Wnt5a inhibits canonical Wnt signaling in hematopoietic stem cells and enhances repopulation

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The mechanisms that regulate hematopoietic stem cell (HSC) fate decisions between proliferation and multilineage differentiation are unclear. Members of the Wnt family of ligands that activate the canonical Wnt signaling pathway, which utilizes β-catenin to relay the signal, have been demonstrated to regulate HSC function. In this study, we examined the role of noncanonical Wnt signaling in regulating HSC fate. We observed that noncanonical Wnt5a inhibited Wnt3a-mediated canonical Wnt signaling in HSCs and suppressed Wnt3a-mediated alterations in gene expression associated with HSC differentiation, such as increased expression of *myc*. Wnt5a increased short- and long-term HSC repopulation by maintaining HSCs in a quiescent G_0 state. From these data, we propose that Wnt5a regulates hematopoiesis by the antagonism of the canonical Wnt pathway, resulting in a pool of quiescent HSCs.

cell cycle | hematopoiesis | hematopoietic stem cell transplantation

Hematopoietic stem cells (HSC) are a rare population of cells that are responsible for life-long generation of all blood cell types (1). To maintain their numbers, HSCs must generate at least one daughter cell that retains the stem cell phenotype. HSCs are capable of rapid proliferation after transplantation and are the only cells capable of both short- (6–8 weeks) and long-term hematopoietic engraftment. The mechanisms that regulate HSC fate between proliferation and differentiation are still unclear, but understanding these mechanisms and developing strategies to manipulate them are imperative for multiple therapeutic goals, such as corrective gene therapy for hematologic disorders.

The Wnt signaling pathway regulates cell-fate decisions at all stages of development in multiple tissues, including embryonic and adult intestinal and skin stem cells (2–5). There are multiple pathways by which Wnt ligands transduce signals, the best characterized of which is the "canonical" pathway (reviewed in ref. 2). In this pathway, the Wnt ligand binds to its cognate receptor Frizzled and the low-density lipoprotein receptor-related protein (LRP) 5/6 coreceptors at the cell surface, resulting in the inhibition of glycogen synthase kinase-3β (GSK-3β). One of the targets of this kinase is β-catenin, the critical factor in relaying canonical Wnt signals. In the absence of Wnt ligand, GSK-3β phosphorylates β-catenin, which targets it for ubiquitination and subsequent degradation. However, when Wnt ligand is bound to its receptors, degradation of β-catenin is suppressed, and β-catenin can translocate to the nucleus where it binds to T cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factors and induces target gene expression. Inappropriate activation of the pathway has been implicated in tumorigenesis; therefore it is necessary that canonical Wnt signaling be precisely controlled [reviewed in (3)].

Reya *et al.*, showed that recombinant Wnt3a could activate canonical Wnt signaling in HSCs and promote *in vitro* expansion and self-renewal of apoptosis-resistant transgenic HSCs (4). Activation of the canonical Wnt signaling pathway has also been demonstrated to promote self-renewal of leukemia stem cells (5). HSCs deficient in the chromatin-binding protein Hmgb3 exhibit increased canonical Wnt signaling which correlated with a bias toward self-renewal (6). These studies suggest that the canonical Wnt signaling pathway plays a critical role in hematopoiesis. However, HSCs deficient in β-catenin possess the same capacity for hematopoietic repopulation as wild-type HSCs (7). Furthermore, long-term constitutive stabilization of β-catenin leads to inhibition of multilineage differentiation and the eventual loss of HSCs (8, 9), indicating that the context in which canonical Wnt signaling is activated may determine the physiological effect and that other factors contribute to regulate canonical Wnt signaling to properly maintain stem cell numbers.

Some members of the Wnt ligand family (e.g., Wnt5a) can activate signaling pathways other than the canonical pathway, depending on which receptors are present at the cell surface (10–13). It has been shown that noncanonical Wnt ligands can inhibit canonical Wnt signaling in transformed cell lines and in *Xenopus* and mouse embryos (10, 14, 15). The mechanism of Wnt5a-mediated inhibition of the canonical pathway is unclear. Wnt5a has been shown to induce *Siah2*, a member of an E3 ubiquitin ligase complex that can target β-catenin for degradation, and *Wnt5a*-deficient mice exhibit increased levels of β-catenin in the distal hind limb (15). However, loss of *Wnt5a* did not affect β-catenin levels in fetal liver cells (11). Wnt5a can also inhibit canonical Wnt signaling downstream of β-catenin stabilization through the calcium-dependent activation of Nemo-like kinase (12) as well as other calcium-independent mechanisms (10).

The canonical *Wnt3a* is expressed in bone marrow mononuclear cells (16). *Wnt5a* is expressed in both fetal liver and bone marrow cells, especially $B220^+$ B cells, and deficiency in *Wnt5a* leads to a cell-autonomous increase in B cell numbers (11, 16–18). *Wnt5a* is also expressed in cultured primary bone marrow stromal cells. In theory, both Wnt3a and Wnt5a can regulate hematopoietic cells by autocrine and paracrine mechanisms. We hypothesized that Wnt5a contributes to the regulation of HSC function through suppression of canonical Wnt signaling. To test this hypothesis, we cultured HSCs under serum-free conditions in the presence of recombinant Wnt3a and/or Wnt5a. We observed that Wnt5a either alone or in combination with Wnt3a, enhanced short- and long-term hematopoietic repopulation of donor HSCs, whereas Wnt3a did not enhance repopulation of HSCs. Wnt5a suppressed changes in gene expression associated with Wnt3a and maintained HSCs in

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Abbreviations: HSC, hematopoietic stem cell; LKSI, lineage-negative, c-kit^{HI}, Sca-1^{HI}, IL-7Rα^-.

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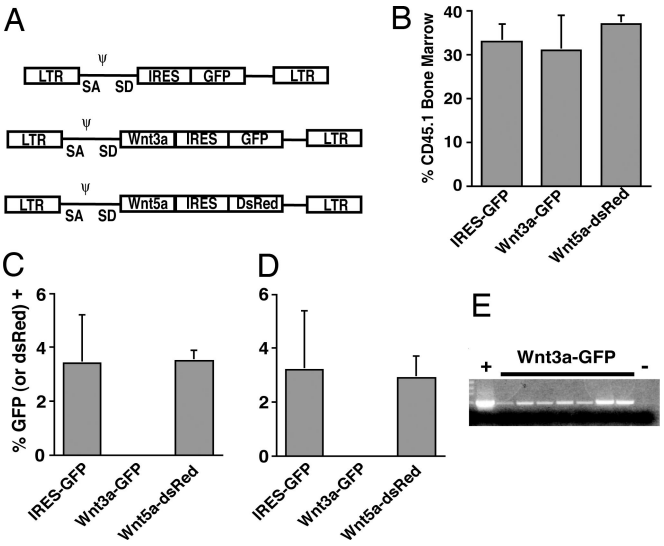

Fig. 1. Enforced expression of Wnt ligands in bone marrow. (*A*) Diagram of the control MSCV MGirL22Y IRES-GFP vector (*Top*), the Wnt3a-IRES-GFP vector (*Middle*), and the Wnt5a-IRES-DsRed vector (*Bottom*). (*B*) Average repopulation of whole bone marrow by CD45.1 bone marrow transduced with either the control vector ($n = 9$), Wnt3a-IRES-GFP ($n = 7$), or Wnt5a-IRES-DsRed ($n = 8$). FACS analysis of whole recipient whole bone marrow was performed 16 weeks after transplant. Data represent the pooled results of two independent experiments. Error bars in *B–D* represent standard deviation, and *P* values were generated by Student's *t* test. (*C*) Average percentage of CD45.1$^+$ bone marrow cells positive for GFP (control and Wnt3a vectors) or DsRed (Wnt5a vector). (*D*) Average percentage of CD45.1$^+$ lin$^-$ bone marrow cells positive for GFP (control and Wnt3a vectors) or DsRed (Wnt5a vector). (*E*) PCR was used to amplify a fragment from the GFP cDNA in recipients of bone marrow transduced by Wnt3a-IRES-GFP. PCR plasmid was used as a positive control (+), and DNA isolated from wild-type bone marrow was used as a negative control (−).

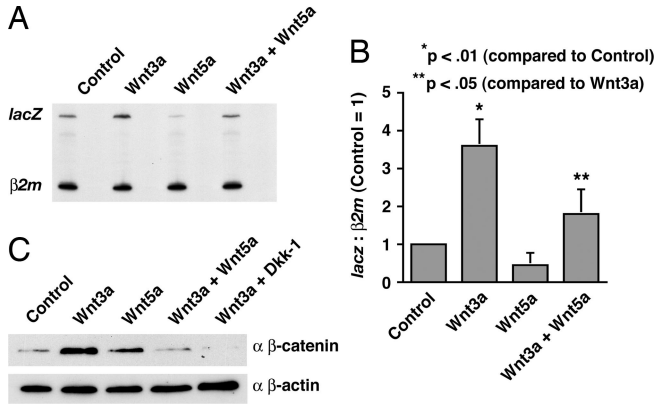

Fig. 2. Inhibition of canonical Wnt signaling in HSCs by Wnt5a. (*A*) Semi-quantitative duplex RT-PCR analysis of *lacZ* mRNA in TOPGAL LKSI cells after a 48-h *in vitro* culture as described in *Materials and Methods*. *LacZ* mRNA levels were quantified within the linear range of amplification by densitometry and normalized to *β2m* expression. (*B*) Average *lacZ* mRNA levels relative to *β2m*. The amount of *lacZ* mRNA in TOPGAL LKSI cells was normalized to *β2m* mRNA (*β2*) by the formula (densitometry value: *lacZ*/densitometry value: *β2m*) ($n = 3$ for all groups). The normalized TOPGAL *lacZ* mRNA level in control LKSI cells was set to 1. The data represent the average results of three independent experiments. Error bars represent standard deviation. *P* values were generated by Student's *t* test. (*C*) Representative Western blot analysis of *β*-catenin levels in lin$^-$ cells after a 48-h *in vitro* culture. The experiment was performed three times with independent cultures.

a quiescent state. Our data suggest a model in which Wnt5a-mediated regulation of canonical Wnt signaling is critical for maintenance of quiescent, transplantable HSCs.

Results

Effect of Enforced Expression of Wnt3a and Wnt5a. To test our model of paracrine function, we transduced stimulated bone marrow cells (CD45.1) with bicistronic retroviral vectors containing Wnt3a (Wnt3a-IRES-GFP) or Wnt5a (Wnt5a-IRES-dsRed) cDNA or a control IRES-GFP cassette. (Fig. 1*A*). Sorted GFP$^+$ or DsRed$^+$ cells were then transplanted along with an equal dose of mock-transduced bone marrow cells (CD45.2) into lethally irradiated recipients. At 16 weeks, we observed no differences in long-term repopulation of bone marrow by donor cells transduced by all three vectors (Fig. 1*B*). This indicates that selection of *ex vivo*-transduced cells based on vector expression did not affect their overall repopulating activity. However, when we analyzed either whole bone marrow (Fig. 1*C*) or lin$^-$ cells (Fig. 1*D*) for cells that expressed the retroviral vector, we observed only control IRES-GFP$^+$ and Wnt5a-IRES-DsRed$^+$ cells. No cells with the Wnt3a-IRES-GFP retrovirus expressed GFP.

Wnt3a-IRES-GFP proviral DNA was present in bone marrow genomic DNA from all recipients (Fig. 1*E*), indicating integration of the vector into HSCs. Together, these data suggest that long-term enforced expression of Wnt3a did not lead to autocrine expansion of HSCs. The absence of GFP$^+$ cells in the mature bone marrow may be due to several mechanisms, including silencing of the Wnt3a retrovirus or a selective disadvantage for long-term repopulation by HSCs transduced by the Wnt3a retrovirus. Our observation that the Wnt5a retrovirus has no effect may be due to the inability of Wnt5a to act through autocrine mechanisms. However, because we have also observed that the bone marrow stroma contains ≈20-fold more *Wnt5a* mRNA than bone marrow mononuclear cells (data not shown), an alternative explanation is that potential autocrine effects of Wnt5a cannot be distinguished from the background of total Wnt5a-derived signals.

Wnt5a Inhibits Activation of Canonical Wnt Signaling. We then tested our hypothesis that Wnt5a would activate noncanonical signaling in HSCs, resulting in inhibition of the canonical Wnt pathway. We cultured primary lineage-negative, c-kit^{HI}, Sca-1^{HI}, IL-7Rα^- (LKSI) cells, which are enriched for HSCs (19), in serum-free media containing stem cell factor (SCF), Fms-like tyrosine kinase 3 ligand (Flt3L), and 100 ng/ml recombinant Wnt3a and/or 500 ng/ml recombinant Wnt5a. We isolated LKSI cells from the TOPGAL mouse, which contains a *lacZ* reporter gene that is specifically expressed when the canonical Wnt pathway is activated. The doses of Wnt3a and Wnt5a were determined in dose–response assays [supporting information (SI) Fig. 7]. We also observed no difference in the biological activity of recombinant Wnt3a with Wnt3a-conditioned medium (SI Fig. 8). We observed that, as expected, recombinant Wnt3a, but not Wnt5a, significantly increased *lacZ* expression (3.6 ± 0.7-fold, $P < 0.01$) (Fig. 2 *A* and *B* and SI Fig. 8). Wnt5a was able to significantly inhibit canonical Wnt signaling induced by Wnt3a by 50% ($P < 0.05$), indicating that Wnt3a and Wnt5a have antagonistic effects on the canonical Wnt pathway in HSCs *in vitro*.

Wnt5a inhibition of canonical Wnt signaling has been described in other cell types and systems (10, 14, 15). To begin to determine the mechanism by which Wnt5a can inhibit canonical Wnt signaling in primitive hematopoietic cells, we performed Western blot analysis to determine the levels of *β*-catenin protein after culture with Wnt3a and Wnt5a (Fig. 2*C*). The data from three independent experiments showed that, as expected, culturing primitive hematopoietic lin$^-$ progenitors with Wnt3a increased total *β*-catenin compared with control, whereas Wnt5a did not, although there was some variability in *β*-catenin levels in the presence of Wnt5a. These data, in combination with that

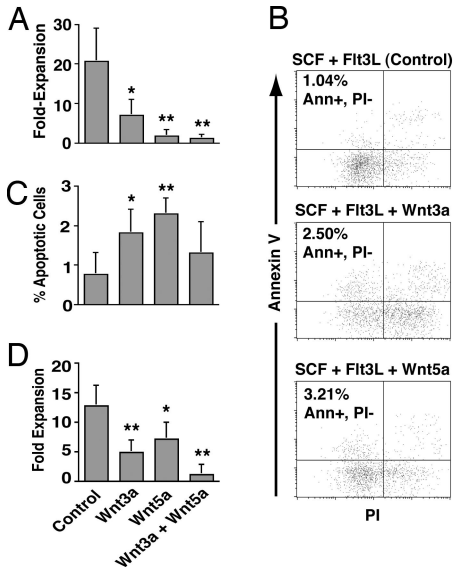

Fig. 3. Analysis of Wnt-mediated HSC expansion and apoptosis. (*A*) Average fold expansion of wild-type LKSI cells after 4 days in culture ($n = 3$ for all conditions). Error bars represent standard deviation. *P* values were generated by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$). (*B*) Representative FACS analyses of apoptotic wild-type LKSI cells cultured for 4 days under control conditions (*Top*), with recombinant Wnt3a (*Middle*), and recombinant Wnt5a (*Bottom*). Actively apoptotic cells actively were defined as Annexin V^+ and PI^-. (*C*) Average percentage of apoptotic wild-type LKSI cells after 4 days in culture ($n = 4$ for all conditions). Error bars represent standard deviation. *P* values were determined by Student's *t* test. (*D*) Average fold expansion of H2K-*BCL-2* LKSI cells after 4 days in culture ($n = 4$ for all conditions). Error bars represent standard deviation. *P* values were generated by Student's *t* test

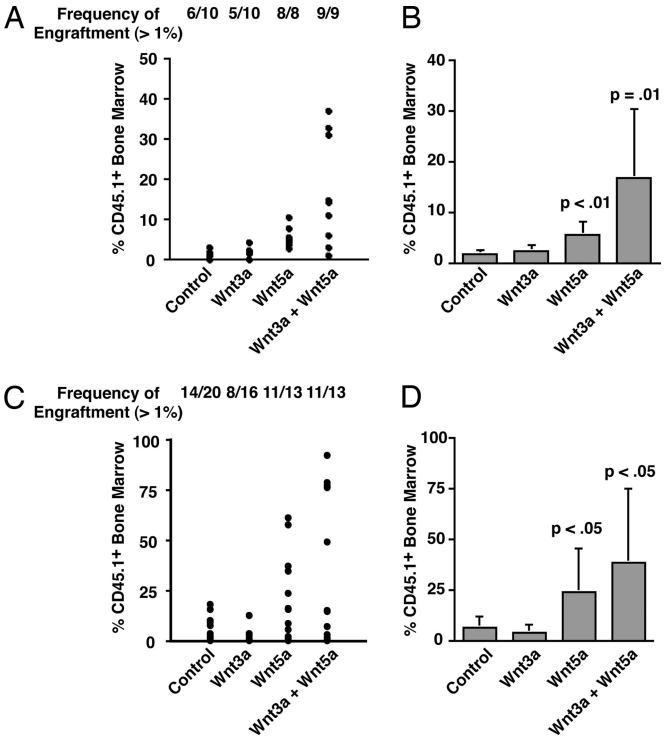

Fig. 4. Analysis of Wnt-mediated effects on hematopoietic repopulation. (*A*) Raw data of short-term (6 weeks) engraftment of cultured LKSI cells. Recipients were defined as positive for engraftment if they contained >1% hematopoietic chimerism. (*B*) Average short-term repopulation of cultured LKSI cells. Data represent the pooled results of two independent experiments. Only recipients with >1% chimerism were included in this analysis. Error bars represent standard deviation. *P* values were generated by Student's *t* test. (*C*) Raw data of long-term (16 weeks) engraftment of cultured LKSI cells. Recipients were defined as positive for engraftment if they contained >1% hematopoietic chimerism. (*D*) Average long-term repopulation of cultured LKSI cells. Data represent the pooled results of three independent experiments. Only recipients with >1% chimerism were included in this analysis. Error bars represent standard deviation. *P* values were generated by Mann–Whitney nonparametric analysis.

presented in Fig. 2*B*, indicate that, in hematopoietic progenitors, Wnt5a primarily signals through noncanonical pathways. Furthermore, culturing lin^- cells with both Wnt3a and Wnt5a decreased β-catenin protein compared with Wnt3a alone, suggesting that Wnt5a inhibits canonical Wnt signaling in primitive hematopoietic cells by promoting the destabilization of β-catenin.

Wnt5a Inhibits HSC Expansion *in Vitro*. We hypothesized that Wnt5a-mediated destabilization of β-catenin would suppress Wnt3a-mediated effects on LKSI cell proliferation. To test this, we cultured wild-type LKSI cells in the presence of Wnt3a and/or Wnt5a. We observed that under serum-free conditions, the presence of Wnt3a significantly reduced total cell expansion 3-fold ($P = 0.05$) after 4 days in culture compared with control conditions (SCF and Flt3L alone) (Fig. 3*A*). However, Wnt5a either alone (12-fold, $P = 0.01$) or in combination with Wnt3a (16-fold, $P < 0.01$) also significantly reduced cell expansion compared with control conditions. There was no difference among the groups in the percentage of cells that retained the LKSI phenotype (data not shown).

To test whether the Wnt-mediated inhibition of cell proliferation could be due to induction of apoptosis, we cultured LKSI cells with either Wnt3a or Wnt5a (Fig. 3 *B* and *C*). Both (Wnt3a: 2.4-fold; Wnt5a: 3-fold) significantly increased the percentage of total cells actively undergoing apoptosis ($P < 0.05$) from 1% to 3%. Therefore, it is probable that other mechanisms in addition to apoptosis contribute toward inhibition of proliferation by Wnt3a and Wnt5a.

To further investigate the role of apoptosis in Wnt-mediated inhibition of cell proliferation, we cultured LKSI cells that were isolated from transgenic mice that overexpressed the human antiapoptotic gene *BCL-2* (20). We observed that culturing *BCL-2*$^+$ LKSI cells with Wnt3a and/or Wnt5a resulted in decreased total cell expansion compared with control conditions ($P < 0.01$) (Fig. 3*D*). Wild type and *BCL-2*$^+$ LKSI cells expanded equally when cultured under either control conditions or with Wnt3a. In contrast, *BCL-2*$^+$ LKSI cells proliferated more in the presence of Wnt5a compared with wild type ($P < 0.01$). These data suggest that the induction of apoptosis by Wnt3a or Wnt5a occurs through different mechanisms, with Wnt5a-mediated induction reliant on pathways governed by BCL-2.

Effect of Wnt3a and Wnt5a on HSC Repopulating Ability. We examined the effects of activating Wnt signaling pathways on HSC function (measured through competitive repopulation assays). Wild-type CD45.1 LKSI cells were cultured for 6 days under serum-free conditions with Wnt3a and Wnt5a before transplantation. Six weeks after transplant, we analyzed recipients' bone marrow for short-term repopulation (mediated by both short-term and long-term HSCs) by cultured donor LKSI cells (Fig. 4*A*). We defined a recipient having >1% $CD45.1^+$ bone marrow cells as positive for hematopoietic chimerism. We did not observe a significant difference in short-term repopulation by control LKSI cells and LKSI cells cultured with Wnt3a (Fig. 4*B*). However, LKSI cells cultured with either Wnt5a alone or both Wnt3a and Wnt5a showed significant 3- to 5-fold increases, respectively, in short-term repopulation compared with control

($P < 0.01$) (Fig. 4*B*). We observed no differences in the homing abilities of LKSI cells cultured under control conditions, with Wnt3a, or with Wnt5a (SI Fig. 9), indicating that Wnt5a does not promote hematopoietic repopulation by enhancing the ability of cultured LKSI cells to home.

To determine the effects of Wnt3a and Wnt5a on primary hematopoietic repopulation mediated solely by long-term HSCs, we analyzed recipients' bone marrow 16 weeks after transplant, using the same criteria for engraftment outlined above. As with short-term repopulation, there was no significant difference in long-term repopulation by control LKSI cells and LKSI cells cultured with Wnt3a (Fig. 4 *C* and *D*). Similarly, we observed significant 3.6-fold and 5.7-fold increases in long-term hematopoietic repopulation by LKSI cells cultured with Wnt5a ($P < 0.01$) and Wnt5a and Wnt3a ($P < 0.01$), respectively. Combined with the data in Fig. 4*B*, this indicates that Wnt3a does not enhance repopulation by either short- or long-term HSCs. Finally, to determine whether Wnt3a prolonged the period in which HSCs could be cultured and still repopulate, we transplanted LKSI cells that had been cultured with and without Wnt3a for 2 weeks and found that neither condition yielded any long-term engraftment (data not shown). These data indicate that short-term treatment with Wnt5a, but not Wnt3a, can increase the primary repopulating ability of cultured HSCs.

Our observations that stimulating the canonical Wnt signaling pathway has an inhibitory effect on cell expansion and no effect on the ability of *in vitro*-cultured LKSI cells to engraft contrasts with previous reports that showed a significant positive effect on HSC function (4, 21). One explanation for the discrepancy between our data and those from other studies is that other signals are necessary to enable Wnt3a to expand HSCs *in vitro*. Because HSC expansion is regulated by apoptosis (20), we transplanted *BCL-2*$^+$ LKSI cells (CD45.1) that were cultured under identical conditions as their wild-type counterparts. Wnt3a inhibited long-term engraftment of *BCL-2*$^+$ HSCs because only 1 of 7 recipients showed any long-term engraftment (1.2% CD45.1$^+$ bone marrow cells) whereas *BCL-2*$^+$ LKSI cells cultured under control conditions engrafted in 7 of 7 recipients (17.8 ± 6.8% CD45.1$^+$ cells) (SI Fig. 10). *BCL-2*$^+$ LKSI cells cultured with Wnt5a showed similar levels of engraftment compared with *BCL-2*$^+$ LKSI cells cultured under control conditions ($P = 0.40$).

Effect of Wnt3a and Wnt5a on HSC Gene Expression. To investigate the mechanism underlying our observations, we examined the effects of Wnt5a on mRNA levels of selected genes in LKSI cells. It has been reported that activation of Notch signaling pathways (22–24) positively regulates HSC self-renewal. We were unable to detect any mRNA of the Notch pathway target gene *Hes-1* in LKSI cells cultured under control conditions. However, *Hes-1* expression was observed when LKSI cells were cultured with Wnt5a. (Fig. 5*A*), suggesting that stimulation of LKSI cells with Wnt5a can activate Notch signaling.

We also examined whether Wnt5a could inhibit expression of other genes that are targets of canonical Wnt signaling. *Myc* is a target of the canonical Wnt pathway, and its overexpression has been shown to inhibit HSC self-renewal (25). Consistent with this, we observed that Wnt3a induced a significant 2.5-fold increase ($P < 0.05$) in *c-myc* mRNA compared with control (Fig. 5*B*) and that Wnt5a abrogated this induction.

Finally, it was demonstrated that the loss of Wnt5a in mouse fetal liver cells resulted in increased expression of the cell cycle regulatory gene *Ccnd1* (cyclin D) and cell proliferation (11). We were unable to observe any evidence of increased levels of *Ccnd1* mRNA levels in LKSI cells cultured with Wnt5a (data not shown). However, we did observe that Wnt5a significantly increased expression of *Cdkn1b* ($P = 0.02$), which encodes for the cell cycle inhibitory protein p27 (Fig. 5*C*), suggesting that Wnt5a-mediated inhibition of cell proliferation may be partially due to increased expression of this gene.

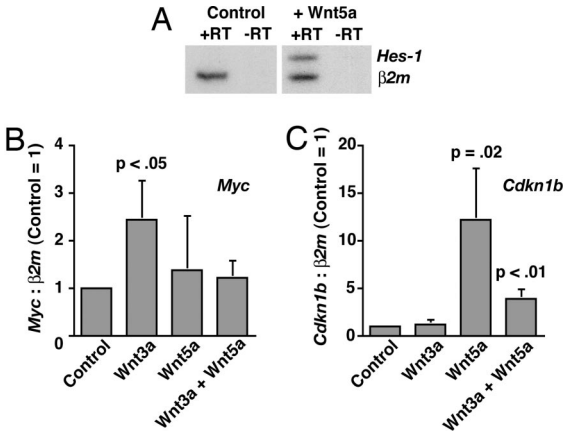

Fig. 5. Analysis of Wnt5a-mediated effects on gene expression. (*A*) Representative duplex RT-PCR analysis of *Hes-1* mRNA in LKSI cells. (*B*) Average *Myc* mRNA levels relative to *β2m* ($n = 3$ for all groups). The data represent the average results of three independent experiments. Error bars represent standard deviation. *P* values were generated by Student's *t* test. (*C*) Average *Cdkn1b* mRNA levels relative to *β2m* ($n = 3$ for all groups).

Wnt5a Maintains HSC Quiescence. The majority of HSCs are in the G_0/G_1 phases of the cell cycle and HSCs within the quiescent G_0 stage are more efficient at hematopoietic repopulation (26–29). We hypothesized that the positive effect of Wnt5a on hematopoietic repopulation of cultured LKSI cells correlated with increased numbers of input cells in the G_0 phase.

First, we determined the overall cell cycle profile of LKSI cells cultured with Wnt3a and Wnt5a. As expected, based on our earlier observations that the Wnt ligands inhibited cell proliferation, we observed that the percentage of cells actively cycling (defined as the percentage of cells in the S, G_2, or M phases) decreased 1.4- to 1.6-fold when cultured with Wnt3a, Wnt5a, or both (Fig. 6*A*). We then analyzed cells cultured with Wnt3a and Wnt5a for differences in the distribution of cells between the G_0 and G_1 phases.

There was no difference between LKSI cells cultured under either control conditions or with Wnt3a in the percentage of G_0/G_1 cells residing in the quiescent G_0 phase (Fig. 6*B*). However, culturing LKSI cells with Wnt5a, either alone or in combination with Wnt3a, led to a 2-fold increase in the percentage of cells in G_0. We observed no difference in the percentage of LKSI cells that incorporated BrdU (nearly 100% for all conditions, data not shown), suggesting that the increased percentage of G_0 cells was not due to Wnt5a indiscriminately inhibiting cell cycle progression of LKSI cells (which are mainly G_0). These data demonstrate that Wnt5a maintained cells in the quiescent G_0 stage *in vitro* and suggest that this is one mechanism by which Wnt5a enhances hematopoietic engraftment.

Discussion

Wnt5a has been demonstrated to enhance formation of multilineage colonies from primitive human progenitor cells as well as the SCID-repopulating ability of human cord blood cells (17, 30). However, the mechanisms by which noncanonical Wnt5a can regulate hematopoietic stem cell function have not been characterized. In this study, we have demonstrated that Wnt5a positively regulates HSC repopulating ability through noncanonical signaling pathways, which include those that inhibit canonical Wnt signaling.

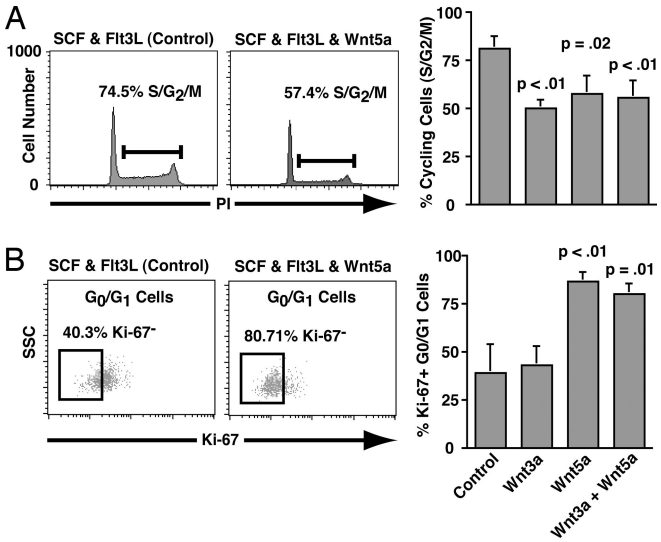

Fig. 6. Analysis of Wnt-mediated effects on HSC cell cycle status. (*A Left*) Representative cell cycle histograms obtained through propidium iodide (PI) staining of wild-type LKSI cells cultured for 4 days under control conditions (*Left*) or recombinant Wnt5a (*Right*). Histograms were analyzed as described in *Materials and Methods*. (*Right*) Average percentage of wild-type LKSI cells in $S/G_2/M$ phases of the cell cycle after 4 days in culture. Data represent the average of three independent experiments for each condition. Error bars represent standard deviation. *P* values were generated by Student's *t* test. (*B Left*) Representative FACS analyses of G_0/G_1 LKSI cells after 4 days in culture under control conditions (*Left*) or recombinant Wnt5a (*Right*). LKSI cells in the G_0/G_1 phase were defined as cells with 2n DNA content as detected by 7-AAD staining (data not shown). G_0/G_1 cells were gated and analyzed for the percentage of cells that were in the G_0 phase, defined as Ki-67$^-$. Regions were drawn based on isotype controls. (*Right*) Average percentage of wild-type G_0/G_1 LKSI cells in the G_0 phase after 4 days in culture. Data represent the average of three independent experiments for each condition. Error bars represent standard deviation. *P* values were generated by Student's *t* test. The approximate percentage of total cells in G_0 under control conditions, with Wnt3a, or with Wnt5a were 10%, 25%, and 35%, respectively (determined by multiplying the percentage of total cells in G_0/G_1 (*A*) by the percentage of G_0/G_1 cells in G_0 (*B*).

Our data indicate that Wnt5a regulates HSC proliferation through multiple mechanisms. The ability of BCL-2 to block Wnt5a-mediated inhibition of proliferation indicates that inducing apoptosis is one mechanism by which Wnt5a regulates hematopoiesis. It is also likely that increased expression of *Cdkn1b* in HSCs cultured with Wnt5a also contributes to reduced cell proliferation. Our data do not suggest that Wnt5a promotes *ex vivo* expansion of HSCs. We propose that short-term treatment of primitive hematopoietic cells with pharmacologic doses of Wnt5a promotes their retention within the quiescent G_0 phase, resulting in increased short-term and long-term repopulating ability (29). We cannot rule out the possibility that the initial stimulation with Wnt5a subsequently promotes HSC self-renewal over the 16 weeks of repopulation, but, because the HSCs present in the primary recipients engraft into a normal environment after the initial stimulation by Wnt5a, we favor the former explanation.

We have shown that Wnt5a attenuates the canonical Wnt pathway in HSCs. Analogous to the ability of Tcf3 to maintain hair follicle stem cells by repressing canonical Wnt signaling (31), Wnt5a may maintain HSC function by inhibiting alterations in gene expression induced by the canonical Wnt pathway, such as increased expression of *myc* (25), that can lead to stem cell differentiation. Wnt5a may also maintain HSC function by mechanisms independent of its ability to inhibit the canonical Wnt pathway, such as activation of Notch signaling (24).

Our results, along with those of Scheller *et al.* and Kirstetter *et al.* (8, 9) indicate that activation of the canonical Wnt pathway by Wnt3a inhibits HSC self-renewal. It is possible that for HSCs to be maintained in the presence of increased canonical Wnt signaling, other pathways need to be activated as well. Trowbridge *et al.* (32) showed that treating mice with an inhibitor to GSK-3b, which regulates several pathways in addition to activating the canonical Wnt pathway, could enhance hematopoietic repopulation. The presence of additional factors required to balance activated canonical Wnt signaling may also explain some of the apparent discrepancies in HSC engraftment potential between our results using recombinant Wnt3a and those of Willert *et al.* and Reya *et al.* (4, 21), some of which were obtained by using culture conditions that included serum. From these data, we propose that there may be additional signals needed to regulate the level of canonical Wnt signaling to maintain HSCs. Because canonical Wnt signaling has been has been demonstrated to be present in primary acute myeloid, chronic myelogenous, and chronic lymphocytic leukemia cells (5, 33–35), we predict that if enhanced canonical Wnt signaling plays a role in leukemic transformation, other complementary signaling pathways must also be activated.

We propose a model in which Wnt5a regulates HSC function in a paracrine fashion through several distinct mechanisms including modulating canonical Wnt signaling and regulating quiescence. Based on this model, we predict that bone marrow stromal cells deficient in Wnt5a would exhibit a reduced capacity to support hematopoiesis. These results also demonstrate that noncanonical recombinant Wnt5a, and not the canonical Wnt3a, may be a useful component in designing therapeutic *in vitro* strategies for maintaining and manipulating functional HSCs.

Materials and Methods

Mice. B6.SJL-*Ptprc*^{a}/BoAiTac (*Ptprc*^{a} encodes for the CD45.1 protein) mice were obtained from Taconic Farms (Germantown, NY). TOPGAL transgenic mice (36) were maintained in the Transgenic Mouse Core of the National Human Genome Research Institute. H2K-*BCL-2* transgenic mice (20) were kindly provided by Irving Weissman (Stanford University School of Medicine, Palo Alto, CA) and rederived on a B6.SJL-*Ptprc*^{a}/BoAiTac background.

Retroviral Transduction of Stimulated Bone Marrow. Wnt3a-IRES-GFP was constructed by cloning a 1.1-kb Asc I-PacI blunt-end insert containing a Wnt3a cDNA fragment (translated region only) into a blunt-end BamHI site in the MGirL22Y IRES-GFP retroviral vector (provided by Brian Sorrentino, St. Jude Children's Research Hospital, Memphis, TN). Wnt5a-IRES-dsRed was constructed by first cloning a 1.1-kb BamHI-XbaI blunt-end insert containing the Wnt5a cDNA fragment (translated region only) into a blunt-end BamHI site in the MSCV-IRES-GFP retroviral vector. An IRES-DsRed cassette was substituted for IRES-GFP by cloning a BglII-NotI IRES-DsRed fragment from pIRES2-DsRed-Express (Clontech, Mountain View, CA) into the matching sites of Wnt5a-IRES-GFP. All constructs were transfected into BOSC (37) cells by standard techniques and cultured for 48 h before collection of viral supernatant. Retroviral transduction of B6.SJL-*Ptprc*^{a}/BoAiTac bone marrow was performed as described (38). Sorted GFP$^+$- or DsRED$^+$-transduced CD45.1 cells were mixed with an equal dose of mock-transduced C57BL/6 bone marrow and injected into the lateral tail vein of lethally irradiated 129Sv $\times$ C57BL/6 F_1 recipients. Bone marrow cells were collected 16 weeks after transplant and stained with allophycocyanin (APC)-conjugated anti-CD45.1 (BD Biosicences, San Diego, CA).

Isolation and Culture of LKSI Cells. For all experiments, LKSI cells were purified as described (39). For all experiments, LKSI cells

were cultured in serum-free StemSpan media (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 30 ng/ml SCF and Flt3L. Recombinant mouse Wnt3a and Wnt5a (R & D Systems, Minneapolis, MN) were added at concentrations of 100 and 500 ng/ml. Length of the culture period differed according to the experiment.

Western Blot Analysis. Lin^- cells were isolated and cultured for 48 h. Whole-cell extracts were then isolated from 2×10^6 cells. Cells were washed twice in PBS and lysed in boiling 2× NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA) before sonication (three times for 10 seconds each). Western blot analysis for β-catenin protein was performed as described (15). Anti-β-actin (Sigma, St. Louis, MO) was used as a loading control.

RT-PCR. Semiquantitative RT-PCR for detecting gene expression levels was performed on LKSI cells after *in vitro* culture for 48 h. This procedure was performed as described previously by using a duplex-PCR with one primer pair amplifying the test gene and the second primer pair amplifying β2-microglobulin as an internal control (39). Limiting dilutions of LKSI mRNA were used to ensure that amplifications remained within the linear range. Primer sets and their annealing temperatures are described in SI Table 1.

***In Vitro* Analysis of HSC Cell Cycle Status and Apoptosis.** To determine the percentage of actively apoptotic cells, LKSI cells were stained after 4 days in culture with APC-conjugated anti-Annexin V (BD Biosicences) and propidium iodide (PI) according to the manufacturer's instructions before analysis by flow cytometry. Cells actively undergoing apoptosis were defined as Annexin V^+ and PI^-. Cell cycle status was determined as described (39). To determine the percentage of cells within the G_0 phase, LKSI cells were stained after 4 days in culture with mouse anti-human fluorescein isothiocyanate (FITC)-conjugated Ki-67 and 7-AAD (BD Biosicences). Cells were fixed for nuclear staining by using Cytofix/Cytoperm buffer according to the manufacturer's instructions (BD Biosicences). Quiescent cells were defined as Ki-67^- and 7-AAD^-. *P* values were generated by Student's *t* test.

Competitive Repopulation of Cultured HSCs. Independent experiments were performed to analyze repopulation at 6 and 16 weeks after transplant. Isolated LKSI cells (from both wild-type and H2K-*BCL2* transgenic mice; CD45.1) were cultured for 6 days before being mixed with primary whole bone marrow cells (CD45.2) at a cell ratio of 1:100. Cells were injected into the tail veins of lethally irradiated (950 cGy) 129Sv × C57BL/6 F_1 recipients. For analysis at 6 weeks, 1×10^4 cultured HSCs were injected. For analysis at 16 weeks, the number of cultured LKSI cells transplanted ranged from 3 to 5×10^3 cells. Hematopoietic repopulation was measured by staining whole bone marrow cells with APC-conjugated anti-CD45.1 and phycoerythrin (PE)-conjugated anti-CD45.2 (BD Biosicences). Successful engraftment was defined as ≥1% CD45.1-positive bone marrow cells. Statistical differences in total repopulation by wild-type LKSI cells were calculated by using the Student's *t* test (6 weeks) and Mann–Whitney test (16 weeks). Statistical differences in total repopulation by H2K-*BCL2* LKSI cells were calculated by using Student's *t* test (16 weeks).

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