

Original Article

Three-dimensional assessment of bystander effects of mesenchymal stem cells carrying a cytosine deaminase gene on glioma cells

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Abstract: Stem cells carrying a suicide gene have emerged as therapeutic candidates for their cytotoxic bystander effects on neighboring cancers, while being non-toxic to other parts of the body. However, traditional cytotoxicity assays are unable to adequately assess the therapeutic effects of bystander cells. Here, we report a method to assess bystander effects of therapeutic stem cells against 3-dimensionally grown glioma cells in real time. U87 glioma cells were stably transduced to express a green fluorescence protein and co-cultivated with mesenchymal stem cells engineered to carry a bacterial cytosine deaminase gene (MSC/CD). Following addition of a 5-fluorocytine (5-FC) prodrug to the co-culture, fluorescence from U87 cells was obtained and analyzed in real time. Notably, the IC₅₀ of 5-FC was higher when U87 cells were grown 3-dimensionally in soft agar medium for 3 weeks, as compared to those grown for one week in two-dimensional monolayer cultures. Additionally, more MSC/CD cells were required to maintain a similar level of efficacy. Since three-dimensional growth of glioma cells under our co-culture condition mimics the long-term expansion of cancer cells *in vivo*, our method can extend to an *in vitro* assay system to assess stem cell-mediated anti-cancer effects before advancing into preclinical animal studies.

Keywords: Bystander effect, gene therapy, suicide gene, 3D culture, stem cell

Introduction

Glioblastoma multiforme (GBM) is the most common form of malignant brain tumor in the central nervous system. Despite extensive efforts to treat GBM, brain resection following a combination of chemotherapy and radiotherapy remains the most effective therapy and only extends the median survival period for glioma patients by 7 to 9 months [1, 2].

Recently, suicide gene therapy has emerged as a therapeutic candidate for treating brain tumors. Suicide genes convert a non-toxic prodrug into an active derivative that exerts cytotoxic effects on cancer cells while minimizing negative effects to other parts of the body. Safety and efficacy are the primary challenges in developing this type of gene-delivery system. Generally, viral vectors are more efficient at transferring genes than non-viral vectors; how-

ever, clinical studies have revealed that efficacy of virus-mediated suicide gene therapy was limited due to inability to disperse and infect targeted cells *in vivo* and the possibility of invoking an immune response [3].

These limitations can be resolved by using stem cells that have a strong tropism to brain tumors as vehicles to selectively deliver the gene-of-interest to tumor sites. For this, stem cells were expanded *in vitro* and engineered to express the therapeutic genes prior to transplantation (*ex vivo* therapy) [4]. The advantage of this type of therapy is that it does not require the direct delivery of suicide genes to cancer cells, but rather relies on the strong bystander effects of the engineered stem cell vehicles.

Cytosine deaminase (CD) has attracted attention for its strong bystander effect compared to other suicide genes, such as herpes simplex

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virus thymidine kinase gene (HSV-tk) [5, 6]. Phosphorylated metabolites of ganciclovir converted by HSV-tk integrate into DNA during replication, subsequently causing cell death. However, these cytotoxic effects rely on intercellular gap junctions since ganciclovir metabolites are unable to diffuse across the plasma membrane. CD converts the nontoxic prodrug 5-fluorocytosine (5-FC) into its potent anticancer derivative 5-fluorouracil (5-FU), which has been used to treat gastrointestinal cancer. Unlike HSV-tk/ganciclovir, the CD/5-FC system has a significant bystander effect that does not require direct cell contact, as 5-FU can readily disperse amongst cells by non-facilitated diffusion [7]. Engineered stem cells that express the CD gene migrate toward cancer sites and produce 5-FU in the presence of 5-FC. 5-FU can then diffuse to neighboring cancer cells and exert its cytotoxic effects by interfering with DNA and RNA synthesis (bystander effect). During this process, the stem cells carrying the CD gene are also subject to these effects and undergo cell death. Hence, the combination of CD and 5-FC systematically increases the local dose of 5-FU around tumor sites and decreases the exposure level of 5-FU to other regions. Recent studies demonstrate that neural stem cells (NSCs) that express the CD gene were able to migrate near the tumor cells, and successfully suppress the growth of intracranial gliomas following 5-FC administration [8]. Likewise, mesenchymal stem cells (MSC) engineered to express CD gene also sufficiently inhibited the growth of the brain tumors in 5-FC-treated rats [9, 10].

Since evaluating bystander effects requires the co-culture of therapeutic stem cells and cancer cells, *in vitro* assays need to be capable of excluding the signals from stem cells and specifically measure the growth or death of cancer cells. Currently, most stem cell-based studies utilize conventional colorimetric assays that include mitochondrial enzyme-based methods [11, 12] and trypan blue exclusion [13], which are unable to distinguish the viability signals of both therapeutic stem cells and tumor cells surviving after suicide and bystander effects, respectively. Alternatively, other studies have used tumor cells pre-labeled with fluorescent dye [12, 14]; however, the fluorescent dye may become diluted in proportion to the tumor growth during the assay period.

In this paper, we describe a method to exclusively measure the surviving signals of glioma cells co-cultured *in vitro* as monolayers in the presence of therapeutic stem cells. We also demonstrate that our assay is also sufficient to assess bystander effects in 3D culture conditions that better emulate *in vivo* microenvironment, and thus narrow the gap between *in vitro* cell culture assays and *in vivo* animal studies.

Materials and methods

Cells and viral vectors

U87MG (ATCC HTB-14, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen, Grand Island, NY, USA) in a 37°C incubator. The LacZ expressing retroviral vector MSCV-puroLacZ was transduced to U87MG with 4 µg/mL polybrene (Sigma, St. Louis, MO, USA). Two days after transduction, U87/LacZ-positive cells were selected in a growth medium containing 1 µg/mL puromycin (Sigma) for two weeks with media changed every three days. Surviving cells were pooled and expanded in fresh growth medium. Similarly, the green fluorescence protein (GFP) expressing lentiviral vector LL3.7 was used to transduce U87MG with 4 µg/mL polybrene [15]. Two days later, transduced cells were sorted using fluorescence-activated cell sorter (BD Biosciences, San Jose, CA, USA) and resulting U87/GFP cells were expanded in growth medium. Human MSCs were obtained from iliac crest bone marrow of healthy donors for future allogeneic transplantation with the approval of the Institutional Review Board of Ajou University, Medical Center (AJIRB-GEN-10-175), as previously described [9, 16]. Parental written informed consent was obtained for the use of bone marrow samples for an experimental study. Briefly, MSCs were maintained in a standard growth medium further supplemented with 20 ng/mL basic fibroblast growth factor (bFGF, Dong-A Pharmaceutical Co., Youngin, Korea). The differentiation potency of MSCs into osteocytes, adipocytes, and chondrocytes was verified. The CD-expressing retroviral vector FIP-CD was used to transduce MSCs at multiplicity of infection of 20 for 8 h with 4 µg/mL polybrene. Two days later, cells were selected in growth medium containing 2 µg/mL puromycin.

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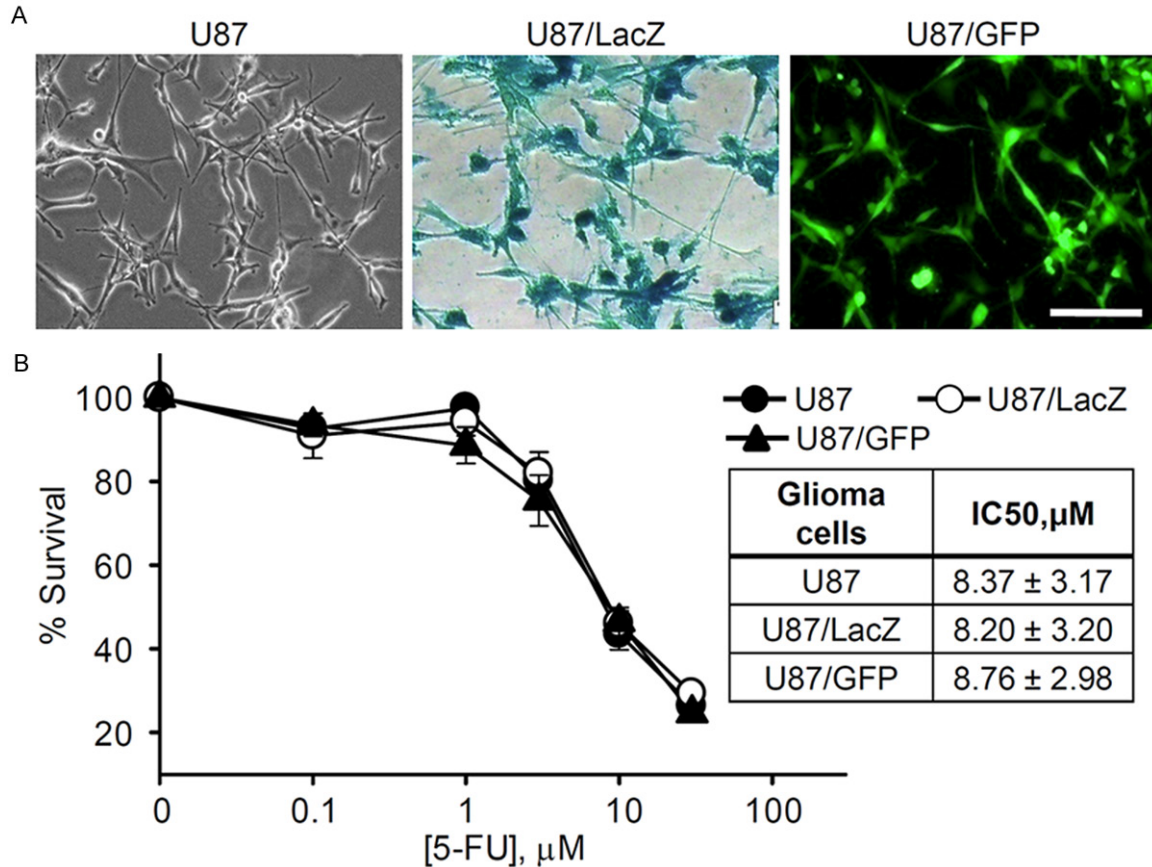


Figure 1. 5-FU sensitivity of reporter gene expressing glioma cells. **A.** Morphology and reporter gene expression in glioma cells. Scale bar = 100 μm. **B.** Cells were cultured in the presence of the indicated concentrations of 5-FU for five days. Cell viability was then measured by MTT assays and presented as relative survival with respect to the value in the absence of 5-FU. The values of IC₅₀ are presented as means ± SE.

cin for two weeks. Surviving cells were pooled, expanded, and maintained in growth medium supplemented with 20 ng/mL bFGF.

MTT assays

U87MG, U87/LacZ, and U87/GFP cells were plated in 12-well plates at a density of 10,000 cells/well. After culturing in standard growth medium for 24 h, old medium was replaced with fresh growth medium containing indicated concentrations of 5-FU (Sigma-Aldrich) every 48 h. On the fifth day, survival rates of the cells were measured with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich). Briefly, cells were incubated in 1 mL of MTT for 1 h. Formazan crystals were then solubilized with 1 mL of DMSO, and absorbance at OD₄₉₀ was measured using a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Data acquired from at least

from 3 independent experiments were presented as a mean ± standard error (SE) relative to that of the untreated control group. Likewise, MSCs or MSC/CDs were plated in 12-well plates at a density of 10,000 cells/well. After 24 h of incubation in growth medium containing indicated concentrations of 5-FC (Kolon Life Science Co., Gwacheon, Korea), the old medium was replaced with fresh medium containing the same concentrations of 5-FC every 48 h. On the seventh day, survival rates of MSC/CDs and MSCs were measured using the MTT assay as previously described. The data at least from 3 independent experiments were presented as a mean ± SE relative to that of the untreated control group.

Two-dimensional (2D) assays for bystander effects

U87/GFP or U87/LacZ cells were co-cultured with MSC/CDs at densities of 10,000:10,000,

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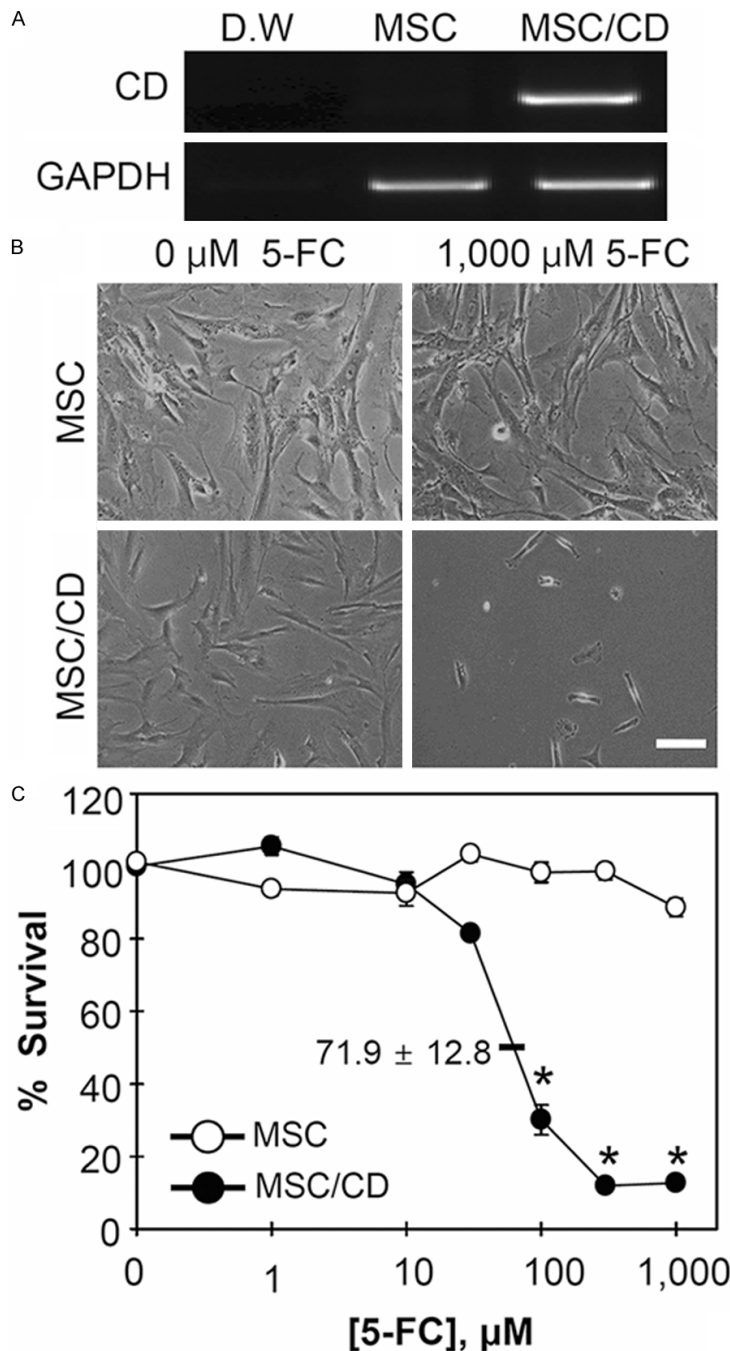


Figure 2. Suicide effects of MSC/CD in the presence of 5-FC. A. RT-PCR indicated expression of CD gene in MSC/CDs. B, C. Cells were incubated in the presence of the indicated concentration of 5-FC for seven days. Surviving cells were quantified by MTT assay and presented as the means \pm SE at each concentration relative to the value in the absence of 5-FC. Result from at least three independent experiments. (* $p < 0.05$, one-way ANOVA).

10,000:3,000, and 10,000:1,000 per well in 12-well plates. After 24-h incubation in growth medium containing indicated concentrations of

5-FC, images of surviving U87/GFP cells were acquired under a fluorescence microscope (MVX-10, Olympus, Japan). The cells were then lysed in 100 μ L of passive lysis buffer (Promega, Madison, WI, USA). Fifty microliters of whole lysate was transferred into each well of 96 well plates, and the fluorescence quantified with a fluorometer (Molecular Devices). Beta-galactosidase viability assays were performed by briefly fixing U87/LacZ cells with 0.625% glutaraldehyde in phosphate-buffered saline (PBS) for 10 min. The cells were then washed with PBS for 5 min 3 times prior to staining with X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) solution consisting of 1 mg/mL X-gal (Koma Biotech, Seoul, Korea), 2 mM $MgCl_2$, and 5 mM potassium ferrocyanide/potassium ferricyanide in PBS for 16 h at 37°C. Alternatively, the cells were lysed in 200 μ L passive lysis buffer, and 20 μ L of whole cell lysates was utilized for β -galactosidase assays using ortho-nitrophenyl- β -galactoside as a substrate. The reaction mixtures were incubated at 37°C for 30 min, and absorbance at OD_{420} was measured. The survival rates for each group with different concentrations were expressed as a mean \pm SE relative to that of untreated control group from three independent experiments.

Three-dimensional (3D) assays for bystander effects

U87/GFP cells were mixed with MSC/CDs at the indicated ratios in 0.4% soft agar mixture in a growth medium using the cell transformation detection assay kit (Millipore, Temecula, CA, USA)

following the manufacturer's recommendations. Briefly, 24-well plates were coated with 100 μ L/well of 0.8% soft agar prepared in

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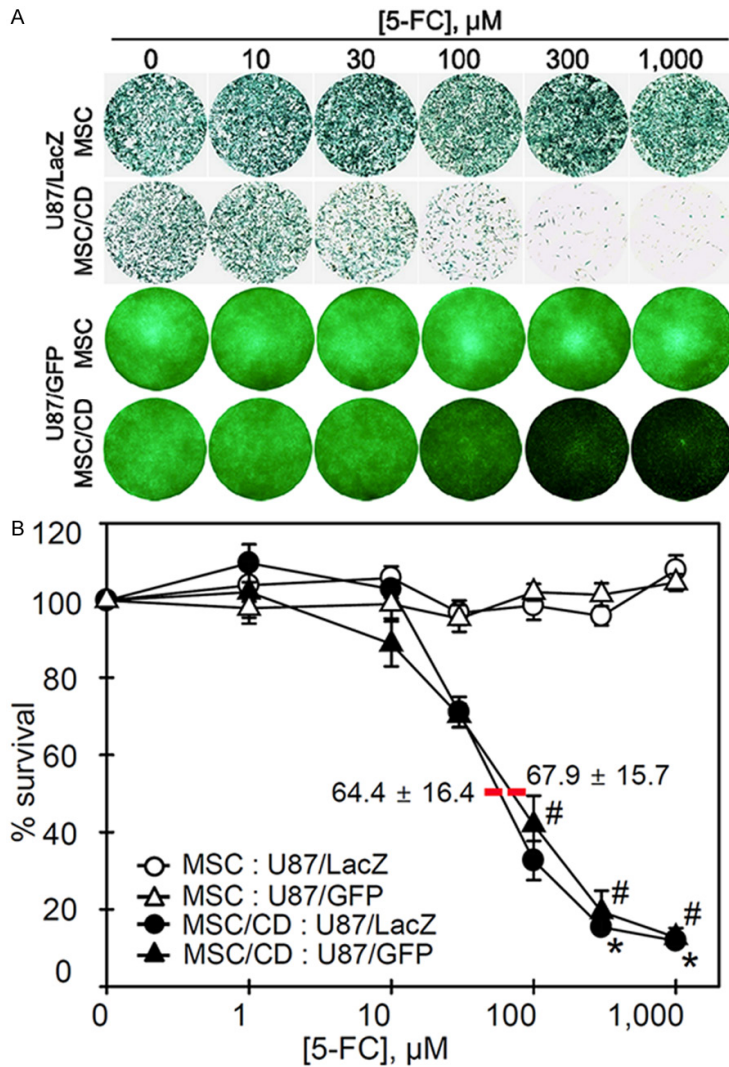


Figure 3. Bystander effects of MSC/CD on U87/LacZ and U87/GFP cells in two-dimensional (2D) co-culture system. A. The U87/LacZ and U87/GFP cells were co-cultured with MSCs or MSC/CDs in growth medium containing indicated concentrations of 5-FC for seven days. B. Viable U87-LacZ and U87/GFP cells were observed by X-gal staining and fluorescence microscopy, respectively. Values are presented as the mean ± SE of results from at least three independent experiments ($^{\#}p < 0.05$ compared to MSC:U87/LacZ group, $^*p < 0.05$ compared to MSC:U87/GFP group; one-way ANOVA).

growth medium, and left to solidify at 4°C for 15 min. After warming to room temperature for 10 min, the indicated numbers of dissociated cells in 100 μL of growth medium were mixed with 100 μL of 0.8% soft agar to make a cell suspension in 0.4% soft agar, which was then plated on top of the solidified bottom layer. For growth assays, an additional 500 μL of growth medium was applied to each well on day 0. For drug sensitivity assays, 500 μL of growth medi-

um containing 5-FC at the indicated concentrations was added to each well 24 h later. The medium was replaced with fresh media containing the same concentration of 5-FC every 48 h. Fluorescent images of U87/GFP spheroids were acquired after three-week cultures using a fluorescence dissecting microscope (Olympus). The fluorescent signals were quantified using ImageJ software (NIH, Bethesda, MD, USA) and presented as relative value with respect to the control in the absence of 5-FC. The surviving cells grown as spheroids were harvested by scraping the upper layer of soft agar and mixing with 250 μL PBS. The soft agar was melted by heating at 80°C for 10 min, and 100 μL of the supernatant of each well was transferred to a new well in a 96 well plate. The fluorescence emitted from GFP was measured with a microplate reader (Molecular Devices) at the excitation and emission wavelengths of 485 nm and 538 nm, respectively.

Bystander effect on U87MG *in vivo*

All animal protocols were approved by the Institutional Animal Care and Use Committee of Ajou University, Medical School (Permit Number: AMC 95), as previously described [9]. Briefly, adult BALB/C nude mice (~20 g, Nara Biotech, Seoul, Korea) were anesthetized and U87/LacZ or U87/GFP (3×10^5 cells in 3 μL of PBS) and a day later, MSC or MSC/CD (3×10^5 cells in 6 μL of PBS), were sequentially transplanted into the striatum (anteroposterior, +0.5 mm; mediolateral, +3 mm; dorsoventral, 4 mm) using a stereotaxic device (Stoelting, Wood Dale, IL, USA). After 24 h, 500 mg/kg of 5-FC was given to each mouse daily via intraperitoneal injection. Three or five weeks later, animals were perfused with 4% paraformaldehyde (PFA), and the

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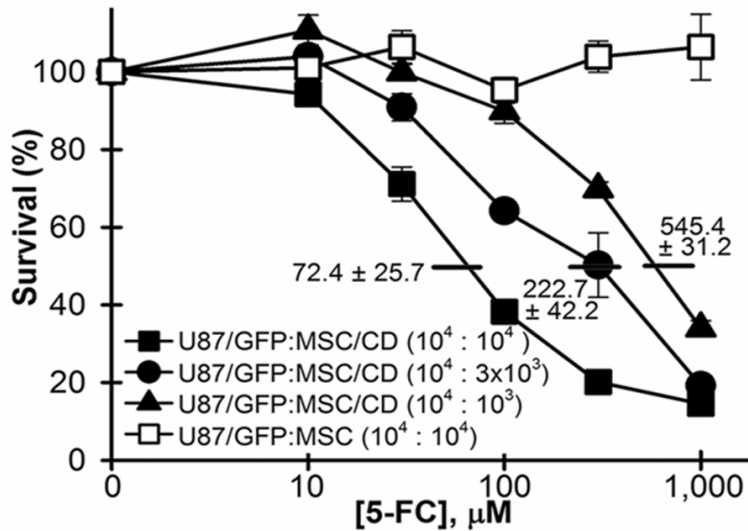


Figure 4. The differential responsiveness of U87/GFP cells to 5-FC with respect to the number of MSC/CDs. U87/GFP cells were plated with MSCs or MSC/CDs and incubated in growth medium containing indicated concentrations of 5-FC for seven days. Surviving U87/GFP cells were quantified by measuring the fluorescence intensity. Values are presented as the mean \pm SE of at least three independent experiments with each concentration relative to that observed in the absence of 5-FC.

brains fixed with 4% PFA at 4°C overnight. Brains were sliced into 1-mm thick sections using a rodent brain matrix (Stoelting). For animals with U87/LacZ grafts, slices were incubated in X-gal staining solution for 16 h at 37°C with the addition of 0.2% NP40. The images of both sides of X-gal-positive or GFP-positive brain slices were acquired with a fluorescence dissecting microscope (MVX10, Olympus), and were analyzed by Image J program (NIH, Bethesda, MD, USA) as previously described [9]:

$$\text{Tumor volume (mm}^3\text{)} = \sum(\text{Number of pixels of tumor area/Number of pixels per unit area}) \times 0.5 \text{ mm}$$

Results

Sensitivity of glioma cells to 5-FU was preserved after transduction of reporter genes

U87/LacZ and U87/GFP glioma cell lines showed fibroblastic morphologies, which were similar to those found in untransduced U87MG cells (Figure 1). MTT assays measuring the mitochondrial activity of surviving U87MG, U87/LacZ, and U87/GFP cells showed IC_{50} values of 8.37, 8.20, and 8.76 μM , respectively. The data indicated that negligible changes

were made by transduction of reporter genes in fibroblastic morphology and 5-FU sensitivity. The results also validated the prerequisite for our approach to accurately distinguish signals of surviving U87MG from remnant MSC/CDs by either β -galactosidase-based enzyme assays or GFP-based fluorometric analysis (see below).

Suicide effect of the therapeutic stem cells

RT-PCR analysis showed consistent expression of CD gene in MSC/CD (Figure 2A). Consistent with our previous report [16], transduction did not alter the morphology (Figure 2B) or the differentiation capability of MSCs (*data not shown*).

To determine the suicide effect of MSC/CDs in the presence of 5-FC, cells were incubated in growth medium containing the indicated concentrations of 5-FC for seven days. MTT assay showed that the survival rate of MSC/CDs decreased in a dose-dependent manner with IC_{50} of 71.9 μM (Figure 2C). In contrast, the MSC survival rate was unaffected in high concentrations of 5-FC under the same conditions. The results validated that the CD expressed in MSC/CDs can convert 5-FC in the medium into 5-FU at levels sufficient to inhibit MSC/CD proliferation through suicide effects.

2D assays for bystander effects against glioma cells

To confirm the bystander effect of MSC/CD with 5-FC against glioma cell lines, U87/LacZ cells were plated with MSCs or MSC/CDs in 1:1 ratio and incubated in growth medium containing the indicated concentrations of 5-FC for seven days. X-gal staining demonstrated that the growth of U87/LacZ cells was noticeably inhibited by 5-FC concentrations $>30 \mu\text{M}$ only when co-cultured with MSC/CDs (Figure 3A). Beta-galactosidase assays indicated that the IC_{50} for U87/LacZ cells was 64.4 μM . Similarly, when U87/GFP cells were co-cultured with MSC/CDs, the cytotoxic bystander effect was evident at

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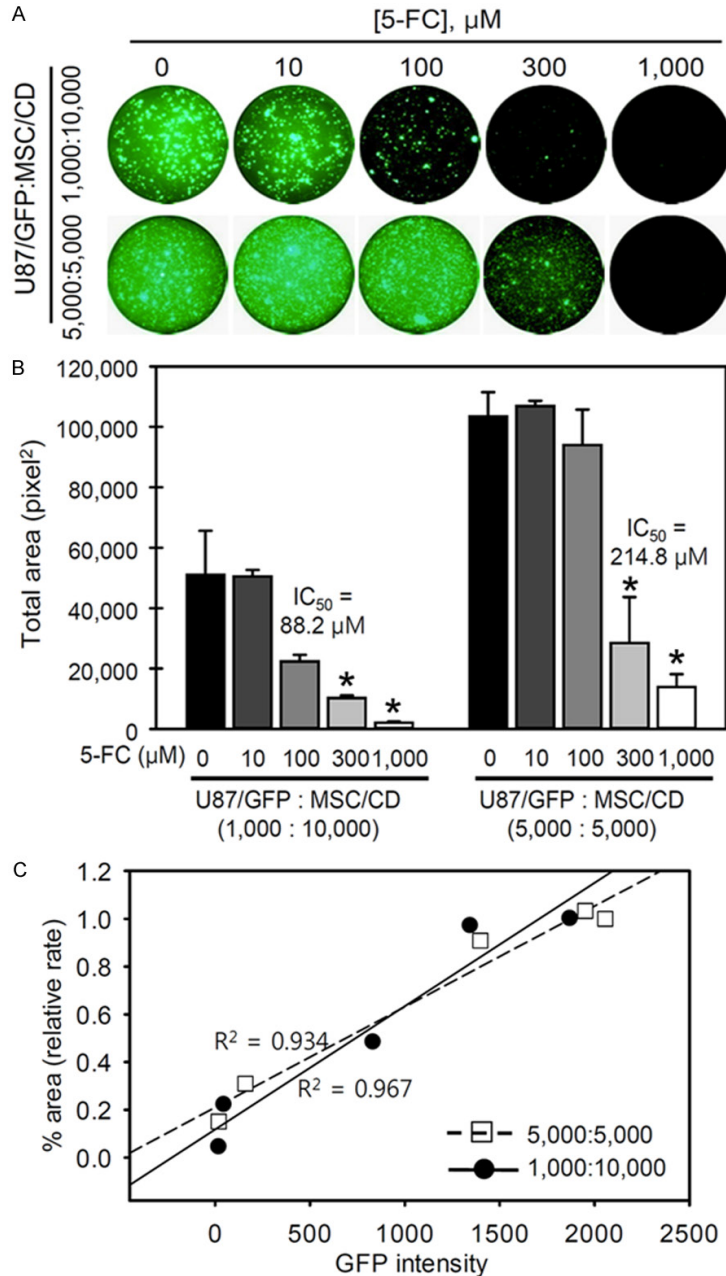


Figure 5. *In vitro* bystander effects of MSC/CDs + 5-FU in 3D spheroid culture system. (A) U87/GFP cells were mixed with MSC/CDs at the ratios shown in 0.4% soft agar and treated with the indicated concentrations of 5-FU. After three weeks, spheroids were observed using fluorescence microscopy. (B) GFP-positive areas were measured as surviving U87/GFP cells using Image J. The values are presented as the means \pm SE ($*p < 0.05$, one-way ANOVA) (C) GFP intensity was measured using fluorometer after harvesting the spheroid U87/GFP by dissolving soft agar. The quantitative fluorescence was correlated with fluorescence images.

concentrations $>30 \mu\text{M}$ (Figure 3A). Fluorometric analysis revealed that the IC_{50} for U87/GFP cells was $67.9 \mu\text{M}$ (Figure 3B). However, when less MSC/CD (3×10^3 or 10^3 cells) were

utilized in the co-culture with 10^4 U87/GFP cells, the bystander effect of 5-FU became evident at concentrations $>100 \mu\text{M}$ with IC_{50} values of 222.7 and $545.4 \mu\text{M}$, respectively (Figure 4). The parental MSCs did not exert any cytotoxic effects on glioma cells even in the presence of this highest 5-FU concentration. U87-GFP fluorometric assays indicated that the bystander effect of CD and 5-FU was dependent not only the concentration of 5-FU, but also on the ratio of therapeutic MSCs to glioma cells.

3D assays to measure bystander effects against glioma cells

To better emulate *in vivo* tumor growth, glioma cells were cultured in a 3D environment using soft agar for longer periods. In semi-solid medium, tumor cells can proliferate as spheroids in an anchorage-independent manner, whereas the therapeutic MSCs cannot. The fluorescence emitted from the multicellular U87/GFP spheroids through soft agar became evident within two weeks, and the cultures were maintained up to three weeks (Figure 5A). Unlike 2D cultures, plating 10^4 cells each of U87/GFP and MSC/CD did not allow proper spheroid formation of U87/GFP (data not shown) due to competition for slowly diffused nutrients in the 3D culture environment. Therefore, we limited the number of cells initially plated to approximately 10^4 cells per well total while varying the ratios of MSC/CD and U87/GFP cells.

After equal numbers of cells ($5,000:5,000$) were co-cultured for three weeks and fluorescent images were acquired. The fluorescence intensities decreased in proportion to 5-FU concentration with an IC_{50} of $214.8 \mu\text{M}$ (Figure 5B). When 10-fold more MSC/CD cells were plated ($10,000 \text{ MSC/CD}:1,000 \text{ U87/GFP}$),

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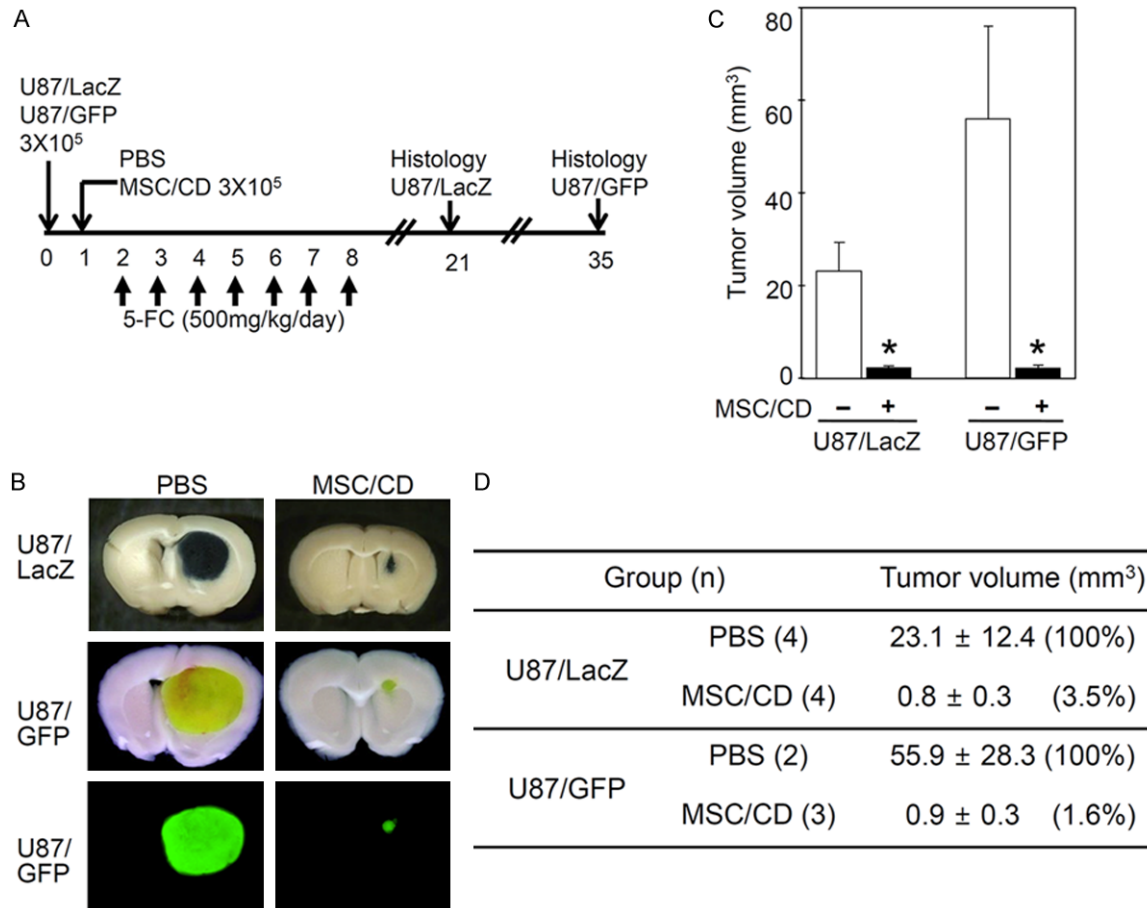


Figure 6. Bystander effects of MSC/CDs +5-FC in orthotopic glioma models. **A.** Schematic diagram used in the present study for in vivo bystander effect. **B.** Representative brain sections showed X-gal and GFP positive tumors from glioma cells. **C, D.** The brain from each animal was serially sectioned in 1-mm thickness and tumor volumes are presented as the mean ± SE (* $p < 0.05$, one-way ANOVA).

the IC_{50} increased to 88.2 μ M, indicating the dependency of bystander effects on the MSC/CD:U87/GFP ratios as seen in the 2D assay. Cells were then harvested by dissolving soft agar at 80°C prior to lysing and fluorometric analysis as described above (see the *Methods*). Importantly, the results obtained from fluorescence images were highly-correlated with the quantitative values obtained from fluorometric assays using total homogenate ($r^2 = 0.934\sim 0.967$, **Figure 5C**). The results validated our assay using two independent methods. It should also be noted that the IC_{50} of 5-FC in three-week soft agar cultures was approximately 3-fold higher than the value obtained in 2D culture after one week. Alternatively, approximately three times more MSC/CDs were required to maintain a similar level of bystander effect (see the *Discussion*).

Bystander effects of MSC/CD in vivo

To determine the bystander effects of MSC/CDs in an orthotopic model, we injected 3×10^5 U87/LacZ or U87/GFP cells into striatum. The next day, we transplanted equal numbers of MSC/CDs near the tumor sites. Animals were intraperitoneally administered with 500 mg/kg 5-FC once a day for one week (**Figure 6A**). Three to five weeks after the transplantation, the brain was removed and subjected to X-gal staining for U87/LacZ or fluorescence analysis for U87/GFP (**Figure 6B**). The tumor area per brain section was measured and expressed as tumor volume (see the *Methods*). The tumor growths of both U87/LacZ and U87/GFP were attenuated by the treatment of 5-FC in animals transplanted with MSC/CDs. The data indicated that, similar to our *in vitro* analyses, the MSC/

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CDs exert bystander effects on the expansion of U87 glioma cells independently of the reporter gene used to pre-label the glioma cells (Figure 6C, 6D).

Discussion

Virus-mediated suicide gene therapy emerged as a potent therapeutic candidate for the treatment of malignant cancers. However, its therapeutic efficacy is contingent on how well the engineered virus travels to the cancer site and its uptake by the targeted cell type. These limitations were resolved by using engineered stem cells as cellular carriers to deliver suicide genes with strong bystander activity. In our case, MSCs carrying CD gene were able to migrate towards the tumor site and impart cytotoxic effects to nearby tumor cells, while minimizing harm to non-tumor cells.

The MTT/MTS assay is based on the colorimetric measurement of formazan products produced by viable cells and it has been widely utilized to evaluate the cytotoxic effects of anti-cancer drugs [17]. We utilized MTT assays to measure the cytotoxic effects of 5-FU on glioma cells (Figure 1) and suicide effects of 5-FC on MSC/CDs (Figure 2). Since the MTT/MTS assay fails to differentiate between the signals of surviving glioma cells from those of other types of cells in the co-culture, we pre-labeled U87MG cells with LacZ or GFP reporter genes. This allowed us to quantify specific signals from surviving glioma cells by β -galactosidase or fluorescence-based assays in the presence of MSC/CD without altering the morphological traits or 5-FU sensitivity of the U87MG glioma cells (Figures 1, 3 and 4). Moreover, the results obtained using the image analysis and the fluorometric assessment exhibited a good correlation (Figure 5), suggesting that the image analysis method may replace the laborious fluorometric method that requires cell lysis, even for multicellular spheroids.

Although MSC/CDs inhibited the growth of the glioma cells both in the 2D and 3D co-cultures (Figures 4 and 5), the greater ratios of MSC/CDs with respect to U87/GFP cells increased the responsiveness of glioma cells to 5-FC, consequently decreasing the IC_{50} values (Figures 4 and 5). The results suggested that more MSC/CD cells—if practically feasible—guarantee a better outcome and that 3D cul-

ture is applicable to determining a proper dosage of therapeutic cells.

Although the bystander effects were observed consistently, the IC_{50} values from 3D cultures were higher compared to that of 2D culture counterparts (Figures 3 and 5). This finding might be attributed to several reasons. First, the availability of a 5-FC to MSC/CDs and diffusion of 5-FU might be confined to the outer cell layer; thus, the cells inside spheroids are less vulnerable to these cytotoxic effects. Second, the expression profiles of survival-related genes might also vary depending on a cell's relative position in 3D spheroids [18, 19]. Finally, a population of U87MG cells underwent immediate cell death in 2D monolayer culture after one week. In contrast, a single U87MG cell formed a multicellular colony by clonal expansion after three weeks of 3D culture. Therefore, 3D culture better mimics the *in vivo* model, in which the glioma cells proliferate over several weeks to form a multicellular mass in the brain (Figure 6).

5-FC is absorbed rapidly from the gastrointestinal tract and is widely distributed throughout the body, including the central nervous system [20]. For humans, a single dose of 37.5 mg/kg yielded a plasma concentration of 70-80 μ g/mL 2 h after administration [21]. The human dose equates to 462.5 mg/kg in mouse after cross-species normalization based on body surface area as previously suggested [22]. Thus, it is very reasonable that a single dose of 500 mg/kg might yield a concentration of approximately 70 μ g/mL (~540 μ M) in mouse plasma and brain, which was higher than the IC_{50} value obtained in 3D culture. With a half-life of 3~6 h, such concentrations appear sufficient to suppress the tumor growth in a mouse brain tumor model (Figure 6).

In summary, transduction of tumor cells with reporter genes allowed for reliable measurement of tumor-specific growth under co-culturing conditions as either 2D monolayer or 3D spheroids. In addition, image analysis could be a convenient method alternative to fluorometric measurement for evaluating anti-cancer effects in 3D culture. The dosage responsiveness of therapeutic MSC/CDs was well conserved in 3D culture, as the bystander effects observed with 3D culture correlated significantly with those obtained in a mouse brain tumor

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model. Taken together, we propose that 3D coculturing in soft agar medium is an efficient method to narrow the gap in translating the results obtained from short-term monolayer culture experiments prior to conducting a pre-clinical animal study that requires a long-term evaluation.

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Disclosure of conflict of interest

None.

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References

- [1] Stupp R, Mayer M, Kann R, Weder W, Zouhair A, Betticher DC, Roth AD, Stahel RA, Majno SB, Peters S, Jost L, Furrer M, Thierstein S, Schmid RA, Hsu-Schmitz SF, Mirimanoff RO, Ris HB and Pless M. Neoadjuvant chemotherapy and radiotherapy followed by surgery in selected patients with stage IIIB non-small-cell lung cancer: a multicentre phase II trial. *Lancet Oncol* 2009; 10: 785-793.
- [2] Stupp R and Weber DC. The role of radio- and chemotherapy in glioblastoma. *Onkologie* 2005; 28: 315-317.
- [3] Kwiatkowska A, Nandhu MS, Behera P, Chioccia EA and Viapiano MS. Strategies in gene therapy for glioblastoma. *Cancers (Basel)* 2013; 5: 1271-1305.
- [4] Ahmed AU, Thaci B, Alexiades NG, Han Y, Qian S, Liu F, Balyasnikova IV, Ulasov IY, Aboody KS and Lesniak MS. Neural stem cell-based cell carriers enhance therapeutic efficacy of an oncolytic adenovirus in an orthotopic mouse model of human glioblastoma. *Mol Ther* 2011; 19: 1714-1726.
- [5] Mesnil M and Yamasaki H. Bystander effect in herpes simplex virus-thymidine kinase/ganciclovir cancer gene therapy: role of gap-junctional intercellular communication. *Cancer Res* 2000; 60: 3989-3999.
- [6] Dachs GU, Hunt MA, Syddall S, Singleton DC and Patterson AV. Bystander or no bystander for gene directed enzyme prodrug therapy. *Molecules* 2009; 14: 4517-4545.
- [7] Huber BE, Austin EA, Richards CA, Davis ST and Good SS. Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. *Proc Natl Acad Sci U S A* 1994; 91: 8302-8306.
- [8] Aboody KS, Brown A, Rainov NG, Bower KA, Liu S, Yang W, Small JE, Herrlinger U, Ourednik V, Black PM, Breakefield XO and Snyder EY. Neural stem cells display extensive tropism for pathology in adult brain: evidence from intracranial gliomas. *Proc Natl Acad Sci U S A* 2000; 97: 12846-12851.
- [9] Chang DY, Yoo SW, Hong Y, Kim S, Kim SJ, Yoon SH, Cho KG, Paek SH, Lee YD, Kim SS and Suh-Kim H. The growth of brain tumors can be suppressed by multiple transplantation of mesenchymal stem cells expressing cytosine deaminase. *Int J Cancer* 2010; 127: 1975-1983.
- [10] Altaner C, Altanerova V, Cihova M, Ondicova K, Rychly B, Baciak L and Mravec B. Complete regression of glioblastoma by mesenchymal stem cells mediated prodrug gene therapy simulating clinical therapeutic scenario. *Int J Cancer* 2014; 134: 1458-1465.
- [11] Kucerova L, Altanerova V, Matuskova M, Tyciakova S and Altaner C. Adipose tissue-derived human mesenchymal stem cells mediated prodrug cancer gene therapy. *Cancer Res* 2007; 67: 6304-6313.
- [12] Yi BR, Kim SU, Kim YB, Lee HJ, Cho MH and Choi KC. Antitumor effects of genetically engineered stem cells expressing yeast cytosine deaminase in lung cancer brain metastases via their tumor-tropic properties. *Oncol Rep* 2012; 27: 1823-1828.
- [13] Wang C, Natsume A, Lee HJ, Motomura K, Nishimira Y, Ohno M, Ito M, Kinjo S, Momota H, Iwami K, Ohka F, Wakabayashi T and Kim SU. Neural stem cell-based dual suicide gene delivery for metastatic brain tumors. *Cancer Gene Ther* 2012; 19: 796-801.
- [14] Matuskova M, Hlubinova K, Pastorakova A, Hunakova L, Altanerova V, Altaner C and Kucerova L. HSV-tk expressing mesenchymal stem cells exert bystander effect on human

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- glioblastoma cells. *Cancer Lett* 2010; 290: 58-67.
- [15] Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, Kopinja J, Rooney DL, Zhang M, Ihrig MM, McManus MT, Gertler FB, Scott ML and Van Parijs L. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat Genet* 2003; 33: 401-406.
- [16] Park JS, Chang DY, Kim JH, Jung JH, Park J, Kim SH, Lee YD, Kim SS and Suh-Kim H. Retrovirus-mediated transduction of a cytosine deaminase gene preserves the stemness of mesenchymal stem cells. *Exp Mol Med* 2013; 45: e10.
- [17] Alley MC, Pacula-Cox CM, Hursey ML, Rubinstein LR and Boyd MR. Morphometric and colorimetric analyses of human tumor cell line growth and drug sensitivity in soft agar culture. *Cancer Res* 1991; 51: 1247-1256.
- [18] Chitcholtan K, Asselin E, Parent S, Sykes PH and Evans JJ. Differences in growth properties of endometrial cancer in three dimensional (3D) culture and 2D cell monolayer. *Exp Cell Res* 2013; 319: 75-87.
- [19] Hongisto V, Jernstrom S, Fey V, Mpindi JP, Kleivi Sahlberg K, Kallioniemi O and Perala M. High-throughput 3D screening reveals differences in drug sensitivities between culture models of JIMT1 breast cancer cells. *PLoS One* 2013; 8: e77232.
- [20] Brunton LL LJ, Parker KL. *Goodman & Gilman's The Pharmacological Basis of Therapeutics*. McGraw-Hill Co.; 2006. pp. 1229-1230.
- [21] Pai MP, Bruce H and Felton LA. Clinical pharmacokinetics of oral controlled-release 5-fluorocytosine. *Antimicrob Agents Chemother* 2010; 54: 1237-1241.
- [22] Reagan-Shaw S, Nihal M and Ahmad N. Dose translation from animal to human studies revisited. *FASEB J* 2008; 22: 659-661.