Additional effects of engineered stem cells expressing a therapeutic gene and interferon-β in a xenograft mouse model of endometrial cancer

BO-RIM YI[1], SEUNG U. KIM[2] and KYUNG-CHUL CHOI[1]

[1]Laboratory of Biochemistry and Immunology, College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk, Republic of Korea; [2]Department of Medicine, Faculty of Medicine, University of British Columbia, Vancouver, British Columbia, Canada

Received March 27, 2015; Accepted May 4, 2015

DOI: 10.3892/ijo.2015.2999

Abstract. Endometrial cancer is the most common gynecologic malignancy in women worldwide. In the present study, we evaluated the effects of neural stem cell-directed enzyme/prodrug therapy (NDEPT) designed to more selectively target endometrial cancer. For this, we employed two different types of neural stem cells (NSCs), HB1.F3.CD and HB1.F3.CD.IFN-β cells. Cytosine deaminase (CD) can convert the non-toxic prodrug, 5-fluorocytosine (5-FC), into a toxic agent, 5-fluorouracil (5-FU), which inhibits DNA synthesis. IFN-β is a powerful cytotoxic cytokine that is released by activated immune cells or lymphocytes. In an animal model xenografted with endometrial Ishikawa cancer cells, the stem cells stained with CM-DiI were injected into nearby tumor masses and 5-FC was delivered by intraperitoneal injection. Co-expression of CD and IFN-β significantly inhibited the growth of cancer (~50-60%) in the presence of 5-FC. Among migration-induced factors, VEGF gene was highly expressed in endometrial cancer cells. Histological analysis showed that the aggressive nature of cancer was inhibited by 5-FC in the mice treated with the therapeutic stem cells. Furthermore, PCNA expression was more decreased in HB1.F3.CD.IFN-β treated mice rather than HB1.F3.CD treated mice. To confirm the *in vitro* combined effects of 5-FU and IFN-β, 5-FU was treated in Ishikawa cells. 5-FU increased the IFN-β/receptor 2 (IFNAR2) and BXA levels, indicating that 5-FU increased sensitivity of endometrial cancer cells to IFN-β, leading to apoptosis of cancer cells. Taken together, these results provide evidence for the efficacy of therapeutic stem cell-based immune therapy involving the targeted expression of CD and IFN-β genes at endometrial cancer sites.

Correspondence to: Dr Kyung-Chul Choi, Laboratory of Biochemistry and Immunology, College of Veterinary Medicine, Chungbuk National University, Chungdae-ro 1, Cheongju, Chungbuk 361-763, Republic of Korea
E-mail: kchoi@cbu.ac.kr

Key words: gene-direct enzyme/prodrug therapy, cytosine deaminase, interferon-β, endometrial cancer, 5-fluorouracil

Introduction

Endometrial cancer is the most common gynecologic malignancy with an estimated 142,200 cases reported during 2013 in developed countries (1). Endometrioids are present in up to 80% of endometrial cancer patients and represent the natural progression of atypical complex hyperplasia due to estrogen imbalance (2). Endometrial cancer is classified into two types, type I and type II, according to aggressiveness, prognosis and molecular characteristics (3). Type I endometrial cancer is thought to have slower growth, hormone sensitivity, low stage and an excellent prognosis (4). Approximately 10% of endometrial cancer cases are type II lesions which are high grade with deep invasion into the underlying myometrium and represent more aggressive malignancies (5). As a result, this type of tumor is thought to be a high-risk lesion and post-operative management reflects the need for more aggressive therapy.

The International Federation of Gynecology and Obstetrics (FIGO) stage, the most important prognostic features of endometrial cancer, is determined by a surgical staging technique for classifying endometrial cancers based on myometrial invasion, histological type and differentiation grade (6). Conventional therapy for most endometrial cancer begins with an abdominal hysterectomy and bilateral salpingo-oophorectomy (7). Various adjuvants such as systemic thermotherapy, external beam pelvic radiotherapy, or vaginal brachytherapy are adopted to the surgical patients (8). Chemotherapy is typically platinum-based and may cause myelosuppression, nausea, and neuropathy in patients although it is generally well tolerated (9,10). Furthermore, vaginal brachytherapy has not been shown to improve survival rates (11). Despite the many medical advances developed over the past 25 years, the incidence of endometrial cancer continues to increase. Thus, new therapies for treating this type of cancer are constantly under development.

As an alternative modality, gene therapy is one of the most prominent cancer treatments and uses genes that encode a functional therapeutic factor (12). Gene-directed enzyme/prodrug therapy (GEPT) has received much attention as an molecular chemotherapy approach for treating

various types of cancer (13). For instance, cytosine deaminase (CD)/5-fluorocytosine (5-FC) and carboxyl esterase (CE)/irinotecan (CPT-11) are GEPT systems involving a suicide enzyme to eradicate tumor cells (14,15). The CD gene product can convert the non-toxic prodrug 5-FC into a toxic agent, 5-fluorouracil (5-FU), which inhibits DNA synthesis. The CE gene product metabolically converts the prodrug CPT-11 into SN-38 which induces apoptosis in cancer cells (16). GEPT systems seem to reduce the toxicity of anticancer drugs in normal tissues compared to conventional therapies (17). However, these systems are hindered by the low gene transfer efficiency of viral vectors and the inability to specifically target cancer cells (18). To overcome these barriers, many researchers are exploring the clinical use of stem cells in gene therapies designed to treat cancer. Stem cells are capable of self-renewal, multilineage differentiation and inherent migration to tumor sites (19).

In the present study, we evaluated the effects of neural stem cell-directed enzyme/prodrug therapy (NDEPT) designed to more selectively target endometrial cancer cells. For this, we employed two different types of neural stem cells (NSCs), HB1.F3.CD and HB1.F3.CD.IFN-β cells. The CD and IFN-β genes were simultaneously expressed by the HB1.F3.CD.IFN-β cells. IFN is a powerful cytotoxic cytokine that is released by activated immune cells or lymphocytes (20). Two different types of INF are thought to exist: i) type I that includes IFN-α, IFN-β and IFN-ω; and ii) type II that includes IFN-γ (21). Type I and type II IFNs interact with the IFN-α/β receptor (IFNAR) and IFN-γ receptor (IFNGR) (22). The antiproliferative action of type I IFNs is possibly mediated by the interaction of IFN with multi-subunit heterodimeric receptors (23). IFNAR consists of IFNAR1, IFNAR2a (soluble form), 2b (short subunit), and 2c (long subunit) on the cell surface, and further activates the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway (24). Another clinical study demonstrated that combination chemotherapies which use IFN such as IFN-α along with 5-FU might be effective for treating advanced hepatocellular carcinoma (HCC) patients (25).

In the present study, we describe the therapeutic effects of genetically engineered stem cells (GESTECs) using an NDEPT system that inhibited the growth of endometrial cancer via a tumor tropic effect in a xenograft mouse model. In a previous study, we demonstrated the therapeutic effect of GESTECs on endometrial cancer cells using an *in vitro* assay (12). In the present research, we evaluated the mechanism underlying the relationship between IFN-β and the induction of apoptosis following 5-FU treatment. Our results suggest that the NDEPT system is useful for treating endometrial cancer and takes advantage of the tumor-specific migration abilities of stem cells.

Materials and methods

Cell lines and culture. Ishikawa cells derived from a human endometrial adenocarcinoma arising from glandular epithelial cells were provided by the Laboratory of Veterinary Biochemistry and Molecular Biology at Chungbuk National University (Cheongju, Republic of Korea). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories, Inc., Logan, UT, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone Laboratories), 100 U/ml penicillin (Cellgro Mediatech Inc., Manassas, VA, USA), 100 μg/ml streptomycin (Cellgro Mediatech), and 10 mM HEPES (Gibco, Carlsbad, CA, USA) at 37˚C in a humidified 5% CO_2 atmosphere. Human HB1.F3.CD and HB1.F3.CD.IFN-β cells were obtained from the University of British Columbia (Vancouver, British Columbia, Canada) and incubated in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, and 0.1% antimycoplasmal agents (Invivogen, Inc., San Diego, CA, USA) at 37˚C in a humidified 5% CO_2 atmosphere. All cell lines were passaged using 0.05% trypsin/0.02% EDTA (Gibco).

Xenograft mouse model. Female NOD/SCID mice (Six-week old; n=24) from the Korea Research Institute of Bioscience and Biotechnology (KRIBB; Cheongwon, Chungbuk, Korea) were used in the present study. All protocols involving animals were reviewed and approved by the Animal Care Committee of Chungbuk National University, and performed in accordance with the guidance for the Care and Use of Experimental Animal. Suspended Ishikawa cells (100 μl)containing $2x10^6$ cells were subcutaneously injected into the dorsal side of NOD/SCID mice.

Assessment of the therapeutic effects of GESTECs in an animal model. At week 5 post-inoculation, the volume of the tumor arising from the Ishikawa cells reached 250 mm^3. The 24 mice were randomly divided into three groups containing 8 mice each: i) HB1.F3.CD and 5-FC; ii) HB1.F3.CD.IFN-β and 5-FC; and iii) a negative control (no treatment with the stem cells and 5-FC). Stem cell suspension (100 μl) containing $4x10^6$ cells was subcutaneously injected into the nearby tumor mass. The stem cells were pre-stained with 2 μM chloromethylbenzamide-1,1'-dioctadecyl-3,3,3'-tetramethyl-indocarbocyanine perchlorate (CM-DiI; Invitrogen-Life Technologies, Carlsbad, CA, USA) prior to injection. The mice were inoculated twice during the experimental period (at 35 and 49 days). Two days after stem cell injection, each animal in the stem cell-treated groups was given an intraperitoneal injection of 5-FC (Sigma-Aldrich) at a dose of 500 mg/kg/day in 100 μl of saline. The mice were sacrificed 48 h after the last 5-FC treatment and the tumor mass was isolated. Tumor sizes were measured by a caliper and calculated every week using the following formula: Length x width x high x 0.5236.

Hematoxylin and eosin (H&E) staining. Paraffin-fixed xenograft tumor tissue sections from the control and NSC-treated mice were used for histophatological analysis. The tumor tissues were collected after sacrifice of the mice, fixed in 10% normal formalin (Sigma-Aldrich), embedded in paraffin blocks, and cut with a microtome (3-μm sections). After deparaffinization and rehydration, the sections were stained with hematoxylin (Sigma-Aldrich) and eosin (Sigma-Aldrich). To prevent sample contamination, the stained sections were hydrated and mounted using mounting solution. The morphology of cancer cells in each slide was examined by light microscopy using a BX51 microscope (Olympus, Tokyo, Japan).

Immunohistochemistry. Paraffin-embedded sections were deparaffinized and rehydrated with xylene, various concentration of ethanol (concentrations of 100, 90, 80 and 70%), and tap water. Antigen retrieval was performed by microwaving for 10 min in a chamber with 0.01 M citrate buffer (pH 6.0; Sigma-Aldrich). After antigen retrieval, the tissue sections were placed in 0.3% methanol/hydrogen peroxidase (Sigma-Aldrich) for 30 min to quench the endogenous peroxidase activity. To block non-specific antibody binding, the section was incubated with 10% normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 1 h. Subsequently, the sections were incubated with a mixture of anti-proliferating cell nuclear antigen (PCNA, 1:100; Abcam Ltd., Cambridge, UK) as the primary antibody in 5% bovine serum albumin (Sigma-Aldrich) for overnight at 4˚C. The next day, the slides were washed three times in 1X phosphate-buffered saline/0.1% Tween-20 (1X PBS-T, pH 7.4; Bio-Rad Laboratories Inc., Hercules, CA, USA) and incubated with the appropriate biotinylated anti-mouse secondary antibodies (1:500; Vector Laboratories) for 30 min at room temperature. The slides were rinsed with PBS-T for 10 min and Vectastain Universal Elite ABC kit reagent (Vector Laboratories) was applied for 30 min. Immunoreactive complexes were detected with a 3,3'-diaminobenzidine (DAB) substrate (Sigma-Aldrich) and counterstained by using hematoxylin. Finally, the slides were mounted with a coverslip and mounting solution. All slides were visualized under a BX51 light microscope and images were captured with digital photography (BX51 microscope).

Fluorescent staining analysis. Paraffin-fixed xenograft tumor tissue sections from the negative control and stem cell-treated mice were used to evaluate the migration of stem cells toward the tumor. After deparaffinization and rehydration of the tumor sections, the specimens were fixed with 4% paraformaldehyde and washed three times in 1X PBS. 4',6-Diamidino-2-phenylindole (DAPI; Sigma-Aldrich) staining was performed to detect the nuclei of cancer cells. DAPI staining solution (200 ng/ml) was dropped onto the slide and incubated in the dark for 10 min at 37˚C. After washing three times, the specimens were mounted onto coverslips with a drop of mounting solution. All sections were examined with an Olympus microscope (IX71 inverted microscope; Olympus) attached to a fluorescence detector.

Real-time PCR. Total RNA was extracted with TRIzol reagent (Invitrogen-Life Technologies), RNA extraction solution, according to the manufacturer's recommendations. Reverse transcription was performed using 1 μg total RNA with murine leukemia virus reverse transcriptase (MMLV-RT; iNtRON Biotechnology, Sungnam, Korea), 10 pM dNTPs (Bioneer Corp., Daejeon, Korea), nonamer random primer (Takara Bio, Shiga, Japan), 5X RT buffer (iNtRON Biotechnology), and RNase inhibitor (iNtRON Biotechnology). The real-time PCR reactions included 2X SYBR-Green Premix (Takara Bio), ROX (Takara Bio) as a reference dye, and reverse and forward primers for uPA, SDF-1α, VEGF, MCP-1 and SCF (Bioneer) as chemoattractant factor genes. Sequences of the primer pairs are listed in Table I. PCR reaction for the chemoattractant factor genes was performed with 40 cycles of denaturation at 95˚C for 15 sec, annealing at 58˚C for 20 sec, and extension at 72˚C for 15 sec. Real-time PCR was carried out in triplicate for each sample. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization and compared to the mRNA levels of urokinase-type plasminogen activator (uPA), stromal cell-derived factor 1 alpha (SDF-1α), vascular endothelial growth factor (VEGF), monocyte chemotactic protein 1 (MCP-1) and stem cell factor (SCF). The mRNA levels of these genes were determined using the $2^{-\Delta\Delta Ct}$ method.

Table I. Sequences of the oligonucleotide primers used for real-time PCR.

mRNA	Sequence (5'-3')
uPA	F: GGCAGGCAGATGGTCTGTAT
	R: TTGCTCACCACAACGACATT
MCP-1	F: CAAGCAGAAGTGGGTTCAGGA
	R: TCTTCGGAGTTTGGGTTTGC
SCF	F: GGCAAATCTTCCAAAAGACTACA
	R: GCCTTCAGAAATATTTGAAAACTTG
VEGF	F: CCAGCACATAGGAGAGATGAGCTT
	R: TCTTTCTTTGGTCTGCATTCACAT
SDF-1α	F: GTGTCACTGGCGACACGTAG
	R: TCCCATCCCACAGAGAGAAG
GAPDH	F: ATGTTCGTCATGGGTGTGAACCA
	R: TGGCAGGTTTTTCTAGACGGCAG

F, forward; R, reverse.

5-FU treatment. To monitor upregulation of the IFNAR2 and BAX genes following 5-FU treatment, the Ishikawa cells were exposed to 5-FU. Briefly, the cells ($1x10^5$ cells/well) were seeded in a 6-well plate and cultured for 1 day in DMEM containing 10% FBS. To serum-starve the cells before 5-FU treatment, the medium was changed to DMEM without FBS and the cells were cultured for 1 day. The next day, the cells were treated with two different concentrations of 5-FU (Sigma-Aldrich; 0.5 and 1.0 μg/ml) for 3, 6, 9 and 24 h.

Reverse transcription (RT)-PCR. To analyze the mRNA levels of IFNAR2 and BAX in the Ishikawa cells following 5-FU treatment, RT-PCR was performed. The reactions contained cDNA template, Taq polymerase (iNtRON Biotechnology), dNTP, 10X PCR buffer (iNtRON Biotechnology), and reverse and forward primers (Table II). PCR amplification was performed for 30 cycles of denaturation at 95˚C for 30 sec, annealing at 58˚C for 30 sec, and extension at 72˚C for 30 sec. The PCR products were loaded onto a 1.5% agarose gel pre-stained with ethidium bromide (EtBr; Sigma-Aldrich). The bands were analyzed with Gel Doc 2000 software (Bio-Rad Laboratories Inc., Hercules, CA, USA). GAPDH was used as an endogenous control for normalization.

Statistical analysis. RNA quantification was performed in triplicate. Statistical differences were identified with the Student's t-test. Results are expressed as the mean ± standard deviation (SD). A one-way ANOVA was used to analyze the

Table II. Sequences of the oligonucleotide primers for reverse transcription (RT)-PCR and the expected product sizes.

mRNA	Sequence (5'-3')	Size (bp)
IFNAR2	F: ATTCATATGATTCGCCTGATTAC R: GACTTTGGGGAGGCTATTTCTTAA	758
GAPDH	F: ATGTTCGTCATGGGTGTGAACCA R: TGGCAGGTTTTTCTAGACGGCAG	351

F, forward; R, reverse.

results of the animal experiments followed by Tukey's multiple comparison test. Data were expressed as the mean ± standard error of the mean (SEM) and P-values <0.05 were considered statistically significant.

Results

Therapeutic effect of GESTECs in a mouse model of endometrial cancer. The schematic experimental plan outlining the strategy to observe the effects of stem cells expressing therapeutic genes is presented in Fig. 1A. Ishikawa endometrial cancer cells were subcutaneously implanted into NOD/SCID mice, and the animals were observed for 4 weeks. When the tumor volume reached 250 mm^3, DM-DiI-stained HB1.F3.CD and HB1.F3.CD.IFN-β cells were subcutaneously injected into the mice at two different times. Two days after stem cell inoculation, the animals that received the stem cells were treated with 500 mg/kg/day of 5-FC. All mice were euthanized at 9 weeks and the tumor masses were preserved in 4% normal formalin solution. Tumor volume was measured from 4 to 9 weeks after inoculation of cancer cells. During the experimental period, tumor growth in the groups treated with stem cells and prodrug was significantly decreased (Fig. 1B). At 9 weeks, tumor mass growth was 50-60% inhibited in the mice treated with HB1.F3.CD or HB1.F3.CD.IFN-β cells (Fig. 1C). Tumor volumes reached 1,400 and 450 mm^3 in the control mice and animals injected with stem cells, respectively.

Histopathological analysis of the endometrial cancer sections. Tumor tissues from the mice were obtained for histopathological analysis. H&E staining showed that the cancer cells grew exuberantly in the negative control animals and stem cell-treated mice with atypia (Fig. 2A). Most of the cells in the specimen of a negative control showed atypia, however, major typical tumor necrosis was not shown in a negative control. In contrast, the mice that received HB1.F3.CD and HB1.F3.CD.IFN-β cells along with 5-FC showed smaller zones of endometrial cancer cell necrosis that exhibited nuclear pyknosis and karyorrhexis as seen in Fig. 2A. In the HB1.F3.CD and 5-FC-treated mice, 5-FU converted by the CD gene product induced pyknosis and karyorrhexis in Ishikawa endometrial cancer cells. Furthermore, karyorrhexic and karyolitic nuclei were observed in the animals injected with the HB1.F3.CD.IFN-β cells in the present study.

To observe the fate of cancer cells in the tumor specimen, we performed immunohistochemistry to detect PCNA, a marker of proliferation (Fig. 2B). In the negative control of the mice, PCNA protein, which indicates S phase of the cell cycle, was shown in the nuclei of almost all tumor cells. However, PCNA levels decreased in the nuclei of cells from mice treated with the stem cells and a prodrug. Although the expression of PCNA protein was reduced in the mice treated with either HB1.F3.CD or HB1.F3.CD.IFN-β cells. Notably, PCNA expression

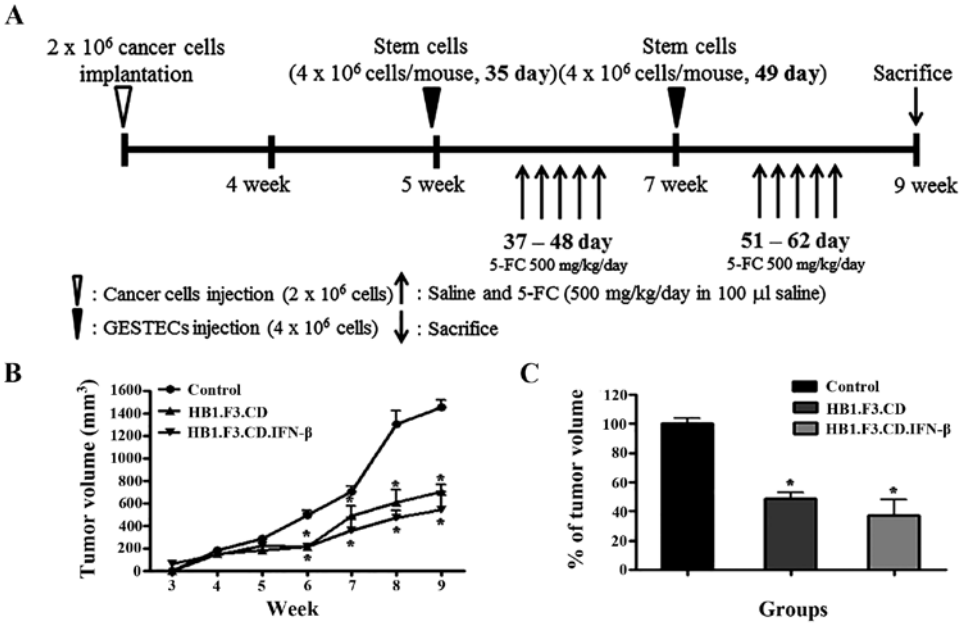

Figure 1. Therapeutic effect of GESTECs in a mouse endometrial cancer xenograft model. (A) Outline of the animal protocol. Ishikawa endometrial cancer cells ($2x10^6$ cells) were subcutaneously injected into female NOD/SCID mice. After 5 weeks, stem cells stained with CM-DiI were injected near the tumor site. The next two days, the mice were treated with 5-FC via i.p. injection. (B) Changes in tumor growth according to treatment with the NDEPT system during the 9-week experimental period. Tumor volume was measured using a caliper and calculated with the formula: 0.5236 x width x length x high. (C) Graph showing the final tumor volumes. *P<0.05 vs. the control.

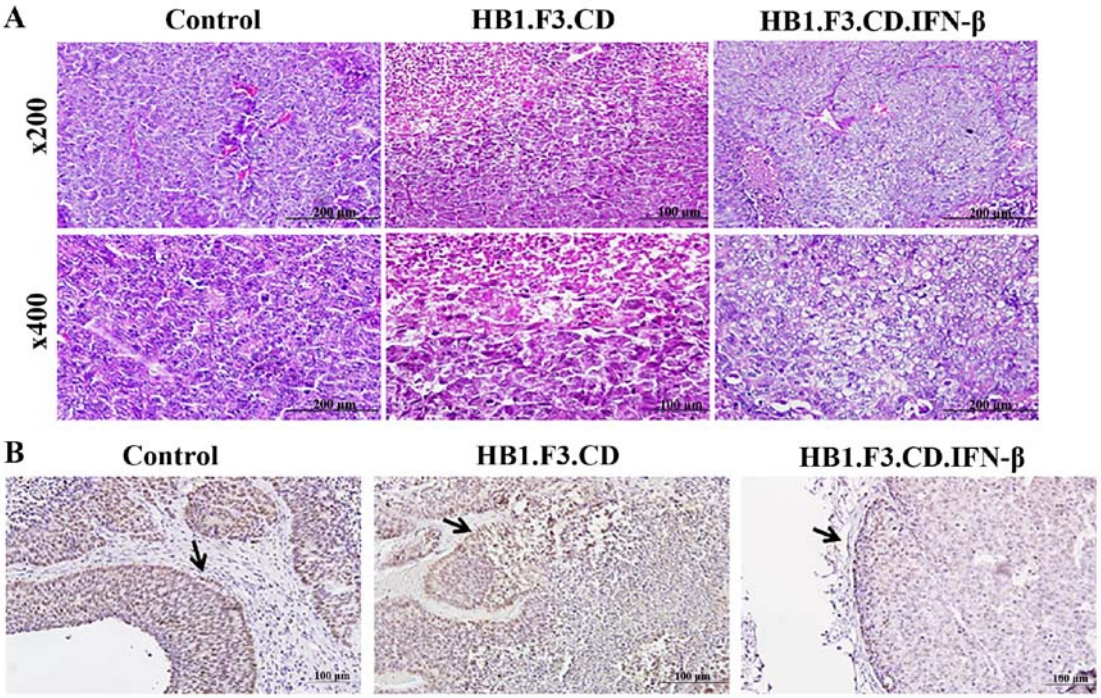

Figure 2. Histological analysis of the tumor mass. (A) H&E staining of the tumor mass fixed in formalin. The tumor tissue was hydrated with ethanol and xylene, embedded in paraffin, and cut into sections with a microtome. (B) Expression of the proliferation marker PCNA. After rehydration, each section was blocked in normal goat serum and incubated with the appropriate primary (anti-PCNA) and biotinylated secondary antibodies (anti-mouse). To detect PCNA protein, the ABC method was used, and DAB with hematoxylin were applied to the slides. Arrow; PCNA expression in each tissue section. Original magnification x200 or x400.

was decreased to a greater extent in the mice treated with HB1.F3.CD.IFN-β cells compared to the mice treated with HB1.F3.CD cells only in the presence of a prodrug.

Effect of stem cell migration in the tumor tissue. Tumor-tropic effects of the stem cells were directly examined in cancer tissue specimens obtained from the mice. For this, the stem cells were stained with CM-DiI before being injected into each mouse. DAPI was used for counterstaining. DAPI-stained nuclei in the tumor cells were observed in all groups of mice as shown in Fig. 3. However, CM-DiI-stained cells were not detected in the control group because stem cells were not subcutaneously injected in these mice (Fig. 3A). On the other hand, we confirmed that stained stem cells were present inside the tumor masses (Fig. 3B and C) of mice that received injections of HB1.F3.CD or HB1.F3.CD.IFN-β. More HB1.F3.CD.IFN-β cells were found in the tumor specimens compared to the HB1.F3.CD cells. HB1.F3.CD.IFN-β cells migrated to endometrial cancer mass in a xenograft mouse model at 1.64 times more than HB1.F3.CD (Fig. 3D).

Expression of chemoattractant ligands in the cancer cells. In addition to the migratory effect observed in the fluorescent analysis, we measured the expression of chemoattractant factors released by the endometrial cancer cells during the experiment period. To identify chemoattractant ligand expression in the Ishikawa cells, we performed real-time PCR specific for various ligands including uPA, SDF-1α, VEGF, MCP-1 and SCF. Ishikawa cells were found to express uPA, SDF-1α, VEGF, MCP-1 and SCF. In particular, VEGF was highly expressed in the endometrial cancer cells (Fig. 3E). Taken together, these results indicate that the VEGF/VEGFR2 pathway as a major signaling cascade promotes stem cell migration in our specific drug delivery system.

Effect of 5-FU on IFNAR2 and BAX expression. Treatment with 5-FU had significant effects on the membrane expression of IFNAR2 and BAX in the Ishikawa cells. To define the role of 5-FU in the Ishikawa cells, we analyzed 5-FU-treated cells by RT-PCR as shown in Fig. 4. Analysis of the IFNAR2 PCR product showed that the gene expression was weakly increased (Fig. 4B). In the presence of 0.5 μg/ml 5-FU, significant changes in IFNAR2 gene expression were not observed at 3, 6, 9 or 24 h. In contrast, IFNAR2 mRNA expression was increased 1.6-fold at 6 h compared to the control of Ishikawa cells. To evaluate the apoptotic effect of 5-FU, the expression of BAX gene was detected by RT-PCR and quantified (Fig. 4C). Initially, BAX expression increased ~1.6-fold at 3 h by treatment with 5-FU (1.0 μg/ml). At 24 h after 5-FU exposure (0.5 μg/ml), BAX levels were increased by 1.4-fold compared to the untreated control in Ishikawa cells.

Discussion

Cases of endometrial cancer are divided into two groups according to aggressiveness, prognosis and various molecular characteristics of the tumors (27). Typically, surgeons use various therapies, such as abdominal hysterectomy, systemic thermotherapy, external beam pelvic radiotherapy, or vaginal brachytherapy, to treat endometrial cancer (27,28). To improve the survival rate and minimize side-effects of the adjuvants in patients, the present study was performed and showed that

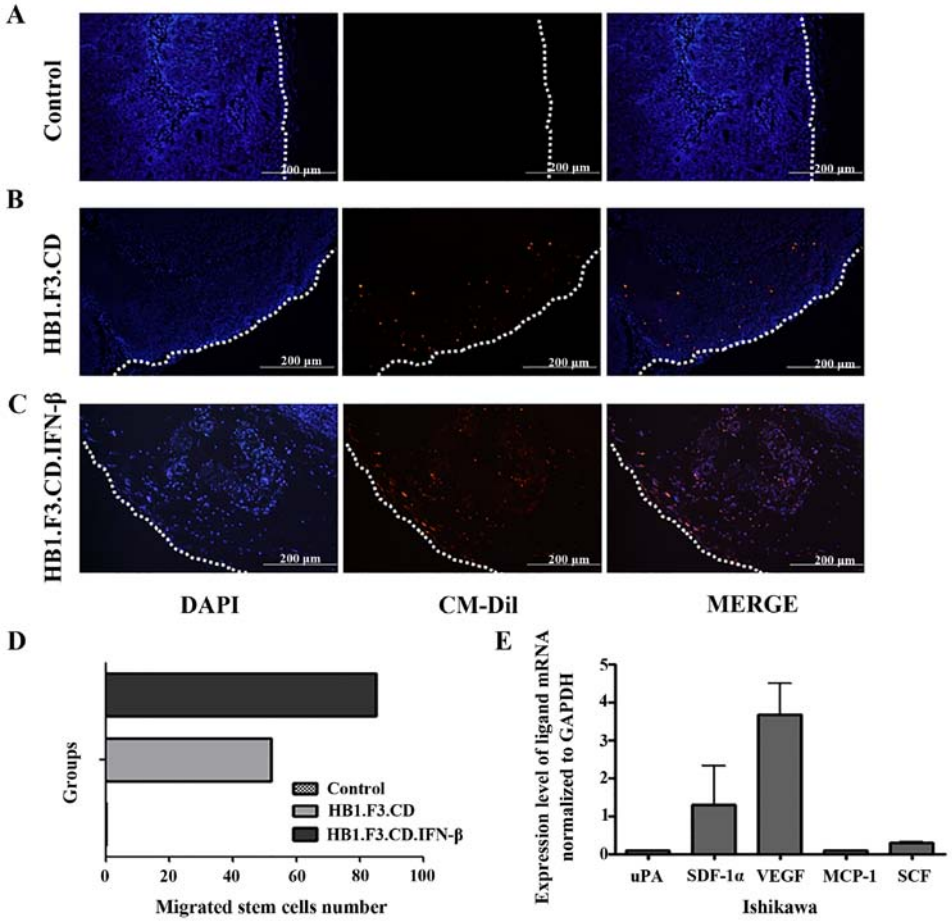

Figure 3. Tumor-tropic effects of the NSCs against endometrial cancer. Tissue specimens were rehydrated and stained with DAPI as a counterstain to CM-DiI. (A) Fluorescence analysis of the control sample. (B) Fluorescence analysis of the HB1.F3.CD cell and 5-FC-treated group. (C) Fluorescence analysis of the HB1.F3.CD.IFN-β cell and 5-FC-treated group. White dotted lines, tumor borderline; blue, DAPI-stained nuclei in the cancer and stem cells; red, CM-DiI-stained stem cells. Original magnification, x200. (D) Quantification of migrated NSCs. (E) Identification of chemoattractant ligand in the endometrial cancer cells. cDNA was produced from total RNA of the cancer cells and real-time PCR was performed with specific primer pairs.

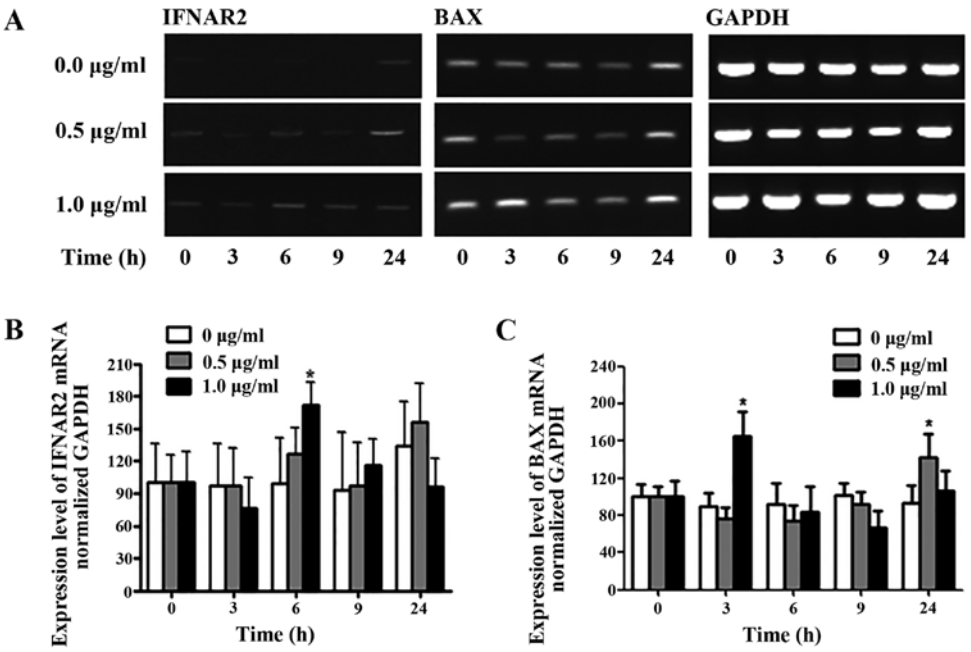

Figure 4. Alteration of IFNAR2 and BAX gene expression according to 5-FU treatment in the endometrial cancer cells. To confirm the apoptotic effects of 5-FU, the cancer cells were treated with two concentrations of the drug (0.5 and 1.0 μg/ml) for 3, 6, 9 and 24 h. Total RNA was extracted and cDNA was synthesized. (A) IFNAR2 and BAX gene expression was analyzed by RT-PCR. (B) Relative expression levels of IFNAR2. (C) Relative expression levels of BAX. *P<0.05 vs. the control treated with saline.

stem cell-based therapy using a therapeutic gene has advantages that can improve endometrial cancer treatment.

To investigate the important role of NDEPT in controlling the progression of endometrial cancer, Ishikawa cells were subcutaneously injected into female SCID mice. At week 5 post-inoculation, HB1.F3.CD and HB1.F3.CD.IFN-β cells along with 5-FC were administered to each mouse. Our xenograft mouse data clearly showed that stem cells expressing CD and/or IFN-β not only reduced the tumor volume but also inhibited tumor growth. The latter effect was more prominent in the HB1.F3.CD.IFN-β cell-treated group. Although HB1.F3.CD.IFN-β cells were genetically engineered to express the CD and IFN-β genes to enhance the therapeutic effects of the stem cells, the volumes of the tumor masses of the mice treated with HB1.F3.CD or HB1.F3.CD.IFN-β cells were not significantly different.

To analyze the tumor mass in greater detail, a histopathological analysis was performed. H&E staining revealed that the tumor mass from animals which received HB1.F3.CD.IFN-β cells and 5-FC had more apoptotic features. Additionally, the expression of PCNA, a marker of proliferation, was observed in all tumor tissues including the negative control group. However, the level of PCNA protein was lower in the mice treated with HB1.F3.CD.IFN-β cells and 5-FC compared to animals that received HB1.F3.CD cells and 5-FC. These results suggest the intriguing possibility that tumor cell necrosis in the center of the tumor mass was induced to a greater extent by IFN-β and 5-FU. Although tumor volume measured by veterinary caliper was not different between two treated mice with HB1.F3.CD and HB1.F3.CD.IFN-β, more damaged or apoptotic cancer cells were found at tumor tissues in the treated mice with HB1.F3.CD.IFN-β cells than HB1.F3.CD cells.

Previously, IFN-α and 5-FU combined therapy was found to have antiproliferative and anti-angiogenic effects in a xenograft mouse model injected with human HCC cells (29,30). Additionally, combination therapy induced extensive DNA fragmentation compared to treatment with wild-type HuH7 cells and inhibition of cell growth by apoptosis induced by Bcl-2 family members after transient transfection of IFNAR2 (31). Based on this finding, we evaluated the 5-FU effect on IFNAR2 and BAX gene expression by RT-PCR. The results of our studies showed that 5-FU appeared to be associated with increased IFNAR2 expression and induced apoptotic signaling pathway involved with BAX expression, a proapopotic gene. IFNAR2 mRNA expression was increased 1.6-fold compared the untreated (control) Ishikawa cells at 6 h. Taken together, the data indicate that 5-FU affected the expression of IFNAR2 mRNA and increased sensitivity to IFN-β produced by the HB1.F3.CD.IFN-β cells. Following the induction of IFNRA2 gene expression, JAK/STAT signaling is activated by IFN-β and induces apoptosis through 5-FU in the endometrial cancer cells.

Stem cells have the capability of therapeutic gene delivery for treating various cancers (32,33). NSCs have a strong tendency to migrate toward gliomas and surround invading tumor cells *in vivo* (34). This characteristic makes NSCs attractive as a vehicle for delivering therapeutic genes (35). In the present study, we confirmed that HB1.F3.CD and HB1.F3.CD.IFN-β cells were observed in the tumors that formed in the mice. This selective tumor tropism of the GESTECs was facilitated by chemoattractant factors secreted by endometrial cancer cells such as uPA, SDF-1α, VEGF, MCP-1 and SCF. Among these, VEGF mRNA expression was significantly increased in Ishikawa cells. In a previous study, we confirmed that three genes encoding the uPA receptor (uPAR), VEGF receptor (VEGFR2), and SCF receptor (c-Kit) were strongly expressed compared to ones encoding SDF-1α receptor (CXCR4) and MCP-1 receptor (CCR2) in the stem cells (36). As an angiogenic factor, VEGF promotes the mobilization and recruitment of endothelial and hematopoietic stem cells into the neo-angiogenic site, thereby accelerating vasculogenesis and angiogenesis (37,38). Tang *et al* (39) reported that cardiac stem cell (CSC) migration may be induced by multiple signaling pathways such as the VEGFR/PI3K/Akt and p38 mitogen-activated protein kinase (MAPK) cascades but not by platelet-derived growth factor receptor (PDGFR). Therefore, we suggest that the migratory effect of NSCs we observed may involve activated level of VEGF/VEGFR2 signaling pathway.

In conclusion, we showed in the present study that our NDEPT could be used to target endometrial cancer cells and more potent therapeutic effects were achieved in the presence of 5-FC. Furthermore, our engineered stem cells significantly inhibited endometrial cancer cell growth with IFNAR2 activation and Bcl-2 family-associated apoptosis following 5-FU conversion by HB1.F3.CD or HB1.F3.CD.IFN-β cells expressing the CD gene. With the HB1.F3.CD.IFN-β cells, more extensive activation of the IFNAR2 gene by 5-FU resulted in significant additional therapeutic effects for treating endometrial cancer.

Acknowledgements

The present study was supported by the Basic Science Research Program though the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (MEST) (2013R1A1A2059092). In addition, this study was also supported by a grant (no. PJ009599) from the Next-Generation BioGreen 21 Program, Rural Development Administration, Republic of Korea. The authors appreciate Dr Kyung-A Hwang for critical writing revision of this manuscript.

References

1. Oldenburg CS, Boll D, Nicolaije KA, Vos MC, Pijnenborg JM, Coebergh JW, Beijer S, van de Poll-Franse LV and Ezendam NP: The relationship of body mass index with quality of life among endometrial cancer survivors: A study from the population-based PROFILES registry. Gynecol Oncol 129: 216-221, 2013.
2. Lachance JA, Darus CJ and Rice LW: Surgical management and postoperative treatment of endometrial carcinoma. Rev Obstet Gynecol 1: 97-105, 2008.
3. Singh M, Spoelstra NS, Jean A, Howe E, Torkko KC, Clark HR, Darling DS, Shroyer KR, Horwitz KB, Broaddus RR, *et al*: ZEB1 expression in type I vs type II endometrial cancers: A marker of aggressive disease. Mod Pathol 21: 912-923, 2008.
4. Havrilesky LJ, Secord AA, Bae-Jump V, Ayeni T, Calingaert B, Clarke-Pearson DL, Berchuck A and Gehrig PA: Outcomes in surgical stage I uterine papillary serous carcinoma. Gynecol Oncol 105: 677-682, 2007.
5. Stefansson IM, Salvesen HB, Immervoll H and Akslen LA: Prognostic impact of histological grade and vascular invasion compared with tumour cell proliferation in endometrial carcinoma of endometrioid type. Histopathology 44: 472-479, 2004.

6. Lax SF, Kurman RJ, Pizer ES, Wu L and Ronnett BM: A binary architectural grading system for uterine endometrial endometrioid carcinoma has superior reproducibility compared with FIGO grading and identifies subsets of advance-stage tumors with favorable and unfavorable prognosis. Am J Surg Pathol 24: 1201-1208, 2000.
7. Amant F, Moerman P, Neven P, Timmerman D, Van Limbergen E and Vergote I: Endometrial cancer. Lancet 366: 491-505, 2005.
8. Trimbos JB, Vergote I, Bolis G, Vermorken JB, Mangioni C, Madronal C, Franchi M, Tateo S, Zanetta G, Scarfone G, *et al*; EORTC-ACTION collaborators. European Organisation for Research and Treatment of Cancer-Adjuvant ChemoTherapy in Ovarian Neoplasm: Impact of adjuvant chemotherapy and surgical staging in early-stage ovarian carcinoma: European Organisation for Research and Treatment of Cancer-Adjuvant ChemoTherapy in Ovarian Neoplasm trial. J Natl Cancer Inst 95: 113-125, 2003.
9. Kelly MG, O'malley DM, Hui P, McAlpine J, Yu H, Rutherford TJ, Azodi M and Schwartz PE: Improved survival in surgical stage I patients with uterine papillary serous carcinoma (UPSC) treated with adjuvant platinum-based chemotherapy. Gynecol Oncol 98: 353-359, 2005.
10. Jutzi L, Hoskins P, Lim P, Aquino-Parsons C, Tinker A and Kwon JS: The importance of adjuvant chemotherapy and pelvic radiotherapy in high-risk early stage endometrial carcinoma. Gynecol Oncol 131: 581-585, 2013.
11. Soumarová R, Homola L, Perková H, Czudek S, Skrovina M and Adamcík L: The role of interstitial brachytherapy in multimodality management of solid tumors. Rozhl Chir 86: 533-539, 2007 (In Czech).
12. Yi BR, O SN, Kang NH, Hwang KA, Kim SU, Jeung EB, Kim YB, Heo GJ and Choi KC: Genetically engineered stem cells expressing cytosine deaminase and interferon-β migrate to human lung cancer cells and have potentially therapeutic antitumor effects. Int J Oncol 39: 833-839, 2011.
13. Yi BR, Park MA, Lee HR, Kang NH, Choi KJ, Kim SU and Choi KC: Suppression of the growth of human colorectal cancer cells by therapeutic stem cells expressing cytosine deaminase and interferon-β via their tumor-tropic effect in cellular and xenograft mouse models. Mol Oncol 7: 543-554, 2013.
14. Seol HJ, Jin J, Seong DH, Joo KM, Kang W, Yang H, Kim J, Shin CS, Kim Y, Kim KH, *et al*: Genetically engineered human neural stem cells with rabbit carboxyl esterase can target brain metastasis from breast cancer. Cancer Lett 311: 152-159, 2011.
15. Kim KY, Yi BR, Lee HR, Kang NH, Jeung EB, Kim SU and Choi KC: Stem cells with fused gene expression of cytosine deaminase and interferon-β migrate to human gastric cancer cells and result in synergistic growth inhibition for potential therapeutic use. Int J Oncol 40: 1097-1104, 2012.
16. Kim KY, Kim SU, Leung PC, Jeung EB and Choi KC: 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, 2010.
17. Yi BR, Hwang KA, Kang NH, Kim SU, Jeung EB, Kim HC and Choi KC: Synergistic effects of genetically engineered stem cells expressing cytosine deaminase and interferon-β via their tumor tropism to selectively target human hepatocarcinoma cells. Cancer Gene Ther 19: 644-651, 2012.
18. Yi BR, Choi KJ, Kim SU and Choi KC: Therapeutic potential of stem cells expressing suicide genes that selectively target human breast cancer cells: Evidence that they exert tumoricidal effects via tumor tropism (Review). Int J Oncol 41: 798-804, 2012.
19. Yi BR, Kim SU and Choi KC: Development and application of neural stem cells for treating various human neurological diseases in animal models. Lab Anim Res 29: 131-137, 2013.
20. Trinchieri G: Type I interferon: Friend or foe? J Exp Med 207: 2053-2063, 2010.
21. Yi BR, Hwang KA, Aboody KS, Jeung EB, Kim SU and Choi KC: Selective antitumor effect of neural stem cells expressing cytosine deaminase and interferon-beta against ductal breast cancer cells in cellular and xenograft models. Stem Cell Res (Amst) 12: 36-48, 2014.
22. Guo B, Chang EY and Cheng G: The type I IFN induction pathway constrains Th17-mediated autoimmune inflammation in mice. J Clin Invest 118: 1680-1690, 2008.
23. Monroe KM, McWhirter SM and Vance RE: Induction of type I interferons by bacteria. Cell Microbiol 12: 881-890, 2010.
24. Sangfelt O and Strander H: Apoptosis and cell growth inhibition as antitumor effector functions of interferons. Med Oncol 18: 3-14, 2001.
25. Damdinsuren B, Nagano H, Wada H, Noda T, Natsag J, Marubashi S, Miyamoto A, Takeda Y, Umeshita K, Doki Y, *et al*: Interferon alpha receptors are important for antiproliferative effect of interferon-alpha against human hepatocellular carcinoma cells. Hepatol Res 37: 77-83, 2007.
26. Olawaiye AB and Boruta DM II: Management of women with clear cell endometrial cancer: A Society of Gynecologic Oncology (SGO) review. Gynecol Oncol 113: 277-283, 2009.
27. Morris L, Stevens MJ, Valmadre S, Martland J and Lee T: Adjuvant intravaginal brachytherapy for uterus didelphys with synchronous endometrial adenocarcinomas and unfavourable vaginal topography. Gynecol Oncol Case Rep 2: 121-123, 2012.
28. Nout RA, Putter H, Jürgenliemk-Schulz IM, Jobsen JJ, Lutgens LC, van der Steen-Banasik EM, Mens JW, Slot A, Stenfert Kroese MC, Nijman HW, *et al*: Five-year quality of life of endometrial cancer patients treated in the randomised Post Operative Radiation Therapy in Endometrial Cancer (PORTEC-2) trial and comparison with norm data. Eur J Cancer 48: 1638-1648, 2012.
29. Hagiwara S, Kudo M, Nakatani T, Sakaguchi Y, Nagashima M, Fukuta N, Kimura M, Hayakawa S and Munakata H: Combination therapy with PEG-IFN-alpha and 5-FU inhibits HepG2 tumour cell growth in nude mice by apoptosis of p53. Br J Cancer 97: 1532-1537, 2007.
30. Wada H, Nagano H, Yamamoto H, Arai I, Ota H, Nakamura M, Damdinsuren B, Noda T, Marubashi S, Miyamoto A, *et al*: Combination therapy of interferon-alpha and 5-fluorouracil inhibits tumor angiogenesis in human hepatocellular carcinoma cells by regulating vascular endothelial growth factor and angiopoietins. Oncol Rep 18: 801-809, 2007.
31. Kondo M, Nagano H, Wada H, Damdinsuren B, Yamamoto H, Hiraoka N, Eguchi H, Miyamoto A, Yamamoto T, Ota H, *et al*: Combination of IFN-alpha and 5-fluorouracil induces apoptosis through IFN-alpha/beta receptor in human hepatocellular carcinoma cells. Clin Cancer Res 11: 1277-1286, 2005.
32. Kim DJ, Yi BR, Lee HR, Kim SU and Choi KC: Pancreatic tumor mass in a xenograft mouse model is decreased by treatment with therapeutic stem cells following introduction of therapeutic genes. Oncol Rep 30: 1129-1136, 2013.
33. Kang NH, Hwang KA, Yi BR, Lee HJ, Jeung EB, Kim SU and Choi KC: 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, 2012.
34. Aboody KS, Brown A, Rainov NG, Bower KA, Liu S, Yang W, Small JE, Herrlinger U, Ourednik V, Black PM, *et al*: Neural stem cells display extensive tropism for pathology in adult brain: Evidence from intracranial gliomas. Proc Natl Acad Sci USA 97: 12846-12851, 2000.
35. Heese O, Disko A, Zirkel D, Westphal M and Lamszus K: Neural stem cell migration toward gliomas in vitro. Neuro Oncol 7: 476-484, 2005.
36. Yi BR, Hwang KA, Kim YB, Kim SU and Choi KC: Effects of genetically engineered stem cells expressing cytosine deaminase and interferon-beta or carboxyl esterase on the growth of LNCaP rrostate cancer cells. Int J Mol Sci 13: 12519-12532, 2012.
37. Pitchford SC, Furze RC, Jones CP, Wengner AM and Rankin SM: Differential mobilization of subsets of progenitor cells from the bone marrow. Cell Stem Cell 4: 62-72, 2009.
38. Simons M and Ware JA: Therapeutic angiogenesis in cardiovascular disease. Nat Rev Drug Discov 2: 863-871, 2003.
39. Tang J, Wang J, Kong X, Yang J, Guo L, Zheng F, Zhang L, Huang Y and Wan Y: Vascular endothelial growth factor promotes cardiac stem cell migration via the PI3K/Akt pathway. Exp Cell Res 315: 3521-3531, 2009.