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β-Catenin Mediates the Establishment and Drug Resistance of MLL Leukemic Stem Cells

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SUMMARY

Identification of molecular pathways essential for cancer stem cells is critical for understanding the underlying biology and designing effective cancer therapeutics. Here, we demonstrated that β-catenin was activated during development of MLL leukemic stem cells (LSCs). Suppression of β-catenin reversed LSCs to a pre-LSC-like stage and significantly reduced the growth of human MLL leukemic cells. Conditional deletion of β-catenin completely abolished the oncogenic potential of MLL-transformed cells. In addition, established MLL LSCs that have acquired resistance against GSK3 inhibitors could be resensitized by suppression of β-catenin expression. These results unveil previously unrecognized multifaceted functions of β-catenin in the establishment and drug-resistant properties of MLL stem cells, highlighting it as a potential therapeutic target for an important subset of AMLs.

INTRODUCTION

Molecular dissection of oncogenic processes that convert normal cells into cancer cells is critical for understanding transformation mechanisms and for designing effective therapeutics. Although significant progress has been made in recent years in isolating tissue-specific cancer stem cells (CSCs) from patient samples (Ailles and Weissman, 2007; Gupta et al., 2009), very little is known about the natural history and molecular pathways responsible for the oncogenic conversion and establishment of CSCs. In acute myeloid leukemia (AML) where leukemic stem cells (LSCs) have been functionally defined (Bonnet and Dick, 1997; Lapidot et al., 1994), we and others have previously shown that fusion proteins encoded by *Mixed Lineage Leukemia* (*MLL*) gene acting as an initiating event can convert normal murine hematopoietic stem cells (HSCs) or myeloid progenitors into AML pre-LSCs (Cozzio et al., 2003; So et al., 2003) by aberrant recruitment of histone modification enzymes such as Dot1L (Okada et al., 2005) and Prmt1 (Cheung et al., 2007) that mediates epigenetic reprogramming to confer stem cell-like properties to the transformed cells (Krivtsov et al., 2006, 2008; Zeisig et al., 2008). In parallel, human pre-LSCs have also recently been identified in childhood acute lymphoid leukemia carrying TEL-AML1 fusion, which is capable of generating pre-LSC clones that may then convert to LSCs with acquisition of additional genetic or epigenetic events (Greaves and Wiemels, 2003; Hong et al., 2008). The molecular pathways responsible for the conversion of pre-LSCs to LSCs with unlimited self-renewal and alleged drug-resistant properties that are believed to be key for maintenance and reinitiation of the disease during relapse (Reya et al., 2001) are still a largely unexplored area critical for not only understanding the biology of the disease but also designing effective cancer therapeutics.

RESULTS

Characterization of MLL Pre-LSC and LSC-Enriched Populations

To gain further insights into the potential pathways responsible for the conversion and establishment of LSCs, we molecularly

Significance

Multiple genetic and/or epigenetic events are required for development of leukemic stem cells (LSCs) carrying unlimited self-renewal and alleged drug-resistant properties for maintenance and initiation of the disease. Here, we show that activation of the Wnt/β-catenin pathway plays critical roles for establishment of MLL LSCs and conferring drug-resistant properties. Genetic ablation of β-catenin completely abolished development of MLL LSCs. Its suppression reversed MLL LSCs to a pre-LSC-like stage and sensitized their in vitro and in vivo responses to GSK3 inhibitor treatment. β-Catenin, which is largely dispensable for normal function of HSCs, is frequently activated in acute myeloid leukemia (AML); thus, identification of essential functions of β-catenin for MLL LSCs reveals it as a potential therapeutic target for selective eradication of AML stem cells.

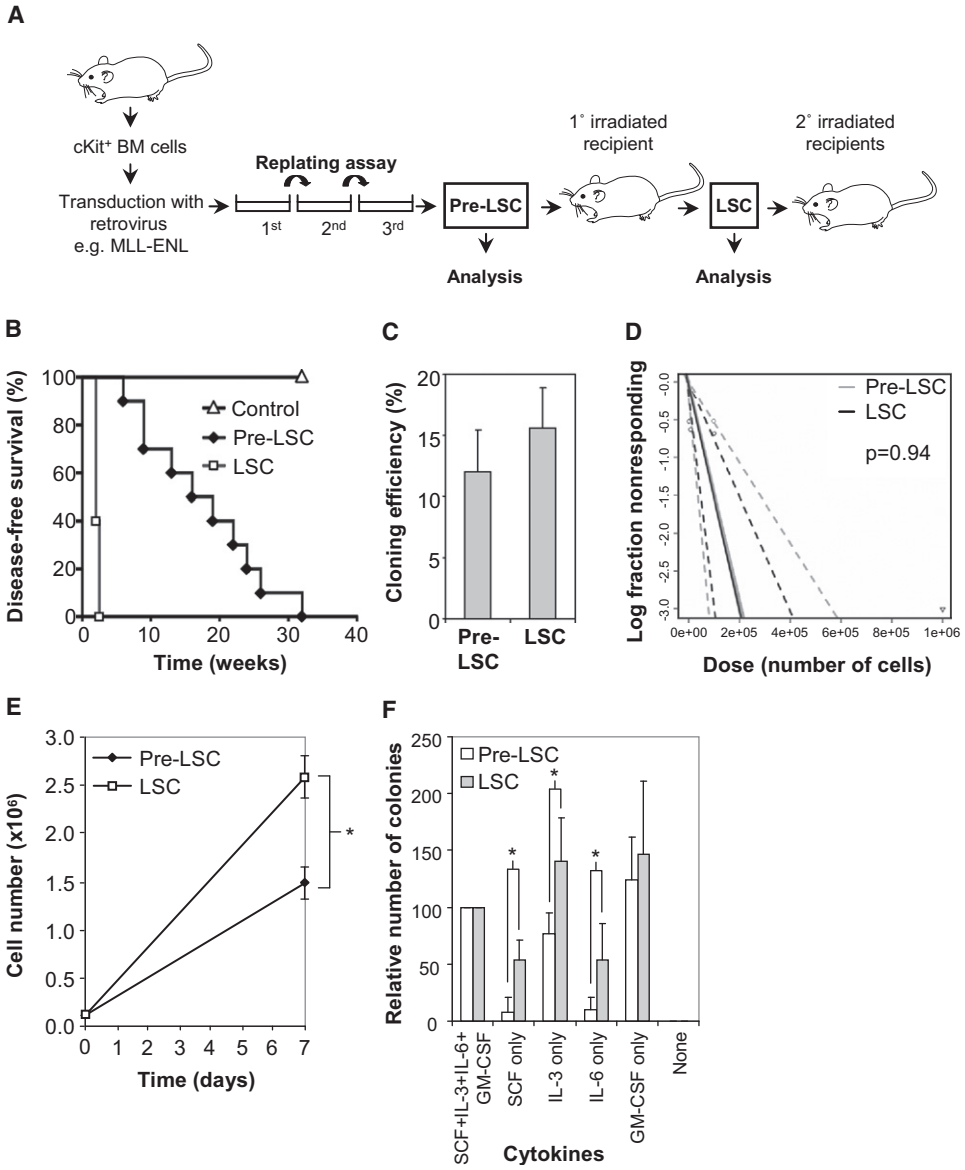

Figure 1. Characterization of MLL Pre-LSC and LSC-Enriched Populations

(A) Schematic diagram illustrating the leukemogenic assay.

(B) MLL-ENL LSCs induce leukemia in mice with significantly shorter latency than pre-LSCs (n = 10 for each cell line). Control indicates mice injected with normal bone marrow cells only.

(C) MLL pre-LSC and LSC-enriched populations had similar cloning efficiencies as determined by the replating assay in methylcellulose medium. Data representative of three independent experiments are shown. There was no significant difference, $p > 0.05$.

(D) LDAs demonstrated that pre-LSCs and LSCs have similar frequencies of leukemia-initiating cells. Dotted lines show 95% confidence intervals.

(E) MLL LSC-enriched population exhibited a higher proliferation rate than pre-LSCs in OP9 stroma coculture. Data representative of three independent experiments are shown ± SD *$p < 0.05$.

(F) Cytokine-dependence assay in methylcellulose showed differences in cytokine requirements between MLL pre-LSC and LSC-enriched populations. Data from three experiments are shown + SD *$p < 0.05$.

dissected and compared the cell populations enriched in MLL pre-LSCs and LSCs, respectively, in our stepwise murine model system (Figure 1A) (So et al., 2003; Somervaille and Cleary, 2006; Wong et al., 2007; Zeisig and So, 2009). In this model, pre-LSCs were identified as early transduced primary cells carrying the initiating event and endowed with the ability to induce leukemia in vivo with a long latency (Figures 1A and 1B), whereas LSCs were defined as leukemic cells harvested from primary mice transplanted with pre-LSCs that had then acquired additional events and were capable of inducing leukemia in secondary mice with a very short latency (Figures 1A and 1B). Despite a major difference in latencies for leukemia induction (pre-LSCs mean survival: 122 ± 19 days; LSCs mean survival: 15 ± 1 days) (Figure 1B), MLL pre-LSC and LSC-enriched populations

Table 1. Summary of LDA Results

	Frequency of Target Cells	95% Confidence Interval
Murine MLL leukemic cells		
—Pre-LSCs	1 in 70,218	26,214–188,087
—LSCs	1 in 67,505	34,600–131,704
Human MLL LTC-IC		
—MLL-1 Empty Vector	1 in 41	28–60
—MLL-1 sh1D	1 in 102	71–148
—MLL-1 sh22	1 in 138	96–200
—MLL-2 Empty Vector	1 in 948	528–1703
—MLL-2 sh1D	1 in 6710	1667–27,010
—MLL-2 sh22	1 in 8930	1805–44,194

exhibited similar morphology and immunophenotypes, and induced AML with very similar, if not identical, phenotypes (see Figures S1A–S1C available online). Most importantly, MLL pre-LSC and LSC-enriched populations had similar (12%–15%) cloning efficiency, which has been previously shown to directly correlate with the abundance of CSCs (Somervaille and Cleary, 2006) (Figure 1C). This is further confirmed by the observation of similar cloning efficiency of c-Kit+/Mac-1+/Gr1+ cells of these two populations, as determined by the single cell-sorting approach (18%–22%) (Figure S1D). To finally assess the frequency of stem cells in these two populations, in vivo transplantation experiments in combination with limiting dilution assays (LDAs) were performed (Hu and Smyth, 2009). Consistent with the results from in vitro clonogenic assay, MLL pre-LSC and LSC-enriched populations possessed very similar frequency of leukemia-initiating cells (approximately 1 in 70,000 versus 1 in 67,000, respectively), and the modest difference was not statistically significant ($p = 0.94$) (Figure 1D, and Table 1; Table S1). The frequency of MLL pre-LSCs was in the same range as one previously reported for a different MLL fusion (MLL-AF9, 1 in 66,000) (Somervaille and Cleary, 2006). The frequency of LSCs was also consistent with the frequency determined for human AML stem cells (1 in 10,000–10,000,000) (Kennedy et al., 2007) and those reported for MOZ-TIFII retroviral-transplanted murine model (1 in 10,000–100,000) (Huntly et al., 2004). Despite the similar frequency of stem cells in these two populations, the MLL LSC-enriched population displayed much faster proliferative rate and was less dependent on cytokines (Figures 1E and 1F). Collectively, these results suggest important qualitative differences between MLL pre-LSCs and LSCs.

Activation of the Wnt/β-Catenin Pathway in the MLL LSC Population

To identify pathways that are potentially responsible for the difference, we performed global expression analysis of MLL pre-LSC and LSC-enriched populations. Although their expression profiles exhibited a very high degree of similarity, pre-LSCs and LSCs did cluster together within their corresponding subgroups. With a 1.5-fold change cutoff, about 1% (<400) of the genes were differentially expressed between MLL pre-LSC and LSC-enriched populations (Figure S2A and Table S2). Metacore software-based pathway analysis on both grouped and matched pair comparisons consistently revealed that multiple critical components of Wnt signaling were activated in MLL LSCs as compared with pre-LSC-enriched populations (Table S2). By RT-qPCR we confirmed upregulation of all these components, including Wnt receptors, frizzled 4/6 as well as their nuclear target, cyclin D2 in MLL LSC-enriched populations (Figure 2A; Figure S2Band Table S2). In addition, MLL LSCs also had elevated level of the key mediator of nuclear Wnt signaling, the β-catenin protein (Figure 2B; Figure S2C). Because β-catenin was previously shown to promote self-renewal of normal HSCs, its expression in lineage-depleted (early) hematopoietic progenitors and lineage-positive (late) mature cells was determined (Figure 2B; Figure S2C). Lineage-negative progenitor cells expressed high levels of β-catenin, which were downregulated during differentiation into lineage-positive cells, consistently suggesting that a higher level of β-catenin may help in maintaining the stemness of hematopoietic cells. To assess the nuclear distribution of activated β-catenin in MLL pre-LSC and LSC populations, immunofluorescent staining using antibody specific for the active form of β-catenin (i.e., non-phosphorylated on Ser37 or Thr41) was performed and showed that a significant portion of LSCs expressed high levels of activated nuclear β-catenin as compared to pre-LSCs, which predominately expressed low level of nuclear β-catenin (Figures 2C and 2D). These differences were statistically significant ($p < 0.05$). To further demonstrate that nuclear β-catenin was indeed transcriptionally active, MLL LSC and pre-LSC populations were transduced with a Wnt pBARVS reporter, where expression of Venus eYFP was driven by 12 copies of TCF consensus-binding sites (Biechele and Moon, 2008). As a result, although the Wnt reporter exhibited a weak transcriptional activity in the MLL pre-LSC-enriched population, we could detect a significantly higher nuclear β-catenin activity in the MLL LSC-enriched population (Figure 2E). The specificity of the assay was further confirmed by: (1) replacement of the Wnt reporter with an identical reporter carrying mutated TCF consensus-binding sites that resulted in a complete abrogation of the YFP signal (Figure 2E); and (2) human HEK293 cells carrying the Wnt reporter transfected together with an exogenous β-catenin, resulting in strong transcriptional activity and expression of the eYFP reporter (Figure S2D). Consistently, both the mRNA and protein expression of its downstream target, cyclin D2 (Huang et al., 2007), were elevated in LSCs relative to pre-LSC-enriched populations (Figure 2F; Figure S2B). Thus, these results support a differential activation of the Wnt-signaling pathway upon progression from MLL pre-LSCs to LSCs.

Suppression of β-Catenin Expression Delayed MLL Leukemia

To determine its functional significance in acute leukemogenesis, β-catenin in MLL-LSCs was specifically knocked down by an shRNA approach. Reduction of β-catenin expression at both mRNA (~85%) and protein (~90%) levels (Figures 3A and 3B; data not shown) did not significantly alter the morphology or immunophenotype of LSC-enriched populations (Figures S3A and S3B). However, it diminished the in vitro cloning efficiency (Figure 3C), cytokine independence in the presence of only stem cell factor (SCF) or interleukin (IL)-6 (Figure 3D), and significantly delayed the disease latency of LSC (Figure 3E), which were reminiscent of the characteristics of pre-LSCs. Moreover, secondary transplantation experiments revealed that β-catenin

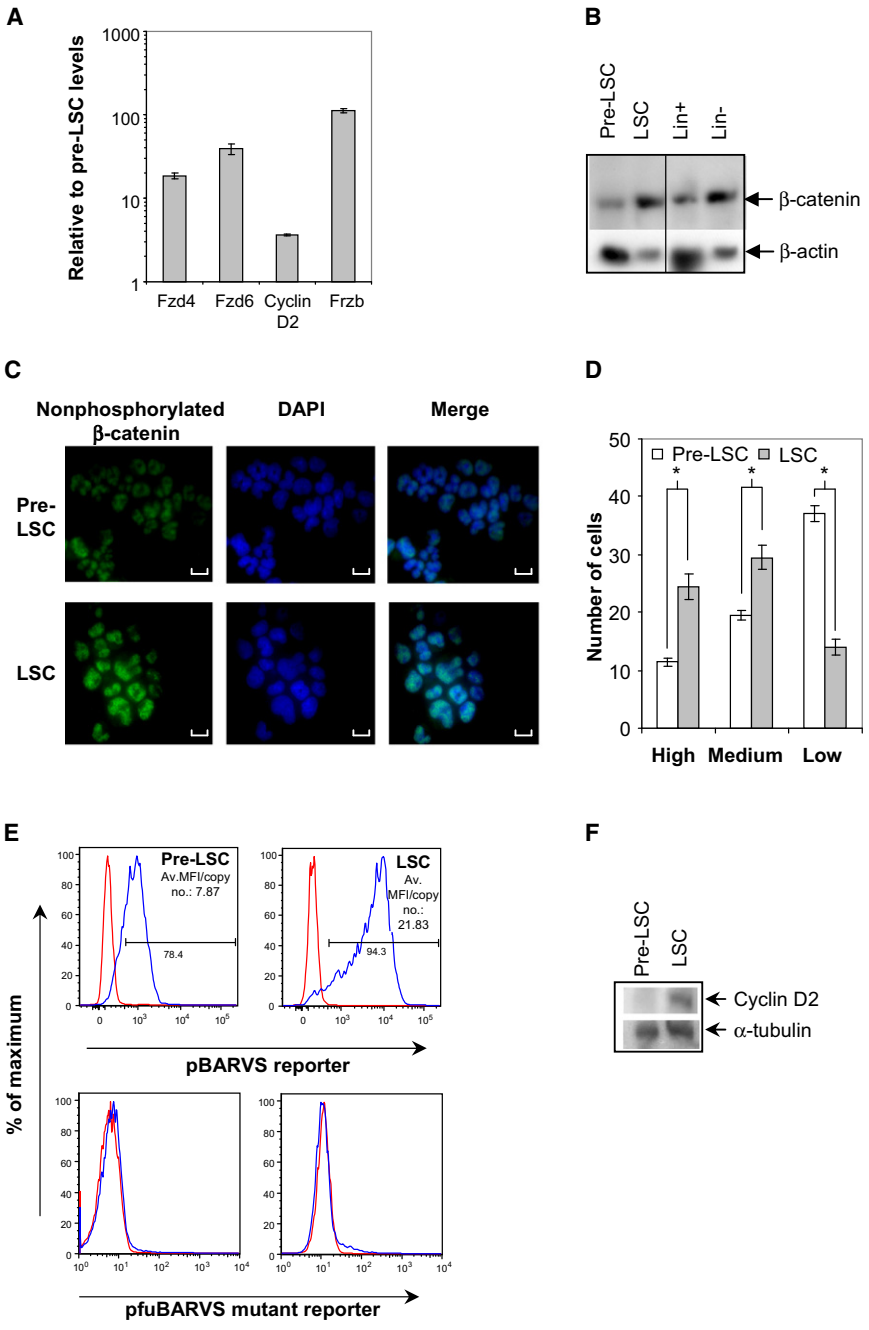

Figure 2. Differential Activation of Wnt/β-Catenin Pathways in MLL LSCs Is Critical for Their Leukemogenic Functions

(A) RT-qPCR validation of differentially expressed genes related to Wnt-signaling pathways identified by global expression array analysis (Figure S2 and Table S2). Data from three experiments are shown ± SD.

(B) Upregulation of total β-catenin in LSC-enriched population compared to pre-LSCs and lineage-positive (Lin+) and lineage-negative (Lin−) cells as determined by western blot.

(C) LSC-enriched population demonstrated higher levels of active nuclear nonphosphorylated β-catenin as determined by immunofluorescence.

(D) Quantification of cells with low, medium, or high levels of nonphosphorylated β-catenin as determined by immunofluorescence. Several fields of cells were scored from two independent experiments ± SD *$p < 0.05$. Scale bar, 10 μm.

(E) Differential upregulation of pBARVS reporter in MLL LSC-enriched population. Average median fluorescent intensities (MFIs) were derived from two independent experiments normalized with the copy number of the reporter in the cells determined by qPCR on genomic DNA. Pre-LSC normalized MFI = MFI: 990/relative copy no.: 127 = 7.87; and LSC normalized MFI = MFI: 9800/relative copy no.: 458 = 21.83. pfuBARVS construct with point mutation in TCF binding sites was also used in the assay and exhibited very similar baseline activity as the untransduced controls.

(F) Upregulation of cyclin D2 protein in LSC-enriched population as determined by western blot.

knockdown also significantly compromised the leukemogenic potential of MLL LSCs, which could otherwise efficiently induce leukemia with a very brief latency and with complete penetrance (Figure 3F). Together with the expression data, these results suggest that activation of β-catenin pathways is functionally critical for the development of LSCs.

β-Catenin Is Required for the Establishment of MLL LSCs

To further test this hypothesis, we conditionally deleted *Ctnnb1* alleles encoding β-catenin at the early pre-LSC stage using floxed *Ctnnb1* bone marrow cells to determine if MLL LSCs could still be established in the absence of β-catenin (Figure 4A). To minimize any long-term toxic effect due to the persistent expression of Cre recombinase, a self-inactivating "hit-and-run" Cre (Silver and Livingston, 2001) was introduced to MLL-ENL-transduced cells. By transient expression of Cre, floxed *Ctnnb1* was deleted in the bone marrow cells and resulted in a complete loss of β-catenin protein expression (Figures 4B and 4C). As determined by in vitro serial replating assays, MLL-ENL still transformed β-catenin-deficient primary hematopoietic cells and generated pre-LSCs in vitro, albeit with lower cloning efficiency as compared with MLL-ENL-transformed cells, which carried intact floxed *Ctnnb1* alleles (Figure 4D). However, it was notable that deletion of β-catenin significantly increased the populations of pre-LSCs that lost the expression of c-Kit, which has been proposed as a marker for enrichment of MLL LSCs (Figure 4E; Figure S4) (Somervaille and Cleary, 2006; Somervaille et al., 2009). To further assess the leukemogenic potential of these pre-LSCs in initiating leukemia, the cells were serially transplanted into syngeneic mice. MLL pre-LSCs with intact

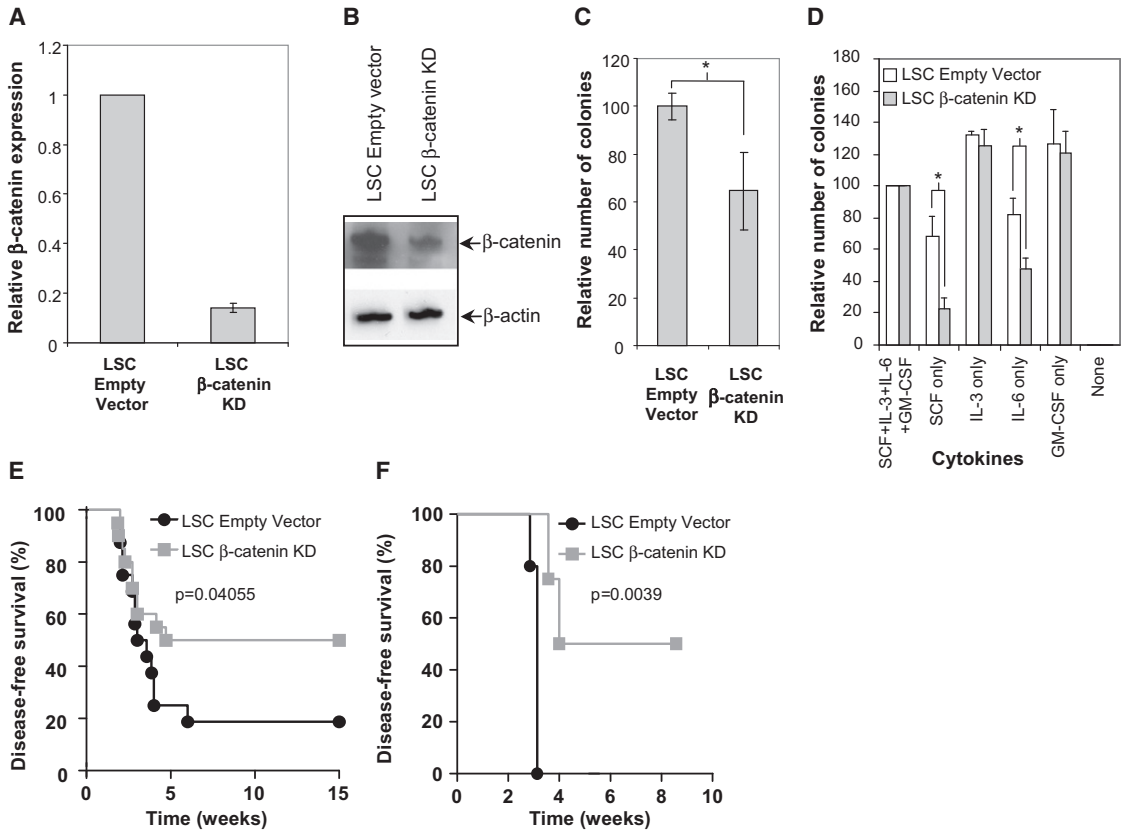

Figure 3. Knockdown of β-Catenin Inhibits Colony Formation and Delays Onset of Leukemia Induced by LSC

(A) RT-qPCR validation of knockdown (KD) of β-catenin. Data representative of three experiments are shown ± SD.

(B) Reduced total β-catenin protein expression after knockdown as determined by western blotting. Knockdown of β-catenin reduced (C) cloning efficiency (data from three independent experiments) and (D) cytokine independence of LSC-enriched population ± SD *$p < 0.05$. Reduced level of β-catenin delayed the onset of leukemia induced by MLL LSCs in both (E) primary (Empty Vector, n = 16; β-catenin KD, n = 20), and (F) secondary transplant mice (Empty Vector, n = 5; β-catenin KD, n = 4).

floxed *Ctnnb1* alleles were able to efficiently induce leukemia in primary and secondary transplanted mice, indicating an efficient conversion of pre-LSCs into full-blown LSCs in the presence of β-catenin. However, the presence of floxed alleles might have a mild impact on the disease progression, as suggested by the relatively small change in disease latency (from 9 to 7 weeks on average) upon the secondary transplant (Figure 4F). In contrast, MLL pre-LSCs with a complete deletion of β-catenin failed to induce leukemia and could no longer be detected in the recipients when mice were sacrificed 20 weeks after transplantation (Figure 4F; data not shown). Together, these results reveal an essential role for β-catenin in the establishment of MLL LSCs.

Suppression of β-Catenin Impaired Human MLL Cells

Although the results from murine models provided strong evidence for a critical function of β-catenin in establishment of murine MLL LSCs, it was not clear whether β-catenin also played an important role in human leukemia. To this end, functional assays were performed using specific shRNAs in combination with three different human systems, including human MLL leukemic cell lines, primary AML cells carrying MLL fusion, and human CD34+ cord blood transduced with MLL fusion. Using shRNAs that effectively knocked down the expression of β-catenin (Figures S5A–S5D), we showed that suppression of β-catenin significantly inhibited the proliferation potentials of HB1119 human leukemic cells carrying MLL-ENL fusion, but not on NB4 leukemic cells carrying the PML-RARα fusion (Figure 5A). Both HB1119 and NB4 leukemic cells were capable of forming leukemic colonies in methylcellulose that could also be serially replated (Figure 5B). Downregulation of β-catenin expression severely inhibited the colony formation ability of HB1119 leukemic cells, whereas β-catenin knockdown did not have significant effect on NB4 leukemic cells (Figure 5B). Suppression of β-catenin exhibited an even more pronounced effect on MLL leukemic cells for their serial replating ability (Figure 5B), which has been used as an in vitro surrogate assay for self-renewal.

Because long-term culturing of leukemic cell lines may have introduced irrelevant genetic/epigenetic events that can complicate the overall interpretation, we also examined the role of β-catenin in patients' primary cells carrying MLL fusions. MLL leukemic cells from two different patients (MLL-1 and MLL-2) were transduced with vector control or β-catenin shRNAs before they were subjected to multiple functional assays, including colony formation, MS5 replating, and LDA. As a result, primary MLL leukemic cells transduced with β-catenin shRNAs

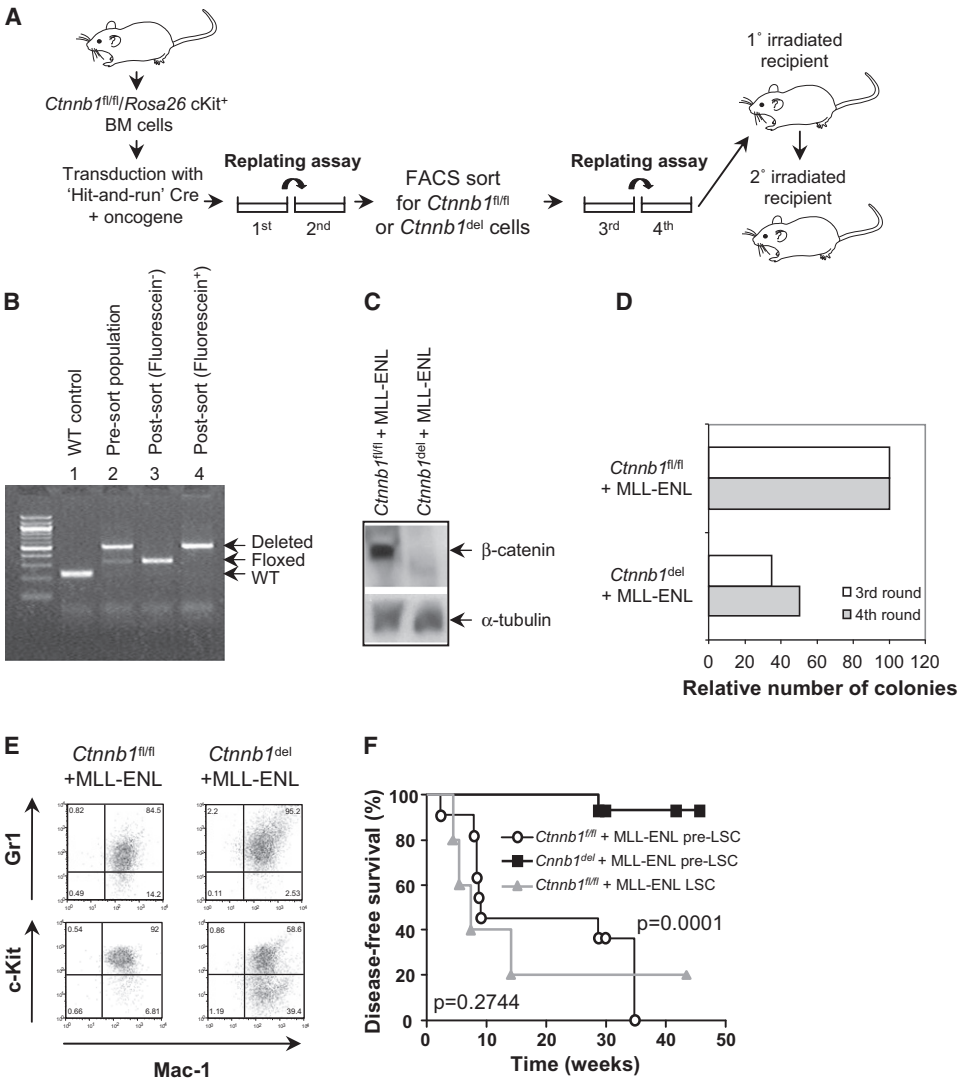

Figure 4. β-Catenin Is Critical for the Establishment of MLL LSCs

(A) Schematic diagram illustrating experimental approaches to assess the functional requirement of β-catenin for establishment of MLL LSCs.

(B) c-Kit+ cells derived from wild-type (lane 1), or *Ctnnb1*$^{fl/fl}$/*Rosa26* floxed-stop-LacZ (lanes 2–4) bone marrows were transduced with retroviruses carrying MLL-ENL and self-inactivating Cre. After the second plating, they were incubated with FDG substrate, which was converted to fluorescein by β-galactosidase activity for FACS. Genotyping of co-transduced cells derived from wild-type bone marrow (lane 1), *Ctnnb1*$^{fl/fl}$/*Rosa26* floxed-stop-LacZ bone marrow prior to FACS (lane 2), post-FACS with fluorescein-negative (lane 3), or fluorescein-positive (lane 4) cells were indicated. Sorted fluorescein-positive cells have Cre-mediated deletion of *Ctnnb1*, whereas fluorescein-negative population maintained the floxed *Ctnnb1* alleles.

(C) Western blot analysis of sorted cells confirmed a complete loss of total β-catenin protein expression in cells derived from fluorescein-positive but not fluorescein-negative cells.

(D) Deletion of β-catenin in MLL-ENL transformed cells inhibited colony formation. Representative data from three independent experiments are shown.

(E) Flow cytometric analysis revealed significant loss of c-Kit expression in MLL-transformed cells derived from β-catenin deficient bone marrow.

(F) Absence of β-catenin abolished in vivo oncogenic potential of MLL-ENL transformed cells (*Ctnnb1*$^{fl/fl}$ + MLL-ENL pre-LSC n = 11; *Ctnnb1*^{del} + MLL-ENL pre-LSC n = 15; *Ctnnb1*$^{fl/fl}$ + MLL-ENL LSC n = 5). *Ctnnb1*$^{fl/fl}$ + MLL-ENL pre-LSCs versus *Ctnnb1*^{del} + MLL-ENL pre-LSCs p = 0.0001. *Ctnnb1*$^{fl/fl}$ + MLL-ENL pre-LSCs versus *Ctnnb1*$^{fl/fl}$ + MLL-ENL LSCs p = 0.2744.

yielded a significantly reduced number of colony-forming unit (CFU)-blasts (Figure 5C), indicating that suppression of β-catenin negatively impacts the cloning efficiency of MLL leukemic cells. A MS5-replating assay was then employed to determine the effect of β-catenin on relatively long-term proliferative potentials of primary MLL cells. Although suppression of β-catenin had little effect on primary MLL leukemic cells in the first 3 weeks of MS5 coculture, a significant reduction of proliferative potentials was observed at 4–5 weeks (Figure 5D), consistent with a putative role of β-catenin in maintaining self-renewal of MLL LSCs. Next, LDAs were performed to determine the frequency of leukemic long-term culture initiating cells (LTC-ICs) upon 3 weeks culture followed by another 3 weeks on MS5 coculture. The frequency of LTC-ICs in MLL-1 and MLL-2 transduced with

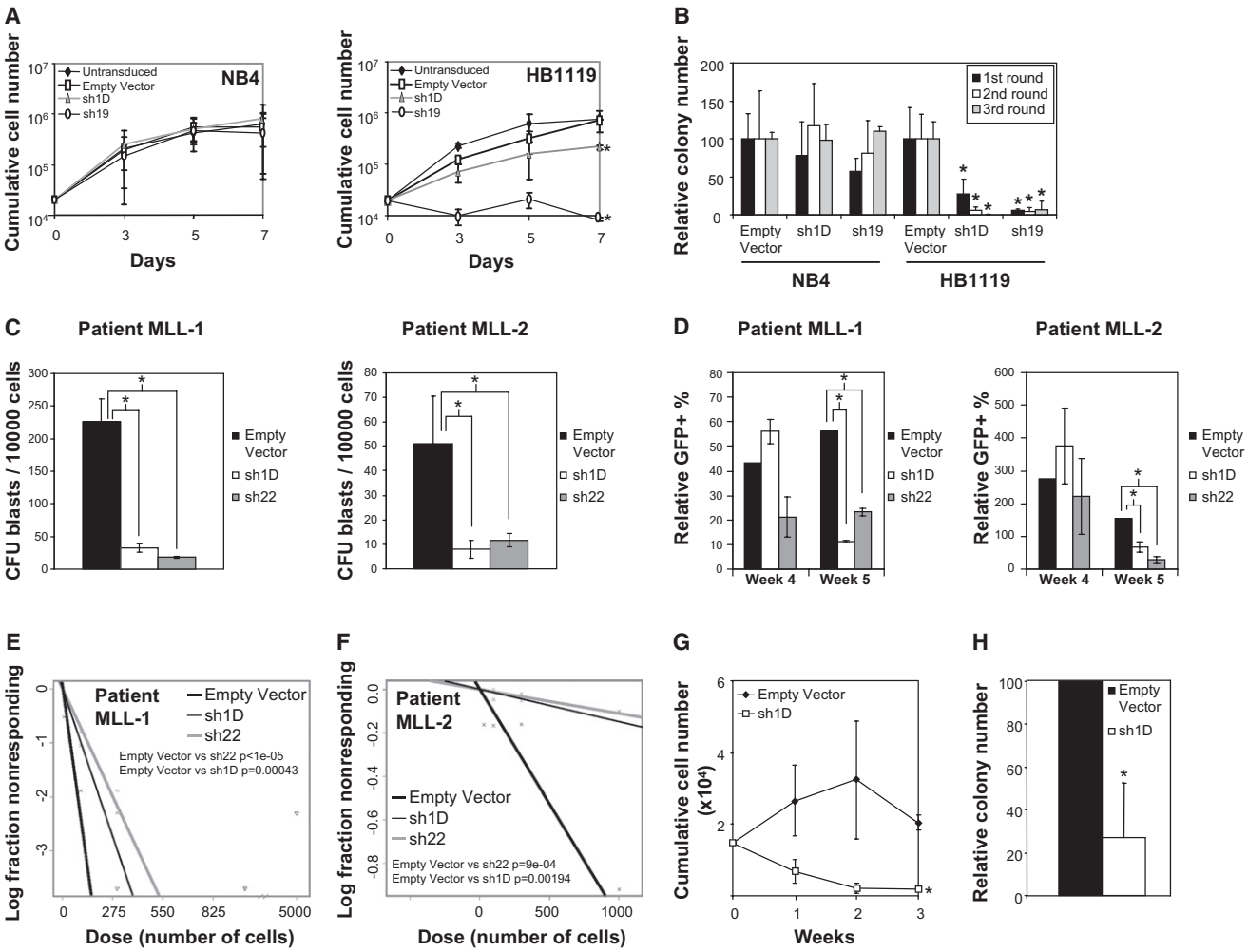

Figure 5. β-Catenin Knockdown Impairs Human MLL Leukemic Cells

(A) β-Catenin knockdown impairs proliferation of HB1119 cells carrying MLL-ENL fusion gene. Cell proliferation was evaluated using MTS assay after 3, 5, and 7 days of culture (sh1D versus Empty Vector, p = 0.048; sh19 versus Empty Vector, p = 0.0032). Data from three independent experiments are shown ± SD. *p < 0.05.

(B) β-Catenin knockdown significantly reduced the cloning efficiency as well as replating ability of HB1119 cells. The number of colonies after each round was normalized against Empty Vector control. Data from three independent experiments are shown ± SD *p < 0.05.

(C) β-Catenin knockdown significantly reduces the frequency of CFU blasts in patients MLL-1 (left panel) and MLL-2 (right panel). Cells were plated in methylcellulose at day 1 after shRNA transduction and counted at day 14. Data for all conditions were then normalized to Empty Vector control using the percentages of GFP+ cells assessed at day 4 (transduction efficiency). Data from three experiments are shown ± SD. *p < 0.05.

(D) β-Catenin knockdown significantly impairs the proliferative potential of AML cells of patient MLL-1 (left panel) and MLL-2 (right panel) between weeks 4 and 5 of co-culturing on MS5 stromal layer. GFP percentages for all conditions are shown in relative to the GFP+ content at day 4, which were set at 100% (Lei et al., 2010). Data from three experiments are shown ± SD. *p < 0.05.

(E and F) Limiting dilution analysis at week 6 of coculture of patients' cells (MLL-1 in E, MLL-2 in F) on MS5 stromal layer indicated that β-catenin knockdown significantly decreased the frequency of long-term initiating cells (also see Table 1).

(G) β-Catenin knockdown impaired proliferation of human CD34+ cord blood (hCB) carrying MLL-ENL fusion gene. Cell proliferation was evaluated by enumeration of viable cells with trypan blue every week for 3 weeks. Data from three experiments are shown ± SD. *p < 0.05.

(H) β-Catenin knockdown reduced the cloning efficiency of MLL-ENL transduced hCB. Number of colonies were normalized versus Empty Vector control (sh1D versus Empty Vector; *p < 0.05). All the data are the means of at least three independent experiments ± SD.

vector control was 1 in 41 and 1 in 948, respectively (Figures 5E and 5F; Table 1). However, specific knockdown β-catenin expression resulted in an average of 3- and 8-fold reduction of LTC-IC frequency in MLL-1 and MLL-2 patients, respectively (Figures 5E and 5F; Table 1), suggesting that β-catenin is required for maintenance of LTC-ICs.

Finally, to assess the potential impact of β-catenin during the early stages of MLL leukemia development, CD34+ human hematopoietic cells derived from cord blood were transduced with MLL-ENL together with vector control or β-catenin shRNA and tested for proliferation and colony formation abilities. MLL-ENL transduced human CD34+ hematopoietic cells efficiently

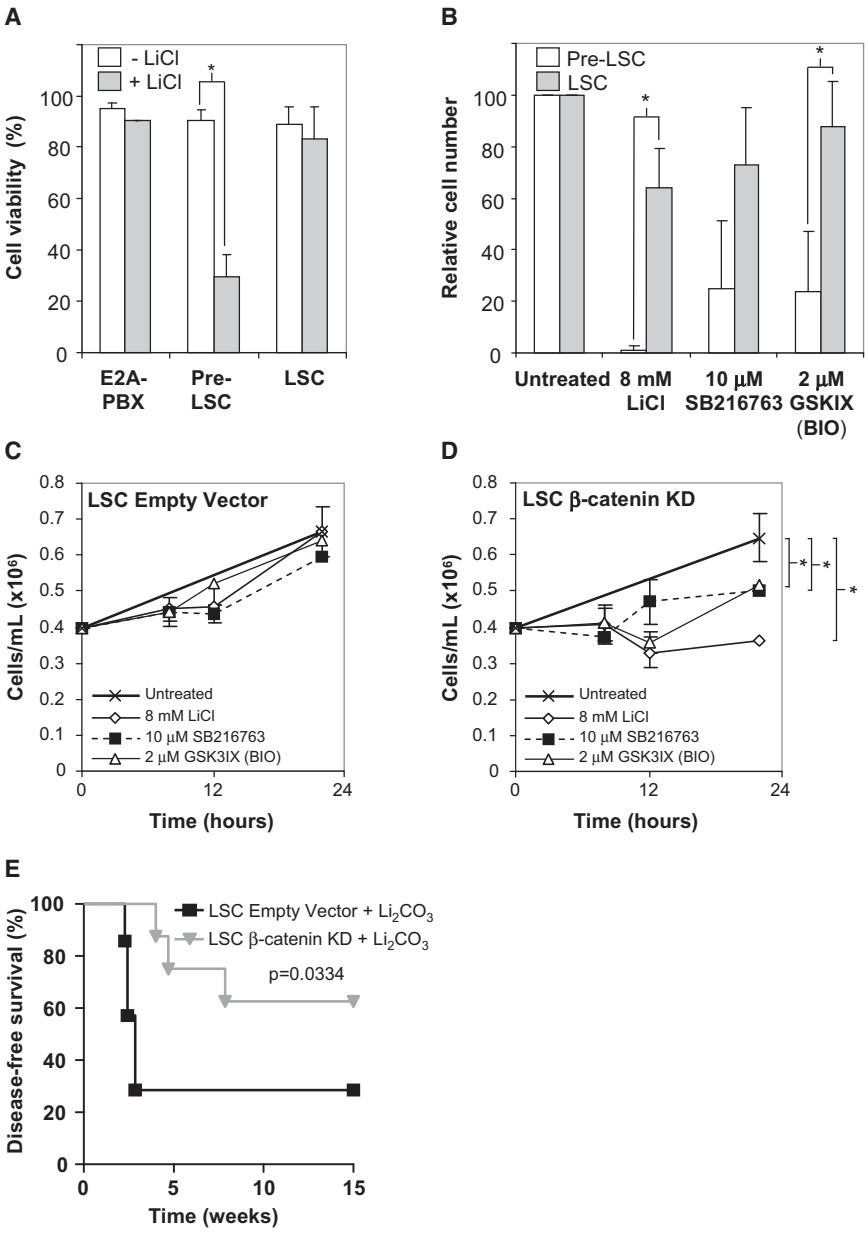

Figure 6. Acquired Drug Resistance of the MLL LSC-Enriched Population to GSK3 Inhibitors Is Dependent on β-Catenin

(A) MLL-LSCs acquired additional drug resistance to GSK3 inhibitors. Differential growth inhibitory effects of LiCl on MLL pre-LSC and LSC-enriched populations after 24 hr treatment. E2A-PBX transformed primary cells were used as a control. Data are means from two experiments ± SD. *$p < 0.05$.

(B) GSK3 inhibitors, LiCl, SB216763, and GSK3IX (BIO) preferentially inhibited the proliferation of MLL pre-LSC but not LSC-enriched populations over 7 days in OP9 coculture. Data are means from two experiments ± SD. *$p < 0.05$.

(C) Modest effect of GSK3 inhibitors on MLL LSCs transduced with Empty Vector control. Representative data are shown from two experiments. There was no significant difference, $p > 0.05$.

(D) Reduction of the β-catenin sensitized MLL LSC-enriched population to GSK3 inhibitors. Representative data are shown from two experiments ± SD. *$p < 0.05$.

(E) GSK3 inhibitor, lithium carbonate, delayed onset of leukemia in mice transplanted with MLL LSCs carrying β-catenin shRNA, but not vector control (Empty Vector, n = 7; β-catenin KD, n = 8).

proliferated and formed colonies in vitro (Figures 5G and 5H). However, β-catenin knockdown significantly suppressed their proliferation on MS5 coculture (Figure 5G) as well as their clonogenic potentials (Figure 5H). Together with the data from established human cell lines and primary AML cells, these results strongly indicate that β-catenin also plays a critical role in human MLL leukemic cells.

Suppression of β-Catenin Resensitized GSK3 Inhibitors Sensitivity of the MLL LSC-Enriched Population

In addition to self-renewal, drug resistance is another important feature of LSCs that forms the critical hypothesis of LSCs as a possible source for relapse (Reya et al., 2001). GSK3 inhibitors have recently been identified as highly specific and effective inhibitors to MLL transformed cells, partially via activation of p27 cell cycle inhibitor (Wang et al., 2008). However, inhibition of GSK3 will at the same time suppress proteasome-mediated inactivation of β-catenin that may counteract their growth inhibitory effect. This led to our hypothesis that GSK3 inhibitors would become less effective in the cells that had high levels and are driven by active β-catenin functions such as MLL LSCs. Therefore, we compared the sensitivity of MLL pre-LSCs and LSCs with differential activation of Wnt/β-catenin signaling to various GSK3 inhibitors at the same concentration as employed in the previous study (Wang et al., 2008). E2A-PBX transformed cells, which have been previously shown to be resistant to GSK3 inhibitors, were used as a control (Wang et al., 2008). As expected, E2A-PBX transformed cells were refractory to LiCl treatment and exhibited only modest inhibition (Figure 6A; Figure S6A). In contrast, the MLL pre-LSC-enriched population was sensitive to LiCl, which rapidly led to a significant cell death within the first 24 hr, as evidenced by its increased sub-G1 population (Figure 6A; Figures S6A–S6C; data not shown). Remarkably, the MLL LSC-enriched population was refractory to the LiCl treatment in both cytokine-supplemented culture and OP9 coculture systems (Figures 6A and 6B; Figures S6A–S6C). Similar but less pronounced inhibitions were also obtained using different GSK3 inhibitors (Figure 6B), indicating that MLL LSCs have acquired additional resistance to GSK3 inhibition. To determine if activation of β-catenin contributed to the resistance, we knocked down β-catenin in the MLL LSC-enriched

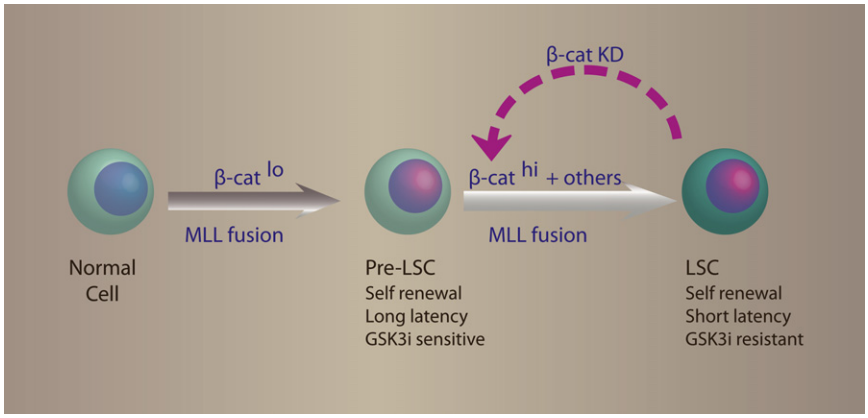

Figure 7. Schematic Diagram Illustrates the Potential Function of β-Catenin during Development of MLL LSCs

MLL fusion transforms normal hematopoietic cells into pre-LSCs expressing low level of β-catenin (β-cat^{lo}). Pre-LSCs with enhanced self-renewal property remains sensitive to GSK3 inhibitor (GSK3i) and induces leukemia in mice with long disease latency. Cooperative genetic and/or epigenetic events including upregulation of Wnt/β-catenin pathway (β-cat^{hi}) convert MLL pre-LSCs into LSCs, which can become GSK3 inhibitor resistant and induce leukemia with very brief latency. Although β-catenin is required for the establishment of MLL LSCs, knockdown of the expression of β-catenin (β-cat KD) in LSCs partially reverses them to a pre-LSC-like stage.

population and repeated the drug treatment. As expected, LSCs transduced with vector control were refractory to GSK3 inhibitor treatment (Figure 6C). However, β-catenin knockdown apparently sensitized MLL LSCs to GSK3 inhibitors, which resulted in 30%–60% suppression of proliferation within 24 hr (Figure 6D). To further investigate if reduced levels of β-catenin had a long-term in vivo effect on the MLL LSC, LSC-enriched population transduced with vector control or β-catenin shRNA was injected into syngeneic mice, which were then fed with normal or Li_2CO_3 diet. As a result, mice transplanted with the control MLL LSC population rapidly developed leukemia, regardless of the treatment, which established that they were resistant to the inhibitor (Figure S6D). In contrast, the Li_2CO_3 diet delayed the disease latency for mice transplanted with the MLL LSC-enriched population expressing the β-catenin shRNA (Figure 6E ; Figure S6E) and, therefore, further supported that β-catenin contributes to the observed drug resistance of MLL LSCs.

DISCUSSION

Although the unlimited self-renewal and acquired drug resistance have been two salient properties of CSCs, very little is known about the underlying mechanisms. In this study, we propose that the activated Wnt/β-catenin pathway is critical for the development and drug-resistant property of MLL LSCs. It is not completely clear at which exact stage (e.g., pre-LSCs versus early LSCs) β-catenin is activated. Given the similar cloning efficiencies and stem cell frequencies yet vastly different disease latencies induced by these two studied populations, we favor the hypothesis that the Wnt-signaling pathway is progressively activated during progression from pre-LSCs to LSCs, but we cannot exclude the possibility that a small number of MLL pre-LSCs primed with activated Wnt signaling expanded exponentially and became dominant during clonal evolution. Although β-catenin is preferentially activated in LSCs, it is likely that a low level of β-catenin is also required for MLL pre-LSCs (Figure 7). This is consistent with the observation that although MLL pre-LSCs and LSCs had similar stem cell frequencies: (1) significant knockdown of the expression of β-catenin in MLL LSCs to a level lower than pre-LSCs resulted in further reduction of colony formation potential (Figure 3); (2) β-catenin knockdown in MLL pre-LSCs reduced their cloning efficiency (data not shown); and (3) deletion of β-catenin in the MLL pre-LSC stage led to reduction of in vitro cloning ability (Figure 4). Although different levels of β-catenin may be required for MLL pre-LSCs and LSCs, ablation of β-catenin at an early transformation stage completely abolished the leukemogenic potential of MLL-transduced cells, clearly indicating that β-catenin is essential for establishment of MLL LSCs from normal hematopoietic cells (Figure 7). Importantly, the role of β-catenin is also conserved in human MLL cells. We demonstrated that inhibition of β-catenin significantly suppressed human leukemic cells carrying MLL fusion in three different model systems. Consistently with the murine data, β-catenin seems to be involved at both early (in CD34+ cells transduced with MLL fusion) and late stages of the leukemic development (in MLL primary leukemic cells at the disease presentation). This function is also preserved even in a long-term passage cell line carrying MLL fusion, suggesting a highly conserved and stable requirement of β-catenin for MLL leukemic cells.

MLL leukemia, particularly in infants, is one of the most aggressive and worst prognostic subgroups among all hematological malignancies. Recent studies showed that its transformation is largely independent of Hedgehog and Bmi1 signaling (Hofmann et al., 2009; and unpublished data). Identification of essential functions of β-catenin in MLL leukemia not only sheds light on the biology of the disease but also provides further opportunities for the development of more effective cancer therapeutics. A very recent study has sophisticatedly shown that GSK3 inhibitors target MLL leukemic cells via suppression of Hox/Pbx/Meis1/Creb activation complex and may also be effective on other Hox-activated leukemias (Wang et al., 2010b). Although these results support the application of GSK3 inhibitors for targeted therapies in AML, their efficacy at eliminating LSCs remains to be demonstrated. In contrast to the MLL pre-LSC-enriched population that was sensitive to GSK3 inhibitor treatment, we showed that MLL LSCs with a high level of nuclear β-catenin activity were refractory to the inhibitors, suggesting the presence of potentially counteracting pathways in LSCs that will allow resistance toward GSK3 or other related inhibitors. Notably, it is unlikely that β-catenin is the only factor that contributes to the conversion of MLL pre-LSCs to LSCs, but suppression of β-catenin in established MLL LSCs diminished

their in vitro cloning efficiency, cytokine independence, in vivo leukemogenic potential, and also GSK3 inhibitor resistance, partially reversing them to a pre-LSC-like stage (Figure 7), providing a strong rationale of targeting β-catenin in MLL leukemia.

β-Catenin is associated with imatinib resistance in chronic myeloid leukemia (CML) (Jamieson et al., 2004) and has recently been shown to be critical for the establishment of aggressive cutaneous CSCs that express high levels of CD34 (Malanchi et al., 2008), which is also upregulated in multiple hematological malignancies including our MLL LSCs (Table S2, and Figures S2E and S2F). The Wnt/β-catenin pathway has been recurrently implicated in different forms of AML (Mikesch et al., 2007) and is activated in self-renewing myeloid progenitors during CML blast crisis (Jamieson et al., 2004), suggesting that activation of β-catenin may be a common pathway shared by different CSCs. However, a complete deletion of β-catenin did not abolish BCR-ABL induced leukemia (Zhao et al., 2007). β-Catenin-deficient CML cells with LSK phenotypes were compromised in transplanting the disease to secondary recipients, but mice still developed transplantable ALL, suggesting a rather limited role of β-catenin in acute leukemogenesis (Zhao et al., 2007). In contrast, the current study provides clear evidence and strong rationales for targeting β-catenin in human acute leukemia associated with MLL fusions. This is also echoed by the recent discovery of an important function of β-catenin in the development of murine AML stem cells from normal granulocyte-macrophage progenitors (GMPs), where β-catenin is required for leukemic transformation by Meis1/Hoxa9 or MLL-AF9 fusion (Wang et al., 2010a). In addition to the functional demonstration of its role in human MLL leukemia, our findings have also further defined the multifaceted function of β-catenin in the establishment of MLL LSCs, in particular for the transition from pre-LSCs to LSCs, and a previously unrecognized function in conferring drug resistance. In contrast to MLL LSCs, β-catenin is largely dispensable for normal HSCs. Bone marrow cells derived from mice deficient in β-catenin or even in combination with γ-catenin do not have any obvious hematopoietic defects under both normal and stress conditions and are capable of fully reconstituting hematopoietic systems upon bone marrow transplantation into syngeneic mice (Cobas et al., 2004; Jeannet et al., 2008; Koch et al., 2008). Thus, identification of essential functions of β-catenin in the establishment of MLL LSCs reveals it as a potential therapeutic target for eradication of AML stem cells but sparing normal HSCs.

EXPERIMENTAL PROCEDURES

Retroviral/Lentiviral Transduction and Transformation Assays

Both RTTAs/LTTAs were performed on primary murine or human hematopoietic cells as previously described, with some modifications (Zeisig and So, 2009; Bonnet, 2009; Schuringa and Schepers, 2009; Wunderlich and Mulloy, 2009).

c-Kit$^+$ cells were isolated from murine bone marrow and were cultured overnight in R10 medium (see Cell Culture details) supplemented with 20 ng/ml SCF, 10 ng/ml IL-3, and 10 ng/ml IL-6. Spinoculation using concentrated viral supernatant was carried out by centrifugation at 800 × g in the presence of 5 μg/ml polybrene (Sigma-Aldrich, Poole, UK) at 32°C for 2 hr. A second spinoculation step was performed on the following day. Cells were plated in M3231 methylcellulose medium (Stem Cell Technologies, Vancouver, Canada) supplemented with recombinant murine 20 ng/ml SCF, 10 ng/ml IL-3, 10 ng/ml IL-6, and 10 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF) and appropriate selection antibiotic on the following day. Colonies were scored after 7 days of culture and replated every 7 days. After the third or fourth round of plating, cells were cultured in R20/20 (see Cell Culture details) to establish cell lines. All recombinant murine cytokines were purchased from PeproTech EC, London, UK.

For human cell studies, frozen AML samples were thawed and prestimulated for 4 hr at 37°C in Stem Span medium (Stem Cell Technologies) supplemented with 20 ng/ml each of recombinant human IL-3, granulocyte-colony-stimulating factor (G-CSF), and thrombopoietin (TPO) (PeproTech, Rocky Hill, NJ, USA) and then incubated overnight in Stem Span medium supplemented with 100 ng/ml SCF, 100 ng/ml Flt3-ligand (Flt3-L), 60 ng/ml IL-3, and 10 ng/ml TPO in the presence of 4 μg/ml polybrene and of viral particles at a multiplicity of infection of 30. Cells were then washed and either cocultured on MS5 stromal layer (see LDA section), plated in triplicate in H4330 MethoCult media (Stem Cell Technologies) for CFU blast leukemic colony assay. For hCB studies, purification and transduction of hCB cells were performed as previously described (Wunderlich and Mulloy, 2009). Briefly, density gradient purified fresh cord blood was selected for CD34+ cells using the human CD34 purification MidiMACS purification kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and subsequently cultivated in hCB expansion medium (IMDM supplemented with 10% heat-inactivated fetal calf serum [FCS], 10^{-4} M β-mercaptoethanol [β-ME], antibiotics, and 10 ng/ml each of human recombinant SCF, TPO, Flt3-L, IL-3, and IL-6) for 24 hr at 37°C and 5% CO_2 prior to spinoculation at 800 × g in the presence of 5 μg/ml polybrene at 32°C for 2 hr with lentiviral supernatant. At 48 hr after transduction, cells were selected with puromycin and blasticidin (for MLL fusion) for 72 hr. Cells were subsequently plated into (1) H4100 methylcellulose (Stem Cell Technologies) containing 20% heat-inactivated FCS, 10^{-4} M β-ME, 20% IMDM, 2 mM L-glutamine, and 10 ng/ml each of human recombinant SCF, IL-6, IL-3, G-CSF (PeproTech EC), and 6 U/ml erythropoietin (EPO) (R&D Systems, Abingdon, UK) (Wunderlich and Mulloy, 2009); and (2) coculture with irradiated MS5 stromal cells in long-term culture (LTC) medium (alpha-MEM supplemented with heat-inactivated 12.5% FCS, heat-inactivated 12.5% horse serum [Sigma-Aldrich], 2 mM L-glutamine, 57.2 μM β-ME, and 20 ng/ml each of human recombinant TPO, IL-3, and G-CSF) (Schuringa and Schepers, 2009). Cultures were kept at 37°C and 5% CO_2 and replated on new MS5 stroma every week. At nominated time points, cell number and viability were evaluated with trypan blue or MTS assay using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay kit according to the manufacturer's instructions (Promega, Southampton, UK).

Immunofluorescence Staining for Active Nonphosphorylated β-Catenin

A total of 5 × 10^4 cells was cytospun onto glass microscope slides and then fixed with 4% formaldehyde in PBS (pH 7.4) for 30 min on ice. Cells were washed in 1× phosphate-buffered saline (PBS) and permeabilized and blocked using 10% FCS/1% bovine serum albumin (BSA)/0.2% TX-100/PBS for 15 min. Anti-human active nonphosphorylated β-catenin (clone 8E7) (Millipore, Watford, UK) was used at 1:50 dilution in 10% FCS/1% BSA/PBS and incubated overnight at 4°C. Slides were washed three times with PBS and subsequently incubated with 1:100 donkey anti-mouse FITC diluted in 10% FCS/1% BSA/PBS for 30 min at room temperature in the dark. Slides were washed five times at 10 min each with PBS. Slides were incubated with DAPI at 100 μg/ml in PBS for 5 min at room temperature. Slides were washed once in PBS for 10 min and briefly air-dried prior to mounting with Vectashield (Vector Laboratories, Peterborough, UK) and a coverslip. Several fields were scored for low, medium, and high intensity staining.

β-Catenin Knockdown

All of the shRNAs constructs expressed the GFP marker. pSiren-RetroQ-β-catenin murine shRNA and control retroviral constructs were previously published (Cawthorn et al., 2007) and were modified to express GFP. For human shRNAs, pGIPZ lentivirus from OpenBiosystems (Epsom, UK) expressing both GFP and puromycin markers were used. The target sequences, from 5′ to 3′, were CAGATGGTGTCTGCTATTGTA (targeting the position 846–866 of human β-catenin gene sh1D), TAGCTGATATTGATGGACAGT (targeting the

position 505–527 of human β-catenin gene sh19), and GCTCCTTCTCT GAGTGGTAAA (targeting the position 396–417 of human β-catenin gene sh22).

β-Catenin/TCF Reporter Assay

Cells were transduced with pBARVS lentiviral reporter, which contains 12 TCF response elements upstream of β-globin linked to Venus (a variant of eYFP) (Biechele and Moon, 2008). Cells were spinoculated as described above. Cells were expanded, and Venus eYFP$^+$ cells were purified by fluorescence-activated cell sorting (FACS). Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN). pBARVS copy number was determined by qPCR using primers described in Supplemental Experimental Procedures. A standard curve was prepared using serial dilutions of pBARVS plasmid. Copy number was expressed relative to *Gapdh* levels, as determined using TaqMan mouse *Gapdh* primer/probe control reagents (Applied Biosystems). A median fluorescence intensity (MFI) and copy ratio were determined from independent experiments to assess relative levels of nuclear β-catenin activity. As a control for specificity, the pfuBARVS reporter, which contained mutated TCF response elements (Biechele and Moon, 2008), was also used to transduce pre-LSCs and LSCs.

LDA

For in vivo LDAs, varying numbers of cells from pre-LSC or LSC-enriched populations were transplanted into sublethally irradiated syngeneic mice and monitored for disease development. For in vitro LDAs, AML samples were cocultured on MS5 stromal layer in the presence of 20 ng/ml recombinant human IL-3, G-CSF, and TPO (Schuringa and Schepers, 2009). After 3 weeks, 20 individual wells per condition were assessed and scored for GFP status and proliferation. AML samples cocultured on MS5 stromal layer were harvested after trypsinization and analyzed for GFP content in the human CD45+ population (mouse antihuman CD45 APC-Cy7; BD PharMingen) at day 3 and from weeks 1 to 5 using the LSRII cytometer (Becton Dickinson). FlowJo software (Tree Star, Switzerland) was used for analysis. Replating was done at week 3 with an equal number of GFP+ sorted cells into a new layer of MS5 stroma.

In Vitro Drug Studies

Drug studies were carried out using cells at 4×10^5 cells/ml in R20/20 or 0.5×10^4 to 1×10^5 cells/ml for OP9 coculture in the absence or presence of 10 μM SB216763 (Sigma-Aldrich), 8 mM lithium chloride (Sigma-Aldrich), and 2 μM GSK3 inhibitor IX (BIO) (Cayman Chemicals). For cell cycle analysis, cells were washed in cold PBS and then fixed with ice-cold 70% ethanol for least 30 min at 4°C. Fixed cells were washed with SM (PBS; 0.8% FCS) and resuspended in 500 μl of 40 μg/ml propidium iodide and 100 μg/ml RNase A (Invitrogen) in SM and incubated at 37°C for 20 min and analyzed by flow cytometry. Cell viability was determined with trypan blue.

Animals and Drug Treatment Studies

All experimental procedures were approved by King's College London, Institute of Cancer Research, and London Research Institute ethics committees and conform to the UK Home Office regulations. *Ctnnb1*$^{fl/fl}$ mice (Brault et al., 2001) were crossed with the *Rosa26* mice reporter line (Soriano, 1999). Compound homozygous mice were used for experiments. For transplantation experiments, C57BL/6 or SJL mice were given 8.5 Gy total body γ-irradiation and injected intravenously with up to 1×10^6 test cells mixed with 0.2×10^6 C57BL/6 or SJL bone marrow mononuclear cells. For drug studies, lithium carbonate treatment commenced on the day after irradiation and injection of cells. Mice were given 0.4% lithium carbonate containing chow (LabDiet 5002; PMI Nutrition International) with 0.9% saline every other day for 1–2 weeks to minimize toxicity from lithium-induced polyuria. Mice were then subsequently maintained on 5 days of lithium carbonate diet and saline alternated with 2 days regular diet and water for the remainder of the experiment.

Microarray Studies

Total RNA from pre-LSCs, LSCs, and clonal derivatives of each were processed using protocols derived from the Affymetrix GeneChip Whole Transcript (WT) Sense Target Labeling Assay Manual. Affymetrix GeneChip Mouse Exon 1.0ST arrays were hybridized and scanned with an Affymetrix GeneChip scanner 3000 running GCOS software. Analyses of whole transcript expression levels were carried out. Normalized data were then filtered for genes displaying a fold change of 1.5 or greater between pre-LSCs and LSCs. Metacore pathway analysis and data mining software (GeneGo) were used to identify pathways of interest.

Real-Time Quantitative PCR

RT-qPCR was performed using SYBR Green reagents on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems) using primers listed in Supplemental Experimental Procedures. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008).

Purification of *Ctnnb1*^{del} Cells

Fluorescein di-β-galactopyranoside (FDG) (Invitrogen) was used as a fluorogenic substrate for the *Rosa26* reporter according to the manufacturer's instructions. Fluorescein-positive and -negative cells were purified by FACS. *Ctnnb1*^{del} and *Ctnnb1*^{fl} alleles were detected by PCR as previously described (Brault et al., 2001) using primers listed in the Supplemental Experimental Procedures.

Cloning Efficiency and Cytokine-Dependence Assays

A total of 1×10^3 cells was plated into M3231 methylcellulose medium (Stem Cell Technologies) containing four cytokines or single cytokines. Cytokines were used at the concentrations as described previously. Colony numbers were enumerated on day 7. Cloning efficiency was also determined by sorting single cells into wells of 96-well plates that contained 100 μl cytokine-supplemented M3231 methylcellulose.

Western Blot Analysis

Membranes were probed with anti-β-catenin antibody (clone 14/Beta-Catenin) (BD) or anti-Cyclin D2 Ab-2 (clone DCS-3.1) (Thermo Fisher Scientific). Western blots were scanned and quantified using ImageJ software from the National Institutes of Health.

Cell Culture

All murine myeloid cell lines were maintained in R20/20 medium (RPMI 1640, 20% FCS, 20% WEHI-conditioned medium as a source of IL-3, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). Conditioned medium was harvested from WEHI cells grown to confluency over 3 days in R10 medium (RPMI 1640, 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) and sterile filtered using a 0.22 μm filter. OP9 stroma cells were maintained in alpha-MEM medium supplemented with 20% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. For coculture studies, OP9 cells were irradiated at 30 Gy to inhibit cell growth and plated at a density of approximately 1×10^4 cells/cm^2 in 12-well plates. Irradiated cells were incubated overnight before hematopoietic cells were seeded. The medium was replenished every other day by a half-medium change.

Flow Cytometric Analysis of Murine Cell Lines and Leukemic Mice

Protocols and reagents used were as previously described (Yeung and So, 2009; Zeisig and So, 2009).

Statistical Analysis

Two-tailed Student's t test was used to determine statistical significance for all bar charts except for the human AML patient data, which was preformed using R (R Development Core Team, 2010). The log-rank test and Gehan-Breslow-Wilcoxon test were used to compare survival curves. For the colony assays comparison, a generalized linear model using a Poisson family with log link using the glm function was used. For the comparison of GFP evolution over time in MS5 cocultures, a negative binomial model, as implemented in glm.nb, was used (package MASS) (Venables and Ripley, 2002). Analysis of deviance tests were carried out with the appropriate call to function ANOVA. LDA plots were produced using the limdil function of package statmod (Smyth et al., 2010), together with a plotting function slightly modified using the plotrix package (Lemon, 2006). p values less than 0.05 were considered statistically significant.

ACCESSION NUMBERS

Microarray data were deposited in the ArrayExpress public database at the European Bioinformatic Institute (http://www.ebi.ac.uk/arrayexpress) with accession number E-MEXP-2618.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at doi:10.1016/j.ccr.2010.10.032.

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