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ORIGINAL ARTICLE

Retrovirus-mediated transduction of a cytosine deaminase gene preserves the stemness of mesenchymal stem cells

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Human mesenchymal stem cells (MSCs) have emerged as attractive cellular vehicles to deliver therapeutic genes for *ex-vivo* therapy of diverse diseases; this is, in part, because they have the capability to migrate into tumor or lesion sites. Previously, we showed that MSCs could be utilized to deliver a bacterial cytosine deaminase (*CD*) suicide gene to brain tumors. Here we assessed whether transduction with a retroviral vector encoding *CD* gene altered the stem cell property of MSCs. MSCs were transduced at passage 1 and cultivated up to passage 11. We found that proliferation and differentiation potentials, chromosomal stability and surface antigenicity of MSCs were not altered by retroviral transduction. The results indicate that retroviral vectors can be safely utilized for delivery of suicide genes to MSCs for *ex-vivo* therapy. We also found that a single retroviral transduction was sufficient for sustainable expression up to passage 10. The persistent expression of the transduced gene indicates that transduced MSCs provide a tractable and manageable approach for potential use in allogeneic transplantation.

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INTRODUCTION

Mesenchymal stem cells (MSCs) have been utilized for the treatment of diverse diseases, including neuropathies such as Parkinson's disease,[1] Huntington's disease,[2] multiple sclerosis,[3,4] amyotrophic lateral sclerosis,[5] ischemic stroke,[6,7] and non-neurological diseases such as myocardial infarction,[8,9] and graft-*versus*-host diseases.[10] The therapeutic effects of MSCs are ascribed to their paracrine functions that include the secretion of beneficial molecules,[11,12] anti-inflammatory factors,[13,14] or extracellular matrix.[15] However, a major challenge is how to render MSCs more disease-specific and enhance their paracrine effects. As MSCs are highly migratory to lesion and tumor sites,[16] it has been suggested they can be used as cellular vehicles to deliver therapeutic genes to target tissues for *ex-vivo* therapy and to overcome targeting problems of conventional gene therapy. To tailor MSCs to be more disease-specific or to modify them as gene carriers, viral vectors are frequently utilized to introduce therapeutic genes into MSCs.

Previously, we showed that MSCs could be utilized as a cellular vehicle to deliver a cytosine deaminase (*CD*) gene to brain tumors.[17] *CD* genes are naturally expressed in bacteria and fungi, but absent in humans. CD can convert a nontoxic prodrug, 5-fluorocytosine (5-FC) into 5-fluorouracil, an anti-cancer drug that has been used for the treatment of gastrointestinal cancers.[18] Cell membranes are highly permeable to 5-fluorouracil, which can enter neighboring cells through simple diffusion and exert cytotoxic effects by interfering with DNA and RNA synthesis (bystander effects). We showed that MSCs infected with a retroviral vector expressing an *Escherichia coli CD* gene could migrate toward brain tumors and suppress tumor growth through bystander

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effects,[17] when animals were systemically administered with 5-FC. In addition to our efforts, other laboratories have utilized MSCs as cellular vehicles to deliver therapeutic genes (including interleukin-12,[19] herpes simplex virus–thymidine kinase,[20] tumor necrosis factor apoptosis ligand,[21] and interferon-β) to brain tumors.[22]

Retroviral vectors are often used to guarantee long-lasting transgene expression. However, these vectors can cause insertional mutagenesis when they integrate into host chromosomes. In clinical trials carried out in Europe, eight of nine patients with X-linked severe combined immunodeficiency (SCID-X1) exhibited clinical improvement after receiving an infusion of CD34+ autologous hematopoietic stem cells that were transduced with retroviral vectors carrying the intact γ-chain gene. However, some patients developed acute leukemia in subsequent years, owing to *in-vivo* cloning and expansion of hematopoietic stem cells that carried insertional mutations.[23] Unlike *ex-vivo* therapy using hematopoietic stem cells, the lifespan of our CD-expressing MSCs is transient *in vivo* because of the suicide effects of CD in combination with 5-FC.[17] Indeed, it has been proposed that suicide genes, such as *HSV-tk*, *CD*, or *inducible caspase-9* can be utilized to ablate abnormal, unwanted cells *in vivo* and increase the safety of gene and cell therapy.[24] Thus, the potential risks of insertional mutagenesis associated with retroviral vectors may not be relevant to CD-expressing MSCs. Nonetheless, it is a prerequisite to ensure the chromosomal stability of genetically modified MSCs before clinical application.

The characteristics and phenotypes of MSCs vary according to the tissue source (bone marrow, adipose tissues and umbilical cord blood), passage cycle and culture conditions.[25] One study reported that MSCs could undergo spontaneous malignant transformation upon *in vitro* cultivation for extended periods of time, although human MSCs tend to be resistant to spontaneous malignant transformation.[26] Transformed human MSCs with epithelial polygonal morphology emerged between 11–106 weeks after most MSCs cells stopped growing, and the transformed cells grew well in an anchorage-independent manner, similar to cancer cells.[27] For murine MSCs, spontaneous transformation is always accompanied by gross chromosomal alterations.[28] Therefore, it is necessary to establish safe criteria with respect to the genomic stability of MSC for planning *ex-vivo* therapy.

In this study we investigated whether the stem cell properties of MSCs were altered after transduction of a bacterial *CD* gene by using a retroviral vector. We also investigated for how long the CD expression was maintained when expanded *in vitro*. We found that proliferation and differentiation potentials, as well antigenicity of CD-expressing MSCs, were similar to those of naive MSCs. We also found that a single transduction with the retroviral vector was sufficient for long-lasting expression of the CD gene *in vitro*. Our results suggest that retroviral vectors provide efficient tools to deliver suicide genes to MSCs for *ex-vivo* gene therapy of cancers.

MATERIALS AND METHODS

Isolation and cultivation of MSCs

Human MSCs were originally derived from the iliac crest bone marrow of healthy 10- to 15-year-old donors undergoing bone-marrow aspiration for future allogeneic transplantation, with approval of the Institutional Review Board of Ajou University, Medical Center, as previously described.[29] Briefly, mononucleate adherent cells were collected and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin (Invitrogen, Grand Island, NY, USA) and 10 ng ml^{-1} basic fibroblast growth factor (Dong-A Pharmaceutical Co., Youngin, Korea).

Retroviral transduction

CD-expressing MSCs were prepared by transducing MSCs with a retroviral vector encoding CD, as previously described.[17] The CD gene was cloned from *E. coli* K12 MG1655 (KRIBB, Daejon, Korea) by PCR (forward primer: 5′-GAA TTC AGG CTA GCA ATG TCG AAT AAC GCT TTA CAA AC-3′; reverse primer: 5′-GGA TCC TCT AGC TGG CAG AC A GCC GC-3′) and then into a pFIP plasmid (ViroMed, Seoul, Korea). A retroviral vector containing pFIP/CD was produced in a FLYRD18-packaging cell line expressing the Moloney murine leukemia virus *gag-pol* gene and the cat endogenous virus *RD114 env* gene. Two days after plating of packaging cells in a density of 1.5×10^6/T-75 flask, the viral supernatants were collected and syringe-filtered using a 0.45-μm filter. MSCs at passage 1 were plated at a density of 1×10^5 cell per 100 mm dish and exposed to retrovirus with 20 multiplicity of infection for 8 h in the presence of 4 μg ml^{-1} polybrene (Sigma-Aldrich, St Louis, MO, USA) and 10 ng ml^{-1} basic fibroblast growth factor.[17] Two days later, cells were subcultured and 2 μg ml^{-1} puromycin (Sigma-Aldrich) was added to the culture for 2 weeks. Surviving cells were pooled and maintained by subculturing every 5–7 days. To compare growth kinetics of MSCs with MSC/CD, cells were counted by trypan blue exclusion test and plated in a density of 1000 cells per cm^2 for the next passage in culture. All cell culture medium was replaced with fresh one every 2 or 3 days.

Differentiation

Adipogenic, osteogenic, and chondrogenic differentiation were performed as previously described[30] with a slight modification. Briefly, cells were plated at a density of 2×10^4 cells per 3.8 cm^2 in a culture medium and grown to confluence. The culture medium was replaced with adipogenic medium supplemented with 0.5 mM isobutylmethylxanthine, 60 μM indomethacine, 1 μM dexamethasone, 10 μg ml^{-1} of insulin for 2 weeks, or osteogenic medium supplemented with 0.1 μM dexamethasone, 60 μM ascorbic acid and 10 mM β-glycerophosphate for 5 weeks. Adipogenic differentiation was verified by accumulation of lipid droplets stained with Oil Red O; osteogenic differentiation and the associated accumulation of extracellular calcium crystals were scored by staining by Alizarin Red S. Chondrogenic differentiation was induced by cultivating 2×10^5 cells in pellets in an induction medium supplemented with 1% fetal bovine serum, 6.25 μg ml^{-1} insulin, 10 ng ml^{-1} transforming growth factor beta 1 (TFG-β1) and 50 ng ml^{-1} ascorbic acid for 6 weeks. Alcian blue was used to stain metachromic extracellular material in the pellet, and then, Nuclear Fast Red was used for counter staining of chondrocytes.

Flow cytometry analysis

To measure the expression of surface antigen, MSCs or CD-expressing MSCs were collected with 0.25% Trypsin/EDTA (Invitrogen) and resuspended in phosphate-buffered sline (PBS) containing 1% bovine

serum albumin. Cells were stained with fluorochrome-conjugated antibodies against STRO-1, HLA-ABC, HLA-DR, CD34, CD45, CD90, CD105, CD11b, CD29, CD49a, CD73, CD117 and isotype controls (Biolegend, San Diego, CA, USA) for 10 min at room temperature (RT). After washing with PBS containing 1% bovine serum albumin, cells were analyzed using BD FACS vantage (BD Biosciences, San Jose, CA, USA) with CellQuestPro software (BD Biosciences). All assays included isotype controls.

Anti-cancer effects

For *in-vitro* suicide effects, cells were plated at a density of 10 000 cells per well in 12-well plates, and 24 h later, 5-FC (Archimica, Flintshire, UK) was added at the indicated concentrations. MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) assays were performed to measure cell viability on day 7. The medium was replaced every 2 days with fresh growth medium containing the indicated concentrations of 5-FC. The values at each 5-FC concentration are expressed relative to those of untreated cells and presented as the means ± s.e. To assess bystander effects *in vitro*, U87MG glioma cells were transduced by a lentiviral vector expressing green fluorescent protein (GFP), and GFP-positive cells were sorted by fluorescence-activated cell sorting. U87MG/GFP cells were cocultured with MSCs or MSC/CD cells at a ratio of 10^4:10^4 in 12-well plates. Twenty-four hours later, 5-FC was added to obtain the indicated concentrations, and the medium was replaced every 2 days thereafter. On day 7, fluorescent images of the remaining U87MG/GFP cells were acquired first by fluorescence microscopy, and then, cells were lysed in Passive Lysis Buffer (Promega, Madison, WI, USA). The fluorescence values of the cell lysates were measured using a fluorometer (Molecular Devices, Sunnyvale, CA, USA) and expressed relative to the value of untreated cells (means ± s.e.).

Immunoassays with anti-CD antibody

E. coli CD was produced as a 48-kDa protein in *E. coli* BL21 by using a pET vector, and purified using a Ni-column. Anti-CD rabbit polyclonal antibody was custom-made by Abfrontier (Seoul, Korea). Cells were lysed in RIPA buffer (50 mM Tris; pH 7.4, 1 M NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS), using a standard protocol. Forty-five micrograms of whole-cell lysates of MSC and MSC/CD cells was separated on poly-acrylamide gel for western analysis of CD, and 30 μg was separated for β-actin. Proteins on the gel were transferred to polyvinylidene difluoride membrane and probed with anti-CD antibody (1:5000) or anti-β-actin antibody (1:5000). Immunoreactivity was visualized using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (1:5000; Zymed, San Francisco, CA, USA) and the SuperSignal Chemiluminescence Substrate kit (PIERCE, Rockford, IL, USA).

For immunocytochemistry, cells grown on coverslips were fixed with 4% paraformaldehyde for 10 min at RT. To block nonspecific binding, cells were incubated in blocking solution (0.1% Triton X-100, 0.1% bovine serum albumin, 10% normal horse serum in PBS) for 2 h at RT and then in the presence of a polyclonal anti-CD antibody (1:500 diluted in blocking solution) at 4 °C overnight. After washing, cells were reacted with Alexa 488-conjugated anti-rabbit IgG antibody (1:200; Molecular Probes, Eugene, OR, USA) at RT for 30 min. Nuclei were counter stained with Hoechst 33258 for 5 min. After washing, fluorescent images were acquired with a fluorescent microscope (Olympus, Shinjuku, Japan).

For flow cytometry analysis, MSC/CD cells were collected, fixed with 4% paraformaldehyde and resuspended in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin. Cells were incubated with anti-CD antibody (1:500) for 30 min at RT, and then with Alexa 488-conjugated anti-rabbit IgG antibody. After washing twice, cells were analyzed as described above.

Table 1 Primers and probes for quantitative PCR

Gene	*Sequences*
CD	
Forward	5′-TGATGAGATCGATGACGAGCAGTC-3′
Reverse	5′-GGGTTGGCGACAAAGTTAATACCG-3′
Probe	56-FAM/5′-TATACCTCA/ZEN/CGCCTGTTCCGCTTGCTGAAA-3′/3IABkFQ
GAPDH	
Forward	5′-GGCCATCCACAGTCTTCTG-3′
Reverse	5′-CAGCCTCAAGATCATCAGCAA-3′
Probe	56-FAM/5′-ATGACCACA/ZEN/GTCCATGCCATCACT-3′/3IABkFQ

Abbreviations: CD, cytosine deaminase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Quantitative PCR

Total RNA was isolated from cells using RNAzol B (Tel-Test, Friendswood, TX, USA) and cDNA synthesized from 1 μg of RNA using the First-strand cDNA synthesis kit (Roche, Mannheim, Germany). Amplification was performed using a Taqman universal PCR master mix kit (Applied Biosystems, Foster City, CA, USA) and 1/20 of the volume of the first-strand cDNA reaction mixture using Roto-Gene Q (Qiagen, Hilden, Germany) and a software provided by the manufacturer. Relative CD gene expression to glyceraldehyde-3-phosphate dehydrogenase in MSCs was calculated with Delta-Delta CT relative quantification and presented relatively with respect to the value of MSC at passage 5 (means ± s.e.). PCR primers and probes are summarized in Table 1.

Chromosomal stability and tumorigenicity tests

For chromosome analysis, 20 cells at metaphase were counted after staining with Leishman stain solution, and 5 cells were analyzed for more detailed karyotyping using CytoVision (Applied Imaging International, San Jose, CA, USA). GTG-banding (G-banding by trypsin and Giemsa stain) with 450 bands of resolution was conducted in a clinical Cytogenetics Laboratory at Ajou University Hospital (http://www.ajoumc.or.kr). To measure *in-vitro* transformation, MSC, MSC/CD and U87MG cells were cultured in soft agar by using a kit according to the manufacturer's protocol (Millipore, Billerica, MA, USA). Briefly, 24-well plates were coated with 0.8% agarose in a mixture of distilled water and growth medium, and 500 μl of base agar was added to a well. Cells were plated in 0.4% agarose solution in a density of 1, 250 cells per well suspended and incubated for 2 weeks, with addition of 250 μl of growth medium twice a week. To measure *in-vivo* tumorigenicity, we suspended 5×10^6 cells in 100 μl of PBS containing 20% Matrigel (BD Biosciences), and subcutaneously inoculated them in Balb/C/nu/nu mice (Nara Biotech, Seoul, Korea). As a positive control, an equal number of U87MG were inoculated. Tumor dimensions were measured with a caliper and the volumes were calculated as $\pi/6 \times$ length $\times$ width $\times$ height. All animal protocols were approved by the Institutional Animal Care and Use Committee of Ajou University School of Medicine.

RESULTS

Retroviral transduction of MSCs

MSCs were isolated from the bone marrow of a human volunteer and expanded *in vitro* as previously reported.[31]

Retroviral vectors expressing the *E. coli CD* gene generated in FlYRD18 packaging cells were added to MSCs at passage 1, and CD-expressing MSCs (MSC/CD) were selected in the presence of puromycin. Initially, the growth rate of MSC/CD cells was temporarily retarded by transduction and selection, but was recovered immediately (Figure 1a). Once they were grown to confluence at day 7, MSCs and MSC/CD cells were exponentially expanded by approximately 19-fold per every 6-day culture (Figure 1b); the growth rates were indistinguishable up to passage 10 between both cell types. These data indicate that neither transduction with retroviral vectors nor CD expression interferes with the proliferation capability of MSCs.

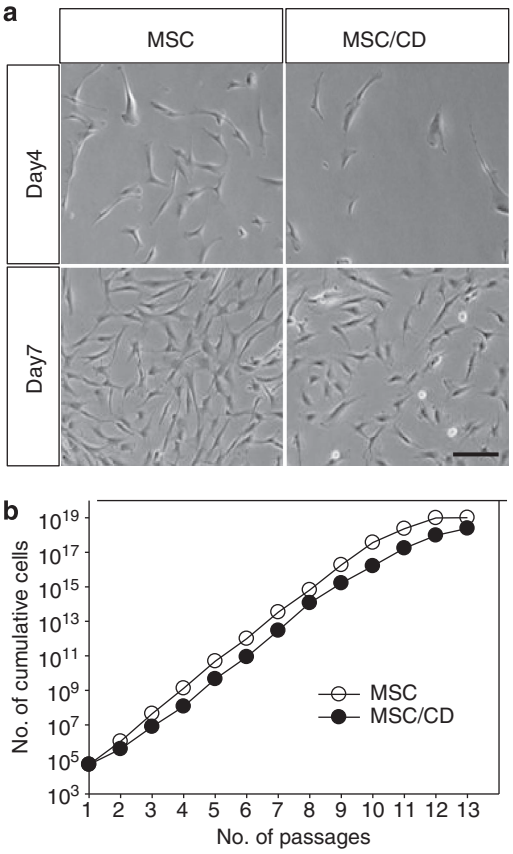


Figure 1 No effects of retroviral transduction on the MSC proliferation capability. (**a**) MSCs at P1 were transduced with a retroviral vector encoding CD. Three days later, cells were subcultured in a density of 5×10^4 per 100 mm dish, and $2\,\mu g\,ml^{-1}$ puromycin was added to the culture for 2 weeks. The medium was replaced every other day. The growth rate of MSC/CD was temporarily retarded at day 4, but was recovered by day 7. Scale bar = 50 μm. (**b**) During puromycin selection, MSC/CD cells were subcultured twice at day 9 and 14. MSCs and MSC/CD cells were counted every 6 or 7 days with trypan blue exclusion tests and plated at a density of $1 \times 10^3\,cm^2$. Medium was replaced every other day. Both MSCs and MSC/CD cells grew at similar rates up to passage 10, but at slower rates after p10.

Characterization of MSC/CD cells

According to the International Society for Cellular Therapy, MSCs can be defined by three criteria, such as adhering to plastic, expressing specific surface antigens and differentiating into mesodermal multilineages.[32] Both naive MSCs and MSCs/CD adhered to plastic culture dishes on which they exhibited similar fibroblastic morphology (Figure 2a). Both cell types retained differentiation potential when induced to differentiate into adipocytes, osteocytes and chondrocytes (Figure 2a). These MSCs and MSCs/CD cells both expressed the typical surface antigen of classical MSCs. Specifically, they were positive for STRO-1, HLA-ABC, CD29, CD49a, CD73, CD90 and CD105, whereas they were negative for HLA-DR, CD45, CD34, CD11b and CD117 (Figure 2b). These results indicate that retrovirus-mediated CD expression does not alter the morphology, multipotency and surface antigenic properties of MSCs.

In vitro anti-cancer effects of MSC/CD

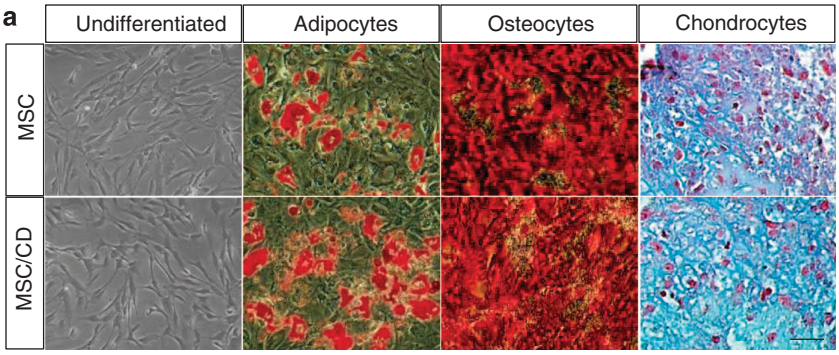
Suicide effects of MSC/CD cells were measured in the presence of 5-FC. MTT assays showed that 5-FC decreased the number of MSC/CD cells with IC_{50} (half maximal inhibitory concentration) values of 60.4 μM (Figure 3a). Parental MSCs were resistant to 5-FC up to a concentration of 1 mM (open circles in Figure 3a), indicating the specificity of the suicide function of CD. The anticancer effects of MSC/CD cells were tested against cocultured U87MG glioma cells stably expressing GFP (U87MG/GFP) to distinguish glioma cells. When cocultured with parental MSCs, U87MG cells continued to proliferate even in the presence of 1 mM 5-FC (Figure 3c). In contrast, when cocultured with MSC/CD cells, the growth of U87MG/GFP cells was suppressed upon 5-FC treatment, with an IC_{50} of 32.8 μM (Figure 3b).

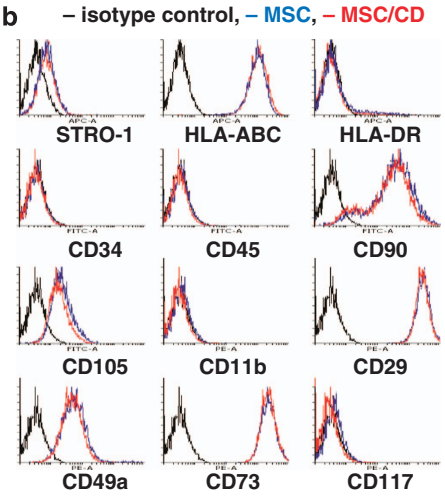
Long-lasting expression of CD in MSC/CD cells

Retroviral vectors integrate into the host genome and induce long-term expression of the gene. However, it is also known that the genes exogenously introduced by retroviral vector can be silenced by methylation of cytomegalovirus promoter[33] or long terminal repeat promoter.[34] To test whether the *CD* gene was persistently expressed after long-term culture, we generated a polyclonal anti-CD antibody. Immunocytochemistry indicated a specific immunoreactivity only in MSCs/CD cells, but not in MSCs (Figure 4a). Fluorescence-activated cell sorting analysis indicated that 94.7% of MSC/CD cells persistently express the *CD* gene at passage 8 (Figure 4b). Quantitative reverse transcription-PCR analysis indicated that MSC/CD cells ranging from passage 2 to passage 9 expressed *CD* mRNA at similar levels (Figure 4c). Western blot analysis indicated that the CD expression level of MSC/CD cells was similar to the level at passage 5 and passage 8 (Figure 4d). The results indicated that expression of the *CD* gene that was transduced at passage 1 using a retroviral vector was sustained after long-term cultivation up to passage 10, by which time the cells had expanded 10^{12}-fold.

Genetic stability of MSC/CD cells

One of the general concerns regarding retroviral vectors is that they can induce genomic instability owing to their integration





Marker	MSC	MSC/CD
STRO1	+/-	+/-
HLA-ABC	++	++
HLA-DR	-	-
CD34	-	-
CD45	-	-
CD90	++	++
CD105	++	++
CD11b	-	-
CD29	++	++
CD49a	++	++
CD73	++	++
CD117	-	-

Figure 2 No effects of retroviral transduction on multipotency and surface antigen profiles. (**a**) Both naive MSCs and MSC/CD were induced to differentiate into adipocytes, osteocytes and chondrocytes for 3, 5 and 6 weeks, and were stained by Oil Red O, Alizarin Red S and Alcian blue/Nuclear Fast Red, respectively. Both MSCs and MSC/CD cells were able to differentiate into three mesodermal lineage cells. Scale bar = 50 μm. (**b**) Both naive MSCs and MSC/CD cells showed the same phenotypes: positive for STRO-1, HLA-ABC, CD90, CD105, CD29, CD49a and CD73, whereas negative for all isotype controls, HLA-DR, CD11b, CD34, CD45 and CD117. CD-retrovirus transduction did not affect the surface antigenicity of MSCs.

into host chromosomes or other effects they exert over long-term *in-vitro* culture. Although CD-expressing MSCs would be eliminated *in vivo* when combined with 5-FC administration, we tested the genomic stability of MSC/CD cells with a G-banding assay that could evaluate microscopic chromosomal aberrations, such as nondisjunction or translocation of chromosomes. Such as MSCs, MSCs/CD cells contained normal 22 autosomal chromosome pairs and XY (Figure 5a). MSC/CD obtained from an independent transduction with retroviral vectors also showed the normal karyotype, even after long-term cultivation *in vitro* (data not shown). The results indicate that retrovirus-mediated transduction or long-term cultivation does not cause microscopic chromosomal aberration. Both MSC and MSC/CD cells cultured to passage 8 failed to form any colonies in soft agar under conditions in which U87MG glioblastoma cells formed numerous colonies (Figure 5b). This suggests that unlike cancer cells, both cell types cannot grow anchorage-independently. Indeed, when both cell types were subcutaneously transplanted to nude mice, they did not form any detectable tumors over a 5-month period, whereas U87MG glioblastoma cells grew to solid tumors with a volume of $5.4 \pm 0.8\,cm^3$ by 53 days (Figures 5c and d).

DISCUSSION

MSCs are currently being evaluated for cell-based therapies of diverse tissue injury and degenerative diseases. MSCs can be obtained via relatively non-invasive methods from diverse tissues and autologously transplanted after being expanded to a large scale *in vitro*. Recently, therapeutic genes have been introduced to render MSCs more tissue- and disease-specific. In this study, we provide evidence that retroviral vectors can be efficiently utilized to introduce therapeutic genes into MSCs, because they allow sustainable expression of therapeutic genes without disturbing the stemness and genetic stability of MSCs during expansion *in vitro*.

MSCs can be identified by their fibroblastic morphology, adhering activity to plasticware and surface antigen profiles, whereas the stemness of MSCs can be defined by their capability of long-term proliferation and differentiation into mesodermal lineage cell types. We proved that our MSCs and MSC/CD cells could be cultivated as adherent cells. Both

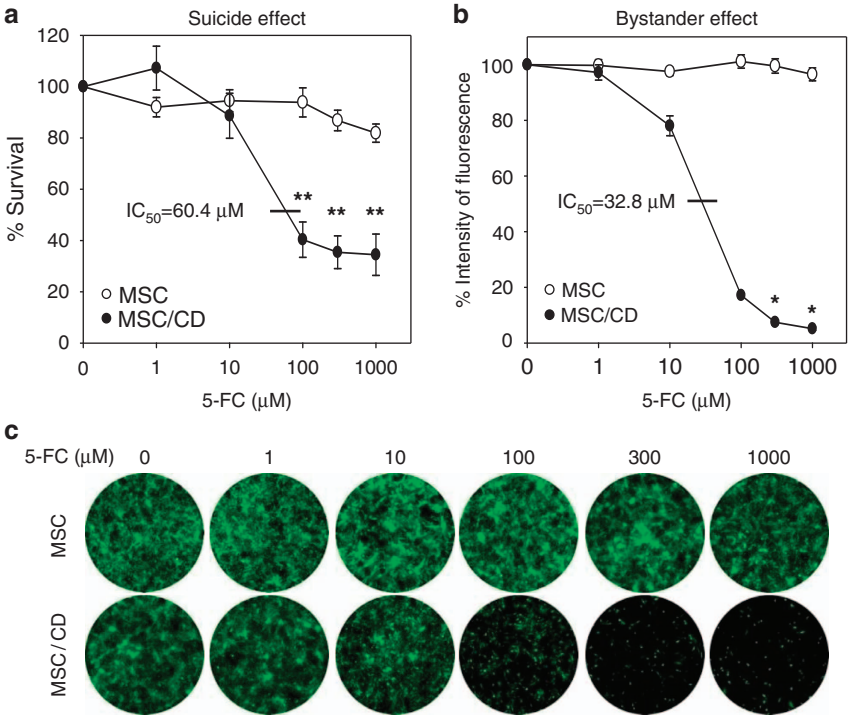


Figure 3 *In-vitro* cytotoxic effects of MSC/CD in combination with 5-FC. (**a**) To quantify the suicide effects, MSC/CD cells were incubated in the presence of the indicated concentrations of 5-FC for 7 days. Surviving MSC/CD cells were quantified by MTT assays. (**b**) To quantify the bystander effects, U87MG glioma cells stably expressing GFP (U87MG/GFP) were cocultured with MSC/CD cells in the presence of indicated concentrations of 5-FC for 7 days. The remaining U87MG/GFP cells were quantified by fluorometry or (**c**) by fluorescent microscopy. MSCs were used as negative controls. The values are presented as the means ± s.e. at each concentration relative to the value in the absence of 5-FC. Results from at least three independent *in vitro* experiments were analyzed using one-way analysis of variance followed by Tukey's honestly significant difference *post-hoc* test (*$P<0.05$; **$P<0.01$).

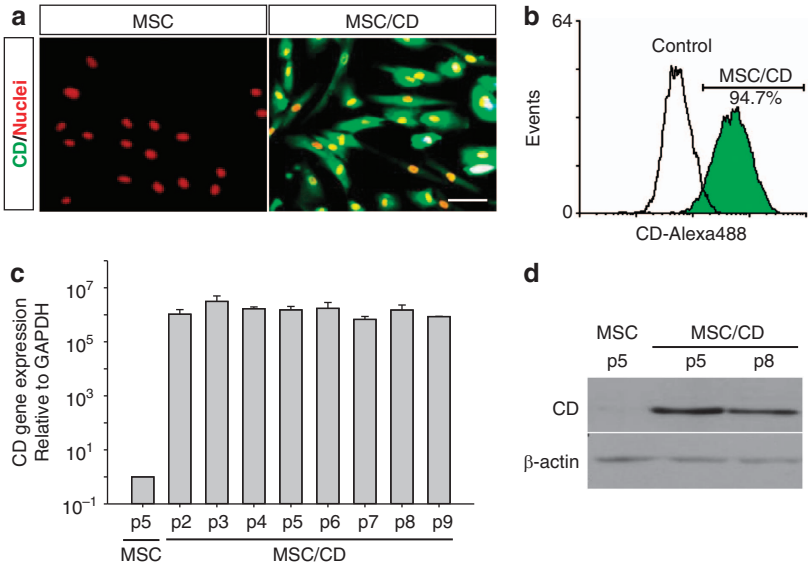


Figure 4 Sustainable CD expression in MSC/CD after long-term culture. CD expression was assessed in MSC/CD during p5–p10. (**a**) Immunocytochemistry with anti-CD antibody indicated CD expression in MSC/CD cells at p10. Scale bar = 50 μm. (**b**) Fluorescence-activated cell sorting analysis indicated that 94.7% of MSC/CD cells expressed CD at p8. (**c**) Real-time reverse transcription-PCR indicated similar levels of CD mRNA expression in MSC/CD at ranging from p2 to p9. (**d**) Western blot analysis revealed similar levels of CD proteins in MSC/CD cells at p5 and p8.

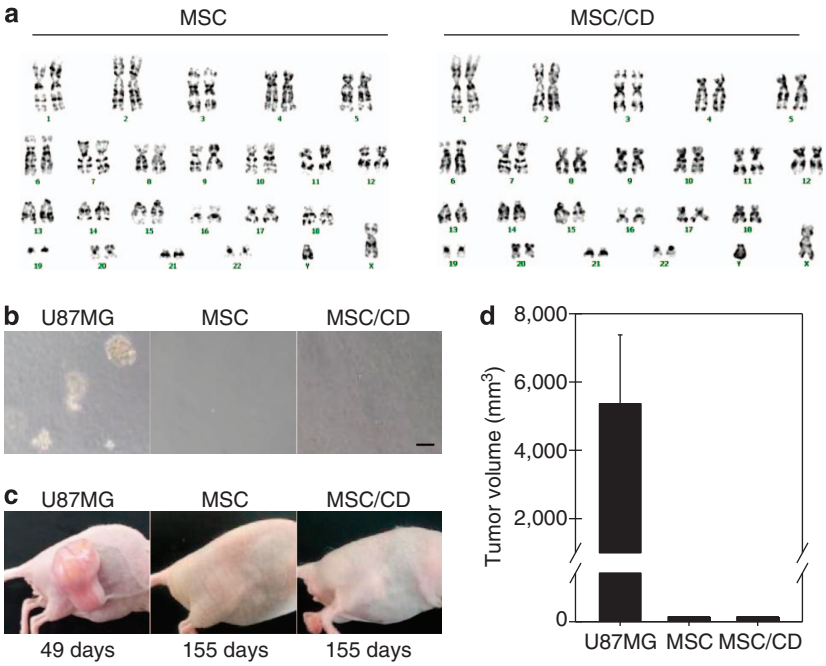


Figure 5 Normal karyotype and non-tumorigenicity of MSC/CD. (**a**) G-banding assay indicated a normal (46, XY) karyotype both in MSC and MSC/CD. (**b**) Unlike U87MG glioma cells, MSC and MSC/CD failed to form spheres in the soft agar assay *in vitro*. Scale bar = 100 μm. (**c**) MSCs and MSC/CD cells did not form tumors 155 days after 5×10^6 cells were subcutaneously injected in nude mice under conditions in which U87MG glioma cells readily formed tumors within 49 days. (**d**) Solid tumors were measured in six spots of U87MG-injected mice at day 53 ($n = 3$), and the tumor volume was calculated according to the following formula: Volume = $\pi/6 \times L \times W \times H$.

cell types equally had a fibroblastic morphology and expressed surface antigens, including STRO-1, HLA-ABC, CD29, CD49a, CD73, CD90 and CD105, but not HLA-DR, CD45, CD34, CD11b and CD117. We also showed that MSCs and MSC/CD cells could grow up to passage 10 without growth retardation. Growth of MSC/CD was temporarily delayed for the first few days because of retroviral transduction. Following selection, the growth rate of MSC/CD cells returned to that observed in naive MSCs. MSCs and MSC/CD cells retained the capability to differentiate into adipocytes, osteocytes and chondrocytes. Our results indicate that neither retrovirus-mediated CD expression nor long-term cultivation alters the stemness of MSCs. Moreover, once MSC/CD cells were selected in the presence of puromycin, the expression level of CD remained stable over multiple passages; indeed, 94–99% surviving cells at passage 8–10 expressed CD when tested by flow cytometry or immunocytochemistry.

In contrast to our study, others have reported that the stemness of MSCs can be influenced by the nature of transduced genes. For example, Ngn1, a proneural transcription factor, converted the mesodermal fate of MSCs into a neural one, and Ngn1-expressing MSCs lost the capability to differentiate into mesodermal lineage cells.[31] Retroviral vectors encoding Wnt-4 or glucocorticoid-induced leucine zipper converted MSCs into pro-osteogenic progenitor cells at the expense of adipogenic capability.[35,36] A tendon-specific transcription factor, scleraxis, converted MSCs into tendon progenitor cells, which failed to differentiate into chondrocytes and osteocytes.[37]

In-vitro expansion of MSCs is required to obtain sufficient cell numbers for cell-based therapy. As mentioned earlier, it was reported that MSCs could undergo spontaneous malignant transformation during extremely extended *in vitro* culture. The transformed MSCs grow like cancers in an anchorage-independent manner[27] and exhibited gross chromosomal aberrations.[27,28] However, most laboratories including ours have demonstrated that MSCs usually stop proliferation after passage 11 (70 days *in vitro* culture) and cannot grow in soft agar in an anchorage-independent manner. Moreover, we could not detect any gross chromosomal aberrations in our MSCs or MSC/CD cells. Therefore, our MSCs and MSC/CD cells below passage 11 with a normal karyotype can be considered non-tumorigenic. Indeed, these cells could not form solid tumors in nude mice. More importantly, our MSC/CD cells undergo cell death owing to the suicide effects of the *CD* gene product in the presence of 5-FC, whereas exerting bystander effects on the cocultured U87MG glioma cells. This effectively eliminates the tumorigenic potential of MSC/CD cells, consistent with the proposal that suicide genes are safety tools that can be used to ablate unwanted abnormal cells *in vivo* once the therapeutic genes have been delivered to their target site.

For practical uses, retroviral vectors may have greater advantages over adenoviral vectors with respect to

introduction of the *CD* suicide gene into MSCs. This is because a single retroviral transduction is sufficient to obtain stable expression of the *CD* gene. Once transduced cells are selected and stored, working cell banks for clinical applications can be easily manufactured in compliance with current good manufacturing practice by simple expansion without additional transduction steps. This property may help to lower medical expenses and make this therapy more economically accessible. In comparison, adenoviral vectors allow transient expression of *CD* genes only for a limited time, because they remain as episomes and are diluted in proportion to the host cell proliferation. Consistent with other reports that MSCs are hypo-immunogenic,[38] our MSCs and MSC/CD cells also lack HLA-DR, a major histocompatibility complex class II, which causes an immune response after allogeneic injection. The hypo-immunogenicity of MSCs together with the sustainable expression of the transduced *CD* genes after expansion in a large quantity further warrant the clinical application of allogeneic transplantation, although autologous transplantation is always preferable to allogeneic.

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