Am J Cancer Res 2015;5(12):3624-3634
www.ajcr.us /ISSN:2156-6976/ajcr0019470

Original Article

Selective expression of constitutively active pro-apoptotic protein BikDD gene in primary mammary tumors inhibits tumor growth and reduces tumor initiating cells

Omar M Rahal[1], Lei Nie[1], Li-Chuan Chan[1,2*], Chia-Wei Li[1*], Yi-Hsin Hsu[1*], Jennifer Hsu[1,3,4], Dihua Yu[1,2], Mien-Chie Hung[1,2,3,4]

[1]Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas, 77030, USA; [2]Graduate School of Biomedical Sciences, The University of Texas Health Science Center at Houston, Houston, Texas, 77030, USA; [3]Center of Molecular Medicine and Graduate Institute of Cancer Biology, China Medical University and Hospital, Taichung, 404, Taiwan; [4]Department of Biotechnology, Asia University, Taichung, 413, Taiwan. [*]Equal contributors.

Received November 9, 2015; Accepted November 29, 2015; Epub November 15, 2015; Published December 1, 2015

Abstract: Our previous study showed that specifically delivering BikDD, a constitutive active mutant of pro-apoptotic protein Bik, to breast cancer cell xenografts in immunocompromised mice has a potent activity against tumor initiating cells (TICs), and that the combination between tyrosine kinase inhibitors (TKI) and BikDD gene therapy yielded synergistic effect on EGFR and HER2 positive breast cancer cells in immunodeficient nude mice. Those encouraging results have allowed us to propose a clinical trial using the liposome-complexing plasmid DNA expressing BikDD gene which has been approved by the NIH RAC Advisory committee. However, it is imperative to test whether systemic delivery of BikDD-expressing plasmid DNAs with liposomes into immunocompetent mice has therapeutic efficacy and tolerable side effects as what we observed in the nude mice model. In this study, we investigated the effects of BikDD gene-therapy on the primary mammary tumors, especially on tumor initiating cells (TICs), of a genetically engineered immunocompetent mouse harboring normal microenvironment and immune response. The effects on TIC population in tumors were determined by FACS analysis with different sets of murine specific TIC markers, $CD49f^{high}CD61^{high}$ and $CD24^{+}Jagged1^{-}$. First we showed *in vitro* that ectopic expression of BikDD in murine N202 cells derived from MMTV-HER2/Neu transgenic mouse tumors induced apoptosis and decreased the number of TICs. Consistently, systemic delivery of VISA-Claudin4-BikDD by liposome complexes significantly inhibited mammary tumor growth and slowed down residual tumor growth post cessation of therapy in MMTV-HER2/Neu transgenic mice compared to the controls. In addition, the anti-tumor effects of BikDD in vivo were consistent with decreased TIC population assessed by FACS analysis and in vitro tumorsphere formation assay of freshly isolated tumor cells. Importantly, systemic administration of BikDD did not cause significant cytotoxic response in standard toxicity assays or body weight changes. Taken together, our findings validated that selective expression of BikDD in the primary mammary tumors in immunocompetent hosts significantly reduced tumor burden and inhibited the residual tumor growth at off-therapy stage by eliminating TICs. Hence, the VISA-Claudin4-BikDD-mediated gene therapy is worthy of further investigation in breast cancer clinical trials.

Keywords: Proapoptotic protein, Bik, BikDD, gene therapy, HER2, primary mammary tumors, tumor initiating cells (TICs), immunocompetent mice

Introduction

Breast cancer is one of the most common cancers among women worldwide, especially in the developed countries. It is well known that human epidermal growth factor receptor 2 (HER2) plays an important role in development and progression of certain types of breast cancers [1]. The HER2-enriched subtype of breast cancer, characterized by overexpression of HER2 receptor or *HER2* gene amplification, comprises approximately 15-25% of breast cancers [1, 2], and more recently, somatic mutations in *HER2* in the absence of overexpression or gene ampli-

fication account for 1-2% of breast cancer cases [3]. Although single or combinatorial anti-HER2 targeted therapies such as monoclonal antibodies (e.g., trastuzumab) or tyrosine kinase inhibitors (e.g., lapatinib) have improved the poor-prognosis associated with this subtype of breast cancer patients, intrinsic or acquired resistance still limits the efficacy to such treatments [4, 5]. Treatment resistance, recurrence, and metastasis in breast cancer has been attributed to a small population of cancer cells termed cancer stem cells or tumor initiating cells (TICs) that are able to self-renew and generate the heterogeneous cell types that make the tumor burden [6-8]. Several studies support a role for HER2 as a regulator of TICs in human breast cancer cell lines [9-12] and MMTV-HER2/Neu mouse mammary tumors [13, 14].

In an earlier work, we demonstrated that BikDD, a double phosphorylation mimic and constitutively active form of the pro-apoptotic protein Bik, synergizes with lapatinib to target breast TICs by inhibition of multiple members of the anti-apoptotic Bcl-2 family [15]. Integration of BikDD into an expression vector complex termed VISA-Claudin4 that selectively and efficiently induces BikDD expression in breast cancer cells, with minimal expression in normal cells, showed that VISA-Claudin4-BikDD specifically enhanced the killing effect of lapatinib on $EGFR^+/HER2^+$ breast cancer cell lines and prevented chemotherapy-induced increase of TICs *in vitro* and *in vivo* [15].

To further study the effects of BikDD gene therapy on the primary mammary tumors and on TICs of breast cancers in immunocompetent hosts, we used well-established MMTV-HER2/Neu transgenic mice, which develop spontaneous mammary tumors with similar features as the human disease [16, 17], as a model to determine whether BikDD effectively inhibits tumor growth and/or eliminates TICs in the tumor burden. In this study, we showed that in N202 cells, a murine mammary carcinoma cell line derived from MMTV-HER2 transgenic mice, ectopic expression of BikDD induced apoptosis and also decreased total cell viability and TIC number *in vitro*. Furthermore, we demonstrated that systemic delivery of VISA-Claudin4-BikDD-liposome complex and specific expression of BikDD proteins in the primary mammary tumor cells suppressed tumor growth and decelerated residual tumor expansion at off-therapy stage concomitantly with decrease of TICs in the genetically engineered mice. Our study provided positive preclinical evidence in mice with normal microenvironment and immunity response to safely move VISA-Claudin4-BikDD gene therapy into clinical trials for breast cancer patients.

Materials and methods

Cell lines and in vitro treatment

The N202 mammary carcinoma cell line was derived from an MMTV-HER-2/Neu transgenic mouse. N202 cells were grown in DMEM supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin. Cell viability was measured by trypan blue exclusion method in N202 cells infected either with pLOVE-vector or pLOVE-BikDD lentiviral supernatants after 96 hours. Viral titer was quantified using a qPCR Lentivirus Titration Titer Kit following the manufacturer's instructions (Abm Inc., Richmond, BC, Canada). For all *in vitro* experiments the cells were analyzed 96 hours post infection.

Animal studies

Female MMTV-HER2/Neu transgenic mice (FVB/N, Tg-MMTV-Neu 202Mul/J; Jackson Laboratory) were used to test the effect of VISA-Claudin4-BikDD gene therapy on mammary tumor growth and the TIC populations. Once mammary tumor volume reached ~100-150 mm^3, mice (8-10 mice per group) were randomly assigned to be administered either pUK21 vector alone (Control group) or pUK21-VISA-Claudin4-BikDD (BikDD group) DNA complexed with liposomes (15 μg DNA/mouse) via tail vein injection using a 29-gauge needle twice per week for total of 3 weeks. DNA:DOTAP liposome complex preparation was made as described previously [15]. HLDC (Hung Lab-modified DOTAP:cholesterol) was produced in our laboratory according to Dr. Nancy Templeton's protocol [18]. Briefly, HLDC (20 mmol/L) stock solution and stock DNA solution were diluted in sterile 5% dextrose water and mixed in equal volumes to make a final solution of 4 mmol/L HLDC and 15 μg DNA in 100 μl of solution. Plasmid DNAs for animal injection were purified by Qiagen Endo-Free Mega Prep

kit (Qiagen, Valencia, CA) with endotoxin level < 10 units/mg (QCL-1000 kit; BioWhittacker, Walkersville, MD). Tumor burden was measured twice a week with a caliper, and tumor volume was calculated by the formula: 0.5 × length × width^2. Mouse survival was monitored after cessation of VISA-Claudin4-BikDD treatment until tumors reached 1500 mm^3 or when mice showed signs of distress.

Preparation of single mammary tumor cell

All animal protocols were reviewed and approved by IACUC committee at the University of Texas MD Anderson Cancer Center. Isolation of single tumor cells from mammary tumors of MMTV-HER2/Neu transgenic mice for FACS analysis and tumorsphere formation assay was performed as described previously with slight modifications [19]. Briefly, the tumor tissues were harvested 24 hours after last injection. The mammary tumors were minced using razor blades and digested in 10 mL digestion media/1 g tissue [digestion media containing DMEM/F12, 1% antibiotic-antimycotic (Invitrogen), and freshly added 100 U/mL collagenase/hyaluronidase (Stemcell Technologies)] at 37°C for 1 hour in a rotary shaker at 200 rpm (pipetted up and down every 15 min). The resultant cells were filtered through 40 μm cell strainers and washed with washing buffer [DMEM/F12 medium, 5% fetal bovine serum (FBS), 1% antibiotic-antimycotic (Invitrogen)]. The dispersed cell suspensions were centrifuged at 800 rpm for 3 min until the supernatant was clear and no red blood cells were visible in the pellet. The single cells were then resuspended in HBSS (Invitrogen) containing 2% FBS and 10 mmol/L HEPES buffer till further analysis.

Flow cytometry of cancer stem cells and normal mammary stem cells

Effect of BikDD on TICs *in vivo* was determined by FACS analysis of freshly isolated tumor cells using previously reported MMTV-HER2/Neu-specific murine TIC markers CD49f^{high}CD61^{high} [13] and CD24$^+$Jagged1$^-$ [14]. We also analyzed TICs by using normal mammary stem cell markers CD24 and CD29 [20]. Briefly, freshly isolated single tumor cells were resuspended in HBSS-5% FBS buffer (1×10^7 cells/ml) and stained with specific antibodies of biotin-conjugated lineage surface markers and FITC- and PE-conjugated TIC surface markers for 30 min on ice. Cells were then washed with HBSS-5% FBS buffer and incubated with streptavidin-APC for 20 min on ice. The cells were pelleted and washed once with HBSS buffer and then subjected to FACS on a Beck man Coulter Gallios Flow Cytometer. Dead and mouse-lineage-positive cells were excluded using 4',6-diamidino-2-phenylindole (DAPI; 3 μM; Molecular Probes) and mouse lineage panel (BD Biosciences), respectively. Results shown for isolated tumor cells are from seven (using TIC markers) and five (using normal stem cell markers) independent experiments, with each experiment representing a different mouse.

Tumorsphere formation assays

Freshly isolated single tumor cells from MMTV-HER2/Neu transgenic mice after last treatment were used for tumorsphere assays. Tumorsphere culture was performed as previously described [21]. In brief, 50,000 cells per well were grown in 24-well Ultra Low Attachment plates (Corning, NY) with 0.5 mL serum-free mammosphere medium (DMEM/F12 with 20 ng/mL basic fibroblast growth factor, 20 ng/mL epidermal growth factor, 10 μg/mL heparin, 1X B27, 100 μg/ml gentamicin, 1% antibiotic-antimycotic). Cells were grown in non-adherent culture dishes and allowed to form tumorspheres. Tumorsphere-forming units (TFU: ≥ 60 μM) were manually counted at day seven. Results shown were from four independent experiments, with each experiment representing cells isolated from a different mouse and performed in six replicates.

Systemic toxicity analysis

After reaching tumor end-point, serum was collected from either control or BikDD-treated mice for analysis of aspartate aminotransferase/serum glutamic-oxaloacetic transaminase (AST/SGOT), alanine aminotransferase/serum glutamic pyruvic transaminase (ALT/SGPT), and blood urea nitrogen (BUN) using assay kits (Roche) following manufacturer's recommendations. The body weight of both mouse groups was recorded twice a week.

Western blot analysis

Western blotting was performed as described [22] with the following antibodies against Bik (Cat #4592S), PARP (#9542S) from Cell Sig-

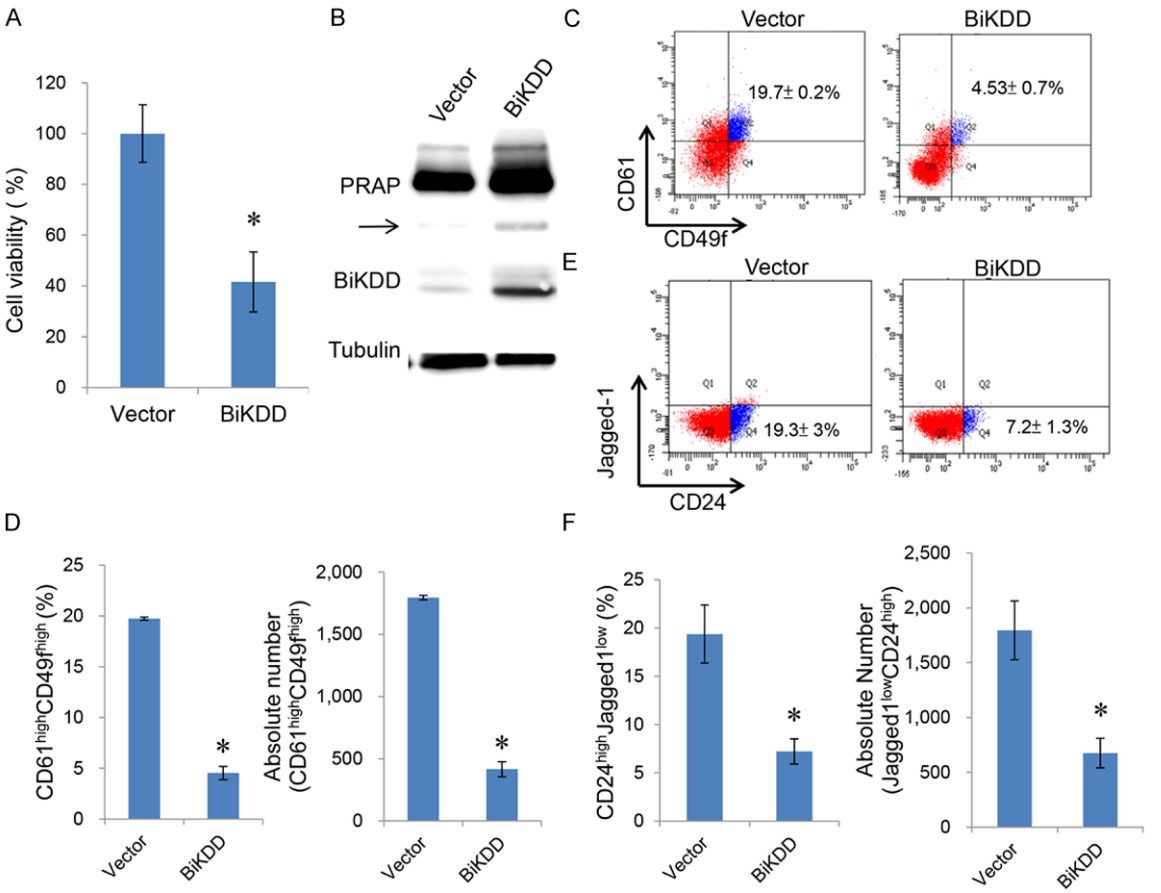

Figure 1. Ectopic expression of BikDD inhibits tumor cell growth and reduces tumor initiating cell (TIL) population in vitro. A. Cell viability of BikDD-expressing and vector control N202 cells was determined 96 hours post infection with either lentivirus of pLOVE or pLOVE-BikDD. B. Western blot analysis of PARP cleavage and BikDD expression in vector control and BikDD-expressing cell lysates was performed with indicated specific antibodies, tubulin is used as cell lysate loading control and arrow indicates PARP cleavage form. C. FACS analysis with TIC markers CD61-FITC and CD49f-PE in vector control and BikDD-expressing N202 cells. D. Quantification of $CD49f^{high}CD61^{high}$ TIC population in percentage and absolute numbers of total live cells. E. FACS analysis with Jagged-1-FITC and CD24-PE in N202 cells. F. Quantification of $CD24^{+}Jagged1^{-}$ TIC population in percentage and absolute numbers of total live cells. Results are mean ± SEM; and experiments were performed in triplicate. *$P < 0.05$.

naling Technology, and α-tubulin (T5168) from Sigma-Aldrich.

Results

Ectopic expression of BikDD inhibits tumor cell growth and reduces TIC population in vitro

Given that breast TICs are resistant to current standard of care therapies and that HER2 is a positive regulator of human breast TICs, we used the murine mammary carcinoma cell line N202 to investigate the effect of BikDD on apoptosis and TIC population. Ectopic expression of BikDD in N202 decreased cell viability by 60% relative to empty vector (**Figure 1A**) and induced apoptosis as measured by PARP cleavage (**Figure 1B**). To test whether ectopic expression of BikDD in tumor cells affects TICs, N202 murine cells were infected with BikDD-lentivirus or empty vector. We then determined TIC populations in the infected cells by FACS analysis using two sets of surface markers $CD49f^{high}CD61^{high}$ and $CD24^{+}Jagged1^{-}$ which were reported to identify TICs in the MMTV/HER2/Neu mouse model [13, 14]. Compared with control N202 cells, the TIC populations as indicated by $CD49f^{high}CD61^{high}$ and $CD24^{+}Jagged1^{-}$ in the BikDD-expressing cells were decreased by 77% (from 19.7% to 4.5% of total cells; **Figure 1C** and **1D**) and 63% (from 19.3% to 7.2% of total cells; **Figure 1E** and **1F**), respectively. These results suggested that BikDD expression in the mammary tumor cells not

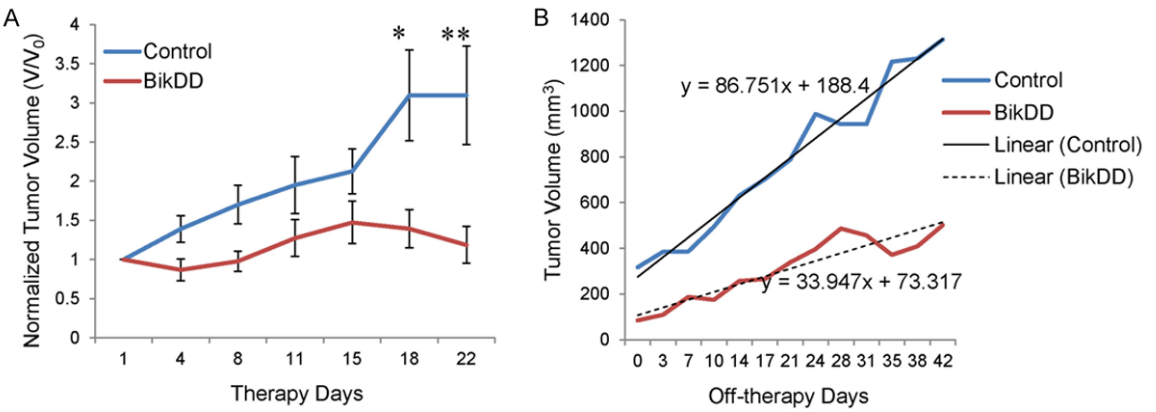

Figure 2. Inhibitory effect of systemic delivery of VISA-Claudin4-BikDD on primary tumor growth in MMTV-HER2/Neu mice. A. MMTV-HER2/Neu mice harboring tumors (100-150 mm^3) were randomly assigned to receive either pUK21 control vector or pUK21-VISA-Claudin4-BikDD DNA/liposome complexes via tail vein injection, twice a week for 3 weeks. Tumor volume (V) was measured every 3-4 days and normalized to the tumor volume prior to therapy (V_0). Error bars, SEM. *P = 0.00936; **P = 0.00672 (n = 18 mice per group). B. Slope of tumor growth of the residual tumors was monitored for 6 weeks after cessation of treatment (off-therapy) and calculated with the equation Y = mx+B (where m is slope).

only induces tumor cell apoptosis but also reduces TICs *in vitro*.

Inhibitory effect of systemic delivery of VISA-Claudin4-BikDD on primary mammary tumor growth in MMTV-HER2/Neu transgenic mice

Transgenic expression of HER2 driven by MMTV promoter in the mammary glands results in spontaneous mammary tumors in more than 50% of mice by ~7 months of age. Since these mammary tumors resemble human HER2-positive breast tumors, MMTV-HER2/Neu transgenic mice represent an excellent model to study novel, clinically relevant anti-cancer therapies that can be translated into breast cancer patients. To investigate the anti-tumor effect of VISA-Claudin4-BikDD in HER2-driven breast cancer, MMTV-HER2/Neu mice harboring tumors (100-150 mm^3) were randomly administered either pUK21 vector (Control group) or pUK21-VISA-Claudin4-BikDD (BikDD group) DNA complexed with liposome via tail vein injection twice a week for a total of 4 weeks, with tumor volume and recurrence assessed during and after treatment cessation. As shown in **Figure 2A**, systemic delivery of VISA-Claudin4-BikDD significantly inhibited tumor growth during the 3-week treatment period in MMTV-HER2/Neu mice compared to the control group with a 62% inhibition at the end of three weeks (P < 0.005). Despite mammary tumors grew back in BikDD-treated mice upon treatment termination, the slope of residual tumor growth in BikDD-treated mice was much lower than those in control-treated mice (**Figure 2B**).

Systemic delivery of VISA-Claudin4-BikDD reduces TICs of primary mammary tumors in vivo

Previously, Korkaya *et al*. provided evidence that HER2 drives self-renewal of human breast cancer stem cells [9]. More recently, Lo *et al.* [13] and Liu *et al.* [14] independently characterized the phenotype of mammary TICs in tumors from MMTV-HER2/Neu transgenic mice by using TIC markers $CD49f^{high}CD61^{high}$ or $CD24^{+}Jagged1^{-}$, respectively. Given that breast TICs are often chemo- and radiation-resistant, it is imperative that ideal therapies not only substantially reduce the tumor burden but also eliminate the small population of TICs. To determine whether inhibitory effects of systemic BikDD therapy on mammary tumor growth *in vivo* (**Figure 2**) are associated with reduction of TICs, the percentage of TICs in freshly isolated tumor cells from mammary tumors of MMTV-HER2/Neu transgenic mice at the end of the 3-week treatment period was quantified by FACS based on the well-established TIC markers [13, 14] within the lineage-negative (Lin^{-}) population ($CD45^{-}$, $TER119^{-}$, $CD31^{-}$). The results indicated that systemic delivery of VISA-Claudin4-BikDD decreased the $CD49f^{high}$CD-

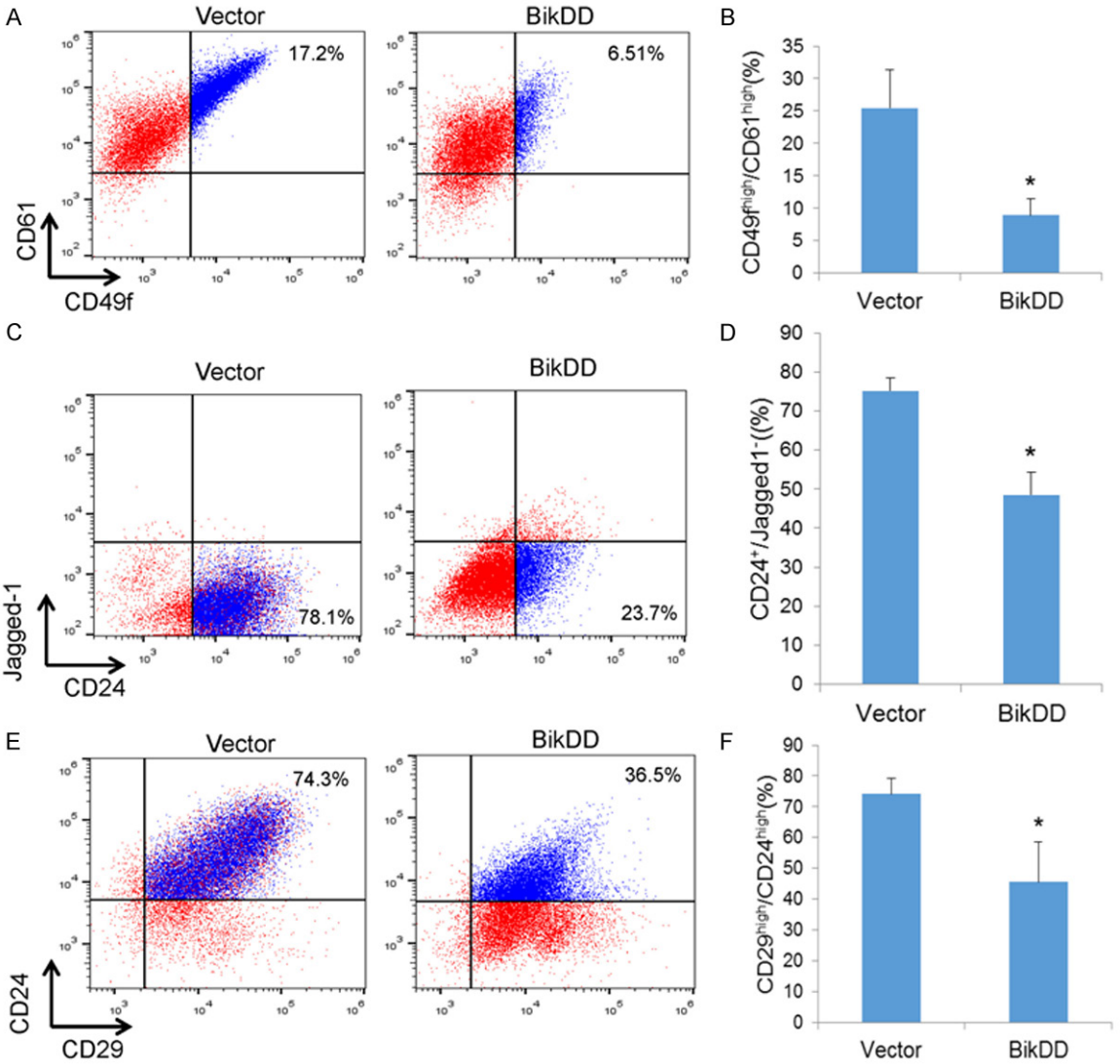

Figure 3. Systemic delivery of VISA-Claudin4-BikDD reduces TICs of mammary tumors *in vivo*. (A) Representative FACS plot of freshly isolated mammary tumor cells from vector (left)- or BikDD (right)-treated MMTV-HER2/Neu mice. The tumors were harvested 24 hours after last injection. CD61 and CD49f staining of the tumor cells was plotted by gating the lineage negative population ($CD45^-$, $TER119^-$, and $CD31^-$) after exclusion of dead cells ($DAPI^+$); (B) Quantification of $CD49f^{high}CD61^{high}$ TIC population. Data shown are from 7 independent mice per group. $^*P = 0.009$. (C) Representative FACS plot of Jagged1 and CD24 TIC markers in freshly isolated mammary tumor cells from MMTV-HER2/Neu mice treated as in (A). (D) Quantification of $CD24^+Jagged1^-$ TIC population. Data shown are from 7 independent mice per group. $^*P = 0.0058$. (E) FACS analysis of freshly isolated tumor cells treated as in (A) was conducted with normal mammary stem cell markers CD29-APC/Cy7 and CD24-FITC. (F) The basal MaSC-enriched ($CD29^{hi}CD24^+$) population in mammary tumor cells is decreased with systemic treatment with BikDD. Data shown are from 4 independent mice per group. $^*P = 0.048$ relative to pUK21 empty vector.

61^{high} TIC population by 65% (**Figure 3A** and **3B**) and the $CD24^+Jagged1^-$ TIC population by 35% (**Figure 3C** and **3D**) as compared to the control group. These findings suggested that BikDD-mediated *in vivo* tumor growth inhibition and tumor regression post off-therapy stage are correlated, at least in part, by the decrease in TICs. TICs in the mammary tissue can be originated from the dysregulated normal mammary stem cells (MaSCs) during an oncogenic environment such as MMTV promoter-driven HER2 overexpression. Therefore, we also analyzed the tumor cells with additional stem cell markers, CD29 and CD24, which are used to characterize MaSCs [20] by FACS on freshly isolated tumor cells. Consistent with the staining pat-

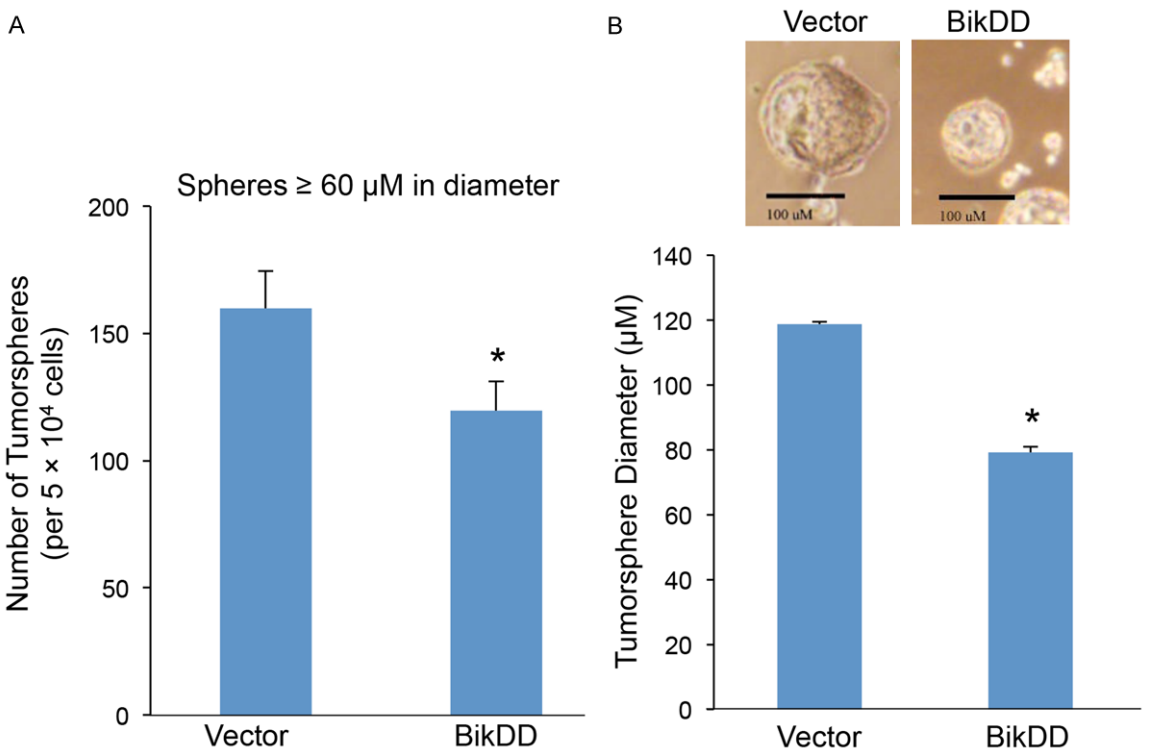

Figure 4. *In vivo* systemic delivery of BikDD reduces tumorsphere formation potential of tumor cells isolated from MMTV/HER2/Neu mice. A. The number of tumorsphere developed from tumor cells isolated from BikDD and vector-treated tumors. Data represent the mean values of the tumorsphere number ± SEM of four independent experiments; *P = 0.036. B. Upper panel, representative photo of different sizes of the tumorspheres derived from BikDD and vector-treated tumor cells; Scale bar: 100 μm; Bottom panel, size difference of the tumorspheres in diameter between BikDD and vector-treated groups, and the bar graph represents the diameter mean values of the tumorsphere ± SEM of at least three independent experiments. *P = 0.001569.

terns of two sets of TIC markers mentioned above, we found that BikDD gene therapy also decreased the percentage of MaSC-enriched $CD29^{hi}CD24^{+}$ population by 39% (from 74.1% to 45.5% of total cells, **Figure 3E** and **3F**). Taken together, these results revealed that administration of VISA-Claudin4-BikDD-liposomes substantially reduced TICs as characterized by different surface markers in the immunocompetent transgenic mouse model. To further evaluate the effects of VISA-Claudin4-BikDD on TIC population in mammary tumor cells isolated from MMTV-HER2/Neu mice after 3-week systemic treatment, we also performed tumorsphere formation assay for TICs based on the ability of a small population of stem/progenitor cells to grow in suspension as spheres [23]. As shown in **Figure 4A**, the number of tumorspheres from freshly isolated tumor cells from BikDD-treated tumors were significantly reduced compared with that from vector-treated ones (BikDD vs Control: 120 ± 11.67 vs 160 ± 14.82). In addition to the reduction in tumorsphere numbers, the size of the tumorspheres formed (μM in diameter) was significantly smaller in the BikDD treated group than vector controls (79.11 ± 1.81 vs 118.76 ± 0.81, P = 0.00152; **Figure 4B**). These findings were highly consistent with the results from FACS analysis of freshly isolated single tumor cells, indicating that the BikDD treatment effectively reduced TIC numbers and attenuated their tumorsphere formation ability.

Evaluation of cytotoxic potential of VISA-Claudin4-BikDD-mediated gene therapy in vivo

In order to evaluate toxicity of BikDD-mediated gene therapy, all tumor-bearing immunocompetent mice were carefully monitored throughout systemic administration of either control vector- or VISA-Claudin4-BikDD-liposomes for any signs of distress or pain. Meanwhile, we also monitored the mice body weight twice a week at the start of treatment. No major signs of distress or pain and acute adverse immune re-

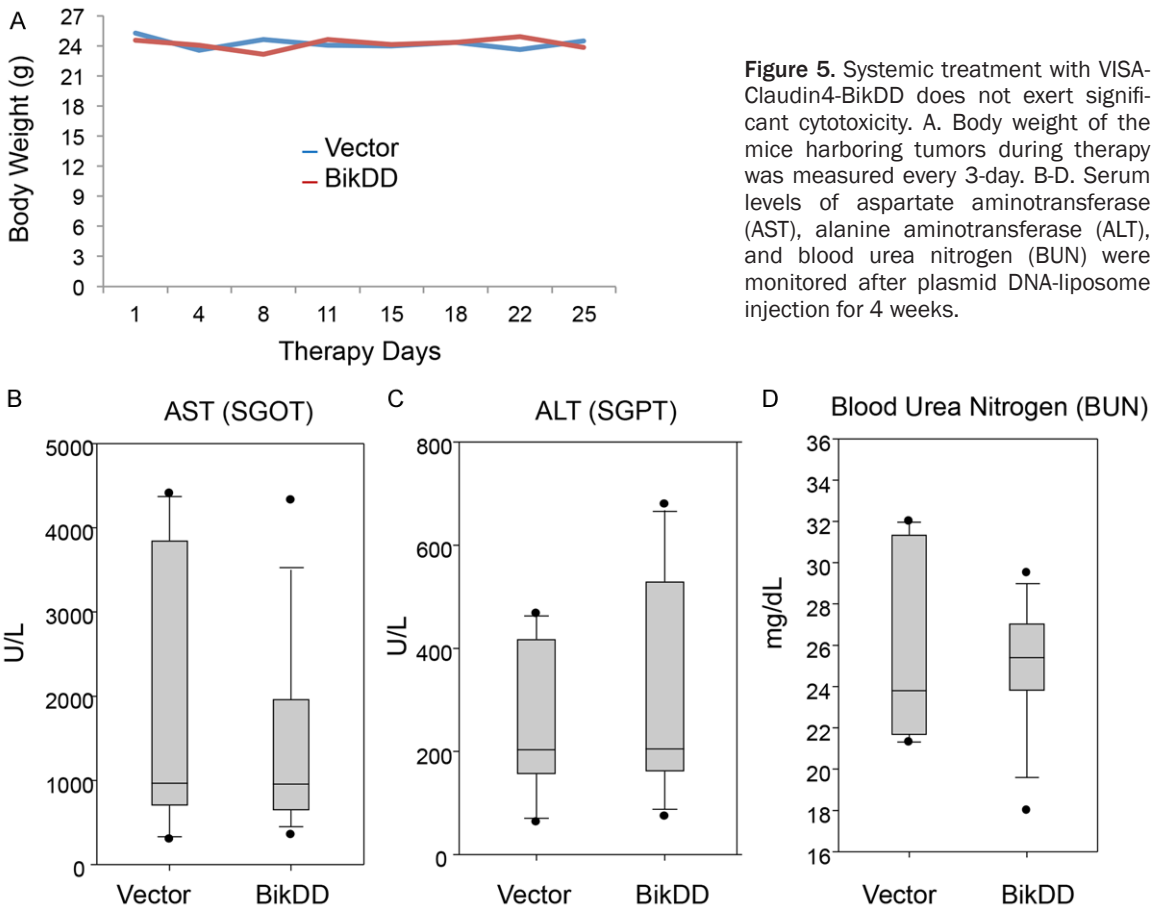

Figure 5. Systemic treatment with VISA-Claudin4-BikDD does not exert significant cytotoxicity. A. Body weight of the mice harboring tumors during therapy was measured every 3-day. B-D. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and blood urea nitrogen (BUN) were monitored after plasmid DNA-liposome injection for 4 weeks.

sponses were observed in mice administered the gene therapy. In addition, no significant changes in body weight were observed in mice after receiving systemic administration of VISA-Claudin4-BikDD (**Figure 5A**). We also collected blood samples at the endpoint of the study from mice from both groups to determine the serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and blood urea nitrogen (BUN) (**Figure 5B-D**). The serum levels of AST, ALT, and BUN did not significantly differ between VISA-Claudin4-BikDD- and vector-treated groups, suggesting that under our experimental conditions, systemic delivery of VISA-Claudin4-BikDD did not have major organ damages, and that VISA-Claudin4-BikDD gene therapy has a relatively safe treatment profile.

Discussion

Like other subtypes of breast cancers, despite improved prognosis with the advent of HER2-targeted agents in patients with HER2-amplified or -overexpressing breast cancer, a significant portion of these patients will relapse and die of breast cancer in part due to intrinsic or acquired resistance to current therapies [4, 5]. The poor clinical outcomes suggest a need for alternative, novel, and efficacious treatment options for chemo- and targeted-therapy-resistant breast cancer patients. Our previous work provided solid evidence demonstrating that *in vitro* and in xenograft mouse models expression of BikDD has a potent anti-tumor activity against $EGFR^+/HER2^+$ human breast cancer cells with minimal toxicity to normal cells. More importantly, BikDD synergized with lapatinib and paclitaxel treatment [15]. In order to safely move BikDD-mediated gene therapy into clinical trial of human breast cancer patients, we further examined the therapeutic efficacy and toxicity of this novel therapy in immunocompetent mouse model MMTV-HER2/Neu transgenic mice. The MMTV-HER2/Neu transgenic mice developed spontaneous mammary tumors exhibiting salient features of human HER2-positive breast cancer [16, 17], and those mammary tumors have been shown to originate from TICs [13, 14]. Using the immunocompe-

tent and oncogene HER2-driven transgenic mammary tumor mouse model, we demonstrated that systemic delivery of VISA-Claudin4-BikDD significantly inhibited primary mammary tumor growth and delayed recurrence in MMTV-HER2/Neu transgenic mice with a concomitant decrease in TIC numbers and tumorsphere-forming efficiency in freshly isolated mammary tumor cells (**Figures 3** and **4**). Although the residual tumors in both groups grew back at off-therapy stage, the slope of tumor growth was significantly lower in BikDD-treated group compared to empty vector-treated cohorts, supporting the notion that TIC cells are sensitive to BikDD gene therapy (**Figure 2B**). Mechanistically, our findings demonstrated that the anti-tumor effects of BikDD gene therapy on tumor cells and primary tumors of MMTV-HER2/Neu transgenic mice are mediated by inducing apoptosis and, at least in part, by eliminating TICs.

The relative resistance of TICs to current chemo- and radiation therapies explains their role in tumor recurrence and metastasis [24, 25]. Previous findings revealed HER2 functions as a regulator of TICs in human and mouse breast cancer cells, and HER2 is amplified or overexpressed in approximately 15-20% of human breast cancers and associated with poor clinical outcome [1-5, 9-12]. Furthermore, limited clinical efficacy of anti-HER2 drugs can be due to an inoperative apoptotic machinery or induction of anti-apoptotic proteins of Bcl-2 family [26, 27]. In clinic, administration of HER2-targeted agents such as trastuzumab and lapatinib in both adjuvant and advanced settings has provided significant benefits; however, the majority of tumors will relapse and lead to incurable metastatic disease. Our studies confirmed that VISA-Claudin4-BikDD gene therapy triggers apoptosis of breast cancer cells including TICs in immunocompetent mice. Of note, systemic delivery of VISA-Claudin4-BikDD gene therapy in our current study did not lead to major signs of distress, body weight changes, or liver and kidney toxicity (**Figure 5**). These results complement our previous work on BikDD gene therapy for breast cancer cells transplanted in immunocompromised mice, providing more preclinical evidence to support that BikDD-mediated gene therapy is a novel, effective, safe anti-tumor therapy with tolerable systemic toxicity.

Acknowledgements

The authors would like to thank Chun-Te Chen, Zhenbo Han, and Jing-Fong Lee their assistance. The study was supported in part by the following grants: Cancer Prevention and Research Institute of Texas (ETRA RP120727); National Institutes of Health grants (CCSG CA16672); National Breast Cancer Foundation; Breast Cancer Research Foundation; Patel Memorial Breast Cancer Endowment Fund; The University of Texas MD Anderson-China Medical University and Hospital Sister Institution Fund; Ministry of Health and Welfare, China Medical University Hospital Cancer Research Center of Excellence (MOHW104-TDU-B-212-124-002, Taiwan); International Research-Intensive Centers of Excellence in Taiwan (MOST 104-2911-I-002-302, Taiwan); and Center for Biological Pathways.

Disclosure of conflict of interest

Mien-Chie Hung has ownership interest (including patents for BikDD). All other authors have no conflicts of interest to declare.

Address correspondence to: Dr. Mien-Chie Hung, Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 108, Houston, TX 77030, USA. Tel: 713-792-3668; Fax: 713-794-3270; E-mail: mhung@mdanderson.org

References

[1] Zhu X and Verma S. Targeted therapy in her2-positive metastatic breast cancer: a review of the literature. Curr Oncol 2015; 22: S19-28.

[2] Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. Nature 2012; 490: 61-70.

[3] Bose R, Kavuri SM, Searleman AC, Shen W, Shen D, Koboldt DC, Monsey J, Goel N, Aronson AB, Li S, Ma CX, Ding L, Mardis ER and Ellis MJ. Activating HER2 mutations in HER2 gene amplification negative breast cancer. Cancer Discov 2013; 3: 224-237.

[4] Gradishar WJ. Emerging approaches for treating HER2-positive metastatic breast cancer beyond trastuzumab. Ann Oncol 2013; 24: 2492-2500.

[5] Kumler I, Tuxen MK and Nielsen DL. A systematic review of dual targeting in HER2-positive breast cancer. Cancer Treat Rev 2014; 40: 259-270.

[6] Luo M, Brooks M and Wicha MS. Epithelial-mesenchymal plasticity of breast cancer stem

cells: implications for metastasis and therapeutic resistance. Curr Pharm Des 2015; 21: 1301-1310.
[7] Korkaya H and Wicha MS. HER2 and breast cancer stem cells: more than meets the eye. Cancer Res 2013; 73: 3489-3493.
[8] Visvader JE and Lindeman GJ. Cancer stem cells: current status and evolving complexities. Cell Stem Cell 2012; 10: 717-728.
[9] Korkaya H, Paulson A, Iovino F and Wicha MS. HER2 regulates the mammary stem/progenitor cell population driving tumorigenesis and invasion. Oncogene 2008; 27: 6120-6130.
[10] Duru N, Fan M, Candas D, Menaa C, Liu HC, Nantajit D, Wen Y, Xiao K, Eldridge A, Chromy BA, Li S, Spitz DR, Lam KS, Wicha MS and Li JJ. HER2-associated radioresistance of breast cancer stem cells isolated from HER2-negative breast cancer cells. Clin Cancer Res 2012; 18: 6634-6647.
[11] Korkaya H, Kim GI, Davis A, Malik F, Henry NL, Ithimakin S, Quraishi AA, Tawakkol N, D'Angelo R, Paulson AK, Chung S, Luther T, Paholak HJ, Liu S, Hassan KA, Zen Q, Clouthier SG and Wicha MS. Activation of an IL6 inflammatory loop mediates trastuzumab resistance in HER2+ breast cancer by expanding the cancer stem cell population. Mol Cell 2012; 47: 570-584.
[12] Ithimakin S, Day KC, Malik F, Zen Q, Dawsey SJ, Bersano-Begey TF, Quraishi AA, Ignatoski KW, Daignault S, Davis A, Hall CL, Palanisamy N, Heath AN, Tawakkol N, Luther TK, Clouthier SG, Chadwick WA, Day ML, Kleer CG, Thomas DG, Hayes DF, Korkaya H and Wicha MS. HER2 drives luminal breast cancer stem cells in the absence of HER2 amplification: implications for efficacy of adjuvant trastuzumab. Cancer Res 2013; 73: 1635-1646.
[13] Lo PK, Kanojia D, Liu X, Singh UP, Berger FG, Wang Q and Chen H. CD49f and CD61 identify Her2/neu-induced mammary tumor-initiating cells that are potentially derived from luminal progenitors and maintained by the integrin-TGFbeta signaling. Oncogene 2012; 31: 2614-2626.
[14] Liu JC, Voisin V, Bader GD, Deng T, Pusztai L, Symmans WF, Esteva FJ, Egan SE and Zacksenhaus E. Seventeen-gene signature from enriched Her2/Neu mammary tumor-initiating cells predicts clinical outcome for human HER2+:ERalpha- breast cancer. Proc Natl Acad Sci U S A 2012; 109: 5832-5837.
[15] Lang JY, Hsu JL, Meric-Bernstam F, Chang CJ, Wang Q, Bao Y, Yamaguchi H, Xie X, Woodward WA, Yu D, Hortobagyi GN and Hung MC. BikDD eliminates breast cancer initiating cells and synergizes with lapatinib for breast cancer treatment. Cancer Cell 2011; 20: 341-356.
[16] Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD and Muller WJ. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc Natl Acad Sci U S A 1992; 89: 10578-10582.
[17] Muller WJ, Arteaga CL, Muthuswamy SK, Siegel PM, Webster MA, Cardiff RD, Meise KS, Li F, Halter SA and Coffey RJ. Synergistic interaction of the Neu proto-oncogene product and transforming growth factor alpha in the mammary epithelium of transgenic mice. Mol Cell Biol 1996; 16: 5726-5736.
[18] Templeton NS, Lasic DD, Frederik PM, Strey HH, Roberts DD and Pavlakis GN. Improved DNA: liposome complexes for increased systemic delivery and gene expression. Nat Biotechnol 1997; 15: 647-652.
[19] Zhang M, Behbod F, Atkinson RL, Landis MD, Kittrell F, Edwards D, Medina D, Tsimelzon A, Hilsenbeck S, Green JE, Michalowska AM and Rosen JM. Identification of tumor-initiating cells in a p53-null mouse model of breast cancer. Cancer Res 2008; 68: 4674-4682.
[20] Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, Wu L, Lindeman GJ and Visvader JE. Generation of a functional mammary gland from a single stem cell. Nature 2006; 439: 84-88.
[21] Montales MT, Rahal OM, Kang J, Rogers TJ, Prior RL, Wu X and Simmen RC. Repression of mammosphere formation of human breast cancer cells by soy isoflavone genistein and blueberry polyphenolic acids suggests diet-mediated targeting of cancer stem-like/progenitor cells. Carcinogenesis 2012; 33: 652-660.
[22] Xie X, Xia W, Li Z, Kuo HP, Liu Y, Li Z, Ding Q, Zhang S, Spohn B, Yang Y, Wei Y, Lang JY, Evans DB, Chiao PJ, Abbruzzese JL and Hung MC. Targeted expression of BikDD eradicates pancreatic tumors in noninvasive imaging models. Cancer Cell 2007; 12: 52-65.
[23] Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ and Wicha MS. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 2003; 17: 1253-1270.
[24] Li X, Lewis MT, Huang J, Gutierrez C, Osborne CK, Wu MF, Hilsenbeck SG, Pavlick A, Zhang X, Chamness GC, Wong H, Rosen J and Chang JC. Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. J Natl Cancer Inst 2008; 100: 672-679.
[25] Phillips TM, McBride WH and Pajonk F. The response of CD24(-/low)/CD44+ breast cancer-initiating cells to radiation. J Natl Cancer Inst 2006; 98: 1777-1785.

[26] Martin AP, Miller A, Emad L, Rahmani M, Walker T, Mitchell C, Hagan MP, Park MA, Yacoub A, Fisher PB, Grant S and Dent P. Lapatinib resistance in HCT116 cells is mediated by elevated MCL-1 expression and decreased BAK activation and not by ERBB receptor kinase mutation. Mol Pharmacol 2008; 74: 807-822.

[27] Xia W, Bacus S, Hegde P, Husain I, Strum J, Liu L, Paulazzo G, Lyass L, Trusk P, Hill J, Harris J and Spector NL. A model of acquired autoresistance to a potent ErbB2 tyrosine kinase inhibitor and a therapeutic strategy to prevent its onset in breast cancer. Proc Natl Acad Sci U S A 2006; 103: 7795-7800.