSC) or low (MPG-β-catenin; L-BC/MSC) levels of β-catenin.

Only a low level of active β-catenin accumulation promotes the proliferation and self-renewal of colony-forming mesenchymal progenitors

We first compared the morphology and proliferation of MSCs expressing high and low levels of β-catenin during in-vitro culture. As shown in Fig. 2A, MSCs expressing high levels of β-catenin (H-BC/MSCs) exhibited a larger size than those expressing low levels of β-catenin (L-BC/MSC), as determined by flow cytometry plots. In addition, the L-BC/MSCs exhibited higher proliferative activity during in vitro culture than the control MSCs, whereas H-BC/MSCs exhibited significantly lower proliferation than the control (Fig. 2B). Similarly, BrdU uptake was increased in L-BC/MSCs but decreased in H-BC/MSCs (Fig. 2C), indicating that MSC proliferation is promoted predominantly under low levels of β-catenin accumulation. Consistent with this finding, the c-myc expression level was increased in L-BC/MSCs but decreased in H-BC/MSCs compared to control MSCs (Fig. 2D). Together, these results show that β-catenin exerts different effects on the proliferation of MSCs depending on its level of expression in MSCs.

Next, we examined the effects on the colony formation (CFU-F) of MSCs by plating equivalent numbers of MSCs in each group. The colony formation of L-BC/MSCs was enhanced compared to that of control MSCs, whereas that of H-BC/MSCs was decreased (Fig. 3A). In addition, the colonies that formed from L-BC/MSCs were larger than those from H-BC/MSCs (Fig. 3B), reflecting the higher proliferative activity of MSCs during the colonization process. However, no difference was observed for the survival and senescence of MSCs expressing high or low levels of β-catenin (Supplemental Fig. 2). Moreover, L-BC/MSCs exhibited higher expression of genes associated with the primitive state of MSCs, such as Oct-4, Nanog, Sox-2, and Nestin (Greco et al., 2007; Tsai et al., 2012), than H-BC/MSCs (Fig. 3C). These results show that only low-level accumulation of β-catenin can support the maintenance and self-renewal of MSC progenitors that are capable of forming colonies.

To further examine these effects, we examined whether differences in the concentration of Wnt ligands could induce similar differences in MSCs by treating non-transduced MSCs

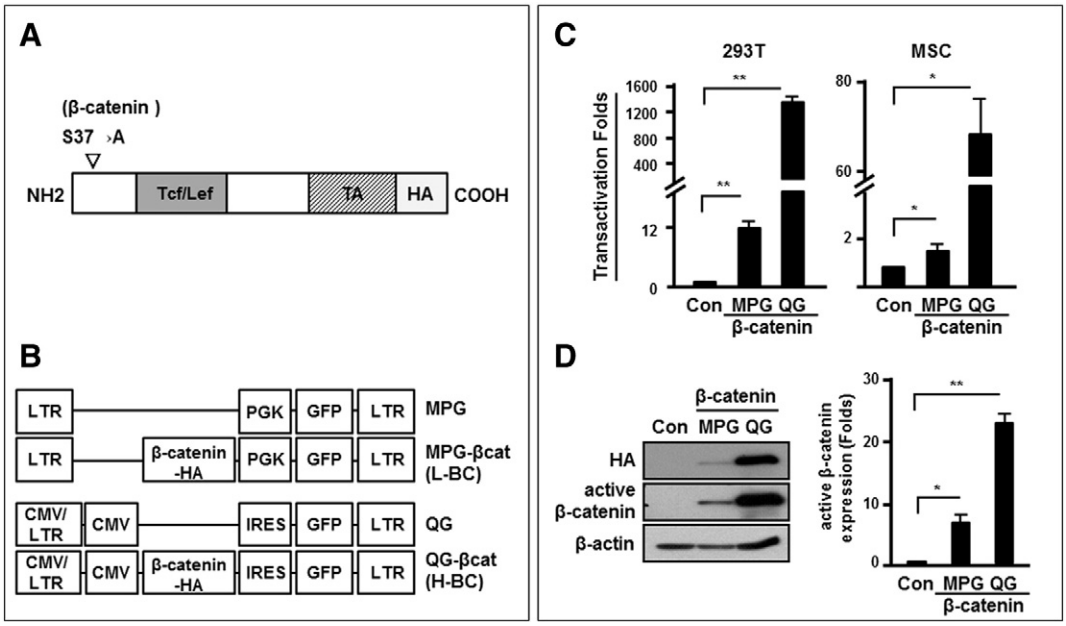

Figure 1 Establishment of MSCs with different intracellular levels of active β-catenin. (A): Schematic representation of the active form of β-catenin replacing serine S37 into Ala. TCF/LEF, T-cell factor-1/ lymphoid enhancing factor-1 binding site; HA, human influenza hemagglutinin-tag. (B): Retroviral constructs encoding green fluorescence protein (GFP) along with β-catenin under different promoters. (C): Comparisons of transactivation potential of β-catenin in 293T cells (left) or primary bone marrow-derived MSCs (right). Transactivation was assayed by luciferase activity in each transduced cell after normalization against β-galactosidase activity. The transactivation of reporter genes by β-catenin was determined by the folds of TOP/ FOP ratio relative to that of control. Mean ± SEM is shown (3 replicates, n = 9). (*: $p < 0.05$, **; $p < 0.01$). (D): Expression of β-catenin proteins in MSCs transduced by each type of retroviral vector. Shown are the representative Western blots using antibodies against indicated proteins (left) and their folds expression levels of active β-catenin protein relative to β-actin compared to control MSCs (right) with SE (3 exp. n = 3). (*: $p < 0.05$, **; $p < 0.01$).

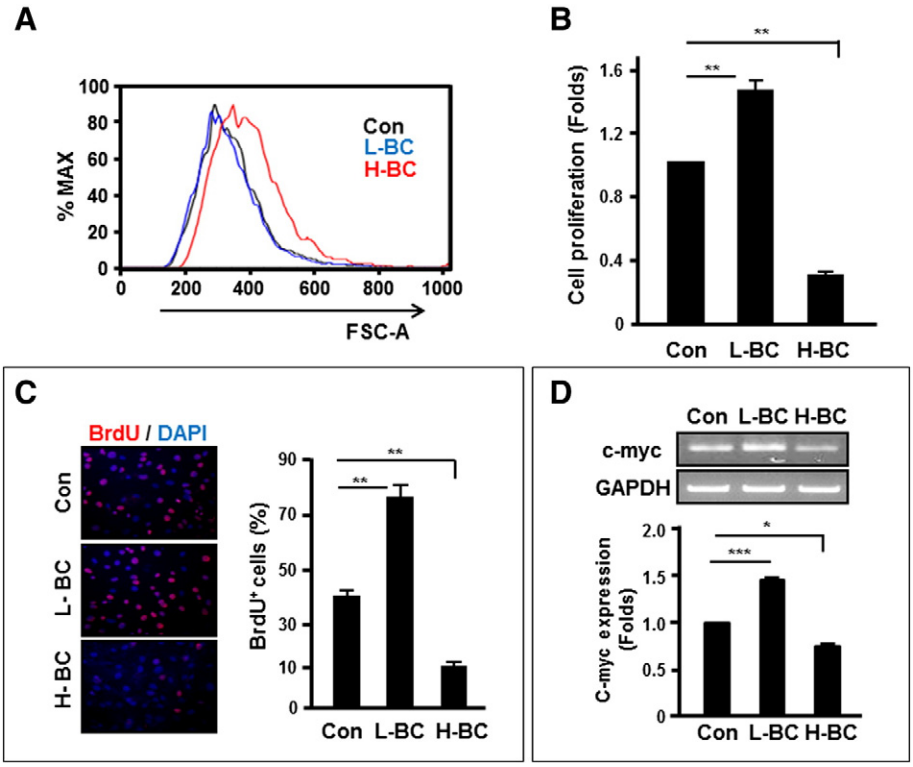

Figure 2 Distinct effects of different levels of β-catenin on MSC proliferation. (A): Representative flow cytometry plots for MSCs with low and high expression of β-catenin. (B,C): Proliferation of MSCs with low and high expression of β-catenin was compared with control transduced MSCs (B) and % of BrdU+ cells among DAPI+ was compared for each group of MSCs (C). Means ± SEM are shown (3 experiments, n = 9, **; $p < 0.01$). (D): Expression of c-myc in MSCs with low and high β-catenin. Shown are the representative RT-PCR plot (upper) and fold expression level normalized to GAPDH levels relative to the control (lower).

with high (100 ng/ml) and low (10 ng/ml) concentrations of Wnt3a ligand. As shown in Figs. 3D and E, high and low concentrations of Wnt3a ligand produced similar differences in the protein levels of β-catenin and transactivation of the TOPFLASH reporter (Figs. 3D and E), which produced similar increases and decreases in CFU-F numbers, respectively (Fig. 3F), reproducing the effects of expressing high and low levels of β-catenin MSCs. Next, we examined the relation between β-catenin levels and CFU-Fs by comparing the levels in fresh bone marrow stromal cells. As shown, the stromal cells expressing leptin receptor, the primitive subsets of MSCS that was shown to be highly enriched with CFU-F (Zhou et al., 2014), exhibited lower level of β-catenin protein than leptin receptor (−) cells, thus showing similar correlation between higher CFU-Fs and lower level β-catenin levels under in-vivo conditions (Fig. 3G).

These results together show that the strength of Wnt/β-catenin signaling in MSCs exerts distinct effects on the proliferation and self-renewal of MSC progenitors, pointing to a dose-specific response of MSCs to Wnt/β-catenin signals.

Differentiation of MSCs is regulated by β-catenin distinctly from colonization and proliferation

We examined the effects of β-catenin levels on differentiation of MSCs towards osteogenic or adipogenic lineages. As shown in Fig. 4A, osteogenic differentiation was increased in L-BC/MSCs compared to control MSCs, and differentiation was further increased with high levels of β-catenin (H-BC/MSC). In contrast, adipogenic differentiation of MSCs was decreased in L-BC/MSCs compared to control MSCs, and this inhibition of adipogenic differentiation was also further decreased under high accumulation of β-catenin (H-BC/MSCs) (Fig. 4B), indicating that the differentiation of MSCs is regulated by Wnt/β-catenin signals in a manner dependent on β-catenin levels.

Interestingly, β-catenin accumulation in proliferating MSCs caused priming of MSCs towards osteogenic lineages and away from adipogenic lineages, i.e., β-catenin-expressing MSCs (at both low and high levels) already had higher expression of osteogenic genes (ALP and OC) with a higher percentage of ALP(+) cells and lower expression of adipogenic genes (PPAR-γ and αP2) than control MSCs even before the induction of differentiation (Fig. 4C, 4D). Similarly, MSCs treated with a high concentration of Wnt3a ligand exhibited higher osteocalcin promoter activity during the proliferation phase (Fig. 4E). In addition, MSCs that had been treated with a high concentration of Wnt3a ligand exhibited enhanced mineral deposition when induced to differentiate (Fig. 4F), indicating that Wnt/β-catenin signals prime MSCs towards osteogenic lineages in a manner proportional to the intensity of the signals.

Moreover, when the MSCs expressing high and low levels of β-catenin were inoculated into subrenal capsule for in-vivo ossification (Chan et al., 2009), L-BC/MSCs exhibited increased levels of osteogenic mineralization than control MSCs, which was further increased in the H-BC/MSCs (Fig. 4G), indicating that the in-vitro findings on effects of

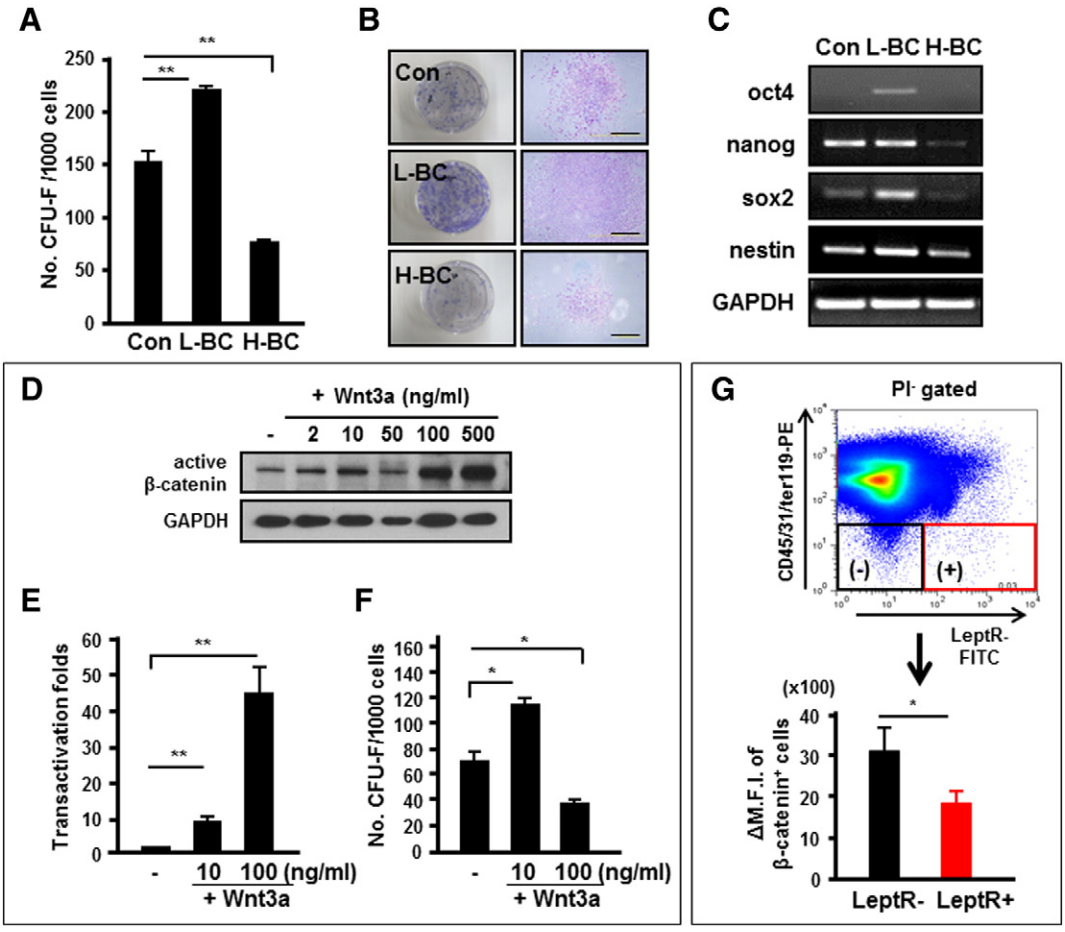

Figure 3 Distinct effects of signal intensity of Wnt/β-catenin on self-renewal of mesenchymal progenitors. (A): Comparisons of CFU-F in MSCs with high and low expression of β-catenin. The number of colonies from 1000 cells of each group of MSCs is shown (mean ± SEM, 3 experiments, n = 9). **; $p < 0.01$. (B): Representative morphology of colonies stained with crystal violet (scale bar = 50 μm). (C): Effects of β-catenin level in MSCs on expression of pluripotency-related genes. Expression of each indicated gene was analyzed by RT-PCR in MSCs transduced with each retroviral vector encoding β-catenin. (D–F): Concentration effects of recombinant Wnt3a ligand on MSCs. (D) The accumulation of β-catenin under each dose of Wnt3a ligand was analyzed by Western blot (E). Transactivation of reporters in MSCs treated with low (10 ng/ml) or high (100 ng/ml) concentrations of recombinant Wnt3a ligand was analyzed by relative folds of TOP/FOP ratios after normalization against β-galactosidase activity. (F) MSCs maintained for 6 days under low and high concentrations of recombinant Wnt3a ligand were plated for colony formation. The mean number of CFU-Fs from 1000 MSC cells is shown with the SEM (3 replicates, n = 3 each). *; $p < 0.05$, **; $p < 0.01$. (G): Comparisons of β-catenin protein levels in subsets of fresh BM stromal cells. Fresh BM cells were analyzed by intracellular staining for β-catenin protein. Shown are the representative flow cytometry plot for Leptin Receptor (+) or (−) cells among MSCs (CD45/31/Ter119-) (upper) and levels of β-catenin protein in each subset of MSCs represented by specific mean fluorescent intensity (Δ M.F.I.) normalized by those of isotype control (n = 3) (lower).

β-catenin levels are similarly reproduced during in-vivo differentiation of MSCs.

Thus, in contrast to the proliferation/self-renewal of MSCs observed under low levels of Wnt/β-catenin, a high level of Wnt/β-catenin drives MSC differentiation from adipogenic to osteogenic lineages, indicating that two distinct aspects of MSC function are modulated by the intensity of Wnt/β-catenin signal.

Distinct niche activity of MSCs according to β-catenin stabilization level

Because previous studies have shown that β-catenin stabilization in MSCs stimulates HSC self-renewal and maintenance (Kim et al., 2009; Nemeth et al., 2009), we next examined whether this hematopoiesis-supporting function of MSCs is distinctively regulated by the strength of Wnt/β-catenin signaling in MSCs. To examine the possibility, L-BC/MSCs or H-BC/MSCs were used as a feeder layer and co-cultured with hematopoietic progenitors (5-FU treated BM cells) for 5 days. When the proliferation of hematopoietic cells ($CD45^+$) was analyzed during co-culture with MSCs, L-BC/MSCs supported the expansion of hematopoietic cells to a level comparable to that in control MSCs, but H-BC/MSCs induced significantly lower proliferation of the hematopoietic cells. In contrast, the frequency of phenotypically defined HSCs ($Lin^-Sca\text{-}1^+c\text{-}kit^+$; LSK) was significantly higher in the co-culture on H-BC/MSCs than in the co-culture on L-BC/MSCs or control/MSCs (Fig. 5B). Thus, whereas the net expansion of undifferentiated hematopoietic

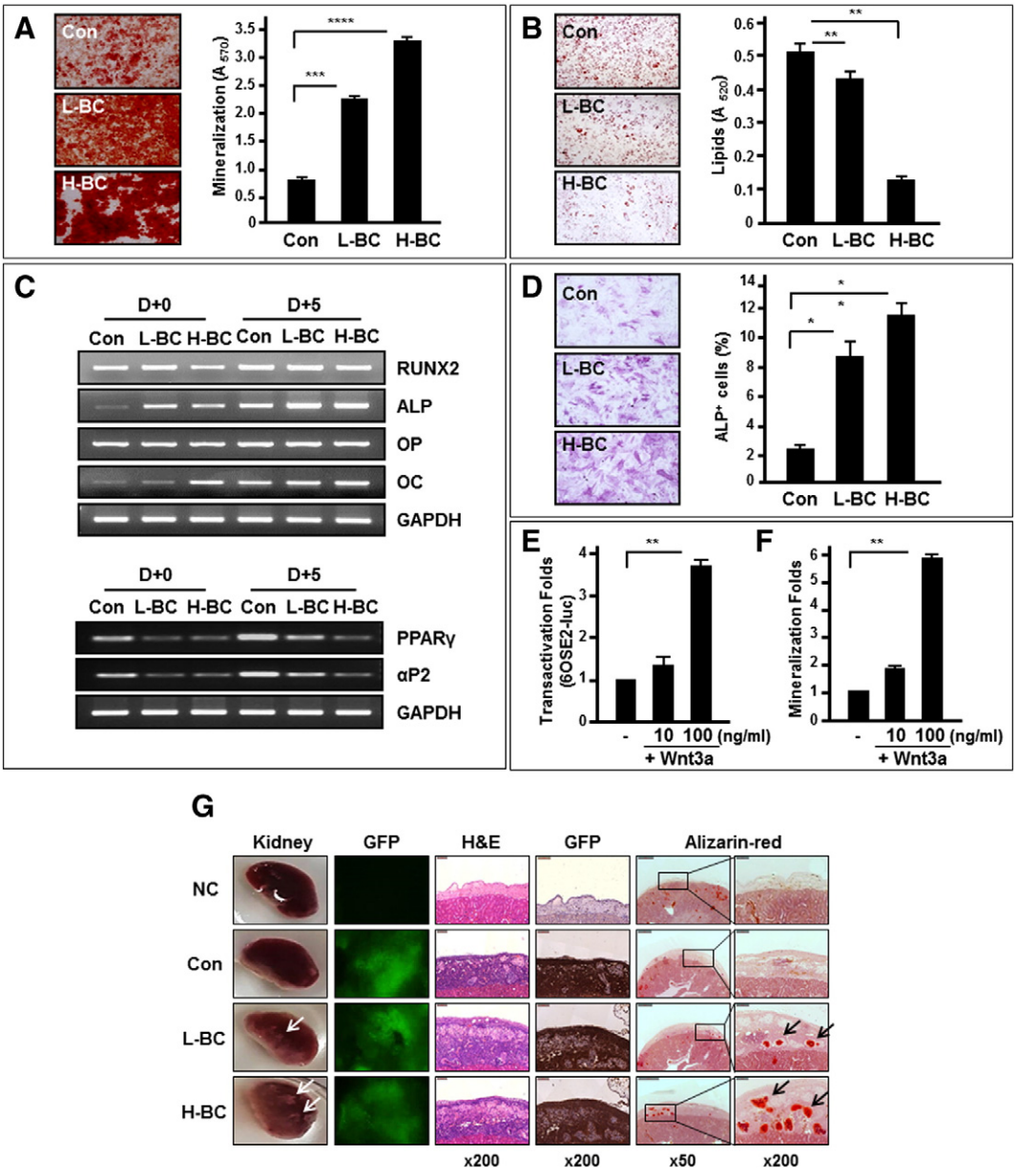

Figure 4 Concentration effects of β-catenin on the osteogenic and adipogenic differentiation of MSCs. (A, B): Effects of β-catenin levels on osteogenic and adipogenic differentiation. Each group MSC was subjected to osteogenic or adipogenic differentiation and stained with Alizarin-Red S or Oil Red O, respectively. Representative staining profiles (left, 100×) and spectrophotometric quantification of osteogenic (A) and adipogenic differentiation (B) are shown (mean ± SEM, from 3 experiments, n = 6). **; $p < 0.01$. ***; $p < 0.001$, ****; $p < 0.0001$. (C): Expression of osteogenic and adipogenic genes. Representative plots for RT-PCR analysis of osteogenic genes (upper) and adipogenic genes (lower) in each group of MSCs before (D + 0) and 5 days after differentiation induction (D + 5) are shown. (D): ALP activity in MSCs expressing β-catenin. Representative images (100×) and % of ALP(+) cells are shown (mean ± SEM, from 3 experiments, n = 9). *; $p < 0.05$, **; $p < 0.01$. (E): Concentration effects of Wnt3a ligand on osteocalcin promoter activity in MSCs. MSCs pre-treated with the indicated concentrations of recombinant Wnt3a were transfected with osteocalcin promoter reporters (6OSE2-luc). The mean ± SEM of the fold transactivation of osteocalcin reporters in each type of treated MSC is shown relative to that in BSA-treated MSCs (3 experiments, n = 9). **; $p < 0.01$. (F): Concentration effects of Wnt3a ligand on osteogenic differentiation. MSCs pre-treated with each indicated concentration of Wnt3a ligand were subjected to osteogenic differentiation. Quantification of mineral deposition 5 days after induction of differentiation is shown (mean ± SEM values from 3 experiments, n = 6). **; $p < 0.01$. (G): Effect of β-catenin dose on the in-vivo osteogenic differentiation. Each indicated MSC group was inoculated into subrenal capsules in a mixture of Matrigel and kidneys were analyzed 2 weeks after injection. Representative images are shown for gross morphology, fluorescence (GFP), hematoxylin & eosin staining, immunohistochemical staining for GFP and Alizarin-red staining for mineralization. NC, group transplanted only with Matrigel without MSCs. The white arrows indicate bonny area in the kidney, and the black arrows indicate mineralized nodules with alizarin red staining. Note comparable level of transplanted (GFP+) cells for each injected MSC group.

cells (LSK) was similarly increased in both types of β-catenin-stabilized MSCs compared to the control group (Fig. 5C), high and low levels of β-catenin promoted distinct modes of self-renewal, predominantly supporting the maintenance of the undifferentiated state or the proliferation of the cells, respectively.

To explore the molecular basis for this distinction in hematopoietic support, we compared these two types of MSCs for expression of microenvironmental cross-talk molecules such as Notch ligand, CXCL-12 (Greenbaum et al., 2013), and Ang-1 (Arai et al., 2004). As shown in Fig. 5D, MSCs expressing high and low levels of β-catenin exhibited comparable levels of expression of Ang-1 (Arai et al., 2004) and CXCL-12 (Greenbaum et al., 2013). In contrast, a difference was observed for molecules involved in Notch signaling such as Jagged-1 or Mfap2 (microfibril-associated glycoprotein 2, proliferin-2) (Choong et al., 2003); these molecules were expressed at a higher level in L-BC/MSCs than in H-BC/MSCs. Together, these results show that different levels of β-catenin in MSCs can provide distinct stromal functions for hematopoietic progenitor cells by presenting distinct spectra of niche cross-talk signals.

Transcriptional reprogramming of MSCs with different amounts of β-catenin accumulation

We examined the gene expression profiles of MSCs with high and low levels of β-catenin using a microarray. We first selected 1000 genes with highly variable expression among the groups and performed hierarchical clustering (Fig. 6A), which clearly segregated the control MSCs from MSCs with β-catenin stabilization. The segregation of expression profiles was also apparent between MSCs with high and low levels of β-catenin accumulation, which displayed differential gene expression in subsets of genes (arrows in Fig. 6A). However, the level of relative expression (β-catenin versus controls) measured for each gene was positively correlated ($r = 0.416$; Fig. 6B) between MSCs with high and low levels of β-catenin accumulation. These findings suggest that low and

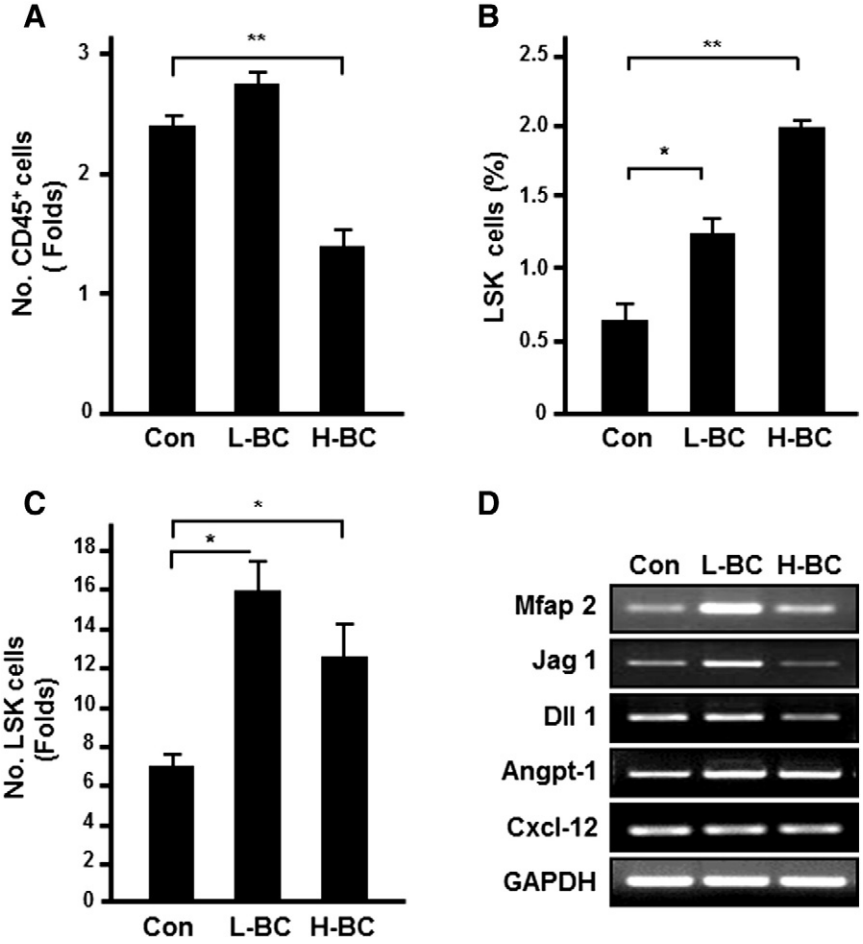

Figure 5 Distinct hematopoietic supporting activity of MSCs according to the β-catenin level in MSCs. 5-FU BMCs were co-cultured on each indicated MSC group for 5 days and analyzed for expansion of hematopoietic progenitors. (A): Fold change of total $CD45^+$ hematopoietic cells compared to input cells (mean ± SEM values from 3 experiments, n = 3). **; $p < 0.01$. (B): Percentage of LSK (Lin^- $Sca\text{-}1^+c\text{-}kit^+$) cells in hematopoietic cells ($CD45^+$) after coculture for 5 days (mean ± SEM values from 3 experiments, n = 3). *; $p < 0.05$ **; $p < 0.01$. (C): Net expansion of LSK cells after co-culture. Fold changes in the total number of LSK cells relative to the input LSK cell number are shown (mean ± SEM values from 3 experiments, n = 3). *; $p < 0.05$. (D): Expression of hematopoietic regulatory factors in each group of MSCs. Expression of indicated genes was analyzed by RT-PCR using GAPDH as an internal control.

high expression of β-catenin in MSCs results in overall similar transcriptomic changes but also differential expression for a subset of genes.

To further characterize the difference in transcriptomes, we used Gene Set Enrichment Analysis (GSEA) to investigate the functional categories of genes that were differentially expressed in MSCs with high and low levels of β-catenin. Supplemental Table 1 lists the 17 and 20 categories of gene sets that were relatively overexpressed in MSCs expressing high and low levels of β-catenin, respectively. When the functional relationship among these GO categories was profiled in a network diagram, 15 and 14 GO categories showed redundancy into common functional groups in MSCs with high and low levels of β-catenin, respectively (Figs. 6C, D). Three gene categories representing signaling pathway/development, metabolism, and muscle fibers were relatively overexpressed in MSCs with high levels of β-catenin compared to those with low levels of β-catenin (Fig. 6C). In contrast, cell cycle- and DNA metabolism-related GO categories were overexpressed in MSCs with low levels of β-catenin compared to those with high levels of β-catenin (Fig. 6D), consistent with the findings of higher proliferation and self-renewal in MSCs expressing low levels of β-catenin. Together, these results show that MSCs with high and low expression of β-catenin induce distinct transcription profiles in subsets of gene groups that belong to distinct functional categories, thus supporting the molecular basis of distinct biological impacts in MSCs with different levels of β-catenin accumulation.

Distinct transcriptional milieu under different levels of β-catenin

Next, we evaluated the molecular basis for the distinct transcription patterns induced by different levels of β-catenin in MSCs. Because transactivation of β-catenin in the promoter region is modulated by protein interactions with co-factors such as LEF or TCF families (Gan et al., 2008; Kikuchi et al., 2006), we first compared the expression of

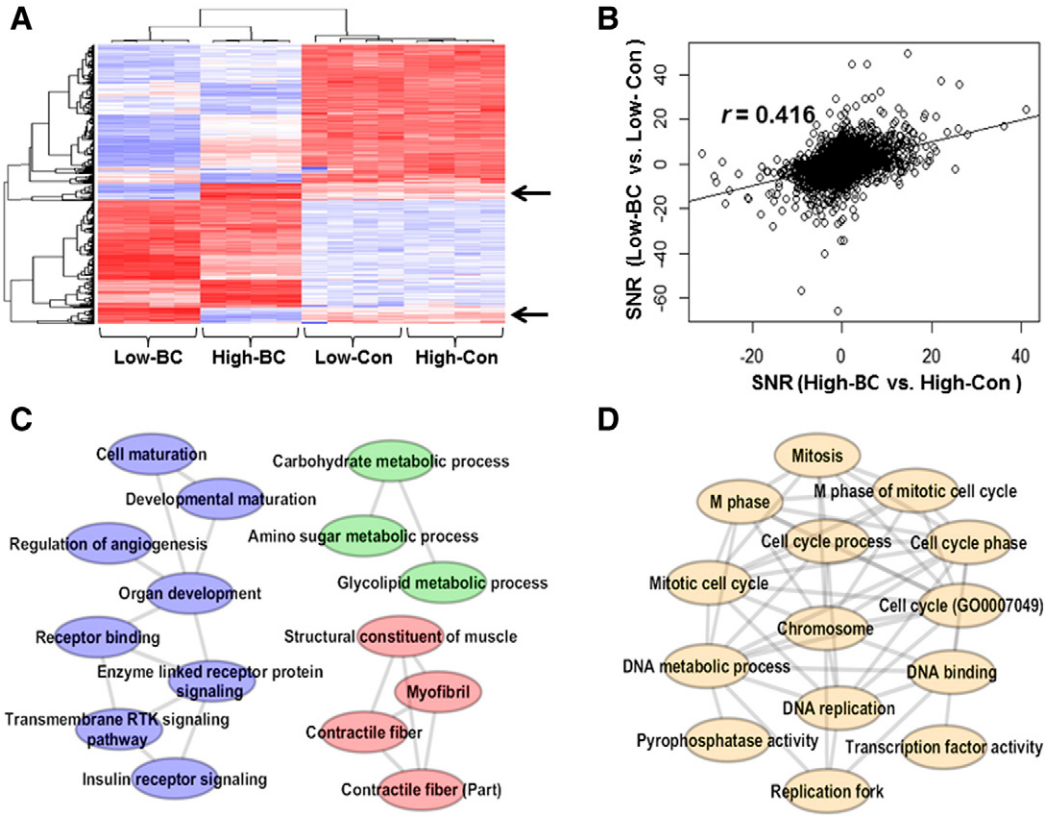

Figure 6 Transcriptomic changes induced by different levels of β-catenin in MSCs. (A): Hierarchical clustering of the 1000 genes that showed the largest changes in expression across the expression profiles. MSCs transduced with retroviral vectors encoding high and low levels of β-catenin are marked with the corresponding controls for each vector (control). Arrows indicate genes that showed differential expression between MSCs with high and low expression of β-catenin. (B): Differential gene expression between MSCs with different levels of β-catenin expression compared to control-transduced MSCs is calculated as the signal-to-noise ratio (SNR). The scatter plots compare the SNR from low-BC/MSCs and high-BC/MSCs for each gene. A fitted line is shown with the correlation level (r). (C): GO categories enriched among genes relatively overexpressed in MSCs with high β-catenin levels compared to those with low β-catenin levels are shown as nodes. Edges indicate significant ($P < 10^{-10}$; Fisher's exact test) overlap of gene contents between two GO categories. (D): GO categories enriched in genes overexpressed in low-BC/MSCs relative to HIGH-BC/MSCs (see Supplemental Table 1 for the full list of 17 and 20 GO categories upregulated in high- and low-BC/MSCs, respectively). The color of the nodes indicates the members of functional subnetworks in each GO category.

these co-factors in each type of MSC. As shown, MSCs with low expression of β-catenin exhibited prominently higher levels of LEF-1 and TCF-1, whereas MSCs with high expression of β-catenin exhibited profound suppression of LEF-1 with modest differences in the levels of TCF3 and TCF4 (Figs. 7A, B). These results suggest that different intracellular levels of β-catenin could result in distinct transcriptional complexes at the promoter to generate different transcription profiles.

To further explore the possibility of variation in the transcriptional milieu under different intracellular levels of β-catenin, we examined the cellular proteins that interact with β-catenin in each condition. We performed a proteomic analysis of intracellular proteins that co-precipitated with β-catenin using nano-scale liquid chromatography/mass spectrometry (Nano LC–MS/MS) (Gaspari and Cuda, 2011). As shown in Supplemental Table 2, extensive differences were seen in the spectra of proteins that co-precipitated with each level of β-catenin, suggesting that β-catenin interacts with different intracellular proteins depending on its intracellular accumulation levels.

Taken together, these results show that the transcription milieu varies according to the intracellular concentration of β-catenin, which thus serves as a distinct transactivation factor that can induce distinct transcription profiles in MSCs and thereby produce distinct functional outcomes.

Discussion

Recent studies have focused on signals that regulate proliferation/differentiation and stromal function under various physiological and pathological conditions. Of these regulatory signals, canonical Wnt signals have been implicated in the regulation of MSCs either by controlling the expression of cross-talk molecules such as Jagged-1 or dll-1 (Kim et al., 2009) or by controlling MSC differentiation into osteogenic or adipogenic lineages (Ling et al., 2009; Takada et al., 2009). Moreover, constitutive stabilization of β-catenin in bone marrow stromal cells was associated with development of myeloproliferative disease (Kode et al., 2014), indicating that canonical Wnt signaling in stromal cells should be fine-tuned for coordinated microenvironmental regulation of hematopoietic activity.

However, little is known regarding the precise biological impact of the signal intensity of canonical Wnt signaling in MSCs. Moreover, the effects of Wnt signaling on the

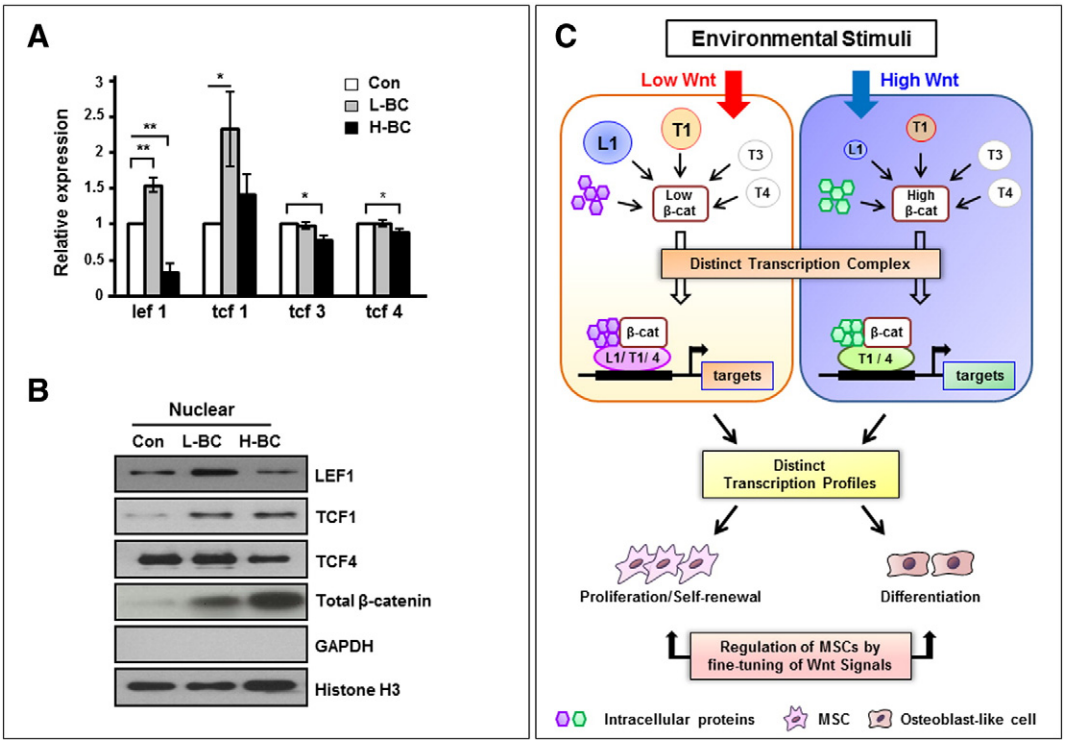

Figure 7 Changes in the expression of co-factors for β-catenin with respect to β-catenin levels. (A): MSCs expressing high and low levels of β-catenin were analyzed for expression of co-factors for β-catenin transactivation. Relative expression levels of each co-factor compared to those in control MSCs were analyzed by real-time quantitative RT-PCR (RQ-PCR) after normalization to GAPDH (mean ± SEM values from 3 experiments, n = 3). *; $p < 0.05$ **; $p < 0.01$. (B): Western blot for co-factors in the nuclear extracts of MSC expressing high and low levels of β-catenin. (C): Hypothetical model for concentration-specific effects of Wnt/β-catenin signaling in MSCs. Differences in the intensity of Wnt/β-catenin signaling induce differences in the transcription milieu for β-catenin, i.e., TCF-1 is dominantly up-regulated in Low-BC, but Lef-1 is profoundly down-regulated in High-BC. In addition, distinct spectra of intracellular proteins (marked with blue and red hexagon) interact with β-catenin. Thus, different protein complexes are formed under high and low levels of β-catenin to induce distinct transcription profiles and therefore generate distinct biological functions in MSCs under these two conditions. * L1: LEF-1, T1: TCF-1, T3: TCF-3, T4: TCF-4. The size of each factor denotes relative expression levels in the cells, and hexagons denote intracellular proteins interacting with β-catenin.

differentiation of MSCs is controversial (Boland et al., 2004; Cook et al., 2014; Gaur et al., 2005; Kawai et al., 2007), raising the possibility that the regulatory effects of canonical Wnt signals in MSCs could be more complex than previously thought.

Therefore, to investigate the possibility that the biological characteristics of MSCs are regulated by fine-tuning of Wnt signaling strength, we established MSCs with high and low expression of β-catenin by transduction of the cells with two different retroviral vectors. From this approach, we found that two distinct aspects of MSC function were controlled by the intensity of Wnt/β-catenin signals, i.e., proliferation and self-renewal of MSCs were promoted only under low levels of Wnt/β-catenin, whereas osteogenic differentiation was promoted in a manner proportional to the intensity of the Wnt signals.

Moreover, MSCs proliferating under low Wnt/β-catenin exhibited higher expression of pluripotency genes such as Oct-4, Sox-2, and Nanog (Greco et al., 2007; Tsai et al., 2012), indicative of a more primitive state, whereas the cells exhibited more committed osteoblast-like properties under high Wnt/β-catenin.

Interestingly, studies have shown that the characteristics of primitive MSCs derived from embryonic or induced pluripotent stem cells as well as MSCs from fetal tissue exhibit higher levels of pluripotent gene expression, higher CFU-F and proliferation together with higher osteogenic, but not adipogenic differentiation potential compared to adult tissue-derived MSCs (Boyd et al., 2009; Chen et al., 2012) (Guillot et al., 2007; Zhang et al., 2009), which was similarly observed among MSCs with early and late passages during serial subculture (Vacanti et al., 2005). Thus, taken these findings together, MSCs under low Wnt/β-catenin levels mimic the primitive MSCs sharing exhibiting common functional characteristics with those MSCs.

Of note, differences in the accumulation levels of β-catenin in MSCs also resulted in distinct stimulatory effects on hematopoietic progenitor cells, i.e., whereas MSCs with low expression of β-catenin better supported the proliferation of hematopoietic cells than MSCs with high expression of β-catenin, maintenance of the undifferentiated state was better supported by MSCs with high levels of β-catenin.

Together, these findings suggest that the MSCs exhibit distinct biological responses to physiological conditions and exert distinct influences on the tissue regenerative process depending on the strength of Wnt/β-catenin signaling in their microenvironment. Although further studies on the physiological significance of such regulation of niche activity are warranted, our findings point to the importance of fine-tuning Wnt/β-catenin signaling for coordination of regulatory effects in MSCs.

At present, the mechanisms underlying the concentration-specific effects of Wnt/β-catenin signaling remain unclear. However, our study shows that β-catenin induces distinct transcriptomic changes depending on the level of β-catenin accumulation, especially for subsets of genes in distinct functional groups of gene ontology. In particular, low levels of β-catenin in MSCs resulted in up-regulation of genes involved in cell cycle and DNA metabolism, whereas high levels of β-catenin caused up-regulation of genes involved in signaling pathway/development and metabolism. Thus, β-catenin can serve as a distinct transactivation molecule depending on its level of accumulation in the cells to induce different transcriptomic changes in the cells.

Although the molecular basis for the distinct transactivation of β-catenin remains unclear, our study raises the possibility that the transcriptional milieu at the target promoters could vary according to the level of β-catenin accumulation. Supporting this possibility, MSCs expressing different levels of β-catenin exhibited different intracellular expression of co-factors, such that high and low levels of β-catenin resulted in different levels of LEF-1 and TCF-1. Thus, it is possible that different levels of β-catenin result in the formation of a distinct transcription complex at the target promoters, thus resulting in different components of the transcription milieu. Moreover, nano-scale LC/mass spectrophotometry showed that β-catenin interacts with different intracellular proteins, as evidenced by the distinct spectrum of co-precipitated proteins specific to each concentration of β-catenin.

Therefore, out study shows that different intracellular levels of β-catenin can affect the transcription milieu, and thus β-catenin may serve as a distinct transactivation molecule to produce distinct biological effects in MSCs (schematically shown in Fig. 7C).

Of note, our finding of distinct transactivation with different intracellular levels of β-catenin could provide a mechanism for broader spectra of stem cell regulation by Wnt signals, as several studies have shown that the regulatory effects of Wnt signals on HSCs are dependent on the intensity of the signals (Luis et al., 2011). Similarly, differentiation of ES cells towards specific lineages was influenced by the strength of Wnt signals (Kielman et al., 2002; Reya and Clevers, 2005), providing a common scheme for regulation by Wnt signals. In this sense, it is plausible that the current paradigm of concentration-specific regulation by Wnt/β-catenin signaling is conserved over a broad extent of tissue regeneration and that various types of stem cells and their niche function can be regulated through fine tuning of Wnt/β-catenin signaling. Further studies are necessary for a more complete understanding of the regulation of various tissue regenerative processes by Wnt/β-catenin signaling.

Nevertheless, our study shows that different intracellular levels of β-catenin in MSCs may result in distinct biological responses of MSCs and that β-catenin serves as a distinct transactivation molecule that regulates MSC function according to Wnt signaling intensity. Our study provides a novel insight into the importance of fine tuning of Wnt/β-catenin signals for coordination of various regenerative biological functions.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2015.02.007.

Author contributions

JAK., HKC.: performed the experiments and generated the data.

SHL., TMK.: performed gene expression analysis and bioinformatics study.

IHO.: designed the current study, supervised the study, and wrote the manuscript.

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