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Discovering alkylamide derivatives of bexarotene as new therapeutic agents against triple-negative breast cancer

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ABSTRACT

Triple-negative breast cancer (TNBC) has been reported to be correlated with high expression of proliferation markers as well as constitutive activation of metastasis-relevant signaling pathways. For many years, breast cancer researchers have been investigating specific and effective methods to treat or to control the development of TNBC, but promising therapeutic options remain elusive. In this study, we have demonstrated that alkylamide derivatives of bexarotene DK-1–150 and DK-1–166 induce apoptotic cell death in TNBC cell lines without causing cytotoxicity in the normal mammary epithelial cell line. Furthermore, the bexarotene derivatives also showed significant effects in inhibiting TNBC cell proliferation and migration, modulating cancer stem cell markers expressions, as well as limiting the epithelial-mesenchymal transition (EMT) activities of TNBC cell lines in terms of downregulating EMT marker and blocking nuclear translocation of β-catenin. Therefore, we propose the alkylamide derivatives of bexarotene as potential candidates for novel anticancer therapeutics against TNBC.

Triple-negative breast cancer (TNBC), characterized by mammary tumors that lack detectable expressions of estrogen receptor (ER), progesterone receptor (PR) and human epithermal growth factor receptor 2 (HER2),[1] contributes to 15–20% of all breast cancer cases diagnosed.[2,3] This breast cancer subtype is associated with high expression of proliferation markers such as Ki-67,[4] and activation of the β-catenin pathway.[5] TNBC is further classified into 6 subtypes based on the gene expression profiles, namely basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR) subtype. Among these TNBC subgroups, mesenchymal (M) and mesenchymal stem-like (MSL) subtypes are enriched in genes associated with the epithelial-mesenchymal transition (EMT) and growth factor signaling pathways.[6] In contrast to other subtypes of breast cancer, TNBC is more aggressive and invasive, more resistant to chemotherapies, and also possesses elevated EMT activity and a higher percentage of cancer stem cell (CSC) population. Such properties of TNBC tumors give rise to resistance to conventional anti-cancer therapies, metastasis and tumor relapse.

Although a variety of single agents and combination regimens are available for breast cancer prevention and/or treatment, none of them are recommended specifically for TNBC.[7–9] Without effective treatments, most TNBC patients with advanced diseases experienced relapse shortly after neoadjuvant chemotherapy, frequently with visceral metastases and a short life expectancy.[10] Particularly, advanced TNBC has a higher rate of early recurrence and distant metastasis to the brain and lungs, as compared to other breast cancer subtypes.[11–13] Thus, identifying effective, tailored and less toxic therapeutic options for TNBC is a pressing need. In this study, we are demonstrating the alkylamide derivatives of bexarotene as a new class of therapeutic agents targeting TNBC, imposing anti-cancer activities through induction of apoptotic cell death, suppression of cell proliferation and migration, as well as limiting EMT and CSC properties of TNBC cells.

The bexarotene alkylamide derivatives (Fig. 1A), DK-1–150 and DK-1–166 were synthesized by reacting acyl chloride of bexarotene with 1-(2-aminoethyl)pyrrolidine or *N*,*N*-diethylethylenediamine, respectively. Cellular toxicity of the alkylamide derivatives of bexarotene was assessed in two of the invasive TNBC cell lines, MDA-MB-231 (MSL subtype) and BT549 (M subtype), via the evaluation of post-treatment cell viabilities (Fig. 1B). In addition to the TNBC cell lines, we also used normal mammary epithelial cell line MCF10A and non-TNBC breast cancer cell MCF-7, in order to examine if the cytotoxic effects induced by bexarotene derivatives are selectively high in TNBC cells. MTT cell viability assay results showed that 5 μM of bexarotene failed to cause significant reduction in cell viabilities in MCF10A, and in the three breast cancer cell lines. However, 5 μM of DK-1–150 or DK-1–166 was

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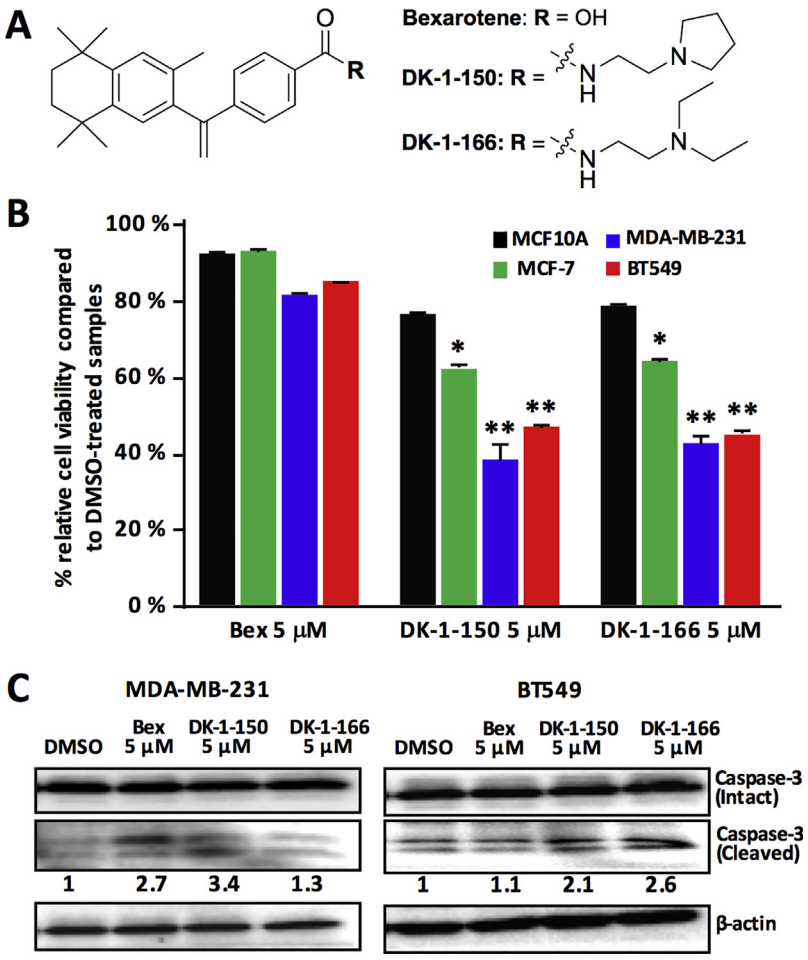

Fig. 1. Bexarotene derivatives display cytotoxicity against TNBC cell lines. A) Chemical structures of bexarotene and its alkylamide derivatives, DK-1–150 and DK-1–166. B) Cytotoxicity evaluation by MTT assay comparing post-treatment cell viabilities among MCF10A and breast cancer cell lines. ($^{*}p < .05$, $^{**}p < .005$). C) Activation of caspase-3 of TNBC cell lines by the bexarotene derivatives. β-Actin was used as endogenous loading control. Densitometry measurement for quantification was performed, and the results were normalized to DMSO-treated samples. Numbers stated underneath indicate expression fold-change values from ImageJ analysis. See Supplementary Fig. 2 for the activation of caspases-8 and -9.

able to induce about 40% reduction in MCF-7 cell viability, and about 60% reduction in cell viability of MDA-MB-231 and BT549 cells. The EC_{50} of bexarotene on TNBC cells is estimated to be higher than 56 μM while the EC_{50} of DK-1–150 and DK-1–166 on TNBC cells are less than 10 μM (Fig. 1B; Supplementary Fig. 1). Importantly, both derivatives showed minimal cytotoxicity on MCF10A. These results suggest the two bexarotene derivatives are more cytotoxic than bexarotene to breast cancer cells, but may not be harmful to the normal breast epithelial cells.

To examine if the cytotoxicity of the derivatives is due to induction of apoptosis, the activation of caspases in TNBC cells was evaluated. Bexarotene and the two derivatives significantly induced activation of caspase-8, caspase-9, and caspase-3 in TNBC cell lines by at least 2 folds (Fig. 1C; Supplementary Fig. 2), suggesting that the derivatives are able to initiate both intrinsic and extrinsic apoptotic cascades, as well as induce both early and late apoptosis in TNBC cells. In contrast, no significant activation of caspases was observed in MCF10A and MCF-7 cell lines, upon treatments with bexarotene or either of the derivatives (Supplementary Fig. 2). Interestingly, BT549 cell line responds better to DK-1–150 or DK-1–166 than to bexarotene of the same dosage (Fig. 1C; Supplementary Fig. 2). Furthermore, both MDA-MB-231 and BT549 cells treated with 5 μM DK-1–150 showed increased levels of cleaved PARP (Fig. 4B), suggesting induction of DNA damage by the derivative through apoptosis mechanism.

To assess the effect of the bexarotene derivatives on breast cancer cell proliferation, we performed adherent colony-forming assay and found that both DK-1–150 and DK-1–166 led to 80% reduction of colony formation in MCF-7 cell line at 5 μM, while bexarotene did not affect the colony-forming capacity of MCF-7 (Fig. 2A). To further evaluate if the derivatives can inhibit anchorage-independent proliferation, we conducted the soft-agar colony-forming assay. Similar to the adherent colony-forming assay, soft-agar colony-forming assay results reflected that both bexarotene derivatives at 2 μM inhibited the formation of colonies by at least 90%, while 10 μM bexarotene failed to inhibit colony formation in MDA-MB-231 cells (Fig. 2B; Supplementary Fig. 3). TNBC is well known for its high metastatic potential. To assess the *in vitro* anti-metastatic activity of bexarotene derivatives on TNBC cell lines, we performed wound-healing assay on MDA-MB-231 cells. While DMSO- or bexarotene (5 μM)-treated MDA-MB-231 cells were able to migrate, and eventually close the "wound gap" after 24 h incubation, cells treated with 2 μM DK-1–150 or 2 μM DK-1–166 had significantly lowered capability of cell migration (Fig. 2C; Supplementary Fig. 4). Collectively, these data prove that the bexarotene derivatives are able to suppress TNBC cell proliferation and migration.

It has been proposed that CSCs, while existing as a minor population, exert resistance to the contemporary anti-cancer therapies, and hence become a potential root of tumor recurrence or

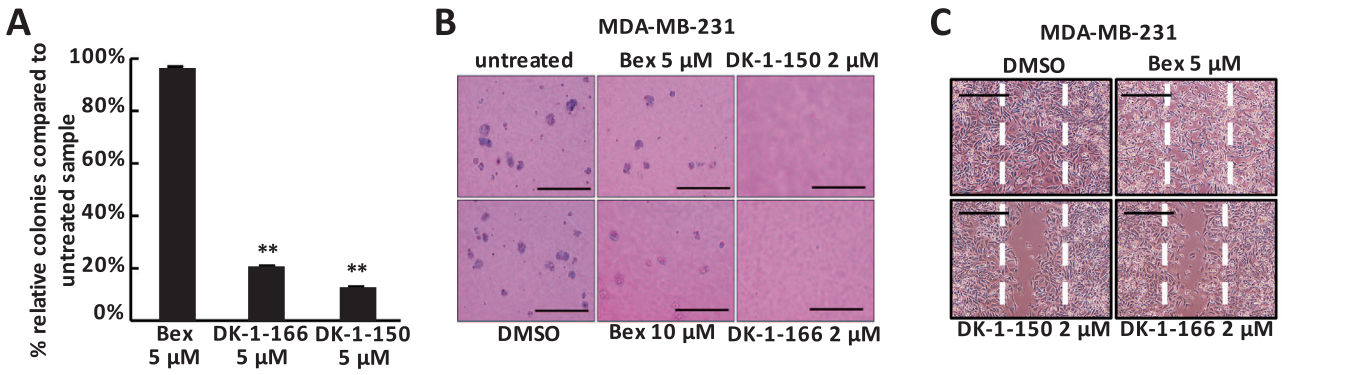

Fig. 2. Suppression of proliferation and migration of breast cancer cells by the alkylamide derivatives of bexarotene. A) Adherent colony-forming assay performed on MCF-7 cells. Number of colonies was counted at the end of 7-day treatment, and the results were normalized with untreated samples ($^{**}p < 0.005$). B) Soft-agar colony-forming assay performed on MDA-MB-231 cells (see quantitative analysis in Supplementary Fig. 3). Scale bar indicates 200 μm. C) Wound-healing assay performed on MDA-MB-231 cells, and the images taken at 24 h. See Supplementary Fig. 4 for the cells treated with higher dose (5 μM) of DK-1–150 or DK-1–166.

metastasis.[14–16] Previous studies have reported that such CSC population is enriched in the tissues or cell lines of triple-negative breast cancer than those of other breast cancer subtypes, and such CSC population contributes to the aggressiveness and poor prognosis of TNBC.[17–19] To assess the derivatives' activities on modulating CSC properties, we conducted the mammosphere formation assay[20,21] that evaluates the self-renewal capability of cancer cells. The result showed that while DMSO-treated and bexarotene-treated MCF-7 cells still retained capability to form mammospheres, cells treated with 2 μM DK-1–150 or 2 μM DK-1–166 failed to form detectable mammospheres (Fig. 3A and B). We have also evaluated the expression levels of CSC-related markers such as c-Myc and KLF4, Nanog, Oct4A and Sox-2. These stemness-associated transcription factors contribute to pluripotency and cannot be replaced by other tumor-promoting oncogenes.[22–24] Western blot analysis on MCF-7 cells indicated that 5 μM DK-1–150 or 5 μM DK-1–166 downregulated protein expressions of c-Myc and KLF4 by about 10–30%; downregulated protein expression of Nanog by about 20%. Particularly, 5 μM DK-1–150 caused about 60% reduction in Sox-2 expression, while 5 μM DK-1–166 induced approximately 50% decrease in Oct4A protein expression (Fig. 3C). Western blot analysis on MDA-MB-231 cells revealed that 5 μM DK-1–150 or 5 μM DK-1–166 downregulated protein expressions of KLF4 by about 50%, while no significant effect was observed in c-Myc expressions in TNBC cells (Fig. 3D). Suppression of KLF4 by DK-1–150 or DK-1–166 is significant as KLF4 has been known to be required to maintain CSC properties and breast cancer cell invasion and migration.[25] There were no substantial changes in both c-Myc and KLF4 expressions in bexarotene-treated TNBC cells. These results suggest that both bexarotene derivatives not only effectively suppress the self-renewal capacity of stem cell-like breast cancer cells, but also selectively downregulate stemness-associated transcription factors in TNBC cell lines.

EMT is a key developmental program that is often activated during cancer invasion and metastasis. It is also suggested that EMT is a key event by which cancer stem cells are generated.[26] As the bexarotene derivatives show CSC-inhibitory activities, we sought to examine if the derivatives can also affect EMT properties of TNBC cell lines. Both DK-1–150 and DK-1–166 at concentration of 5 μM were able to induce downregulation of mesenchymal markers, vimentin and slug in TNBC cell lines at a similar or slightly better effectiveness, as compared to 5 μM bexarotene. Moreover, the two TNBC cell lines respond differently to the two bexarotene derivatives, in terms of changes in the mesenchymal markers expression. The suppression of slug by the derivatives was observed in BT549, but not in MDA-MB-231. In addition, MDA-MB-231 responded well to both derivatives, but BT549

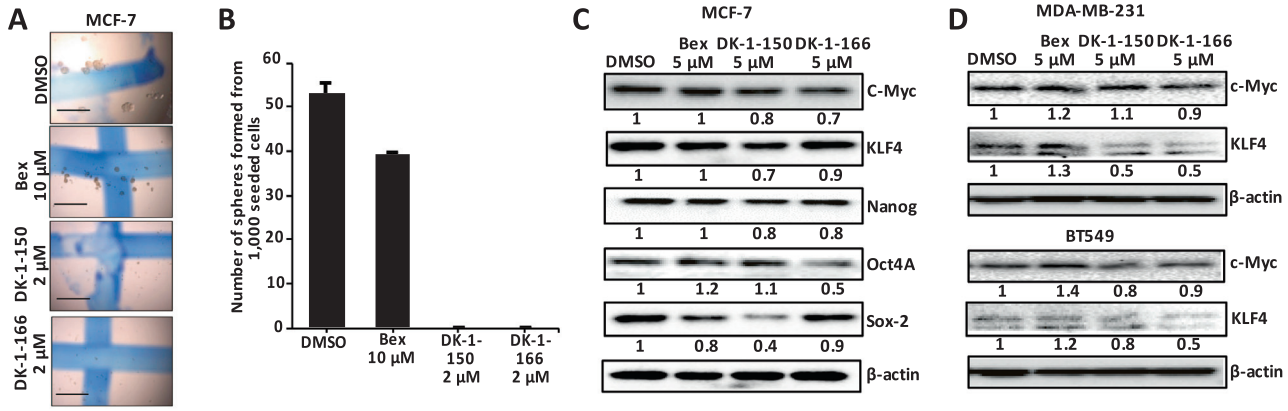

Fig. 3. Modulation of cancer stem cell (CSC) properties of breast cancer cell lines by bexarotene derivatives. A) Mammosphere-formation assay performed on MCF-7 cells. Scale bar indicates 200 μm. (B) Number of spheres larger than or equal to 60 μm was counted for quantitation. C, D) MCF-7 cells (C) and MDA-MB-231 cells (D) were subjected to Western blot analysis on post-treatment expressions of selected CSC markers. β-Actin was used as endogenous loading control. The basal level expressions of Nanog, Oct4A and Sox-2 in MDA-MB-231 cells were under the detection limit in Western blot analysis. Densitometry measurement with Image J software for quantification was performed, and the results were normalized to DMSO-treated samples. Numbers stated underneath indicate expression fold-change values.

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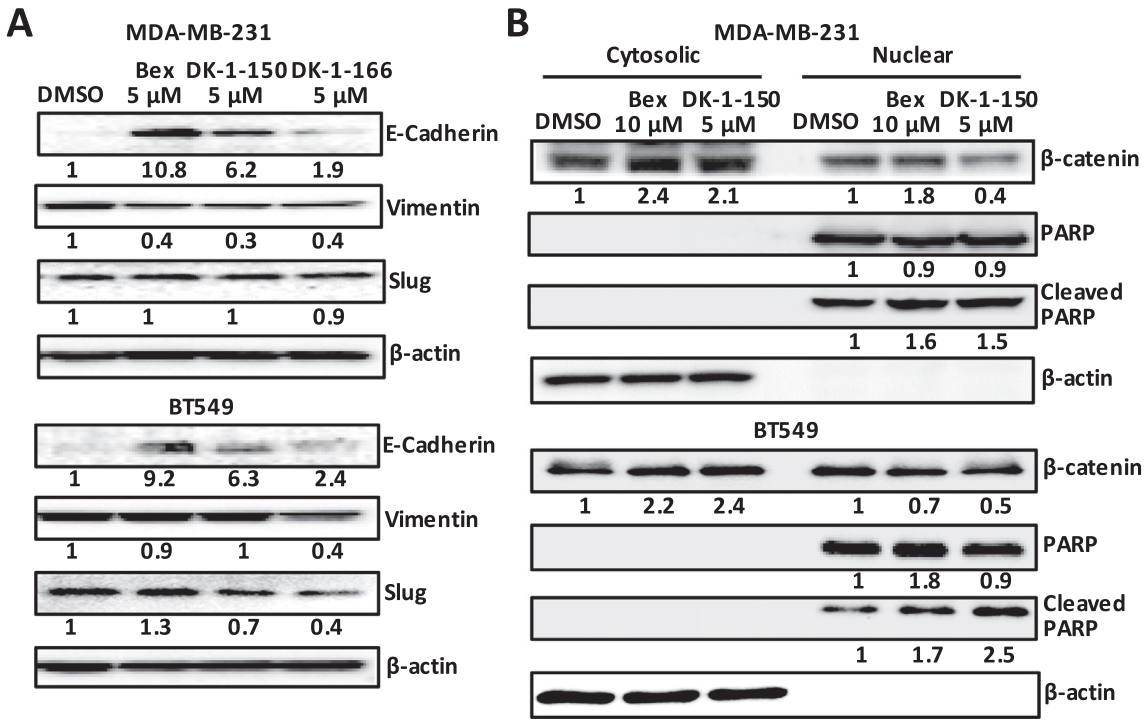

Fig. 4. Epithelial-mesenchymal transition (EMT) activities of TNBC cell lines are affected by bexarotene derivatives. A) TNBC cell lines were subjected to Western blot analysis on post-treatment expressions of selected epithelial and mesenchymal markers. B) Post-treatment TNBC cell lines were subjected to cytosolic and nuclear fractionation, followed by Western Blot analysis on translocation of β-catenin. PARP was used as fraction purity indicators for nuclear fraction. β-Actin was used as endogenous loading control. Densitometry measurement for quantification was performed, and the results were normalized to DMSO-treated samples. Numbers stated underneath indicate expression fold-change values from ImageJ analysis.

responded better to DK-1–166 than to DK-1–150 by almost 2 folds (Fig. 4A). Interestingly, both MDA-MB-231 and BT549 treated with DK-1–150 (5 μM) showed increased level of epithelial marker, *E*-Cadherin, while DK-1–166 (5 μM) displayed a slight increase in *E*-Cadherin expressions in the TNBC cell lines. These results indicate that the derivatives can induce the acquisition of epithelial properties and the concurrent loss of mesenchymal properties. Next, we examined the nuclear localization of β-catenin in the presence of the derivatives. Translocation of β-catenin into the nucleus facilitates the transcription of mesenchymal proteins, while suppressing the expressions of epithelial proteins.[27] We observed that in both MDA-MB-231 and BT549, 5 μM DK-1–150 is more effective than 10 μM bexarotene in restraining β-catenin in the cytosol and preventing its translocation into the nucleus (Fig. 4B). Collectively, our results suggest that the bexarotene derivatives are capable of limiting mesenchymal characteristics, while promoting epithelial feature in TNBC cell lines.

In conclusion, the results of our study provide strong evidence that the alkylamide derivatives of bexarotene effectively target TNBC by inducing apoptotic cell death, suppressing proliferation and migration, and inhibiting CSC and EMT properties of TNBC cells. The overall results proved that both DK-1–150 and DK-1–166 work more effectively than their parental molecule bexarotene as anti-breast cancer therapeutic agents against TNBC cells. We envision that detailed structure and activity relationship (SAR) studies would result in more potent derivatives. Identification of cellular target(s) of the derivatives are underway to elucidate their mechanism of action.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2017.12.033.

References

1. Dent R, Trudeau M, Pritchard KI, et al. Triple-negative breast cancer: clinical features and patterns of recurrence. *Clin Cancer Res*. 2007;13:4429.
2. Bauer KR, Brown M, Cress RD, Parise CA, Caggiano V. Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: a population-based study from the California cancer Registry. *Cancer*. 2007;109:1721.
3. Rakha EA, Elsheikh SE, Aleskandarany MA, et al. Triple-negative breast cancer: distinguishing between basal and nonbasal subtypes. *Clin Cancer Res*. 2009;15:2302.
4. Viale G, Rotmensz N, Maisonneuve P, et al. Invasive ductal carcinoma of the breast with the "triple-negative" phenotype: prognostic implications of EGFR immunoreactivity. *Breast Cancer Res Treat*. 2009;116:317.
5. Geyer FC, Lacroix-Triki M, Savage K, et al. B-Catenin pathway activation in breast cancer is associated with triple-negative phenotype but not with CTNNB1 mutation. *Mod Pathol*. 2011;24:209.
6. Lehmann BD, Bauer JA, Chen X, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest*. 2011;121:2750.
7. D'Agostino RB. Changing end points in breast-cancer drug approval–the Avastin story. *N Engl J Med*. 2011;365:e2.
8. von Minckwitz G, Martin M. Neoadjuvant treatments for triple-negative breast cancer (TNBC). *Ann Oncol*. 2012;23(Suppl. 6):vi35.
9. Hudis CA, Gianni L. Triple-negative breast cancer: an unmet medical need. *Oncologist*. 2011;16:1.
10. Liedtke C, Mazouni C, Hess KR, et al. Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. *J Clin Oncol*. 2008;26:1275.
11. Sørlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *PNAS*. 2001;98:10869.
12. Dent R, Hanna WM, Trudeau M, Rawlinson E, Sun P, Narod SA. Pattern of metastatic spread in triple-negative breast cancer. *Breast Cancer Res Treat*. 2009;115:423.

13. Sørlie T. Introducing molecular subtyping of breast cancer into the clinic? *J Clin Oncol*. 2009;27:1153.
14. Dick JE. Stem cell concepts renew cancer research. *Blood*. 2008;112:4793.
15. Clevers H. The cancer stem cell: premises, promises and challenges. *Nat Med*. 2011;17:313.
16. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature*. 2001;414:105.
17. Idowu MO, Kmieciak M, Dumur C, et al. CD44(+)/CD24(−/low) cancer stem/progenitor cells are more abundant in triple-negative invasive breast carcinoma phenotype and are associated with poor outcome. *Hum Pathol*. 2012;43:364.
18. Ma LH, Wang F, Shi H, et al. Enriched CD44+/CD24− population drives the aggressive phenotypes presented in triple-negative breast cancer (TNBC). *Cancer Lett*. 2014;353:153.
19. Liu TJ, Sun BC, Zhao XL, et al. CD133+ cells with cancer stem cell characteristics associates with vasculogenic mimicry in triple-negative breast cancer. *Oncogene*. 2013;32:544.
20. Manuel Iglesias J, Beloqui I, Garcia-Garcia F, et al. Mammosphere formation in breast carcinoma cell lines depends upon expression of E-cadherin. *PLoS One*. 2013;8:e77281.
21. Shaw FL, Harrison H, Spence K, et al. A detailed mammosphere assay protocol for the quantification of breast stem cell activity. *J Mammary Gland Biol Neoplasia*. 2012;17:111.
22. Hadjimichael C, Chanoumidou K, Papadopoulou N, Arampatzi P, Papamatheakis A, Kretsovali A. Common stemness regulators of embryonic and cancer stem cells. *World Journal of Stem Cells*. 2015;7:1150.
23. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006;126:663.
24. Theunissen TW, Jaenisch R. Molecular control of induced pluripotency. *Cell Stem Cell*. 2014;14:720.
25. Yu F, Li J, Chen H, et al. Kruppel-like factor 4 (KLF4) is required for maintenance of breast cancer stem cells and for cell migration and invasion. *Oncogene*. 2011;30:2161.
26. Kong D, Li Y, Wang Z, Sarkar FH. Cancer stem cells and epithelial-to-mesenchymal transition (EMT)-phenotypic cells: are they cousins or twins? *Cancers (Basel)*. 2011;3:716.
27. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer*. 2002;2:442.