

Glycogen synthase kinase 3 β missplicing contributes to leukemia stem cell generation

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Recent evidence suggests that a rare population of self-renewing cancer stem cells (CSC) is responsible for cancer progression and therapeutic resistance. Chronic myeloid leukemia (CML) represents an important paradigm for understanding the genetic and epigenetic events involved in CSC production. CML progresses from a chronic phase (CP) in hematopoietic stem cells (HSC) that harbor the BCR-ABL translocation, to blast crisis (BC), characterized by aberrant activation of β -catenin within granulocyte-macrophage progenitors (GMP). A major barrier to predicting and inhibiting blast crisis transformation has been the identification of mechanisms driving β -catenin activation. Here we show that BC CML myeloid progenitors, in particular GMP, serially transplant leukemia in immunocompromised mice and thus are enriched for leukemia stem cells (LSC). Notably, cDNA sequencing of Wnt/ β -catenin pathway regulatory genes, including adenomatous polyposis coli, GSK3 β , axin 1, β -catenin, lymphoid enhancer factor-1, cyclin D1, and c-myc, revealed a novel in-frame splice deletion of the GSK3 β kinase domain in the GMP of BC samples that was not detectable by sequencing in blasts or normal progenitors. Moreover, BC CML progenitors with misspliced GSK3 β have enhanced β -catenin expression as well as serial engraftment potential while reintroduction of full-length GSK3 β reduces both in vitro replating and leukemic engraftment. We propose that CP CML is initiated by BCR-ABL expression in an HSC clone but that progression to BC may include missplicing of GSK3 β in GMP LSC, enabling unphosphorylated β -catenin to participate in LSC self-renewal. Missplicing of GSK3 β represents a unique mechanism for the emergence of BC CML LSC and might provide a novel diagnostic and therapeutic target.

blast crisis chronic myeloid leukemia | wnt pathway | xenograft | self-renewal | cancer stem cells

Chronic myeloid leukemia (CML) was the first cancer shown to be initiated at the hematopoietic stem cell (HSC) level by a pathognomonic chromosomal abnormality the Philadelphia chromosome, which produces a constitutively active protein tyrosine kinase—P210BCR-ABL (1–6). CML was also the first malignancy treated with a molecularly targeted agent imatinib, which inhibits the BCR-ABL tyrosine kinase (3). However, most CML patients treated with BCR-ABL inhibitors harbor cells with low-level BCR-ABL transcripts, suggesting that these cells may be susceptible to further transforming events that promote relapse (3).

Several studies indicate that relapse and disease progression derive from a rare population of cancer stem cells (CSC), the only cells within the cancer that can recapitulate the tumor in transplant models (1, 7–16). Recent evidence suggests that CSC are generated by a sequence of heritable events, both epigenetic and via mutations that alter progenitor self-renewal, survival, and differentiation (16). To date, CSC have been identified in human acute myelogenous leukemia (AML), breast cancer, several brain tumors, head and

neck squamous cell carcinomas, pancreatic, and colon cancer (1, 7–16). Preliminary studies suggest that CSC of the hematopoietic system, also called leukemia stem cell (LSC), have subverted the properties normally ascribed to HSC such as self-renewal capacity (1, 9–11, 16). By studying 100 CML blood and marrow samples, we previously discovered that a cell population sharing the same immunophenotype as granulocyte-macrophage progenitors (GMP) expressed high levels of BCR-ABL and had activated the Wnt/ β -catenin self-renewal pathway (1). Candidate LSC had enhanced replating capacity, an in vitro surrogate measure of self-renewal potential that was inhibited by a specific Wnt pathway antagonist-axin (1).

Here we studied (i) the capacity of candidate blast crisis (BC) LSC to self-renew in immunocompromised mice and (ii) the mechanisms driving Wnt/ β -catenin self-renewal pathway activation.

Results

We developed an in vivo bioluminescent BC LSC model via intrahepatic transplantation of candidate BC LSC transduced with lentiviral luciferase (GLF) into neonatal immunocompromised (RAG2^{-/-} γ c^{-/-}) mice (17). Controls included CML HSC and blast (Lin⁺) cells, as well as normal HSCs and progenitors (Tables S1 and S2A). Weekly in vivo bioluminescence imaging demonstrated enhanced engraftment of GLF-transduced BC progenitors (CD34⁺CD38⁺Lin⁻) which, compared to CD34⁺CD38⁻Lin⁻ cells, yielded twice as many myeloid progenitors, prominent myeloid engraftment in all hematopoietic tissues, and 1.7-fold higher β -catenin expression (Figs. S1 and S2a). Moreover, mice transplanted with lentiviral luciferase (GLF)-transduced BC CML progenitors developed signs of leukemia, including wasting, piloerection, and lethargy (Fig. 1A). These mice expressed BCR-ABL in all hematopoietic tissues and formed bioluminescent BCR-ABL⁺ myeloid cell-enriched tumors typical of BC granulocytic sarcomas (Fig. 1B, C, and D). While only one tumor formed in mice ($n = 8$) transplanted with the HSC enriched 34⁺38⁻Lin⁻ fraction, 9 tumors arose in mice ($n = 15$) transplanted with BC CML progenitors and no tumors arose in mice ($n = 4$) transplanted with Lin⁺ cells (Fig. S2b).

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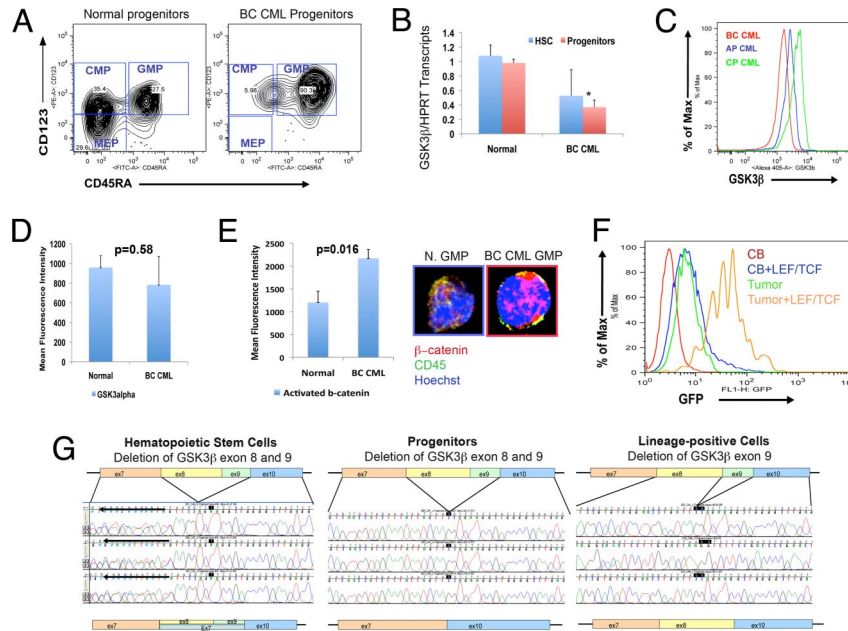


Fig. 3. Aberrant *GSK3β* expression by BC CML progenitors. (A) FACS plot demonstrating characteristic expansion of the GMP compartment in BC CML compared with normal blood and marrow samples. FACS analysis performed on normal ($n = 9$) and CML CP ($n = 5$), CML AP ($n = 6$), and BC CML ($n = 6$) blood and bone marrow samples revealed that, while the proportion of HSC did not expand with progression to BC, there was a significant increase in GMP compared with normal controls ($P = 1.93 \times 10^6$; two-tailed Student's t test). (B) HSC and progenitors were FACS sorted from normal or BC CML $CD34^+$ ($n = 3$) blood samples and *GSK3β* transcript levels measured by quantitative RT-PCR. There was a significant difference ($P < 0.05$; two-tailed Student's t test) in *GSK3β* transcript levels between normal (mean $0.98 \pm \text{S.E.M. } 0.05$) and BC CML progenitors ($0.36 \pm \text{S.E. } 0.04$). (C) FACS histograms of CML CP ($n = 3$), AP ($n = 2$), and BC ($n = 3$) progenitors revealed a decrease in *GSK3β* protein expression with progression to BC. (D) FACS analysis performed on normal blood ($n = 5$), and BC CML ($n = 5$) revealed that there was not a significant difference ($P = 0.58$; two-tailed Student's t test) in *GSK3α* protein expression as measured by mean fluorescence intensity \pm SEM in BC CML samples compared with normal blood. Mean fluorescence intensity of isotype control (Rabbit IgG) was subtracted from all of the samples. (E *Left*) FACS analysis performed on normal blood ($n = 6$), and BC CML ($n = 5$) revealed that there was a significant difference ($P = 0.016$; two-tailed Student's t test) in activated β -catenin levels as measured by mean fluorescence intensity \pm SEM in BC CML compared with normal blood. Isotype control (Mouse IgG₁) was subtracted from all of the samples. (E *Right*) Confocal fluorescence microscopic analysis revealed that normal GMP had little activated nuclear β -catenin whereas BC CML GMP expressing misspliced *GSK3β* had high levels of nuclear β -catenin (green: CD45 membrane marker, blue: Hoechst nuclear stain, red: activated β -catenin). (F) In 6 experiments, BC GMP from CML samples ($n = 2$) or tumor ($n = 1$) derived from BC GMP transplanted mice and normal cord blood GMP ($n = 3$) were transduced with a lentiviral LEF/TCF GFP reporter for activated β -catenin. BC GMP samples had significantly higher GFP expression ($P = 0.037$, two-tailed Student's t test) than normal cord blood GMP ($n = 3$) treated in the same manner. Results are expressed as percentage of maximum fluorescence intensity. (G *Left*) BC CML HSC in 5 of 8 patient samples subjected to cDNA sequencing analysis had demonstrable misspliced *GSK3β* transcripts. Nucleotide sequence data represents 2 species of *GSK3β* transcript in HSC: misspliced *GSK3β* and FL-*GSK3β*. (Middle) BC CML progenitors in 5 of 8 samples had prominent misspliced *GSK3β* transcripts in the ORF of the cDNA. (Right) BC CML lineage-positive (blast) cells showing a deletion of *GSK3β* exon 9 in the ORF of the cDNA that was also detectable in normal samples.

were subjected to direct DNA sequencing to identify mutations in critical *Wnt/β-catenin* signaling pathway genes including *β-catenin*, *GSK3β*, *axin 1*, *adenomatous polyposis coli (APC)*, *cyclin D1*, *lymphoid enhancer factor-1 (LEF-1)*, and *c-myc (18–25)*. A comprehensive cDNA sequencing analysis revealed a novel exon 8 and 9 deleted misspliced isoform of *GSK3β (m-GSK3β)* in progenitors in 4 of 7 myeloid BC CML samples and 1 of 4 CP CML samples (Table S2 A and B). In addition to its role in regulation of metabolic pathways such as insulin signaling, *GSK3β* is a critical component of the β -catenin destruction complex and thus, *GSK3β* deregulation would be expected to enhance β -catenin activation (19, 21–23). While cDNA sequencing analysis demonstrated that *m-GSK3β* was a prominent isoform in BC GMP, BC HSC harbored lower levels of *m-GSK3β* (Table S2B and Fig. 3G). These *m-GSK3β* transcripts lacking the FRAT and axin binding domains encoded by exons 8 and 9 were not detected by sequencing in CML blasts or normal sample populations (Tables S2 A and B) (23). Splice isoform specific Q-PCR confirmed higher levels of *m-GSK3β* transcripts in BC GMP compared with $CD34^+CD38^-$ cells, which may explain, in part, the functional hierarchy in leukemic transplantation potential. This analysis also detected low levels of *m-GSK3β* in BC Lin^+ cells and 3 additional CP progenitor samples (Fig. S4 A and B). Alternative *GSK3β* splice isoforms,

deleted in exon 9, exon 11, or exon 9 and 11, were detected in the blasts as well as CP CML and normal peripheral blood, marrow, and cord blood, where they represented the predominant transcripts (Tables S2 A and B and Fig. S5). While exon 9- and 11-deleted *GSK3β* splice isoforms were previously identified in neurons of Parkinson's disease patients (18), exon 8 and 9 truncated transcripts found in CML progenitors have not been described to date. The capacity to down regulate β -catenin signaling may have been impaired as a result of both the absence of axin 2 expression (Fig. S4C) and deregulation of *GSK3β*. These data suggest that in the molecular context of LSC, an *m-GSK3β* isoform predominates that cannot phosphorylate β -catenin.

Transplantation of *m-GSK3β*-expressing BC progenitors produced high levels of BCR-ABL⁺ myeloid cell engraftment in hematopoietic tissues and tumors in primary and secondary recipient mice (Fig. 4 A and B). Western blot analysis demonstrated that *m-GSK3β* protein expression in tumors derived from BC progenitor transplanted mice (Fig. 4C). While lentiviral *m-GSK3β* transduction of CP CML progenitors led to increased levels of activated β -catenin expression, lentivirally-enforced expression of full-length *GSK3β* reduced β -catenin expression. In addition, CP CML progenitors lentivirally cotransduced with GLF and misspliced *GSK3β* had increased replating capacity

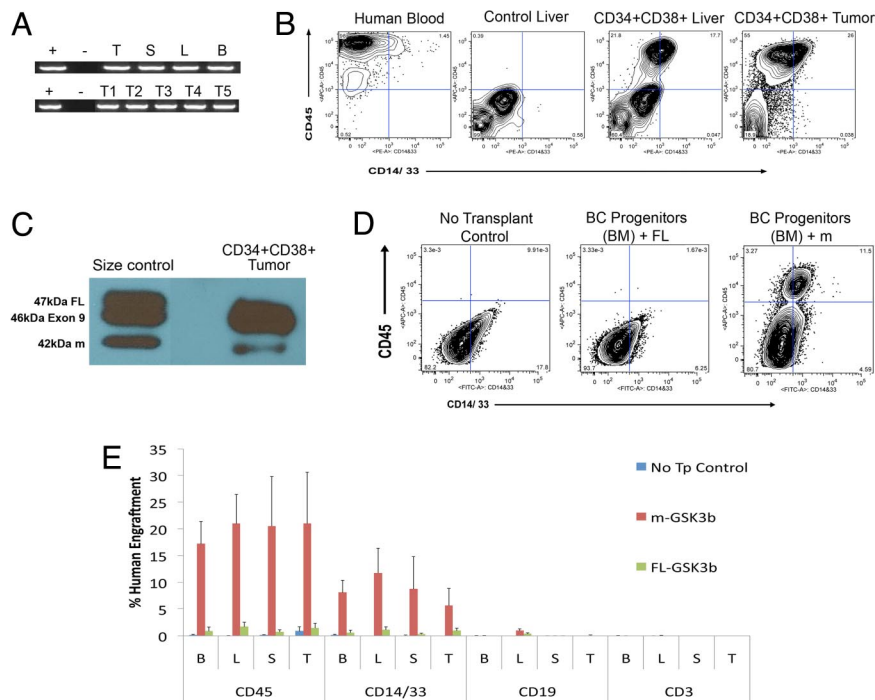


Fig. 4. Enhanced engraftment of misspliced GSK3 β -expressing CML progenitors. (A) P210 BCR-ABL RT-PCR transcript levels in hematopoietic tissues from mice transplanted with human CD45⁺ cells sorted from BC CML progenitor transplanted mice (10^5 cells; $n = 7$ mice). Tissues include thymus (T), spleen (S), liver (L), bone marrow (B) and tumors (T1-T5) (B) FACS analysis of 2^o myeloid progenitor engrafted mice transplanted with human CD45⁺ cells sorted from bone marrow, liver, and spleen of mice that was originally transplanted with BC CML progenitor cells ($n = 3$ experiments). (C) Western blot of K562 cells (size control, left lane) transduced with GSK3 β full length (FL), GSK3 β exon 9 deleted (Exon 9), or GSK3 β exon 8 and 9 deleted (m) and of 34⁺CD38⁺ CML BC derived tumor (right lane), shows that mGSK3 β protein is expressed in the tumor cells. (D) Representative FACS plots of long-term (week 11–12) human CD45 and CD14/33 bone marrow (BM) engraftment in no transplant control (Left), BC progenitors transduced with FL-GSK3 β (Middle) and misspliced GSK3 β ($n = 3$) transduced BC CML progenitor transplanted mice (Right). (E) FACS analysis revealed a decreased percentage of long-term human CD45 cells in the PI negative fraction \pm S.E.M. in hematopoietic organs including bone marrow (B; $P = 0.019$), liver (L; $P = 0.025$), spleen (S; $P = 0.0109$), and thymus (T; $P = 0.10$) of mice transplanted with FL-GSK3 β ($n = 3$) compared with m-GSK3 β transduced ($n = 3$) BC CML progenitors. Reduced human CD14 and CD33 engraftment was also noted in bone marrow ($P = 0.03$), liver ($P = 0.08$), spleen ($P = 0.024$), and thymus ($P = 0.22$) while there was no appreciable engraftment of human CD3 or CD19 cells in BC CML progenitor transplanted mice.

(Fig. S4E). Conversely, lentiviral overexpression of full-length GSK3 β reduced activated β -catenin expression (Fig. S4D) and leukemic engraftment by BC progenitors in all hematopoietic tissues (Fig. 4D and E). Thus, deregulation of GSK3 β through missplicing may be a key event in the evolution of LSC.

These studies suggest that decreases in functional GSK3 β at specific stages of hematopoiesis may represent an important mechanism triggering aberrant β -catenin activation, nuclear entry, and enhanced self-renewal capacity (18–23). In CML, the combined effects of BCR-ABL-mediated stabilization of β -catenin (24) and possibly decreased expression of other negative regulators of the Wnt/ β -catenin pathway, such as axin 2, may accentuate the effects of m-GSK3 β on β -catenin activation (Fig. S4C).

Discussion

Our finding that enforced overexpression of full-length (FL) GSK3 β decreases β -catenin expression and engraftment of m-GSK3 β expressing BC progenitors validates the potential of repaired splicing to have some therapeutic benefit. GSK3 β acts in different cells and has many important substrates; here we show that in the leukemic progression of an HSC clone and its GMP progeny, one effect of decreasing its activity is to enhance the activity of β -catenin in these cells. Extrapolation of this activity in these cells to other situations might be enhanced by an assessment of the other activities of the enzyme in the target cells.

Recently, loss of β -catenin was shown to inhibit engraftment of mouse myeloid leukemia and normal stem cells, underscoring the importance of β -catenin in normal and LSC self-renewal (25). Previous studies in mice revealed that a master regulator of hematopoiesis, Ikaros, has splice isoforms specific to HSC, progenitors, and progeny cells (26). The mechanism(s) responsible for alternative splicing of GSK3 β have yet to be elucidated. Previously, SNPs were shown to create alternative splice acceptor sites responsible for generating GSK3 β splice isoforms (18). Alternatively, these transcripts could arise through epigenetic fixation of splice isoforms, mutations in a yet unknown splicing element, or spliceosomal errors (26–28). Of particular significance to the pathogenesis of CML, enforced expression of BCR-ABL in cord blood progenitors was previously reported to induce increased expression of a number of genes involved in alternative splicing (27). Changes in splicing have been shown to play a functionally significant role in tumorigenesis, either by inactivating tumor suppressors or by gain of function of proteins promoting tumor development (28). In addition, oncogenic splicing events may generate novel epitopes that can be recognized by the host's immune system as cancer-specific and may serve as targets for immunotherapy. Finally, the identification of LSC-specific splice isoforms of GSK3 β may be a useful indicator of disease progression and should be evaluated as a therapeutic target for eradicating the reservoir of LSC in advanced phase CML.

Materials and Methods

FACS Analysis and Sorting. Normal blood and marrow samples were purchased from the San Diego Blood Bank or All Cells. CML blood and marrow samples

were donated by Stanford University and MD Anderson Cancer Center patients according to Institutional Review Board (Princess Margaret Hospital) approved protocols. HSC, progenitor, and Lin⁺ cells were purified via FACS as previously described (1).

Lentiviral Transduction and Transplantation. Equal numbers of normal or CML HSC, progenitors and Lin⁺ cells were FACS sorted, transduced with lentiviral vectors according to established methods (1, 29), and transplanted intrahepatically into neonatal RAG2^{-/-}γc^{-/-} mice (1, 17). Weekly bioluminescence imaging was performed with an in vivo imaging system (IVIS 200; Caliper Inc.). When moribund or at 8–12 weeks posttransplantation, mice were euthanized and single cell suspensions of hematopoietic organs and tumors were analyzed for human cell engraftment via FACS (*SI Methods*). BCR-ABL transcripts in transplanted mouse hematopoietic tissues were detected by PCR with a one step RT-PCR kit (Qiagen) (1, 30) BCR-ABL product sizes for b2a2 and b3a2 were 383bp and 458bp, respectively (30).

GSK3α, GSK3β, β-catenin, and Target Gene Analysis. Normal or CML CD34⁺ cells were stained with a rabbit anti-human GSK3α (#9338, Cell Signaling Technology), rabbit anti-human GSK3β (#9315, Cell Signaling Technology) or anti-activated β-catenin monoclonal antibody (clone 8E7, Upstate Technologies) (1). To further assess β-catenin activation, sorted GMP (700–5,000 cells/well) were transduced with a lentiviral LEF/TCF GFP reporter and analyzed for GFP expression by FACS after 6–7 days in culture (2). Quantitative RT-PCR to detect axin2, c-myc, GSK3β transcripts, and the GSK3β isoforms in normal versus CML HSC and progenitors was performed with SYBR Greener two-Step Q-RT-PCR Kit (Invitrogen) (*SI Methods*) (1). To determine misspliced GSK3β protein expression, primary BC CML progenitors, tumors from BC progenitor transplanted mice or 293FT cells transduced with lentiviral FL-GSK3β or m-GS3β for 48 h, were collected and 5 μg of the whole cell lysate were probed with GSK3β antibody (Cell Signaling) in Western blot analysis.

Wnt Pathway Mutation Screening. Genomic DNA mutation and cDNA analysis of β-catenin, APC, axin 1, c-myc, LEF-1, cyclin D1, and GSK3β was conducted with SURVEYOR mismatch cleavage analysis using the WAVE-HS System (Transgenomic) followed by bidirectional sequence analysis on an ABI 3100 sequencer (Applied Biosystems, Inc.) (*SI Methods*).

Statistical Analysis. Statistical analyses were performed with the aid of FlowJo, Caliper, and Excel software.

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