Metastatic Consequences of Immune Escape from NK Cell Cytotoxicity by Human Breast Cancer Stem Cells

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Abstract
Breast cancer stem-like cells (BCSC) are crucial for metastasis but the underlying mechanisms remain elusive. Here, we report that tumor-infiltrating natural killer (NK) cells failed to limit metastasis and were not associated with improved therapeutic outcome of BCSC-rich breast cancer. Primary BCSCs were resistant to cytotoxicity mediated by autologous/allogeneic NK cells due to reduced expression of MICA and MICB, two ligands for the stimulatory NK cell receptor NKG2D. Furthermore, the downregulation of MICA/MICB in BCSCs was mediated by aberrantly expressed oncogenic miR20a, which promoted the resistance of BCSC to NK cell cytotoxicity and resultant lung metastasis. The breast cancer cell differentiation–inducing agent, all-trans retinoic acid, restored the miR20a–MICA/MICB axis and sensitized BCSC to NK cell–mediated killing, thereby reducing immune escape–associated BCSC metastasis. Together, our findings reveal a novel mechanism for immune escape of human BCSC and identify the miR20a–MICA/MICB signaling axis as a therapeutic target to limit metastatic breast cancer. Cancer Res; 74(20): 5746–57. ©2014 AACR.

Introduction
The escape of cancer cells from immune surveillance has been considered as a prerequisite for metastasis (1), the major cause of death in breast cancer. Cancer cells are highly heterogeneous and a small fraction of cancer cells with stem cell properties, known as tumor-initiating cells, or cancer stem cells (CSC), may be responsible for tumorigenesis (2), angiogenesis (3) and metastasis (4). Recently, selection by adaptive immunity has been shown to generate or enrich cancer cells with stemness (5–9), suggesting the presence of intimate interaction between CSCs and the skewed adaptive immunity in patients with cancer. However, the role of innate immunity in affecting CSCs and their metastasis remains unclear.

Cancer immunoediting, defined by phases of elimination, equilibrium, and escape, consists of both tumor-limiting and -promoting effects (10). Selection by adaptive and innate immunity components, together with clonal evolution of malignant cells, contributes to the generation of cancer cells with better survival advantages and functional heterogeneity (10). Recent studies using mice deficient in adaptive immunity demonstrate that cancer immunoediting may occur in the absence of adaptive immune cells through IFNγ-producing natural killer (NK) cells (11). Thus, we hypothesize that improper immunoediting by NK cells may result in the failure of cancer cells with stem cell property to respond to anticancer immunity and, thus, promote cancer metastasis.

As innate immune effectors, NK cells are one of the major infiltrating immune cells in breast cancer and are endowed with the capability of recognizing nascent transformed cells to prevent tumorigenesis and subsequent metastasis (12–15). Breast cancer stem-like cells (BCSC) are resistant to therapies and are highly metastatic (16–18). However, it remains unclear whether BCSCs succumb to NK cell cytotoxicity. In this study, we attempt to address this issue and report that BCSCs escape from NK cell cytotoxicity and metastasize through miR20a–mediated downregulation of MICA and MICB, two ligands for the NK cell–activating receptor NKG2D.

Materials and Methods
Breast cancer specimens and immunohistochemistry
Breast cancer tissues were obtained from 591 consecutive consented patients who underwent surgical resection at the Breast Surgery Center at Southwest Hospital from 2006 to 2007, with approval from the Institutional Ethics Committee. All specimens were formalin-fixed and paraffin-embedded in the Institute of Pathology at Southwest Hospital. The median age of patients at diagnosis was 45 years (range, 23–87 years). The patients received radical mastectomy, or modified radical mastectomy.
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mastectomy, or breast-conserving surgery. The axillary lymph nodes were routinely examined for metastasis. Tumor size, location, distal metastasis, and final tumor–node–metastasis stage were determined. The histologic diagnosis was made according to the WHO standard. The expression of the breast cancer marker aldehyde dehydrogenase 1 (ALDH1) and the NK cell marker CD56 was examined by immunohistochemistry (IHC; Supplementary Materials and Methods).

Cell sorting by FACS

Primary breast cancer cells at 90% confluence were suspended in ALDEFLUOR assay buffer containing an ALDH substrate and incubated for 45 minutes at 37°C (ALDEFLUOR assay kit; Stem Cell Technologies; refs. 18, 19). Negative control cells were incubated with an ALDH inhibitor, diethyleniamino-benzaldehyde (DEAB). The cells were then suspended in assay buffer for sorting ALDH+ and ALDH− cells on a BD Aria II sorter (BD Biosciences). ALDH+ cells were maintained as mammospheres for further analysis.

Culture of peripheral blood NK cells and NK cell cytotoxicity assay

Peripheral blood NK cells (pNK cells) from patients with breast cancer and healthy donors were isolated for direct analysis or for further expansion (Supplementary Materials and Methods). pNK cell cytotoxicity was measured by a 51-chromium (51Cr) release assay. The target cells (1 × 106) were labeled with 250 μCi Na251CrO4 for 2 hours at 37°C, seeded into 96-well round bottom plates at 5,000 per well, and cocultured with pNK cells (effector cells) at indicated effectortarget (E/T) ratios. K562 cells were served as a positive control for killing. After incubation for 4 hours at 37°C, cell supernatants were collected, and spontaneous and maximal releases of 51Cr were measured. The percentage of 51Cr release (cytotoxicity) was calculated as 100 × (experimental release – spontaneous release)/maximum release (spontaneous release). In blocking experiments, pNK cells were pretreated with 20 μg/mL anti-NKG2D-neutralizing mAb (R&D Systems) for 20 minutes at room temperature before the assay.

Cell death of targets induced by NK cell cytotoxicity was also examined by flow cytometry (Supplementary Materials and Methods).

IFNγ quantification by ELISA

Briefly, target cells were seeded in laminin-coated 96-well plates and cultured for 24 hours. pNK cells (5 × 105/mL) were incubated with the targets (2.5 × 105/mL) in a final volume of 200 μL of fresh NK cell medium (Supplementary Materials and Methods) for 24 hours (E/T = 2:1). Blockade of NKG2D receptor before incubation was performed using an anti-NKG2D-neutralizing mAb (R&D Systems). To test whether cancer cells released IFNγ, we included target cells alone as control. After centrifugation, the supernatant was collected to determine the level of IFNγ using a human IFNγ ELISA Kit (Invitrogen). Because the levels of IFNγ secretion varied among donors, we showed the representative data using NK cells derived from one donor for presentation, and all of the assays were repeated using NK cells from at least three different donors.

Clearance by NK cells and lung metastasis

The elimination of target cells by NK cells in vivo and immunoavasion-associated metastasis were assessed in a short-term lung clearance assay and an experimental lung metastasis model in NK cell (mNK)–depleted NOD/SCID mice transferred with human pNK cells (Supplementary Materials and Methods).

Statistical analysis

All experiments were performed at least in triplicates and representative data were shown. The correlation between the number of CD56+ NK cells or ALDH1 positivity and clinicopathologic parameters was assessed by the nonparametric Wilcoxon rank-sum test, the χ2 test, or the Fisher exact test as appropriate. Overall survival (OS) and metastasis-free survival (MFS) rates were plotted as Kaplan–Meier curves and analyzed using the log-rank test. The Cox proportional hazard regression model was used to determine the influence of various parameters on patient survival in univariate and multivariable analysis. The Student t test or ANOVA was used when appropriate. All data were analyzed using SPSS 13.0 statistical software. Data were shown as the means ± SD. A P value of <0.05 was considered statistically significant.

Results

Failure of tumor-infiltrating NK cells to prevent the metastasis of BCSC-rich human breast cancers

We first examined the presence of CD56+ NK cells and BCSCs in breast cancer specimens from 591 patients using ALDH1 as a stem cell marker (18–20). On the basis of the IHC staining, 212 cases of breast cancers were ALDH1high and 379 cases were ALDH1low (Supplementary Table S1). High level of infiltrating CD56+ cells was detected in 33.0% of cancers. Of note, we frequently detected codistribution of CD56+ cells in the areas with either non-BCSCs (ALDH1low) or BCSCs (ALDH1high) cancer cells (Fig. 1A). In ALDH1low cancers, the level of infiltrating CD56+ cells was lower in cancer tissues with ≥4 lymph node metastasis (LNM) than those with <4 LNM (Fig. 1B). Although close localization of infiltrating CD56+ cells with BCSCs was observed (Fig. 1C), there was no significant difference in the number of tumor-infiltrating CD56+ cells between cases with ≥4 LNM and those with <4 LNM in ALDH1high tumors (Fig. 1D).

We then analyzed the correlation of infiltrating CD56+ cells with patient survival in ALDH1low and ALDH1high subgroups. Significantly longer OS and MFS were found in the patients with higher level of infiltrating CD56+ cells than those with lower level of infiltrating CD56+ cells in ALDH1low, but not ALDH1high cancers (Fig. 1E and F). Moreover, increased level of infiltrating CD56+ cells in tumors was a favorable prognostic factor for OS and MFS in ALDH1low, but not the ALDH1high patient subgroup (Supplementary Table S2). Furthermore, increased level of infiltrating CD56+ cells was able to independently predict fewer distal metastases in the ALDH1low tumors (Supplementary Table S3). However, in the ALDH1high subgroup, no significant difference in the relative risk to develop metastasis was observed in patients with CD56+ tumors as compared with those with CD56− tumors. These
results indicate that high level of infiltrating NK cells in breast cancer fails to prevent ALDH1<sup>high</sup> BCSCs from metastasis in patients.

**BCSCs are resistant to NK cell cytotoxicity and contribute to lung metastasis**

We isolated ALDH<sup>+</sup> cells from primary breast cancer cells and found that they exhibited potent self-renewal capacity with multiple differentiation potentials and markedly enhanced tumorigenicity in vivo (Supplementary Fig. S1), confirming the BCSC property of ALDH<sup>+</sup> cells (18, 19). We next examined activation and degranulation of NK cells cocultured with either BCSCs or non-BCSCs (ALDH<sup>−</sup> cells). Flow cytometric analysis showed reduced expression of CD107a, a surface marker for NK cell degranulation, on human pNK cells cocultured with BCSCs (Fig. 2A and Supplementary Fig. S2A). Because IFNγ released by activated NK cells is important for immunoregulation, we determined the level of IFNγ and found significantly reduced production of IFNγ by pNK cells cocultured with BCSCs (Fig. 2B and Supplementary Fig. S2B). Thus, primary BCSCs are unable to activate normal NK cells.

**Figure 1.** Infiltrating NK cells fail to limit metastasis of human breast cancers with high level of BCSCs. A, in ALDH1<sup>low</sup> cancers (top), low or high levels of infiltrating CD56<sup>+</sup> cells (bottom) were detected by IHC staining on serial sections, demonstrating codistribution of NK cells with non-BCSCs. B, average numbers of infiltrating CD56<sup>+</sup> cells in ALDH1<sup>low</sup> cancers with ≥4 or <4 LNM. C, in ALDH1<sup>high</sup> cancers (top), low or high levels of infiltrating CD56<sup>+</sup> cells (bottom) were also revealed, showing codistribution of NK cells with BCSCs. D, average numbers of infiltrating CD56<sup>+</sup> cells in ALDH1<sup>high</sup> cancers with ≥4 or <4 LNM. E and F, Kaplan–Meier plot of OS and MFS of the patients with ALDH1<sup>low</sup> (E) or ALDH1<sup>high</sup> cancers (F), stratified by the levels of infiltrating CD56<sup>+</sup> cells. Mean ± SD (B and D); bar, 100 μm.
To examine the capacity of NK cells to destroy BCSCs, we used pNK cells from both patients with breast cancer and healthy donors. Activated pNK cells showed a weaker lytic activity on autologous BCSCs than non-BCSCs (Fig. 2C). BCSCs were also resistant to killing by either long-term expanded or fresh pNKs from healthy donors (Fig. 2D and Supplementary Fig. S2C and S2D). Pretreatment of NK cells with an anti-CD16 mAb inhibited both degranulation and cytotoxicity by NK cells that were cocultured with BCSCs or non-BCSCs (Fig. 2A and D, Supplementary Fig. S2A, S2C, and S2D), indicating the specificity of the function of NK cells. Moreover, the levels of cell death in BCSCs incubated with either long-term expanded or fresh pNKs were significantly lower than those in non-BCSCs (Fig. 2E and F).

Figure 2. Resistance of BCSCs to the cytotoxicity of autologous or allogeneic pNK cells and the contribution of BCSCs to lung metastasis. A, detection of CD107a on pNK cells by FACS in pNK cells cocultured with primary BCSCs or non-BCSCs (E:T = 5:1). K562 cells served as positive control. Alternatively, pNK cells were pretreated with anti-CD16 mAb (3 μg/mL) to demonstrate the specificity for the function of NK cells. pNK represents human peripheral blood NK cells that were expanded for 2 to 3 weeks. Fresh pNK represents freshly isolated NK cells. B, IFN-γ released by pNK cells in the coculture supernatant (E:T = 2:1). C, lysis of primary BCSCs and non-BCSCs by autologous pNK cells as measured by 51Cr release. Data from six patients are shown (triple repeats). D, lysis of BCSCs and non-BCSCs by allogeneic pNKs from healthy donors. Anti-CD16 mAb was applied to specifically inhibit NK cell activity. E, flow cytometric analysis of target cell death cultured with pNK. The middle panel shows representative flow cytometric graph and total dead cells (%). F, the percentages of ALDH+ cells (left) or efficiency of sphere formation (right) of cancer cells that survived NK cell–mediated lysis. G, the relative survival ratio of BCSCs or non-BCSCs (DiD-labeled) in relation to HeLa cells (GFP-labeled, internal control) in a short-term lung clearance assay in NOD/SCID mice (n = 3). H, the numbers of pulmonary metastasis established by BCSCs and non-BCSCs (n = 3); mean ± SD (A–I); *P < 0.05; **P < 0.01; ns, no significance.

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freshly isolated pNK cells were reduced (Fig. 2E and Supplementary Fig. S2E). In addition, short-term exposure to pNK cells did not significantly affect cell-cycle distribution and proliferation of BCSCs (Supplementary Fig. S2F and S2G). These results suggest that the resistance of BCSCs to NK cell cytotoxicity is due to reduced cell death. To test possible immunoselection of BCSCs versus non-BCSCs by NK cells, we cultured primary cancer cells with allogeneic pNK cells and found that the residual viable cancer cells were enriched with BCSCs, as indicated by increased number of ALDH1+ cells and high efficacy of tumorsphere formation (Fig. 2F and Supplementary Fig. S2H). Thus, BCSCs are more resistant than non-BCSCs to lysis by NK cells.

To test the consequences of BCSC resistance to NK cell cytotoxicity, we used an in vivo short-term lung clearance assay of cancer cells in mice (Supplementary Fig. S3A; refs. 21, 22). Although no significant difference of cell survival rates was found between BCSCs and non-BCSCs i.v. injected into NOD/SCID mice depleted of NK (mNK) cells (mNK cell-depleted mice), less BCSCs were eliminated in the mice transferred with human pNK cells (Fig. 2G and Supplementary Fig. S3B). These results suggest that circulating BCSCs are resistant to elimination by NK cells in vivo. In a lung metastasis model (Supplementary Fig. S3C), i.v. injected BCSCs formed higher numbers of metastases in the lungs of normal mice as compared with non-BCSCs (Fig. 2H and Supplementary Fig. S3D). In contrast, there was no difference in the number of metastatic foci formed by either BCSCs or non-BCSCs in mNK cell-depleted mice. However, i.v. injected BCSCs established more metastatic foci in the lungs of mice adoptively transferred with human pNK cells. The results indicate that BCSCs are resistant to NK cell cytotoxicity in vivo, thus are prone to form lung metastasis.

Reduced expression of MICA and MICB by BCSCs contributes to the resistance to NK cell cytotoxicity and the resultant metastasis

Tumor-infiltrating immune cells release cytokines such as IFNγ to regulate leukemic stem cells (8). However, we found that the expression of IFNγ receptor α chain, an essential component of IFNγ receptor for ligand binding and trafficking, was comparable between BCSCs and non-BCSCs (Supplementary Fig. S4A). Because NK cell receptor ligands expressed by target cells determine the sensitivity to NK cell cytotoxicity, we compared the expression of inhibitory receptor ligands HLA-A/B/C, HLA-E, HLA-G by BCSCs and non-BCSCs and also found no significant difference (Supplementary Fig. S4B). We then compared the levels of activating receptor ligands (Supplementary Fig. S4C–S4E) on BCSCs and non-BCSCs, and found that the expression of both MICA and MICB, two ligands for the activating receptor NKG2D, was significantly lower on BCSCs as compared with non-BCSCs (Fig. 3A and Supplementary Fig. S4F).

To examine whether the lower expression of MICA and MICB was responsible for the resistance of BCSCs to NK cell cytotoxicity, we transfected BCSCs with MICA and MICB (Supplementary Fig. S4G). Coculture of transfected BCSCs with pNK cells significantly increased the expression of CD107a by pNK cells, which was inhibited by pretreatment of pNK cells with an NKG2D-blocking mAb (Fig. 3B and Supplementary Fig. S4H). In addition, these cocultured pNK cells showed increased IFNγ secretion (Fig. 3C). BCSCs with increased MICA or MICB, but not nontransfected parental BCSCs or vector control, were significantly more sensitive to allogeneic pNK-mediated lysis (Fig. 3D and Supplementary Fig. S4I). Increases in IFNγ secretion and NK cell–mediated cytotoxicity were inhibited by blocking NKG2D but not NKp30 receptor on NK cells (Fig. 3C and D, Supplementary Fig. S4I). Short-term lung clearance assay showed a reduced survival of MICA- or MICB-transfected BCSCs in the lungs of mNK cell-depleted mice transferred with human pNK cells. In contrast, blocking NKG2D on NK cells promoted the survival of BCSCs (Fig. 3E and Supplementary Fig. S4J). Moreover, BCSCs transfected with NKG2D ligands formed fewer metastatic foci in the lungs of these mice. The NKG2D-blocking mAb restored the lung metastasis of MICA- or MICB-transfected BCSCs (Fig. 3F and Supplementary Fig. S4K). These results indicate that lower levels of MICA and MICB contribute to BCSC resistance to NK cell lysis, thus increasing their lung metastasis.

To further examine the relevance of MICA and MICB to tumor cell sensitivity to NK cell cytotoxicity, we used siRNA to downregulate the ligands originally expressed at higher levels on non-BCSCs (siRNA–non-BCSCs; Supplementary Fig. S5A). Coculture of pNK cells with siRNA–non-BCSCs reduced their CD107a expression (Fig. 4A and Supplementary Fig. S5B), accompanied by reduced IFNγ secretion (Fig. 4B), suggesting that cancer cells with lower levels of MICA or MICB are unable to trigger NK cell killing. These nonactivated NK cells, therefore, were less effective in lysing siRNA–non-BCSCs, but not nontransfected non-BCSCs or scramble siRNA control (Fig. 4C). NKG2D mAb, but not isotype IgG or NKp30 mAb, cast the effect similar to MICA/MICB knockdown on the IFNγ secretion and cytotoxicity (Fig. 4B and C), suggesting an important role of MICA/MICB–NKG2D interaction in the immune surveillance of non-BCSCs by NK cells. Moreover, siRNA–non-BCSCs with downregulated NKG2D ligands showed improved survival (Fig. 4D and Supplementary Fig. S5C), and, thus, metastasized more frequently to the lungs of mNK cell–depleted mice transferred with human pNK cells (Fig. 4E and Supplementary Fig. S5D). These in vivo results demonstrate that loss of MICA or MICB results in enhanced survival and metastasis of breast cancer cells.

High level of miR20a in BCSCs downregulates MICA/B and promotes metastasis

The mechanisms underlying the loss of MICA and MICB in BCSCs were explored. We failed to detect differences in the mRNA levels of either MICA or MICB between BCSCs and non-BCSCs (Supplementary Fig. S5E). However, the level of one microRNA, miR20a, was found significantly higher in BCSCs than non-BCSCs (Fig. 5A). We, therefore, hypothesized that miR20a might be involved in the downregulation of MICA and/or MICB on BCSCs (Supplementary Fig. S5B). Dual luciferase reporter assays showed that integration of MICA and MICB (Fig. 5B) 3′ untranslated region (UTR) markedly inhibited the luciferase activity. However, the inhibition was reversed when miR20a-binding sites were mutated, suggesting
Figure 3. The expressions of NGK2D ligands MICA and MICB on BCSCs and their contribution to NK cell cytotoxicity and tumor metastasis. A, representative data of MICA and MICB expression by primary BCSCs and non-BCSCs (left). Right, quantitative data from six cases of primary cancers. B, detection of CD107a on pNK cells cocultured with BCSCs overexpressing MICA or MICB (E:T = 5:1). To abrogate MICA/B-NKG2D interaction, pNK cells were pretreated with an NKG2D-blocking mAb (20 μg/mL). C, IFNγ levels in the coculture supernatant of pNK cells with NKG2D ligands transfected with BCSCs (E:T = 2:1). Experimental controls included pNK cells alone, or nontransfected BCSCs alone, or NKp30 mAb (2 μg/mL)- or NKG2D mAb-pretreated pNK cells with BCSCs. D, the cytotoxicity of pNK cells to BCSCs (mean ± SD). Controls included K562 cells, nontransfected or vector-transfected tumor cells, or pNK cells pretreated with NKG2D or NKp30 mAb, E, the survival of BCSCs overexpressing MICA or MICB in vivo (n = 3). F, numbers of lung metastatic nodules formed by BCSCs (n = 3). Mean ± SD (A–C, E, and F); *, P < 0.05.
that endogenous miR20a binds to 3’-UTR of MICA and MICB to regulate their expression. These results were confirmed by the findings that the miR20a inhibitor (Supplementary Fig. S6C) markedly upregulated the expression of MICA and MICB on BCSCs (Fig. 5C). Thus, miR20a appears to interfere with the posttranscriptional modification of MICA and MICB.

To study the contribution of increased miR20a in BCSCs to their escape from NK cell killing, the cells were transfected with an miR20a inhibitor (miRi-BCSCs). After coculture with miRi-BCSCs, pNK cells expressed a significantly higher level of CD107a (Fig. 5D) and secreted increased amounts of IFNγ (Fig. 5E). miRi-BCSCs were also more sensitive to the cytotoxicity (Fig. 5F and Supplementary Fig. S6E) and the clearance by NK cells in vivo (Fig. 5G and Supplementary Fig. S6F). Masking the NKG2D receptor on NK cells reversed the effect of the miR20a inhibitor. Moreover, inhibition of miR20a reduced the metastasis of BCSCs in the lungs of the mice, which was also reversed by blockade of the NKG2D receptor on pNK cells (Fig. 5H and Supplementary Fig. S6G). Although the miR20a inhibitor slightly reduced sphere formation, ALDH expression, and proliferation by BCSCs (Supplementary Fig. S6H and S6I), inhibition of miR20a alone did not significantly increase the apoptosis of the cells without the presence of NK cells (Supplementary Fig. S6J). Inhibition of miR20a alone did not significantly attenuate cell survival and metastatic capacity of BCSCs in the absence of NK cells (Fig. 5G and H and Supplementary Fig. S6F and S6G). Therefore, miR20a downregulates MICA and MICB and increases the capacity of BCSCs to metastasize by evading NK cell clearance.
Targeting the miR20a–MICA/B axis by all-trans retinoic acid sensitizes BCSCs to NK killing and prevents metastasis

Because differentiation therapy targeting CSC represents a promising therapeutic approach (2, 23, 24), we tested the capacity of all-trans retinoic acid (ATRA), a well-established differentiation-inducing agent for CSCs from leukemia and glioblastoma (25, 26), to affect immune evasion by BCSCs. ATRA markedly enhanced the expression of both MICA and MICB proteins (Fig. 6A) but not their mRNA levels (Fig. 5B).
Supplementary Fig. S7A) by BCSCs. Moreover, ATRA treatment downregulated miR20a (Fig. 6B), whereas its restoration with miR20a mimics (miR20a M) in ATRA-treated BCSCs (Supplementary Fig. S7B) reduced MICA and MICB on BCSCs (Fig. 6C). These results suggest that ATRA regulates MICA and MICB expression by repressing aberrantly expressed miR20a.

The enhancing effect of ATRA on MICA and MICB expression prompted us to investigate the potential of induced differentiation to sensitize BCSCs to NK cell cytotoxicity, thus preventing metastasis. BCSCs treated with ATRA showed reduction of miR20a and were more sensitive to NK cell–mediated lysis. Restoration of miR20a in ATRA-treated BCSCs or blocking NKG2D on pNK cells attenuated the sensitivity of ATRA-treated BCSCs to the lysis by NK cells (Fig. 6D). Furthermore, pretreatment of BCSCs with ATRA reduced their survival (Fig. 6E and Supplementary Fig. S7C) and metastatic capacity (Fig. 6F and Supplementary Fig. S7D) in mice transferred with human pNK cells. The effect of ATRA was reversed by introduction of exogenous miR20a in BCSCs or blockade of NKG2D on NK cells. In addition, ATRA treatment resulted in measurable reduction in the propagation and stem-like properties of BCSCs (Supplementary Fig. S7E and S7F). However, ATRA alone neither induced significant apoptotic death of BCSCs (Supplementary Fig. S7G), nor attenuated their survival/metastatic capacity in vivo (Fig. 6E and F), indicating that ATRA inhibits BCSC metastasis mainly through facilitating elimination of BCSCs by NK cells. Moreover, ATRA showed less effect on the expression of miR20a and MICA/MICB as well as the sensitivity to NK cell cytotoxicity in non-BCSCs (Supplementary Fig. S7H–S7J), suggesting that ATRA preferentially targets BCSCs. These observations indicate that targeting the miR20a–MICA/B axis by induction of differentiation sensitizes BCSCs to NK cell killing and prevents their metastasis.

**Discussion**

CSCs are the cellular origin of multiple malignant properties in cancer, including unrestrained self-renewal (2), neovascularization (3), resistance to chemo- or radiotherapy (2, 27), dissemination (4), and evasion of antitumor immunity (5–9). The metastasis of BCSCs is a result of epithelial–mesenchymal transition (EMT) induced by genetic/epigenetic alterations or microenvironmental cues (4). In this study, we demonstrate that BCSCs evade NK cell surveillance to establish
tumor metastasis, representing a revelation of novel mechanisms by which BCSCs establish distant metastasis. Tumor heterogeneity has been recognized as the consequence of genetic diversity within cancer and the presence of CSCs (2). Immunoediting may also promote cancer progression and select tumor cells with enhanced malignancy (10). The potential for CSCs to avoid destruction by adaptive immunity has been documented in the context of CTL-mediated cytolysis (5–7, 9, 28). In addition, IFNγ released by CTL promotes the proliferation of CSCs and contributes to leukemia progression (8). These observations demonstrate that tumor metastasis is associated with dynamic regulation of CSC evasiveness from adaptive immunity.

The impact of innate immunity on cancer progression and enrichment of CSCs has largely been unclear. It has been reported that several types of normal stem cells were efficiently lysed by fresh allogeneic NK cells (29–31). Similarly, stem-like cells in oral squamous carcinoma (31), colorectal carcinoma (32), and glioblastoma (33) were susceptible to NK cell–mediated cytolysis. However, leukemic stem cells were resistant to allogeneic NK cell cytotoxicity in vitro (34). Evasion of BCSCs from NK cell surveillance was implicated in trastuzumab resistance, in which treatment of MCF-7 cells with trastuzumab resulted in enrichment of CD44+CD24− cells by NK cells (35), although BCSCs and remaining cells from other cell lines were equally sensitive to allogeneic NK cell–mediated lysis in vitro (13). Our findings, using primary breast cancer cells, demonstrate that ALDH1+ BCSCs were resistant to both autologous and allogeneic NK cell cytotoxicity, and that cancer cells surviving NK cell killing were enriched with ALDH1− BCSCs. Therefore, CSCs from neoplastic tissues of different origin exhibit diverse sensitivity to lysis by NK cells. Understanding this diversity has important implication for the development of rational immune therapy against CSCs.

NK cell anergy is detected in a great number of patients with cancer, especially those with late-stage cancer (15, 36). By using in vitro expanded anergic NK cells, we demonstrate that immunoevasion of BCSCs from NK cell cytotoxicity is essential for establishment of breast cancer metastasis. Therefore, resistance to NK cell lysis may be an intrinsic property of BCSCs to initiate metastasis in the patients with dysfunctional NK cells. In cancer tissues, we observed codistribution of CD56− cells with ALDH1+ BCSCs, indicating that these cells may play wider roles in tumor microenvironment. IFNγ released by immune cells regulates the biology of CSCs in chronic myeloid leukemia (8). Nevertheless, no significant difference of IFNγ receptor α chain expression between BCSCs and non-BCSCs indicates that the immune evasion of BCSCs is not likely due to the differential activation of IFNγ signaling by infiltrating NK cells.

We found that the lack of stimulatory signals in BCSCs for NK cells was associated with reduced expression of MICA and MICB, two ligands for the NK-activating receptor NK2D (37), in BCSCs. MICA and MICB are stress-inducible antigens rarely expressed by healthy cells but are frequently upregulated on transformed or virus-infected cells (38). Reduced expression of MICA/B facilitates the evasion of epithelial tumors from surveillance by NK cells and subsets of T cells (39). We demonstrated that reduced MICA/B expression by BCSCs contributed to their resistance to NK cell cytolysis and enhanced metastatic capacity in vivo. Therefore, it is plausible that reduction in MICA/B expression promotes BCSC metastasis by evading NK cell killing.

Despite the lower expression of MICA/B proteins by BCSCs, no substantial difference in MICA/B mRNA levels was detected between BCSCs and non-BCSCs, suggesting posttranscriptional regulation of MICA/B in tumor cells. Small noncoding RNAs, miRNAs, are important posttranscriptional regulators in CSCs (40, 41). miRNAs targeting MICA or MICB have been identified in studies of viral infection and IFNγ treatment (42–45). As a member of the oncogenic miR17-92 cluster, the expression of miR20a is elevated in several malignancies (46–48). We demonstrated that overexpression of miR20a reduced the levels of both MICA and MICB in BCSCs, resulting in their immune evasion with increased metastasis. Thus, miR20a is a pivotal regulator of immune evasion by BCSCs, and represents a potential target for strengthening immunosurveillance to inhibit tumor metastasis.

Promoting differentiation is a promising therapeutic approach against CSCs. Induced differentiation of CSCs results in their loss of chemoresistance and tumorigenicity (24). One such clinically applied differentiation agent is ATRA, which is well-known for its potent differentiation-inducing effect on CSCs from leukemia and glioblastoma (25, 26). In the present study, BCSC differentiated by ATRA showed diminished immune evasiveness with reduced expression of miR20a but enhanced MICA/B expression. Thus, the resistance of BCSCs to NK cell surveillance is reversible by differentiation and increasing the levels of ligands for NK cell stimulating receptor NK2D.

In conclusion, our present study reveals that an aberrant miR20a–MICA/B axis is responsible for the escape of BCSCs from innate immunity, which is critical for increased metastatic capacity of BCSCs. Thus, targeting the miR20a–MICA/B axis may represent a promising immune therapeutic strategy to prevent breast cancer metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Conception and design: B. Wang, Z. Wang, X.-W. Bian
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