Activation of Wnt/β-Catenin Protein Signaling Induces Mitochondria-mediated Apoptosis in Hematopoietic Progenitor Cells

Received for publication, January 12, 2012, and in revised form, May 13, 2012 Published, JBC Papers in Press, May 15, 2012, DOI 10.1074/jbc.M112.342089

Ming Ming1, Sheng Wang1, Wenshu Wu5, Vitalyi Senyuk1, Michelle M. Le Beau4, Giuseppina Nucifora5, and Zhijian Qian1,2,4

From the 1Department of Medicine and the 2Cancer Research Center, University of Illinois, Chicago, Illinois 60621, the 3Department of Hematology/Oncology and the Comprehensive Cancer Center, University of Chicago, Chicago, Illinois 60637, and the 4Children’s Hospital Oakland Research Institute, Oakland, California 94609

Background: Wnt/β-catenin signaling plays a strong role in maintaining homeostasis of hematopoietic progenitor cells (HPCs).

Results: Activated β-catenin deregulates the survival of HPCs by induction of the mitochondrial apoptotic pathway.

Conclusion: Wnt/β-catenin signaling is critical for HPC pool maintenance.

Significance: This study clarifies the role of the Wnt/β-catenin signaling in HPCs by showing that it has negative impact on the function and survival of the cells.

The canonical Wnt/β-catenin signaling is activated during development, tumorigenesis, and in adult homeostasis, yet its role in maintenance of hematopoietic stem/progenitor cells is not firmly established. Here, we demonstrate that conditional expression of an active form of β-catenin in vivo induces a marked increase in the frequency of apoptosis in hematopoietic stem/progenitor cells (HSCs/HPCs). Activation of Wnt/β-catenin signaling in HPCs in vitro elevates the activity of caspases 3 and 9 and leads to a loss of mitochondrial membrane potential (∆Ψm), indicating that it induces the intrinsic mitochondrial apoptotic pathway. In vivo, expression of activated β-catenin in HPCs is associated with down-regulation of Bcl2 and expression of Casp3. Bone marrow transplantation assays reveal that enhanced cell survival by a Bcl2 transgene re-establishes the reconstitution capacity of HSCs/HPCs that express activated β-catenin. In addition, a Bcl2 transgene prevents exhaustion of these HSCs/HPCs in vivo. Our data suggest that activation of the Wnt/β-catenin pathway contributes to the defective function of HPCs in part by deregulating their survival.

The canonical Wnt/β-catenin pathway plays an important regulatory role in hematopoiesis; however, its involvement in regulation of HSC function is controversial. Mice with deletion of β-catenin (1) or β-catenin and γ-catenin (2) had no obvious HSC defects. In contrast, other investigators showed the existence of hematopoietic abnormalities in mice with deletion of β-catenin from fetal life (3). Retroviral expression of activated β-catenin enhances the self-renewal of Bcl2 transgenic HSCs in vitro, and the Wnt signaling inhibitor Axin represses HSC proliferation and decreases reconstitution capacity of HSCs (4). However, others reported that purified Wnt protein in vitro (5) or inhibition of GSK3β in vivo (6) promotes the self-renewal of HSCs. Baba et al. (7, 8) showed that constitutive activation of β-catenin impairedmultilineage differentiation, whereas others found that expression of constitutively active β-catenin in hematopoietic cells in vivo leads to loss of HSC repopulation and multilineage differentiation block (9, 10).

The role of β-catenin in the regulation of cell proliferation and apoptosis is cell context-dependent. β-Catenin is an enhancer of proliferation and survival in tumor cells (11, 12) but can also induce apoptosis in a variety of cells. Forced expression of an activated β-catenin lacking the NH2 terminus promoted proliferation and apoptosis in mouse intestinal epithelial cells (13). However, in fibroblasts, the overexpression of β-catenin induced apoptosis independently of its transactivation function with LEF1 (14). In enzastaurin-treated multiple myeloma cells, it resulted in cell death-dependent up-regulation of JUN and TP73 (15).

Taken together, these contradictory reports emphasize the difficulty of understanding the role of the Wnt/β-catenin pathway in HSCs/HPCs. Here, we have revisited this controversy and have examined the effects of activated β-catenin in hematopoietic progenitor cells in vitro and in vivo. In vitro our studies reveal that activation of the Wnt/β-catenin signaling induces a mitochondria-dependent apoptotic pathway. In vivo we find that it promotes apoptosis of HPCs by suppressing Bcl2...
Activated β-Catenin Induces Apoptosis

and inducing Casp3. In addition, we show that an enhanced survival signal is required for maintenance and function of HSC/HPCs with activated β-catenin in vivo. Our studies suggest that the negative effect of Wnt/β-catenin signaling in HPCs results from the deregulation of their survival.

MATERIALS AND METHODS

Mice—C57BL/6 and B6.SJL mice were purchased from Tac- onic (Hudson, NY) for breeding in the mouse facility at the University of Illinois at Chicago. The mice (6–12 weeks) were used for bone marrow (BM) collection. All animal protocols were approved by the Animal Care and Use Committee of the University of Illinois at Chicago. Vav-Bcl2 transgenic mice were kindly provided by Dr. Jerry Adams (The Walter and Eliza Hall Institute of Medical Research, Bundoora, Victoria Australia). β-Catenin\(^{\text{box/flox}}\) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Ctnnb1(Ex3)\(^{\text{flox/flox}}\) mice have been described (16) and were obtained from Dr. Kathleen Goss (University of Chicago). Effective deletion of floxed exons was monitored by PCR. The sequence of the primers is listed in supplemental Table S1.

Quantitative Real-time PCR—To evaluate the expression of candidate genes deregulated in mutant β-catenin mice, HPCs were sorted from Mx1-cre Ctnnb1\(^{(Ex3)}\)/\(^{\text{Δ/Δ}}\) and control mice (\(n = 3\)) 4 days after three doses of pl-pC injection. RNA was isolated with TRIzol reagent (Invitrogen) and quantified. Relative abundance of specific candidate gene transcripts normalized to β-actin was determined by quantitative RT-PCR by SYBR Green incorporation. The primer sequence for each gene is listed in supplemental Table S1.

Retroviral Vectors and BM Infection—The pBabe-β-cat-S33Y vector encoding an activated form of β-catenin was kindly provided by Dr. Eric Fearon (University of Michigan School of Medicine, Ann Arbor, MI). The BamHI-EcoRI DNA fragment encoding β-cat-S33Y was cloned into the MSCV-IRESCAT (MIGR1) vector. The Bcl2 cDNA was subcloned into MSCV-EYFP vector. Using Effectene transfection reagent (Qiagen), high titer retrovirus was produced by transient transfection of 293T cells with the pCL ecotropic packaging plasmid and the appropriate retroviral vectors (17). The MSCV-Cre-IRESCAT and MSCV-puro-Cre vectors were provided by Dr. Jiwang Zhang (Loyola University, Maywood, IL). The BM cells isolated from mice treated with 5-fluorouracil (150 mg/Kg) for 4 days, the lineage-negative BM cells, and the day 14.5 fetal liver cells were infected with the appropriate retrovirus by spinoculation as described previously (18).

Cell Culture—BM cells were collected by flushing murine femurs and tibias with IMDM containing 2% fetal bovine serum (FBS). To isolate lineage-negative BM cells, the cells were stained with biotinylated mouse antibodies specific for lineage markers (CD3, B220, IgM, Ter119, Gr-1, Thy1.1, IL-7R, and CD19) followed by PE-Cy5-conjugated streptavidin, Kit-APC-eFluo780, Sca-PE, CD48-PE-Cy7, and CD150-APC staining. Flow cytometry was performed at the University of Illinois at Chicago facility using FACSCalibur or CyAn flow cytometers. Cell cycle analysis with DAPI staining was performed as described previously (20). Cell cycle analysis with Hoechst 33421 was performed as described (4). For detection of apoptosis, BM cells were cultured overnight at a density of 0.2 × 10^6/ml. The next day, the cells were stained with annexin V (BD Biosciences) and 7-AAD or DAPI following the manufacturer’s instructions. Caspase 3 and caspase 9 staining was performed using kits from BD Biosciences and BioVision according to the manufacturer’s instructions. All data were analyzed by the Flowjo software (TreeStar).

Transplantation Assays—BM cells and day 14.5 fetal liver cells from C57BL/6 or BM cells from Vav-Bcl2-transgenic mice were infected with MIGR1 or β-catenin retrovirus and were transplanted into lethally irradiated (960 radians) B6.SJL mice by retro-orbital injection. After transplantation, the mice were closely monitored. FACS analysis of EGFP-positive cells in PB was performed every month after transplantation.

Mitochondrial Membrane Potential (ΔΨ\(_{\text{m}}\)) Measurement—Tetramethylrhodamine methyl ester perchlorate (TMRM) (Sigma) was used as an indicator of ΔΨ\(_{\text{m}}\). Live BM cells were washed once with PBS and stained with freshly prepared 10 μM TMRM for 30 min at 37 °C. The cells were then washed twice with PBS and suspended in PBS for flow cytometric analysis.

Western Blot Analysis—Cell extracts were prepared using lysis buffer containing 1% Triton X-100, 2 mM EDTA, 100 mM NaCl, 10% glycerol, 50 mM Tris-HCL, and a protease inhibitor mixture (Sigma). Mouse anti-β-catenin (BD Transduction Laboratories) and mouse anti-tubulin (Millipore, Billerica, MA) were used for Western blot analysis, which was performed as described previously (21).

Luciferase Reporter Assay—The luciferase assay was performed with the Dual-Glo\(^{\text{TM}}\) luciferase assay system (Promega).

Statistical Analysis—Statistical significance was calculated using the two-tailed Student’s t test (Excel, Microsoft, Redmond, WA).

RESULTS

Activation of β-Catenin Induces Apoptosis in Vivo—To determine whether in vivo activation of β-catenin induces apoptosis of HSCs/HPCs, we used the Cre-loxP system to activate β-catenin in hematopoietic stem/progenitor cells in vivo.
Activated β-Catenin Induces Apoptosis

We generated Mx1-cre<sup>+</sup> Ctnnb1<sup>Ex3fl/+</sup> mice by crossing Ctnnb1<sup>Ex3fl/+</sup> mice with transgenic mice expressing cre recombinase under control of the type I interferon-inducible Mx1 promoter. In Mx1-cre<sup>+</sup> Ctnnb1<sup>Ex3fl/+</sup> mice, the exon 3 of Ctnnb1 is flanked by loxP sites. Induction of exon 3 deletion by injection of interferon-α inducer pl-pC leads to expression of activated β-catenin. By PCR analysis, Mx1-cre<sup>+</sup> Ctnnb1<sup>Ex3fl/+</sup> mice treated with pl-pC had Cre-mediated deletion of exon 3 of Ctnnb1 in the majority of BM cells (Fig. 1A). After 4 days of treatment with three doses of pl-pC every other day, we characterized the HPCs and Lin<sup>−</sup>c-Kit<sup>−</sup>Sca<sup>−</sup><sup>−</sup> cells (Lin<sup>−</sup>LSKs, stem-enriched population) in these mice. The frequency (Fig. 1B, left panel) as well as the total number (Fig. 1B, right panel) of HPCs and LSKs were decreased 3–10-fold in BM cells from Mx1-cre<sup>+</sup> Ctnnb1<sup>Ex3Δ/+</sup> when compared with control mice. The LSKs and HPCs expressing activated β-catenin displayed a 3–4-fold increase in apoptosis when compared with control cells (Fig. 1C). Our data suggest that in vivo activation of Wnt/β-catenin by stabilized β-catenin induces apoptosis in HPCs and LSKs, leading to the depletion of these cells.

To gain insight into the molecular mechanism underlying the increased apoptosis of HPCs that express the mutant β-catenin, we examined several genes regulating the apoptotic and survival pathways. HPC cells were isolated from the Mx1-cre<sup>+</sup> Ctnnb1<sup>Ex3Δ/+</sup> and control mice 4 days after three doses of pl-pC injection. In sorted Ctnnb1-mutant HPCs, Bcl2 is down-regulated (>3-fold, Fig. 1D, black bars) whereas Casp3 is up-regulated (>3-fold, Fig. 1D, black bars) when compared with HPCs of control mice (Fig. 1D, gray bars). However, the expression of other genes involved in cell apoptosis and survival was not significantly altered.

Activation of Wnt/β-Catenin Induces Apoptosis in Hematopoietic Progenitors in Vitro—To determine whether these effects are also seen in primary BM cells expressing the active form of β-catenin (β-cat-S33Y) in vitro, we cloned β-cat-S33Y in the retroviral vector MIGR1 and expressed the protein in primary BM cells (supplemental Fig. S1A). As a control for endogenous expression of the protein at a physiological level, we used Apc<sup>−/−</sup> BM cells. Apc is an essential component of β-catenin destruction complex, and its loss leads to retention of the protein in the cytoplasm (11). Western blot analyses showed no significant difference between β-cat-S33Y-transduced Lin<sup>−</sup> cells and Apc<sup>−/−</sup> Lin<sup>−</sup> cells, indicating that the retrorval expression of β-cat is within a normal range (supplemental Fig. S1B). To confirm that β-cat-S33Y activates the Wnt/β-catenin signaling, we performed luciferase reporter assays. The Wnt signaling was stimulated by this mutant β-catenin (supplemental Fig. S1C). In addition, we found that in β-cat-S33Y-EGFP<sup>+</sup> HPCs (Lin<sup>−</sup>Kit<sup>+</sup>) or Lin<sup>−</sup> cells, the frequency of early apoptotic cells (Fig. 2A) and the level of caspase 3 (Fig. 2B) were increased significantly when compared with MIGR1-transduced HPCs and Lin<sup>−</sup> cells.
Activated β-Catenin Induces Apoptosis

The Wnt/β-catenin pathway can be activated by the addition of LiCl, which inhibits GSK-3β, a component of the degradation complex, thereby stabilizing β-catenin. In methylcellulose-based medium, treatment of BM cells with LiCl, but not with equimolar amounts of NaCl, repressed colony formation (supplemental Fig. S2A), and the number of early apoptotic BM cells was significantly increased (supplemental Fig. S2B). Similarly, in liquid culture, the early apoptotic Lin- c-Kit+ progenitor cells (Fig. 3A) and progenitor cells with an elevated caspase 3 activity (Fig. 3B) were increased significantly after LiCl treatment when compared with NaCl treatment. The frequency of apoptosis was increased more significantly at high concentration of LiCl than at low concentration of LiCl, suggesting that LiCl-induced apoptosis is dosage-dependent (Fig. 3A).

Indomethacin is a Cox inhibitor that blocks the Wnt signaling pathway by repressing Ctnmb (22). To confirm that the effects of LiCl are dependent on β-catenin, we treated HPCs with LiCl alone or with indomethacin. The results showed that LiCl-induced apoptosis is reduced by indomethacin (supplemental Fig. S3). Next, we isolated BM cells from β-catenin−/− mice in which exons 2–6 can be excised by Cre expression (23) with LiCl alone or with indomethacin. The results showed that LiCl treatment resulted in a higher activity of caspase 9 than what was observed in the control HPCs (supplemental Fig. S5). The proper function of the mitochondria was tested with TMRM, which detects the mitochondrial membrane potential (ΔΨm). HPCs treated with LiCl and TMRM showed a significant drop in fluorescence with increasing concentration of LiCl (Fig. 4B, black bars), indicating that a loss of ΔΨm occurred in the LiCl-treated HPCs. To determine whether expression of Bcl2 protects HPCs from apoptosis, Lin− BM cells isolated from Mx1-Cre CreERT2 CreERT2 mice were infected with MSCV-puro and MSCV-EYFP, MSCV-puro-Cre and MSCV-EYFP, or MSCV-puro-Cre and MSCV-EYFP-Bcl2. The excision of exon 3 of Ctnmb1 in Mx1-cro-Ctnmb1CreERT2BM cells was induced by Cre recombinase expressed in a retroviral vector. As we expected, Lin− cells expressing activated β-catenin (Fig. 4C, middle bar) displayed a frequency of apoptosis higher than the control cells in vitro. However, Bcl2 could repress apoptosis (Fig. 4C, right bar). To confirm the role of Bcl2 in blocking apoptosis induced by activated β-catenin, Lin− BM cells, isolated from Vav-Bcl2 mice, were infected with the β-catenin-S33Y or the MIGR1 retrovirus. Flow cytometric analysis of the cells revealed that the frequency of annexin V+ cells and the activity of caspase 3 were slightly lower in β-catenin-S33Y-positive Lin− BM cells than in MIGR1-transduced Lin− BM cells (Fig. 4, D and E) and that LiCl treatment did not affect apoptosis in HPCs that expressed the Bcl2 transgene (Fig. 4F). Together, our data suggest that Bcl2 can reduce apoptosis induced by activated β-catenin.

A Bcl2 Transgene Rescues the Defects of HSCs/HPCs Expressing β-catenin-S33Y—We hypothesized that apoptosis induced by activated β-catenin contributes to the defective function of HSCs/HPCs with active Wnt/β-catenin signaling. If this is the case, then Bcl2 expression should rescue the function of these HSCs/HPCs. Therefore, we isolated CD45.2+ BM cells from C57BL/6 donors 4 days after treatment with 5-fluorouracil; these cells and the BM cells were donor-derived (CD45.2+). More than 90% of the PB cells from the recipients were CD45.2+ BM cells infected with MSCV-puro-Cre and MSCV-EYFP-Bcl2. The excision of exon 3 of Ctnmb1 in Mx1-cro-Ctnmb1CreERT2BM cells was induced by Cre recombinase expressed in a retroviral vector. As we expected, Lin− cells expressing activated β-catenin (Fig. 4C, middle bar) displayed a frequency of apoptosis higher than the control cells in vitro. However, Bcl2 could repress apoptosis (Fig. 4C, right bar). To confirm the role of Bcl2 in blocking apoptosis induced by activated β-catenin, Lin− BM cells, isolated from Vav-Bcl2 mice, were infected with the β-catenin-S33Y or the MIGR1 retrovirus. Flow cytometric analysis of the cells revealed that the frequency of annexin V+ cells and the activity of caspase 3 were slightly lower in β-catenin-S33Y-positive Lin− BM cells than in MIGR1-transduced Lin− BM cells (Fig. 4, D and E) and that LiCl treatment did not affect apoptosis in HPCs that expressed the Bcl2 transgene (Fig. 4F). Together, our data suggest that Bcl2 can reduce apoptosis induced by activated β-catenin.

FIGURE 2. Expression of β-catenin-S33Y in BM cells induces apoptosis in vitro. A, the fraction of apoptotic Lin− c-Kit+ cells expressing MIGR1 (left panel) or MIGR1-β-catenin-S33Y (right panel) was determined by FACs. The frequency of early apoptotic cells (annexin V+DAPI− or annexin V−7-AAD−) is shown (three independent experiments; mean ± S.D.; *, p < 0.05; **, p < 0.01). B, the activity of caspase 3 in primary progenitor-enriched cells (Lin−) expressing MIGR1 (left panel) or β-catenin-S33Y (right panel). The percentage of cells with active caspase 3 is shown (three independent experiments, mean ± S.D.; *, p < 0.05). All analyses were performed on a gated EGFP+ cell population.

Is Inhibited by Bcl2—There are two major apoptotic pathways, the mitochondrial “intrinsic” and transmembrane “extrinsic” pathway. To determine whether the mitochondrial apoptotic pathway is activated by Wnt signaling, we examined the activity of caspase 9 in HPCs that expressed β-catenin-S33Y or that were treated with LiCl. We found that caspase 9 was activated in β-catenin-positive HPCs (Fig. 4A, black bar) and that LiCl treatment resulted in a higher activity of caspase 9 than what was observed in the control HPCs (supplemental Fig. S5). The proper function of the mitochondria was tested with TMRM, which detects the mitochondrial membrane potential (ΔΨm). HPCs treated with LiCl and TMRM showed a significant drop in fluorescence with increasing concentration of LiCl (Fig. 4B, black bars), indicating that a loss of ΔΨm occurred in the LiCl-treated HPCs. To determine whether expression of Bcl2 protects HPCs from apoptosis, Lin− BM cells isolated from Mx1-Cre CreERT2 CreERT2 mice were infected with MSCV-puro and MSCV-EYFP, MSCV-puro-Cre and MSCV-EYFP, or MSCV-puro-Cre and MSCV-EYFP-Bcl2. The excision of exon 3 of Ctnmb1 in Mx1-cro-Ctnmb1CreERT2BM cells was induced by Cre recombinase expressed in a retroviral vector. As we expected, Lin− cells expressing activated β-catenin (Fig. 4C, middle bar) displayed a frequency of apoptosis higher than the control cells in vitro. However, Bcl2 could repress apoptosis (Fig. 4C, right bar). To confirm the role of Bcl2 in blocking apoptosis induced by activated β-catenin, Lin− BM cells, isolated from Vav-Bcl2 mice, were infected with the β-catenin-S33Y or the MIGR1 retrovirus. Flow cytometric analysis of the cells revealed that the frequency of annexin V+ cells and the activity of caspase 3 were slightly lower in β-catenin-S33Y-positive Lin− BM cells than in MIGR1-transduced Lin− BM cells (Fig. 4, D and E) and that LiCl treatment did not affect apoptosis in HPCs that expressed the Bcl2 transgene (Fig. 4F). Together, our data suggest that Bcl2 can reduce apoptosis induced by activated β-catenin.

A Bcl2 Transgene Rescues the Defects of HSCs/HPCs Expressing β-catenin-S33Y—We hypothesized that apoptosis induced by activated β-catenin contributes to the defective function of HSCs/HPCs with active Wnt/β-catenin signaling. If this is the case, then Bcl2 expression should rescue the function of these HSCs/HPCs. Therefore, we isolated CD45.2+ BM cells from C57BL/6 donors 4 days after treatment with 5-fluorouracil; these cells and the BM cells were donor-derived (CD45.2+). More than 90% of the PB cells from the recipients were donor-derived (CD45.2+), indicating a successful reconstruction (data not shown). Five weeks after transplantation, the percentage of EGFP+ cells in total BM cells was determined by flow cytometry. Before transplantation (in vitro) and 5 weeks after transplantation, in the control mice, the percentage of MIGR1-transduced BM cells was comparable (Fig. 5A, gray bars). In contrast, before transplantation, the percentage of β-catenin-S33Y-EGFP-positive cells was about 14%, but the cells were hardly detected in the recipient mice at 5 weeks after transplantation (Fig. 5A, black bars). To confirm these results, we infected day 14.5 fetal liver cells with β-catenin-S33Y and MIGR1 retroviruses followed by transplantation. The percentage of MIGR1-transduced fetal liver cells was comparable.
Activated β-Catenin Induces Apoptosis

FIGURE 3. β-Catenin mediates the induction of HPC apoptosis after LiCl treatment. A, early apoptotic cells (annexin V–DAPI−) of Lin−Kit+ BM cells treated with increasing concentration of NaCl or LiCl (three independent experiments; mean ± S.D.; * p < 0.05) for 24 h. Black bars indicate cells treated with NaCl and used as controls; gray bars indicate cells treated with LiCl. B, Lin− BM cells were treated with 15 mM NaCl or 15 mM LiCl for 24 h. The histogram depicts the mean percentage of cells with active caspase 3 (three independent experiments; mean ± S.D.) ** p < 0.01. C, the frequency of early apoptotic Lin− Kit+ BM cells isolated from β-catenin−/− mice with expression of MIGR1 (gray bars) or EYFP-Cre to excise β-catenin (black bars). The BM cells were treated with 10 mM LiCl for 24 h (three independent experiments; mean ± S.D.; *, p < 0.05).

FIGURE 4. Activation of the Wnt/β-catenin pathway initiates the mitochondria-dependent apoptotic program, which can be inhibited by Bcl2. A, the histogram shows the quantification of active caspase 9 in control cells (gray bar) and in cells expressing β-cas -S33Y (black bar). The Lin− kit+ BM cells were infected with MIGR1 or β-cas -S33Y retrovirus. The analysis was performed on gated EGFP+ cells. The results are from three independent experiments (mean ± S.D.; ** p < 0.01). B, the histogram depicts the mean percentage of TMRM-positive cells in three independent experiments (mean ± S.D.; ** p < 0.01). The gated Lin−/Th−Kit− BM cells treated with NaCl and LiCl for 18 h and stained with TMRM were analyzed by flow cytometry. C, flow cytometric analysis of the early apoptotic cells (annexin V−7-AAD+) in Mx1−cre β-catenin−/− BM cells, co-infected with MSCV-puro and MSCV-EYFP (left bar), with MSCV-puro-Cre and MSCV-EYFP (middle bar), or with MSCV-puro-Cre and MSCV-EYFP-Bcl2 (right bar). The EGFP+ cells were analyzed after 7 days of selection in medium containing 1 μg/ml puromycin (mean ± S.D.; *, p < 0.05). D and E, flow cytometric analysis of early apoptotic cells (annexin V−7-AAD+) in Bcl2 transgenic BM cells of Bcl2 transgenic β-catenin−/− mice. F, the frequency of early apoptotic BM cells isolated from Bcl2 transgenic BM cells infected with MIGR1 (gray bars) or EGFP-Cre (black bars). The BM cells were treated with 10 mM LiCl for 24 h (three independent experiments; mean ± S.D.; *, p < 0.05).
DISCUSSION

Whether β-catenin acts as a proapoptotic factor in hematopoietic cells has not been conclusively defined. Previous studies revealed that expression of activated β-catenin did not alter the frequency of apoptosis in HSC population (9, 10). However, Li and colleagues (25) showed that activated β-catenin enhances apoptosis of HSCs in vitro. The goal of this study was to help to solve, if possible, the existing discrepancies on β-catenin in HSCs/HPCs. Our results include strong data, obtained in vitro and in vivo, which support the apoptotic effect that this protein exerts on HPCs. In addition, our results show the molecular mechanisms by which this occurs and indicate that regulation...
of survival by β-catenin is critical for the maintenance of functional HSCs/HPCs.

By using an Mx1-cre/loxP system to induce the deletion of exon 3 of Ctnnb1 gene, leading to activated β-catenin in vivo, we showed that a significant decrease in the number of HPCs and LSKs and a markedly increased frequency of apoptosis occur in these cells. Consistently, Cre-dependent activation of β-catenin in Ctnnb1fl(fl)fl/H9252 HPCs enhances apoptosis in vitro. The result was confirmed by retroviral expression of activated β-cat-S33Y in HPCs. However, the frequency of apoptosis is induced by activated β-catenin more significantly in vivo than it is in vitro. In our opinion, it is likely that the in vivo microenvironment could contribute to the survival of HPCs. Bcl2 is a survival factor, and ectopic expression of Bcl2 prevents apoptosis in HSCs (26). Caspase 3 plays a central role in the execution of cell apoptosis (27). Down-regulation of Bcl2 and induced expression of Casp3 occur in Ctnnb1 mutant HPCs in vivo. The expression of p53 is not altered in the mutant HPCs (Fig. 1D). Thus, increased apoptosis in Ctnnb1 mutant HPCs may be a consequence of the combined deregulation of Bcl2, Casp3, and perhaps other genes involved in apoptosis and survival of HPCs, which are still unidentified and which could be regulated directly or indirectly by β-catenin. Our in vitro study suggests that activation of Wnt/β-catenin signaling activates caspase 3 and caspase 9 and reduces the mitochondrial membrane potential, leading to a mitochondria-mediated apoptosis. We previously showed that loss of Apc induces apoptosis in HSCs/HPCs (19). Apc is a multifunction, cell context-dependent protein (28), and it is still unclear whether β-catenin is a critical function mediator of Apc. Our data suggest that β-catenin is likely to mediate, at least in part, the function of Apc in regulating the survival of HPCs.

Although previous studies suggested that the retroviral expression of active β-catenin in Bcl2 transgenic HSCs enhanced their self-renewal, there was no indication on the potential pathways utilized by β-cat-S33Y (4). It was therefore unclear whether the effects of β-catenin on self-renewal depended on Bcl2 expression, and the mechanism by which Bcl2 cooperates with β-catenin in maintenance of HSCs/HPCs remained not well defined. In this study, we show that retroviral expression of active β-catenin disrupts the reconstitution capacity of HSCs/HPCs but that Bcl2 transgenic HSCs/HPCs expressing activated β-catenin maintain their reconstitution capacity. We further show that a Bcl2 transgene inhibits the apoptosis induced by activated β-catenin in vitro and prevents the exhaustion of HSCs/HPCs in vivo. We believe that taken together, our studies clarify the existing contradictory data on the effects of activated β-catenin in HSCs/HPCs not only by supporting the notion that activation of Wnt/β-catenin negatively impacts the function of HSCs/HPCs, but more importantly, by strongly indicating that an enhanced survival signal is required for the survival of these cells. It is very likely that regulation of the function of HSCs by Wnt/β-catenin is dosage-dependent (29). Our study suggests that the negative impact of Wnt/β-catenin in HSCs/HPCs is due, at least in part, to an increased apoptosis induced by high dosage of Wnt signaling.

Finally, the activation of Wnt/β-catenin signaling and the overexpression of Bcl2 have been frequently associated with acute myeloid leukemia (30–34). Our data suggest that Wnt/β-catenin may cooperate with survival signaling in promoting the self-renewal or proliferation of leukemia stem/progenitor cells in some acute myelogenous leukemia patients.

REFERENCES

1. Cobas, M., Wilson, A., Ernst, B., Mancini, S. J., MacDonald, H. R., Klemper, R., and Radtke, F. (2004) β-Catenin is dispensable for hematopoiesis and lymphopoiesis. J. Exp. Med. 199, 221–229
2. Jeannet, G., Scheller, M., Scarpellino, L., Duboux, S., Gardiol, N., Back, J., Kuttler, F., Malanchi, I., Birchmeier, W., Leutz, A., Huesken, J., and Held, W. (2008) Long-term, multilineage hematopoiesis occurs in the combined absence of β-catenin and γ-catenin. Blood 111, 142–149
3. Zhao, C., Blum, J., Chen, A., Kwon, H. Y., Jung, S. H., Cook, J. M., Lagoo, A., and Reya, T. (2007) Loss of β-catenin impairs the renewal of normal and CML stem cells in vivo. Cancer Cell 12, 528–541
4. Reya, T., Duncan, A. W., Ailles, L., Domen, J., Scherer, D. C., Willert, K., Hintz, L., Nusse, R., and Weissman, I. L. (2003) A role for Wnt signaling in self-renewal of hematopoietic stem cells. Nature 423, 409–414
5. Willert, K., Brown, J. D., Danenberg, E., Duncan, A. W., Weissman, I. L., Reya, T., Yates, J. R., 3rd, and Nusse, R. (2003) Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature 423, 448–452
6. Trowbridge, J. I., Xenocostas, A., Moon, R. T., and Bhatia, M. (2006) Glycogen synthase kinase-3 is an in vivo regulator of hematopoietic stem cell repopulation. Nat. Med. 12, 89–98
7. Baba, Y., Barrett, J., Taniguchi, T., and Kinde, P. W. (2005) Constitutively active β-catenin confers multilineage differentiation potential on lymphoid and myeloid progenitors. Immunity 23, 599–609
8. Baba, Y., Yokota, T., Spits, H., Garrett, K. P., Hayashi, S., and Kinde, P. W. (2006) Constitutively active β-catenin promotes expansion of multipotent hematopoietic progenitors in culture. J. Immunol. 177, 2294–2303
9. Kastner, P., Anderson, K., Porse, B. T., Jacobsen, S. E., and Nerlov, C. (2007) Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. Nat. Immunol. 7, 1048–1056
10. Scheller, M., Huelsen, J., Rosenbauer, F., Taketo, M. M., Birchmeier, W., Tenen, D. G., and Leutz, A. (2006) Hematopoietic stem cell and multilineage defects generated by constitutive β-catenin activation. Nat. Immunol. 7, 1037–1047
11. He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998) Identification of c-MYC as a target of the APC pathway. Science 281, 1509–1512
12. Tetsu, O., and McCormick, F. (1999) β-Catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 398, 422–426
13. Wong, M. H., Rubinfeld, B., and Gordon, J. I. (1998) Effects of forced expression of an NH2-terminal truncated β-catenin on mouse intestinal epithelial homeostasis. J. Cell Biol. 141, 765–777
14. Kim, K., Pang, K. M., Evans, M., and Hay, E. D. (2000) Overexpression of β-catenin induces apoptosis independent of its transcriptional function with LEF-1 or the involvement of major G1 cell cycle regulators. Mol. Biol. Cell 11, 3509–3523
15. Raab, M. S., Breitkreutz, I., Tomon, G., Zhang, J., Hayden, P. J., Nguyen, T., Fruehauf, J. H., Lin, B. K., Chauhan, D., Hideshima, T., Munshi, N. C., Anderson, K. C., and Podar, K. (2009) Enhanced expression of FHL2 leads to abnormal myelopoiesis and leukemic transformation of a myelomonocytic precursor by retinoic acid. Cancer Res. 69, 8560–8569
16. Harada, N., Tamai, Y., Ishikawa, T., Sauer, B., Takaku, K., Oshima, M., and Taketo, M. M. (1999) Intestinal polyposis in mice with a dominant stable mutation of the β-catenin gene. EMBO J. 18, 5931–5942
17. Qian, Z., Mao, L., Fernald, A. A., Yu, H., Luo, R., Jiang, Y., Anastasi, J., Valk, P. J., Delwel, R., and Le Beau, M. M. (2009) Enhanced expression of HFL2 leads to abnormal myelopoiesis in vivo. Leukemia 23, 1653–1657
18. Lavau, C., Szilvassy, S. J., Slany, R., and Cleary, M. L. (1997) Immortalization and leukemic transformation of a myelomonocytic precursor by retrovirally transduced HRX-ENL. EMBO J. 16, 4226–4237
19. Qian, Z., Chen, L., Fernald, A. A., Williams, B. O., and Le Beau, M. M. (2008) A critical role for Apc in hematopoietic stem and progenitor cell
Activated β-Catenin Induces Apoptosis

20. Zhang, J., Grindley, J. C., Yin, T., Jayasinghe, S., He, X. C., Ross, J. T., Haug, J. S., Rupp, D., Porter-Westpfahl, K. S., Wiedemann, L. M., Wu, H., and Li, L. (2006) PTEN maintains hematopoietic stem cells and acts in lineage choice and leukemia prevention. *Nature* **441**, 518–522

21. Qian, Z., Okuhara, D., Abe, M. K., and Rosner, M. R. (1999) Molecular cloning and characterization of a mitogen-activated protein kinase-associated intracellular chloride channel. *J. Biol. Chem.* **274**, 1621–1627

22. Goessling, W., North, T. E., Loewer, S., Lord, A. M., Lee, S., Stoick-Cooper, C. L., Weidinger, G., Puder, M., Daley, G. Q., Moon, R. T., and Zon, L. I. (2009) Genetic interaction of PGE2 and Wnt signaling regulates developmental specification of stem cells and regeneration. *Cell* **136**, 1136–1147

23. Brault, V., Moore, R., Kutsch, S., Ishibashi, M., Rowitch, D. H., McMahon, A. P., Sommer, L., Boussadia, O., and Kemler, R. (2001) Inactivation of the β-catenin gene by Wnt1-Cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development. *Development* **128**, 1253–1264

24. Ogilvy, S., Metcalf, D., Print, C. G., Bath, M. L., Harris, A. W., and Adams, J. M. (1999) Constitutive Bcl-2 expression throughout the hematopoietic compartment affects multiple lineages and enhances progenitor cell survival. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 14943–14948

25. Perry, J. M., He, X. C., Sugimura, R., Grindley, J. C., Haug, J. S., Ding, S., and Li, L. (2011) Cooperation between both Wnt/β-catenin and PTEN/PI3K/Akt signaling promotes primitive hematopoietic stem cell self-renewal and expansion. *Genes Dev.* **25**, 1928–1942

26. Domen, J., and Weissman, I. L. (2000) Hematopoietic stem cells need two signals to prevent apoptosis; BCL-2 can provide one of these, Kitl/c-Kit signaling the other. *J. Exp. Med.* **192**, 1707–1718

27. Porter, A. G., and Jánicek, R. U. (1999) Emerging roles of caspase-3 in apoptosis. *Cell Death Differ.* **6**, 99–104

28. Fodde, R., Smits, R., and Clevers, H. (2001) APC, signal transduction, and genetic instability in colorectal cancer. *Nat. Rev. Cancer* **1**, 55–67

29. Luis, T. C., Naber, B. A., Roozen, P. B., Brugman, M. H., de Haas, E. F., Ghazvini, M., Fibbe, W. E., van Dongen, J. J., Fodde, R., and Staal, F. J. (2011) Canonical Wnt signaling regulates hematopoiesis in a dosage-dependent fashion. *Cell Stem Cell* **9**, 345–356

30. Chang, E. J., Hwang, S. G., Nguyen, P., Lee, S., Kim, J. S., Kim, J. W., Henkart, P. A., Bottaro, D. P., Soon, L., Bonvini, P., Lee, S. J., Karp, J. E., Oh, H. J., Rubin, J. S., and Trepel, J. B. (2002) Regulation of leukemic cell adhesion, proliferation, and survival by β-catenin. *Blood* **100**, 982–990

31. Simon, M., Grandage, V. L., Linch, D. C., and Khwaja, A. (2005) Constitutive activation of the Wnt/β-catenin signaling pathway in acute myeloid leukemia. *Oncogene* **24**, 2410–2420

32. Tickenbrock, L., Schwäble, J., Wiedehage, M., Steffen, B., Sargin, B., Choudhary, C., Brands, C., Berdel, W. E., Müller-Tidow, C., and Serve, H. (2005) Flt3 tandem duplication mutations cooperate with Wnt signaling in leukemic signal transduction. *Blood* **105**, 3699–3706

33. Ysebaert, L., Chicanne, G., Demur, C., De Toni, F., Prade-Houdellier, N., Ruidavets, J. B., Mansat-De Mas, V., Rigal-Huguet, F., Laurent, G., Payrastre, B., Manenti, S., and Racaud-Sultan, C. (2006) Expression of β-catenin by acute myeloid leukemia cells predicts enhanced clonogenic capacities and poor prognosis. *Leukemia* **20**, 1211–1216

34. Del Principe, M. I., Del Poeta, G., Venditti, A., Bussisano, F., Maurillo, L., Mazzone, C., Bruno, A., Neri, B., Inno Consalvo, M., Lo Coco, F., and Amadori, S. (2005) Apoptosis and immaturity in acute myeloid leukemia. *Hematology* **10**, 25–34