Antitumor effects of genetically engineered stem cells expressing yeast cytosine deaminase in lung cancer brain metastases via their tumor-tropic properties

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Abstract. Although mortality related with primary tumors is approximately 10%, metastasis leads to 90% of cancer-associated death. The majority of brain metastases result from lung cancer, but the metastatic mechanism remains unclear. In general, chemotherapy for treating brain diseases is disrupted by the brain blood barrier (BBB). As an approach to improve treatment of lung cancer metastasis to the brain, we employed genetically engineered stem cells (GESTECs), consisting of neural stem cells (NSCs) expressing a suicide gene. Cytosine deaminase (CD), one of the suicide genes, originating from bacterial (bCD) or yeast (yCD), which can convert the non-toxic prodrug, 5-fluorocytosine (5-FC), into 5-fluorouracil (5-FU), can inhibit cancer cell growth. We examined the therapeutic efficacy and migratory properties of GESTECs expressing yCD, designated as HB1.F3.yCD, in a xenograft mouse model of lung cancer metastasis to the brain. In this model, A549 lung cancer cells were implanted in the right hemisphere of the mouse brain, while CM-DiI pre-stained HB1.F3.yCD cells were implanted in the contralateral brain. Two days after the injection of stem cells, 5-FC was administered via intraperitoneal injection. The tumor-tropic effect of HB1.F3.yCD was evident by fluorescent analysis, in which red-colored stem cells migrated to the lung tumor mass of the contralateral brain. By histological analysis of extracted brain, the therapeutic efficacy of HB1.F3.yCD in the presence of 5-FC was confirmed by the reduction in density and aggressive tendency of lung cancer cells following treatment with 5-FC, compared to a negative control or HB1.F3.yCD injection without 5-FC. Taken together, these results indicate that HB1.F3.yCD expressing a suicide gene may be a new therapeutic strategy for lung cancer metastases to the brain in the presence of a prodrug.

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Introduction

Metastasis is an insidious movement of cancer cells from primary tumor sites to distant organs and tissues, including the brain, liver, and bones, via blood and lymphatic vessels. Metastasis accounts for over 90% of lethality in cancer patients (1). Particularly, brain metastasis is the most common intracranial neoplasm and arises in 10-40% of all cancer patients (2). Because metastases in the brain may rapidly compromise central nervous system (CNS) function, it is a significant cause of cancer-related morbidity and mortality worldwide (3). The most common origins of brain metastases include primary cancers of the lung, breast and skin (4). Lung cancer, the most prevalent cancer in men, is the leading cause of cancer-related death in the developed world (5). Of all malignancies, primary lung cancer has the highest incidence for brain metastasis and approximately 40% of all patients with lung cancer develop brain metastasis, followed by breast cancer (6). For treating these brain metastases, therapies usually include surgery, chemotherapy, and radiotherapy, but these therapies have many side effects in the nervous system (7). Therefore, brain metastasis is a very critical problem in the overall management of lung cancer.

In this study, seeking a treatment for lung cancer metastasis to the brain, neural stem cells (NSCs) derived from human fetal telencephalon were used for a genetically engineered stem cell (GESTEC)-based therapy (8). GESTECs were engineered to include the gene for a direct prodrug/enzyme (GEPT) system producing bioactive enzymes that convert a relatively

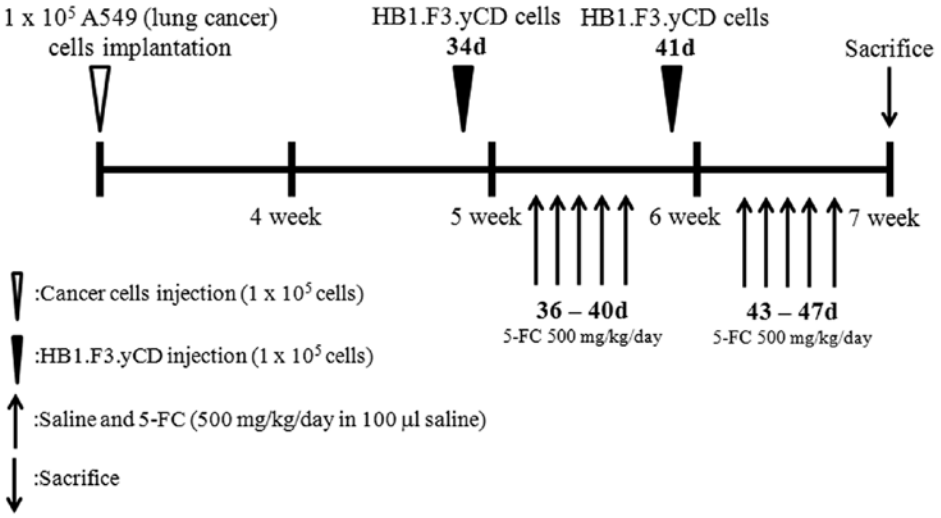

Figure 1. *In vivo* experimental plan. Human non-small cell lung adenocarcinoma A549 cells ($1x10^5$ cells in 10 μl saline) were implanted into the brain of 7-week-old SCID mice (n=15). After 34 to 41 days of implantation, HB1.F3.yCD cells ($1x10^5$ cells in 10 μl saline) and 5-FC (500 mg/kg/day in 100 μl saline) were introduced via contralateral and intraperitoneal injection, respectively. The mice were divided into three groups. In group 1, the mice were treated with 10 or 100 μl saline as a negative control. In group 2, the mice were treated with HB1.F3.yCD cells and 100 μl saline in the absence of 5-FC. In group 3, the mice were treated with HB1.F3.yCD and 5-FC (500 mg/kg/day in 100 μl saline). At 2 days following the last 5-FC injection, all of the mice treated with lung cancer cells were euthanized.

non-toxic prodrug into a toxic agent (9). Cytosine deaminase (CD)/5-fluorocytosine (5-FC), one of the most widely used GEPT systems, is expressed in bacteria like *Escherichia coli* or yeast like *Saccharomyes cerevisiae*. In both organisms, the encoded CD catalyzes the conversion of a 5-FC prodrug into a toxic agent, 5-fluorouracil (5-FU), which is able to inhibit cancer cell growth (10,11) by impairing DNA synthesis and promoting apoptosis (12). However, yeast CD (yCD) appears to be far more efficient in the conversion of 5-FC to 5-FU than bacterial CD (bCD) in both *in vitro* and *in vivo* models (13). In addition, a variety of stem cells as well as NSCs exert tumor tropic effects (14,15). Thus, we previously produced yCD-transfected GESTECs, designated as HB1.F3.yCD, as a superior delivery system to treat metastatic lung cancer to brain or other tissues.

In this study, we employed an immortalized HB1.F3.yCD to selectively target metastatic lung cancer to the brain. The metastatic breast cancer to the brain was proved by using HB1.F3.bCD in the previous study (16). We confirmed the therapeutic efficacy and migratory property of HB1.F3.yCD for lung cancer metastasis to the brain *in vivo*. Overall, these results suggest that GESTECs expressing a therapeutic gene (yCD) can be an excellent therapeutic system to effectively target metastatic lung cancer to the brain or other organs.

Materials and methods

Cell culture. The A549 human non-small cell lung adenocarcinoma cell line was purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). These cells were cultured in containing Dulbecco's modified Eagle's Medium (DMEM; Hyclone Laboratories, Logan, UT, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories), 1% penicillin and 1% streptomycin (Cellgro Mediatech, Manassas, VA, USA), 1% HEPES (Invitrogen Life Technologies, Carlsbad, CA, USA), and plasmocin (InvivoGen, San Diego, CA, USA). HB1.F3.yCD engineered stem cells were cultured in the same medium.

5-FC and 5-FU effects on lung cancer cell growth. To confirm 5-FC and 5-FU effects, A549 cells ($1x10^5$ cells/well) were seeded in 6-well plates containing the aforementioned medium modified to contain 5% FBS. Twenty-four hours after cancer cell incubation, 5-FC (0.5 mmol/l; Sigma-Aldrich, St. Louis, MO, USA) and 5-FU (0.5 mmol/l; Sigma-Aldrich) were added to each well for 4 days. These results were observed by inverted microscopy using an IX71 microscope (Olympus, Tokyo, Japan). Next, to ascertain the effects of various concentrations of 5-FU (0.005-1.0 mmol/l) on lung cancer cells, $5x10^3$ A549 cells were cultured in a 96-well plate for 1 day. 5-FU of different concentrations was added to each well and incubation was continued for 4 days. A cell viability assay was conducted. Briefly, 10 μl of 5 mg/ml 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) stock solution dissolved in phosphate-buffered saline (PBS) was added to each well and the plate was incubated for 4 h in darkness at 37˚C. Insoluble formazan crystals that formed were dissolved in 100 μl dimethyl sulfoxide (DMSO; Junsei Chemical, Co., Ltd., Tokyo, Japan) and the absorbance was measured at 540 nm using a VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA). Each experiment was conducted in duplicate (n=6).

In vitro therapeutic efficacy of HB1.F3.yCD. To explain the therapeutic efficacy of HB1.F3.yCD, A549 cells ($4x10^3$) were cultured in a 96-well plate and incubated at 37˚C for one day. Twenty-four hours after incubation HB1.F3.yCD cells ($8x10^3$) were co-cultured with A549. A day later, 5-FC diluted in saline (100, 200, 300, 400, and 500 μg/ml) was added in culture wells and incubation continued for 4 days. To confirm cell viability,

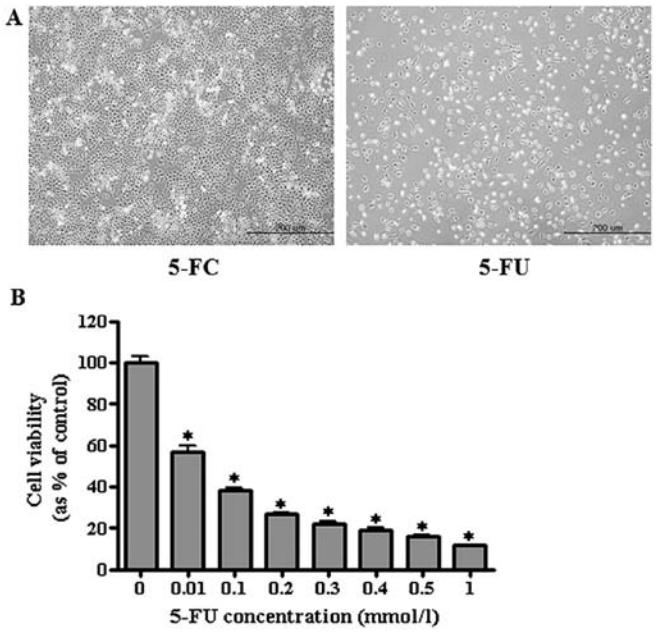

Figure 2. Effects of 5-FC and 5-FC on the cell viability of human lung cancer cells. (A) A549 lung cancer cells (A549, $1x10^5$ cells per well) were seeded in 6-well plates for 24 h. After seeding, 5-FC and 5-FU (0.5 mmol/l) were treated in lung cancer cells cultured for 4 days. Each well was observed by microscopy. Magnification, x100. (B) A549 lung cancer cells ($5x10^3$ cells per well) were seeded in 96-well plates for 24 h. Increasing concentrations of 5-FU at 0.01, 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 mmol/l were treated in cultured 96-well plate for 4 days. After 4 days, the MTT assay was performed to determine cell viability. *P<0.05 vs. a negative control.

MTT solution was added in each well for 4 h and absorbance was measured at 540 nm. The experiments were performed in duplicate.

Animal model of brain metastasis. All animal experiments were approved by the Animal Care Committee of Chungbuk National University. All animals were acclimated at 24-26˚C and 40-60% relative humidity under a 12 h light/dark cycle and frequent ventilation. Fifteen male SCID mice (7-week-old) were obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB, Ochang, Korea). After being anesthetized, SCID mice each received A549 ($1x10^5$ cells in 10 μl saline) via injection into white matter at a depth of 2 mm [anterior/posterior (AP) +1.0 mm, medial/lateral (ML) +1.7 mm, dorsal/ventral (DV) -3.2 mm].

In vivo therapeutic efficacy of HB1.F3.yCD. Four weeks after implantation, saline (10 μl) or HB1.F3.yCD ($1x10^5$ cells in 10 μl saline) was introduced via contralateral injection (AP +1.0 mm, ML -1.7 mm, DV -3.2 mm). HB1.F3.yCD cells were preincubated with CellTracker™ CM-DiI (Invitrogen Life Technologies). Briefly, stem cells were treated with 5 μM CM-DiI for 5 min at 37˚C and for 15 min at 4˚C. After two washes in PBS, the defined number of stem cells was injected into the brain. One hundred microliters or 500 mg/kg/day 5-FC in 100 μl was administered via intraperitoneal injection. The total number of mice were divided into three groups (n=5 per group). Group 1 was treated with 10 μl saline as a negative control. Group 2 was treated with HB1.F3.yCD in the absence of 5-FC in 100 μl saline. Group 3 was treated with HB1.F3.yCD in the presence of 5-FC (500 mg/kg/day) in 100 μl saline. Two days following the last injection with 5-FC, all mice were euthanized and brains were collected. The *in vivo* experimental scheme using a xenograft model is shown in Fig. 1.

Histopathological and fluorescence analysis. The extracted brain was sliced in 4-6 mm-thick sections and fixed in 10% normal formalin solution (Sigma-Aldrich), embedded in paraffin, and sectioned at a thickness of 5 μm using a microtome. Tissues were stained using hematoxylin and eosin following the general protocol and observed by microscopy using a model BX51 microscope (Olympus).

To confirm tumor-tropism effects of stem cell via fluorescence analysis, CM-DiI pre-stained HB1.F3.yCD was injected in mouse brain. After brain sections were obtained as described above, each slide was treated with 10% normal formalin solution for 10 min and washed with PBS two times. 4',6-Diamidino-2-phenylindole (DAPI) solution was added dropwise to each slide and incubated for 10 min at 37˚C in darkness. Stained slides were observed using an IX71 inverted microscope (Olympus).

Statistical analysis. Data of each experiment are shown as the mean ± standard deviation (SD). To evaluate the significance of each *in vitro* group, statistical analysis was performed by the one-way ANOVA Tukey's test using the GraphPad Prism software (v5.0; GraphPad Software, San Diego, CA, USA). P<0.05 were considered to indicate statistically significant differences.

Results

Cytotoxic effects of 5-FC and 5-FU for lung cancer cells. In the 6-well plate culture system, we confirmed the effects of 5-FC and 5-FU on the viability of lung cancer cells. When A549 cells were treated with 0.5 mmol/l of 5-FC, there was no change in the cell growth (Fig. 2A). However, treatment with 0.5 mmol/l 5-FU resulted in significant inhibition of A549 lung cancer cell growth as shown in Fig. 2A. To confirm the effects of 5-FU at various concentrations (0.01, 0.1, 0.2, 0.3, 0.4, 0.5 and 1.0 mmol/l), the cell viability was measured by an MTT assay following culture of A549 cells in 96-well plates. Inhibition of lung cancer cell growth was observed in a dose-dependent manner and the cell viability was suppressed up to approximately 80% as seen in Fig. 2B.

Therapeutic efficacy of HB1.F3.yCD and 5-FC. Based on the effect of 5-FU on A549 cell growth, we employed a co-culture system in which cancer and stem cells were seeded in the same wells, and cell viability was measured by the MTT assay. In the presence of 5-FC and HB1.F3.yCD, the viability of A549 lung cancer cells was reduced 20-40%, and the maximal inhibition was observed at 500 μg/ml of 5-FC in the presence of HB1.F3.yCD (Fig. 3). The growth of A549 lung cancer cells was not altered following treatment with a maximal dose of 5-FC (500 μg/ml) in the absence of HB1.F3.yCD (data not shown).

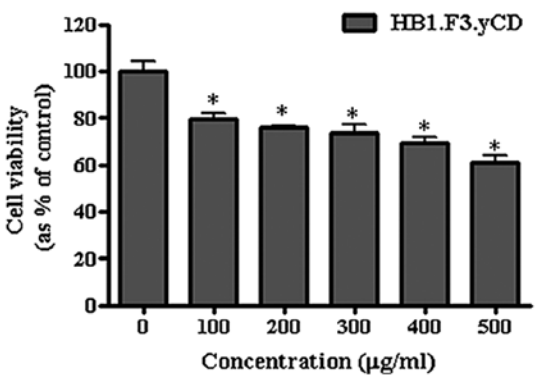

Figure 3. *In vitro* therapeutic efficacy of HB1.F3.yCD cells. A549 lung cancer cells ($4x10^3$ cells per well) were cultured in 96-well plates for one day. Following implantation of HB1.F3.yCD cells ($8x10^3$ cells per well), increasing concentrations of 5-FC at 0, 100, 200, 300, 400 and 500 μg/ml were added into each well for 4 days. Cell viability was measured by the MTT assay. *P<0.05 vs. control (0 μg/ml).

In vivo migratory capacity of HB1.F3.yCD cells. We confirmed the *in vivo* migratory property of HB1.F3.yCD cells toward lung metastatic cancer to the brain. Lung metastases to the brain were mimicked by implantation of A549 cells into the right hemisphere and CM-DiI pre-stained HB1.F3.yCD cells into the left hemisphere. DAPI counterstaining was performed and the migration of CM-DiI-labeled stem cells was traced via microscopy. By fluorescence analysis, red-colored HB1.F3.yCD cells were observed in the contralateral brain as shown in Fig. 4, indicating that injected HB1.F3.yCD cells have a migratory property towards A549 lung cancer cells injected into brain *in vivo*.

In vivo therapeutic efficacy of HB1.F3.yCD cells. To confirm the therapeutic effect of HB1.F3.yCD cells in the mouse brain, a xenograft model was employed as previously described (9,16). At 2 days following the last 5-FC injection, the brains of lung cancer cell-bearing SCID mice were extracted and examined by histological and fluorescence analyses. The implanted lung tumor mass in the brain was not reduced in the control mice in the presence of HB1.F3.yCD without 5-FC or in the absence of HB1.F3.yCD with 5-FC. In contrast, a significant reduction of lung tumor mass was observed in the brain of SCID mice treated with HB1.F3.yCD in the presence of 5-FC (500 mg/kg/day) as demonstrated in Fig. 5. The implanted lung cancer cells and normal mouse brain cells were distinguished, and the nuclear to cytoplasmic ratio was increased in lung cancer cells compared to normal brain cells observed by histological analysis. In addition, the density of lung cancer cells in the formed tumor masses was significantly decreased by HB1.F3.yCD expressing yCD in the presence of 5-FC (Fig. 5).

Discussion

Brain metastases are the most common malignant tumor of the CNS outnumbering primary brain tumors such as glioma and glioblastoma (17). Although the major requirements for metastasis to distant sites remain incompletely understood, clinically, brain metastases most commonly arise from lung, breast and skin cancers (18,19). Such factors have not yet been identified for brain metastasis and the most common treatment approaches for brain metastasis are surgery, radiotherapy, chemotherapy and stereotactic radiotherapy (20). However, these therapies carry the risk of development of neurological and cognitive deficits and, in the case of chemotherapy, the

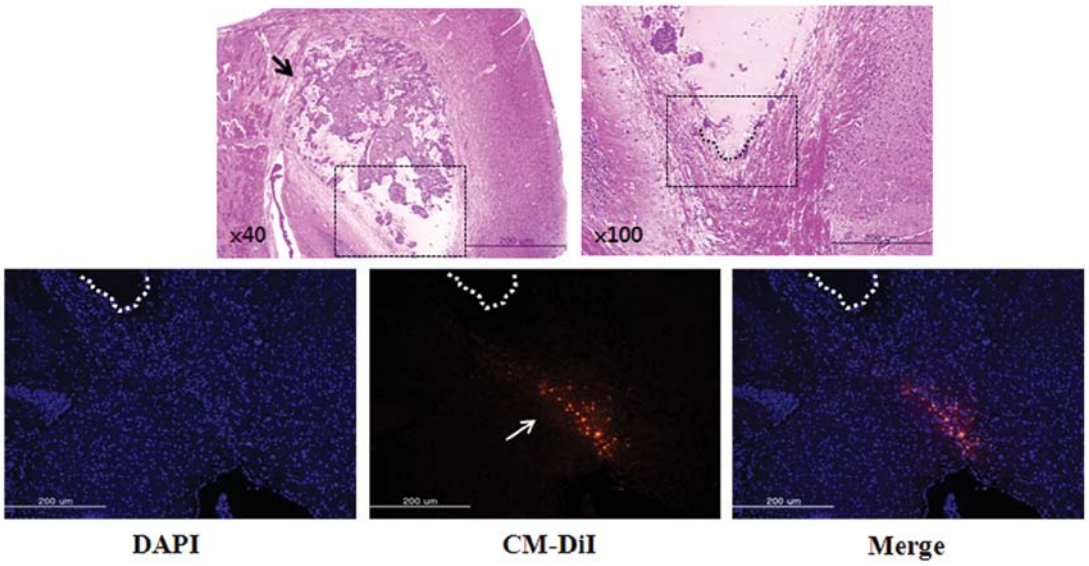

Figure 4. *In vivo* migratory ability of stem cells towards lung cancer metastasis in the brain. The upper panels show brain tissue treated with HB1.F3.yCD and 5-FC by hematoxylin and eosin staining. The lower panels present the brain tissues following injection with A549 lung cancer cells and HB1.F3.yCD by fluorescence analysis. To observe migratory effects, brain sections were stained by DAPI for fluorescence analysis. Red, pre-stained HB1.F3.yCD; blue, stained brain and HB1.F3.yCD cells; black and white dotted line, the boundary of tumor and normal brain cells; black arrow, tumor mass by H&E staining; white arrow, migrated stem cells.

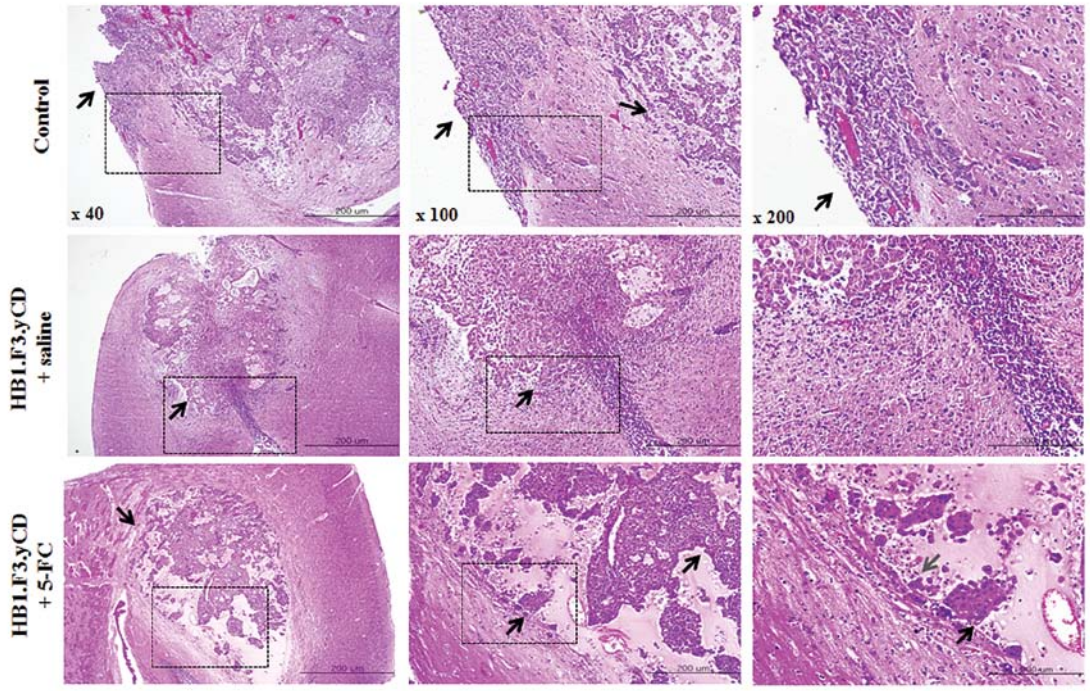

Figure 5. *In vivo* therapeutic efficacy of HB1.F3.yCD cells. After tumor formation in the right hemisphere of the mouse brain, HB1.F3.yCD cells pre-stained with CM-DiI were implanted into the contralateral region of the brain. After 2 days of injection, 5-FC (500 mg/kg/day) was intraperitoneally injected into the SCID mice. The extracted brains were analyzed for tumor formation by HB1.F3.yCD cells in the presence of a prodrug, 5-FC. The dotted rectangle areas are enlarged in the images to the right by microscopy. Black arrow, injected A549 lung cancer cells; gray arrow, dying lung cancer cells.

blood-brain barrier (BBB) is disrupted, enhancing brain metastases (21,22).

Stem cells have been proposed as the basis of alternative therapy. In particular, NSCs can differentiate to neural cell lineages, including astrocytes and oligodendrocytes, *in vitro* and *in vivo* (23). Immortalized NSCs expressing a selected suicide gene can inhibit tumor growth in prostate, lung, and endometrial cancer, as well as brain diseases (9,16). In addition, NSCs have a tumor-tropic effect that includes their migration toward tumor cells (24). A GESTECs based GEPT system, including CD/5-FC and carboxyl esterase (CE)/irinotecan (CPT-11), can effetely deliver therapeutic genes and decrease growth of tumor cells *in vitro* and *in vivo* (25,26). The CD/5-FC system, a GEPT system, is used for bCD and yCD genes, which convert non-toxic prodrug 5-FC into toxic 5-FU. 5-FU inhibits RNA processing and DNA synthesis in cancer cells and ultimately results in cell death (27). Therefore, stem cells, particularly NSCs, may be an excellent vehicle as a delivery system of therapeutic genes.

In this study, we confirmed the therapeutic efficacy and migratory ability of HB1.F3.yCD cells expressing the yCD gene in *in vitro* and *in vivo* models. Presently, lung cancer cells were very sensitive to even low concentrations (0.01 mmol/l) of 5-FU, but 5-FC was not effective in the growth of lung cancer cells. HB1.F3.yCD cells showed a 40% inhibition of cell viability for lung cancer cells in the presence of 5-FC (500 μg/ml) and their therapeutic efficacy was confirmed in the *in vitro* model.

Using a mouse model of lung cancer cell metastasis to the brain, we also verified the effect of HB1.F3.yCD cells in brain metastasis by tumor tropism *in vivo*. After implantation of lung cancer cells into the right hemisphere, CM-DiI-pre-stained HB1.F3.yCD cells were injected into the contralateral brain of SCID mice following injection with A549 lung cancer cells. The red-colored HB1.F3.yCD cells appeared to migrate to the area of lung metastasis in the brain, as observed by fluorescence analysis. This migratory property of HB1.F3.yCD cells can be caused by various chemoattractant factors secreted by cancer cells including stromal cell-derived factor-1, vascular endothelial growth factor, and monocyte chemoattractant protein-1 (28). Secreted ligands may be recognized by HB1.F3.yCD cells, and a tumor specific migratory property may be stimulated by these stem cells (29).

Among them, the use of HB1.F3.yCD cells in the presence of 5-FC showed that the generated tumor mass of lung tumor metastasis was significantly reduced compared to a negative control or HB1.F3.yCD in the absence of 5-FC. In histological analysis, we further confirmed the inhibitory effect of HB1.F3.yCD cells on lung tumor formation in the mouse brain. In particular, the number of lung tumor cells was evidently reduced, and the aggressive character of the tumor cells was decreased by the injection of HB1.F3.yCD in the presence of 5-FC. Taken together, these results indicate that lung cancer metastasis to the brain can be cured by HB1.F3.yCD cells in the conversion of a prodrug, 5-FC, to its metabolic active form, 5-FU, via their tumor tropic effect.

In summary, the viability of lung cancer cells was inhibited by co-culturing with HB1.F3.yCD in the presence to 5-FC *in vitro*. In addition, the therapeutic efficacy and migratory ability of HB1.F3.yCD cells were verified in a mouse xenograft model *in vivo*. Although pre-stained HB1.F3.yCD cells were injected into the contralateral area of the brain following

formation of lung cancer, they can migrate to the tumor mass of lung cancer cells and kill them via tumor tropism. These results suggest that HB1.F3.yCD cells have tumor-tropic and tumoricidal effects in the presence of a prodrug, 5-FC, suggesting that these GESTECs may be an effective therapeutic strategy for lung cancer metastasis to brain.

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