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Genetic and Pharmacologic Inhibition of β-Catenin Targets Imatinib-Resistant Leukemia Stem Cells in CML

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SUMMARY

A key characteristic of hematopoietic stem cells (HSCs) is the ability to self-renew. Genetic deletion of β-catenin during fetal HSC development leads to impairment of self-renewal while β-catenin is dispensable in fully developed adult HSCs. Whether β-catenin is required for maintenance of fully developed CML leukemia stem cells (LSCs) is unknown. Here, we use a conditional mouse model to show that deletion of β-catenin after CML initiation does not lead to a significant increase in survival. However, deletion of β-catenin synergizes with imatinib (IM) to delay disease recurrence after imatinib discontinuation and to abrogate CML stem cells. These effects can be mimicked by pharmacologic inhibition of β-catenin via modulation of prostaglandin signaling. Treatment with the cyclooxygenase inhibitor indomethacin reduces β-catenin levels and leads to a reduction in LSCs. In conclusion, inhibiting β-catenin by genetic inactivation or pharmacologic modulation is an effective combination therapy with imatinib and targets CML stem cells.

INTRODUCTION

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasia (MPN) characterized by massive proliferation of phenotypically normal mature myeloid cells. This disease is initiated by a reciprocal translocation of chromosomes 9 and 22 resulting in the generation of a constitutively active fusion kinase, BCR-ABL (Ben-Neriah et al., 1986; Druker, 2008). This oncogenic fusion is capable of transforming hematopoietic stem cells (HSCs) and is sufficient to initiate MPN in murine bone marrow (BM) transplantation models (Daley et al., 1990). Targeted therapy using small molecule inhibitors of BCR-ABL such as imatinib (IM) (Druker et al., 1996, 2001) or second generation kinase inhibitors such as dasatinib or nilotinib (Kantarjian et al., 2010; Saglio et al., 2010) has revolutionized therapy for CML, but in the overwhelming majority of patients, the disease clone is not eliminated, an effect that has been attributed to a persistent leukemia stem cell (LSC) pool that is inherently resistant to these targeted therapies. LSCs in CML share immunophenotypic features with normal HSCs, reside in the BM, and are resistant to IM treatment, despite inhibition of BCR-ABL (Corbin et al., 2011; Hu et al., 2009). Upon discontinuation of IM therapy, these LSCs are able to re-establish CML and to cause disease relapse (Savona and Talpaz, 2008).

A key characteristic of stem cells is their ability to self-renew. Several genes and signaling pathways control the fine balance between self-renewal and differentiation in HSCs and potentially also in LSCs. One such pathway is the canonical Wnt pathway (Jamieson et al., 2004; Majeti et al., 2009; Malhotra and Kincade, 2009; Müller-Tidow et al., 2004; Reya et al., 2003; Zhao et al., 2007). β-catenin, the pathway's central effector molecule, is negatively regulated via phosphorylation by a multiprotein complex including APC, Axin, GSK-3β, and casein kinase (Behrens et al., 1998; Rubinfeld et al., 1996). Several compounds interacting with this pathway in a variety of cancers are currently being investigated in preclinical studies (Chen et al., 2009; Huang et al., 2009; Peterson et al., 2009). Prostaglandin E_2 is known to promote stabilization of β-catenin in colon cancer (Castellone et al., 2005) and can be modified by inhibition of its upstream regulator cyclooxygenase (COX). Recently, interference of prostaglandin signaling has been shown to target the Wnt/β-catenin axis in HSCs (Goessling et al., 2009) and acute myeloid leukemia (AML) stem cells (Wang et al., 2010). Abrogation of β-catenin by the cyclooxygenase inhibitor indomethacin led to a 100-fold decrease in AML-initiating cells in secondary recipients. Moreover, indomethacin treatment of fully developed, MLL-AF9-induced leukemia led to reduction of β-catenin levels and caused reduction of LSC frequency. These data indicate that certain subtypes of AML retain dependency on Wnt signaling.

Recent studies have shed light on the impact of Wnt/β-catenin activity on development of BCR-ABL-induced MPN in several CML mouse models. Deletion of β-catenin in HSC development (using vav-Cre) (Zhao et al., 2007) or concurrently with activation

of BCR-ABL (by a retroviral fusion BCR-ABL-Cre) (Hu et al., 2009) in *Ctnnb1*$^{fl/fl}$ knockout mouse BM led to impaired leukemogenesis. These studies clearly indicate that β-catenin plays a role in the development of normal HSCs and BCR-ABL-induced CML. However, β-catenin is not required for maintenance of normal self-renewal in fully developed HSCs, which prompts the question whether β-catenin is required for maintenance of BCR-ABL-induced CML LSCs. This question is critical for therapeutic targeting of LSCs in chronic-phase CML patients. Furthermore, given that all patients with CML are treated with tyrosine kinase inhibitors, it is important to determine the effects of pathway modulation in this context. We therefore aimed to investigate the impact of β-catenin modulation in established and IM-treated BCR-ABL-induced CML.

RESULTS

Genetic Deletion of β-Catenin Reduces Bone Marrow and Peripheral Blood CML Cells

In order to address whether β-catenin is required for LSC maintenance, we aimed to delete β-catenin in BM cells after the engraftment of BCR-ABL-transduced stem cells in primary recipient mice. BM from mice with the genotypes *Ctnnb1*$^{fl/fl}$ Esr1-Cre^{+}, *Ctnnb1*$^{+/fl}$ Esr1-Cre^{+}, and *Ctnnb1*$^{+/+}$ Esr1-Cre^{+} were transduced with a retrovirus encoding human p210-BCR-ABL and GFP (MSCV-BCR-ABL-IRES-GFP) and injected into wild-type syngeneic recipient mice. Recipient mice were then followed for establishment of disease by assessment of GFP^{+} cells in the peripheral blood after BM transplantation. Upon confirmation of GFP^{+} cells (>3%) in peripheral blood, tamoxifen (TAM) was introduced via intraperitoneal injection (generally starting day +14). Mice were then followed for survival, and unexpectedly, recipient mice of *Ctnnb1*$^{fl/fl}$ Esr1-Cre^{+} (*Ctnnb1*$^{-/-}$) BM showed similar survival to treated wild-type or untreated controls, with all mice succumbing to a CML-like disease between 3 and 5 weeks after BM transplantation (Figure 1A). There was no significant survival difference detectable between the different treatment cohorts, although there was a trend for the *Ctnnb1*$^{-/-}$ group ($p = .12$). β-catenin excision was shown by PCR in *Ctnnb1*$^{-/-}$ recipients, demonstrating excision in the majority of GFP-sorted cells (Figure S1 available online). Upon analysis of moribund animals, we recognized that organ infiltration (especially hemorrhagic pulmonary infiltrates) was comparable in all treatment groups (Figure 1C). In contrast, there was a clear difference detectable with regard to BM and blood involvement. White blood counts were significantly lower in the *Ctnnb1*$^{-/-}$ ($p = .0149$*) group compared to TAM-treated WT controls (Figure 1B). BM samples of each mouse showed decreased GFP^{+} cells in *Ctnnb1*$^{-/-}$ mice (Figure 1D). This difference was noted in both the total GFP^{+} fraction as well as the GFP^{+}/Sca1^{+} fraction of individually analyzed BM samples from each group (Figure 1D). Significantly, it is this GFP^{+}Sca1^{+} population that is enriched for LSCs in that transplantation of these cells is sufficient to reconstitute MPN in secondary recipients (Hu et al., 2009). These data suggest that conditional β-catenin deletion after onset of BCR-ABL-induced disease predominantly reduces more immature hematopoietic cells, including phenotypic LSCs, as compared to more mature cells that have infiltrated organs such as the lung.

Combination of IM and β-Catenin Deletion Results in Delayed CML Recurrence after IM Discontinuation

Tyrosine kinase inhibitors such as imatinib (IM) are established as the first-line therapy for patients with CML, resulting in high rates of hematologic, cytogenetic, and molecular remissions. However, withdrawal of IM, even in patients that had achieved a complete molecular remission, results in relapsed disease in the majority of patients (Ross et al., 2010). This has been hypothesized to reflect a resistant and quiescent LSC population that can regenerate disease in the event of treatment interruption (Mahon et al., 2010; Ross et al., 2010; Rousselot et al., 2007). Based on this, we hypothesized that the combination of IM therapy, which targets most cells other than the LSC population, with Wnt/β-catenin pathway modulation, which should target more immature LSC-like cells, might delay relapse after IM withdrawal. Transplantation-related reconstitution/engraftment of genetically modified cells is always an issue when investigating leukemia development. Therefore, our experiment (Figure 2) was specifically designed to take the transplantation-related reconstitution/engraftment out of the equation. To address this, we examined whether activation or inhibition of Wnt/β-catenin signaling could have an effect on disease relapse after discontinuation of IM treatment. Wnt/β-catenin signaling is known to regulate the cell cycle in HSCs and constitutive activation is associated with loss of quiescence and stem cell exhaustion (Gothot et al., 1998; Kirstetter et al., 2006; Passegué et al., 2005; Scheller et al., 2006). To model constitutive activation of β-catenin, we used a validated model of enhanced Wnt signaling, Apc^{min} mice (Lane et al., 2010). We assessed inactivation by using *Ctnnb1*$^{fl/fl}$ Esr1-Cre^{+} mice or the appropriate wild-type mice (*Ctnnb1*$^{+/+}$ Esr1-Cre^{+}, *Ctnnb1*$^{+/+}$ Esr1-Cre^{-}) as BM donors. 5-FU-treated BM cells of donor mice (as indicated above) were infected with the p210-BCR-ABL retroviral construct and injected into syngeneic recipients. After engraftment, IM treatment was initiated followed by TAM treatment to induce β-catenin deletion (Figure 2A). After 3 weeks, IM treatment was discontinued and mice were monitored for disease progression. All mice were analyzed for disease burden after the first animal had to be sacrificed according to the animal facility guidelines. BM, spleen, and peripheral blood were analyzed for signs of MPN. All wild-type animals investigated at that early time point had a significant amount of disease burden, so the first animal is not likely to be an outlier. White blood counts were significantly lower in animals where β-catenin was deleted as compared to wild-type-recipient controls ($p = .048$*); however, recipients of Apc^{min} BM showed similar levels compared to wild-type recipients (Figure 2B, left). Given the short time after IM discontinuation, no significant differences in spleen size were detected (Figure 2B, right). Investigating the BM, however, revealed greater than 100-fold reduction of GFP^{+} cells and GFP^{+}Lin^{-} cells in mice that received *Ctnnb1*$^{fl/fl}$ Esr1-Cre^{+} BM and that were treated with TAM (Figures 2C and 2D). Recipients of Apc^{min} BM showed similar levels of leukemic cells and a minor reduction in the lineage-negative (Lin^{-}) population. These results indicate that the combination of IM with genetic deletion of β-catenin reduces the amount of leukemic disease burden and delays disease relapse caused by persistent immature leukemia cells in vivo. Therefore, combined tyrosine kinase inhibition and β-catenin

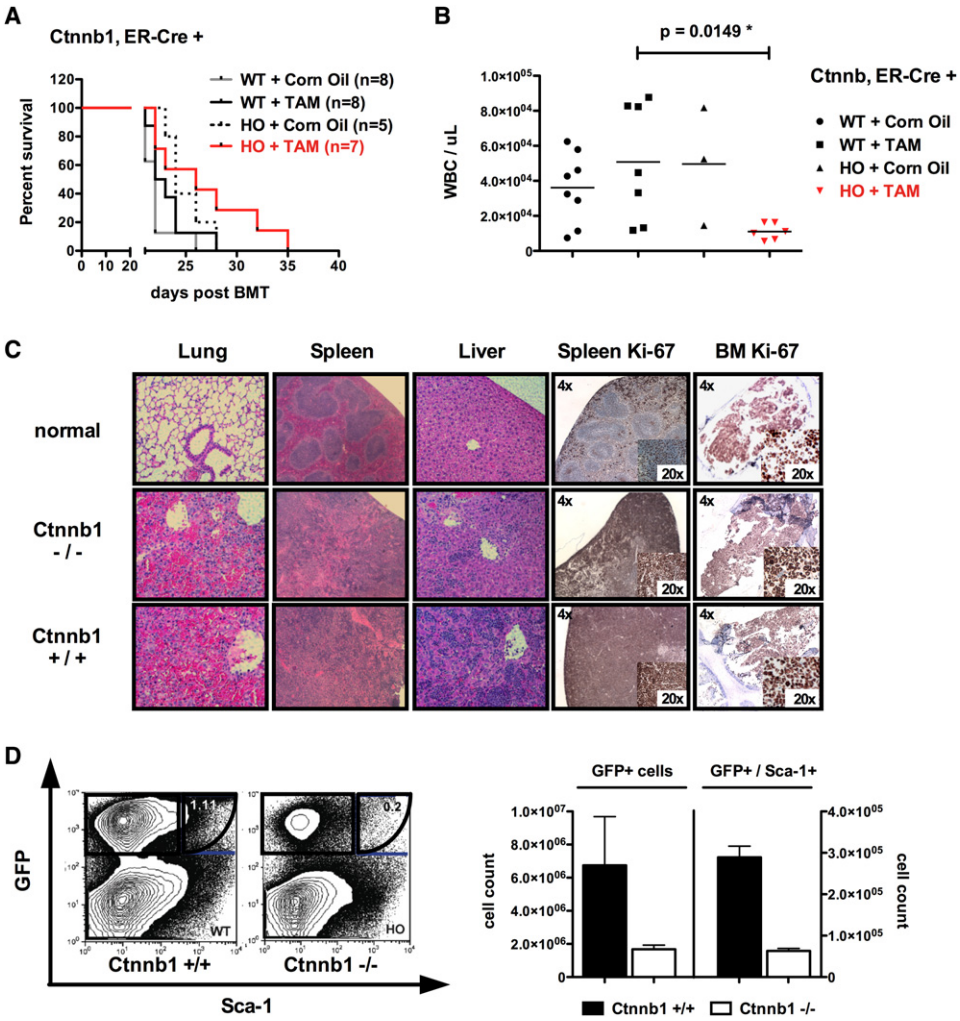

Figure 1. Genetic Deletion of β-Catenin after Engraftment of BCR-ABL-Positive Disease Leads to Reduction of Leukemia Cells in Blood and BM but Does Not Prolong Survival of Recipient Mice

(A) After injection of 70,000 sorted GFP$^+$ cells plus 1 × 10^6 supporter cells, no significant difference in survival was evident in primary recipient mice of homozygous floxed (HO) or wild-type (WT) BM cells. β-catenin (Ctnnb1) was excised after administration of tamoxifen (TAM) and corn oil served as an "empty control."

(B) Blood counts of moribund animals display significant differences between recipients of homozygous (*Ctnnb1*$^{fl/fl}$ Cre$^+$) (p = .0149*) and wild-type (*Ctnnb1*$^{+/+}$ Cre$^+$) controls.

(C) Organ infiltration (predominantly lung infiltration) was the cause of death in the majority of recipient mice without differential infiltration patterns between the groups investigated (displayed are representative HE stains of each genetic group). Loss of β-catenin did not influence proliferation (Ki67) of the majority of transformed cells in spleen and BM. Error bars indicate standard deviation (SD) of three cohorts investigated. Excision control PCR on sorted GFP$^+$ bone marrow cells from moribund primary recipient mice is indicated in Figure S1.

(D) BM analysis reveals reduction in GFP$^+$ (~3-fold reduction in *Ctnnb1*$^{-/-}$ recipients) and GFP$^+$Sca1$^+$ (~3-fold reduction in *Ctnnb1*$^{-/-}$ recipient mice) cells. The left panel demonstrates GFP % and the right panel absolute number of GFP$^+$ cells.

deletion was an effective combined therapy to delay disease progression.

Combination of Imatinib Treatment and β-Catenin Deletion Targets CML Leukemia Stem Cells

To determine whether combination of IM with genetic deletion of β-catenin could eliminate the LSC population and improve survival, we evaluated the ability of CML LSCs to maintain disease in a serial BM transplantation assay, considered the most stringent test of self-renewal in vivo. Disease was induced by transduction of BM from mice with the following genotypes: *Ctnnb1*$^{fl/fl}$ Esr1-Cre$^+$, *Ctnnb1*$^{+/fl}$ Esr1-Cre$^+$, and *Ctnnb1*$^{+/+}$ Esr1-Cre$^+$. Then we injected 70,000 GFP$^+$ sorted cells together with 1 × 10^6 supporter cells in primary mice. Primary recipients were sacrificed after onset of disease and 150,000 LSC-enriched GFP$^+$Sca1$^+$ cells were injected into lethally irradiated secondary recipients with 1 × 10^6 supporter cells as indicated above. IM treatment was initiated 7 days after transplantation into secondary recipient mice and continued for 21 days, combined with tamoxifen (TAM) administration on days 14–18. Secondary recipients were sacrificed 2 days after discontinuation of IM treatment (day 30) for immunophenotypic analysis of BM and

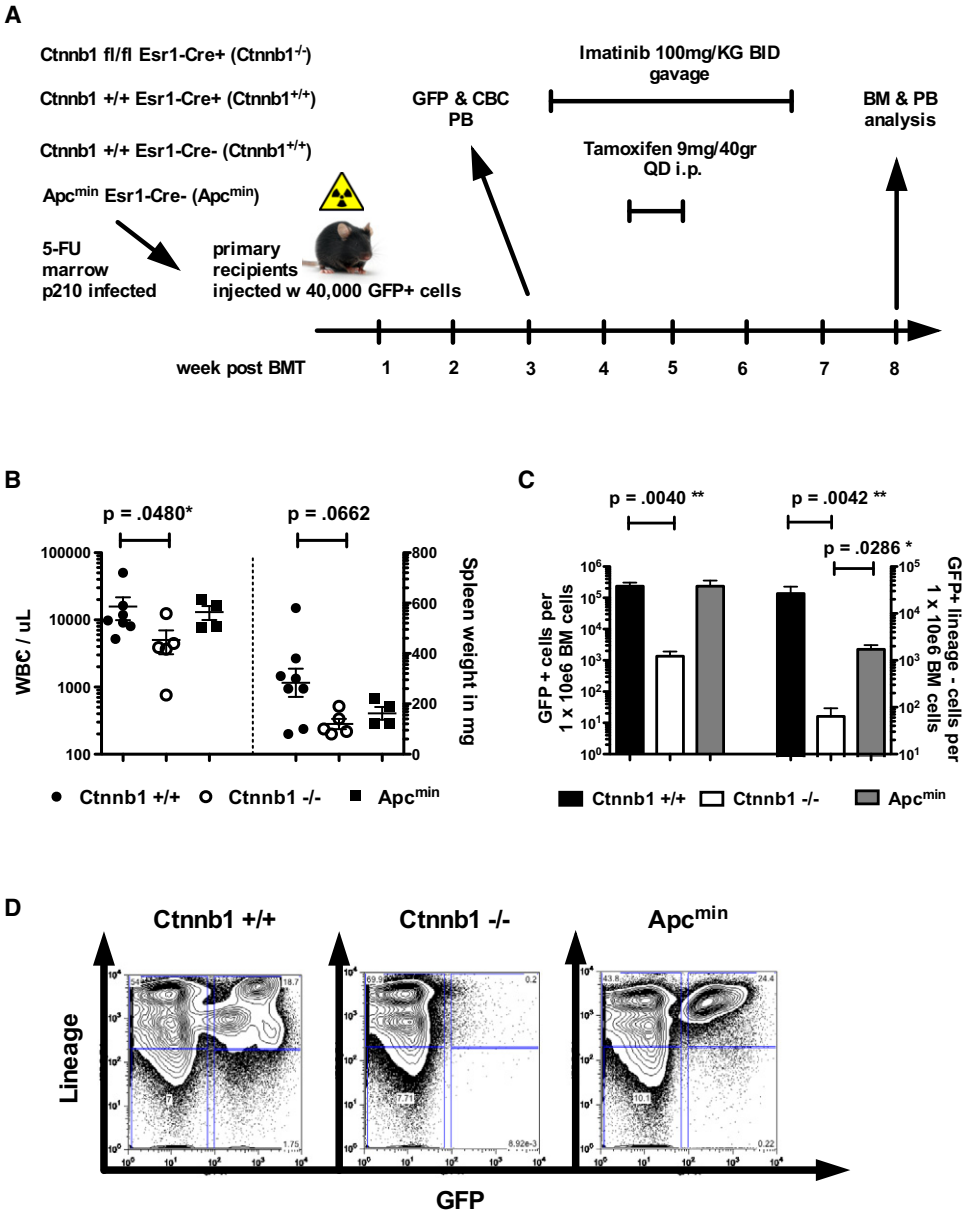

Figure 2. Conditional Deletion of β-Catenin Followed by Withdrawal of IM Treatment Leads to Delayed Disease Recurrence

(A) Treatment schedule of primary recipient mice after transplantation with 40,000 GFP$^+$ cells.

(B–D) Endpoint analysis of white blood count (WBC) and BM and spleen weight have been performed at disease onset in the first relapsed animal after discontinuation of treatment. At this point all remaining animals were sacrificed and analyzed. Disease burden (BCR-ABL-transformed GFP$^+$ cells) was analyzed as a correlate for relapse ("cytogenetic or hematologic relapse after treatment discontinuation").

(B) WBCs are significantly reduced in recipients of *Ctnnb1*$^{-/-}$ BM, compared to *Ctnnb1*$^{+/+}$ controls. Apc^{min} mice do not display any differences in WBC or spleen weight compared to *Ctnnb1*$^{+/+}$ animals.

(C and D) BM analysis reveals significant reduction of GFP$^+$ and GFP$^+$LSK cells in the *Ctnnb1*$^{-/-}$ recipient cohort. Apc^{min} mice show similar amounts of GFP$^+$ cells as WT controls with a minor reduction in the GFP$^+$Lin$^-$ compartment. Error bars indicate standard deviation of WBCs; n > 4 per cohort displayed.

transplantation of unfractionated BM cells into tertiary recipients (Figure 3A). We analyzed BM for GFP$^+$ or GFP$^-$Lin$^-$Sca1$^+$Kit$^+$ (LSK) cells (Figure 3B, representative plot). The percentage and total numbers of GFP$^+$Lin$^-$ and GFP$^+$LSK (enriched for LSC activity) cells in the TAM-treated animals showed a significant decrease compared to control treated animals (Figure 3C). When 1.5 × 10^6 whole BM cells isolated from treated secondary recipients were injected into lethally irradiated tertiary recipients, this resulted in significantly prolonged survival of recipients of *Ctnnb1*$^{-/-}$ BM (Figure 3D) compared to *Ctnnb1*$^{+/+}$ controls (p = .0019**). Secondary recipients of heterozygous *Ctnnb1*$^{+/-}$ BM showed a slight but not significant reduction of GFP$^+$LSK cells. When this BM was transplanted into tertiary recipients, these animals had a slight but not significant survival benefit as

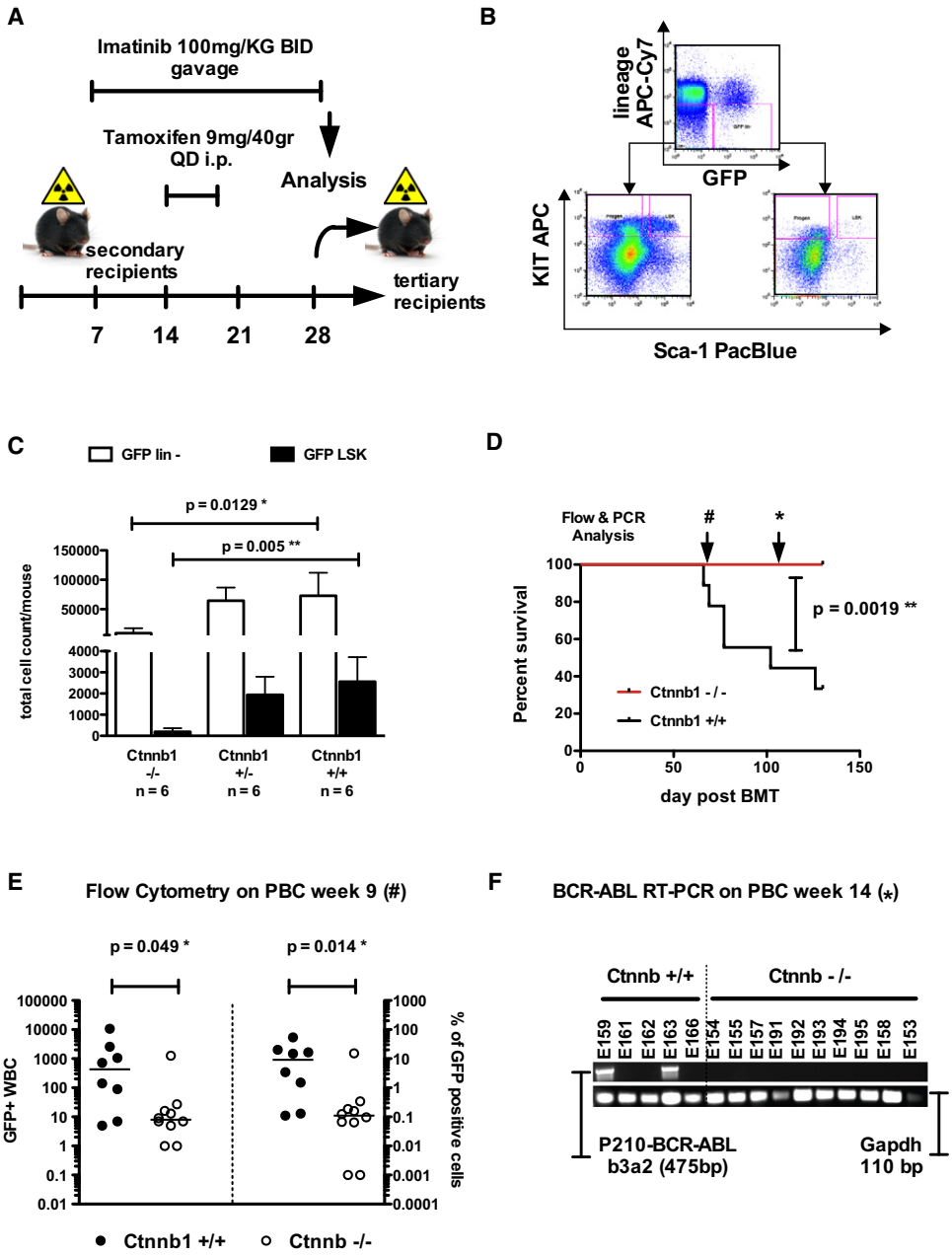

Figure 3. Combination of Genetic Deletion of β-Catenin with IM Treatment Leads to Significant Reduction of CML LSCs in a Serial BM Transplantation Assay

(A) Treatment schedule of secondary recipient mice after transplantation with 150,000 GFP$^+$Sca1$^+$ cells plus supporter cells.

(B) Representative analysis plot of GFP$^+$Lin$^-$ and GFP$^+$LSK compartments of secondary recipient mice.

(C) Genetic deletion of β-catenin (Ctnnb1) leads to a significant decrease of GFP$^+$Lin$^-$ (p = .0129*) and GFP$^+$ LSK (p = .005**) cells in secondary recipient mice when combined with imatinib (IM) treatment. Deletion of β-catenin in combination with IM led to a reduction of GFP$^+$Lin$^-$ cells from 72,988 in *Ctnnb1*$^{+/+}$ recipient animals to 9,231 in *Ctnnb1*$^{-/-}$ recipient animals (calculated as percentage of total harvested BM cells). These numbers correlated with the reduction of GFP$^+$LSK cells from 2,560 to 194, respectively. Error bars indicate the standard deviation of n = 6 mice investigated in the cohort displayed.

(D) Transplantation of 1.5 × 10^6 whole BM cells leads to a significant survival advantage in tertiary recipients of *Ctnnb1*$^{-/-}$ BM when compared to *Ctnnb1*$^{+/+}$ controls. Transplantation of higher cell numbers (6 × 10^6 whole BM cells) is displayed in Figure S2.

(E and F) Analysis of disease burden at different time points by flow cytometry (E) or nested RT-PCR (F) showed a significant decrease in disease load in *Ctnnb1*$^{-/-}$ tertiary recipient mice. These results could be recapitulated with Mx1-Cre (Figures S2C–S2E).

compared to control (data not shown). In order to directly compare disease burden in tertiary recipient mice, we investigated the peripheral blood for GFP$^+$ cells after the development of lethal disease in the first animal (Figures 3D and 3E). A significant difference in total number and percentage of GFP$^+$ cells was evident at that time between recipient animals of

Ctnnb1$^{+/+}$ and *Ctnnb1*$^{-/-}$ BM (Figure 3E). Because several mice developed disease in the *Ctnnb1*$^{+/+}$ cohort but none of the *Ctnnb1*$^{-/-}$ became sick, we aimed to analyze the molecular disease burden as determined by BCR-ABL transcript detection in the peripheral blood by nested-RT-PCR. PCR primers for the human p210 BCR-ABL construct were used together with murine Gapdh controls. None of the ten living animals that received *Ctnnb1*$^{-/-}$ BM had any b3a2-transcript detectable, while two out of five remaining animals that received *Ctnnb1*$^{+/+}$ BM showed positivity for a BCR-ABL transcript (Figure 3F). The other five animals in the *Ctnnb1*$^{+/+}$ cohort had already succumbed to MPN, and BM and spleen infiltration by GFP$^+$ (BCR-ABL$^+$) cells had been confirmed.

We repeated the experiment with a higher cell number of unfractionated BM cells for injection into tertiary recipients. With 6×10^6 whole BM cells, one out of five mice developed GFP$^+$ MPN in the *Ctnnb1*$^{-/-}$ recipient group whereas all of the *Ctnnb1*$^{+/+}$ recipients succumbed to disease (Figure S2A). Moreover, all of the recipients of heterozygous floxed BM (*Ctnnb1*$^{+/-}$) developed lethal MPN but with a slightly longer latency than the wild-type recipient control. Analysis of disease burden on day 36 after transplantation revealed significantly lower GFP$^+$ cells in the peripheral blood of *Ctnnb1*$^{-/-}$ mice compared to wild-type and heterozygous controls (Figure S2B).

Although only one of five animals in the *Ctnnb1*$^{-/-}$ (6×10^6 cells injected) cohort had succumbed to disease, we used transplants performed at limiting cell number to determine the estimated LSC frequency. With the two data points available (dilutions of 6×10^6 and 5×10^5), we estimate an approximate 35-fold decrease in LSCs. Taken together, our data show a dramatic reduction in LSC frequency.

Because there have been recent reports that highlight Esr1-Cre-related toxicity on myelopoiesis (Higashi et al., 2009), we aimed to clarify the impact of β-catenin deletion on the reduction or disappearance of LSCs in our model, so we repeated the genetic model applied with the well-characterized Mx1-Cre model (Figures S2C–S2E). IM treatment was started on day 14 (100 mg/KG per gavage BID). Mice were injected on days 15, 17, and 19 with poly(I:C) at a dose of 12.5 μg/g body weight. After a treatment period of 21 days of IM, mice were sacrificed. We analyzed the BM (as indicated below) to determine the amount of immunophenotypic LSCs (GFP$^+$LSK cells). This analysis showed an approximately 10-fold reduction in GFP$^+$LSK cells. In order to determine the number of functional LSCs, we transplanted dilutions of total BM (5×10^5 and 6×10^6 cells) into secondary recipient mice. Animals in the 6×10^6 *Ctnnb1*$^{+/+}$ cohort developed lethal MPN within 45 days after transplantation, whereas only 25% of the *Ctnnb1*$^{-/-}$ mice died of MPN. When the graft was diluted to 5×10^5 cells, only 20% of the *Ctnnb1*$^{+/+}$ but none of the mice with a *Ctnnb1*$^{-/-}$ genotype developed disease. This functional data set with the established Mx1-Cre system suggests a ~10-fold reduction of LSCs (Figure S2E).

Indomethacin Is Effective in Modulating β-Catenin Levels and Synergizes with IM in CML

To address whether modulation of Wnt/β-catenin signaling can be achieved pharmacologically in CML stem cells, we aimed to inhibit or enhance β-catenin levels in the human BCR-ABL-positive cell line K562. Although inhibition of β-catenin may contribute to reduction of LSCs, stimulation of β-catenin activity could potentially bring LSCs into cycle and therefore make them more susceptible to IM treatment (Scheller et al., 2006). Moreover, there is evidence that inhibition of GSK3, which should activate β-catenin, is an effective approach for AML (Holmes et al., 2008; Wang et al., 2008). As it is known that inhibition of prostaglandin signaling via COX_2 inhibitors can decrease β-catenin level in HSCs and LSCs, we utilized indomethacin to inhibit and the GSK3 inhibitor BIO ((2′Z,3′E)-6-Bromoindirubin-3′-oxime) to stimulate Wnt/β-catenin pathway activity.

Treatment of BCR-ABL-rearranged K562 cells with either indomethacin or IM led to reduction of β-catenin protein levels, but the combination of both drugs led to almost complete abrogation of β-catenin in vitro (Figure 4A). In contrast, incubation with the GSK3β inhibitor BIO caused pronounced accumulation of β-catenin. These stimulative BIO effects could be partially reversed by coincubation with indomethacin and IM. Next we developed a flow cytometry-based assessment of β-catenin so that we could assess levels in small numbers of cells. When we assessed β-catenin levels in K562 cells treated with BIO and indomethacin, we found similar results to our immunoblot experiments (Figure 4B, left).

Because BIO and indomethacin proved to effectively modulate β-catenin in the cell culture model, and indomethacin even synergized with IM in β-catenin suppression, we aimed to confirm these modulatory effects and capacity in the LSC-enriched population from mice with BCR-ABL-induced MPN. Mouse BM cells were transduced with the BCR-ABL retrovirus after 5-FU treatment and injected into primary (lethally irradiated) recipient mice as described above. Primary recipient mice were sacrificed on day 20 after transplantation and BM was harvested and sorted for GFP$^+$Sca1$^+$ cells. This LSC-containing population was treated with either DMSO, IM, indomethacin, or BIO. Flow cytometry revealed increased β-catenin levels in BIO-treated GFP$^+$Sca1$^+$ cells, and a reduction of β-catenin could be detected in IM- or indomethacin-treated cells (Figure 4B, right). Additionally, we treated GFP$^+$Sca1$^+$ cells ex vivo with BIO, PGE_2, indomethacin, or DMSO and then plated them in methylcellulose. This revealed decreased colony formation upon indomethacin treatment whereas enhancement of β-catenin with BIO or PGE_2 did not show any significant change in colony formation (Figure 4C). When we tested primary recipient mice for induction of cell cycle by GSK3 inhibition, we found that administration of BIO led to induction of cell cycle in GFP-negative LSK, but the induction was even more pronounced in GFP-positive LSKs (Figure S3A). Because we aimed to link indomethacin treatment with effects on β-catenin levels in CML LICs, we performed an ELISA-based assay for detection of cAMP levels on GFP$^+$Sca1$^+$ cells. Low levels of cAMP were detected in the DMSO-treated controls; however, treatment with indomethacin lowered the cAMP levels in the GFP$^+$Sca1$^+$ leukemia-initiating fraction (as indicated by increase in absorbance; Figure S3B). Although this is not a functional assay, decrease of cAMP levels by indomethacin treatment in the leukemia-initiating population links the functional data to the mechanism of PGE_2 stimulation of HSCs, which was through stimulation of cAMP levels and PKA phosphorylation of β-catenin (Goessling et al., 2009). These data show that indomethacin works together with IM to suppress

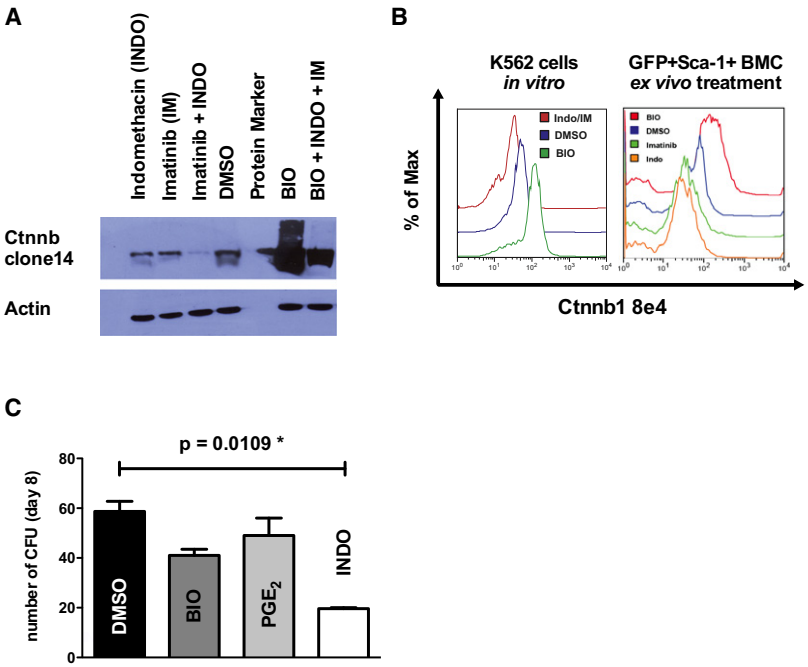

Figure 4. Modulation of Prostaglandin Signaling Leads to Alteration of β-Catenin Levels and Influences Colony Formation

(A) Western blotting on whole-cell lysates of K562 cells. Incubation of K562 cells with either IM (1 μM) or indomethacin (40 μM) led to reduction of β-catenin protein levels, while combined treatment revealed profound decrease of β-catenin. Increase of β-catenin levels were detectable upon GSK3β inhibition with BIO (0.5 μM) and partially reversible by cotreatment with IM and indomethacin.

(B) Using low cell numbers, these results could be validated with intracellular flow cytometry in K562 cells (left) and in GFP^+Sca1^+ leukemic cells from primary mice (right).

(C) When plated in methylcellulose, indomethacin-treated GFP^+Sca1^+ cells sorted from primary recipient mice (n = 3 mice/group, error bars indicate SD) showed significantly impaired colony formation compared to DMSO-, BIO-, or PGE_2-treated cells (p = .0109). Evaluation of β-catenin modulation in CML LICs derived from recipient mice after drug treatment is shown in Figure S3.

β-catenin in vitro and in vivo, prompting further assessment of the combination.

Imatinib plus Indomethacin Prolongs Survival in a Serial Murine BM Transplantation Model

Because inhibition of PGE_2 signaling suppressed β-catenin levels in CML cells, we aimed to elucidate whether cotreatment with indomethacin and IM could effectively reduce the number of LSCs and could lead to delayed onset of disease in a serial transplantation approach. As described above, 150,000 GFP^+Sca1^+ cells isolated from mice with a primary BCR-ABL-induced CML were injected into secondary recipients along with supporter BM cells. These mice were treated with IM (d7–d28) and indomethacin or vehicle control (d14–d18, d21–d25) (Figure 5A). After 28 days, all mice were sacrificed and analyzed in detail (Figure 5B). Normal spleen size and white blood counts of treated secondary recipients indicated efficacy of IM only and of IM plus indomethacin treatment (Figures S4A and S4B). Strikingly, we observed a significant reduction of GFP^+LSK cells in the indomethacin-plus-IM-cotreated animals compared to IM-only controls (Figure 5C). However, the numbers of normal LSK cells (GFP^-, derived from supporter BM) were not affected by any cotreatment (data not shown).

To investigate the functional relevance of this reduction, we injected 2 × 10^6 whole BM cells into tertiary recipient mice and monitored these animals for onset of disease. All tertiary recipients of the IM-only-treated BM developed lethal disease within 36 days after BM transplantation. However, recipients of IM-plus-indomethacin-cotreated BM showed delayed onset of disease and a significantly prolonged survival of up to 62 days after transplantation (Figure 6A, p < .0001***). In contrast to previous reports that the deletion of β-catenin via vav-Cre before transformation with BCR-ABL had resulted in development of acute lymphoblastic leukemia rather than CML (Zhao et al., 2007), we observed that both treatment groups developed morphologically and immunophenotypically similar MPN (Figures 6B and 6C), despite delayed onset of disease in the indomethacin-plus-IM-treated group. Because activation of canonical Wnt signaling in our genetic model (using Apc^{min} mice) had not shown any significant reduction in disease burden, we also aimed to elucidate whether pharmacological stimulation of β-catenin could have an effect on LSC frequency or survival of tertiary recipient mice. Stimulation of β-catenin by cotreatment with the GSK3β inhibitor BIO resulted in a minor decrease of GFP^+Lin^- and GFP^+LSK cells in secondary animals (Figure S4C). However, this did not translate into any survival benefit of tertiary recipients (Figure S5).

In order to correlate these findings of our mouse model with human data, we analyzed a previously published data set that investigated $CD34^+$ cells from IM-naive chronic-phase CML patients to predict responses upon IM treatment (McWeeney et al., 2010). Interestingly, supervised analysis comparing responders versus nonresponders revealed the prostaglandin-endoperoxide synthase (*PTGS2*, also called cyclooxygenase-2) among the top candidates. *PTGS2* expression levels were significantly lower in the "responder" cohort (p = 0.0067, Figure 6D), thereby confirming that inhibition of PTGS2/COX2 may translate into molecular responses (and therefore targeting LSCs). Moreover, we found a molecular signatures database (MSigDB) gene set defined by genes down- or upregulated in $CD4^+$ T lymphocytes after stimulation with prostaglandin E2 (MSigDB gene set CHEMNITZ_RESPONSE_TO_PROSTAGLANDIN_E2, M2214) to be significantly enriched in the responder versus nonresponder associated gene expression pattern (p = 0.0284). Finally, clustering of the CML samples based only on genes involved in prostanoid hormones (MSigDB gene set REACTOME_PROSTANOID_HORMONES, M14714) correlated with response/nonresponse, and thus showed decreased expression levels

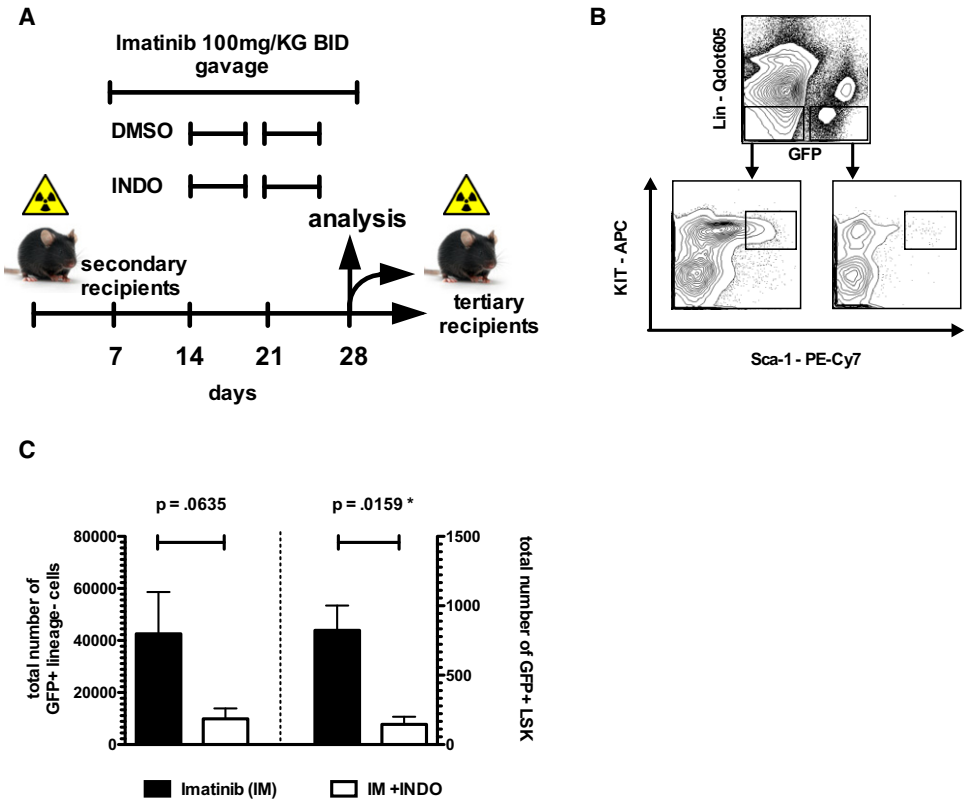

Figure 5. Pharmacologic Inhibition of PGE_2 Signaling in Combination with IM Treatment Leads to Reduction of LSCs in a Serial BM Transplantation Assay In Vivo

(A) Treatment schedule of secondary recipient mice ($n \geq 8$ mice/cohort) injected with 150,000 GFP^+Sca1^+ cells out of primary recipient mice.

(B) Representative analysis plot of GFP^+Lin^- and GFP^+LSK compartments of secondary recipient mice after treatment.

(C) Cotreatment of secondary recipient mice with IM and indomethacin leads to reduction of GFP^+Lin^- cells ($p = .0635$) and to significant depletion of GFP^+LSK cells ($p = .0159$) in the BM. GFP^+Lin^- cells were reduced from 42,560 (IM-only group) to 9,867 (IM+INDO-treated cohort). This translated into a significant reduction of GFP^+LSK cells from 823 to 146, respectively. (Error bars indicate SD of GFP-positive cells; $n > 4$ per cohort investigated.) Evaluation of secondary recipients after drug combination therapy is displayed in Figure S4.

of prostaglandin-related genes to be correlated with treatment response ($p = 0.0067$, Figure 6E).These data provide evidence for an approach to target CML stem cells through the inhibition of Wnt/β-catenin signaling in combination with tyrosine kinase inhibitors by using a widely available and affordable clinical therapeutic (indomethacin).

DISCUSSION

The Wnt/β-catenin pathway has been shown to be necessary for HSC development but not for HSC maintenance in fully developed stem cells (Koch et al., 2008; Zhao et al., 2007). Also, β-catenin has been shown to be necessary for development of LSCs in a mouse model of BCR-ABL-induced MPN (Hu et al., 2009; Zhao et al., 2007). However, these data do not address the critical question as to whether β-catenin is required for maintenance of LSCs after the establishment of BCR-ABL-induced disease. This is a critical question that will help guide development of therapeutics that target the Wnt/β-catenin pathway in patients with CML who are maintained on tyrosine kinase inhibitors. We hypothesized that inhibition of canonical Wnt signaling could potentially affect the maintenance of IM-resistant CML stem cells, thereby providing synergy when used in conjunction with IM treatment. To address this question, we conditionally inactivated β-catenin after engraftment of BCR-ABL-transformed HSCs/LSCs. Although we and others have had extensive experience with the hematopoietic-specific Mx1-Cre recombinase, the induction of cre expression in this model is induced by an interferon response through the injection of poly(I:C) in mice. Interferon was the standard therapy (alone or in combination with cytarabine) for CML for almost a decade and is known to induce complete cytogenetic remissions (Guilhot et al., 1997). Therefore, using Mx1-Cre in a conditional β-catenin knockout model could potentially confound our results through the antileukemic effects of elevated interferon levels. We therefore chose in the first set of experiments a tamoxifen (TAM)-inducible estrogen-receptor Cre recombinase. However, because there have been recent reports that highlight Esr1-Cre-related toxicity on myelopoiesis (Higashi et al., 2009), we repeated our experiments using the Mx1-Cre system with consistent results. A caveat when using the retroviral transduction model for BCR-ABL is the requirement of cell division for retroviral integration. Therefore, only dividing HSCs will be transformed by the oncogene. However, by 2 weeks after transplantation of these

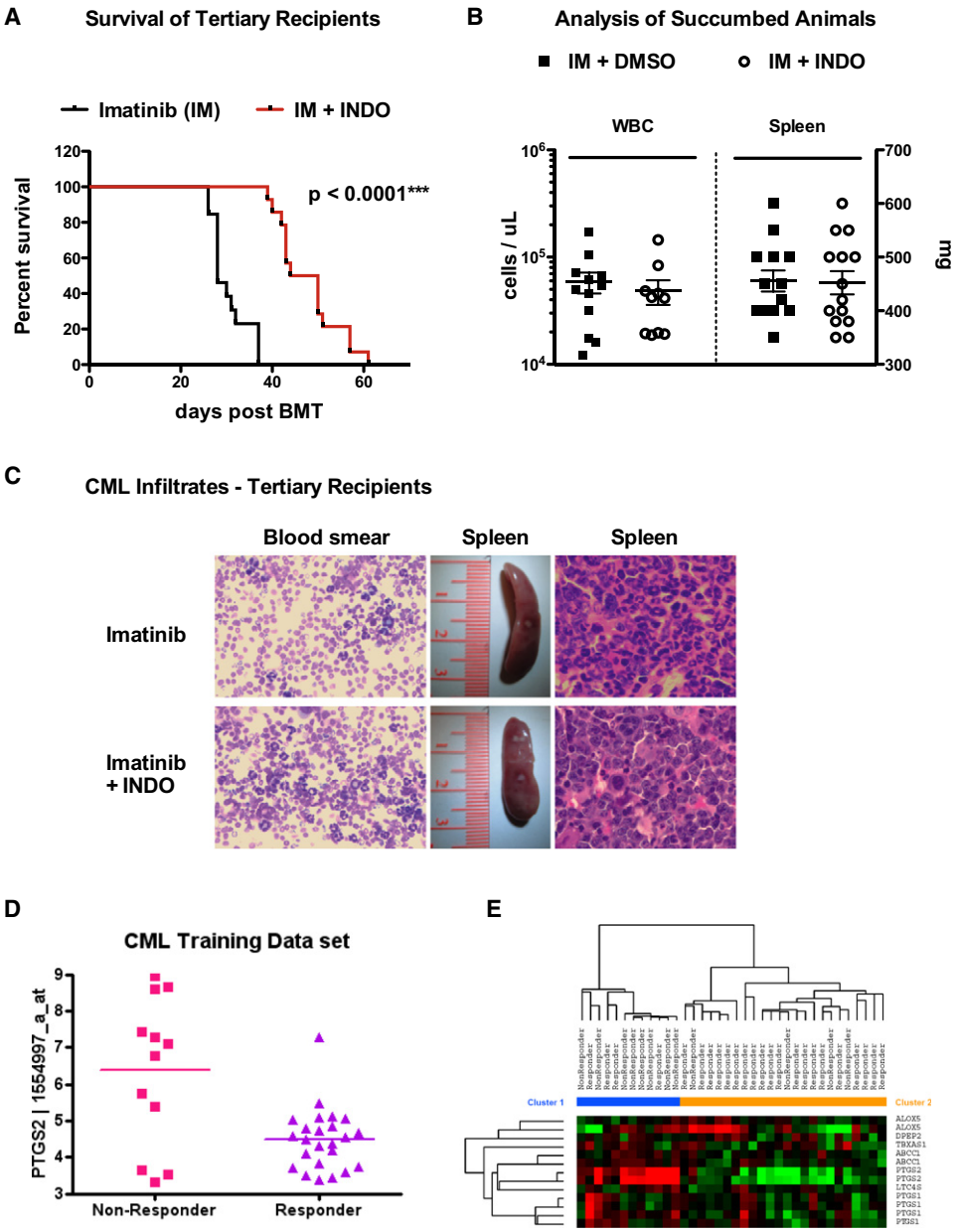

Figure 6. Prostaglandin Signaling Is Relevant as a Target in CML LSCs and Has Prognostic Relevance in Human CML

(A–C) Pharmacologic inhibition of PGE_2 signaling in combination with IM treatment leads to delayed onset of disease in tertiary recipient mice. Injection of 1.5×10^6 whole BM cells leads to delayed onset of disease in tertiary recipients of IM-plus-indomethacin-treated BM cells in comparison to IM-only-treated controls (A). Endpoint analysis of moribund animals revealed no difference in disease phenotype in the different treatment arms with regard to white blood count, spleen weight (B), or peripheral blood/organ involvement (C). Error bars indicated standard deviation of all mice ($n > 13$) investigated in both cohorts investigated.

(D and E) Gene expression profiling of CML patient-derived $CD34^+$ cell samples (D, E) reveals a prognostically relevant PGE_2 signature. Expression levels of prostaglandin synthetase 2 correlate with molecular response in the data set investigated ($p = 0.0004$) (McWeeney et al., 2010). Moreover, sample clustering based on the "REACTOME_PROSTANOID_HORMONES" MSigDB gene set correlates also with response/nonresponse in this data set ($p = 0.0067$) (E). Figure S5 shows survival of tertiary recipient mice after combination of imatinib and BIO in secondary donor animals.

transformed preleukemic stem cells, a notable fraction of GFP^+ LSK cells remains in G0 in cell cycle analysis before Esr1-Cre- or Mx1-Cre-mediated excision of β-catenin was initiated. Upon IM monotherapy, an increasing fraction of stem cells remains quiescent in the BM. This indicates—at least in part—that also a temporarily quiescent fraction of stem cells can be affected by genetic deletion of β-catenin.

Unexpectedly, conditional deletion of β-catenin did not result in significant prolongation of survival in primary recipient mice and all recipients died as a result of organ infiltration. However,

in the peripheral blood and BM, a clear reduction of GFP^+ cells was detectable. Thus, these data support the tenet that deletion of β-catenin may result in reduction of LSCs, but may spare the differentiated progenitors, most probably because of a lack of dependence on β-catenin signaling for survival of this population. Furthermore, these results may reflect differences in the experimental design compared to previously reported results—when β-catenin was deleted in early development (Zhao et al., 2007) or simultaneously to transformation with BCR-ABL with a fusion retrovirus vector (Hu et al., 2009). In our model, differentiated progenitor cells had already infiltrated different organ sites prior to β-catenin deletion. These data demonstrate that therapeutic approaches which largely target LSCs will need to be combined with therapeutics that predominately target progenitors (like Imatinib) in order to abrogate the detrimental effects of the high progenitor burden.

In order to target LSC populations and to simulate a clinically relevant setting for patients with CML that targets residual disease in tyrosine kinase inhibitor-treated patients, we combined IM treatment with genetic modulation of β-catenin. Based on previous studies, there was rationale to test β-catenin loss of function in combination with IM. However, there is growing evidence that activation of Wnt signaling results in alteration of HSCs. Wnt/β-catenin activation by stabilization of β-catenin has been shown to lead to HSC exhaustion due to cell cycle entry and consecutive apoptosis after initial expansion of the HSC pool (Scheller et al., 2006). Less pronounced activation with a GSK3β inhibitor led to increase of the HSC pool and enhanced repopulation capacity (Trowbridge et al., 2006). These data suggested that activation of Wnt signaling might lead to increased proliferation of the LSC pool and therefore increase susceptibility to IM treatment. However, it was unclear whether activation or inhibition of this pathway would be most beneficial in the context of IM therapy. Thus, we decided to assess either loss-of-function or constitutive activation models of the Wnt/β-catenin pathway. We induced CML by transformation of murine stem and progenitor cells with the human p210-BCR-ABL oncogene. IM treatment was initiated after engraftment of transformed cells and confirmed with either genetic deletion (by administration of tamoxifen in *Ctnnb1*$^{fl/fl}$ Esr1-Cre^+ BMC) or activation (Apc^{min} BM) of Ctnnb1. Whereas activation of β-catenin seemed to leave the disease-initiating population largely unaffected, deletion of β-catenin led to reduction of LSCs as displayed by delayed and reduced recurrence of disease. The specific effect of β-catenin deletion on LSC self-renewal was manifest by an inability of *Ctnnb1*$^{-/-}$ marrow to maintain disease through subsequent generation of recipients in a serial BM transplantation assay. Genetic deletion in combination with IM treatment led to abrogation of LSCs in secondary (treated) recipients and delayed disease kinetics in tertiary recipient mice. Not only did we observe delayed onset of disease, there was also a 10- to 35-fold reduction in LSC frequency from secondary mice transplanted with β-catenin-depleted leukemia compared to WT controls, demonstrating a striking and potent effect on LSCs that might be utilized for clinical gain.

As targeted therapies are currently developed for a variety of solid tumors (Alvarez et al., 2010; De Roock et al., 2011) and leukemia (Fischer et al., 2010; Mason et al., 2009), we aimed to investigate whether canonical Wnt signaling could become a "druggable" target in patients with minimal residual disease CML. Activation of canonical Wnt signaling is known to be an important oncogeneic event in solid tumors, especially in colon carcinomas (Behrens, 2000; Oving and Clevers, 2002) and long-term treatment with COX-inhibitors/nonsteroidal-anti-inflammatory drugs (NSAID) has been found to reduce the relative risk for colon carcinoma development (Gupta and Dubois, 2001; Rothwell et al., 2010). Recently, reduction of tumor incidence has been reported for long-term use of the COX-inhibitor acetylsalicylic acid (aspirin) for a variety of cancers, providing provocative evidence that dysregulated Wnt signaling may be a common feature of many malignancies (Rothwell et al., 2011). In the hematopoietic system, perturbations in the COX-Prostaglandin-Wnt cascade have been shown to have important effects on HSC and LSC function (Goessling et al., 2009; Wang et al., 2010). Stimulation of Wnt signaling with PGE_2 has been shown to enhance HSC homing, survival, and proliferation (Frisch et al., 2009; Hoggatt et al., 2009; Goessling et al., 2009). Conversely, reduction of β-catenin levels with the COX inhibitor indomethacin significantly decreases MLL-AF9 LSC frequency (Wang et al., 2010). To elucidate the role of pharmacological β-catenin modulation in this CML model, we investigated both stimulation (via the GSK3β inhibitor BIO) and inhibition (via the COX inhibitor indomethacin) of β-catenin in combination with IM treatment in vivo. As indicated by small spleen sizes and normal white blood counts, imatinib was an effective therapy against transformed progenitors and differentiated cells in secondary recipients. Given the difficulty with limiting dilution transplantation experiments in this model, we used survival of tertiary recipient mice as an indicator of LSC function. This experimental design has been used as an alternative for assessment of LSC function in CML and AML LSC models (Hu et al., 2009; Zhao et al., 2007). With this serial transplantation assay, we did not observe biologically significant results on LSC function by the combination of BIO (leading to Wnt activation) and IM. However, our results indicate that reduction of the β-catenin level via indomethacin leads to delayed onset of disease (in tertiary recipients) as a result of LSC reduction in the secondary recipient mice. This finding is consistent with analyses of gene-expression data sets that were previously published on $CD34^+$ cells of CML patients and have been correlated with response to IM treatment. Patients that show enrichment for prostaglandin signaling-related signatures show inferior molecular responses upon IM therapy.

In summary, we have demonstrated that targeting β-catenin by genetic deletion or drug treatment is an effective combination therapy with IM and eradicates LSCs that remain after IM treatment. This leads to a delayed recurrence of CML after IM discontinuation, providing support for the notion that targeting LSCs will be an effective combination approach with tyrosine kinase inhibitors in this and perhaps other diseases. Recent reports indicate that adult HSCs do not require fully active β-catenin for maintenance (Koch et al., 2006; Luis et al., 2011), and our data show that that pharmacologic inhibition of β-catenin leads to reduction of GFP^+ (BCR-ABL-transformed) LSK cells, thus suggesting a therapeutic index. However, this difference has not been addressed yet in a human setting. Therefore, establishing a therapeutic index between normal HSCs and LSCs in human CML after β-catenin modulation and IM treatment will

be a crucial prerequisite for Wnt signaling inhibitors in clinical trials. These findings prompt an important question as to why CML LSCs are dependent upon β-catenin whereas normal HSCs are less dependent on this pathway. The answer to this question will be the focus of much future work, and is likely to point to further vulnerabilities in cancer stem cells that can be targeted for therapeutic purposes. Finally, these data provide a rational template and preclinical model for the development and utilization of β-catenin-targeted therapies to eradicate LSCs in patients with CML.

EXPERIMENTAL PROCEDURES

Bone Marrow Infection and Transplantation

Murine BM transplantation was performed as previously described (Hu et al., 2009). Donor mice (C57BL/6, Charles River Labs, Wilmington, MA) were injected with 150 mg/KG 5-FU i.v. and BM was harvested on day 5. BM cells were infected by colocalization of virus supernatant (containing IL-3, IL-6, and SCF) (Peprotech, Rocky Hill, NJ) with mouse BM cells on retronectin-coated plates. Infection was repeated after 24 hr. One day after second infection, GFP^+ cells were sorted with a FACSAria and used for transplantation. For transplantation, GFP^+ BM cells were transplanted via lateral tail vein injection into lethally irradiated (1,100 cGy, split-dose) 4- to 6-week-old C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) in the presence of 1 million freshly isolated erythrocyte-lysed "supporter" BM cells. For serial transplantation experiments, BM of primary recipient mice was harvested and 150,000 GFP^+Sca1^+ sorted cells were injected into secondary recipients. Tertiary recipient mice were injected with whole BM cells, harvested from secondary recipients after treatment was discontinued.

Drug Treatment of Mice

Imatinib was dissolved in PBS solution at a stock solution of 100 mg/ml and administered by oral gavage twice daily at a single dose of 100 mg/kg. Indomethacin and BIO (6-Bromoindirubin-3′-oxime) (both Sigma, St. Louis, MO, USA) were diluted in DMSO and administered by i.p. injection at a dose of 4 mg/kg/day and 50 μg/kg/day, respectively. DMSO was injected as a negative control.

Methylcellulose Colony Formation Assay

For investigation of colony formation in methylcellulose, BM cells of primary CML mice were harvested on day 21 after BMT and sorted for GFP^+Sca1^+ cells. Cells were treated ex vivo with either BIO (0.5 μM), PGE_2 (10 μM, Sigma), or indomethacin (40 μM) for 48 hr and 1,000 cells were plated subsequently in 1.1 ml of methylcellulose (M3234, Stem Cell Technologies), supplemented with cytokines (IL-3, IL-6, SCF). Colony numbers were counted on day 8 after plating.

Flow Cytometry

For immunophenotype analysis, peripheral blood cells, BM, or spleen cells were resuspended in PBS/1% FBS after erythrocyte lysis (PharmLyse, BD PharMingen, San Diego, CA). Sorting and analysis of LSK cells or $Sca1^+$ cells was performed as previously described (Krivtsov et al., 2009). Intracellular flow cytometry was performed as described previously (Kalaitzidis and Neel, 2008) with a primary antibody against Ctnnb1 (Clone 8e4, A.G. Scientific, San Diego, CA) followed by a secondary Alexa 594-labeled antibody (A31623, Invitrogen, Carlsbad) and analyzed with a LSRII (Becton Dickinson) cytometer. Analysis was performed with FlowJo software (Treestar, Ashland, OR).

Western Blotting

Western blot experiments were performed as described previously (Heidel et al., 2006) with antibodies for total β-catenin (BD Transduction Lab, No. 61054).

PCR Assays

For qualitative detection of BCR-ABL, a nested-PCR assay was performed with 200 μl of whole blood. RNA was prepared with TRIzol Reagent (Invitrogen, Carlsbad, CA). cDNA was transcribed from 1 μg of RNA with the Tetro cDNA Synthesis Kit (Bioline, Taunton, MA). 40 cycles of PCR were conducted. Primers are indicated in the Supplemental Information. This resulted in amplification of a 475 bp fragment (b3a2 breakpoint) of the human p210 BCR-ABL construct.

Statistics and Analysis of Gene Expression Data

For survival analysis, Kaplan-Meier curves were plotted with GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA). Differences between survival distributions were analyzed with the logrank test. Statistical analyses were performed with Student's t test (normal distribution) or Mann-Whitney U test (when normal distribution was not given). p less than .05 was considered statistically significant ($^*p < .05$, $^{**}p < .01$, and $^{***}p < 0.001$).

Published gene expression data (GSE14671) (McWeeney et al., 2010) and curated gene sets (M2214 and M14714) were downloaded from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and the Molecular Signatures Database (MSigDB v3.0, http://www.broadinstitute.org/gsea/msigdb/index.jsp), respectively. Gene expression data were normalized and analyzed as previously reported (Lück et al., 2011).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at doi:10.1016/j.stem.2012.02.017.

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