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BikDD Eliminates Breast Cancer Initiating Cells and Synergizes with Lapatinib for Breast Cancer Treatment

Jing-Yu Lang¹, Jennifer L. Hsu^{1,6,7}, Funda Meric-Bernstam², Chun-Ju Chang¹, Qingfei Wang¹, Yi Bao¹, Hirohito Yamaguchi¹, Xiaoming Xie^{1,5}, Wendy A. Woodward³, Dihua Yu¹, Gabriel N. Hortobagyi⁴, and Mien-Chie Hung^{1,6,7,*}

¹Department of Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030

²Department of Surgical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030

³Department of Radiation Oncology, Division of Radiation Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030

⁴Department of Breast Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030

⁶Graduate Institute of Cancer Biology, Center for Molecular Medicine, China Medical University, Taichung, Taiwan 40402

⁷Asia University, Taichung, Taiwan

SUMMARY

Breast cancer initiating cells (BCICs), which can fully recapitulate the tumor origin and are often resistant to chemo- and radiotherapy, are currently considered as a major obstacle for breast cancer treatment. Here, we show that *BikDD*, a constitutively active mutant form of proapoptotic gene, *Bik*, effectively induces apoptosis of breast cancer cells and synergizes with lapatinib. Most importantly, BikDD significantly reduced BCICs through co-antagonism of its binding partners Bcl-2, Bcl-xL and Mcl-1, suggesting a potential therapeutic strategy targeting BCICs. Furthermore, we developed a cancer-specific targeting approach for breast cancer that selectively expresses BikDD in breast cancer cells including BCICs, and demonstrated its potent antitumor activity and synergism with lapatinib *in vitro* and *in vivo*.

INTRODUCTION

Cancer initiating cells (CICs), also termed cancer stem cells, are a small subpopulation of cancer cells within tumors with characteristics of resistance to cancer treatments and re-

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*Correspondence: mhung@mdanderson.org.

⁵Current address: Department of Breast Oncology, Sun Yat-Sen University Cancer Center, Guangzhou, People's Republic of China;

SUPPLEMENTAL INFORMATION

Supplemental information includes supplemental experimental procedures and six figures.

The authors have declared that no conflict of interest exists. In memoriam, Mrs. Serena Lin-Guo for her courageous fight against breast cancer.

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growth of new tumors, and are currently considered as a major obstacle for cancer therapies (Gupta et al., 2009; Li et al., 2008; Park et al., 2009; Yu et al., 2007). Breast cancer initiating cells (BCICs), identified by Clarke MF et al. in an enriched CD44⁺/CD24⁻ subfraction of cells, form mammospheres in suspension culture and are often resistant to chemo- and radiotherapy (Al-Hajj et al., 2003; Dontu et al., 2003; Li et al., 2008; Yu et al., 2007). Although lapatinib has been shown to be able to stabilize this small population of BCICs in the clinic (Li et al., 2008), there are no drugs currently available that can effectively reduce BCICs in patients. Moreover, due to the resistant nature of BCICs to chemo- and radiotherapy, treatments using these conventional methods have been shown to increase the BCIC population from 9% to 74% in tumors of the treated patients, and the loss of responsiveness of BCICs to these treatments will eventually lead to tumor relapse (Li et al., 2008; Yu et al., 2007). Therefore, it is imperative to develop anticancer drugs targeting BCICs.

One key mechanism that accounts for chemo-resistance in cancer initiating cells is low susceptibility to apoptosis (Park et al., 2009). For example, evasion of apoptosis through deregulated mitochondrial apoptosis machinery is essential for tumor initiation and progression, enabling cancer cells to undergo unchecked proliferation, maintain survival, and acquire drug resistance (Datta et al., 1999; Fesik, 2005; Hanahan and Weinberg, 2000; McCubrey et al., 2006; Mills et al., 2008). The Bcl-2 family is the primary regulator of the mitochondrial apoptosis pathway and is classified into three subgroups according to their Bcl-2 homology (BH) domains: the multidomain antiapoptotic subgroup (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Bfl-1/A1), the multidomain proapoptotic subgroup (Bax, Bak), and the BH3-only proapoptotic subgroup (Bid, Bim, Bad, Bik, Noxa, Puma, Bmf, Hrk) (Certo et al., 2006). An intricate balance of members within these three subgroups of Bcl-2 family determines the outcome of death signals. Interestingly, several reports have indicated that Bcl-2 and Bcl-xL, which are antiapoptotic members of the Bcl-2 family, may be involved in the survival of leukemia and glioblastoma cancer initiating cells (Domen and Weissman, 2000; Konopleva et al., 2006; Liu et al., 2006).

Unlike normal cells, expression of proapoptotic members and their antiapoptotic counterparts are often mismatched to bypass apoptosis in breast cancer cells. For instance, Bcl-2, Bcl-xL and Mcl-1 are overexpressed in breast cancer cells which is correlated with high tumor grade and poor prognosis of breast cancer patients (Ding et al., 2007; Hamilton and Piccart, 2000; Olopade et al., 1997; Silvestrini et al., 1994). In addition, overexpression of Bcl-2, Bcl-xL, and/or Mcl-1 has also been implicated in the development of drug resistance in the clinic after chemotherapy such as paclitaxel, doxorubicin, cisplatin, and bortezomib (Bauer et al., 2005; Ellis et al., 1998; Gomez-Bougie et al., 2007; Oltersdorf et al., 2005; Tabuchi et al., 2009; Teixeira et al., 1995; van Delft et al., 2006). Since the overall expression pattern of Bcl-2, Bcl-xL, and Mcl-1 appears to inversely correlate with apoptotic response following drug treatment, an antagonist that targets all of these antiapoptotic proteins would be expected to have the greatest proapoptotic efficacy and a broader and more effective application in different breast cancer cells. However, it is not yet clear whether this approach will be applicable to BCICs, which are a major determinant for tumor recurrence due to their resistant nature to traditional therapies.

Lapatinib, a dual EGFR/HER2 small molecule tyrosine kinase inhibitor, is one of the drugs currently used in the clinic for treating HER2-positive breast cancer patients, which stabilizes but does not reduce the BCIC population in patients (Geyer et al., 2006; Li et al., 2008). To achieve their clinical efficacy, anti-HER2 drugs such as trastuzumab and lapatinib greatly depend on their ability to promote apoptosis in cancer cells through HER2 inhibition. For instance, despite inhibition of p-EGFR, p-HER2, p-Erk1/2, and p-Akt, an inoperative apoptosis machinery renders breast cancer cells ineffective to trastuzumab or lapatinib-

induced apoptosis (Burris et al., 2005; Mohsin et al., 2005). The acquired resistance of breast cancer cells to lapatinib has been attributed to overexpression of Bcl-2 and Mcl-1, suggesting that lapatinib-induced apoptosis requires inactivation of antiapoptotic Bcl-2 family proteins (Martin et al., 2008; Xia et al., 2006).

To develop a therapeutic approach that can promote apoptosis in breast cancer including BCICs and enhance treatment efficacy of lapatinib, we used a mutant form of BH3-only proapoptotic protein Bik (BikDD), in which the mutations T33D and S35D were made to mimic the constitutively phosphorylated form with enhanced binding affinity to its multiple binding partners Bcl-2, Bcl-xL, Bcl-w, and Mcl-1 (Chen et al., 2004; Day et al., 2006; Li et al., 2003; Xie et al., 2007; Zou et al., 2002). We first examined the role of BikDD targeting BCICs compared to individual knockdown or co-silencing of Bcl-2, Bcl-xL, and Mcl-1 using shRNAs. Further, we examined the therapeutic effect of BikDD driven by engineered breast cancer-selective promoter and in combination with lapatinib in breast cancer cells, including BCICs.

RESULTS

BikDD effectively induces apoptosis in breast cancer cells and reduces the CD44⁺/CD24⁻ population, mammosphere formation, and cancer initiation activity

To examine the proapoptotic effect of BikDD, a potent mutant form of the proapoptotic Bik (Chen et al., 2004; Day et al., 2006; Li et al., 2003; Xie et al., 2007; Zou et al., 2002) in BCICs, we infected breast cancer cells using a pLOVE lentivirus system as described (Blleloch et al., 2007), with the pLOVE vector carrying the *BIKDD* gene or a empty control vector. Cells that were infected with viruses carrying *BIKDD* significantly induced apoptosis in MDA-MB-468 cells. Specifically, using cell number counting, MTT and clonogenic assays, we showed that expression of BikDD significantly inhibited the growth and clonogenicity of MDA-MB-468 cells compared with the vector control (all $p < 0.01$, Figure 1A). We further found that apoptotic bodies were present in large numbers in *BIKDD*-infected, but not vector control-infected cells (Figure 1B, left panel), and a high level of cleaved PARP, which is indicative of apoptosis, was detected in *BIKDD*-infected cells via immunoblotting analysis (Figure 1B, right panel). Interestingly, expression of BikDD reduced the CD44⁺/CD24⁻ population to 29% of the control in addition to the proapoptotic effect in total population of MDA-MB-468 cells described above ($p < 0.01$, Figure 1C). Consistent with previous studies (Gupta et al., 2009; Li et al., 2008), chemodrugs such as paclitaxel increased this CD44⁺/CD24⁻ population by almost 3-fold ($p < 0.01$, Figure 1C). Consequently, BikDD decreased mammosphere formation of MDA-MB-468 parental cells to 13% compared with the control vector ($p < 0.01$, Figure 1C bottom left). Next, we examined whether BikDD can block mammosphere formation of mammosphere-selected tumor cells. We found that BikDD expression via lentivirus infection reduced the number of re-formed mammospheres of MDA-MB-468 mammosphere cells that was acquired from MDA-MB-468 parental cells through mammosphere formation culture *in vitro* ($p < 0.01$, Figure 1D). Likewise, reduction of CD44⁺/CD24⁻ population and numbers of mammosphere formation by BikDD was also found in BT474 human breast cancer cells (Figure 1E). Similar results were also obtained from MCF7/HER2 cell line (data not shown).

In addition to human breast cancer cell lines, we also examined the effect of BikDD in patient and mouse MMTV-Neu transgenic primary breast tumor cells. First, we examined the killing effect of BikDD in radiation-treated patient primary breast tumor cells. As shown in Figure 2A, patient primary breast tumor cells post radiation therapy contained 29.25% of CD44⁺/CD24⁻ population. Under BikDD treatment, the percentage of CD44⁺/CD24⁻ population was reduced to 9.75%, i.e. 33% of the control (or 67% inhibition, Figure 2A). BikDD also decreased the mammosphere formation of these primary breast tumor cells to

25% of the control (or 75% inhibition, Figure 2B). We also obtained similar results from primary cell culture of another patient breast tumor xenograft model in which patient breast tumor specimen was directly passaged in nude mice and showed that the CD44⁺/CD24⁻ population was reduced to 49% of the control (Figure S1A). In addition, we also examined the killing effect of BikDD in MMTV-Neu primary tumor cells that were obtained from MMTV-Neu NDL2-5 transgenic mouse (Siegel et al., 1999). We found that BikDD also significantly reduced the percentage of either CD24⁺/CD29⁺ (Figure 2C, top) or CD24⁺/CD49f⁺ (Figure 2C, bottom) populations, which are biomarkers for mouse breast stem cells (Liu et al., 2007; Shackleton et al., 2006; Stingl et al., 2006). BikDD was also able to block the mammosphere formation of MMTV-Neu primary tumor cells and dramatically reduced the number of formed MMTV-Neu primary mammospheres obtained by mammosphere culture (both $p < 0.01$, Figures 2D and 2E). Thus, these results suggest that BikDD not only induces apoptosis in breast cancer cell lines but also eliminates the CD44⁺/CD24⁻ population and mammospheres in primary breast cancer cells.

Next, we asked whether BikDD also inhibits cancer initiation activity. To this end, we first selected for mammospheres from MDA-MB-468 parental cells (referred to as “1° mammosphere cells”), and then the 1° mammosphere cells were infected with lentivirus expressing BikDD or vector control. Two days after infection, the survived cells were trypsinized, counted, and injected into NOD/SCID mice with the indicated numbers of cells (Group B). In this experiment, only 500 1° mammosphere cells were sufficient to induce tumor formation (Figure 2F). In contrast, the 1° mammosphere cells survived after BikDD treatment exhibited a significant reduction in tumorigenicity (Group B), suggesting that BikDD treatment reduced the BCIC population. To ensure this phenomenon can be observed in the secondary transplants (Al-Hajj et al., 2003; Dick, 2003; Haase et al., 1995; Lapidot et al., 1996), we harvested the tumor tissues from the vector-control group (since virtually no tumor was developed in the BikDD-treated group) to isolate primary tumor cells, and selected for mammospheres (referred to as “2° mammosphere cells”). Again, these 2° mammosphere cells were infected with lentivirus expressing BikDD or vector-control. Two days after infection, the survived cells were trypsinized, separated into single-cell suspension, counted, and then injected into NOD/SCID mice with indicated numbers of cells (Group C). As shown in Figure 2F, these 2° mammosphere cells from the vector-control group still maintained their ability to induce tumor in mice with only 500 cells. However, the 2° mammosphere cells survived after BikDD treatment no longer produced tumor (Group C). Together, these results suggest that the selected mammosphere cells maintained their cancer initiation activity by secondary transplants (vector-control group), and BikDD expression in those selected mammosphere cells significantly inhibited their cancer initiation activity, most likely by reduction of BCIC population.

Furthermore, in order to address whether BikDD can inhibit cancer initiation activity during an *in vivo* tumorigenicity assay, we adopted a gene therapy protocol (described in details later in Figure 6C), which allows us to assay the cancer initiation activity from tumor xenografts growing in mice after BikDD treatment. To this end, we treated mice bearing MDA-MB-468 tumor xenografts with control vector-liposome or VISA-claudin4-BikDD-liposome complexes. After treatment stopped, we harvested tumor tissues from mice, created a single-cell suspension, and then passaged them into new animals with indicated numbers of cells (Figure S1B). We found tumor cells from mice treated with VISA-claudin4-BikDD-liposome complexes also demonstrated significantly reduced tumorigenesis in new animals compared to that from mice treated with vector-control-liposome complexes. Specifically, no mice developed tumor in BikDD-treated groups that received 10⁴ and 10⁵ cells isolated from tumor of the VISA-claudin4-BikDD-treated mice (Figure S1B). In addition, we also observed a reduction in CD44⁺/CD24⁻ population and the number of mammosphere formed upon *in vivo* VISA-claudin4-BikDD treatment

(Figures S1C and S1D). Taken together, these results indicate that BikDD indeed eliminates BCICs under a gene therapy setting.

Co-silencing of multiple Bcl-2 antiapoptotic members mimics the proapoptotic effect of BikDD in BCICs

In our previous work, BikDD was shown to have enhanced binding affinity to Bcl-2 antiapoptotic proteins such as Bcl-2 and Bcl-xL compared with the wild-type Bik (Li et al., 2003). Therefore, we examined the effect of its major binding partners including Bcl-2, Bcl-xL and Mcl-1 in BCICs using an shRNA knockdown approach. Interestingly, co-silencing Bcl-2, Bcl-xL, and Mcl-1 reduced the CD44⁺/CD24⁻ population to 25% of the control MDA-MB-468 cells (Figure 3A) and consequently decreased mammosphere formation of MDA-MB-468 cells to 10% of the control (Figure 3B). However, we found that individual knockdown of each of the three molecules only had a partial killing effect in CD44⁺/CD24⁻ population and number of mammospheres, suggesting that Bcl-2 antiapoptotic proteins may have functional redundancy in the BCIC survival (Figures 3A and 3B). When BikDD was expressed by infection in these cells as described above (Figures 1A and 1B), we showed that it reduced CD44⁺/CD24⁻ population and mammosphere formation to 27% and 10%, respectively, compared with vector control group, which is comparable to the killing effect of co-silencing its three major binding partners Bcl-2, Bcl-xL and Mcl-1. Furthermore, BikDD did not enhance the killing effect against BCICs post co-silencing of all three molecules (Figure S2). The specificity of shRNAs against these three molecules was validated by immunoblotting (Figure 3C), supporting that the reduction in CD44⁺/CD24⁻ population and number of mammospheres were indeed due to the loss of these protein expression. Similar results were also obtained from another set of shRNAs against these three antiapoptotic Bcl-2 family proteins (see Supplemental Information and also data not shown), in order to exclude the off-target issue of shRNAs. We also carried out the same experiment in BT474 cells and showed similar results to that of MDA-MB-468 described above (data not shown). Taken together, we determined that efficient induction of apoptosis in BCICs requires silencing of all three antiapoptotic Bcl-2 proteins, which suggests that co-antagonism of multiple Bcl-2 antiapoptotic proteins by BikDD may have a better killing effect against BCICs than targeting individual antiapoptotic proteins, which is likely due to their functional redundancy in the survival of BCICs.

The engineered VISA-claudin-4 promoter is robustly and selectively activated in breast cancer cells *in vitro* and *in vivo*

To explore BikDD gene therapy, we search for an expression vector that allows *BIKDD* gene to selectively express in breast cancer cells. In our previous work, we developed a transcriptional targeting vector “VISA” (VP16-GAL4-WPRE integrated systemic amplifier) based on an engineered expression vector, which can enhance a cancer-specific promoter activity by several hundred-fold and prolong duration of gene expression without loss of its cancer specificity. This system has been successfully applied in pancreatic, lung, and ovarian cancer preclinical models (Sher et al., 2009; Xie et al., 2009; Xie et al., 2007). In this study, we also utilized this powerful targeting approach to express BikDD in breast cancer cells under a promoter that is activated in breast cancer cells.

By comparing the mRNA expression level between breast cancer and normal tissues in the public SAGE and cDNA microarray libraries, we identified several candidate promoters including claudin-4, fatty acid synthase (FASN), MMP1, and StarD10 (Figure S3A). After compared their promoter activities in multiple breast cancer cell lines, we found that the claudin-4 promoter exhibited highest activity among all other promoters tested. Moreover, this promoter had little activity in normal cell lines and several cancer types (Figure 4A), suggesting its selectivity for breast cancer.

Although the claudin-4 promoter was selectively expressed in breast cancer cells, its activity level was only about 1–4% of CMV promoter activity (Figure S3B). Therefore, we integrated the claudin-4 promoter into the VISA system (Figure 4B). Indeed, the engineered VISA-claudin4-Luc vector showed robust luciferase activity in almost all of the 13 breast cancer cell lines tested and was comparable to or higher than that of the CMV promoter (Figure 4C). In addition, its activity was nearly silent in normal cell lines compared with that of the CMV promoter (Figure 4C), which further supports its breast cancer selectivity. Moreover, the expression ratio of luciferase from VISA-claudin4-Luc to CMV-Luc at day 5 was consistently higher than at day 2 in the five breast cancer cell lines tested (Figure 4D), suggesting that the prolonged duration of gene expression from the VISA vector, which was likely due to the presence of WPRE, an RNA stabilizing element, in the VISA system as reported previously (Xie et al., 2007).

To further examine the breast cancer-specific expression *in vivo*, we treated mice with CMV-Luc or VISA-claudin4-Luc plus DOTAP/Cholesterol liposome as the gene delivery system via tail vein injection at a single dose of 2.5 mg/kg in 4T1 tumor-bearing syngeneic mice (Templeton et al., 1997; Xie et al., 2007). The mammary tumors were inoculated in the fourth inguinal mammary gland to prevent overlapping signals from lung and heart in which CMV promoter was highly activated. We found that the luciferase activity of VISA-claudin4-Luc was selectively localized in the tumor region, while the CMV-Luc activity was primarily observed in the lung with weak signals in the tumor area after 48 hours (Figure 4E). This result was further confirmed by examining these individual organs, which demonstrated that the signal of VISA-claudin4-Luc was indeed from the tumor itself and not from other organs (Figure 4F). Collectively, we conclude that the VISA-claudin4-Luc vector is robustly and selectively expressed in breast cancer cells both *in vitro* and *in vivo*.

The VISA-claudin4-BikDD expression efficiently inhibits breast cancer cells growth *in vitro* and *in vivo* and has less toxicity than CMV-BikDD

To investigate the therapeutic efficacy of BikDD, we incorporated *BIKDD* into VISA-claudin4 vector (Figure 5A) and examined its inhibitory effect on cell growth in a panel of breast cancer and normal cell lines. Compared with CMV-BikDD, VISA-claudin4-BikDD demonstrated comparable or stronger inhibitory effects on cell growth of different breast cancer cell lines (Figure 5B). In contrast, it had virtually little or no effect on the growth of human normal cell lines MCF10A, 184A1, and WI38 (Figure 5B). This killing effect was unlikely a result of non-therapeutic component of the VISA system such as GAL4/VP2 synthetic transcription factor because VISA-claudin4-Luc has virtually no effect on the growth of breast cancer cells (Figure 4C and data not shown). Thus, VISA-claudin4-BikDD efficiently and selectively killed breast cancer cells *in vitro*, consistent with the luciferase expression data (Figure 4C).

To evaluate the antitumor efficacy of VISA-claudin4-BikDD *in vivo*, we tested one syngeneic mouse breast tumor and multiple human breast tumor orthotopic xenograft models. First, we used the 4T1-Luc syngeneic mouse mammary orthotopic model and found that both CMV-BikDD and VISA-claudin4-BikDD, at 0.75mg/kg, twice per week for 3 weeks, greatly reduced 4T1 tumor growth and prolonged mouse survival *in vivo* (Figures 5C–E, both $p < 0.01$). Consistently, both CMV-BikDD and VISA-claudin4-BikDD effectively reduced tumor growth and prolonged mice survival in a human breast orthotopic xenograft model (Figures 5F and 5G, both $p < 0.01$). Notably, VISA-claudin4-BikDD suppressed tumor growth and prolonged mice survival more significantly than CMV-BikDD (Figures 5D, 5F and 5G, both $p < 0.05$).

Similar results were also obtained from other breast cancer orthotopic models such as MCF7/HER2, BT474, and MDA-MB-468 (see later in Figure 6C, all $p < 0.05$). In addition,

expression of BikDD mRNA containing the WPRE RNA stabilizing element is readily detectable and can be distinguished from endogenous wild-type Bik in tumor tissues of MCF7/HER2 and BT474 xenograft models after VISA-claudin4-BikDD treatment but not in untreated mice (data not shown), indicating that expression of the therapeutic gene in the tumors after treatment. We also found that VISA-claudin4-BikDD produced less toxicity compared with CMV-BikDD *in vivo* (Figure 5H), without obvious changes in AST, ALT, and BUN as determined by liver and kidney functional assays, respectively (Figure S4). These results indicate that VISA-claudin4-BikDD efficiently inhibited tumor growth of breast cancer xenografts *in vivo*.

The VISA-claudin4-BikDD synergizes with lapatinib in EGFR⁺/HER2⁺ breast cancer cell lines *in vitro* and *in vivo*

As previously reported, the clinical efficacies of anti-HER2 agents such as lapatinib and trastuzumab are greatly limited by either inoperative apoptosis machinery or overexpression of Bcl-2 antiapoptotic proteins following lapatinib treatment that may be improved by inactivation of Bcl-2 antiapoptotic proteins (Burris et al., 2005; Martin et al., 2008; Mohsin et al., 2005; Xia et al., 2006). Therefore, we examined whether BikDD or inhibition of Bcl-2 antiapoptotic members may enhance the therapeutic effect of lapatinib in breast cancer cells. In this study, we found that VISA-claudin4-BikDD effectively sensitized BT474 and MDA-MB-453 (HER2⁺), and MDA-MB-468 and BT20 (EGFR⁺) cells to lapatinib (Figure 6A, see also Figures S5A and S5B for EGFR/HER2 expression and combination index). It should be mentioned there was virtually no added killing effect under the combination of VISA-claudin4-BikDD plus lapatinib in the EGFR⁻/HER2⁻ MCF7 human breast cancer cell line or in MCF10A normal human mammary epithelial cell line (Figure 6A). The lack of sensitization of VISA-claudin4-BikDD to lapatinib in MCF7 is most likely due to low expression of EGFR and HER2 (Figure S5A) while the lack of activity in the normal cell lines is most likely due to the absence of VISA-claudin4 expression activity in normal cells (Figure 4C). Moreover, we determined that this is not a result of low transfection efficiencies since all cell lines tested using the electroporation method had similar GFP expression level (data not shown).

We further examined whether inactivation of Bcl-2 antiapoptotic proteins by siRNAs sensitized EGFR⁺/HER2⁺ breast cancer cells to lapatinib. We found that co-silencing of Bcl-2, Bcl-xL and Mcl-1 indeed sensitized EGFR⁺/HER2⁺ breast cancer cells to lapatinib that was comparable to BikDD alone, which surpassed individual knockdown of them (Figure 6B, top) but not in EGFR⁻/HER2⁻ MCF7 cell line (Figure 6B, bottom left). However, when HER2 was expressed in the MCF7 cells (Wang et al., 2004), co-silencing of these three Bcl-2 antiapoptotic members was able to sensitize the cells to lapatinib again (Figure 6B, bottom right), suggesting that co-antagonism of Bcl-2, Bcl-xL and Mcl-1 by three specific siRNAs or BikDD alone can overcome the functional redundancy of Bcl-2 antiapoptotic proteins and synergized with lapatinib.

The sensitization effect of VISA-claudin4-BikDD plus lapatinib treatment was further supported by the enhanced expression level of cleaved PARP (a major apoptosis signal) under combinational therapy. Combination treatment of VISA-claudin4-BikDD plus lapatinib enhanced the expression level of cleaved PARP in HER2⁺ MCF7/HER2 and EGFR⁺ MDA-MB-468 cells to about 44.9% or 162.8% more than either single treatment alone using both VISA-claudin4-Luc and pUK21 plasmid as negative controls (Figure S5C). These results suggest that the enhanced proapoptotic effect under a combination of VISA-claudin4-BikDD plus lapatinib may contribute to their synergistic effects on the growth of EGFR⁺/HER2⁺ breast cancer cells.

To further examine the therapeutic efficacy of VISA-claudin4-BikDD plus lapatinib combination *in vivo*, we treated mice bearing HER2⁺ BT474 human breast cancer xenografts with VISA-claudin4-BikDD and/or lapatinib. Lapatinib was administered orally at a dose of 50 mg/kg every other day, and VISA-claudin4-BikDD was intravenously injected as indicated by arrows in Figure 6C. VISA-claudin4-BikDD or lapatinib alone significantly inhibited tumor growth compared with the vector alone group (Figure 6C, both $p < 0.05$). However, the combination treatment of VISA-claudin4-BikDD plus lapatinib demonstrated even much better therapeutic efficacy than single-agent treatment of either VISA-claudin4-BikDD or lapatinib alone (Figure 6C, both $p < 0.05$). A more significant synergy between VISA-claudin4-BikDD and lapatinib was observed when MDA-MB-468 cells was transfected by VISA-claudin4-BikDD once before transplanted into nude mice, followed by lapatinib treatment every other day ($p < 0.01$, Figure S5D). Similar results were also obtained from EGFR⁺ MDA-MB-468 orthotopic xenograft model and HER2⁺ MCF7/HER2 human breast cancer orthotopic xenograft model (Figure 6C, both $p < 0.05$). Thus, targeted expression of VISA-claudin4-BikDD sensitized breast cancer cells to lapatinib *in vitro* and *in vivo* and provided additional benefit when combined with lapatinib, ensures that normal cells or tissues are spared from its potent proapoptotic effect.

In addition, we also explored whether VISA-claudin4-BikDD can sensitize breast cancer cells to other clinical used drugs since Bcl-2 antiapoptotic proteins have been shown to contribute to the development of drug resistance following chemotherapy (Ellis et al., 1998; Oltersdorf et al., 2005; Tabuchi et al., 2009). Among them, we found that VISA-claudin4-BikDD also sensitized multiple breast cancer cell lines to paclitaxel *in vitro* including the EGFR⁻/HER2⁻ MCF7 human breast cancer cell line (Figure S5E). However, it should be mentioned that BikDD sensitized the HER2⁺ SKBr3 human breast cancer cell line to lapatinib but not paclitaxel (data not shown), suggesting that the combination of VISA-claudin4-BikDD plus lapatinib may have potential benefits in EGFR⁺ or HER2⁺ breast cancer cells while the combination of VISA-claudin4-BikDD plus paclitaxel may be more useful for EGFR⁻/HER2⁻ breast cancer cells.

VISA-claudin4-BikDD treatment efficiently reduces CD44⁺/CD24⁻ population even after paclitaxel treatment and markedly attenuates tumor growth at off-therapy stage

Next, we examined the activity of engineered VISA-claudin4 promoter in BCICs. To determine if VISA-claudin4 vector could drive gene expression in BCICs, we used VISA-claudin4-Luc to measure its promoter activity. As shown in Figure 7A, luciferase expression of VISA-claudin4-Luc in the CD44⁺/CD24⁻ population and mammosphere cells of MDA-MB-468, MCF7/HER2, MDA-MB-435, BT549, and HBL100 breast cancer cell lines was comparable or even higher compared with the total population, indicating the engineered claudin4 promoter is activated in BCICs, even in claudin-low cell lines (Figure S6). Next, we examined the therapeutic effect of BikDD in MDA-MB-468 tumor orthotopic xenograft mouse model. MDA-MB-468 tumor-bearing mice were treated with empty vector, lapatinib, paclitaxel, VISA-claudin4-BikDD, and VISA-claudin4-BikDD plus lapatinib or paclitaxel. Consistent with the *in vitro* data, BikDD treatment significantly reduced the percentage of CD44⁺/CD24⁻ population of MDA-MB-468 tumor to 48% compared to vector control (set as 100%) while paclitaxel treatment increased this population by about 3-fold as expected at day 26 of last treatment (both $p < 0.05$, Figure 7B). Interestingly, BikDD blocked the expansion of the CD44⁺/CD24⁻ population under paclitaxel treatment ($p < 0.01$, Figure 7B). A reduction in the CD44⁺/CD24⁻ population in VISA-claudin4-BikDD or VISA-claudin4-BikDD plus paclitaxel at day 26 led to significant tumor growth inhibition compared with paclitaxel alone after drug cessation from day 26 to day 49 (off-therapy stage) with a growth rate ratio determined from the relative slope of the tumor growth curves of 0.44, 0.45, and 1.45, respectively (Figures 7C and 7D). The relative slopes were determined by measuring

the slope of tumor growth curves in each group during the off-therapy stage from day 26 to day 49 (calculated from the exponential trendlines in Microsoft Excel) compared to control vector (Figure 7D). 49 days after first drug treatment, VISA-claudin4-BikDD plus paclitaxel exhibited better tumor growth suppression compared with VISA-claudin4-BikDD alone ($p < 0.05$, Figure 7C left). We also found similar results from combination treatment of VISA-claudin4-BikDD and lapatinib (Figures 7B–D). Collectively, these results indicate that BikDD driven by VISA-claudin4 vector potently reduced the CD44⁺/CD24⁻ population *in vivo* even after chemotherapy and efficiently attenuated tumor growth after cessation of drug treatment, suggesting that VISA-claudin4-BikDD treatment may serve as a potential therapeutic approach to kill BCICs, which is considered as a major barrier for breast cancer treatment.

Taken together, the current study suggests a potential therapeutic approach for breast cancer treatment by showing: 1) BikDD effectively reduced BCICs through co-antagonism of its major binding partners Bcl-2 antiapoptotic proteins; 2) the engineered VISA-claudin4 promoter was selectively activated in breast cancer cells including BCICs; 3) targeted expression of BikDD driven by VISA-claudin4 vector demonstrated potent anti-tumor activities in multiple syngeneic and orthotopic mouse models, synergized with lapatinib and paclitaxel, and attenuated tumor growth at off-therapy stage by reducing BCICs. Therefore, it is worthy of moving VISA-claudin4-BikDD into a clinical trial, which will provide potential benefit in breast cancer patients by triggering apoptosis in both non-BCICs and BCICs.

DISCUSSION

There is increasing evidence to support that conventional cancer therapies which are able to kill the bulk of differentiated cancer cells within tumors have failed to eliminate cancer initiating cells (Gupta et al., 2009; Park et al., 2009). Residual cancer cells such as BCICs will reform new tumor and eventually lead to tumor relapse in patients. At present, there are no effective treatments available for treating BCICs in the clinic, which are urgently needed for relapsed breast cancer patients. However, it is worth mentioning that Gupta et al. recently identified a compound that can specifically kill BCICs (Gupta et al., 2009). Thus, agents as such which can target BCICs are worthy of moving into clinical trials.

In this study, we found that antiapoptotic Bcl-2 family of proteins play an important role in maintaining survival of BCICs, and elimination of these chemo-resistant cancer initiating cells required co-antagonism of Bcl-2 antiapoptotic proteins either through BikDD expression or co-silencing of Bcl-2, Bcl-xL and Mcl-1 (Figures 1–3). We have provided evidence to show that co-antagonism of Bcl-2 antiapoptotic proteins via BikDD not only markedly reduced BCICs, but also effectively killed non-BCICs in multiple breast cancer cell lines, which surpassed knockdown of them individually. Since multiple Bcl-2 antiapoptotic members were redundantly and highly expressed in several breast cancer cell lines including BT474 and MDA-MB-468 (Figure S5A), this could explain why co-antagonism of Bcl-2 antiapoptotic proteins had better therapeutic effect than inhibition of them individually. Inhibition of the CD44⁺/CD24⁻ population and mammosphere formation by BikDD expression in patient and mouse primary tumor cells suggests its potential clinical therapeutic value. Moreover, the killing effect of BikDD against BCICs is likely mediated through co-inhibition of the three major binding partners Bcl-2, Bcl-xL and Mcl-1 because BikDD did not further enhance the killing effect against BCICs post co-silencing of these three molecules which were validated in both MDA-MB-468 and BT474 cell lines (Figure S2).

Practically, it would be advantageous to develop a breast cancer therapy that can target breast cancer cells including BCICs through inhibition of antiapoptotic Bcl-2 family of proteins. To this end, we engineered a breast cancer targeting VISA-claudin4 vector to express the therapeutic gene selectively in breast cancer cells including BCICs. Although claudin-4 protein were reported to be expressed in breast tissue during lactation and other cancer types (Hewitt et al., 2006), the expression level of claudin-4 was significantly lower in breast normal tissues compared to breast cancer according to the data obtained from the public SAGE and microarray database (70:1200 ratio), which was further supported by other studies (Kominsky et al., 2004; Soini, 2004). In addition, we also examined its promoter activity in claudin-low cell lines that have been shown to correlate with BCICs (Hennessy et al., 2009; Kuo et al., 2009; Neve et al., 2006). This 1100-bp truncated fragment of claudin-4 promoter, which was originally cloned from claudin-low MDA-MB-435 cells, exhibited comparable or higher activity in claudin-low subtype compared with luminal and basal A subtypes, with little expression in normal breast cancer cell lines (Figure S6). Moreover, it still processed comparable promoter activities in the CD44⁺/CD24⁻ population and mammosphere cells compared with parental cells after constructing it into the VISA system even in the claudin-low subtype breast cancer cell lines (Figure 7A), suggesting that this region of the claudin-4 promoter is an excellent candidate for transcriptionally-targeted breast cancer therapy.

Next, we demonstrate significant antitumor activities of VISA-claudin4-BikDD in a mouse syngeneic and multiple human orthotopic xenograft breast cancer models *in vitro* and *in vivo* (Figures 5C–G, and Figure 6C), which strongly suggest its feasibility for breast cancer treatment. For breast cancer treatment, anti-HER2 drugs such as lapatinib and trastuzumab have been widely used in HER2⁺ breast cancer patients (Geyer et al., 2006; Li et al., 2008). However, their clinical efficacy appeared to be limited by inoperative apoptotic machinery or overexpression antiapoptotic members of Bcl-2 family proteins after drug treatment (Martin et al., 2008; Xia et al., 2006). Indeed, BikDD sensitized HER2⁺ breast cancer cells to lapatinib, which was mimicked by co-inhibition of antiapoptotic Bcl-2 family proteins by three combined siRNAs but not individually. Moreover, BikDD also sensitized EGFR⁺ breast cancer cells to lapatinib, which has been reported to be less effective under lapatinib treatment compared with HER2⁺ cells (Konecny et al., 2006). In addition to that, we also found that BikDD can sensitize multiple breast cancer cell lines to paclitaxel including EGFR⁻/HER2⁻ MCF7 cells (Figure S5E), suggesting that the combination of VISA-claudin4-BikDD plus paclitaxel may provide some benefits in EGFR⁻/HER2⁻ breast cancer cells. Thus, the evidence shown here greatly supports our notion that apoptosis-promoting agents like BikDD delivered by the VISA-claudin4-vector would be expected to provide additional therapeutic benefits to lapatinib treatment in EGFR⁺/HER2⁺ breast cancer cells and may be potentially used for EGFR⁺/HER2⁺ breast cancer patients following our pancreatic VISA-based targeting vector (Xie et al., 2007), which is in the process of being moved into human clinical trials.

Moreover, we demonstrated that the engineered VISA-claudin4 promoter was activated in the CD44⁺/CD24⁻ population and mammosphere cells (Figure 7A) (Casagrande et al., 2011). BikDD treatment not only significantly reduced the BCIC population of MDA-MB-468 *in vitro* and *in vivo* but also dramatically blocked the CD44⁺/CD24⁻ population expansion under paclitaxel treatment while paclitaxel alone greatly enhanced this population after drug treatment (Figures 1C and 7B). Of note, BikDD was also able to reduce the CD44⁺/CD24⁻ population and mammosphere formation in radiation-treated patient breast tumor cells (Figures 2A and 2B). Reduction of CD44⁺/CD24⁻ population by BikDD or its combination with paclitaxel or lapatinib greatly attenuated tumor growth at off-therapy stage from day 26 to day 49 post first drug treatment (Figures 7C and 7D). These results strongly suggest that BikDD can eliminate BCICs, even after chemotherapy, and VISA-claudin4-

BikDD gene therapy may provide an effective strategy to inhibit breast tumor growth including BCICs.

In summary, our study uncovered the important role of Bcl-2 antiapoptotic proteins in the survival of BCICs, and co-inactivation of antiapoptotic Bcl-2 family proteins is required to reduce BCICs and sensitize EGFR⁺/HER2⁺ breast cancer cells to lapatinib, suggesting a potential therapeutic strategy targeting BCICs. As it has been shown that non-BCICs can also transit into BCICs at a significant rate under certain condition (Gupta et al., 2009), it would be advantageous to develop such a therapeutic agent that can target both population of breast cancer cells. By using our newly developed VISA-claudin4-BikDD for treating breast cancer, it is likely that therapeutic efficacy will be enhanced and potential side effects prevented as we have shown that BikDD targets both non-BCICs and BCICs and demonstrates virtually no toxicity in normal cells.

EXPERIMENTAL PROCEDURES

Patient primary tumor specimen

Patient breast tumor specimen was collected within 1 hour after surgery under the guidelines approved by the Institutional Review Board at MD Anderson Cancer Center, and written informed consent was obtained from patients in all cases at time of enrollment. The tumors were minced into small pieces, and were digested by collagenase/hyaluronidase for 3–16 hours (Stemcell Inc. #07912). Cells were further cultured in Matrigel-coated 6-well plate using MEGM complete medium (Lonza/Clonetics, #cc-3051).

In vivo mouse models

All animal procedures were conducted under the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at MD Anderson Cancer Center. Female BALB/cA normal and athymic mice (Harlan laboratories Inc, Houston, TX) were used as hosts for tumor xenografts. Mice were divided according to the mean value of tumor volume or photon signals in each group. For MCF7/HER2 and BT474 cell lines, 17 β -estradiol with biodegradable carrier-binder (Innovative Research of America, Sarasota, FL) was inoculated under the mice skin 3 days before tumor injection. Lapatinib was administered orally at 50 mg/kg every other day (Scaltriti et al., 2007). Paclitaxel was injected intravenously at 3 mg/kg every other day. VISA-claudin4-BikDD plus liposome mixture was injected intravenously at 0.75 mg/kg as indicated by arrows in the figures. Tumor was measured twice weekly with a caliper, and tumor volume was calculated by the formula: $\pi/6 \times \text{length} \times \text{width}^2$.

Statistical Analyses

Data were analyzed by the Student's T-test except Cox-Mantel test on Survival rates using Statistica 6.0 software. The remaining experimental procedures can be found in Supplemental Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Bcl-2 proteins are critical for breast cancer initiating cells (BCICs) survival
- BikDD eliminates BCICs through co-antagonism of Bcl-2 antiapoptotic proteins
- Development of breast cancer-selective expression vector that also targets BCICs
- BikDD sensitizes breast cancer cells to lapatinib

SIGNIFICANCE

The Bcl-2 family of proteins is essential for tumorigenesis and development of resistance to chemotherapies. However, in this study, we uncovered an important role of Bcl-2 antiapoptotic proteins in the survival of BCICs. Due to the functional redundancy of Bcl-2 antiapoptotic proteins, co-inhibition of these proteins is required to optimally reduce BCICs and sensitize breast cancer cells to lapatinib. We also demonstrated that inactivation of multiple Bcl-2 antiapoptotic proteins by their proapoptotic partner, BikDD, through an expression vector selective for breast cancer produced a profound killing effect and synergized with lapatinib in animal models. Taken together, the current study provides a therapeutic strategy worthy of future clinical trials, with extra benefit for breast cancer patients by triggering apoptosis in BCICs.

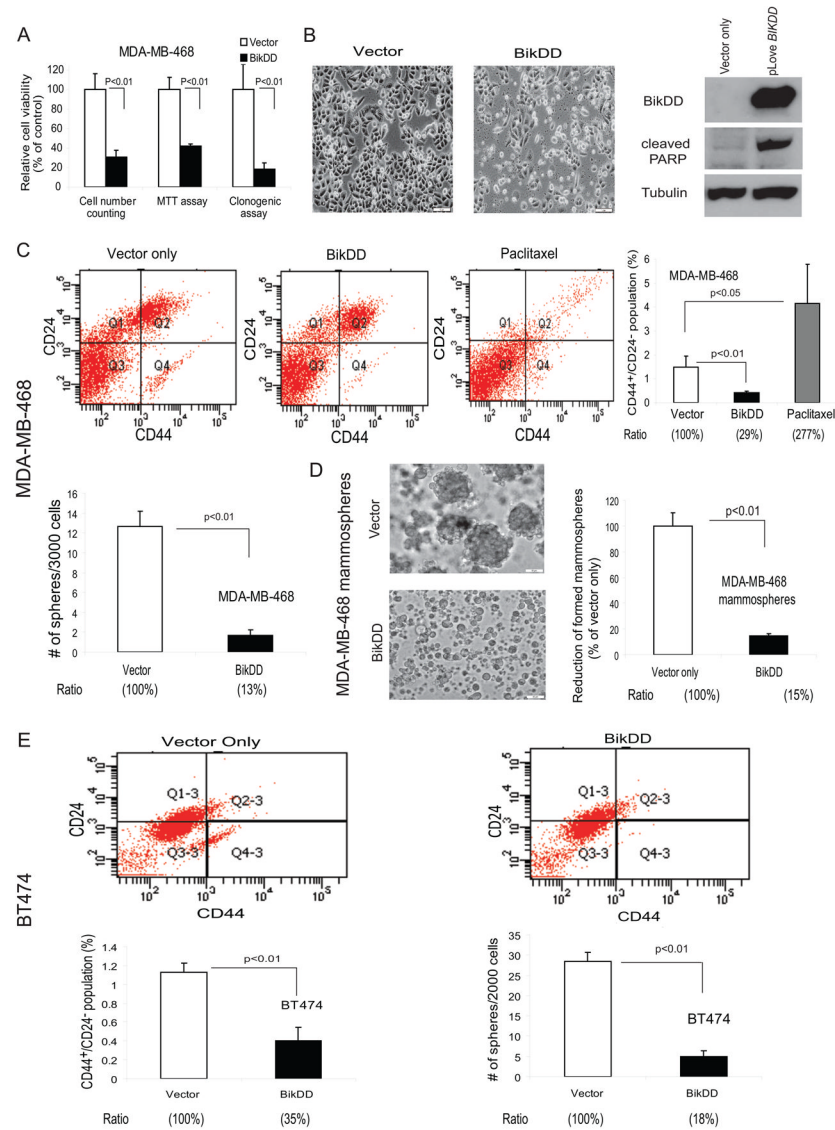


Figure 1. BikDD effectively reduced the CD44⁺/CD24⁻ population and mammosphere formation of breast cancer cells

(A) MDA-MB-468 cells were infected with lentivirus containing *BIKDD* or vector alone, and were analyzed by cell numbers, MTT and clonogenicity assays (setting at 100% in vector group). (B) Cells were imaged, and were immunoblotted by N-terminal Bik antibody and cleaved PARP antibody, respectively. Scale bar indicates 10 μ m. (C) 200,000 MDA-MB-468 cells were analyzed by flow cytometry 7 days post infection or paclitaxel treatment once at 1.0 μ g/ml dose using fluorescent-conjugated CD44 and CD24 antibodies. The data represent the mean of three independent experiments. For mammosphere formation assay, indicated cells were used and the mammospheres was counted under microscope with size ≥ 100 μ m. (D) MDA-MB-468 mammospheres established as above were infected with lentivirus containing either vector alone or *BIKDD*, and the number of re-formed mammospheres were counted within 7 days. Scale bar indicates 50 μ m. (E) BT474 cells eliminated the CD44⁺/CD24⁻ population and mammosphere formation of BCICs. All error bars indicate SD.

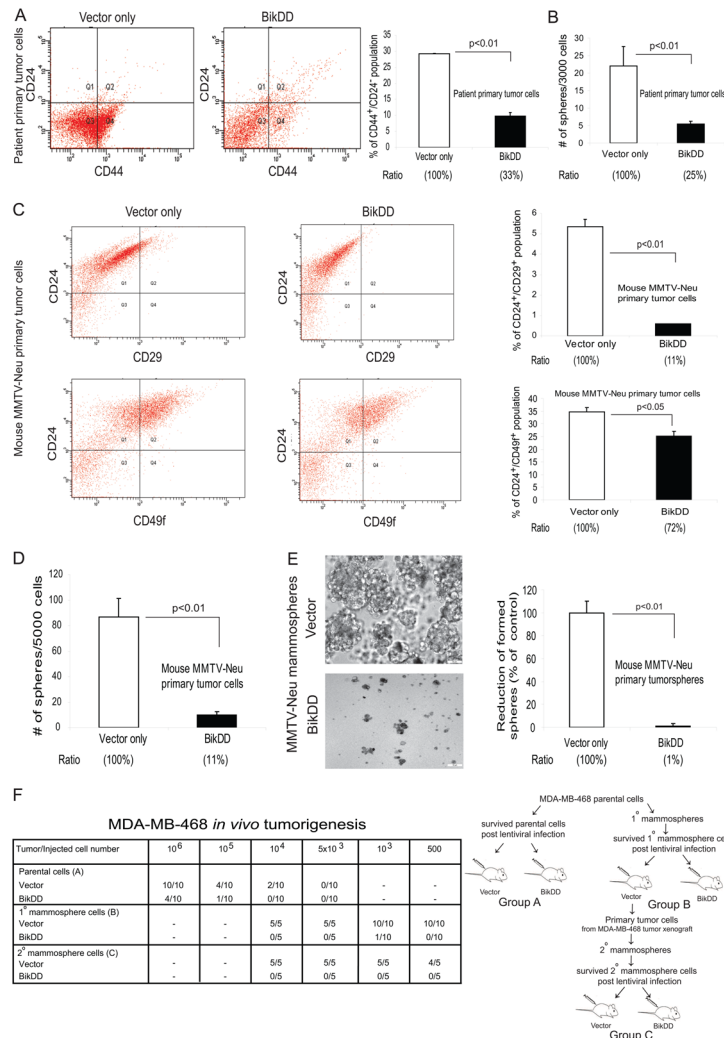


Figure 2. BikDD significantly reduced the BCICs of patient and mouse MMTV-Neu primary tumor cells and blocked *in vivo* tumorigenesis of mammosphere cells (A–B) patient primary breast tumor cells were isolated from radiated-patient primary tumor after surgery, as described in experimental procedures. After BikDD treatment, cells were subjected to FACS analysis. 3,000 cells were used for mammosphere formation, and mammospheres were counted with size at >100 μ m. (C) Mouse MMTV-Neu primary tumor cells were isolated from primary tumor in MMTV-Neu transgenic mouse and subjected to FACS analysis using either mouse CD24⁺/CD29⁺ (top) or CD24⁺/CD49f⁺ (bottom) fluorescent-conjugated antibodies after BikDD treatment. (D) For mammosphere formation, 5,000 cells were used and mammospheres were counted with size >100 μ m. (E) MMTV-Neu primary mammospheres were treated with either vector alone or BikDD via lentivirus infection, and mammospheres were counted after reformation. Scale bar indicates 50 μ m. (F) Indicated numbers of parental MDA-MB-468 cells and mammosphere cells with or without BikDD treatment, were inoculated into the mammary fat pad of NOD/SCID mice, and mice were monitored for the next months for tumorigenesis. All error bars indicate SD. See also Figure S1.

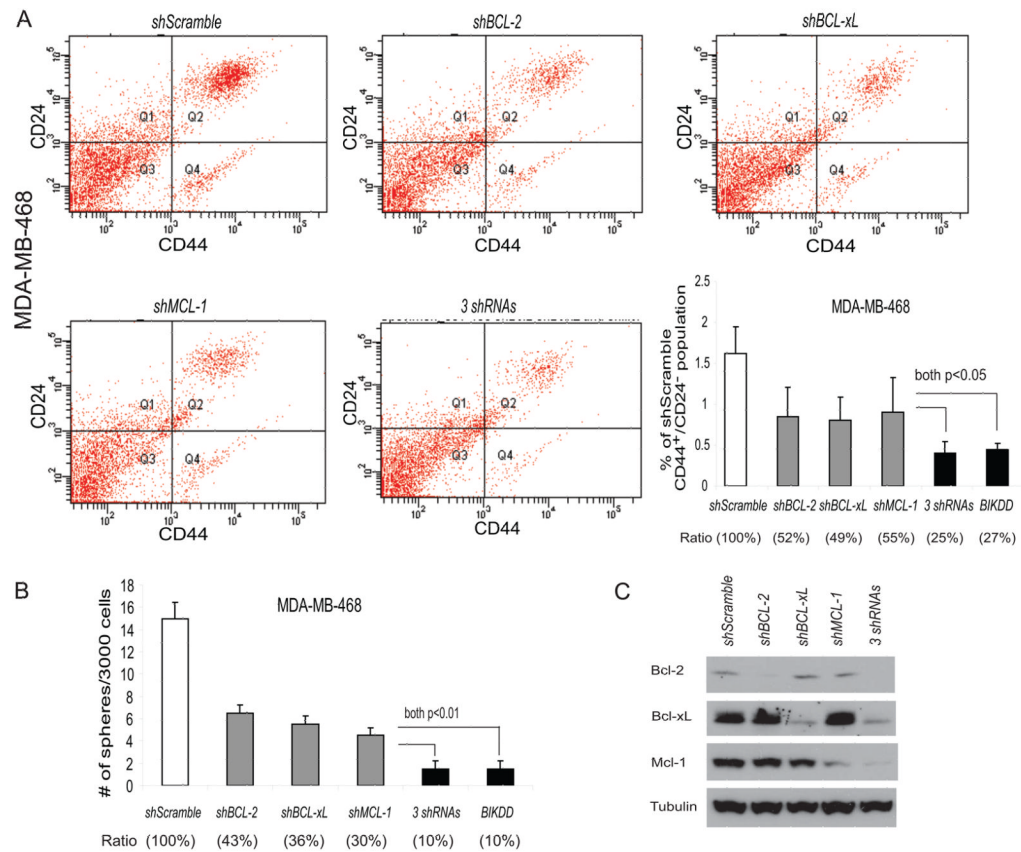


Figure 3. Co-silencing of multiple Bcl-2 antiapoptotic proteins mimicked the proapoptotic effect of BikDD in BCICs

(A) MDA-MB-468 cells were infected with lentivirus containing *BIKDD* or shRNAs against scramble, *BCL-2*, *BCL-xL*, *MCL-1*, or all of them, respectively. Cells from each group were analyzed by flow cytometry 7 days post virus infection. (B) Indicated numbers of cells were subjected to mammosphere culture post infection up to two weeks. (C) The protein expression levels of Bcl-2, Bcl-xL, Mcl-1, and tubulin were detected 7 days after virus infection. All error bars indicate SD. See also Figure S2.

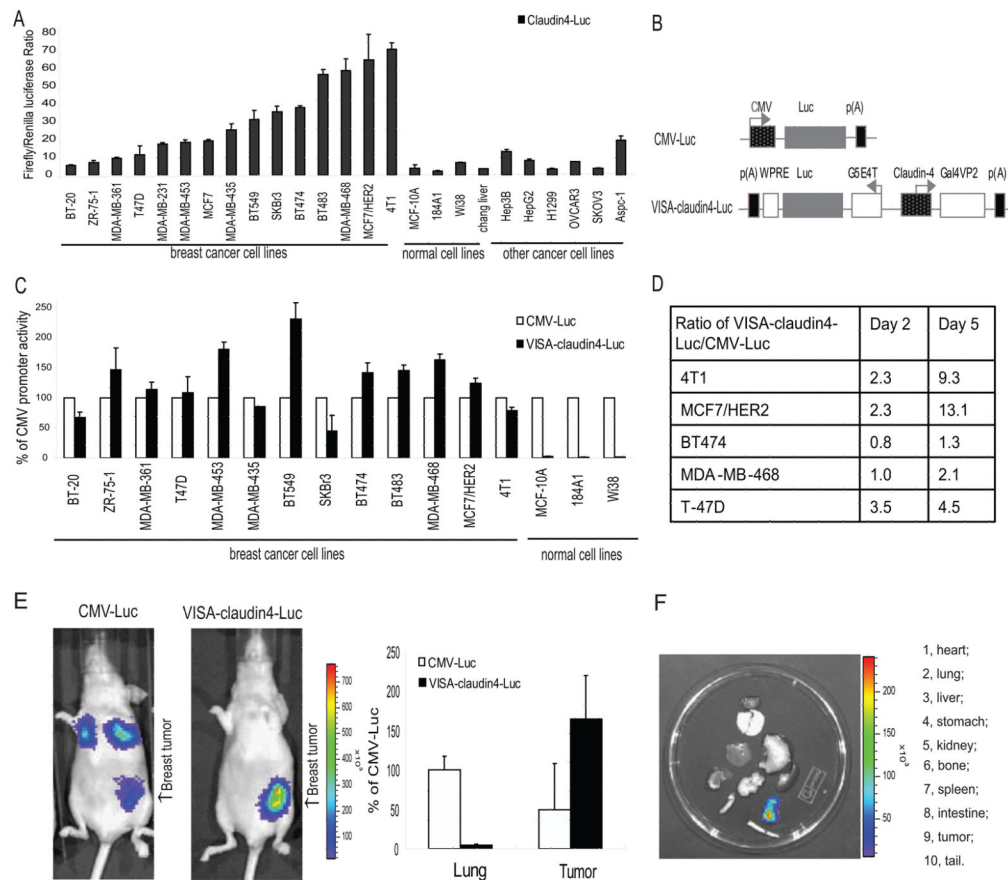


Figure 4. The engineered VISA-claudin4-Luc was robust and selectively expressed in breast cancer cells *in vitro* and *in vivo*

(A) The claudin-4 promoter was selectively activated in breast cancer cell lines. (B) Schematic diagram of engineered claudin-4-based constructs. (C) The promoter activity of engineered VISA-claudin4-Luc was robust and selectively activated in breast cancer cell lines *in vitro*. (D) The promoter activity of VISA-claudin4-Luc was expressed much longer than that of CMV promoter in breast cell lines. The ratio of VISA-claudin4-Luc/CMV-Luc at day 2 and day 5 was presented in table. (E) The engineered VISA-claudin4-Luc was robust and selectively expressed in tumor *in vivo*. Mice were subjected to luciferase imaging 48 hours after injection with 2.5 mg/kg DNA plasmid per mice in 100 μ l plasmid:liposome complex, 5 mice per group. The photon signals were quantified, and the percentage of the photon signals as compared with the CMV-Luc (setting at 100%) was presented. (F) Mice were then dissected, and tumor tissue and other specified organs were removed out and subjected to *ex vivo* imaging. All error bars indicate SD. See also Figure S3.

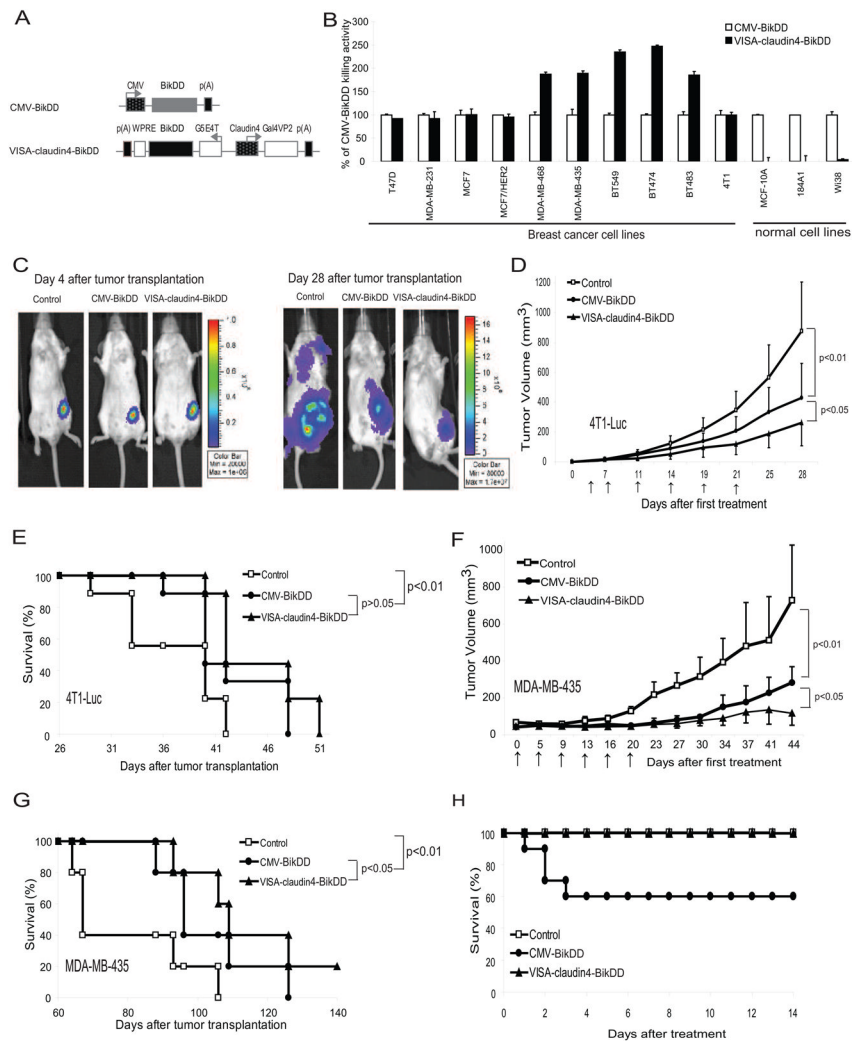


Figure 5. VISA-claudin4-BikDD potently suppressed the growth of breast cancer cells *in vitro* and *in vivo*

(A) Schematic diagram of engineered claudin-4-based constructs. (B) VISA-claudin4-BikDD selectively inhibited the growth of multiple breast cancer cell lines *in vitro*. The percentage of growth inhibition compared to CMV-BikDD is presented. (C–D) VISA-claudin4-BikDD greatly suppressed tumor growth and dissemination of 4T1 mouse breast orthotopic synergetic tumors. Mice were injected with 0.75 mg/kg VISA-claudin4-BikDD or CMV-BikDD as indicated by arrows, respectively. (E) Mice survival was monitored after 28 days post tumor transplantation. (F) VISA-claudin4-BikDD at 0.75 mg/kg significantly reduced the tumor growth of MDA-MB-435 human breast cancer orthotopic xenograft in nude mice. (G) Mice survival was monitored after 60 days post tumor transplantation. (H) VISA-claudin4-BikDD is much safer than CMV-BikDD with intravenous treatment of 5 mg/kg plasmid/Liposome complex. All error bars indicate SD. See also Figure S4.

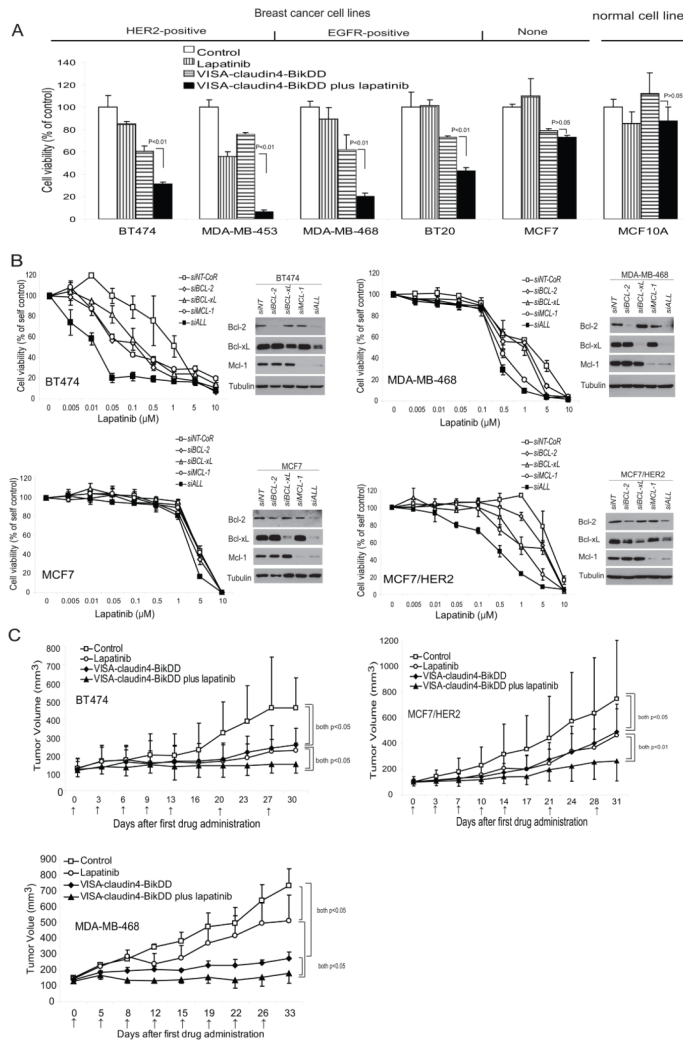


Figure 6. VISA-claudin4-BikDD has synergistic effects with lapatinib in breast cancer cells through antagonism of Bcl-2 antiapoptotic proteins
 (A) VISA-claudin4-BikDD at 0.5 μg sensitized multiple EGFR⁺/HER2⁺ breast cancer cell lines to 1 μM lapatinib, but not in the EGFR⁻/HER2⁻ MCF7 breast cancer cell line and the MCF10A normal breast cell line. The percentage of the cell viability as compared with itself without lapatinib treatment (setting at 100%) was presented. (B) Cancer cells were transiently transfected with siRNAs against *BCL-2*, *BCL-xL*, *MCL-1*, or all of them, respectively, and were subjected to lapatinib at indicated concentrations for 72 hours, and the OD_{570nm} was measured 4 hours after MTT staining. (C) VISA-claudin4-BikDD has synergistic effects with lapatinib in multiple human breast xenograft mouse models. Mice were treated with 0.75 mg/kg VISA-claudin4-BikDD-liposome complexes intravenously as indicated by arrows. Lapatinib was orally administered at 50 mg/kg every other day. All error bars indicate SD. See also Figure S5.

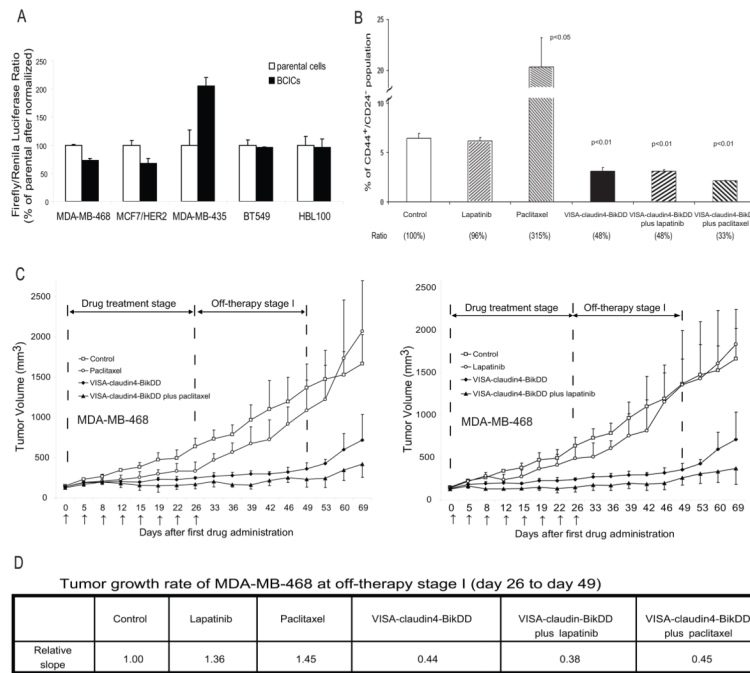


Figure 7. BikDD treatment efficiently reduced the CD44⁺/CD24⁻ population of MDA-MB-468 mammosphere-induced tumors in mice, while VISA-claudin4-Luc was activated in BCICs (A) BCICs from MDA-MB-468, MCF7/HER2, MDA-MB-435, BT549 and HBL100 cells isolated by either FACS sorting or mammosphere culture, were transiently transfected with VISA-claudin4-Luc, with pRL-TK as internal control. The luciferase signal was measured in each population of unsorted parental cells and BCICs, normalized with DNA concentration of firefly/renilla luciferase to exclude the potential different transfection rate. The percentage of the luciferase signals as compared with unsorted total population (setting at 100%) was presented. (B) The CD44⁺/CD24⁻ population of MDA-MB-468 tumor in the different groups were determined at Day 26 (the day of last drug treatment). The P-values shown were obtained by comparison with the control group. (C) For off-therapy study, we monitored tumor growth in different groups after treatment stopped at Day 26 post first drug treatment, and the tumor growth rate (slope) of off-therapy stage (day 26 to day 49) was calculated by exponential trendline in Excel software (D). All error bars indicate SD. See also Figure S6.