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Article

A Pan-BCL2 Inhibitor Renders Bone-Marrow-Resident Human Leukemia Stem Cells Sensitive to Tyrosine Kinase Inhibition

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SUMMARY

Leukemia stem cells (LSCs) play a pivotal role in the resistance of chronic myeloid leukemia (CML) to tyrosine kinase inhibitors (TKIs) and its progression to blast crisis (BC), in part, through the alternative splicing of self-renewal and survival genes. To elucidate splice-isoform regulators of human BC LSC maintenance, we performed whole-transcriptome RNA sequencing, splice-isoform-specific quantitative RT-PCR (qRT-PCR), nanoproteomics, stromal coculture, and BC LSC xenotransplantation analyses. Cumulatively, these studies show that the alternative splicing of multiple prosurvival *BCL2* family genes promotes malignant transformation of myeloid progenitors into BC LSCS that are quiescent in the marrow niche and that contribute to therapeutic resistance. Notably, sabutoclax, a pan-BCL2 inhibitor, renders marrow-niche-resident BC LSCs sensitive to TKIs at doses that spare normal progenitors. These findings underscore the importance of alternative *BCL2* family splice-isoform expression in BC LSC maintenance and suggest that the combinatorial inhibition of prosurvival BCL2 family proteins and BCR-ABL may eliminate dormant LSCs and obviate resistance.

INTRODUCTION

Human leukemia stem cells (LSCs), first described in acute myeloid leukemia (AML) (Lapidot et al., 1994), subvert stem cell properties, such as quiescence, enhanced self-renewal, and survival, which renders them resistant to conventional therapy (Guzman et al., 2002; Visvader, 2011). Chronic myeloid leukemia (CML) represents an important paradigm for dissecting the molecular evolution of LSCs during leukemic progression and the role of LSCs in therapeutic resistance because CML was the first malignancy to be targeted with therapy that selectively inhibits the aberrant kinase responsible for CML initiation (Druker et al., 2001). Although BCR-ABL-targeted tyrosine kinase inhibitors (TKIs) eradicate the bulk of BCR-ABL1-expressing cells, they frequently fail to eliminate quiescent, niche-resident LSCs that drive relapse (Abe et al., 2008; Barnes and Melo, 2006; Chomel et al., 2011; Corbin et al., 2011) and blast crisis (BC) transformation after TKI discontinuation (Chomel and Turhan, 2011; Cortes et al., 2004; Deininger, 2008; Stuart et al., 2009). Despite improvements in overall survival (Druker et al., 2006), no curative pharmacologic therapy for CML exists, partly because the genetic and epigenetic drivers of human BC LSC generation remain to be elucidated.

In human BC CML, and in many cases of AML, LSCs are enriched within the $CD34^+CD38^+Lin^-$ compartment, which is composed predominantly of granulocyte-macrophage progenitors (GMPs) (Eppert et al., 2011; Goardon et al., 2011; Jamieson et al., 2004) with an aberrant self-renewal capacity. Serial transplantation experiments show that as few as 1,000 GMPs serially transplant human BC CML (Abrahamsson et al., 2009). Moreover, GMP LSCs have been identified in transgenic mouse models of both BC CML (Jaiswal et al., 2003) and AML (Krivtsov et al., 2006), suggesting that malignant transformation of progenitors into LSC, through aberrant acquisition of stem cell properties, is a key driver of leukemic progression.

Evidence from primary patient samples demonstrates that chronic phase (CP) CML is a clonal disorder (Martin et al.,

1980) that originates from BCR-ABL (Daley et al., 1990)-expressing hematopoietic stem cells (HSCs) (Jamieson et al., 2004). Although necessary for CP initiation, BCR-ABL expression is not sufficient to drive BC transformation (Radich et al., 2006). Both mouse transgenic models and xenotransplantation data show that the activation of stem cell signaling pathways, including the Wnt/β-catenin pathway (Abrahamsson et al., 2009; Jamieson et al., 2004; McWeeney et al., 2010; Zhao et al., 2007), the hedgehog signaling pathway (Zhao et al., 2009), and the intrinsic apoptotic pathway regulated by the *BCL2* gene family (Jaiswal et al., 2003), promote BC transformation. Malignant transformation of BCR-ABL1-expressing GMPs into self-renewing BC LSCs ($CD34^+CD38^+Lin^-$) occurs, in some cases, as a consequence of the alternative splicing of GSK3β, a negative regulator of Wnt/β-catenin, hedgehog signaling, and MCL1 (Abrahamsson et al., 2009; Ding et al., 2007). Whereas recent reports reveal that mutations in splicing genes promote the progression of myeloid malignancies to acute leukemia (Yoshida et al., 2011), alternative-splicing-mediated alterations in the transcriptome may also enable BC transformation in a malignant microenvironment.

Because CML becomes increasingly refractory to TKIs during progression to BC (Karbasian Esfahani et al., 2006; Sawyers et al., 2002), understanding the epigenetic mechanisms that drive BC LSC maintenance and contribute to therapeutic resistance is essential. In addition, several studies suggest that LSC quiescence induction by the stem cell niche is a major component of therapeutic resistance (Barnes and Melo, 2006; Corbin et al., 2011; Forsberg et al., 2010; Holyoake et al., 1999; Saito et al., 2010). Although recent evidence shows that increased expression of BCL2 family members contributes to CML pathogenesis (Aichberger et al., 2005; Dai et al., 2004; Tauchi et al., 2003), the precise nature of BCL2 splice-isoform usage had not been examined, even though a number of isoforms have antithetical functions (Akgul et al., 2004).

Prosurvival *BCL2* family genes contribute to leukemogenesis (Beverly and Varmus, 2009), CML progression (Jaiswal et al., 2003), TKI resistance (Aichberger et al., 2005; Horita et al., 2000; Jaiswal et al., 2003; Konopleva et al., 2002; Sánchez-García and Grütz, 1995), and HSC and progenitor cell survival (Domen and Weissman, 2003; Milyavsky et al., 2010) by direct inhibition of mitochondrial outer-membrane permeabilization. Expression of *BCL2* family genes has also been linked to bone-marrow-niche-dependent TKI resistance in vitro (Bewry et al., 2008). However, whether prosurvival *BCL2* family gene splice-isoform expression promotes human BC LSC maintenance has not been elucidated. Moreover, the role of niche-dependent *BCL2* family gene expression has not been delineated in the context of BC LSC quiescence induction and TKI resistance in vivo. Thus, we compared *BCL2* family expression in fluorescence-activated cell sorting (FACS)-purified CML progenitors from normal, CP, and BC patients and in BC LSCs engrafted in different hematopoietic niches. We also investigated whether BC LSCs could be targeted with sabutoclax, a pan-BCL2 inhibitor capable of inhibiting BCL2, MCL1, BFL1, and $BCLX_L$. Finally, the capacity of pan-BCL2 inhibition to overcome niche-dependent TKI resistance was assessed both in vitro and in BC LSC xenograft models as a paradigm for understanding the potential utility of sabutoclax in the sensitization of quiescent cancer stem cells (CSCs) to antiproliferative agents in a broad array of malignancies.

RESULTS

Prosurvival *BCL2* Isoform Expression Increases during BC Transformation

Although several studies have linked *BCL2* gene upregulation with CML progression, most have focused on BCR-ABL-expressing cell lines (Amarante-Mendes et al., 1998; Gesbert and Griffin, 2000; Sánchez-García and Grütz, 1995) or bulk $CD34^+$ cells (Aichberger et al., 2005; Horita et al., 2000; Radich et al., 2006) rather than self-renewing human BC LSCs ($CD34^+CD38^+Lin^-$) that promote BC transformation. Even though many *BCL2* family genes encode splice variants with both proapoptotic and antiapoptotic functions (Moore et al., 2010), relatively little is known about the pattern of *BCL2* family gene isoform expression in human BC LSCs. Therefore, we utilized splice-isoform-specific quantitative RT-PCR (qRT-PCR) and whole-transcriptome RNA sequencing (RNA-seq) to analyze *BCL2* family isoform expression in FACS-purified progenitors from primary normal (n = 6), CP (n = 8), and BC (n = 9) human samples (Table S1 available online). Notably, BC LSCs expressed significantly higher levels of *BCR-ABL* and prosurvival $BCL2_L$, $MCL1_L$, $BCLX_L$, and $BFL1_L$ splice-isoforms than did CP progenitors (Figure 1A), as well as higher $BCL2_L$, $BCLX_L$, and $BFL1_L$ than did normal progenitors (Figures S1A and S1B). Both qRT-PCR and RNA-seq revealed a relative abundance of antiapoptotic *MCL1*-long compared with proapoptotic short isoforms in BC LSCs (Figures 1B, 1C, S1A, and S1B). These data suggest that prosurvival *BCL2* family gene isoforms are globally upregulated during CML BC transformation.

Because BCR-ABL induces *BCL2* family gene expression in CML cell lines (Aichberger et al., 2005; Horita et al., 2000; Sánchez-García and Grütz, 1995), we examined whether prosurvival *BCL2* family overexpression coincided with *BCR-ABL* amplification in sorted CML progenitors. A striking correlation was observed between *BCR-ABL* and $BCLX_L$ levels in CML progenitors, which was confirmed in lentiviral BCR-ABL-transduced progenitors (Figure 1D), suggesting that increased $BCLX_L$ expression is driven by BCR-ABL amplification in BC LSCs, as previously reported (Aichberger et al., 2005; Horita et al., 2000; Sánchez-García and Grütz, 1995). Expression of other prosurvival *BCL2* family gene isoforms did not correlate with BCR-ABL, indicating that upregulation occurs through BCR-ABL-independent mechanisms. Consistent with qRT-PCR results, an increase in BCL2 and MCL1 proteins was detected by FACS analysis in BC LSCs compared with CP progenitors (Figures 1E and S1C). Notably, BCL2 protein expression was higher in serially transplantable $CD34^+CD38^+Lin^-$ BC LSCs than in normal or CP $CD34^+CD38^-Lin^-$ and $CD34^+CD38^+Lin^-$ cells (Figure S1D). Moreover, increased expression of both BCL2 transcript and protein levels correlated with the expansion of $CD123^+$ GMP BC LSCs (Figures S1–S1F), suggesting that BCL2 overexpression portends CML progression. In addition to the increased prosurvival *BCL2* family gene expression detected by RNA-seq (Figure S1G), an apoptosis qRT-PCR array demonstrated that BC LSCs harbored distinct expression patterns of prodeath *BCL2* family genes as well as TP53 and

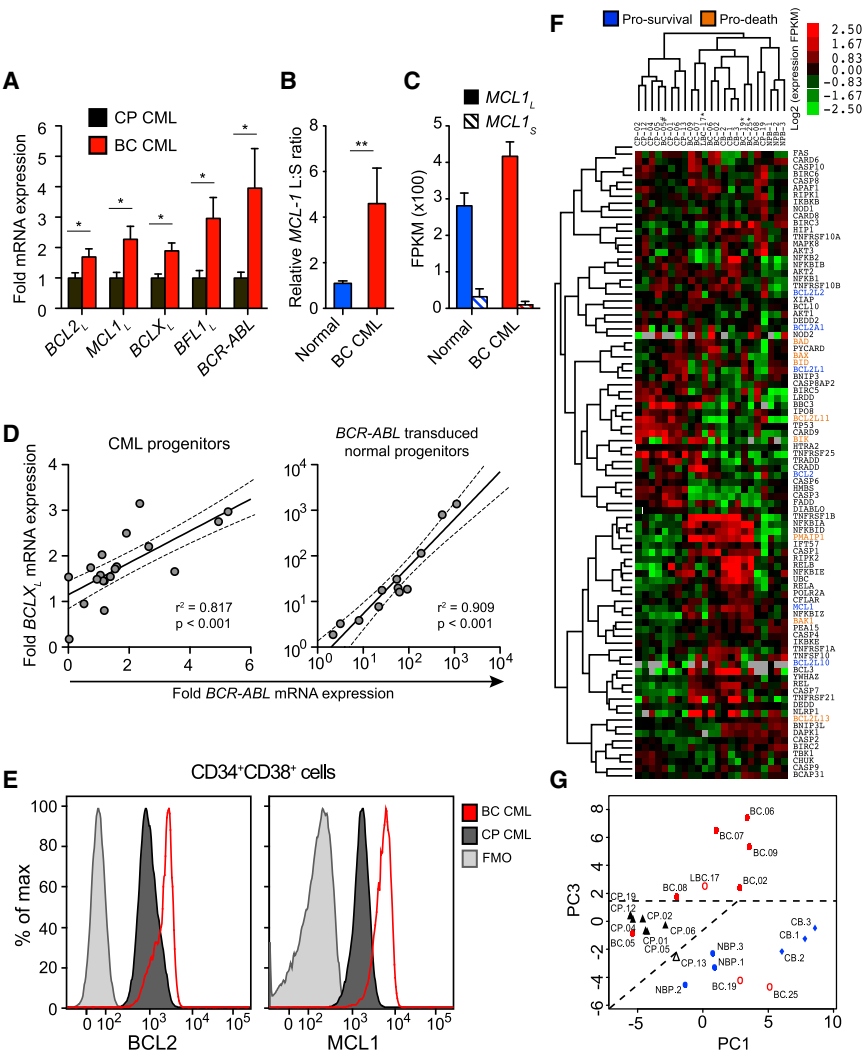

Figure 1. Prosurvival BLC2 Isoform Expression Increases Curing Blast Crisis Transformation

(A) Quantitative RT-PCR (qRT-PCR) of prosurvival (long isoforms) *BCL2*, *MCL1*, *BCLX*, *BFL1*, and *BCR-ABL* messenger RNAs (mRNAs) in primary chronic phase (CP; black, n = 13) and blast crisis (BC; red, n = 11) progenitors. Values are normalized to human *HPRT* mRNA expression. Graphs show mean ± SEM; *, $p < 0.05$ by unpaired t test.

(B) Relative ratio of *MCL1* long to short isoforms in normal (n = 7) and BC (n = 8) progenitors. Graph shows mean ± SEM; **, $p < 0.01$ by unpaired t test.

(C) Whole-transcriptome sequencing of fluorescence-activated cell sorting (FACS)-sorted progenitor cells from a normal and a BC sample showing quantification of $MCL1_L$ and $MCL1_S$ isoforms in each sample. Graph shows fragments per kilobase of exon per million fragments mapped (FPKM) ± 95% confidence interval.

(D) Correlation between *BCR-ABL* mRNA expression and $BCLX_L$ mRNA expression in progenitors. Primary CP and BC samples (n = 20; left). *BCR-ABL* transduced normal progenitor colonies (n = 12; right). Both graphs depict best-fit line and 95% confidence intervals by Pearson correlation analysis.

(E) Representative FACS histograms of BCL2 and MCL1 protein expression in CP progenitors (black) versus BC progenitors (red). Fluorescence minus one (FMO) controls are shown in gray.

(F) Full-transcriptome RNA sequencing analysis of survival-pathway genes in FACS-sorted ($CD45^+CD34^+CD38^+Lin^-$) progenitors from three normal cord blood (CB), three normal adult peripheral blood (NP), eight CP, one lymphoid BC (LBC), and eight BC samples. The heat map depicts log_2-fold FPKM. *BCL2* family genes are highlighted and their functions are indicated by blue (prosurvival) or orange (prodeath) coloring.

(G) Unsupervised principal component analyses of the survival pathway genes for FACS-sorted $CD45^+CD3\ 4^+CD38^+Lin^-$ progenitors from three CB, three NP, eight CP, one LBC, and eight BC samples.

See also Figure S1 and Tables S1 and S2.

TNF superfamily receptors, such as the Fas ligand and other components of the extrinsic apoptotic machinery, compared with normal progenitors (Table S2). To gain further insight into the role of survival regulators in BC transformation, we performed RNA-seq analysis on FACS-purified $CD34^+CD38^+Lin^-$ normal, CP, and BC samples (Table S1). Both heatmap (Figure 1F) and unsupervised principal component (Figure 1G) analysis revealed that survival-related gene expression distinguished BC LSCs from CP LSCs as well as TKI-treated and normal progenitor samples. Together, these data suggest that a distinct survival gene signature predicts LSC generation and BC transformation.

Quiescent BC LSCs Engraft the Bone Marrow Niche and Are TKI Resistant

Previous research demonstrated a link between BCL2 family member expression and the arrest of cells in G_0 or G_1 of the cell cycle (Zinkel et al., 2006; Vairo et al., 1996). In T and B cells of BCL2 transgenic mice, higher BCL2 expression correlated with a higher G_0 or G_1 fraction, a lower S phase fraction, and decreased BrdU incorporation (O'Reilly et al., 1997a; O'Reilly et al., 1997b; O'Reilly et al., 1996). Moreover, enforced BCL2 expression was recently shown to restore quiescence of progenitors in a mouse model of myelodysplastic syndrome (Slape et al., 2012). Seminal studies also show that quiescent LSCs are TKI resistant (Barnes and Melo, 2006; Bewry et al., 2008; Holyoake et al., 1999; Saito et al., 2010).

To analyze the capacity of various hematopoietic niches to maintain dormant LSCs, human BC $CD34^+$ cells, labeled with a membrane-bound fluorescent dye, DiR, which is retained by nondividing cells, were transplanted into neonatal $RAG2^{-/-}\gamma_c^{-/-}$ mice (Abrahamsson et al., 2009). Within 10 weeks, transplanted mice developed BC CML typified by myeloid sarcoma formation as well as robust liver, spleen, blood, and bone marrow

engraftment (Figure S2; Table S3). Notably, FACS analysis revealed that marrow-engrafted BC LSCs harbored higher levels of DiR fluorescence than those in other niches (Figure 2A), corresponding to a distinct population of G_0 ($Ki67^{low}7\text{-}AAD^{low}$) progenitors (Figures 2B and 2C) in the marrow. Confocal fluorescence microscopic and immunohistochemical (IHC) analysis revealed dormant pHis-$H3^-$Ki-67^{low} human $CD45^+CD34^+CD38^+$ cells adjacent to the marrow endosteal region (Figures 2D, 2E, S3A, and S3B), as previously reported in AML LSC xenograft models (Saito et al., 2010). Moreover, FACS analysis revealed that $CD34^+CD38^+CD123^+CD45RA^+Lin^-$ (GMP) BC LSCs, previously shown to harbor the greatest serial-transplantation potential, were more prevalent in the marrow than in other hematopoietic niches (Figure S3C). In addition, cell-cycle FACS analysis revealed that a proportion of quiescent BC LSCs was enriched in the marrow compared to the splenic niche (Figures S3D and S3E).

To examine the capacity of TKIs to eliminate quiescent self-renewing BC LSCs, $RAG2^{-/-}\gamma c^{-/-}$, mice were transplanted with human BC $CD34^+$ cells and treated orally with dasatinib, a potent BCR-ABL-targeted TKI (Figure S4A). Transplantation resulted in robust engraftment of human $CD45^+$ cells (Figures 3A, 3B, S2A and S2B) and BC LSCs ($CD34^+CD38^+Lin^-$) in medullary and extramedullary microenvironments (Figures 3B–3D and S3). Although dasatinib treatment (50 mg/kg) significantly reduced the $CD45^+$ leukemic burden compared with vehicle-treated controls (Figures 3E and S4B–S4D), a dasatinib-resistant BC LSC population persisted in the marrow (Figures 3C, 3F, and S4E). Following dasatinib treatment, nanoproteomic analysis of FACS-purified-marrow-derived BC LSCs revealed a significant reduction in the phosphorylation of CRKL, a direct substrate of the BCR-ABL kinase (Figures 3G and 3H; Goldman and Brender, 2000), indicative of adequate BCR-ABL kinase inhibition. However, cell-cycle FACS analysis demonstrated an increase in quiescence (Figures 3I–3J), suggesting that quiescent BC LSCs are resistant to BCR-ABL kinase inhibition and enriched in the marrow niche, thereby providing a reservoir for relapse.

Marrow-Niche-Engrafted BC LSCs Have a Prosurvival Gene Signature

Because *BCL2* overexpression has been linked to apoptosis and TKI resistance in mouse transgenic models and cell lines (Amarante-Mendes et al., 1998; Domen and Weissman, 2003; Konopleva et al., 2002), we hypothesized that prosurvival *BCL2* family gene expression is enhanced in marrow-engrafted BC LSCs and that they harbor greater TKI resistance than those in other niches. Comparative apoptosis qRT-PCR array analysis performed on FACS-purified $CD45^+CD34^+CD38^+Lin^-$ cells revealed that, while *BCLX*, *BFL1*, and *BCLW* were not differentially expressed, *BCL2* was significantly upregulated in marrow compared with spleen tissue (Figures 4A–4B), as was the expression of the prosurvival isoforms of MCL1 and BFL1 (Figures S5A and S5B), thereby favoring BC LSC survival. Similarly, RNA-seq revealed increased *BCL2* and decreased *BIM* expression in marrow-engrafted BC LSCs compared to BC LSCs before transplantation (Figure S5C). To further support these findings, gene set enrichment analysis (GSEA) of RNA-seq data demonstrated that cell-cycle checkpoint and cell-cycle arrest genes were upregulated in FACS-purified BC LSCs compared with their normal counterparts (Figure S5D). Finally, BCL2 protein expression was significantly higher in marrow-engrafted BC LSCs than in non-LSCs (human $CD45^+CD34^-$ cells) in the same niche and correlated with a decreased sensitivity to dasatinib treatment (Figures S5E and S5F). Thus, marrow-niche-resident BC LSCs express high levels of prosurvival *BCL2* family gene isoform expression, leading to enhanced TKI resistance.

Both IHC and confocal fluorescence microscopic analysis demonstrated that human BCL2 and MCL1 protein expression (Figure 4C) colocalized with human CD34- and CD38-expressing cells in the marrow endosteal niche (Figure 4D). Interestingly, BCL2- and MCL1-expressing human BC $CD34^+$ cells were enriched in the femoral epiphysis, a preferential site for homing, proliferation, and survival of human leukemia cells following xenotransplantation (Figure S5G; Ninomiya et al., 2007). Dasatinib treatment increased BCL2 and MCL1 expression and reduced Ki67 (Figures S5H and S5I), consistent with FACS analyses showing an increase in the proportion of quiescent BC LSCs after TKI treatment (Figure 3I–3J). Although TKIs effectively eliminate LSCs in extramedullary microenvironments, they fail to eradicate quiescent, BCL2- and MCL1-expressing BC LSCs from the marrow niche.

Sabutoclax Inhibits BC LSC Survival

Detection of increased prosurvival *BCL2* isoforms in primary BC samples as well as enhanced BCL2 and MCL1 expression in marrow-engrafted BC LSCs, particularly following dasatinib treatment (Figure S4), provided the impetus for testing the LSC inhibitory capacity of sabutoclax, an optically pure derivative of apogossypol that inhibits all prosurvival BCL2 family proteins (Wei et al., 2009; Wei et al., 2010; Figure 5A). Sabutoclax treatment increased the apoptosis of BC LSCs in a dose-dependent manner in vitro, as measured by cleaved capase-3 and propidium iodide staining (Figure 5B). Because BC LSCs were TKI-resistant in the marrow niche, the anti-LSC efficacy of sabutoclax was tested in a genetically engineered SL and M2 stromal coculture system that secretes human SCF, IL-3, and G-CSF and supports the long-term survival of self-renewing BC LSCs (Hogge et al., 1996; Figure S6A). Despite the induction of prosurvival *BCL2* family gene expression in BC LSC-supportive stromal cocultures (Figure S6B), sabutoclax reduced LSC survival and colony-forming capacity (Figures 5C, 5D and S6A) at doses that spared normal progenitors (Figures 5C and 5D; Table S4). Moreover, lentiviral-mediated short-hairpin RNA knockdown of *BCL2* reduced the colony-forming capacity of BC LSCs but not of normal progenitors (Figures 5E and 5F). However, *BCL2* knockdown did not completely abrogate BC LSC colony formation, suggesting that inhibition of multiple BCL2 family proteins, including MCL1, is required in order to eradicate BC LSCs in supportive niches.

To further assess the role of BCL2 in BC LSC survival, ABT-737, a potent BCL2 and $BCLX_L$ inhibitor, was utilized in parallel stromal coculture experiments. Fluorescence polarization assays demonstrated that sabutoclax and ABT-737 dissociate a BIM peptide from BCL2 and $BCLX_L$ at nanomolar concentrations. However, only sabutoclax effectively displaces BIM from MCL1 and BFL1 (Figures S6C and S6D; Table S4). Because

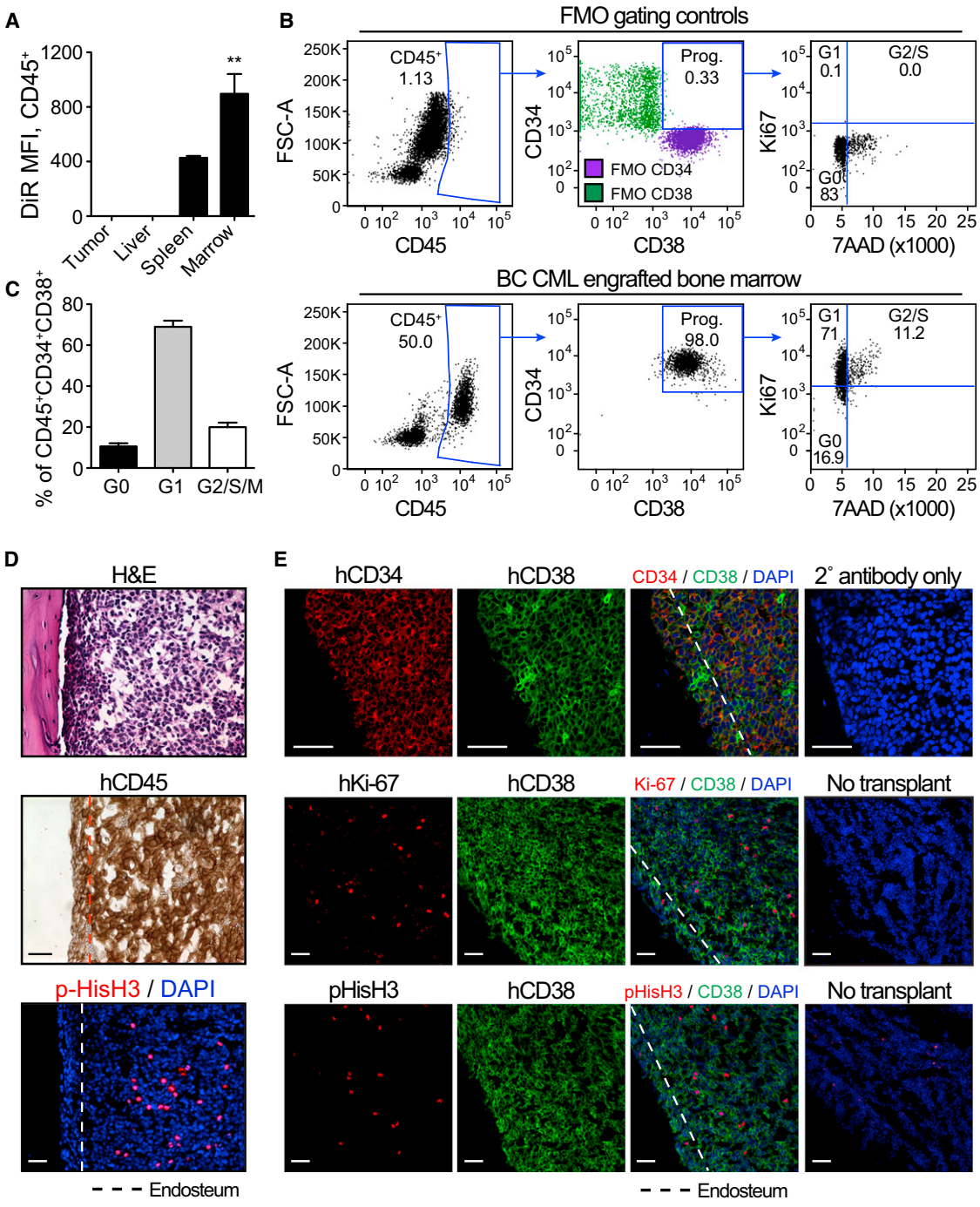

Figure 2. Quiescent BC LSCs Engraft the Bone Marrow Niche

(A) Retained DiR fluorescence of BC progenitors ($CD45^+CD34^+CD38^+Lin^-$) engrafted in tumor (n = 4), liver (n = 4), spleen (n = 3), and bone marrow (n = 2) 18 weeks after DiR surface staining and transplant. Graph shows mean ± SEM; ***, $p < 0.001$ by ANOVA and Tukey post hoc analysis.

(B) Representative FACS plots showing gating and cell-cycle analysis of live (propidium iodide negative [Pi^-]), bone marrow-engrafted BC progenitors ($CD45^+CD34^+CD38^+Lin^-$). FMO gating controls are shown in the top row, and engrafted bone marrow is shown in the bottom row.

(C) Quantification of BC progenitors in untreated marrow in the different phases of the cell cycle. n = 10 engrafted bones. Graph shows mean ±SEM.

(D–E) Histological analysis of engrafted bone marrow showing hematoxylin and eosin (H&E), human CD34, CD38 and Ki67, and pHis-H3 staining. The dotted lines delineate the endosteum (~50 μm from the bone edge). Scale bars represent 50 μm.

See also Figures S2 and S3 and Table S3.

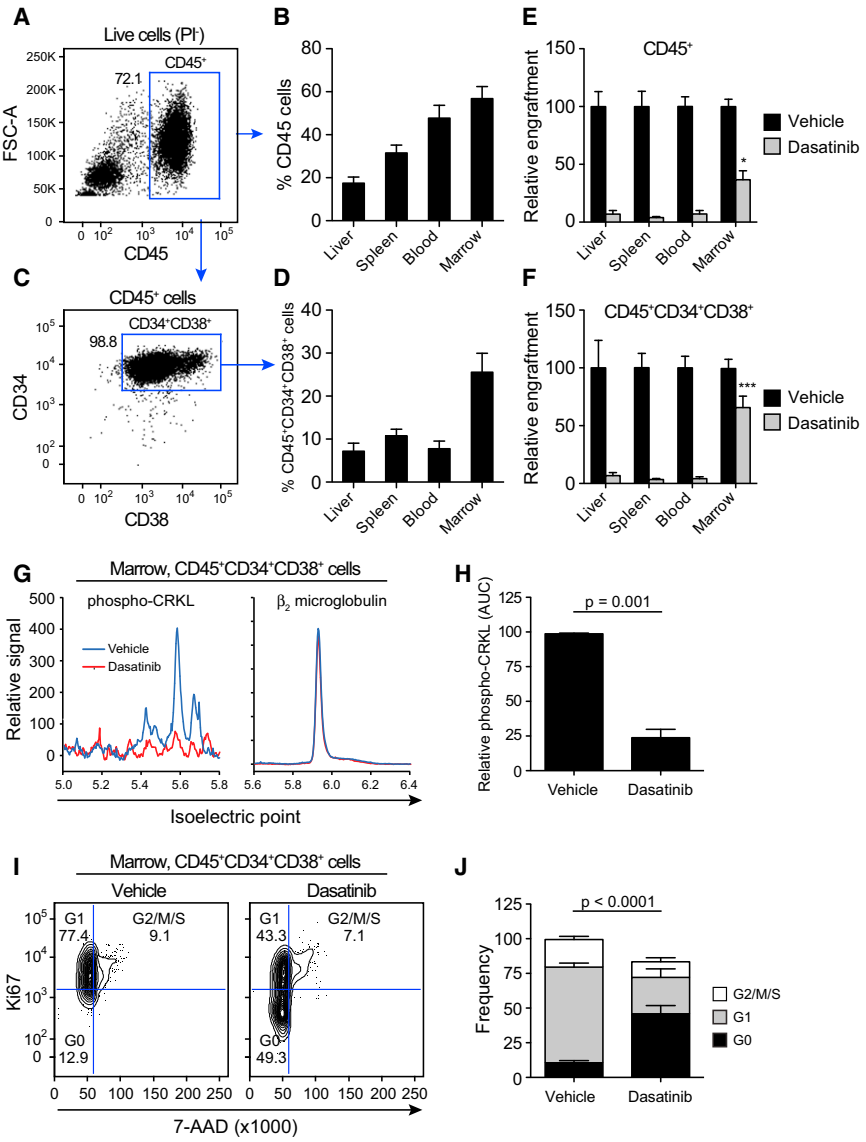

Figure 3. Marrow-Engrafted Quiescent BC LSCs Are TKI Resistant

(A) Representative FACS plots (live cells [PI^-]) showing human CD45 engraftment in mouse bone marrow. Percentages shown are based on parental population as indicated.

(B) Total human CD45 engraftment in liver, spleen, blood, and bone marrow of BC-transplanted mice (n = 31) for 3 different CML BC patients samples. Graphs show mean ± SEM.

(C) Representative FACS plots showing human BC progenitors ($CD45^+CD34^+CD38^+Lin^-$) engraftment in mouse bone marrow.

(D) Total engraftment of progenitors in the hematopoietic organs of BC transplanted mice (n = 31) for 3 different CML BC patient samples. Values are a back-gated calculation, based on the percentage of $CD45^+$ cells = (percentage of $CD45^+$ cells) × (percentage of $CD34^+CD38^+$ cells). Graphs show mean ± SEM.

(E) Relative engraftment (percent of vehicle treated controls) of human CD45 cells in the hematopoietic organs following treatment with vehicle (n = 13) or dasatinib (50 mg/kg, n = 14). Statistical analysis is shown comparing the residual engraftment in each tissue (gray bars) and depicts the results of ANOVA with Tukey post hoc comparisons; *, $p < 0.05$. Graphs show mean ± SEM.

(F) Relative engraftment (percentage of vehicle treated controls) of progenitors in the hematopoietic organs following treatment with vehicle (n = 19) or dasatinib (n = 19). Statistical analysis is shown comparing the residual engraftment in each tissue (gray bars) and depicts the results of ANOVA with Tukey post hoc comparisons; ***, $p < 0.001$. Graphs show mean ± SEM.

(G) Representative proteomics plots showing the analysis of phosphorylated CRKL (left) and β_2 microglobulin (right) in BC progenitors sorted from engrafted mouse bone marrow and after treatment with either vehicle or dasatinib.

(H) Quantification of the total area under the curve (AUC) of phosphorylated CRKL peaks in vehicle- (n = 5) and dasatinib- (n = 5) treated samples. All values are normalized to β_2 microglobulin protein expression in the same sample. Graphs show mean ± SEM.

(I) Representative FACS-cell-cycle plots of live $CD45^+$ BC progenitors engrafted in marrow following vehicle and dasatinib treatment.

(J) Cell-cycle status of live $CD45^+$ BC progenitors sorted from the marrow and following treatment with vehicle (n = 10) or dasatinib (n = 10). All graphs in Figure 3 show mean ± SEM. All statistical analyses are by unpaired t test unless otherwise specified.

See also Figure S4.

ABT-737 resistance is associated with increased *MCL1* and *BFL1* expression (Vogler et al., 2009a; Yecies et al., 2010) and both qRT-PCR and transcriptome data showed that BC LSCs express multiple BCL2 family members, including *MCL1* and *BFL1* (Figures S5A and S5B), the anti-LSC efficacy of sabutoclax and ABT-737 was compared. Sabutoclax reduced BC LSC survival more than ABT-737 did at all doses tested in stromal cocultures (Figures S6F and S6G; Table S4), even though the activity looked comparable in stroma-independent K562 cells (Figure S6H), thereby underscoring the importance of the niche in BCL2 family member induction. Hence, eradication of niche-dependent BC LSCs is predicated on the inhibition of multiple BCL2 family proteins, including MCL1 and BFL1.

Sabutoclax Sensitizes Marrow-Niche-Engrafted BC LSCs to Dasatinib

To examine the necessity of prosurvival *BCL2* family expression for BC LSC maintenance, we tested the efficacy of sabutoclax in inhibiting BC LSC survival in the marrow compared with the splenic niche (Figure 6A). In BC $CD34^+$ cell-engrafted mice, FACS analysis revealed that sabutoclax (5 mg/kg) reduced LSC burden (Figure 6B; Table S5) commensurate with a decrement in human BCL2- and MCL-expressing cells in the marrow (Figures 6C and 6D). Moreover, sabutoclax treatment increased G_2/S (Figure 6E) and $TUNEL^+$ apoptotic cells (Figure 7A), indicative of both cell-cycle and apoptosis induction. Consistent with in vitro results, no significant reduction was observed in normal

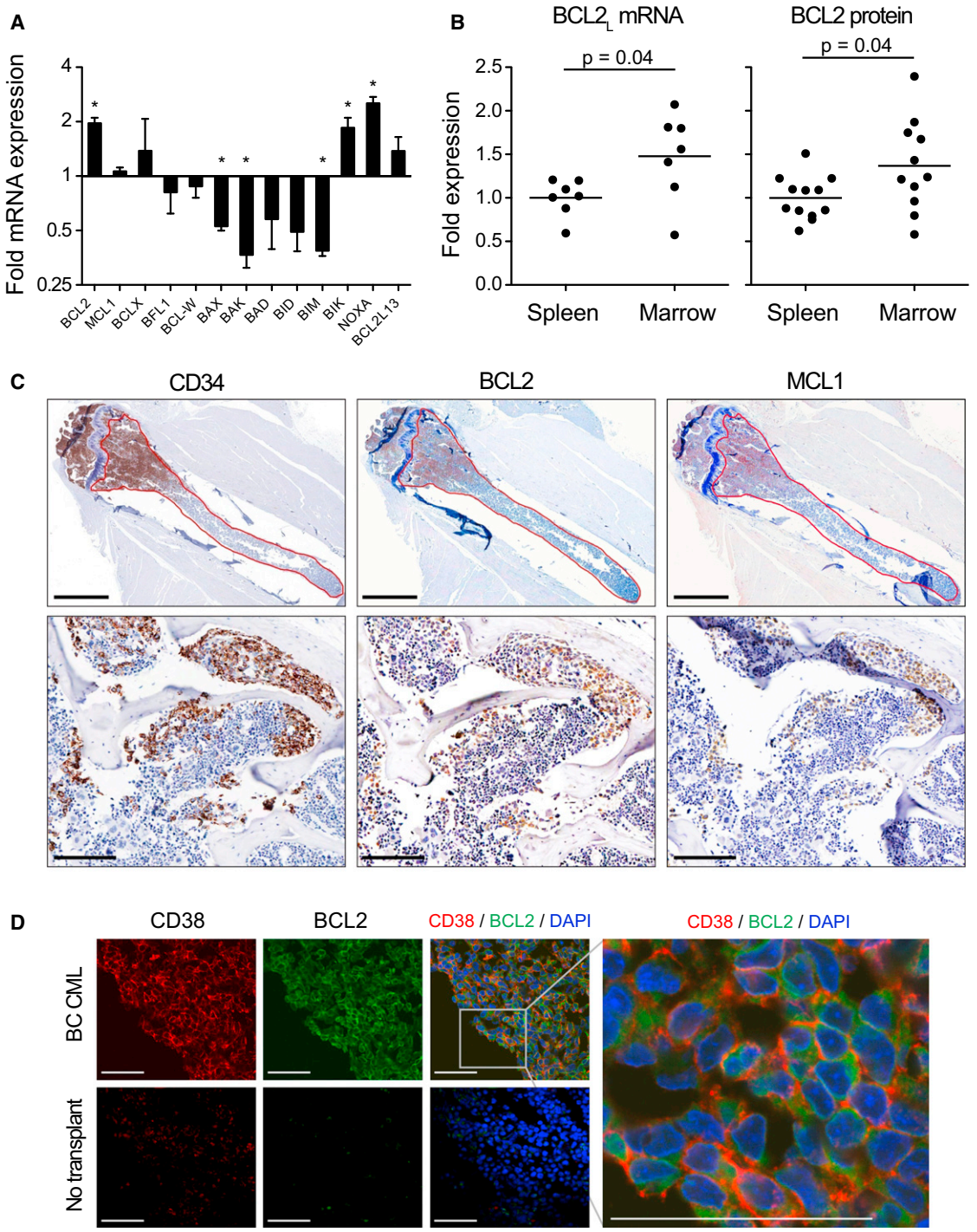

Figure 4. Enhanced BCL2 Expression in Marrow-Niche-Engrafted BC LSCs

(A) BCL2 family RT^2 Profiler PCR Array System data of FACS-sorted progenitors from engrafted mice (n = 3). The graph depicts fold expression in marrow-engrafted progenitors relative to spleen-engrafted progenitors, which are set at 1. Graphs show mean ± SEM; $p < 0.05$ by unpaired t test.

(B) *BCL2L* mRNA isoform expression and BCL2 protein expression in marrow- versus spleen-engrafted BC progenitors. Statistical analyses are by unpaired t test.

(C) Representative immunohistochemical (IHC) analysis of gross (top) and endosteal (bottom) engraftment of human CD34$^+$, BCL2$^+$, and MCL1$^+$ cells in mouse bone marrow. Scale bars represent 1 mm in low-magnification images and 100 μm in high-magnification images.

(D) Representative images showing immunofluorescence analysis of hCD38$^+$ and hBCL2$^+$ cells in BC CML-engrafted marrow compared to nontransplant control marrow. Scale bars represent 50 μm. The boxed area shows a closeup of the endosteal region.

See also Figure S5.

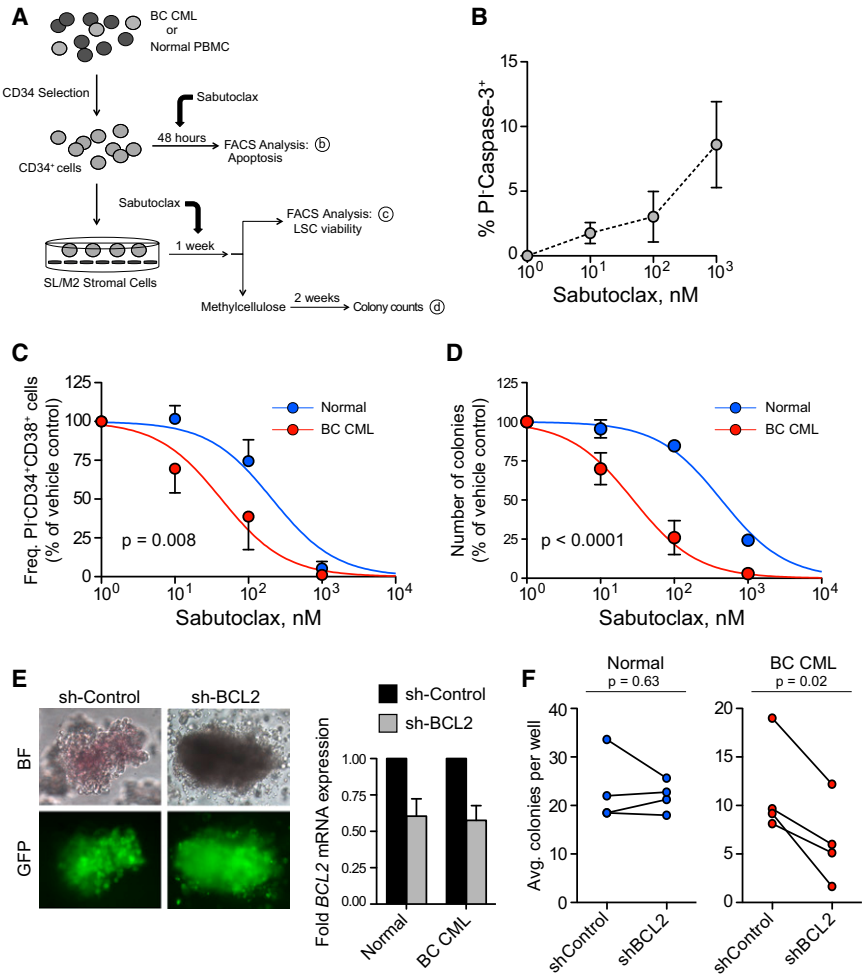

Figure 5. Sabutoclax Inhibits BC LSC Survival

(A) In vitro experimental design used in the present studies.

(B) FACS analysis of activated caspase-3$^+$ CML progenitors following in vitro culture with BI-97C1 (sabutoclax). Graph shows mean ± SEM from three independent experiments.

(C) FACS analysis of normal (blue) and BC (red) progenitors cultured on SL and M2 bone marrow stroma in the presence of sabutoclax. All values are normalized to vehicle treated control. Graph shows mean ± SEM from five different normal and BC samples.

(D) Total colonies formed by normal (blue) and BC (red) progenitors following sabutoclax treatment. All values are normalized to vehicle-treated control. Graph shows mean ± SEM from three different normal and three BC samples. (C) and (D) show best-fit lines and statistical comparisons by nonlinear regression analysis.

(E) Representative colonies from control or shBCL2 transduced progenitors (left). qRT-PCR of $BCL2_L$ mRNA in normal or BC cells following transduction with control and shBCL2 lentivirus (n = 3 colonies for each sample type; right). Graph shows mean ± SEM.

(F) Number of colonies formed by FACS-sorted normal (blue) or BC (red) progenitors following transduction with control or shBCL2 lentivirus. Graph shows average colonies per well for four different normal and BC samples and statistical analysis by paired t test.

See also Figure S6 and Tables S4 and S5.

progenitor engraftment in the marrow after sabutoclax treatment (Figures S7B–S7D), suggesting that a reasonable therapeutic index exists between BC LSCs and normal HSCs.

For quantification of the TKI-sensitizing effects of sabutoclax in the presence of human BC-LSC-supportive cytokines not present in mouse marrow, human BC LSCs from sabutoclax- or vehicle-treated mice were FACS-sorted into SL and M2 stromal cocultures in the presence of dasatinib (Figure S7A). In this ex vivo assay, sabutoclax-pretreated progenitors were more sensitive to dasatinib than were vehicle-pretreated controls (Figure S7B). For further examination of the synergistic effects of sabutoclax and dasatinib, BC LSC-engrafted mice were treated with lower-dose sabutoclax, dasatinib, or a combination of both, followed by FACS-mediated LSC analysis. Whereas lower-dose dasatinib and sabutoclax alone had no significant effect on marrow BC LSC engraftment, combination treatment significantly reduced marrow LSC survival (Figure 7E; Table S6). These results suggest that sabutoclax sensitizes quiescent BCL2- and MCL1-expressing BC LSCs to dasatinib-mediated cell death. Finally, the capacity of combined treatment to eradicate self-renewing BC LSCs was assessed by transplanting treated marrow into secondary recipients and monitoring survival time. Mice transplanted with combination-treated marrow had a significant survival advantage compared to those that received dasatinib-treated marrow (Figure 7F). Sabutoclax-mediated TKI sensitization was dose (Table S6) and route-of-administration dependent, with greater bioavailability provided by intravenous dosing, as shown by pharmacokinetic studies (Figure S7C). More clinically applicable intravenous dosing resulted in a significant reduction in BC LSCs after combination sabutoclax and dasatinib therapy (Figure S7D) at doses that spared normal hematopoietic progenitors (Figures 7C and 7D). Overall, our data demonstrate that dasatinib alone, although effective in reducing bulk leukemic cell burden, does not eradicate marrow-niche-resident BC LSCs. In contrast, combined dasatinib and sabutoclax therapy significantly inhibits both primary and serial LSC engraftment, indicative of abrogation of both TKI resistance and BC LSC self-renewal.

DISCUSSION

Malignant transformation of human myeloid progenitors into BC LSCs through alternative splicing represents a molecular mechanism driving CML BC transformation and therapeutic resistance. By analyzing FACS-sorted, serially transplantable CD34$^+$ CD38$^+$Lin$^-$ cells from primary patient samples, we show that BC LSCs harbor increased expression of multiple prosurvival *BCL2* family genes compared to both CP and normal progenitors. This prosurvival gene expression is further upregulated upon coculture with human LSC-supportive cytokine-secreting bone

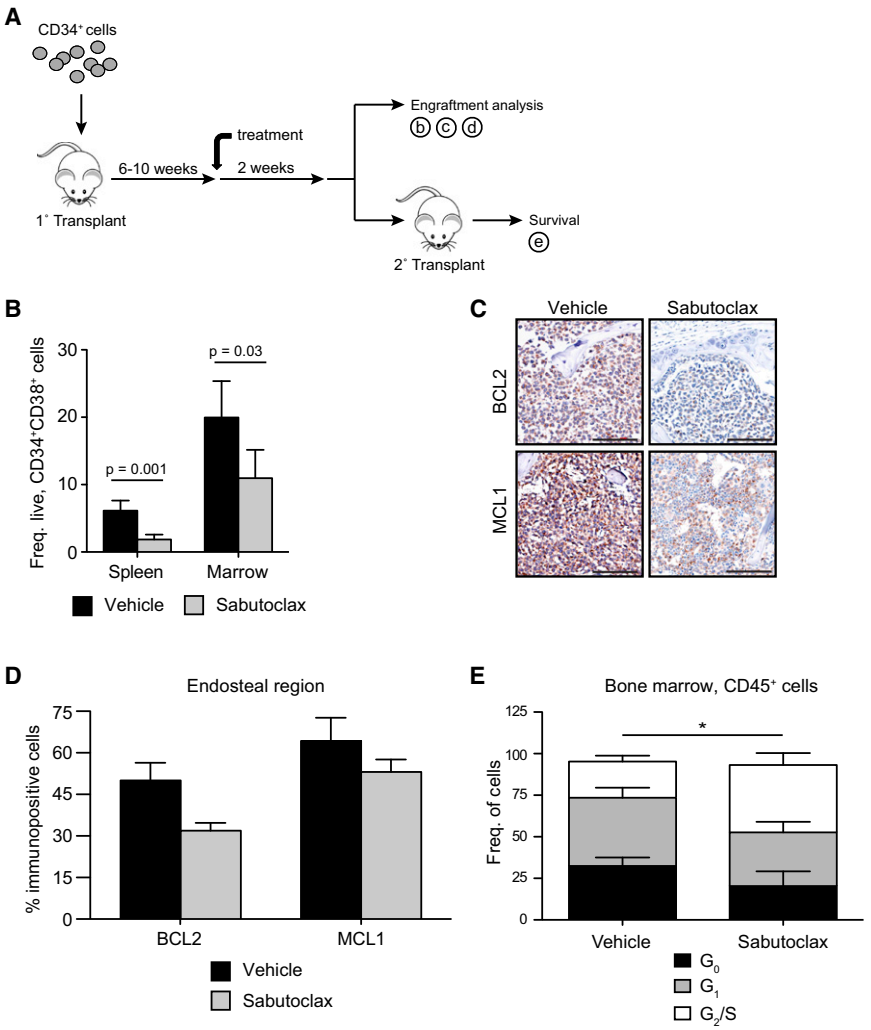

Figure 6. Sabutoclax Sensitizes Marrow-Niche-Engrafted BC LSCs to Dasatinib

(A) In vivo experimental design used in the present studies.

(B) Engraftment of BC progenitors in spleen and bone marrow following vehicle (n = 27) and sabutoclax treatment (5 mg/kg, n = 26). Graph shows mean ± SEM for three different BC patient samples and statistical analysis by Mann-Whitney test.

(C) Representative IHC analysis of BCL2 and MCL1 staining in engrafted bone marrow following vehicle and sabutoclax treatment. Scale bars represent 100 μm.

(D) Quantification of $BCL2^+$ and $MCL1^+$ cells in the endosteum of engrafted bone marrow following vehicle (n = 3) and sabutoclax (n = 3) treatment. Graph shows mean ± SEM.

(E) FACS-cell-cycle analysis of $CD45^+$ bone-marrow-engrafted BC cells in vehicle- (n = 6) and sabutoclax- (n = 5) treated mice. Graph shows mean ± SEM. *, $p < 0.05$ by unpaired t test.

See also Figure S7 and Table S6.

marrow stroma and upon engraftment in the bone marrow niche. These data are consistent with previous reports demonstrating increased BCL2 family expression in CML cells (Aichberger et al., 2005; Horita et al., 2000; Sánchez-García and Grütz, 1995) and upregulation via niche-dependent signals (Bewry et al., 2008). However, our study is unique in that we show that prosurvival *BCL2* family splice-isoform upregulation is present in self-renewing BC LSCs and that niche-dependent BCL2 family expression is associated with TKI resistance in vivo. This study represents an important whole-transcriptome and splice-isoform-specific, qRT-PCR-based elucidation of isoform-specific *BCL2* family gene expression signatures in CML LSCs, which is important given that the *BCL2* family is spliced into variants with antithetical functions (Akgul et al., 2004; Bingle et al., 2000) and has potential clinical significance with regard to predicting leukemic progression.

In a robust $RAG2^{-/-\gamma c-/-}$ xenograft model of human BC CML, we demonstrate that BC LSCs are protected from TKI-mediated cell death when engrafted in the marrow microenvironment as opposed to extramedullary hematopoietic niches, suggesting that LSCs are subject to marrow-specific cytoprotection independent of BCR-ABL, as demonstrated by nanoproteomic phospho-CRKL analysis. Although dasatinib treatment effectively reduces leukemic burden in engrafted mice, it does not fully eliminate BC LSCs, as evidenced by the fact that mice serially transplanted with dasatinib-treated bone marrow quickly develop BC CML. These data add to previous findings that CML BC LSCs also depend on BCR-ABL-independent survival mechanisms (Corbin et al., 2011). Our findings expand on this concept by identifying prosurvival *BCL2* family isoform expression as an important niche-specific survival mechanism and molecular target for CML BC LSC sensitization to TKI therapy. Although lentiviral BCR-ABL transduction experiments suggest that $BCLX_L$ expression is BCR-ABL dependent, our in vivo studies suggest that marrow microenvironmental cues promote splice-isoform switching that favors the expression of multiple prosurvival BCL2 family splice-isoforms in BC LSC, thereby providing the impetus for elucidating these extrinsic factors in future studies.

Both cell-cycle and immunofluorescence analyses demonstrate that quiescent CML BC LSCs engraft the marrow niche and are enriched in the endosteal region, consistent with previous AML xenograft studies (Saito et al., 2010). Moreover, IHC analyses show that endosteal niche-resident BC LSCs express prosurvival BCL2 and MCL1. Strikingly, dasatinib treatment does not eliminate quiescent bone marrow BC LSCs. These quiescent BC LSCs harbor enhanced engraftment potential (Barnes and Melo, 2006), which may explain why mice serially transplanted with dasatinib-treated marrow still develop BC CML.

Notably, BC LSCs in stromal coculture and in the marrow are sensitive to sabutoclax, a pan-BCL2 inhibitor, in a dose dependent manner (Wei et al., 2009; Wei et al., 2010). Sabutoclax

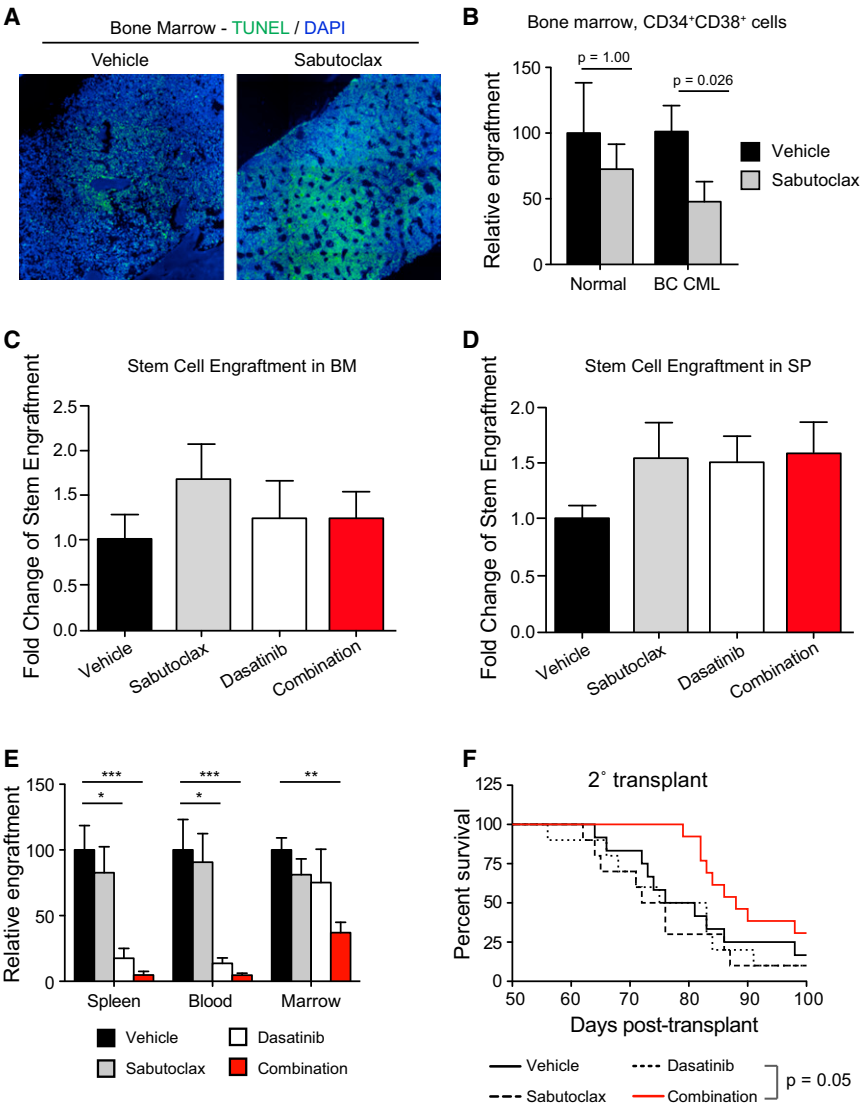

Figure 7. Bone Marrow BC LSCs Are Sensitive to Sabutoclax

(A) Representative TUNEL staining of BC-engrafted bone marrow following treatment with vehicle or sabutoclax.

(B) Relative engraftment of normal and BC progenitors in bone marrow following vehicle and sabutoclax treatment (5 mg/kg). Normal: vehicle n = 6, sabutoclax n = 6; BC: vehicle n = 26, sabutoclax n = 27. Graph shows mean ± SEM and statistical analysis by Mann-Whitney test.

(C) Fold change of normal stem cells in the bone marrow after treatment with vehicle (n = 8); sabutoclax (10 mg/kg IV, biweekly for 4 doses [n = 8]); dasatinib (50 mg/kg, daily oral gavage, 14 doses total [n = 11]); or a combination of sabutoclax and dasatinib (n = 10). Graph shows mean ± SEM.

(D) Fold change of normal stem cell in spleen after treatment with vehicle (n = 8); sabutoclax (10 mg/kg IV, biweekly for 4 doses [n = 8]); dasatinib (50 mg/kg, daily oral gavage, 14 doses total [n = 11]); or a combination of sabutoclax and dasatinib (n = 10). Graph shows mean ± SEM.

(E) Relative engraftment of BC progenitors in spleen, blood, and bone marrow following treatment with vehicle (n = 9), sabutoclax (1.25 mg/kg, n = 9), dasatinib (25 mg/kg, n = 9), and sabutoclax in combination with dasatinib (n = 11). Graph shows mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by Kruskal-Wallis test with Dunn's post hoc analysis.

(F) Survival of mice after being serially transplanted with vehicle (n = 11)-, sabutoclax (n = 9)-, dasatinib (n = 9)-, and combination (n = 13)-treated whole bone marrow. Statistical analysis was performed by log-rank test.

also sensitizes marrow-niche BC LSCs to TKI treatment, suggesting that marrow-specific TKI protection is predicated, at least in part, on BCL2 family expression in the niche and can be overcome with a pan-BCL2 inhibitor. Also, unlike dasatinib, sabutoclax targets quiescent self-renewing LSCs. This is further evidenced by our observation that sabutoclax combined with dasatinib significantly improves survival of serially transplanted mice.

Although BCL2 inhibition has been previously explored in CML, most studies have focused on CML cell lines (Kuroda et al., 2006; Meng et al., 2007) or $CD34^+$ cells grown in culture (Mak et al., 2012) rather than self-renewing CML BC LSCs in selective niches. Moreover, published reports do not address the potential antithetical roles of BCL2 family splice-isoforms or the role of the microenvironment in promoting LSC survival. Treatment with ABT-737 (Kuroda et al., 2006; Mak et al., 2012), a potent BCL2 and $BCLX_L$ inhibitor, does not inhibit $MCL1_L$ or BFL1 (Oltersdorf et al., 2005; Wei et al., 2010), both of which accelerate leukemogenesis (Beverly and Varmus, 2009), mediate resistance (Chen et al., 2007; Vogler et al., 2009a; Yecies et al., 2010), and are upregulated in CML progenitors during progression from CP to BC. Because inhibition of both subfamilies of prosurvival BCL2 family proteins is necessary for apoptosis initiation (Vogler et al., 2009b), inhibition strategies that include MCL1 would be expected to be more successful than those that target BCL2 alone (Placzek et al., 2010). Recently, paired-end DNA sequencing analysis revealed an intronic deletion polymorphism in the proapoptotic gene *BIM* (BCL2-like 11), which generated a splice-isoform lacking the BH3 domain and preventing BIM-induced apoptosis in response to TKI therapy (Ng et al., 2012). Thus, pan-BCL2 inhibition may prove to be more effective at targeting TKI-resistant BC LSCs that naturally express multiple BCL2 family proteins in response to niche-dependent stimuli in vivo.

BCL2 family genes are regulated in a wide variety of hematologic malignancies (Beverly and Varmus, 2009; Reed, 2008) and solid tumors (Placzek et al., 2010). Moreover, CSC identified in several tumor types (Hermann et al., 2010) could conceivably rely on the expression of multiple prosurvival BCL2 family isoforms, making them candidates for pan-BCL2 inhibition as a vital addition to combination CSC eradication therapy. Our findings may also have relevance for the elimination of therapeutically

recalcitrant solid tumor CSCs where metastasis and survival in the metastatic niche are mediated by prosurvival BCL2 family expression (Mehlen and Puisieux, 2006). Thus, pan-BCL2 inhibition with sabutoclax could provide an important component of combination therapies that target a broad array of CSCs residing in protective niches.

EXPERIMENTAL PROCEDURES

Additional details and methods can be found in the Supplemental Experimental Procedures.

Patient Sample Preparation and FACS Sorting

Normal cord blood and adult peripheral blood samples were purchased from All Cells. CML samples were obtained from consenting patients at the University of California San Diego, Stanford University, the University of Toronto Health Network, MD Anderson, and the University of Bologna according to protocols approved by the institutional review board. $CD34^+$ cells were initially purified by magnetic bead separation (MACS), followed by FACS progenitor purification with human-specific CD34 and CD38 antibodies, as previously described (Abrahamsson et al., 2009; Jamieson et al., 2004). Peripheral blood mononuclear cells were extracted from peripheral blood after Ficoll density centrifugation and were then $CD34^+$ selected, stained with fluorescent conjugated antibodies, and analyzed and purified with the FACSAria and FlowJo software as described previously (Abrahamsson et al., 2009; Jamieson et al., 2004).

BCL2 Family Gene Splice-isoform Analysis

Normal or CML $CD34^+$ cells were stained with a mouse antihuman BCL2 (Dako) monoclonal antibody and analyzed by FACS. qRT-PCR was performed with the SYBR GreenER Two-Step qRT-PCR Kit (Invitrogen) for the detection of *BCL2*, *MCL1*, *BCLX*, and *BFL1* isoforms in FACS-sorted normal versus CML progenitors. Quantitative *BCL2* isoform and apoptosis gene analysis was also performed in FACS-sorted normal and CML progenitors by whole-transcriptome RNA-seq.

BCL2 genes were also analyzed in engrafted CML cells. In brief, 20,000–50,000 $CD34^+CD38^+Lin^-$ cells were FACS-sorted from engrafted tissues and analyzed with the use of isoform-specific qRT-PCR, as above, or with the use of an RT-PCR apoptosis-pathway OpenArray nanoplate (Invitrogen). BCL2 protein was also measured in engrafted tissue cells as described above.

Quantitative RT-PCR

20,000–50,000 hematopoietic progenitor cells were sorted from the indicated cell populations with the use of FACS; total RNA was isolated and complementary DNA was synthesized as described previously (Abrahamsson et al., 2009; Jamieson et al., 2004). qRT-PCR was performed in duplicate on an iCycler with the use of SYBR GreenER qPCR SuperMix (Invitrogen), 5 ng of template mRNA, and 0.4mM of each forward and reverse primer. Splice-isoform-specific primers were designed for *BCL2*, *MCL1*, *BCLX*, and *BFL1*, and isoform specificity was confirmed by the sequencing of each PCR product. Messenger RNA (mRNA) levels for each transcript were normalized to HPRT and compared by the delta-delta Ct method.

SL and M2 Coculture and In Vitro Drug Treatment

Normal or CML $CD34^+$ cells were selected and plated on confluent, mitomycin-C-treated SL and M2 cells along with different doses of BI-97C1 (sabutoclax). After 1 week of culture, human progenitor cells were quantified by FACS and cells were plated in methylcellulose for colony-forming assays. Colonies were scored after 2 additional weeks in culture.

Lentivirus Transduction

BCL2 mRNA expression was silenced with the use of shBCL2-encoding SMARTvector 2.0 lentiviral particles (Thermo-Dharmacon, no. SK-003307). The efficiency of shBCL2 and control lentiviral vectors was tested by transduction of 293T and K562 cell lines. Knockdown of ~50% of BCL2 transcripts was confirmed by qRT-PCR. Cells transduced with lentiviral shBCL2 and shControl were FACS-sorted into Methocult media (20–50 cells per well of a 96-well plate, 5–10 wells per condition). Total colonies were counted for each condition after 2 weeks of culture, and BCL2 knockdown was measured in the colonies.

Statistical Analysis

Statistical analyses were performed with the aid of Microsoft Excel, SAS 9.2, and GraphPad Prism software, as indicated in the figure legends.

SUPPLEMENTAL INFORMATION

Supplemental Information contains Supplemental Experimental Procedures, seven figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2012.12.011.

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