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Reversion to an embryonic alternative splicing program enhances leukemia stem cell self-renewal

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Formative research suggests that a human embryonic stem cell-specific alternative splicing gene regulatory network, which is repressed by Muscleblind-like (MBNL) RNA binding proteins, is involved in cell reprogramming. In this study, RNA sequencing, splice isoform-specific quantitative RT-PCR, lentiviral transduction, and in vivo humanized mouse model studies demonstrated that malignant reprogramming of progenitors into self-renewing blast crisis chronic myeloid leukemia stem cells (BC LSCs) was partially driven by decreased MBNL3. Lentiviral knockdown of MBNL3 resulted in reversion to an embryonic alternative splice isoform program typified by overexpression of CD44 transcript variant 3, containing variant exons 8–10, and BC LSC proliferation. Although isoform-specific lentiviral CD44v3 overexpression enhanced chronic phase chronic myeloid leukemia (CML) progenitor replating capacity, lentiviral shRNA knockdown abrogated these effects. Combined treatment with a humanized pan-CD44 monoclonal antibody and a breakpoint cluster region - ABL proto-oncogene 1, nonreceptor tyrosine kinase (BCR-ABL1) antagonist inhibited LSC maintenance in a niche-dependent manner. In summary, MBNL3 down-regulation–related reversion to an embryonic alternative splicing program, typified by CD44v3 overexpression, represents a previously unidentified mechanism governing malignant progenitor reprogramming in malignant microenvironments and provides a pivotal opportunity for selective BC LSC detection and therapeutic elimination.

CD44v3 | MBNL3 | RNA splicing | self-renewal | adhesion molecules

Since the discovery of induction of stem cell characteristics in somatic cells by enforced expression of four transcription factors (1, 2), human pluripotent stem cell research has provided key insights into human development. Comparative DNA and RNA sequencing (RNAseq) studies have revealed that human-specific distal regulatory elements, RNA editing, and alternative splicing play key roles in human embryonic stem cell (hESC) self-renewal and cell fate determination (3–6). Several of the phosphoproteins regulated during differentiation are components of the posttranscriptional RNA modification machinery, including double-stranded RNA-specific adenosine deaminase (ADAR) and serine/arginine-rich splicing factor 7 (SFRS7), thereby highlighting the importance of RNA processing alterations in hESC cell fate determination (5). Another key stem cell regulatory protein, β-catenin, is involved in hESC pluripotency and in the transcriptional regulation of adhesion molecules such as CD44 (5).

Increased CD44 expression and splice isoform switching have been linked to enhanced metastatic potential and a poor prognosis in several types of cancer (7, 8). Alternative splicing of 9 out of 19 exons in human CD44 pre-mRNA results in expression of different transcript variants, leading to variation in the length and function of the extracellular domain [for National Center for Biotechnology Information (NCBI)-designated CD44 nomenclature, see *SI Materials and Methods* and Fig. 1*E*]. Binding of CD44 to stem cell niche-related extracellular matrix molecules, such as hyaluronan (HA) (9) and osteopontin (OPN), is in part predicated on the specific transcript variants expressed (10), and OPN–CD44 signaling promotes aggressive tumor growth (11). Although CD44 expression has been shown to promote both chronic and acute myeloid leukemia stem cell (LSC) maintenance in mouse models (12–14), CD44 splice isoform switching and cognate ligand expression as well as malignant reprogramming of human progenitors into self-renewing LSCs had not been elucidated.

Previously, we showed that progression from chronic phase (CP) to blast crisis (BC) chronic myeloid leukemia (CML) involved malignant reprogramming of BC progenitors into LSCs as a result of β-catenin activation (15). Sequencing analysis of BC LSCs revealed GSK3β missplicing (16), ADAR1 RNA editase activation (17), and BCL2 splice isoform switching (18). Although similarities between hESC and LSC transcriptional programs had previously been reported in a mouse model of AML, embryonic splice isoform patterns were not examined (19, 20). Subsequently, seminal RNAseq studies revealed that decreased expression of a muscleblind-like (MBNL) gene regulatory network enhanced hESC-specific alternative splicing and reprogramming (6).

Although overlapping gene expression patterns between hESCs and LSCs (19) have been reported, stem cell regulatory gene splice isoform differences and their functional consequences were not

Significance

In this study, we show for the first time, to our knowledge, that Muscleblind-like 3 (MBNL3) down-regulation in therapy-resistant human blast crisis leukemia stem cells (LSCs) is associated with activation of a human embryonic stem cell alternative splicing gene regulatory network involved in reprogramming and expression of a CD44 splice isoform, CD44 transcript variant 3. Targeting blast crisis LSCs with a human CD44 monoclonal antibody in combination with a potent breakpoint cluster region - ABL proto-oncogene 1, nonreceptor tyrosine kinase (BCR-ABL1) antagonist impairs LSC self-renewal in a niche-dependent manner and provides a compelling rationale for devising novel clinical combination LSC eradication strategies.

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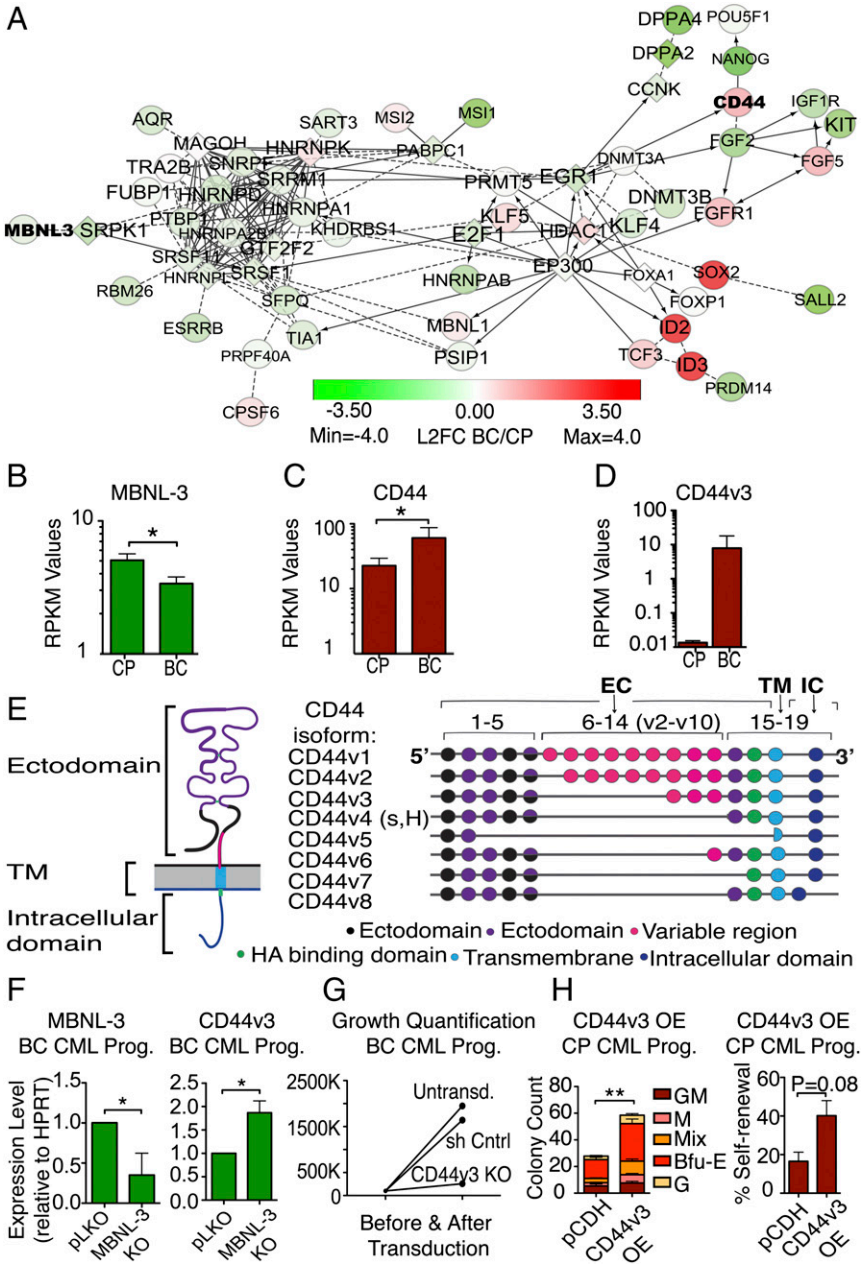

Fig. 1. Reversion to an embryonic splicing program contributes to malignant reprogramming. (*A*) Cytoscape network of previously published MBNL and ESC-specific alternative splicing program-related transcripts (6). Nodes are colored according to log twofold change of fragments of kilobase of exon per million fragments mapped (FPKM) in BC over FPKM in CP. Solid lines represent gene interactions derived from the Reactome Functional Interaction (FI) annotations. Dashed lines represent predicted interactions from the Reactome FI annotations. (*B*) RNAseq analysis of MBNL3 expression in BC CML progenitor cells compared with progenitors from CP CML patient samples ($P = 0.038$; $n = 8$). (*C*) RNAseq analysis of CD44 expression in BC CML progenitor cells compared with progenitors from CP CML patient samples ($P = 0.013$; $n = 8$). (*D*) RNAseq analysis of CD44v3 expression in BC CML progenitor cells compared with CP CML progenitors. (*E*) Model of CD44 exon organization. Colors of circles represent receptor placement or function as follows: black, the ectodomain; dark blue, intracellular (IC) domain; green, the HA binding domain; light blue, transmembrane (TM) domain; pink, variable exons (10, 21). (*F*) qRT-PCR analysis of MBNL3 ($P = 0.049$, *Left*) and CD44v3 ($P = 0.037$, *Right*) expression after MBNL3 KO in BC CML progenitors compared with pLKO ($n = 3$). (*G*) Growth quantification of BC CML progenitors transduced with lentiviral shRNA for CD44v3 shows that knock-down of CD44v3 impacts the cell growth compared with untransduced and sh-control ($n = 3$). (*H*) Lentiviral overexpressing (OE) of CD44v3 in CP CML progenitor increased hematopoietic colony formation (*Left*) ($P = 0.003$), specifically of G ($P = 0.028$), M ($P = 0.008$), mixed ($P = 0.009$), and Bfu-E ($P = 0.008$) colonies. CD44v3 OE CP patient samples showed a trend of increased self-renewal capacity ($P = 0.081$) as measured by secondary colony formation (*Right*) ($n = 3$).

investigated as a mechanism of malignant reprogramming of progenitors into LSCs. Thus, we performed comprehensive RNAseq analysis of alternative splicing regulatory gene and adhesion molecule expression on progenitors from untreated CP and BC CML samples and adult normal peripheral blood (NPB) (Table S1). Because previous studies have shown that MBNL gene knockdown is associated with reversion to embryonic alternative splicing cassette exon use (6) and alternative splicing patterns influencing the capacity of human CD44 variants to bind to HA (21) and other ligands, we reasoned that reversion to an embryonic alternative splicing pattern could promote malignant reprogramming by enhancing survival and self-renewal.

Results

Reversion to an Embryonic Alternative Splicing Program in BC CML. RNAseq analysis demonstrated that CML progression from CP to BC was associated with decreased expression of MBNL3 and other key regulators of an embryonic alternative splicing program (Fig. 1*A*). This coincided with increased expression of fibroblast growth factor receptor-1 (FGFR1) and fibroblast growth factor-5 (FGF5), which are involved in embryonic development, stem cell differentiation and proliferation, and tumor growth, as well as increased expression of Musashi RNA binding protein-2 (MSI2), which has been linked to LSC maintenance in a mouse model of BC CML (22, 23). Pluripotency genes such as *SOX2* and *KLF5* were up-regulated together with CD44 during BC transformation (Fig. 1*A*). Loss of MBNL3 expression coincided with CD44 overexpression in BC compared with CP progenitors (Fig. 1 *B* and *C*, Fig. S1*A*, and Table S2). The predominant NCBI-designated splice variant of CD44 detected by RNAseq, in BC compared with CP progenitors, CD44v3 (containing variable exons 8–10), showed expression levels similar to pluripotent undifferentiated hESCs (Fig. 1 *D* and *E* and Fig. S1 *B* and *C*). Lentiviral shRNA knockdown of MBNL3 (Fig. 1*F* and Fig. S1 *D* and *H*) in CML progenitors resulted in increased CD44v3 transcripts (Fig. 1*F*) and enhanced OCT4 expression (Fig. S1*H*).

Changes in CD44v1 were not statistically significant (Fig. S1*G*). Overexpression of CD44v3 in CP CML progenitors was associated with self-renewal and reduced MBNL3 levels (Fig. 1 *G* and *H* and Fig. S1*I*), whereas knockdown of CD44v3 reduced expansion of progenitors (Fig. 1*G*). Thus, when expressed in the context of the breakpoint cluster region - ABL proto-oncogene 1, non-receptor tyrosine kinase (BCR-ABL1) oncogene, CD44v3 may increase cell expansion and enhance malignant reprogramming of progenitors into self-renewing LSCs. Also, CD44v3 overexpression enhanced hematopoietic colony formation, resulting in a trend toward increased replating capacity, an in vitro surrogate measure of self-renewal (Fig. 1*H*). Prosurvival MCL1 and BCLX long isoforms increased BC LSCs (Fig. S1*K*). Finally, our results linking CD44v3 to activation of the pluripotency network prompted chromatin immunoprecipitation (ChIP) analysis to study OCT4 occupancy of the CD44 promoter. Notably, we observed a trend toward an increase in the recruitment of OCT4 to the CD44 promoter after CD44v3 overexpression (Fig. S1*J*). Rescue experiments confirmed specificity by knocking down CD44v3 and lentivirally reintroducing CD44v3 24 h later (Fig. S1*L*).

CD44v3 Expression Promotes Stem Cell Maintenance. To elucidate the role of CD44v3 in the maintenance of stem cell self-renewal capacity, we investigated the effect of CD44v3 on pluripotent hESCs compared with LSCs. Splice isoform-specific quantitative RT-PCR (qRT-PCR) was used to compare CD44v3 expression in undifferentiated hESC and embryoid bodies. Notably, hESCs harbored significantly higher CD44v3 than their differentiated counterparts (Fig. 2*A*). Although undifferentiated hESCs had low MBNL3 expression, it increased upon hESC differentiation (Fig. 1*B*). Similar to CML progenitors, lentiviral overexpression of CD44v3 in hESCs resulted in OCT4 enrichment on the CD44 promoter (Fig. 2*C*) and up-regulation of the H4K16Ac active mark (Fig. 2*D*). Previous studies have suggested that β-catenin enhances OCT4 activity and therefore reinforces pluripotency, which corroborates our study (24). Similar to hESCs, BC LSCs harbored low levels of MBNL3 compared with their CP progenitor counterparts. Overexpression of CD44v3 in hESCs was associated with increased OCT4 and SOX2, but not CD44v1 (Fig. S2*A*). Also, splice isoform switching favoring prosurvival MCL1 and BCLX long isoform expression (Fig. 2*E*) resulted in increased proliferation (Fig. S2*B*). In contrast, lentiviral knockdown of MBNL3 decreased hESC proliferation (Fig. S2*C*). Confocal fluorescence microscopic analysis confirmed OCT4 up-regulation in hESCs following CD44v3 overexpression (Fig. 2*F*). Moreover, CD44v3 overexpression was associated with increased β-catenin (Fig. 2*G*). Enrichment of OCT4 was detected on the β-catenin promoter in CD44v3-transduced hESCs, suggesting that CD44v3 is a marker of stem cells (Fig. 2*H*) that have increased proliferation (Fig. 2*I*) and decreased differentiation potential (Fig. S2*B*).

Although CD44v3 overexpression enhanced hESC maintenance, knockdown of CD44v3 abrogated these effects (Fig. 2*J*).

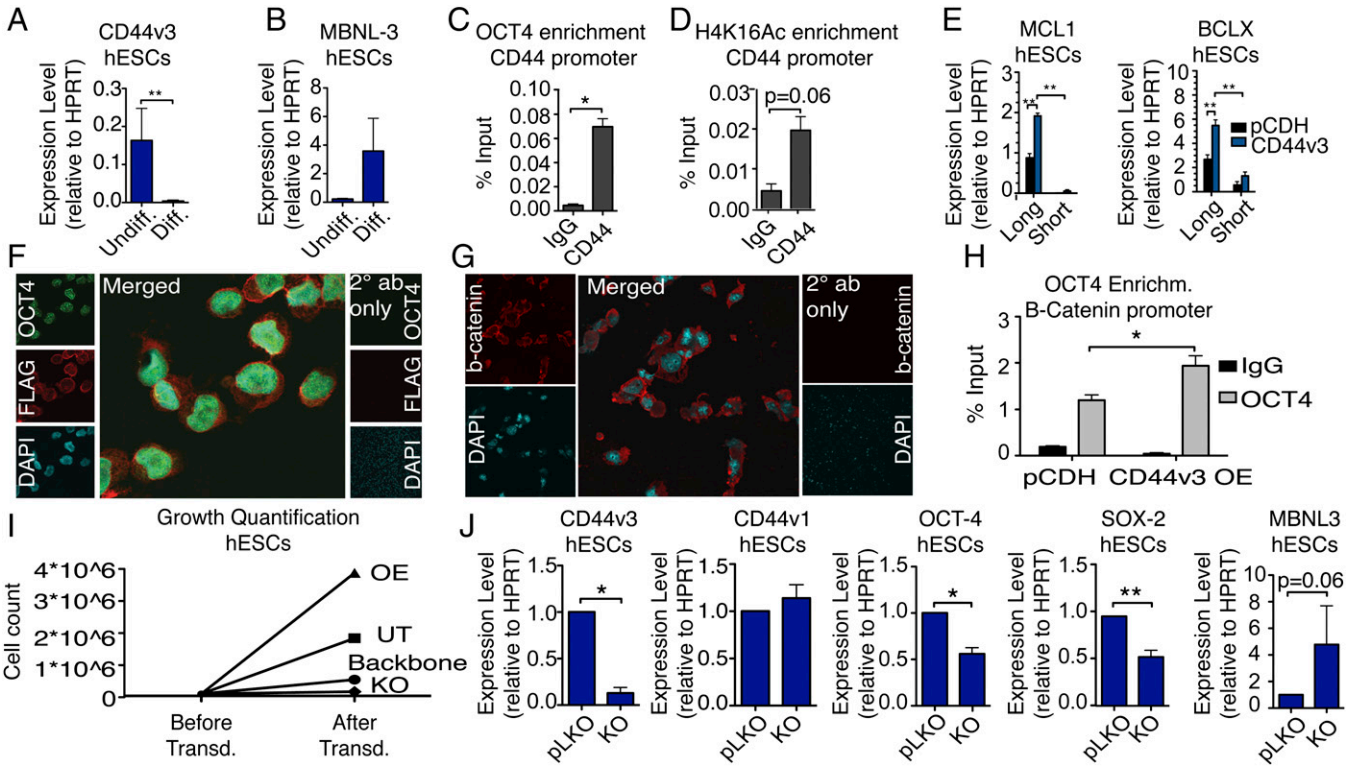

Fig. 2. CD44v3 promotes pluripotent stem cell maintenance. (*A*) CD44v3 expression in undifferentiated hESCs ($n = 6$; $P = 0.006$) compared with their differentiated counterparts, embryoid bodies ($n = 3$). (*B*) MBNL3 expression in undifferentiated hESCs compared with embryoid bodies ($n = 3$). (*C*) OCT4 enrichment on CD44 promoter in CD44v3 OE hESCs ($n = 3$). (*D*) Active histone mark H4K16Ac analysis enriched on the CD44 promoter in CD44v3 OE hESCs ($n = 3$). (*E*) Prosurvival MCL1 long isoform expression in hESCs following lentiviral CD44v3 OE compared with pCDH backbone-transduced controls ($P = 0.0415$). Shown are the difference between long and short isoform in CD44v3 OE samples ($P = 0.0262$; $n = 3$) and BCLX long isoform expression in hESCs following CD44v3 compared with pCDH expression ($P = 0.040$). Shown is the difference between long and short isoforms in CD44v3 OE samples ($P = 0.011$; $n = 3$). (*F*) Immunostained hESCs after lentiviral OE of CD44v3. Cells express FLAG (CD44v3) and OCT4. The cell nucleus is stained with DAPI. (*G*) Confocal fluorescence microscopic analysis of β-catenin expression in hESCs after lentiviral OE of FLAG-tagged CD44v3 compared with pCDH. (*H*) OCT4 enrichment on the β-catenin promoter in hESCs OE CD44v3 ($P = 0.0343$) compared with pCDH backbone ($n = 3$). (*I*) CD44v3 OE and CD44v3 KO effects of cell proliferation compared with untransduced and backbone-transduced controls ($n = 3$). (*J*) qRT-PCR analysis of pluripotency transcripts, including OCT4 ($P = 0.0133$), SOX2 ($P = 0.0034$), and MBNL3 ($P = 0.0583$) of CD44v3 KO hESCs, compared with pCDH and CD44v1 KO ($n = 3$).

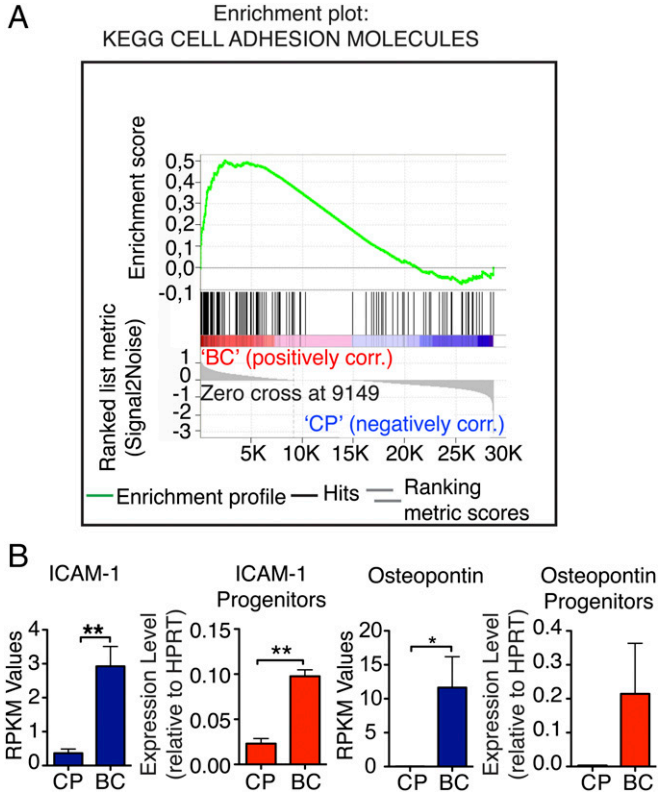

Fig. 3. BC LSCs have a unique adhesion molecule gene expression pattern. (*A*) GSEA of cell adhesion molecule expression in BC compared with CP progenitors. (*B*) OPN expression in BC progenitors compared with CP as analyzed by RNAseq (*Middle Right*) and qPCR ($n = 3$; *Far Right*). ICAM-1 expression is increased in BC CML progenitors compared with CP CML progenitors as analyzed by RNAseq (*Far Left*) and qPCR ($n = 3$; *Middle Left*).

Finally, a rescue experiment of CD44v3 confirmed specificity following knockdown of CD44v3 and lentiviral reintroduction of CD44v3 24 h later (Fig. S2*D*). Together, these data suggest that a shared CD44 splice variant expression pattern promotes both hESC and BC LSC maintenance.

BC LSCs Have a Unique Adhesion Molecule Gene Expression Pattern. The CD44 adhesion ligands OPN, HA (9), intracellular adhesion molecule 1 (ICAM-1), and the HA-mediated motility receptor (RHAMM) (25) were examined by RNAseq and qRT-PCR. In BC LSCs, CD44 was among the most highly expressed adhesion molecules along with OPN and ICAM-1 (Fig. S3 *A–C*). Of the 76 cell adhesion-related genes analyzed, eight were up-regulated in BC compared with CP progenitors, including connective tissue growth factor (CTGF), L-selectin (SELL), and integrin alpha L chain (ITGAL) (Fig. S3 *B* and *C*). Notably, additional transcripts encoding proteins known to interact with HA, including versican (VCAN), ICAM-1, OPN (SPP1), and CD44, were more highly expressed by BC compared with CP progenitors (Fig. S3 *A–C*). A gene set enrichment analysis (GSEA) showed enrichment of adhesion molecule expression in BC compared with CP progenitors (Fig. 3*A*). Also, both RNAseq and qRT-PCR showed that BC progenitors expressed significantly higher levels of OPN and ICAM-1 compared with CP progenitors, whereas RHAMM levels were unchanged (Fig. 3*B* and Fig. S3 *E* and *F*). Overexpression of CD44v3 in CML progenitors was associated with increased OPN ($R^2 = 0.731$) and ICAM-1 ($R^2 = 0.714$) but not with RHAMM ($R^2 = 0.093$) expression (Fig. S3*E*). Similarly, CD44v3 overexpression in hESCs correlated with OPN ($R^2 = 0.81$) and ICAM-1 ($R^2 = 0.84$) ligand expression but not RHAMM ($R^2 = 0.21$) (Fig. S3*F*). These data thereby support hESC and LSC adhesion to a HA-rich niche (Fig. S4*A*).

CD44 and BCR-ABL1 Inhibition Reduces BC LSC Survival. To determine if targeted inhibition of CD44 and BCR-ABL1 could abrogate human BC LSC ($Lin^-CD34^+CD38^+$) survival in vivo, recombinase activating gene-2 ($RAG2^{-/-}\gamma c^{-/-}$) neonatal mice

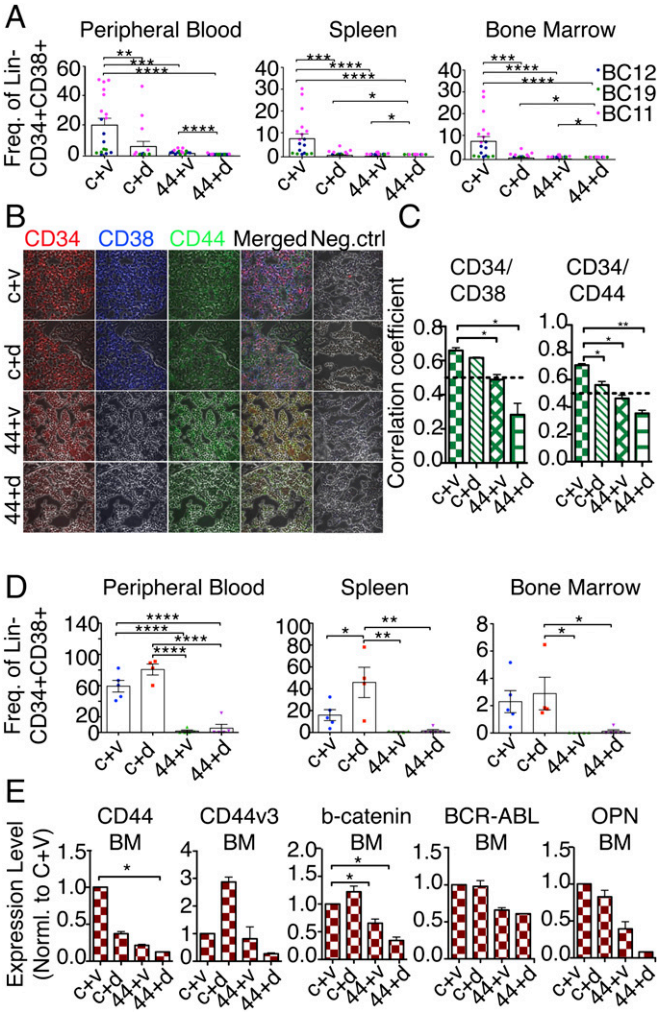

Fig. 4. Disruption of BC LSC ligand interactions with CD44 and BCR-ABL inhibition. (*A*) Human BC CML LSC engraftment in peripheral blood, spleen, and BM from mice transplanted with patient BC11 (magenta; $n \geq 5$ per treatment group), BC12 (blue; $n \geq 5$ per treatment group), and BC19 (green; $n \geq 4$ per treatment group). Combination therapy significantly reduced BC LSCs in the hematopoietic tissues of all three patient models. Graphs depict mean frequency BC LSCs (Live, Lin^-, $CD45^+CD34^+CD38^+$) out of live cells ± SEM and values for individual mice. Pooled data are from five separate experiments. (*B*) Confocal fluorescence microscopic analysis of femur sections stained for CD34, CD38, and CD44. Mice treated with CD44 mAb alone or in combination with dasatinib showed a decrease in CD38 and CD44-positive cells. (*C*) Confocal fluorescence microscopic analysis revealed colocalization of CD34, CD38, and CD44 expression in the control group (C + V) and dasatinib-treated group (C + D). Dashed line, colocalization cutoff value of 0.5. (*D*) Frequency of human BC CML cells (Lin^-CD45^+) in peripheral blood, spleen, and BM from secondary recipient mice transplanted with human CD34+ BM cells from primary patient BC11 engrafted mice after treatment ($n \geq 4$ per treatment group). Graphs show mean ± SEM and values for individual mice. (*E*) qRT-PCR gene expression of CD44, CD44v3, β-catenin, BCR-ABL1, and OPN in human $CD34^+$ BC CML cells isolated from the BM of secondary recipient mice. Graphs show transcript levels normalized to control group.

were xenotransplanted with LSCs from three separate BC patient samples (Fig. S4 *B–D*). Human BC LSC-engrafted mice were treated for 14 d with an IgG1 control antibody, a clinical grade CD44 monoclonal antibody (mAb), a potent BCR-ABL1 inhibitor, dasatinib, or the combination of CD44 antibody and dasatinib. Then, FACS analysis was performed to determine BC LSC frequency in peripheral blood, spleen, and bone marrow (BM) (Fig. 4*A*). Although CD44 mAb or dasatinib treatment alone reduced human BC LSC frequency in peripheral blood and splenic niches (Fig. 4*A* and Figs. S5 *A–C* and S6 *A* and *B*) as well as myeloid sarcoma formation (Fig. S5*D*), only combination treatment significantly reduced BC LSC survival in the BM niche in all three patient models (Fig. 4*A* and Figs. S5 *A–C* and S6 *A* and *B*). Similarly, single agent CD44 mAb treatment of CP and BC CML in vitro did not have a significant effect on cell survival or differentiation (Fig. S6 *C* and *D*). These data suggest that only combined CD44 and BCR-ABL1 inhibition reduces human BC LSC survival.

Disruption of BC LSC Ligand Interactions with CD44 and BCR-ABL Inhibition. The effect of combined CD44 and BCR-ABL1–targeted therapy on CD34, CD38, CD44, HA, and OPN expression and interaction in engrafted mouse femurs was analyzed by qRT-PCR and confocal fluorescence microscopy using colocalization software (Fig. 4*B* and Fig. S7*C*). Analysis of $CD34^+$ and $CD38^+$ cells showed that these markers colocalized in control IgG- (R_r = 0.64), CD44 mAb alone- (R_r = 0.58), and dasatinib alone-treated mice (R_r = 0.61), indicative of the persistence of BC LSCs (Fig. 4 *B* and *C* and Fig. S7*C*). Conversely, CD34 and CD38 colocalization was disrupted in BM following combination treatment (R_r = 0.28). Colocalization of $CD34^+$ cells with CD44 and OPN was observed in the control IgG-, dasatinib alone-, and CD44 mAb alone-treated mice but was disrupted in femurs from combination-treated mice (R_r = 0.34 and R_r = 0.39, respectively) (Fig. S7*C*). As expected, colocalization of $CD34^+$ cells with HA was abrogated in the CD44 mAb- or combination-treated group, indicating that CD44 mAb RG7356 blocks CD44–HA ligand interaction (R_r = 0.42) (Fig. S7*B*). Following both CD44 mAb and combination treatment, qRT-PCR analysis of $CD34^+$ cells isolated from BM and spleen revealed a significant reduction in CD44 and CD44v3 expression in BM (Fig. S7*A*) and spleen (Fig. S7*B*). Only the spleen showed significantly reduced levels of ICAM-1, RHAMM, and OPN following combination therapy (Fig. S7*B*). These results demonstrate that dasatinib and CD44 mAb therapy exert distinct effects on BC LSC–ligand interactions in different niches.

CD44 and BCR-ABL1 Inhibition Reduces BC LSC Self-Renewal. To evaluate the effect of CD44 inhibition on BC LSC self-renewal in vivo, we performed serial transplantation of human $CD34^+$ cells from the BM of BC LSC engrafted mice after 14 d of treatment. Although dasatinib alone did not eradicate self-renewing BC LSCs, recipients of cells from CD44 mAb-treated mice or in those treated with both CD44 mAb and dasatinib displayed a reduction in BC LSC self-renewal in peripheral blood, spleen, and BM (Fig. 4*D*). Notably, there was no significant reduction in BCR-ABL1 transcript levels following serial transplantation of dasatinib alone-treated BM, thereby highlighting the inability of dasatinib to eradicate BC LSCs (Fig. 4*E*). Transcript levels of CD44, OPN, BCR-ABL1, β-catenin, and CD44v3 expression were significantly reduced in engrafted spleens (Fig. S7*D*) in CD44 mAb alone- or combination-treated mice. Although there was a significant reduction in total CD44 and β-catenin expression in the BM of CD44 mAb combination therapy transplant recipients, expression levels of CD44v3, BCR-ABL1, and OPN were not significantly reduced. These results suggest that CD44 plays a vital role in BC LSC self-renewal and combined CD44-targeted therapy can reduce their self-renewal capacity in a niche-dependent manner (Fig. S4*A*).

Materials and Methods

RNAseq. RNAseq was performed as described previously (26). Paired-end Chastity-passed reads were aligned to the human reference sequence plus exon junction sequences constructed from all transcripts identified in RefSeq, EnsEMBL, and University of California, Santa Cruz (UCSC) databases using Burrow–Wheeler Aligner software (27) with default parameters.

hESC Culture and Lentiviral Knockdown and Overexpression. hESCs were cultured in modified mTeSR1 (mTeSR1, a defined medium for hESC culture) on Matrigel-coated plates. Before transduction, cells were treated with 6 μg/mL polybrene in 500 μL mTeSR1 for 15 min. Subsequently, 50,000 cells were transduced for 24 h, washed, and cultured for 72 h in mTeSR1 medium. hESC research was performed in accordance with embryonic stem cell research oversight committee (ESCRO) guidelines.

Primary Patient Sample Preparation. $CD34^+$ cells were selected using magnetic beads from Ficoll-purified mononuclear cells. One day before overexpression and knockdown, cells were cultured in 96-well plates (50 K per well) in StemPro medium with cytokines (18). Cells were lentivirally transduced and collected 72 h posttransduction.

Lentiviral Knockdown and Overexpression. We developed a lentiviral human CD44v3 overexpression vector in pCDH-EF1-MCS-T2A-copGFP (Fig. S1*E*) and both CD44v3 and MBNL3 knockdown vectors in Mission pLKO.1-puro (Fig. S1 *D* and *F*).

ChIP. The commercially available LowCell#ChIP kit from Diagenode was used. Cross-linking of cells was performed using 1% formaldehyde.

In Vivo Experiments. Intrahepatic xenotransplantion of $RAG2^{-/-}\gamma c^{-/-}$ mice with human BC CML progenitors was performed within 1–3 d of birth. Mice were used in treatment experiments once PB engraftment reached 1% by FACS analysis. All mouse experiments were conducted according to Institutional Animal Care and Use Committee (IACUC) specifications, which adheres to guidelines set forth by the National Institutes of Health (NIH). Patient samples were donated for biobanking after obtaining written informed consent in accordance with institutional review board-approved protocols.

Discussion

Alternative pre-mRNA splicing, which is more common in human than primitive pluripotent stem cells (6), regulates membrane protein structure and function as well as cancer stem cell evolution (16, 19). We have shown, for the first time to our knowledge, that decreased expression of MBNL3, a repressor of embryonic alternative splicing and stem cell reprogramming, is associated with activation of a pluripotency network and with up-regulation of a CD44 splice isoform, CD44v3 (NCBI designation, compare Fig. 1*B*), in human BC LSCs. In keeping with activation of a stem cell reprogramming network, CD44v3 up-regulation is associated with increased expression of pluripotency transcription factors, including OCT4, SOX2, and β-catenin, in addition to the prosurvival long isoforms of MCL1 and BCLX, resulting in increased self-renewal and apoptosis resistance. In addition, CD44v3 expression correlated with higher levels of ICAM-1 and OPN, suggesting that an embryonic alternative splicing program promotes stem cell adhesion. Up-regulation of both CD44 and its ligand, OPN, has been documented in many cancers and correlates with a poor prognosis (28). Although an increased (13, 18) LSC burden has also been linked to a poor prognosis (29), LSC isoform-specific expression data have been lacking.

In the BC LSC context, these molecules promote homing and adhesion of cells in the HA-rich BM niche, where LSCs are retained in a dormant state and thus are impervious to tyrosine kinase inhibitors that target dividing cells. Indeed, previous studies have demonstrated that dasatinib alone is not enough to effectively target cancer stem cells in the BM (18). Others have shown that BCR-ABL1–expressing cancer stem cells in mice are dependent on CD44 for homing as well as retention in the BM (12). Our data suggest that CD44v3 is linked to enhance expression of OPN and ICAM-1 on BC progenitors, which are important for interactions in the local microenvironment. Thus, it is conceivable

that BC LSCs, in addition to adhesion molecule up-regulation and interaction with ligands, contribute to their own niche in an autocrine fashion by producing OPN and HA, resulting in enhanced adhesion as well as survival. We therefore hypothesized that a pan–anti-CD44 mAb disrupts the homeotypic adhesion of LSCs and retention in malignant niches, forcing them to enter the blood stream, where dasatinib effectively can target them. Although CD44 mAb and dasatinib treatment significantly reduces BC LSC self-renewal in the splenic niche and lowers self-renewal in BM, some BCR-ABL1– and CD44v3-expressing cells persist in the BM niche following combination therapy, suggesting that a CD44v3-specific mAb may be more effective at eradicating BC LSCs from the more recalcitrant BM niche (Fig. S4*A*).

Both BC LSC self-renewal and survival appear to be predicated on reversion to an embryonic alternative splicing program typified by CD44v3 expression. Down-regulation of MBNL3 in CML activates an embryonic alternative splicing program and malignant reprogramming of progenitors into self-renewing BC LSCs that express CD44v3. In addition, we observed that the CD44 target gene, β-catenin, interacts with the pluripotency regulator OCT4 in LSCs, as has been reported in mouse ESCs (24). However, the precise mechanisms through which β-catenin activates OCT4 remain unclear and will be the subject of future investigations.

Previous studies highlighted the importance of CD44v isoform expression in colon (30), gastric (31), and hematological malignancies (32) but did not completely elucidate mechanisms governing human variant CD44 expression or their cell type- and context-specific effects on function. Recently, CD44 variable exons 8–10 (present in CD44 isoforms 1, 2, and 3) have been linked to gastric cancer stem cells (33) and associated with tumor progression in bladder cancer (34). In a mouse thymoma cell line, CD44v10 was found to bind more readily to OPN than other adhesion molecules, such as HA, and promoted tumor-infiltrating leukocyte recruitment upon local inflammation (32).

Although other aggressive myeloid malignancies, like AML, have been shown to harbor a distinctive CD44 population that promotes expansion of myeloid lineage cells (35), we show that malignant reprogramming of CML progenitors, by repression of MBNL3, enables them to adopt features of an embryonic alternative splicing program. This culminates in the up-regulation of pluripotency factors, such as SOX2 as well as inhibitor of DNA binding 2 and 3 (ID2 and ID3), which inhibit differentiation. In conclusion, a human CD44-specific mAb, RG7356, impairs tyrosine kinase inhibitor-resistant BC LSC survival and self-renewal in a niche-dependent manner, particularly when combined with the tyrosine kinase inhibitor dasatinib. These results provide the impetus for devising novel combination strategies aimed at eradicating BC LSCs. Finally, we have uncovered a previously unidentified mechanistic link between de-repression of embryonic alternative splicing of CD44v3 and pluripotency programs that provides a unique prognostic and therapeutic opportunity.

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