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# Selective antitumor effect of neural stem cells expressing cytosine deaminase and interferon-beta against ductal breast cancer cells in cellular and xenograft models

Bo-Rim Yi[a], Kyung-A. Hwang[a], Karen S. Aboody[b], Eui-Bae Jeung[a], Seung U. Kim[c,d], Kyung-Chul Choi[a,*]

[a] *Laboratory of Veterinary Biochemistry and Immunology, College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk, Republic of Korea*
[b] *Department of Neurosciences and Neurosurgery, City of Hope National Medical Center and Beckman Research Institute, Duarte, CA, USA*
[c] *Division of Neurology, Department of Medicine, University of British Columbia, Vancouver, BC, Canada*
[d] *Medical Research Institute, Chung-Ang University College of Medicine, Seoul, Republic of Korea*

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**Abstract** Due to their inherent tumor-tropic properties, genetically engineered stem cells may be advantageous for gene therapy treatment of various human cancers, including brain, liver, ovarian, and prostate malignancies. In this study, we employed human neural stem cells (HB1.F3; hNSCs) transduced with genes expressing *Escherichia coli* cytosine deaminase (HB1.F3.CD) and human interferon-beta (HB1.F3.CD.IFN-β) as a treatment strategy for ductal breast cancer. CD can convert the prodrug 5-fluorocytosine (5-FC) to its active chemotherapeutic form, 5-fluorouracil (5-FU), which induces a tumor-killing effect through DNA synthesis inhibition. IFN-β also strongly inhibits tumor growth by the apoptotic process. RT-PCR confirmed that HB1.F3.CD cells expressed CD and HB1.F3.CD.IFN-β cells expressed both CD and IFN-β. A modified transwell migration assay showed that HB1.F3.CD and HB1.F3.CD.IFN-β cells selectively migrated toward MCF-7 and MDA-MB-231 human breast cancer cells. In hNSC-breast cancer co-cultures the viability of breast cancer cells which were significantly reduced by HB1.F3.CD or HB1.F3.CD.IFN-β cells in the presence of 5-FC. The tumor inhibitory effect was greater with the HB1.F3.CD.IFN-β cells, indicating an additional effect of IFN-β to 5-FU. In addition, the tumor-tropic properties of these hNSCs were found to be attributed to chemoattractant molecules secreted by breast cancer cells, including stem cell factor (SCF), c-kit, vascular endothelial growth factor (VEGF), and VEGF receptor 2. An *in vivo* assay performed using MDA-MB-231/luc breast cancer mammary fat pad xenografts in immunodeficient mice resulted in 50% reduced tumor growth and increased long-term survival

* Corresponding author at: Laboratory of Veterinary Biochemistry and Immunology, College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk 361-763, Republic of Korea. Fax: +82 43 267 3150.
*E-mail address:* kchoi@cbu.ac.kr (K.-C. Choi).


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in HB1.F3.CD and HB1.F3.CD.IFN-β plus 5-FC treated mice relative to controls. Our results suggest that hNSCs genetically modified to express CD and/or IFN-β genes can be used as a novel targeted cancer gene therapy.


## 1. Introduction

Malignant breast cancer may present with symptoms that include a change in breast size or shape, swelling, and/or thickening within the breast tissue (Bevers et al., 2009). When these symptoms are detected early, there is a better chance of successful treatment by means of surgery, chemotherapy and/or hormone therapy resulting in increased survival rates (Lv et al., 2010). Histopathologically, breast cancers are classified into two types depending on the area of origin: ductal and lobular carcinomas. More than 80% of breast cancer cases are ductal carcinoma originating from malignant epithelial cells within the mammary ducts or tubes (Vincent-Salomon and Thiery, 2003). Various risk factors that may be involved in the development of breast cancer include gender, age, female hormone exposure, family history, and genetic factors (Vogel et al., 2007). Specifically, the high risk genes include breast cancer types 1 and 2 susceptibility proteins (BRCA1 and BRCA2), p53, phosphatase and tensin homolog (PTEN), and cell cycle checkpoint kinase 2 (CHEK2) (Lynch et al., 2008). These proteins encoded by BRCA1, BRCA2, p53, PTEN, and CHEK2 genes are involved in control of cell cycle, proliferation, differentiation, or DNA repair and apoptosis of somatic cells (Lim et al., 2009; Wallace et al., 2011).

In this study, we investigated neural stem cell-directed enzyme/prodrug therapy (NDEPT) or a suicide gene therapy designed to more selectively target breast cancer cells and to minimize toxicity to normal tissues (Yi et al., 2012). This system induces cell death in tumor cells by the bystander effect of a suicide enzyme which converts an inactive prodrug into toxic metabolites (Hernandez-Alcoceba et al., 2006). One NDEPT is the cytosine deaminase (CD)/5-fluorocytosine (5-FC) system that has been applied to several types of cancers, including clinical trials for colorectal and prostate cancers and glioblastoma multiform (Fuchita et al., 2009). The CD metabolically converts the prodrug 5-FC into the toxic metabolite 5-fluorouracil (5-FU), which inhibits DNA synthesis and induces apoptosis in cancer cells (Danks et al., 2007). Specifically, cancer cell death results from the cytotoxic activity of 5-fluoro-deoxyuridine-monophosphate (5FdUMP) and 5-fluoro-deoxyuridine-triphosphate (5-FUTP), subsequently converted from 5-FU (Longley et al., 2003). Although 5-FU is a strong antitumor agent and used as chemotherapy for breast, stomach, and colon cancer (Zoli et al., 2005), it has several undesirable neurological and hematological side effects including mucositis, abdominal cramps, vomiting, and tiredness (Volate et al., 2009). Therefore, the development of a more selective delivery system to target the gene product to sites of tumor and not to normal tissues is urgently needed for more effective gene therapy.

Genetically engineered stem cells may have an advantage for gene therapy due to their inherent migration to tumor sites (Kang et al., 2012a; Kang et al., 2012b; Kang et al., 2012d). In this study, we employed HB1.F3 human neural stem cells (hNSCs) obtained from 15 week fetal telencephalon and immortalized using a retroviral vector encoding v-*myc*, and cloned (Yi et al., 2011b). hNSCs have been shown to effectively migrate to the target tumor site, drawn to various chemokines and growth factors such as stem cell factor (SCF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and c-kit that are secreted by proliferative by tumor cells (Aboody et al., 2008). We used two NDEPT paradigms in our breast cancer studies: 1) HB1.F3.CD expressing *Escherichia coli* CD and 2) HB1.F3.CD.IFN-β additionally expressing human IFN-β (Yi et al., 2011a). We previously showed that HB1.F3.CD or HB1.F3.CE (carboxylesterase) cells significantly inhibit the growth of SKOV-3 human ovarian cancer cells in the presence of the prodrug 5-FC or camptothecin-11 (CPT-11), respectively (Kim et al., 2010). Of translational significance, HB1.F3.CD NSCs plus 5-FC is currently in first-in-human phase I clinical trials for recurrent brain tumors (Aboody et al., 2011).

In the current study, CD and IFN-β genes were fused and transduced into HB1.F3 cells for stable expression, to examine the potential cytotoxic effect of the therapeutic products 5-FU (in the presence of 5-FC) and IFN-β on breast cancer cells. Interferons (IFNs) are cytotoxic cytokines that are released by lymphocytes of host cells responding to viral infection and activate immune cells by up-regulating antigen presentation (Yoshiji et al., 2005). IFNs are mainly divided two groups: type I IFN and type II IFN (Sottini et al., 2009). IFN-alpha (IFN-α), IFN-beta (IFN-β), and IFN-omega (IFN-ω) belong to type I IFN class, and IFN-gamma (IFN-γ) belongs to type II IFN (Uze and Monneron, 2007). In humans, IFN-α, IFN-ω and especially IFN-β expression strongly inhibit tumor cell growth and induce apoptosis by Jak-Stat1 intracellular signaling pathways, including immunomodulatory and anti-angiogenic effects, *in vitro* and *in vivo* (Matsuzuka et al., 2009). These functions of the type I IFN family are mediated by a common cell surface receptor known as type I IFN receptor (Pestka et al., 2004; Rosewicz et al., 2004). Despite the strong anti-proliferative effect of IFN-β on cancer cell growth, the underlying mechanism has not been successfully elucidated *in vivo* because of its exceptionally short half-life (Oie et al., 2006). We therefore investigated whether CD.IFN-β expressing hNSCs could migrate to breast cancer cells and exert a more potent therapeutic effect in the presence of 5-FC in a co-culture system *in vitro* and a mouse mammary fat pad xenograft model *in vivo*.

## 2. Materials and methods

### 2.1. Cell culture

Two human breast cancer cell lines, MDA-MB-231 and MCF-7, were purchased from Korean cell line bank (KCLB, Seoul, Korea). Also, MDA-MB-231/luc cells expressing firefly

luciferase as a bioluminescent reporter gene were purchased from KOMA Biotechnology (Seoul, Korea). Three types of NSCs (HB1.F3, HB1.F3.CD, and HB1.F3.CD.IFN-β) generated by us and primary bovine fibroblasts (bovine FBs) were used in these studies (Aboody et al., 2006). Briefly, HB1.F3.CD line was constructed using the retroviral pBabePuro to include the CD cDNA and the cells were selected with 3 μg/ml puromycin. The clonal HB1.F3.CD.IFN-β line was derived from HB1.F3.CD cells. An expression plasmid was constructed using the retroviral pLHCX backbone to include the human IFN-β cDNA. The viral supernatant and HB1.F3.CD cells were incubated in the presence of polybrene (8 μg/ml). The transduced cells were then selected with 5 μg/ml hygromycin (Lee et al., 2009). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone Laboratories, Logan, UT, USA) plus 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories), 100 U/ml penicillin G and 100 μg/ml streptomycin (Cellgro Mediatech, Manassas, VA, USA), 10 mM HEPES (Invitrogen Life Technologies, Carlsbad, CA, USA) and anti-mycoplasmal agents (Invivogene, San Diego, CA, USA) at 37 °C in 5% $CO_2$ and 95% air in a humidified cell incubator.

## 2.2. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from hNSCs and breast cancer cells using TriZol reagent (Invitrogen Life Technologies). Following the manufacturer's protocols, 1 μg of total RNA quantified *via* spectrophotometer (Optizen, Mecasys, Deajeon, Korea) was reverse transcribed into complementary DNA (cDNA) using murine leukemia virus reverse transcriptase (MMLV-RT; iNtRON Biotechnology, Sungnam, Korea), 200 pM nonamer random primer (TaKaRa Bio, Shiga, Japan), 10 pM dNTP (Bioneer, Deajeon, Korea), RNase inhibitor (iNtRON Biotechnology), and 5× RT buffer. To confirm the expression of bacterial CD and human IFN-β genes in hNSCs, cDNA (1 μl) from total RNA of hNSCs was amplified using the respective primers by PCR. PCR was also conducted using the cDNA of cancer cells to amplify the chemoattractant ligands and receptors, including SCF, c-kit, VEGF, and VEGF receptor 2 (VEGFR2) with sense and antisense primers based on the published sequence of the genes, respectively. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a positive control and PCR mixture without a cDNA template negative control. The sense and antisense oligo-sequences and the expected size of RT-PCR products are described in Table 1. The PCR mixture was composed of each 10 pmol sense and antisense primer (Bioneer), 2 unit Taq polymerase (iNtRON Biotechnology), 10× PCR buffer (iNtRON Biotechnology), 5 pmol dNTP mixtures (Bioneer) and template (cDNA). The PCR reactions were performed for 30 cycles in denaturation for 30 s at 95 °C, annealing for 30 s at 58 °C, and extension for 30 s at 72 °C. The reaction products (8 μl) were analyzed on a 1.5% agarose gel pre-stained with ethidium bromide (EtBr; Sigma-Aldrich, St. Louis, MO, USA). The gels were scanned by a Gel Doc 2000 apparatus (Bio-Rad Laboratories, Hercules, CA, USA) and compared to a 100 bp ladder of DNA (iNtRON Biotechnology).

## 2.3. *In vitro* migration assay

To confirm the migration ability of our genetically modified hNSCs toward breast cancer cells, a modified Transwell migration assay was performed. On the first day, breast cancer cells (MCF-7 or MDA-MB-231; 1 × $10^5$ cells/well) and bovine FB cells as a control (1 × $10^5$ cells/well) were seeded in 24-well plates. After incubation for 6 h at 37 °C, the medium was changed to serum free medium for 24 h. Before incubation, bottoms of transwell (8 μm; BD Biosciences, Franklin Lakes, NJ, USA) pre-coated with fibronectin (250 μg/ml; Sigma-Aldrich Corp., St. Louis, MO, USA) were inserted in 24-well plates. On the second day, hNSCs (1 × $10^5$ cells/well) were labeled with CM-DiI (Invitrogen Life Technologies) and plated in the upper transwell chamber. CM-DiI staining was performed for a general protocol. Briefly, hNSCs were trypsinized in a culture dish and treated with CM-DiI solution (2 μM working solution) for 5 min at 37 °C and then for an additional 15 min at 4 °C. After labeling, cells were washed with phosphate buffered saline (PBS) and resuspended in fresh medium. Pre-stained hNSCs were seeded in upper chamber of transwell and incubated in

**Table 1** Sequences of the primers employed and the expected sizes of the PCR products.

| mRNA | | Oligo-sequence (5′- > 3′) | Expected size (bp) |
|---|---|---|---|
| CD | Sense | GCGCGAGTCACCGCCAGCCACACCACGGC | 559 |
| | Antisense | GTTTGTAATCGATGGCTTCTGGCTGC | |
| IFN-β | Sense | AAAGAAGCAGCAATTTTCAG | 296 |
| | Antisense | TTTCTCCAGTTTTTCTTCCA | |
| SCF | Sense | ACTTGGATTCTCACTTGCATTT | 505 |
| | Antisense | CTTTCTCAGGACTTAATGTTGAAG | |
| VEGF | Sense | AAGCCATCCTGTGTGCCCCTGATG | 377 |
| | Antisense | GCTCCTTCCTCCTGCCCGGCTCAC | |
| VEGFR2 | Sense | ACGCTGACATGTACGGTCTAT | 438 |
| | Antisense | GCCAAGCTTGTACCATGTGAG | |
| c-kit | Sense | GCCCACAATAGATTGGTATTT | 570 |
| | Antisense | AGCATCTTTACAGCGACAGTC | |
| hIFNAR2 | Sense | ATTCATATGATTCGCCTGATTAC | 758 |
| | Antisense | GACTTTGGGGAGGCTATTTCTTAA | |
| GAPDH | Sense | ATGTTCGTCATGGGTGTGAACCA | 351 |
| | Antisense | TGGCAGGTTTTTCTAGACGGCAG | |

37 °C. On the third day, breast cancer and bovine FB cells were stained with 4′,6-diamidino-2-phenylindole solution (DAPI, 200 ng/ml; Invitrogen Life Technologies) and incubated in each 24-well for 10 min at 37 °C in the dark. Each well was washed two times with 1× PBS and the cells stained with CM-DiI and DAPI were detected by fluorescent microscopy using an IX71 inverted microscope and the number of red color hNSC on the bottom of transwell was counted (Olympus, Tokyo, Japan).

## 2.4. Cell viability assay

The cytotoxic effects of 5-FC, 5-FU, and three types of hNSCs (HB1.F3, HB1.F3.CD, HB1.F3.CD.IFN-β) on breast cancer cells were determined in the first two experiments. The first experiment was done as follows: on day 1, MCF-7 and MDA-MB-231 cells (4000 cells/well) were seeded in 96-well plates (Thermo Fisher Scientific, Roskilde, Denmark). On day 2, three types of hNSCs (8000 cells/well) were added to the breast cancer cells. On day 3, saline with a range of 5-FC (Sigma-Aldrich Corp.) or 5-FU (Sigma-Aldrich Corp.) concentrations (100, 200, 300, 400, and 500 μg/ml) was added for 4 days. On day 7, the MTT (3-(4-,5-dimethylthiazol-2-yl)-2, 5-dyphenyl tetrazolium bromide) assay was performed to measure cell viability. Ten microliters of MTT (Sigma-Aldrich Corp.) stock solution (5 mg/ml in PBS) was added to each well of a 96-well plate and incubated for 4 h at 37 °C. Precipitated formazan crystals were dissolved in dimethyl sulfoxide (DMSO; Junsei Chemical, Tokyo, Japan). Absorbance of each well was measured at 540 nm using a VERSA man ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA). The second experiment was performed in exactly the same manner, but, on day 2, increasing numbers of hNSCs (8,000, 16,000, and 24,000 cells/well) were added to breast cancer cells in 96-well plates and on day 3, 5-FC (500 μg/ml) was added. MTT assay was performed as previously described. Each experiment was carried out in duplicate with six individual samples (n = 6).

## 2.5. 5-FU treatment

MDA-MB-231 cells were seeded in 6-well plate for one day. Next day, 5-FU diluted with PBS (0, 0.5, 1.0, and 5.0 μg/ml) was treated in each well plate. Total RNA of 5-FU treated breast cancer cells was extracted at 0, 3, 6, and 24 h and RT-PCR was performed to detect human interferon alpha/beta receptor 2 (hIFNAR2).

## 2.6. Breast cancer xenograft models

Thirty-six 6 week old female BALB/c nude mice were purchased from Central Laboratory Animal (SLC, Shizuoka, Japan) and the experiment progressed according to the protocols approved by the Animal Care Committee of Chungbuk National University. Briefly, mice were acclimatized in controlled room for at least 1 week prior to the experiments (at 24–24 °C and 40–60% relative humidity under a 12 h light/dark cycle and frequent ventilation). Since acclimation of animals, breast cancer cells expressing luciferase, MDA-MB-231/luc ($2 \times 10^6$ cells/mouse), were injected into the left mammary fat pad of the mice. Tumor size of these orthotopic xenograft models was measured by using a caliper every week and tumor volume was calculated by 0.5236 × length × width × high.

## 2.7. *In vivo* therapeutic efficacy of HB1.F3.CD and HB1.F3.CD.IFN-β hNSCs

To identify therapeutic efficacy in mammary fat pad xenograft mouse model, mice were divided into four experimental groups; Group 1 was a negative control group treated with 0.85% saline instead of the hNSCs plus 5-FC. Group 2 was treated with HB1.F3 hNSCs plus 5-FC (500 mg/kg/day). Group 3 was treated with HB1.F3.CD hNSCs plus 5-FC (500 mg/kg/day). Group 4 was treated with HB1.F3.CD.IFN-β hNSCs plus 5-FC (500 mg/kg/day). These experiments progressed for 4 weeks post-NSC injection. When tumor volume reached 250 $mm^3$, we injected $8 \times 10^6$ NSCs next to the breast tumor mass. These hNSCs were pre-labeled with a CM-DiI cell tracker prior to injection, for ease of detection post-sacrifice. hNSCs were injected to the adjacent breast cancer mass for a total of three times during the experimental period. Two days after the injection of hNSCs, the Group 1 received intraperitoneal (i.p.) injections of normal saline (100 μl), while Groups 2, 3, and 4 received i.p. injections of 5-FC (500 mg/kg/day in 100 μl saline) everyday for 5 days. Forty-eight hours after the last 5-FC treatment, mice were sacrificed and tumor or skin tissues were harvested for analysis.

## 2.8. *In vivo* bioluminescent imaging

Bioluminescent imaging was performed with a charge-coupled (CCD) camera having photon emitting properties *via* a particular enzyme (luciferase)-substrate (D-luciferin) reaction (Day et al., 2004). For *in vivo* imaging, breast cancer xenografts implanted with MDA-MB-231/Luc were given the luciferase substrate, D-luciferin (Promega, Madison, WI, USA) through an i.p. injection (150 μg/kg) and anesthetized with 1–3% isoflurane. Ten minutes later, bioluminescent tumor cells were detected by the CCD camera mounted in a light-tight box (IVIS™ Xenogen; Caliper Life Sciences, Hopkinton, MA, USA) and tumor regions quantified as total photon counter and photons/s using Living Image controlled imaging analysis software (Caliper Life Sciences).

## 2.9. Histopathology

For histopathological analysis, breast cancer xenografts and skin tissues excised from the mice at necropsy were fixed at 10% normal formalin solution (Sigma-Aldrich Corp.). These fixed samples were cut into 4–6 mm thick sections, embedded in paraffin, and microtome sectioned at a thickness of 5 μm. Prepared slides were stained using hematoxylin (Sigma-Aldrich Corp.) and eosin (Sigma-Aldrich Corp.) staining methods. In stained slides, skin and surrounding tissue basis structure and cancer cells were observed by light microscopy using a model BX51 microscope (Olympus).

## 2.10. DAPI staining

To observe the distribution of hNSC's (Di-I fluorescent red) in the breast cancer mass, 4′,6-diamidino-2-phenylindole (DAPI –

nuclei fluorescent blue) staining was performed in prepared tumor section slides. After rehydration, slides were fixed with 10% normal formalin (Sigma-Aldrich Corp.) solution for 10 min, washed using PBS two times, and treated with DAPI for 10 min at 37 °C. Slides were mounted with coverslips and observed by fluorescent microscopy using an IX71 inverted microscope (Olympus).

### 2.11. Statistical analysis

Data from each experiment are presented as the mean ± standard deviation (S.D.) or the mean ± standard error of the mean (SEM). To evaluate the significance of each *in vitro* and *in vivo* group, statistical analysis was performed by one-way ANOVA Tukey's test using Prism Graph Pad (v5.0; GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Identification of CD and human IFN-β genes

The expression of *E. coli* CD and human IFN-β genes was measured by RT-PCR. CD cDNA was detected at 296 bp in both HB1.F3.CD and HB1.F3.CD.IFN-β cells and human IFN-β cDNA at 559 bp in only HB1.F3.CD.IFN-β cells (Fig. 1), thus demonstrating the effective and stable transduction of these genes into HB1.F3 hNSCs. The GAPDH gene was detected at 351 bp in HB1.F3, HB1.F3.CD, and HB1.F3.CD.IFN-β cells (Fig. 1).

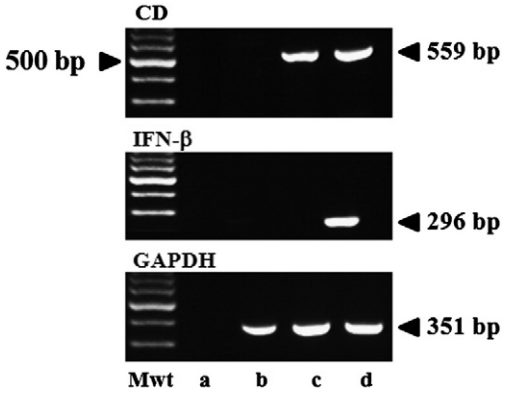


**Figure 1** RT-PCR of *E. coli* CD and human IFN-β gene expression in engineered hNSCs. After total RNA extraction from HB1.F3.CD and HB1.F3.CD.IFN-β cells, cDNAs were synthesized by reverse transcription (RT) reaction. The cDNA was amplified by PCR and PCR product was confirmed *via* 1.5% agarose gel electrophoresis. *E. coli* CD and human IFN-β cDNA were detected at 559 bp and 296 bp, respectively. GAPDH was used to a positive control and detected at 351 bp. PCR mixture without a cDNA template is a negative control. Mwt; 100 bp molecular weight marker, a; Negative control (without cDNA template), b; HB1.F3, c; HB1.F3.CD, d; HB1.F3.CD.IFN-β.

### 3.2. *In vitro* migration of hNSCs toward breast cancer cells

A modified Transwell migration assay was performed to measure the capacity of the hNSCs to migrate toward breast cancer cells. hNSCs (DiI labeled) demonstrated significant migration to MCF-7 and MDA-MB-231 cells, but not to bovine FB cells (Fig. 2A). The number of migrated hNSCs to MCF-7 or MDA-MB-231 breast cancer or bovine FB cells on the bottom of transwell was counted using fluorescent microscopy. Results confirmed significant migration of hNSCs to breast cancer cells *vs.* control non-tumorigenic fibroblasts. Each experiment was performed independently and presented as mean ± S.D. for verification of migrated hNSC number compared to migrated bovine FB (Fig. 2B). It was noted that there was no significant difference in the migration between the HB1.F3, HB1.F3.CD and HB1.F3.CD.IFN-β hNSCs. These results suggest that transgene introduction into stem cells does not affect their migration to breast cancer cells.

In addition, a potential interaction of chemoattractant ligands and their receptors would be a major reason for tumor-tropic effect of engineered stem cells *in vitro* and *in vivo*. Using a general protocol of RT-PCR, we confirmed the expressions of chemoattractant factors, *i.e.*, SCF, VEGF, VEGFR2, and c-kit, in breast cancer and HB1.F3 cells. The chemoattractant ligands, SCF and VEGF, were strongly expressed in MDA-MB-231 and MCF-7 breast cancer cells, while their receptors including c-kit and VEGFR2 were detected in HB1.F3 cells as shown in Fig. 2C.

### 3.3. Therapeutic efficacy of HB1.F3.CD and HB1.F3.CD.IFN-β hNSCs with 5-FC *in vitro*

To determine the cytotoxic effects of 5-FC and 5-FU on breast cancer cells, the MTT assay was performed. Although treatment with 5-FC did not induce any effect of cancer cell growth, 5-FU markedly decreased cell proliferation by approximately 80% (Fig. 3A). Next, using co-culture system with MDA-MB-231 or MCF-7 cells, we measured the cytotoxic activity of hNSCs on breast cancer cells at different concentrations of 5-FC (100 to 500 μg/ml). Cell proliferation of breast cancer cells was significantly inhibited by HB1.F3.CD and HB1.F3.CD.IFN-β cells in the presence of 5-FC. When the number of hNSCs was fixed, the inhibition effect was increased with increased concentrations of 5-FC. When we compared the cytotoxic effects between HB1.F3.CD.IFN-β and HB1.F3.CD hNSCs presence of 5-FC on breast cancer cells, significantly stronger inhibition was observed by HB1.F3.CD.IFN-β cells compared to HB1.F3.CD cells ($P < 0.05$ for MDA-MB-231 cells). These data indicate that IFN-β has an additional cytotoxic effect to 5-FU as seen in Fig. 3B. Specifically, the cell viability of MDA-MB-231 cells was decreased by 60% when treated with HB1.F3.CD.IFN-β cells as compared to a decrease in cell viability by 40% when treated with the HB1.F3.CD cells under the same conditions ($P < .05$).

### 3.4. Upregulation of hIFNAR2 expression in MDA-MB-231 cells

To confirm the change of hIFNAR2 expression, serial diluted 5-FU (0, 0.5, 1.0, and 5.0 μg/ml) was treated in 6-well plate

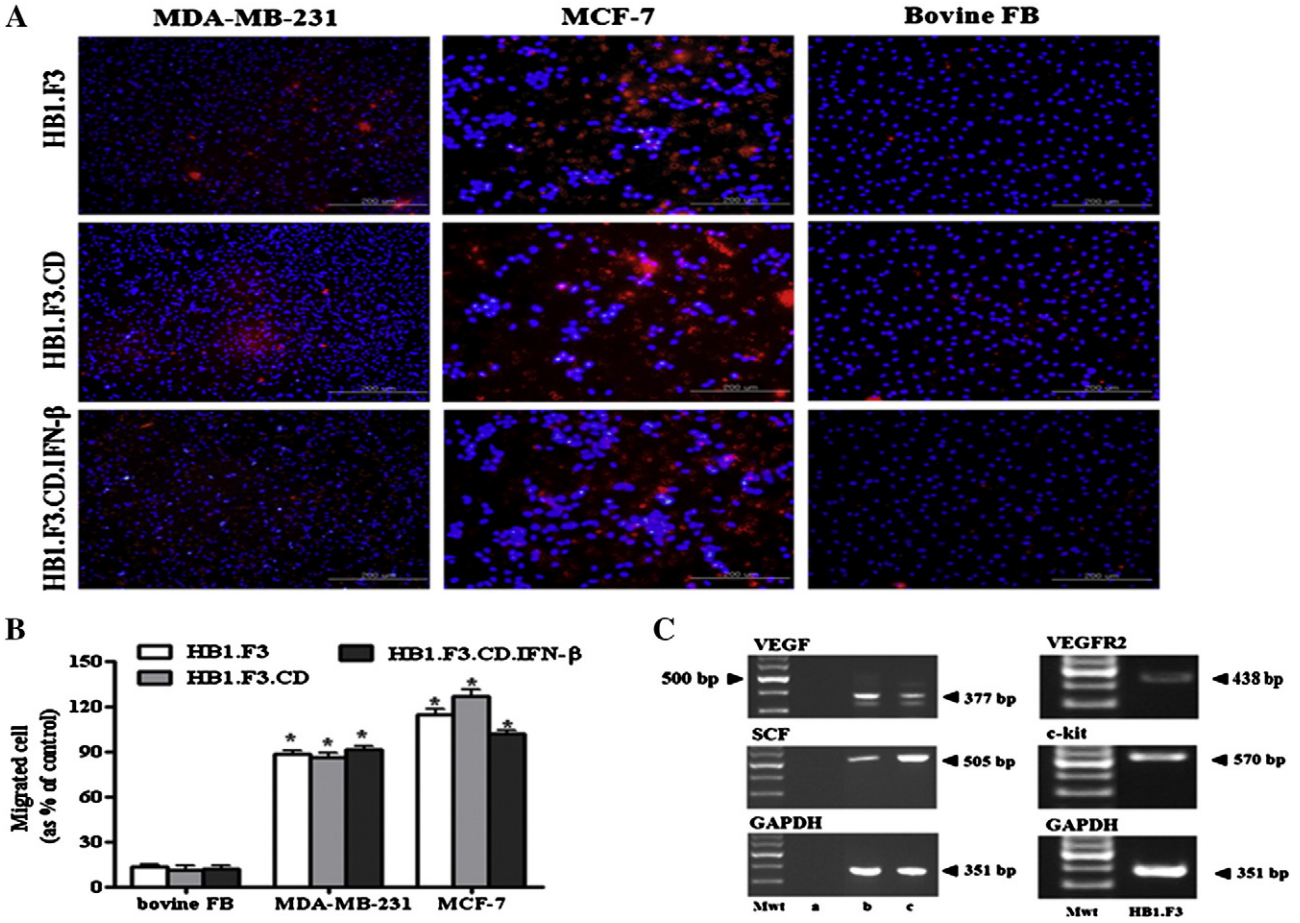


**Figure 2** *In vitro* migration of hNSCs toward breast cancer cells. MDA-MB-231 and MCF-7 breast cancer cells or bovine FB cells (as a control) were seeded with 10% FBS culture media in 24-well plates ($1 \times 10^5$ cells/well). Next day, CM-DiI pre-labeled hNSCs: HB1.F3, HB1.F3.CD, and HB1.F3.IFN-β ($1 \times 10^5$ cells/well) were added to each upper Transwell chamber for 24 h. The cancer cells and bovine FB in the 24-well plate were stained with DAPI. A, The migration of hNSCs to MDA-MB-231, MCF-7 and bovine FB cells was detected under the microscope. Blue: DAPI stained nuclei of breast cancer or bovine FB cells in the each well, Red: CM-DiI hNSCs (HB1.F3, HB1.F3.CD, and HB1.F3.CD.IFN-β). B, The number of migrated hNSCs to breast cancer or bovine FB cells was counted using fluorescent microscopy. Each experiment was performed independently and presented as mean ± S.D. *$P < 0.05$ *vs.* a bovine FB. C, Chemoattractant molecules, *i.e.*, SCF, VEGF, VEGFR2, and c-kit, were identified in breast cancer cells and stem cells (HB1.F3) by RT-PCR. Scale bar: 200 μm, Mwt: 100 bp molecular weight marker a; negative control without cDNA template, b; MDA-MB-231, c; MCF-7.

seeded in MDA-MB-231 cells and total RNA was extracted from MDA-MB-231 cells after 0, 3, 6, and 24 h. In normal state (not treated 5-FU), IFNAR2 gene was weakly or rarely expressed in MDA-MB-231 cells according to treatment time and dose. Enhanced mRNA expression levels for hIFNAR2 were observed in breast cancer cells treated with even small amount of 5-FU (0.5, 1.0, and 5.0 μg/ml) as shown in Fig. 4A. Also, expression of this gene was increased in a time-dependent manner. When treated with 1.0 μg/ml of 5-FU, the expression of hIFNAR2 began to increase from 6 h until 24 h. Finally, 5-FU induced an enhanced expression of hIFNAR2 gene up to about 2.3-fold in MDA-MB-231 cells (Fig. 4B).

### 3.5. Tumor mass measurement in xenograft models

Four weeks after the injection of MDA-MB-231/luc cells to female BALB/c nude mice, a breast tumor was formed in left mammary fat pad of each mouse. Overall experimental schedule is presented in Fig. 5A. From the sixth week during the experiment period, the growth of each breast tumor mass was inhibited by HB1.F3.CD or HB1.F3.CD.IFN-β cells with treatment of 5-FC (500 mg/kg/day). The tumor mass was inhibited by approximately 50% in both groups compared to control group (Figs. 5B and C). This result clearly demonstrates the therapeutic efficacy of HB1.F3.CD and HB1.F3.CD.IFN-β cells *in vivo*. But, HB1.F3.CD.IFN-β cells did not seem to have the additional effect in decreasing the tumor volume compared to HB1.F3.CD cells, as demonstrated *in vitro*.

### 3.6. Bioluminescence imaging of breast cancer growth and survival rate in xenograft models

We also observed tumor state of xenografts more vividly using the bioluminescence imaging assay. Because MDA-MB-231/luc cells implanted into a mouse emit the photons in the presence of D-luciferin, the value of emitted photons measured by CCD

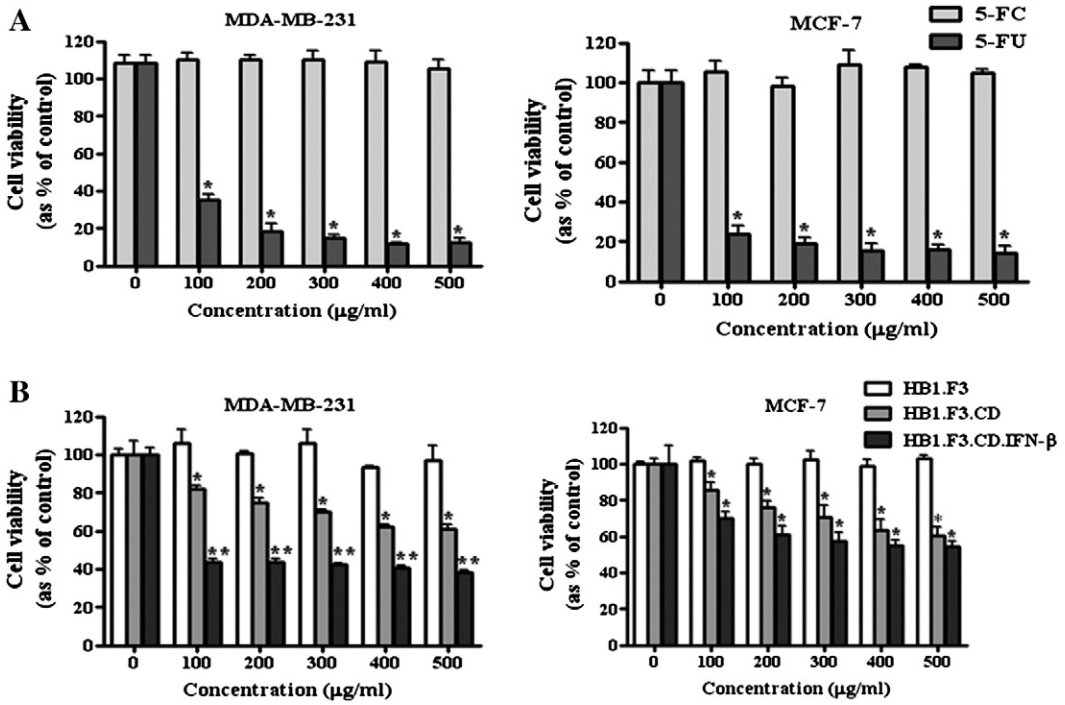


**Figure 3** Therapeutic efficacy of HB1.F3.CD and HB1.F3.CD.IFN-β with 5-FC *in vitro*. After MDA-MB-231 or MCF-7 ($4 \times 10^3$ cells/well) were seeded in 96-well plates for 24 h, stem cells were co-cultured in the presence of 5-FC for another 24 h. After 4 days, the cell viability was measured by MTT assay. A, The cytotoxic effect on breast cancer cells of various concentrations of 5-FC or 5-FU (100, 200, 300, 400, and 500 μg/ml) was measured. B, The breast cancer cells were cultured with HB1.F3, HB1.F3.CD, or HB1.F3.CD.IFN-β hNSCs ($8 \times 10^3$ cells/well) and treated with increasing concentrations of 5-FC (100, 200, 300, 400, and 500 μg/ml). Each experiment was carried out independently and presented as mean ± S.D. *$P < 0.05$ *vs.* HB1.F3, **$P < 0.05$ *vs.* HB1.F3.CD.

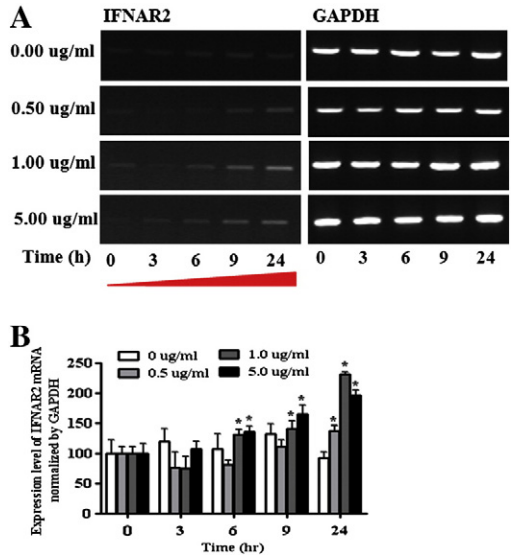


**Figure 4** Potentiation of human interferon alpha/beta receptor 2 (hIFNAR2) activation by 5-FU. Various 5-FU diluted with PBS (0, 0.5, 1.0, and 5.0 μg/ml) were treated in 96-well plate seeded MDA-MB-231. Total RANs of MDA-MB-231 cells were extracted in each time (0, 3, 6, 9, and 24 h). cDNA synthesized from each sample was performed the PCR reaction using by IFNAR2 specific primer. A, hIFNAR2 gene was confirmed by 1.5% agarose gel electrophoresis pre-stained with EtBr. B, Quantification of PCR product was shown in a graft by Quantity One analysis system. Date are represented as mean ± SD. *, $P < 0.05$ *vs.* 0 h as a control.

camera in bioluminescent imaging precisely represented the tumor size in living animals. Results show that the photon quantity was significantly decreased in mice injected with HB1.F3.CD and HB1.F3.CD.IFN-β cells, while it continuously increased in the mice injected with saline or HB1.F3 cells only (Figs. 6A and B). In addition, the treatment with HB1.F3.CD.IFN-β cells resulted in a significant decrease in the tumor volume as compared to HB1.F3.CD cells, indicating the additional antitumor activity of IFN-β in the presence of 5-FU in an *in vivo* mouse xenograft model.

When we measured the survival rate, the mice treated with HB1.F3.CD.IFN-β cells survived significantly longer than any other groups ($P < 0.05$). Whereas the mice treated with HB1.F3.CD.IFN-β cells plus 5-FC showed a survival rate of 80% at 14 weeks, the mice with HB1.F3.CD cells showed 30% survival at 13 weeks, and all died at 14 weeks. All the mice of the negative control and HB1.F3 treated groups died by week 8 as shown in Fig. 6C. These data further confirm the stronger anti-cancer effect *in vivo* of HB1.F3.CD.IFN-β cells as compared to the HB1.F3.CD cells in the presence of 5-FC. These data support the more potent effect of CD/5-FC plus IFN-β than CD/5-FC alone in the killing effect against ductal breast cancer cells.

### 3.7. Histological and fluorescent analysis of breast cancer mass and circumferential tissues

To assess alterations in the histopathological aspects of the tumor mass and its surrounding tissues, we previously

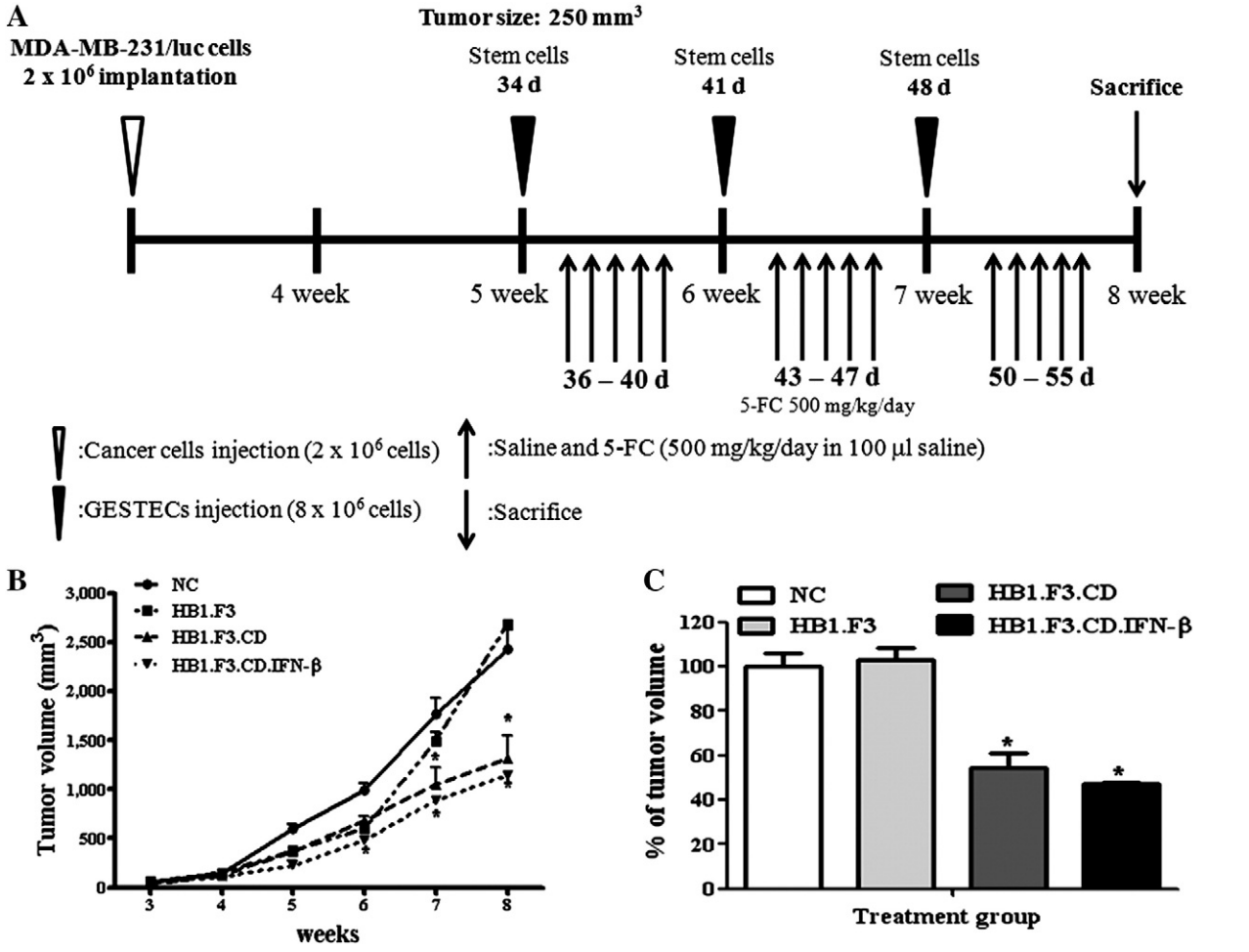


**Figure 5** Experimental schedule of *in vivo* test and measurement of tumor mass volume. A, Xenograft models were manufactured by implanting MDA-MB-231/luc cells to female BALB/c nude mice. Four week after cancer cell injection, pre-stained hNSCs ($8 \times 10^6$ cells/mouse) were injected nearby tumor mass. Two days later, 5-fluorocytosine (5-FC, 500 mg/kg/day) was treated every day. And the animals were sacrificed 2 days after last injection. B, Formed tumor sizes were measured for 8 weeks and the tumor volumes were calculated by 0.5236 × length × width × high. C, Tumor volumes measured at the final week were shown in the graph. Data points are represented as mean ± SEM. *, $P < 0.05$ *vs.* NC. NC, negative control.

observed the normal tissue structure of the mammary fat pad in a mouse (*i.e.*, epidermis, dermis, fat, and muscle layer) as shown in Fig. 7A. In control and HB1.F3 treated groups, the normal structure of mammary fat pad was ravaged by the invasive growth of breast cancer cells. However, we confirmed that the growth of implanted tumor cells was inhibited inside the tumor mass and the tissue structure was maintained relatively intact in the mice implanted with HB1.F3.CD and HB1.F3.CD.IFN-β cells plus 5-FC (Fig. 7B). Additionally, in the mice with HB1.F3.CD.IFN-β cells, the nuclear size of the tumor cells was decreased and macrophages and master cells related with immune response emerged at the dermis and fat layers compared to control and the mice injected with HB1.F3 only. To explain these phenomena (*i.e.*, immune response in the marginal skin, tumor growth inhibition, and the preservation of tissue structure by hNSCs), we performed a fluorescent analysis using CM-DiI pre-labeled hNSCs injected adjacent to the ductal breast tumor mass. We found the positioning of the red fluorescent hNSCs inside the tumor mass as demonstrated in Fig. 8, indicating that these hNSCs effectively distributed through the tumor mass and exerted their anti-tumor activity by the products of delivered genes. It is also noteworthy that more HB1.F3.CD.IFN-β cells were detected at tumor sites than HB1.F3.CD cells following treatment with 5-FC, despite the same number hNSCs injected.

## 4. Discussion

Conventional cancer therapies (radiotherapy, hormone therapy, and chemotherapy) for breast cancer have many undesirable side effects (McArthur et al., 2011; Piccart, 2003). Gene therapy delivered by a packaging line or viruses with activated 'suicide' genes appears to selectively treat cancers, however the packaging line stayed at or near injection sites and virus only reached neighboring tumor cells. The inability of virus to deliver therapeutic genes to invading and distant tumor sites greatly limits its effectiveness. The enzyme/prodrug distribution and efficacy of gene delivery mediators, *i.e.*, adenovirus and lentivirus, is also questionable (Pastorakova et al., 2006). A

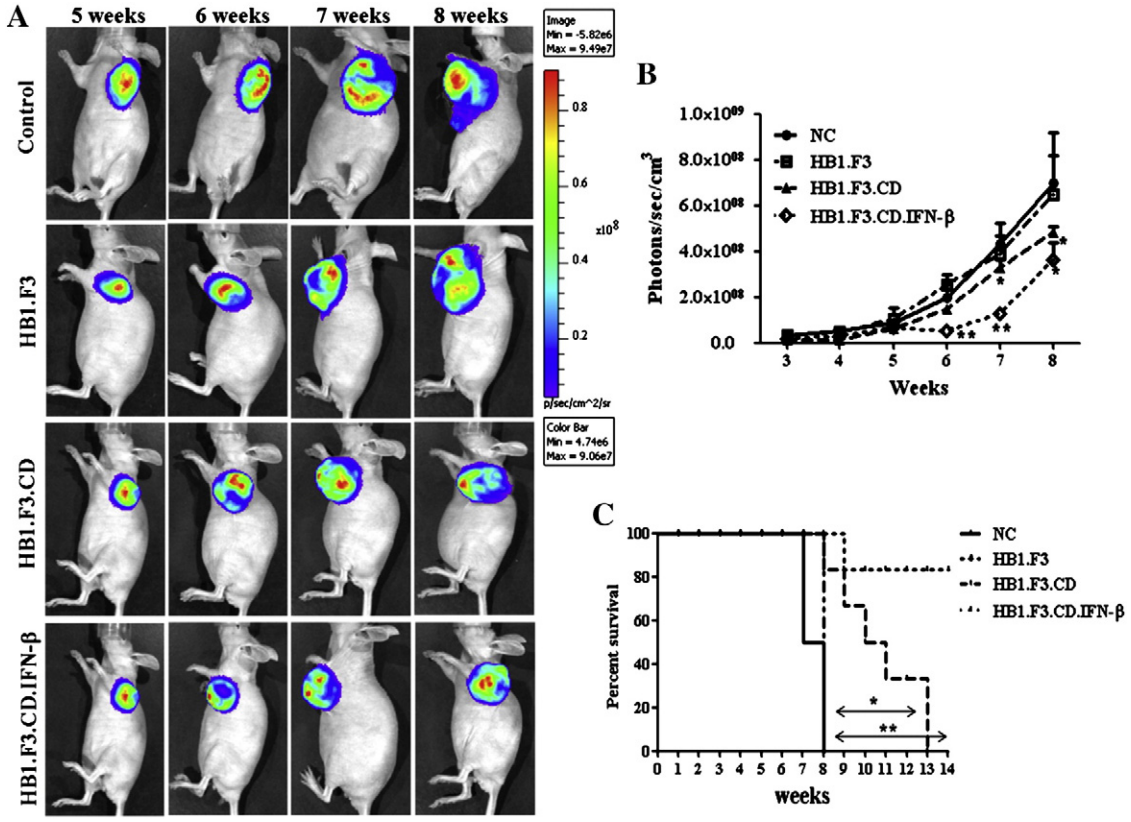


**Figure 6** Bioluminescent imaging by luciferase in breast cancer xenografts and survival rate. A, D-luciferin, a substrate of luciferase (150 μg/kg) was injected *via* intraperitoneal injection in mice. At 10 min after injection, the converted D-luciferin was measured in the value of emitted photons. During the experimental period, alteration of tumor size in each group was shown by bioluminescent imaging by CCD camera. B, Photon values of tumor mass were displayed in the graph. C, The survival rate for the breast cancer xenografts was measured during the experimental period, and expressed as a percent of the initial number of mice in each group. The mice injected with HB1.F3.CD.IFN-β cells showed 80% survival rate at the end of experiment. Data are represented as mean ± SEM. *, $P < 0.05$ *vs.* a negative control, **$P < 0.05$ *vs.* HB1.F3.CD. NC: negative control.

tumor specific delivery system is critical for enhancing the selective effectiveness of well-known prodrugs for *de novo* various tumors. NSCs overcome these limitations of current gene therapy systems by targeting invasive and distant tumor foci and expressing the 'suicide' genes locally. To minimize systemic toxicities, NDEPT has received much attention for cancer-targeted therapy (Kim et al., 2010; Nawa et al., 2008). These gene therapies use viral vectors as vehicles to transport prodrug activating or 'suicide' genes, which convert a non-toxic prodrug to a toxic agent (Anderson, 2000). This leads to a bystander effect, where not only the suicide gene transduced vehicle cells, but also the untransfected neighboring target cells are eradicated (Kang et al., 2012d). Typical enzyme/prodrug therapy systems are CD/5-FC, CE/CPT-11, and thymidine kinase (TK)/ganciclovir (GCV) (Kang et al., 2012c; Shah, 2012). In previous studies, the antitumor effects of CD/5-FC and CE/CPT-11 were demonstrated in animal models of disseminated neuroblastoma (Kim et al., 2010), intracranial medulloblastoma (Kim et al., 2006a), and glioma (Kang et al., 2012b; Longley et al., 2003).

Recently, inherently tumor-tropic stem cells have received a great deal of attention for their clinical potential to treat diverse human cancers. For example, hNSCs are reported to be very effective in delivering various therapeutic agents in preclinical models of malignant brain tumors including medulloblastoma and glioma (Kim et al., 2006a, 2006b; Thu et al., 2009) and other invasive tumors (Kim et al., 2010; Longley et al., 2003). Mesenchymal stem cells (MSCs) have also been employed as a systemic delivery vehicle for therapeutic genes to breast cancer because they can home to tumor sites and evade host immune responses (Conrad et al., 2011; El Haddad et al., 2011; Niess et al., 2011).

In the current studies, we employed HB1.F3 cells, an immortalized human clonal NSC line, as a therapeutic gene delivery vehicle to target breast cancer and to disseminate gene products throughout tumor sites (Kim et al., 2002). HB1.F3, one of NSCs, is homogeneous since they are generated from a single clone and can be stably expanded to large numbers *in vitro*. It is of interest to note that the HB1.F3 can migrate to subcutaneous xenografts of various non-neural solid tumors types, including prostate, breast, and melanoma, indicating a broader range of potential clinical application (Aboody et al., 2006). To investigate therapeutic applications to breast cancer, we engineered HB1.F3 cells to stably express the therapeutic genes CD (HB1.F3.CD) and human IFN-β (HB1.F3.CD.IFN-β). We hypothesized that this NDEPT approach has the potential to

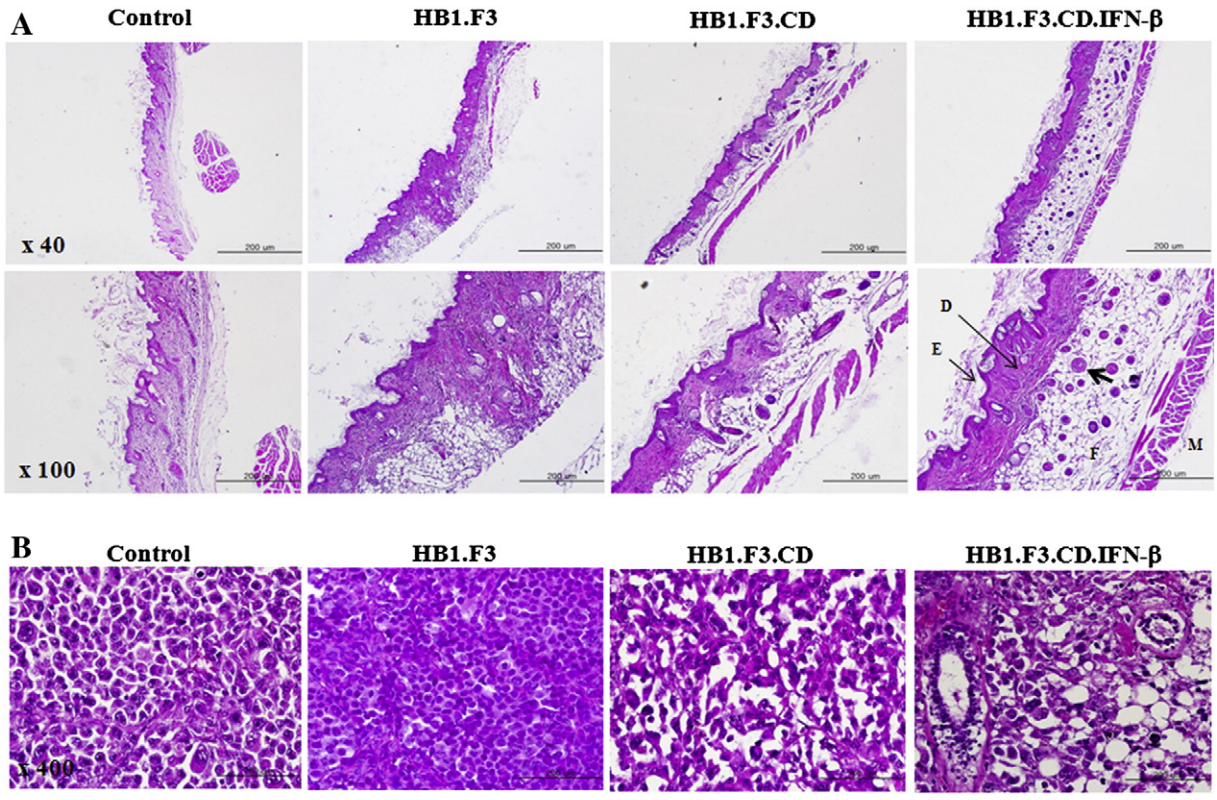


**Figure 7** Histological analysis of breast tumor mass and skin extracted from mice. Breast cancer tissues excised from the mice at necropsy were fixed at 10% normal formalin solution. These fixed samples were cut into 4–6 mm thick sections, embedded in paraffin, and microtome sectioned (5 μm). Tissue sections were stained with hematoxylin and eosin (H&E). A, Tumor structure of mammary pad of the mice in each group. E; epidermis, D; dermis, F; fat layer, M; muscle layer, large arrow; immune responded cells. Magnifications ×40 and ×100. B, High power views of H&E stained tumor sections. Magnification ×400.

improve clinical outcome, while minimizing toxic side effects. We first verified the tumor-tropism of our genetically modified NSC lines in a modified Transwell assay, which demonstrated significant directed migration to MCF-7 and MDA-MB-231 human breast tumor lines, but not to non-tumorigenic bovine FB cells. Although the specific molecular mechanism of NSC tumor-tropism has not been clearly elucidated, numerous chemokines are likely involved. Zhao et al. have recently demonstrated the role of IL-6 in NSC migration toward breast cancer metastases (Zhao et al., 2012). In this study, we confirmed that MCF-7 and MDA-MB-231 breast cancer cells strongly expressed chemoattractant ligands including SCF and VEGF, while the HB1.F3 cells showed the corresponding receptors, c-kit and VEGFR2, likely contributing to their tropic and migratory capacity. In particular, MDA-MB-231 cells expressed high levels of VEGF, which is a chemoattractant factor and a primary stimulant of angiogenesis (Di Domenico et al., 2010). Further study is required to verify the role of these factors in the mechanism of tumor cell recognition and/or tumor tropism by stem cells.

Next, in the cell viability study, HB1.F3.CD.IFN-β and HB1.F3.CD cells showed a high rate of cytotoxic activity on breast cancer cells, particularly MDA-MB-231 cells, in the presence of the prodrug 5-FC. Greater growth inhibition was observed for HB1.F3.CD.IFN-β cells compared to HB1.F3.CD, indicating that an additional cytotoxic effect of IFN-β resulting from the immunotherapy along with 5-FU generated from CD/5-FC may provide a 'double-punch' strategy of chemotherapy. 5-FU has been reported to induce the expression of IFN α/β receptor (IFNAR), which plays a role in increasing the cytokine activity of IFN-β (Oie et al., 2006; Wagner et al., 2004). In our study, enhanced mRNA expression of hIFNAR2 was observed at the concentrations of 5-FU (0.5, 1.0, and 5.0 μg/ml). Thus, the susceptibility of breast cancer cells to IFN-β appears to be increased by the 5-FU generated from CD/5-FC in this system. It has been previously reported that other immune responses or apoptotic factors may be involved in NDEPT-induced antitumor effects (Qiao et al., 2000). Thus, further studies are warranted to clarify the underlying mechanism of IFN-β with CD/5-FC system.

Finally, we confirmed the therapeutic efficacy of our NSC-mediated approach *in vivo* in a mammary fat pad ductal breast cancer xenograft model. NSCs expressing CD +/− IFN-β significantly inhibited growth of tumor masses. Specifically, in both bioluminescent imaging and long-term survival analysis, the HB1.F3.CD.IFN-β group showed a stronger inhibitory effect on tumor growth than the HB1.F3.CD group, consistent with the cell viability assay *in vitro*. The effect of increased lifespan can be induced by transduced human IFN-β in HB.F3.CD.IFN-β because of the adaptive immune system in a xenograft mouse model.

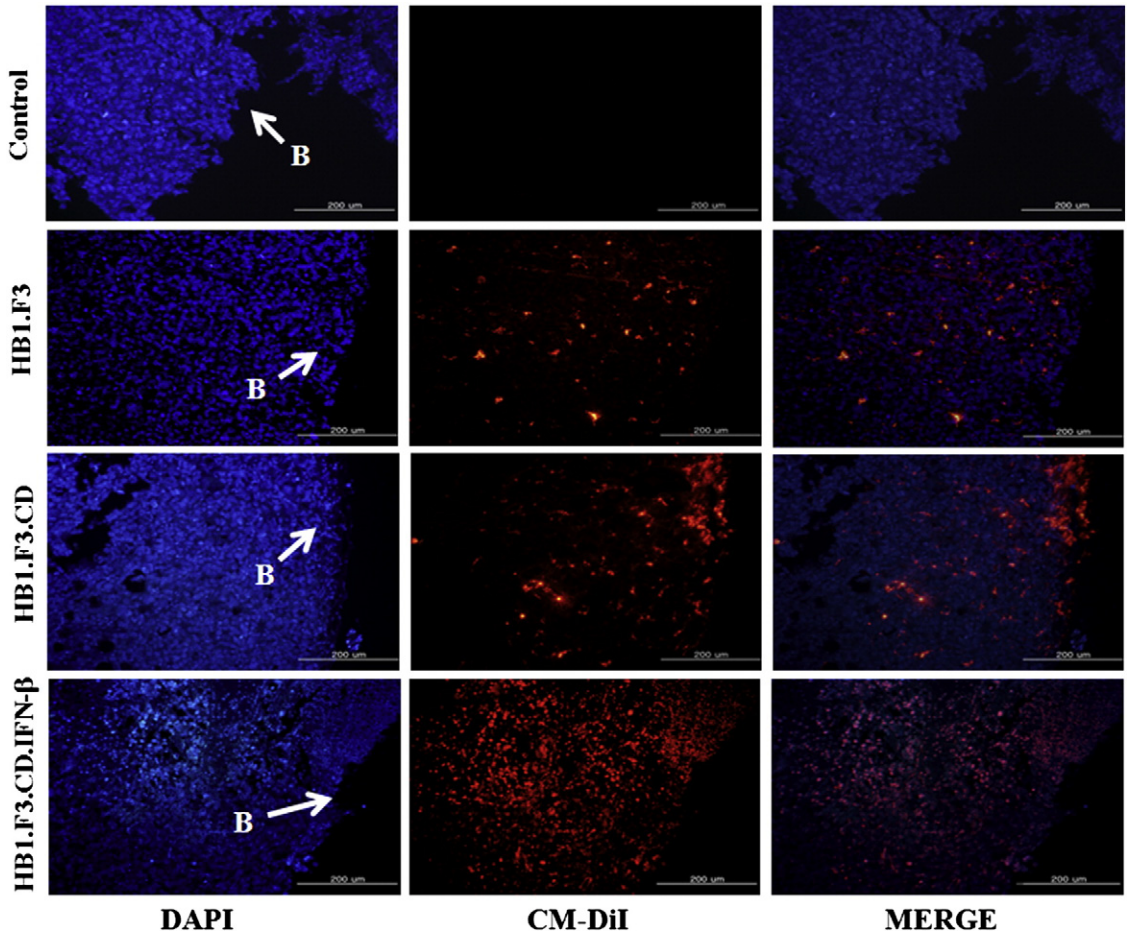


**Figure 8** Fluorescent analysis of breast tumor mass. hNSCs were pre-labeled with CM-DiI cell tracker prior to injection. After preparing tumor section slides, DAPI counterstaining was performed. Stained slides were mounted with coverslips and observed by a microscopy. Magnification ×100, Blue: DAPI stained nuclei of breast cancer cells (dense blue) and hNSCs, Red: CM-DiI labeled hNSCs, B: boundary.

Histological analysis of tumor sections of mice that received the therapeutic NSCs displayed preservation of normal structures, *i.e.*, epidermis, dermis, fat, and muscle layer. On the other hand, the tissue structures of control mice injected with non-modified HB1.F3 cells were damaged by the progressive growth of breast cancer cells. Therefore, we suggested that modified NSCs have a strong therapeutic potential for prolongation of life-span.

In summary, we confirm that HB1.F3.CD and HB1.F3.CD.IFN-β cells selectively migrated toward breast cancer cells. This is likely due to various chemokines expressed by the tumor cells and their cognate receptors expressed on the stem cells, such as SCF/c-kit and VEGF/VEGFR2, respectively. The viability of breast cancer cells was significantly reduced by co-culture with HB1.F3.CD and HB1.F3.CD.IFN-β in the presence of the prodrug 5-FC. More effective inhibition was observed by HB1.F3.CD.IFN-β compared to HB1.F3.CD. *In vivo* mouse mammary fat pad xenograft efficacy studies revealed that HB1.F3.CD.IFN-β cells exerted more potent anticancer effect by simultaneously expressing fusion gene products of CD and IFN-β. Taken together, these results suggest that our engineered NSCs may provide a novel ductal breast cancer treatment approach using a double-punch CD/5-FC enzyme/prodrug and IFN-β immunotherapy strategy.

## Disclosure of Conflict of interest

KSA and SUK are co-founders and shareholders of TheraBiologics Inc., a clinical stage biopharmaceutical company supporting the development of NSC-mediated therapies for cancers.

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## References

Aboody, K.S., Bush, R.A., Garcia, E., Metz, M.Z., Najbauer, J., Justus, K.A., Phelps, D.A., Remack, J.S., Yoon, K.J., Gillespie, S., Kim, S.U., Glackin, C.A., Potter, P.M., Danks, M.K., 2006. Development of a tumor-selective approach to treat metastatic cancer. PLoS One 1, e23.

Aboody, K.S., Najbauer, J., Danks, M.K., 2008. Stem and progenitor cell-mediated tumor selective gene therapy. Gene Ther. 15, 739–752.

Aboody, K., Capela, A., Niazi, N., Stern, J.H., Temple, S., 2011. Translating stem cell studies to the clinic for CNS repair: current

state of the art and the need for a Rosetta Stone. Neuron 70, 597–613.

Anderson, W.F., 2000. Gene therapy scores against cancer. Nat. Med. 6, 862–863.

Bevers, T.B., Anderson, B.O., Bonaccio, E., Buys, S., Daly, M.B., Dempsey, P.J., Farrar, W.B., Fleming, I., Garber, J.E., Harris, R.E., Heerdt, A.S., Helvie, M., Huff, J.G., Khakpour, N., Khan, S.A., Krontiras, H., Lyman, G., Rafferty, E., Shaw, S., Smith, M.L., Tsangaris, T.N., Williams, C., Yankeelov, T., 2009. NCCN clinical practice guidelines in oncology: breast cancer screening and diagnosis. J. Natl. Compr. Cancer Netw. 7, 1060–1096.

Conrad, C., Husemann, Y., Niess, H., von Luettichau, I., Huss, R., Bauer, C., Jauch, K.W., Klein, C.A., Bruns, C., Nelson, P.J., 2011. Linking transgene expression of engineered mesenchymal stem cells and angiopoietin-1-induced differentiation to target cancer angiogenesis. Ann. Surg. 253, 566–571.

Danks, M.K., Yoon, K.J., Bush, R.A., Remack, J.S., Wierdl, M., Tsurkan, L., Kim, S.U., Garcia, E., Metz, M.Z., Najbauer, J., Potter, P.M., Aboody, K.S., 2007. Tumor-targeted enzyme/prodrug therapy mediates long-term disease-free survival of mice bearing disseminated neuroblastoma. Cancer Res. 67, 22–25.

Day, J.C., Tisi, L.C., Bailey, M.J., 2004. Evolution of beetle bioluminescence: the origin of beetle luciferin. Luminescence 19, 8–20.

Di Domenico, M., Ricciardi, C., Fusco, A., Pierantoni, G.M., 2010. Anti-VEGF therapy in breast and lung mouse models of cancers. J. Biomed. Biotechnol. 2011, 947928.

El Haddad, N., Heathcote, D., Moore, R., Yang, S., Azzi, J., Mfarrej, B., Atkinson, M., Sayegh, M.H., Lee, J.S., Ashton-Rickardt, P.G., Abdi, R., 2011. Mesenchymal stem cells express serine protease inhibitor to evade the host immune response. Blood 117, 1176–1183.

Fuchita, M., Ardiani, A., Zhao, L., Serve, K., Stoddard, B.L., Black, M.E., 2009. Bacterial cytosine deaminase mutants created by molecular engineering show improved 5-fluorocytosine-mediated cell killing *in vitro* and *in vivo*. Cancer Res. 69, 4791–4799.

Hernandez-Alcoceba, R., Sangro, B., Prieto, J., 2006. Gene therapy of liver cancer. World J. Gastroenterol. 12, 6085–6097.

Kang, N.H., Hwang, K.A., Kim, S.U., Kim, Y.B., Hyun, S.H., Jeung, E.B., Choi, K.C., 2012a. Potential antitumor therapeutic strategies of human amniotic membrane and amniotic fluid-derived stem cells. Cancer Gene Ther. 19, 517–522.

Kang, N.H., Hwang, K.A., Yi, B.R., Lee, H.J., Jeung, E.B., Kim, S.U., Choi, K.C., 2012b. Human amniotic fluid-derived stem cells expressing cytosine deaminase and thymidine kinase inhibits the growth of breast cancer cells in cellular and xenograft mouse models. Cancer Gene Ther. 19, 412–419.

Kang, N.H., Lee, W.K., Yi, B.R., Park, M.A., Lee, H.R., Park, S.K., Hwang, K.A., Park, H.K., Choi, K.C., 2012c. Modulation of lipid metabolism by mixtures of protamine and chitooligosaccharide through pancreatic lipase inhibitory activity in a rat model. Lab. Anim. Res. 28, 31–38.

Kang, N.H., Yi, B.R., Lim, S.Y., Hwang, K.A., Baek, Y.S., Kang, K.S., Choi, K.C., 2012d. Human amniotic membrane-derived epithelial stem cells display anticancer activity in BALB/c female nude mice bearing disseminated breast cancer xenografts. Int. J. Oncol. 40, 2022–2028.

Kim, S.U., Nakagawa, E., Hatori, K., Nagai, A., Lee, M.A., Bang, J.H., 2002. Production of immortalized human neural crest stem cells. Methods Mol. Biol. 198, 55–65.

Kim, S.K., Kim, S.U., Park, I.H., Bang, J.H., Aboody, K.S., Wang, K.C., Cho, B.K., Kim, M., Menon, L.G., Black, P.M., Carroll, R.S., 2006a. Human neural stem cells target experimental intracranial medulloblastoma and deliver a therapeutic gene leading to tumor regression. Clin. Cancer Res. 12, 5550–5556.

Kim, S.U., Park, I.H., Kim, T.H., Kim, K.S., Choi, H.B., Hong, S.H., Bang, J.H., Lee, M.A., Joo, I.S., Lee, C.S., Kim, Y.S., 2006b. Brain transplantation of human neural stem cells transduced with tyrosine hydroxylase and GTP cyclohydrolase 1 provides functional improvement in animal models of Parkinson disease. Neuropathology 26, 129–140.

Kim, K.Y., Kim, S.U., Leung, P.C., Jeung, E.B., Choi, K.C., 2010. Influence of the prodrugs 5-fluorocytosine and CPT-11 on ovarian cancer cells using genetically engineered stem cells: tumor-tropic potential and inhibition of ovarian cancer cell growth. Cancer Sci. 101, 955–962.

Lee, D.H., Ahn, Y., Kim, S.U., Wang, K.C., Cho, B.K., Phi, J.H., Park, I.H., Black, P.M., Carroll, R.S., Lee, J., Kim, S.K., 2009. Targeting rat brainstem glioma using human neural stem cells and human mesenchymal stem cells. Clin. Cancer Res. 15, 4925–4934.

Lim, L.Y., Vidnovic, N., Ellisen, L.W., Leong, C.O., 2009. Mutant p53 mediates survival of breast cancer cells. Br. J. Cancer 101, 1606–1612.

Longley, D.B., Harkin, D.P., Johnston, P.G., 2003. 5-fluorouracil: mechanisms of action and clinical strategies. Nat. Rev. Cancer 3, 330–338.

Lv, H., Pan, G., Zheng, G., Wu, X., Ren, H., Liu, Y., Wen, J., 2010. Expression and functions of the repressor element 1 (RE-1)-silencing transcription factor (REST) in breast cancer. J. Cell. Biochem. 110, 968–974.

Lynch, H.T., Silva, E., Snyder, C., Lynch, J.F., 2008. Hereditary breast cancer: part I. Diagnosing hereditary breast cancer syndromes. Breast J. 14, 3–13.

Matsuzuka, T., Rachakatla, R.S., Doi, C., Maurya, D.K., Ohta, N., Kawabata, A., Pyle, M.M., Pickel, L., Reischman, J., Marini, F., Troyer, D., Tamura, M., 2009. Human umbilical cord matrix-derived stem cells expressing interferon-beta gene significantly attenuate bronchioloalveolar carcinoma xenografts in SCID mice. Lung Cancer 70, 28–36.

McArthur, H.L., Mahoney, K.M., Morris, P.G., Patil, S., Jacks, L.M., Howard, J., Norton, L., Hudis, C.A., 2011. Adjuvant trastuzumab with chemotherapy is effective in women with small, node-negative, HER2-positive breast cancer. Cancer 117, 5461–5468.

Nawa, A., Tanino, T., Luo, C., Iwaki, M., Kajiyama, H., Shibata, K., Yamamoto, E., Ino, K., Nishiyama, Y., Kikkawa, F., 2008. Gene directed enzyme prodrug therapy for ovarian cancer: could GDEPT become a promising treatment against ovarian cancer? Anti Cancer Agents Med. Chem. 8, 232–239.

Niess, H., Bao, Q., Conrad, C., Zischek, C., Notohamiprodjo, M., Schwab, F., Schwarz, B., Huss, R., Jauch, K.W., Nelson, P.J., Bruns, C.J., 2011. Selective targeting of genetically engineered mesenchymal stem cells to tumor stroma microenvironments using tissue-specific suicide gene expression suppresses growth of hepatocellular carcinoma. Ann. Surg. 254, 767–774 (discussion 774–765).

Oie, S., Ono, M., Yano, H., Maruyama, Y., Terada, T., Yamada, Y., Ueno, T., Kojiro, M., Hirano, K., Kuwano, M., 2006. The up-regulation of type I interferon receptor gene plays a key role in hepatocellular carcinoma cells in the synergistic antiproliferative effect by 5-fluorouracil and interferon-alpha. Int. J. Oncol. 29, 1469–1478.

Pastorakova, A., Hlubinova, K., Altaner, C., 2006. Treatment of human tumor cells by combine gene therapy harnessing plasmids expressing human tumor necrosis factor alpha and bacterial cytosine deaminase suicide gene. Neoplasma 53, 478–484.

Pestka, S., Krause, C.D., Walter, M.R., 2004. Interferons, interferon-like cytokines, and their receptors. Immunol. Rev. 202, 8–32.

Piccart, M., 2003. The role of taxanes in the adjuvant treatment of early stage breast cancer. Breast Cancer Res. Treat. 79 (Suppl. 1), S25–S34.

Qiao, J., Black, M.E., Caruso, M., 2000. Enhanced ganciclovir killing and bystander effect of human tumor cells transduced with a

retroviral vector carrying a herpes simplex virus thymidine kinase gene mutant. Hum. Gene Ther. 11, 1569–1576.

Rosewicz, S., Detjen, K., Scholz, A., von Marschall, Z., 2004. Interferon-alpha: regulatory effects on cell cycle and angiogenesis. Neuroendocrinology 80 (Suppl. 1), 85–93.

Shah, K., 2012. Mesenchymal stem cells engineered for cancer therapy. Adv. Drug Deliv. Rev. 64, 739–748.

Sottini, A., Capra, R., Serana, F., Chiarini, M., Caimi, L., Imberti, L., 2009. Interferon-beta therapy monitoring in multiple sclerosis patients. Endocrinol. Metab. Immune Disord. Drug Targets 9, 14–28.

Thu, M.S., Najbauer, J., Kendall, S.E., Harutyunyan, I., Sangalang, N., Gutova, M., Metz, M.Z., Garcia, E., Frank, R.T., Kim, S.U., Moats, R.A., Aboody, K.S., 2009. Iron labeling and pre-clinical MRI visualization of therapeutic human neural stem cells in a murine glioma model. PLoS One 4, e7218.

Uze, G., Monneron, D., 2007. IL-28 and IL-29: newcomers to the interferon family. Biochimie 89, 729–734.

Vincent-Salomon, A., Thiery, J.P., 2003. Host microenvironment in breast cancer development: epithelial-mesenchymal transition in breast cancer development. Breast Cancer Res. 5, 101–106.

Vogel, K.J., Atchley, D.P., Erlichman, J., Broglio, K.R., Ready, K.J., Valero, V., Amos, C.I., Hortobagyi, G.N., Lu, K.H., Arun, B., 2007. BRCA1 and BRCA2 genetic testing in Hispanic patients: mutation prevalence and evaluation of the BRCAPRO risk assessment model. J. Clin. Oncol. 25, 4635–4641.

Volate, S., Hudson, R., Wang, D., Muga, S., Wargovich, M., 2009. TJ-41 induces apoptosis and potentiates the apoptotic effects of 5-FU in breast cancer cell lines. J. Oncol. 2009, 895381.

Wagner, T.C., Velichko, S., Chesney, S.K., Biroc, S., Harde, D., Vogel, D., Croze, E., 2004. Interferon receptor expression regulates the antiproliferative effects of interferons on cancer cells and solid tumors. Int. J. Cancer 111, 32–42.

Wallace, J.A., Li, F., Leone, G., Ostrowski, M.C., 2011. Pten in the breast tumor microenvironment: modeling tumor-stroma coevolution. Cancer Res. 71, 1203–1207.

Yi, B.R., Kang, N.H., Hwang, K.A., Kim, S.U., Jeung, E.B., Choi, K.C., 2011a. Antitumor therapeutic effects of cytosine deaminase and interferon-{beta} against endometrial cancer cells using genetically engineered stem cells *in vitro*. Anticancer Res. 31, 2853–2861.

Yi, B.R., O, S.N., Kang, N.H., Hwang, K.A., Kim, S.U., Jeung, E.B., Kim, Y.B., Heo, G.J., Choi, K.C., 2011b. Genetically engineered stem cells expressing cytosine deaminase and interferon-beta migrate to human lung cancer cells and have potentially therapeutic anti-tumor effects. Int. J. Oncol. 39, 833–839.

Yi, B.R., Hwang, K.A., Kang, N.H., Kim, S.U., Jeung, E.B., Kim, H.C., Choi, K.C., 2012. Synergistic effects of genetically engineered stem cells expressing cytosine deaminase and interferon-beta *via* their tumor tropism to selectively target human hepatocarcinoma cells. Cancer Gene Ther. 19, 644–651.

Yoshiji, H., Kuriyama, S., Noguchi, R., Yoshii, J., Ikenaka, Y., Yanase, K., Namisaki, T., Kitade, M., Yamazaki, M., Tsujinoue, H., Fukui, H., 2005. Combination of interferon-beta and angiotensin-converting enzyme inhibitor, perindopril, attenuates the murine liver fibrosis development. Liver Int. 25, 153–161.

Zhao, D., Najbauer, J., Annala, A.J., Garcia, E., Metz, M.Z., Gutova, M., Polewski, M.D., Gilchrist, M., Glackin, C.A., Kim, S.U., Aboody, K.S., 2012. Human neural stem cell tropism to metastatic breast cancer. Stem Cells 30, 314–325.

Zoli, W., Ulivi, P., Tesei, A., Fabbri, F., Rosetti, M., Maltoni, R., Giunchi, D.C., Ricotti, L., Brigliadori, G., Vannini, I., Amadori, D., 2005. Addition of 5-fluorouracil to doxorubicin-paclitaxel sequence increases caspase-dependent apoptosis in breast cancer cell lines. Breast Cancer Res. 7, R681–R689.