A role for Wnt signalling in self-renewal of haematopoietic stem cells

Tannishtha Reya*†, Andrew W. Duncan*†, Laurie Ailles‡, Jos Domen§, David C. Scherer‡, Karl Willert||, Lindsay Hintz*, Roel Nusse|| & Irving L. Weissman‡

* *Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710, USA*
‡ *Departments of Pathology and Developmental Biology, Stanford University School of Medicine, Stanford, California 94035, USA*
§ *Departments of Medicine and Immunology, Duke University Medical Center, Durham, North Carolina 27710, USA*
|| *Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, California 94035, USA*
† *These authors contributed equally to this work*

Haematopoietic stem cells (HSCs) have the ability to renew themselves and to give rise to all lineages of the blood; however, the signals that regulate HSC self-renewal remain unclear. Here we show that the Wnt signalling pathway has an important role in this process. Overexpression of activated β-catenin expands the pool of HSCs in long-term cultures by both phenotype and function. Furthermore, HSCs in their normal microenvironment activate a LEF-1/TCF reporter, which indicates that HCSs respond to Wnt signalling *in vivo*. To demonstrate the physiological significance of this pathway for HSC proliferation we show that the ectopic expression of axin or a frizzled ligand-binding domain, inhibitors of the Wnt signalling pathway, leads to inhibition of HSC growth *in vitro* and reduced reconstitution *in vivo*. Furthermore, activation of Wnt signalling in HSCs induces increased expression of *HoxB4* and *Notch1*, genes previously implicated in self-renewal of HSCs. We conclude that the Wnt signalling pathway is critical for normal HSC homeostasis *in vitro* and *in vivo*, and provide insight into a potential molecular hierarchy of regulation of HSC development.

The HSC has the ability to perpetuate itself as well as to differentiate into mature blood cells of all lineages. In the mouse, long-term self-renewing HSCs make up approximately 0.007% of bone marrow and can be isolated by their expression of undetectable levels of lineage markers (such as B220, CD3, Mac-1), high levels of c-Kit and Sca-1, and low levels of Thy-1 (refs 1, 2). Although HSCs have been purified successfully and their phenotypic and functional properties well characterized[1–4], a fundamental question that remains is how their self-renewing growth is regulated. On the basis of a screen of genes expressed in HSCs—where we noted that members of the LEF-1/TCF family were expressed (K. Li, S. Cheshier and I.L.W., unpublished data)—and our previous finding that Wnt signalling can influence lymphocyte progenitor cell proliferation[5], we have investigated whether Wnt signalling influences HSC development.

β-Catenin expression leads to self-renewal of HSCs *in vitro*

We first determined the effects of activating downstream components of the Wnt pathway on HSC function. We activated Wnt signalling in HSCs sorted via fluorescence-activated cell sorting (FACS) (c-Kit^{+} Thy-1.1^{lo} Lin$^{-/lo}$ Sca-1^{+} (KTLS) cells) by retrovirally[6] transducing them with constitutively active β-catenin[7]. Successful transduction of HSCs with retroviruses requires induction of cell cycle entry through the use of multiple growth factors, which can promote differentiation of stem cells *in vitro*. To minimize the pro-differentiation stimuli encountered by HSCs during infection before experiments of interest, we used HSCs from H2K-BCL-2 transgenic mice, which proliferate to steel factor (SLF) alone[8]. Sorted BCL-2 transgenic HSCs were infected with retroviruses encoding either β-catenin–IRES–GFP (β-catenin, internal ribosome entry site and green fluorescent protein) or IRES–GFP alone, and GFP expression was detected in 45–55% of HSCs, which persisted for the entire *in vitro* culture period (data not shown). GFP-positive (GFP^{+}) HSCs were sorted to determine growth kinetics *in vitro* and the ability to reconstitute the immune system *in vivo*.

Short-term growth characteristics of HSCs expressing β-catenin or control vector were determined by cell cycle analysis[9]. In Fig. 1a, whereas 34% of the HSCs infected with control vector were in S/G2/M phases of the cell cycle, 58% of the HSCs expressing activated β-catenin were in the same phases of the cell cycle (Fig. 1a). To determine whether activated Wnt signalling increased long-term growth, HSCs expressing β-catenin were grown *in vitro* in serum-free medium in the presence or absence of growth factors. Medium containing limiting amounts of SLF allowed the growth of β-catenin-transduced HSCs consistently for at least 8 weeks (Fig. 1b). During this period the GFP^{+} cells underwent eight–nine population doublings to generate at least 100 times the number of input cells. In contrast, HSCs infected with control vector showed minimal growth beyond a two-week period (Fig. 1b). On complete withdrawal of SLF during long-term culture, β-catenin-infected HSCs grew for at least 4 weeks, and in some experiments could be maintained and passaged for as long as 1–2 months. In contrast the control transduced HSCs did not survive beyond 48 h (data not shown).

To determine whether growth in response to activated β-catenin was accompanied by differentiation, the morphological characteristics of these cells were analysed at the end of a two-week period. This time point was chosen to be able to compare the differentiation status of control and β-catenin-transduced HSCs, as the lifespan of HSCs transduced with control vector was limited. Cells infected with control vector were found to have a myelo-monocytic appearance. In contrast, 65–75% of the β-catenin-transduced HSCs had a high nuclear to cytoplasm ratio (Fig. 1c). Consistent with this, although most (75–80%) of the HSCs infected with control vector were positive for lineage markers (Fig. 1d, left panel), only 5–10% of cells infected with β-catenin expressed high levels of lineage markers (predominantly Mac-1, an integrin expressed on fetal HSCs[10] and regenerating HSCs[11]). In fact, 60% of HSCs infected with β-catenin were lineage-negative and expressed high levels of c-Kit and Sca-1 (Fig. 1d, middle panel) and almost half of these also expressed low levels of Thy-1.1 (right panel). Thus, at least 30% of the cells in β-catenin-transduced cultures had retained the phenotype of HSCs; that is, c-Kit^{+} Thy1.1^{lo} Lin^{-} Sca-1^{+} (KTLS cells). This indicated that the expression of activated β-catenin maintained haemato-

© 2003 Nature Publishing Group

poietic stem cells in an immature state, while simultaneously allowing these cells to proliferate, thus expanding the HSC pool 20–48-fold on the basis of the total numbers of cells generated (see Supplementary Information).

To determine whether β-catenin-transduced HSCs retained the functional characteristics of HSCs, and to determine whether there was indeed a functional expansion of HSCs, we tested the ability of β-catenin-transduced HSCs to give rise to sustained production of myeloid, B- and T-cell lineages in lethally irradiated mice when transplanted in limiting numbers[1,3]. Specifically, HSCs were infected with control or β-catenin virus, cultured for 1 week *in vitro*, transplanted in varying doses down to 125 cells into irradiated mice, and analysed after 11 weeks. As shown in Table 1, 125 β-catenin-transduced HSCs were sufficient to reconstitute the haematopoietic system of all of the irradiated mice. The average level of chimaerism observed was 61% (range of 27.6–86.8%). Furthermore, each of the transplanted mice displayed clear reconstitution along myeloid, T and B lineages (Table 1 and Fig. 2). In contrast, mice receiving 125 HSCs transduced with control vector failed to reconstitute (Table 1). The reconstitution observed at limiting dilution suggested a functional HSC expansion of 8–80-fold in short-term cultures and 96-960-fold in long-term cultures (Supplementary Information). That β-catenin-transduced HSCs lead to higher levels of chimaerism was observed in five independent experiments using different doses of cells and different culture periods (up to 2 months), indicating that β-catenin prevents HSC differentiation while promoting proliferation, thus inducing self-renewal in serum-free medium.

The expansion of HSCs owing to activated β-catenin probably reflects upstream Wnt signals, as we have demonstrated recently that purified Wnt3a causes self-renewal in both BCL-2 transgenic[12] and wild-type HSCs (Supplementary Figs 1 and 2). Specifically, singly plated HSCs generate sixfold or more numbers of progeny in the presence of Wnt3a compared with control conditions[12] (Supplementary Figs 1 and 2). These daughter cells not only maintain an immature phenotype, but also display a 5–50-fold expansion of HSC function as determined by transplantation analysis of the progeny of single HSCs after expansion *in vitro*[12] (Supplementary Figs 1 and 2).

HSCs *in vivo* normally signal through LEF-1/TCF elements

To determine whether the Wnt signalling pathway is physiologically relevant to HSCs, we tested whether HSCs *in vivo* use signals associated with the Wnt/β-catenin pathway. HSCs were infected with LEF-1/TCF reporter driving expression of destabilized GFP (TOP–dGFP) or with control reporter with mutated LEF-1/TCF binding sites (FOP–dGFP), and then transplanted into lethally irradiated mice (L. Ailles and I.L.W., manuscript in preparation). Recipient bone marrow was examined after 14 weeks to determine whether donor HSCs demonstrated reporter activity. In the example shown, donor-derived HSCs infected with TOP–dGFP expressed GFP in 28% of the cells (Fig. 3a; range observed 4–28%, mean 11.8%), whereas HSCs from the recipient mouse were negative for GFP (Fig. 3b; range observed 2.3–3.2%, mean 2.7%). Moreover, HSCs transduced with the control reporter did not express GFP significantly, demonstrating that functional LEF-1/TCF binding sites were required for HSC expression of GFP (Fig. 3c). In all cases, no reporter activity was observed in the non-HSC myeloid progenitor fraction (Fig. 3a–c, thin line). As a control, we also tested whether the TOP–dGFP reporter was turned on in response to Wnt3a-mediated signalling in HSCs *in vitro*. Thus, HSCs transduced with either TOP–dGFP or FOP–dGFP were stimulated with Wnt3a, and the extent of GFP expression was monitored. As shown in Fig. 3e, Wnt3a-treated HSCs showed significant reporter activity, demonstrating that the reporter is turned on in response to Wnt stimulus, but not in control conditions. Increased reporter activity was observed when the reporter construct driving non-destabilized GFP was used. These data demonstrate that HSCs in their normal microenvironment respond to endogenous Wnt signalling during self-renewal and/or stimulation into cell cycle, and also support the interpretation that the Wnt3a stimulus that caused increased self-renewal[12] signals through the canonical Wnt pathway.

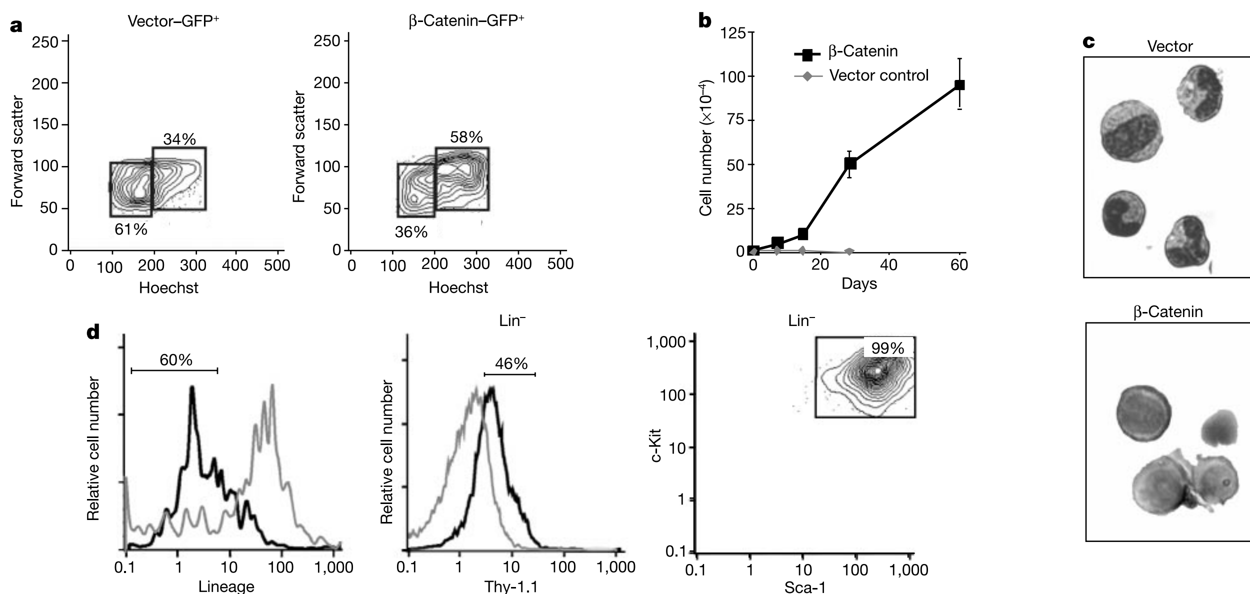

Figure 1 Activated β-catenin promotes growth of HSCs *in vitro* and maintains the immature phenotype of HSCs in long-term cultures. HSCs were infected with activated β-catenin–IRES–GFP or control GFP retrovirus, and subjected to cell cycle analysis after 60 h. **a**, β-Catenin-infected cultures display an increased number of blasting cells (right box, S/G2/M) compared with control. **b**, For long-term growth studies, 10,000 infected HSCs were plated in 1 ng ml^{-1} SLF and monitored over 60 days. Results are from one of five experiments. **c**, Giemsa staining reveals myeloid characteristics in control cells and HSC morphology (high nucleus to cytoplasm ratio) in β-catenin-infected cells. **d**, Control cells (grey lines) are largely lineage-positive, whereas most β-catenin-infected cells (black lines) are lineage-negative (Lin^{-}) or have low levels (left panel). β-Catenin-infected Lin^{-} cells have characteristics of HSCs, including low Thy-1.1 (middle panel), and high c-Kit and Sca-1 (right panel).

© 2003 Nature Publishing Group

Table 1 **Reconstitution efficiency of β-catenin- or vector-transduced HSCs**

Transplantation		Cell type	Frequency of reconstitution (%)	Chimaerism (%)	
Number	Infection			Average	Range
125	Vector	PB	0 (2/2)	0	0
125	β-Catenin	PB	100 (3/3)	61.2	27.6–86.8
125	β-Catenin	B	100 (3/3)	58.1	37.9–78.8
125	β-Catenin	T	100 (3/3)	19.2	16.0–23.4
125	β-Catenin	M	100 (3/3)	15.0	2.6–25.7

HSCs infected with activated β-catenin or control vector were cultured for a week and cells were injected into groups of three to four lethally irradiated host mice along with 300,000 competing syngeneic bone marrow cells. Cells were isolated from peripheral blood and analysed by flow cytometry after 11 weeks. Average reconstitution rates and ranges of donor cells are shown for peripheral blood (PB), B cell (B), T cell (T) and myeloid (M) lineages. (See also Fig. 2.)

HSCs require intact Wnt signalling

To test whether Wnt signalling is required for normal HSC growth, we used a soluble form of the frizzled cysteine-rich domain (CRD) that inhibits the binding of Wnt proteins to the frizzled receptor[13,14] (Supplementary Fig. 3). Wild-type HSCs were incubated with growth factors in the presence of IgG–CRD domain fusion protein or control IgG, and cell proliferation was monitored. The presence of the CRD domain inhibited growth of HSCs fourfold compared with control conditions (Fig. 4a). This inhibition provides direct evidence of a Wnt signal modulating HSC survival and proliferation, as soluble CRD acts at the level of Wnt binding the frizzled molecules. Because only HSCs were present, the Wnt signal is probably derived from some or all of the HSCs in the cultures, and is required despite the presence of multiple other growth factors. These results can be interpreted to mean that all HSC mitoses are the result of Wnt signalling, even if the primary signals are not Wnt.

We also inhibited Wnt signalling through an independent inhibitor by ectopically expressing axin in HSCs[15]. Axin increases β-catenin degradation and acts as an intracellular inhibitor of Wnt signalling[16–19]. Live axin-infected wild-type HSCs were re-sorted 48 h after infection and plated in limiting numbers to assay growth in response to a combination of growth factors. Although control-infected cells proliferated 2.3-fold over 60 h, axin-infected cells showed a sevenfold reduction in the total growth response (Fig. 4b). Axin had an inhibitory effect on growth of BCL-2 transgenic HSCs as well (data not shown), which suggests that expression of BCL-2 cannot protect cells from loss of Wnt signalling. To determine whether axin expression had an effect on cell survival, GFP$^+$ cells were analysed at the end of the infection period using propidium iodide exclusion. Whereas 80% of the control-infected cells were negative for propidium iodide, only 38% of axin-infected HSCs were negative for propidium iodide (Fig. 4c), indicating that axin expression has significant effect on cell survival by blocking β-catenin function.

To determine whether Wnt signalling is required for haematopoietic stem cell responses *in vivo*, we injected axin- or control-transduced viable HSCs into lethally irradiated mice and analysed the level of reconstitution after 10 weeks. Mice transplanted with control-infected HSCs displayed on average sevenfold greater chimaerism (reconstitution range 5–11.6%) than mice transplanted with axin-infected HSCs (reconstitution range 0–1.8%) (Fig. 4e). A representative example of contribution from axin- or vector-infected HSCs in transplanted mice is shown in Fig. 4d. These data show that inhibition of the Wnt pathway reduces reconstitution, suggesting that Wnt signalling is required for normal development of HSCs *in vivo*. This finding, together with the finding

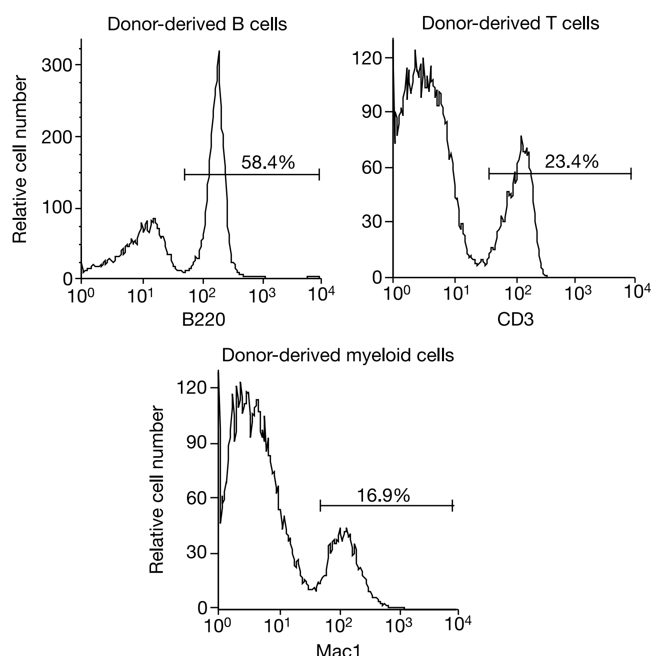

Figure 2 Activated β-catenin-transduced HSCs reconstitute lethally irradiated mice and give rise to multiple haematopoietic lineages. The diagrams show the contribution to each lineage as determined by FACS analysis in a representative sample.

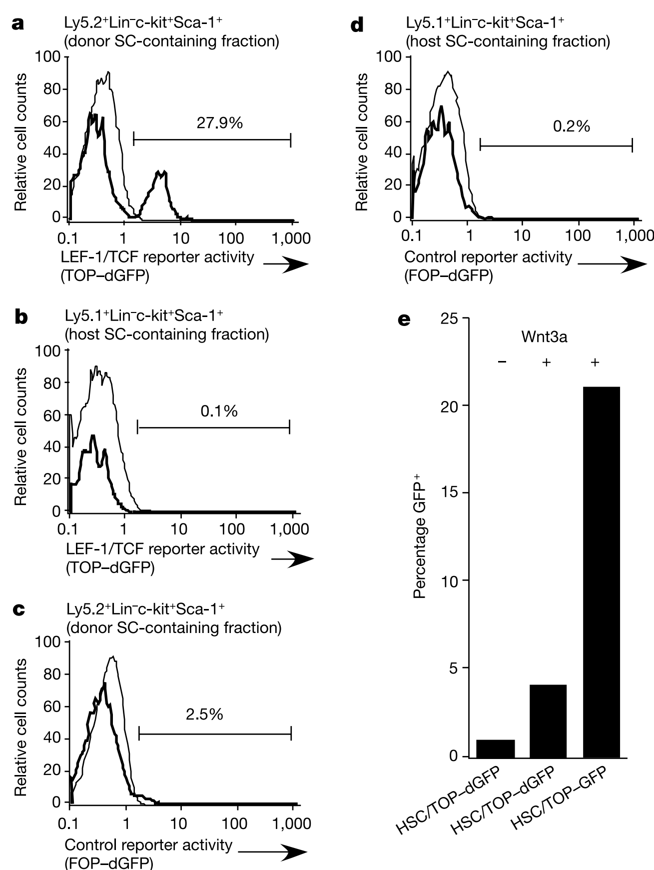

Figure 3 HSCs respond to Wnt signalling in native bone marrow microenvironment. HSCs were infected with a lentiviral reporter containing either LEF-1/TCF binding sites linked to destabilized GFP (TOP–dGFP), or mutated LEF-1/TCF binding sites linked to destabilized GFP (FOP–dGFP). Infected HSCs were transplanted into three lethally irradiated recipient mice, and analysed after 14 weeks. The data shown represent two independent experiments. **a**, **b**, GFP expression is shown in donor-derived (**a**) or host-derived (**b**) HSCs. **c**, **d**, Donor-derived HSCs carrying mutated LEF-1/TCF reporter (**c**) as well as the recipient mouse HSCs (**d**) are GFP negative. Expression of GFP in donor-derived Lin$^-$ c-Kit$^+$ Sca-1$^-$ cells (non-HSCs) is shown by thin lines (**a**–**d**). **e**, HSCs infected with TOP–dGFP or TOP–GFP (a non-destabilized GFP) were stimulated *in vitro* with control medium or with 100 ng ml^{-1} Wnt3a, and the extent of GFP expression measured.

 © 2003 Nature Publishing Group

that HSCs respond to Wnt signalling *in vivo* (Fig. 3), indicates that Wnt/β-catenin signalling is an important physiological mediator of HSC-derived haematopoiesis.

β-catenin upregulates HoxB4 and Notch1 in HSCs

We wished to determine whether Wnt signalling might be regulating HSC self-renewal by upregulating genes previously implicated in HSC self-renewal. To this end we tested upregulation of HoxB4 and Notch1 (refs 20, 21). By using real-time polymerase chain reaction (PCR) analysis on HSCs infected with either β-catenin or control vector, we found that HoxB4 was upregulated an average of 3.5-fold and Notch1 was upregulated 2.5-fold (Fig. 5a). In contrast, Gapdh expression was not differentially regulated as a consequence of β-catenin expression, and was used as a control (Fig. 5b). These data show that genes so far identified as regulators of HSC self-renewal may be related and perhaps act in a molecular hierarchy.

Discussion

Our study shows that components of the Wnt signalling pathway can induce proliferation of purified KTLS bone marrow HSCs while significantly inhibiting their differentiation, thereby resulting in functional self-renewal. We find that expression of β-catenin in HSCs results in increased growth with significantly reduced differentiation *in vitro* for a period of at least many weeks. HSCs transduced with β-catenin give rise to sustained reconstitution of myeloid and lymphoid lineages *in vivo*, when transplanted in limiting numbers. We also find that Wnt signalling is required for the growth response of normal HSCs to other cytokines, as overexpression of axin leads to reduced stem cell growth both *in vitro* and *in vivo*. Furthermore, the inhibition of HSC growth with frizzled-CRD and the finding that Wnt3a causes expansion of HSCs supports the interpretation that the effects of β-catenin and axin reflect upstream Wnt activity. Finally, studies with HSCs containing a LEF-1/TCF reporter indicate that HSCs *in vivo* respond to endogenous Wnt stimulation. The expression of a number of Wnt proteins in the bone marrow[5] and frizzled receptors in bone-marrow-derived progenitors and HSCs supports this possibility[22].

Most growth factors that act on HSCs in culture induce no or limited expansion[23] or are unable to prevent differentiation[8,24]. Thus, one of the most notable findings of our work is the induction of proliferation and the prevention of HSC differentiation by the Wnt signalling pathway. Other signals that increase proliferation of HSCs include Notch[20] and sonic hedgehog[25]. Moreover, the cyclin-dependent kinase inhibitor p21$^{Cip1/Waf1}$ (ref. 26) and the transcription factor HoxB4 (ref. 21) have been shown to be involved in regulating self-renewal of HSCs. Notably, Wnt signalling has been shown to interact with many of these pathways in a variety of organisms[27–30], and our data show that both HoxB4 and Notch1 are upregulated in response to Wnt signalling in HSCs. This raises the possibility that the effects of Wnt signalling on HSCs are mediated through HoxB4 and/or Notch1. Whereas HoxB4 could act directly on these HSCs as demonstrated previously[21], Notch1 action would require nearby Notch1 ligands.

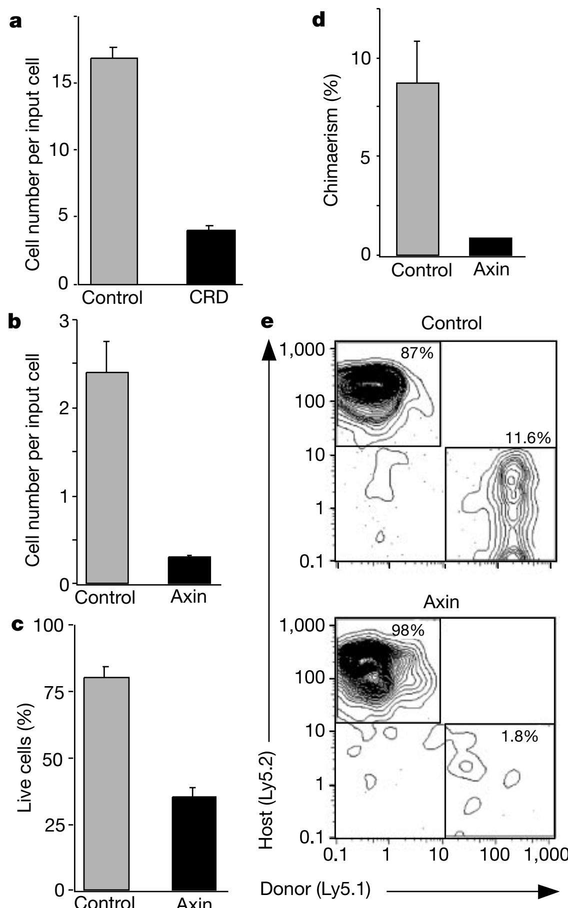

Figure 4 Inhibition of Wnt signalling reduces growth of HSCs *in vitro* and inhibits reconstitution *in vivo*. **a**, HSCs (20 cells per well) were cultured for 60 h in medium containing mitogenic factors and either IgG–CRD or control IgG. **b**, HSCs were infected with virus encoding axin–IRES–GFP or GFP alone. Growth of infected HSCs in the presence of mitogenic factors was monitored over 60 h. **c**, The number of live cells was determined by propidium iodide staining. **d**, **e**, The development of HSCs *in vivo* was determined by injecting 1,000 control or axin-infected cells per mouse into groups of four lethally irradiated, allelically marked (Ly5.2) host mice along with 300,000 competing syngeneic bone marrow cells. Cells were isolated from peripheral blood and analysed by flow cytometry after >10 weeks. Donor-derived (Ly5.1$^+$) cells were monitored in the peripheral blood of hosts; analysis from a representative recipient and average reconstitution is shown.

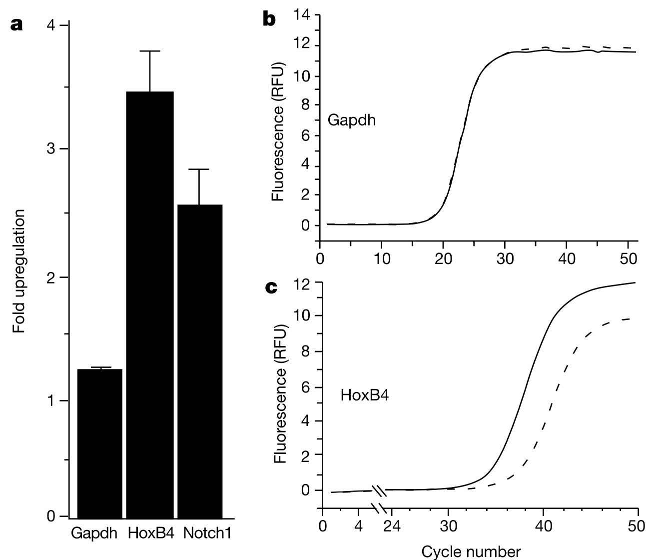

Figure 5 HSCs expressing β-catenin upregulate HoxB4 and Notch1. **a**, Purified wild-type HSCs were infected with activated β-catenin–IRES–GFP or control vector–IRES–GFP, and infected cells sorted based on GFP expression at 48 h. The RNA isolated from these cells was reverse transcribed and expression of HoxB4 and Notch1 was analysed by real-time PCR analysis. Results are averaged over five independent PCR reactions. **b**, **c**, Representative graphs of real-time PCR analysis demonstrating equal amounts of Gapdh (**b**) and differential amounts of HoxB4 (**c**) products from β-catenin-transduced HSCs (solid line) and control-transduced HSCs (dashed line). RFU, relative fluorescence units.

© 2003 Nature Publishing Group

The ability of Wnt3a to induce expansion of HSCs is consistent with previous studies showing increased numbers of haematopoietic progenitors from mouse fetal liver and human bone marrow cells stimulated with Wnt-containing supernatants[31,32]. These studies, although consistent with ours, are more difficult to interpret as they did not use purified HSCs, lacked *in vivo* reconstitution analysis, and did not provide evidence of a physiological requirement for Wnt signalling for HSCs. Components of the Wnt pathway have also been shown to promote proliferation of primitive cells in the skin[33,34], the gut[35,36] and the brain[37], and to inhibit differentiation to a variety of lineages in embryonic stem cells[38], raising the possibility that Wnt signalling may be used as a general cue for self-renewal in stem and/or progenitor cells from diverse tissues. Its role as a self-renewal signal does not, however, preclude its involvement in differentiation of stem cells in certain contexts[39].

Our findings may have important implications for human haematopoietic cell transplantation. We have found that soluble Wnt3a protein induces proliferation of highly purified human bone marrow HSCs in the absence of any other growth factor (T.R., T. Miyamoto and I.L.W., unpublished observation). Induction of HSC growth by Wnt signalling may allow *in vitro* expansion of a patient's own or an allogeneic donor's HSCs, and could provide an increased source of cells for future transplantation. Finally, we have previously raised the hypothesis that self-renewal is a property that could be dangerous, as an adequate definition of cancer stem cells is poorly regulated self-renewal of a particular stage of a developmental lineage[40]. The demonstration here that the Wnt/β-catenin pathway may have a role in haematopoietic stem cell self-renewal leads us to propose that this pathway should be studied for a role in self-renewal of cancer stem cells. □

Methods

Mice

C57Bl/Ka Ly5.1, Thy-1.1 (wild-type and BCL-2), C57Bl/Ka Ly5.2, Thy-1.1, and AKR/J mice were used at 6–10 weeks of age. Mice were bred and maintained on acidified water in the animal care facility at Stanford and Duke University Medical Centers.

HSC isolation

We sorted HSCs from mouse bone marrow as described[41]. All cell sorting and FACS analysis was carried out on a FACSVantage (Becton Dickinson) at the Stanford shared FACS facility and the Duke Cancer Center FACS facility. Cells were sorted and reanalysed on the basis of expression of c-Kit, Sca-1, low levels of Thy-1.1, and low to negative levels of lineage markers (Lin).

Cell cycle analysis

Retrovirally transduced HSCs were collected from cultures and stained with Hoechst 3342 (Molecular Probes) at 37 °C for 45 min in Hoechst medium[9]. Cells were then washed and analysed by Flow cytometry to determine the cell cycle profile of GFP^+ cells.

Viral production and infection

Virus was produced by triple transfection of 293T cells with murine stem cell virus constructs along with gag-pol and vesicular stomatitis virus G glycoprotein constructs. Viral supernatant was collected for three days and concentrated 100-fold by ultracentrifugation at 50,000*g*. For viral infection, 10,000 HSCs were sorted into wells of a 96-well plate and cultured overnight in the presence of SLF (30 ng ml^{-1}) for BCL-2 transgenic HSCs (used in Fig. 1, Fig. 2 and Table 1), or SLF (30 ng ml^{-1}) plus TPO (30 ng ml^{-1}) for wild-type HSCs (all other figures and Supplementary figures). After 12 h, concentrated retroviral supernatant was added to the cells at a 1:1 ratio. Cells were then incubated at 32 °C for 12 h and 37 °C for 36 h before GFP^+ cells were sorted for *in vitro* and *in vivo* assays. Lentiviruses used were produced as previously described. Briefly, 293T cells were transfected with the transfer vector plasmid, the VSV-G envelope-encoding plasmid pMD.G, and the packaging plasmid CMVΔR8.74 (ref. 42). The supernatant was collected and concentrated by ultracentrifugation. All cytokines were purchased from R&D systems.

In vitro HSC proliferation assays

Freshly purified or virally transduced HSCs were plated at 1 to 20 cells per well in Terasaki plates. Cells were sorted into wells containing serum-free medium (X-vivo15, BioWhittaker) supplemented with 5×10^{-5} M 2-mercaptoethanol and the indicated growth factors. Proliferation was monitored by counting the number of cells in each well at defined intervals. For longer-term cultures, transduced HSCs were plated in 96-well plates in the absence or presence of SLF (1 ng ml^{-1}), and the number of cells generated was monitored by cell counting at defined intervals. For inhibition of growth by CRD or axin, cells were cultured in the presence of mitogenic factors (SLF (30 ng ml^{-1}), Flt-3L (30 ng ml^{-1}), interleukin-6 (10 ng ml^{-1})).

In vivo analysis of HSC function

Virally transduced HSCs were cultured *in vitro* and injected retro-orbitally into groups of 4–6 congenic recipient mice irradiated with 9.5 Gy using a 200-kV X-ray machine, along with 300,000 rescuing host total bone marrow or Sca-1-depleted bone marrow cells. Host mice were given antibiotic water after irradiation. Transplanted mice were bled at regular periods to determine the percentage of the haematopoietic compartment contributed by donor cells. Donor and host cells were distinguished by allelic expression of CD45 (Ly5) or expression of the BCL-2 transgene.

Lentiviral reporter assays

The enhanced GFP (eGFP) or the d2-eGFP gene (destabilized, half-life of 2 h; Clontech) was cloned downstream of a LEF-1/TCF-responsive promoter, containing three LEF-1/TCF binding motifs and a TATA box[43]. This cassette was then cloned into a self-inactivating lentiviral vector plasmid, and virus was produced as described above.

For *in vivo* assays, HSCs were transduced with reporter lentiviruses and cultured in X-Vivo15 with glutamate, 5×10^{-5} M 2-mercaptoethanol, and a cocktail of cytokines including 10 ng ml^{-1} interleukin-11, 10 ng ml^{-1} TPO, 50 ng ml^{-1} SCF, 50 ng ml^{-1} Flt-3L. Cells were incubated at 37 °C for 6 h overnight and transplanted into lethally irradiated congenic recipients. Lethally irradiated mice received 500 transduced HSCs along with rescue bone marrow. For analysis, haematopoietic progenitor cells were analysed for reporter activation 14–24 weeks after transplantation.

For *in vitro* assays, purified HSCs were sorted directly into medium (IMDM/10% FBS plus interleukin-11, TPO, SCF and Flt-3L, as above) and plated at 500–1,000 cells per well in 96-well plates. Individual wells were transduced with the appropriate lentiviral reporter and stimulated with or without purified Wnt3a (about 100 ng ml^{-1}). Cells were collected 5 days later, stained with propidium iodide to exclude non-viable cells, and analysed for GFP expression.

Real-time PCR analysis

A total of 75,000 HSCs cultured in 96-well plates containing X-Vivo15, 5×10^{-5} M 2-mercaptoethanol and 100 ng ml^{-1} SLF were infected with either β-catenin or control lentiviruses. After two days in culture, transduced cells were isolated on the basis of GFP expression. RNA was prepared using Trizol (Invitrogen) and linearly amplified using a modified Eberwine synthesis[44]. Each amplified RNA was converted to the first strand and analysed for differential gene expression by real-time PCR. Complementary DNAs were mixed with FastStart Master SYBR Green polymerase mix (Roche), primers (Supplementary Information) and real-time PCR was performed using a LightCycler (Roche).

Received 12 December 2002; accepted 27 March 2003; doi:10.1038/nature01593.
Published online 27 April 2003.

1. Spangrude, G. J., Heimfeld, S. & Weissman, I. L. Purification and characterization of mouse hematopoietic stem cells. *Science* **241,** 58–62 (1989); erratum **244,** 1030 (1989).
2. Uchida, N. & Weissman, I. L. Searching for hematopoietic stem cells: evidence that Thy-1.1^{lo} Lin^- $Sca\text{-}1^+$ cells are the only stem cells in C57BL/Ka-Thy-1.1 bone marrow. *J. Exp. Med.* **175,** 175–184 (1992).
3. Morrison, S. J. & Weissman, I. L. The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity* **1,** 661–673 (1994).
4. Weissman, I. L. Stem cells: units of development, units of regeneration, and units in evolution. *Cell* **100,** 157–168 (2000).
5. Reya, T. *et al.* Wnt signaling regulates B lymphocyte proliferation through a LEF-1 dependent mechanism. *Immunity* **13,** 15–24 (2000).
6. Hawley, R. G., Fong, A. Z., Burns, B. F. & Hawley, T. S. Transplantable myeloproliferative disease induced in mice by an interleukin 6 retrovirus. *J. Exp. Med.* **176,** 1149–1163 (1992).
7. Barth, A. I., Stewart, D. B. & Nelson, W. J. T cell factor-activated transcription is not sufficient to induce anchorage-independent growth of epithelial cells expressing mutant beta- catenin. *Proc. Natl Acad. Sci. USA* **96,** 4947–4952 (1999).
8. Domen, J. & Weissman, I. L. 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 (2000).
9. Cheshier, S. H., Morrison, S. J., Liao, X. & Weissman, I. L. *In vivo* proliferation and cell cycle kinetics of long-term hematopoietic stem cells. *Proc. Natl Acad. Sci. USA* **96,** 3120–3125 (1999).
10. Morrison, S. J., Hemmati, H. D., Wandycz, A. M. & Weissman, I. L. The purification and characterization of fetal liver hematopoietic cells. *Proc. Natl Acad. Sci. USA* **92,** 10302–10306 (1995).
11. Randall, T. D. & Weissman, I. L. Phenotypic and functional changes induced at the clonal level in hematopoietic stem cells after 5-fluorouracil treatment. *Blood* **89,** 3596–3606 (1997).
12. Willert, K. *et al.* Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* advance online publication, 27 April 2003 (doi:10.1038/nature01611).
13. Hsieh, J. C., Rattner, A., Smallwood, P. M. & Nathans, J. Biochemical characterization of Wnt-frizzled interactions using a soluble, biologically active vertebrate Wnt protein. *Proc. Natl Acad. Sci. USA* **96,** 3546–3551 (1999).
14. Cadigan, K. M., Fish, M. P., Rulifson, E. J. & Nusse, R. Wingless repression of *Drosophila* frizzled 2 expression shapes the Wingless morphogen gradient in the wing. *Cell* **93,** 767–777 (1998).
15. Zeng, L. *et al.* The mouse Fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. *Cell* **90,** 181–192 (1997).
16. Sakanaka, C., Weiss, J. B. & Williams, L. T. Bridging of β-catenin and glycogen synthase kinase-3β by axin and inhibition of β-catenin-mediated transcription. *Proc. Natl Acad. Sci. USA* **95,** 3020–3023 (1998).
17. Ikeda, S. *et al.* Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3β and β-catenin and promotes GSK-3β-dependent phosphorylation of β-catenin. *EMBO J.* **17,** 1371–1384 (1998).
18. Yamamoto, H. *et al.* Axil, a member of the Axin family, interacts with both glycogen synthase kinase 3β and β-catenin and inhibits axis formation of *Xenopus* embryos. *Mol. Cell Biol.* **18,** 2867–2875 (1998).
19. Hart, M. J., de los Santos, R., Albert, I. N., Rubinfeld, B. & Polakis, P. Downregulation of β-catenin by

 © 2003 Nature Publishing Group

human Axin and its association with the APC tumour suppressor, β-catenin and GSK3 β. *Curr. Biol.* **8,** 573–581 (1998).
20. Varnum-Finney, B. *et al.* Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. *Nature Med.* **6,** 1278–1281 (2000).
21. Antonchuk, J., Sauvageau, G. & Humphries, R. K. HOXB4-induced expansion of adult hematopoietic stem cells *ex vivo*. *Cell* **109,** 39–45 (2002).
22. Ivanova, N. B. *et al.* A stem cell molecular signature. *Science* **298,** 601–604 (2002).
23. Miller, C. L. & Eaves, C. J. Expansion *in vitro* of adult murine hematopoietic stem cells with transplantable lympho-myeloid reconstituting ability. *Proc. Natl Acad. Sci. USA* **94,** 13648–13653 (1997).
24. Audet, J., Zandstra, P. W., Eaves, C. J. & Piret, J. M. Advances in hematopoietic stem cell culture. *Curr. Opin. Biotechnol.* **9,** 146–151 (1998).
25. Bhardwaj, G. *et al.* Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. *Nature Immunol.* **2,** 172–180 (2001).
26. Cheng, T. *et al.* Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science* **287,** 1804–1808 (2000).
27. Hing, H. K., Sun, X. & Artavanis-Tsakonas, S. Modulation of wingless signaling by Notch in *Drosophila*. *Mech. Dev.* **47,** 261–268 (1994).
28. Maloof, J. N., Whangbo, J., Harris, J. M., Jongeward, G. D. & Kenyon, C. A Wnt signaling pathway controls hox gene expression and neuroblast migration in *C. elegans*. *Development* **126,** 37–49 (1999).
29. Hooper, J. E. Distinct pathways for autocrine and paracrine Wingless signalling in *Drosophila* embryos. *Nature* **372,** 461–464 (1994).
30. Ingham, P. W. & Hidalgo, A. Regulation of wingless transcription in the *Drosophila* embryo. *Development* **117,** 283–291 (1993).
31. Austin, T. W., Solar, G. P., Ziegler, F. C., Liem, L. & Matthews, W. A role for the Wnt gene family in hematopoiesis: expansion of multilineage progenitor cells. *Blood* **89,** 3624–3635 (1997).
32. Van Den Berg, D. J., Sharma, A. K., Bruno, E. & Hoffman, R. Role of members of the Wnt gene family in human hematopoiesis. *Blood* **92,** 3189–3202 (1998).
33. Zhu, A. J. & Watt, F. M. β-catenin signalling modulates proliferative potential of human epidermal keratinocytes independently of intercellular adhesion. *Development* **126,** 2285–2298 (1999).
34. Gat, U., DasGupta, R., Degenstein, L. & Fuchs, E. *De novo* hair follicle morphogenesis and hair tumors in mice expressing a truncated β-catenin in skin. *Cell* **95,** 605–614 (1998).
35. Korinek, V. *et al.* Depletion of epithelia stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nature Genet.* **19,** 1–5 (1998).
36. van de Wetering, M. *et al.* The β-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* **111,** 241–250 (2002).
37. Chenn, A. & Walsh, C. A. Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. *Science* **297,** 365–369 (2002).
38. Kielman, M. F. *et al.* Apc modulates embryonic stem-cell differentiation by controlling the dosage of β-catenin signaling. *Nature Genet.* **32,** 594–605 (2002).
39. Huelsken, J., Vogel, R., Erdmann, B., Cotsarelis, G. & Birchmeier, W. β-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. *Cell* **105,** 533–545 (2001).
40. Reya, T., Morrison, S. J., Clarke, M. F. & Weissman, I. L. Stem cells, cancer, and cancer stem cells. *Nature* **414,** 105–111 (2001).
41. Domen, J., Cheshier, S. H. & Weissman, I. L. The role of apoptosis in the regulation of hematopoietic stem cells: Overexpression of BCL-2 increases both their number and repopulation potential. *J. Exp. Med.* **191,** 253–364 (2000).
42. Dull, T. *et al.* A third-generation lentivirus vector with a conditional packaging system. *J. Virol.* **72,** 8463–8471 (1998).
43. Korinek, V. *et al.* Constitutive transcriptional activation by a β-catenin-Tcf complex in $APC^{-/-}$ colon carcinoma. *Science* **275,** 1784–1787 (1997).
44. Wang, E., Miller, L. D., Ohnmacht, G. A., Liu, E. T. & Marincola, F. M. High-fidelity mRNA amplification for gene profiling. *Nature Biotechnol.* **18,** 457–459 (2000).

Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements We thank W. J. Nelson and A. Barth for providing the β-catenin construct; G. Nolan and L. Naldini for providing the retroviral and lentiviral packaging systems; F. Costantini for providing the axin construct; J. Nathans for the Fzd8-CRD IgG construct; H. Clevers and B. Vogelstein for providing the modified LEF-1/TCF elements used in the reporter constructs; A. Carlton for help with proliferation assays; and P. Feliciano for generation of retroviral constructs. We are grateful to L. Jerabek and P. Shahi for laboratory management and technical help; L. Hidalgo for animal care; S. Smith and V. Braunstein for preparation of antibodies; M. Cook for cell sorting; and M. Krangel for use of the Light Cycler. D.C.S. is a fellow of the American Cancer Society, L.A. is a fellow of the Canadian Institute of Health Research and R.N. is an investigator of the Howard Hughes Medical Institute. This work was supported by NIH grants awarded to I.L.W., and funds from the Cancer Research Institute, Leukemia Research Foundation and the NIH awarded to T.R.

Competing interests statement The authors declare competing financial interests: details accompany the paper on **www.nature.com/nature**.

Correspondence and requests for materials should be addressed to T.R. (t.reya@duke.edu).

© 2003 Nature Publishing Group