Journal of Gastroenterology and Hepatology JGHF

doi:10.1111/j.1440-1746.2009.05862.x

GASTROENTEROLOGY

Cytosine deaminase-producing human mesenchymal stem cells mediate an antitumor effect in a mouse xenograft model

Mi-Hyeon You,[1]* Wang-Joon Kim,[1†] Wooyoung Shim,[1†] Sang-Rim Lee,[‡] Gwang Lee,[†,§] Sangdun Choi,[†] Dae-Yong Kim,* Yong Man Kim,[¶] Hyunsoo Kim[¶] and Sang-Uk Han[†,‡]

*Department of Veterinary Pathology, Seoul National University, Seoul, [†]Department of Molecular Science and Technology, [‡]Department of Surgery, [§]Institute for Medical Science, Ajou University, Suwon, and [¶]FCB-Pharmicell, Sungnam, Korea

Key words
cytosine deaminase, gastric cancer, gene therapy, mesenchymal stem cell, suicide effect.

Accepted for publication 4 March 2009.

Correspondence
Sang-Uk Han, Department of Surgery, Ajou University School of Medicine, San 5, Wonchon-dong, Yeongtong-gu, Suwon 443-721, Korea. Email: hansu@ajou.ac.kr

[1]These authors contributed equally to this work.

Abstract

Background and Aim: Stem cell transplantation offers potential gene therapy for brain tumors. However, this approach has received little attention as a treatment for gastrointestinal tumors. In the present study, we explored the possibility of human bone marrow-derived mesenchymal stem cells (hMSC) producing cytosine deaminase (CD), followed by systemic 5-fluorocytosine (5-FC) administration, showing an antitumor effect on a mouse gastric cancer xenograft.

Methods: We first explored the ability of hMSC, coated with fluorescent dye, to migrate to human gastric cancer MKN45 cells *in vitro* and *in vivo*. We then used hMSC in which a gene expressed the prodrug-activating enzyme CD, which can convert the prodrug 5-FC into the cytotoxic agent 5-fluorouracil (5-FU), and further investigated the potential of these cells to deliver the CD gene and to reduce tumor growth in nude mice. The migratory capacity of hMSC was confirmed by an *in vitro* migration assay, as well as in an *in vivo* model of nude mice bearing subcutaneous tumors of MKN45 cells when hMSC were injected.

Results: The migration ability of hMSC towards MKN45 cells was confirmed by migration assay. Effective conversion of 5-FC to 5-FU by hMSC transfected with the CD gene (CD-hMSC) showed therapeutic anticancer potential in a MKN45 cell co-culture system, as confirmed by thin layer chromatography. Nude mice bearing MKN45 tumors were intravenously injected with CD-hMSC, followed by systemic 5-FC treatment (500 mg/kg/day) for 7 days. Tumor volumes and weights of mice injected with CD-hMSC decreased significantly after treatment with 5-FC. However, the 5-FC-treated group without CD-hMSC injection showed neither a decrease in tumor volume nor bodyweight loss.

Conclusion: The CD-hMSC system showed anticancer therapeutic potential, and minimized the side-effects of 5-FU.

Introduction

Cancer remains a major cause of death worldwide.[1,2] Annually, about 1.18 million, 598 000, and 700 000 deaths are attributable to lung, liver, and stomach cancer, respectively. When cancer progresses to more advanced stages, prognosis is poor and both tumor recurrence and metastasis are to be expected. 5-fluororacil (5-FU) has been the chemotherapeutic choice for the majority of solid tumors, including gastric and colon cancers. However, the serious side-effects of this drug and the high doses required for effectiveness have limited the use of 5-FU as a chemotherapeutic agent. Therefore, therapeutic 5-FU strategies affording fewer side-effects are strongly in demand and, recently, so called 'molecular chemotherapy' has been proposed as one of several potential alternatives.[3–5]

Molecular chemotherapy is based on the concept of target-specific therapeutic suicide gene delivery using a cell-based system. As mesenchymal stem cells (MSC) have the property of tracking tumor cells, molecular chemotherapy using MSC as the vehicle can mediate antitumor effects in tumor lesions.[6,7] Because MSC are acquired from a patient's body and can easily be expanded in culture, they are suitable for the approach described. Furthermore, MSC are easily transfected with plasmids and transduced using adenoviral, retroviral and lentiviral vectors.[3,4]

Several types of suicidal genes have been studied and used for therapeutic purposes. One of the most widely used genes is

bacterial cytosine deaminase (CD),[8–10] which deaminates the prodrug 5-fluorocytosine (5-FC) into the cytotoxic agent 5-fluorouracil (5-FU), in turn creating an antitumor effect.[11,12] The CD/5-FC system is very effective in the treatment of human cancer because non-toxic 5-FC given systemically can be converted to the cytotoxic agent 5-FU by the CD gene product located in the vicinity of the cancer. The drug 5-FU is finally converted to 5-fluoro-deoxyuridine-monophosphate (5-FdUMP), which inhibits thymidylate synthase (TS) activity; TS is an essential enzyme in DNA synthesis.[13–15] This approach is, therefore, more effective than direct administration of cytotoxic 5-FU, because of the alleviation of systemic toxicity.

In the present study, we explored the possibility that the injection of human bone-marrow-derived mesenchymal stem cells (hMSC) producing CD, followed by systemic 5-FC administration, might show an antitumor effect on a mouse gastric cancer xenograft. The tumor-tracking ability of hMSC and the cytotoxic effects of 5-FU were also studied *in vitro* and *in vivo*.

Methods

Culture of human bone marrow-derived mesenchymal stem cells

Cultured hMSC were supplied by FCB-Pharmicell, Sungnam, Korea. All hMSC isolation and culture procedures were carried out under GMP conditions as previously reported.[16,17] Briefly, hMSC were isolated from 20 mL aspirates of the iliac crests of human donors and expanded in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 units/mL penicillin, 100 g/mL streptomycin, and 0.25 g/mL amphotericin B (Invitrogen Biotechnology, Eugene, OR, USA). The hMSC were used at passage 6. A human gastric cancer cell line (MKN45), a fibroblast cell line (NIH3T3) and a keratinocyte cell line (HaCaT) were purchased from the Korean Cell Line Bank. These cells were cultured in RPMI-1640 medium containing 10% (v/v) FBS, 100 units/mL penicillin, and 100 g/mL streptomycin, at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Labeling of hMSC with the fluorescent dye CM-DiI

The fluorescent dye chloromethylbenzamido-1,19-dioctadecyl-3,3,39,39-tetramethylindocarbocyanine perchlorate (CM-DiI, Invitrogen) was dissolved in dimethylformamide (Sigma, St. Louis, MO, USA) to a concentration of 2.5 mg/mL and added to culture medium to a final concentration of 10 μg/mL.[18] hMSC (6×10^6 cells) were incubated in 25 mL of medium containing CM-DiI in a T-175 flask for 48 h. Cells were washed twice in PBS, incubated with dye-free medium for 2 h, and used in experiments. hMSC labeled with CM-DiI (linked to a red or green fluorescence marker) were examined by fluorescence microscopy (Olympus, Tokyo, Japan) using an ultraviolet filter.

In vitro migration assay

The migration ability of hMSC towards MKN45 cells was assessed using BD Biocoat™Matrigel™invasion chambers (BD Biosciences, San Jose, CA, USA). Filters (8 mm in diameter) coated with matrigel (BD Biosciences) were placed between upper and lower chamber wells. hMSC were seeded in the upper well at a density of 2×10^5 cells/well. After incubation in a humidified incubator at 37° in 95% air and 5% CO_2 for 24 h, contents in the upper well and the filters were removed. Non-migrated cells were scraped off the upper side of the filter and the filters were then stained with hematoxylin. We randomly selected four areas of the filters and counted the numbers of cells in these regions. To measure migrated cells in the lower well, hMSC were incubated with CM-DiI (red color), whereas MKN45, NIH3T3 and HaCaT cells were incubated with CM-DiI (green color). Harvested MKN45 cells were placed in the lower well at a density of 2×10^5cells/well. NIH3T3 and HaCaT cells were used as controls. After 24 h incubation, cells in lower wells were harvested and photographed using fluorescence microscopy (Olympus) and an ultraviolet filter.

Construction of plasmids and transfection

After polymerase chain reaction (PCR) amplification, the *Escherichia coli* CD gene was cloned into the multiple cloning site of pcDNA 3.1(-) (Invitrogen). The primer sequences were: forward, 5′-CACTCGAGATGTCGAATAACGCTTTAC-3′ and reverse, 5′-CGAATTCTCAACGTTTGTAATCGATGG-3′. The PCR-derived CD fragment included a *Xho*I site 5′ to the first ATG and an *Eco*RI site distal to the stop codon. DNA of pcDNA 3.1(-) and the CD fragment were ligated with T4 ligase (Takara Biotechnology, Sjiga, Japan). The correctness of the pcDNA-CD clone was confirmed by sequencing and comparison with the GenBank sequence AY552602.1. The constructed pcDNA-CD plasmid was then transfected into hMSC using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Cytosine deaminase conversion assay *in vitro*

To analyze CD activity, we carried out thin layer chromatography (TLC).[16] pcDNA-CD-transfected hMSC (CD-hMSC) and non-transfected hMSC were seeded into 60 mm culture dishes and incubated for 24 h. The cells were trypsinized and resuspended in 100 μL of PBS. The cells were frozen and thawed at least five times, and the supernatant after centrifugation was used. To initiate the conversion reaction, 5-FC (Sigma) was added to the supernatants to 200 mmol; aliquots were then spotted onto a TLC plate (Merck, Dreieich, Germany) and run in butanol : water (86:14) to separate 5-FU from 5-FC. Spots of 100 mmol 5-FC and 5-FU (Sigma) were used as standards. To quantify 5-FC conversion ratio, TLC plates were scanned using LAS-1000 (Fujifilm, Tokyo, Japan) and conversion ratio was analyzed using Multi Gauge 3.0 software (Fujifilm, Tokyo, Japan).

MTT assay

MKN45 cells and hMSC were co-cultured in 96-well plates (Nunc, Roskilde, Denmark) at 10^4 cells/well, in 200 μL medium containing 10% (v/v) FBS. After 24 h incubation, the medium was changed to FBS-free medium and cells were treated with either 5-FC or PBS. The MTT assay was carried out as briefly described below. MTT powder (Sigma) was dissolved in PBS and filtered. MTT solution was added to the cells on plates and incubation

continued for 4 h at 37°C. Supernatants were removed and 200 μL of 0.04 M HCl in isopropanol was added to each well. Optical densities were measured at 550 nm using an ELISA-Reader (Molecular Devices, Sunnyvale, CA, USA). MTT assays were carried out in triplicate.

Cell administration to athymic nude mice

The use of animals in the present study was approved by the Animal Care and Use Committee of Seoul National University and all procedures were carried out in accordance with institutional guidelines. Seven-week-old female athymic nude mice (*Cg-Foxn1 nu/ CrljBgi*) were purchased from the Orient Company, Seongnam-si, Korea. To induce tumor xenografts, 2×10^6 MKN45 tumor cells suspended in 200 μL of HBSS were inoculated subcutaneously into athymic nude mice by using a 27-gauge needle without anesthesia. In a preliminary study, we confirmed that all mice inoculated with 2×10^6 MKN45 tumor cells developed macroscopic nodules in the flank region about 4 days postinjection. To investigate whether hMSC could migrate to and track MKN45 tumor xenografts, 1×10^6 CM-Dil-labeled hMSC were injected through the lateral vein of tumor-bearing mice which were subcutaneously injected with 2×10^6 MKN45 4 days earlier. The total number of CM-Dil labeled hMSC are calculated when the numerical density N_v and the volume of structure V are known.[19] The N_A of CM-Dil labeled hMSC in a tumor was measured on maximal sphere area for each block. $N = V \times N_v$. We acquired N_v by $N_v = N_A/D$, $V = 4/3\pi r^3$ ($r = \pi D/4$) (A, area; r, mean diameter of sphere; D, sphere diameter), $V = 4/3\pi r^3$ ($r = \pi D/4$).

Determination of antitumor effects of 5-FU and 5-FC

When tumor sizes reached about 130 mm^3 in volume, mice were divided into six groups as follows:

1. Group 1: No tumor cell injection, three animals.
2. Group 2: Tumor cells only, seven animals.
3. Group 3: Tumor cells + CD-hMSC + i.v. 5-FU, seven animals
4. Group 4: Tumor cells + CD-hMSC + i.v. 5-FC, seven animals
5. Group 5: Tumor cells + two injections of CD-hMSC + i.v. 5-FC, seven animals
6. Group 6: Tumor cells + hMSC + i.v. 5-FC, seven animals

The mice of groups 3, 4 and 5 received 2×10^6 CD-hMSC suspended in 200 μL of HBSS through the lateral tail vein on day 3, whereas mice belonging to group 6 received 2×10^6 hMSC on the same day. One day later, mice of group 3 were treated i.p. with 30 mg/kg/day of 5-FU for 8 days, whereas animals of groups 4, 5 and 6 were treated with 500 mg/kg/day of 5-FC. In addition, mice of group 5 received 2×10^6 CD-hMSC instead of 500 mg/kg/day of 5-FC, as a means of boosting. Mice injected with MKN45 tumor cells alone (group 2) and mice with no tumor cell injection (group 1) served as controls. Tumor growth was monitored every day by calipers and tumor volume was calculated according to the formula V (mm^3 = (L X W^2) x 0.5, in which V = volume, L = length and W = width.[20]

Statistical analysis

Results were analyzed using one-way analysis of variance (ANOVA; GraphPad Software, La Jolla, CA, USA) to assess the statistical significance of overall differences in tumor volumes between all treatment groups and to evaluate MTT assay data. When the one-way ANOVA test showed $P < 0.05$, data were further analyzed by Dunnett's t-test to assess statistical differences between treatment and control groups. Data are expressed as mean ± SD. Differences were considered statistically significant only when $P < 0.05$.

Results

hMSC migrate towards MKN45 cells *in vitro*

The migration of hMSC towards MKN45 cells was assessed using invasion chambers. Migration was dramatically influenced by the number of MKN45 cells in the lower chamber (Fig. 1a–d). To confirm these results, we repeated the migration study using hMSC labeled with the fluorescence marker CM-Dil. As shown in Figure 1E, labeled hMSC (stained in red) were able to migrate down towards MKN45 cells. Culture medium alone, cultured NIH3T3 (stained in green) or HaCaT cells (stained in green), failed to induce migration of hMSC.

CD-transfected hMSC show strong CD activity *in vitro*

TLC results showed that CD-hMSC expressed high enzyme activity (Fig. 2a), whereas no significant conversion of 5-FC to 5-FU was detected in non-transfected hMSC. After 4 h of incubation of CD-hMSC with 5-FC, more than 80% of total 5-FC was converted into 5-FU. In addition, conversion of 5-FC by CD-hMSC was incubation time dependent. Cytotoxicity of CD-hMSC was evaluated directly by an *in vitro* co-culture experiment. After 24 h of co-culture of MKN45 and CD-hMSC, 5-FC was added. No cytotoxicity was observed in control co-cultures when 5-FC was omitted or normal hMSC were used (Fig. 2b–e). However, as shown in Figure 2F, MKN45 cells markedly decreased in number within 24 h because of cytotoxicity induced by the combined effect of 5-FC and CD-hMSC. To determine the cytotoxic effect of the CD gene, we measured MKN45 cell viability using the MTT assay (Fig. 2g). There was no significant difference when only CD-hMSC or 5-FC was present. However, the viability of MKN45 cells was significantly decreased in the presence of CD-hMSC and 5-FC (89% reduction compared with control, $P < 0.001$). This result clearly showed that the suicidal selective cytotoxicity effect was mediated by CD-hMSC in the presence of 5-FC.

Transplanted hMSC migrated to MKN45 tumor regions

To investigate whether hMSC could migrate to and track MKN45 tumor xenografts, dye-labeled hMSC were injected intravenously into tumor-bearing mice. The mice were euthanized either 24 h or 72 h after injection. Subcutaneous tumor nodules and heart, liver, spleen, lung, stomach and kidney tissues were harvested and

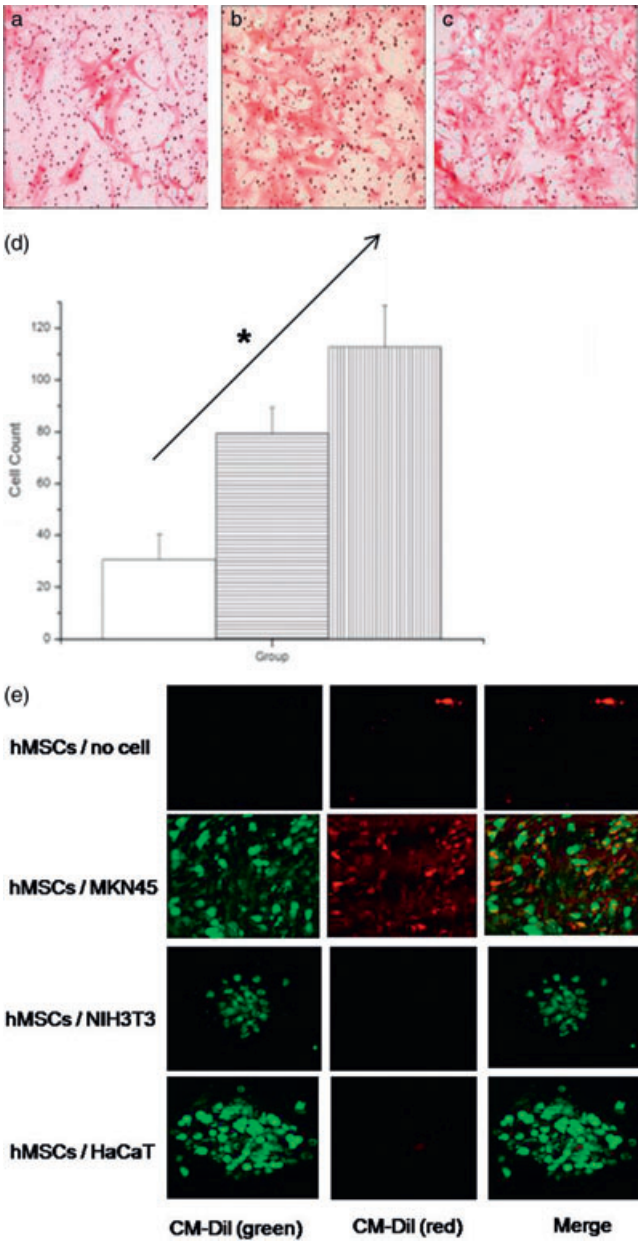

Figure 1 *In vitro* migration assay. The migratory ability of human bone marrow-derived mesenchymal stem cells (hMSC) towards MKN45 cells was determined using a modified transwell invasion assay. (a) hMSC on a filter without any MKN45 cells in the lower well. (b) hMSC on a filter with MKN45 cells in the lower well (cell ratio 1 : 1). (c) hMSC on a filter with MKN45 cells in the lower well (cell ratio 1 : 3). The mean numbers of cells on filters in the hMSC/no MKN45 group, the hMSC/MKN45 (1 : 1) group, and the hMSC/MKN45 (1 : 3) group, were 30.75, 79.25 and 112.75, respectively. (d) The invasion by hMSC of MKN45-containing wells increased dramatically in a dose-dependent manner. (e) Effective tracking of hMSC to MKN45 cells after invasion through the matrigel. Green color: cells in lower wells; red color; hMSC migrated from the upper wells. Only MKN45, a cancer cell line, in the lower well induced migration of hMSC from the upper well towards the lower well (magnification: × 200).

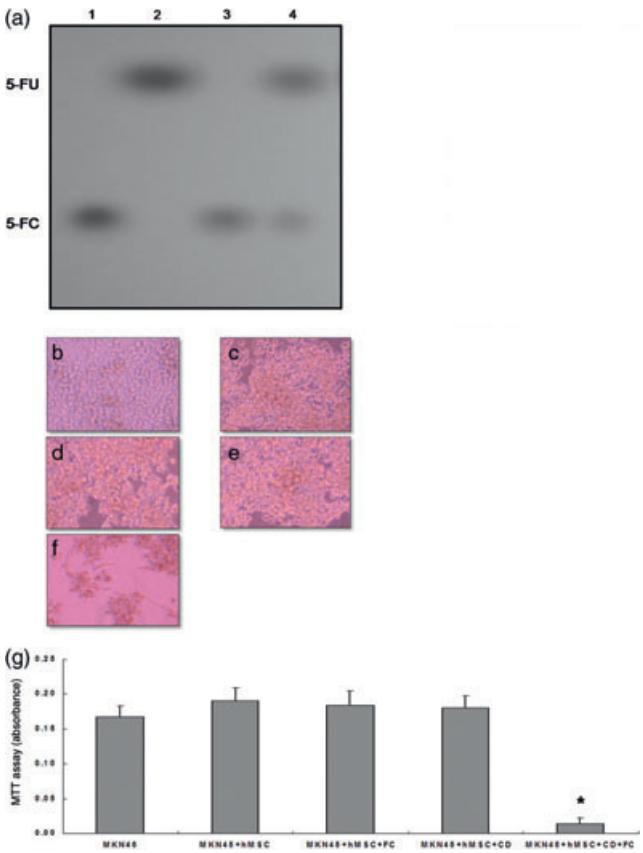

Figure 2 A. *In vitro* cysosine deaminase (CD) activity. CD activity was determined by thin layer chromatography (TLC) to measure the conversion of 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU) by CD present in the lysates of normal human bone marrow-derived mesenchymal stem cells (hMSC) and CD-hMSC. Lanes 1 and 2 are 100 mmol each of 5-FC and 5-FU standard controls, respectively. Significant changes were not observed after 5-FC incubation with normal hMSC (lane 3), however, the conversion of 5-FC to 5-FU took place in CD-hMSC (lane 4). Cell sensitivity to 5-FU and selective cytotoxicity effects were mediated by CD-hMSC in the presence of 5-FC on MKN45 cells. CD-hMSC and MKN45 cells were co-cultured to confirm the cytotoxic suicidal effect of the CD gene and cell sensitivity to 5-FC. As a control, MKN45 cells only (b) and MKN45 cells + normal hMSC (c) were cultured under the same conditions. (d) The MKN45 cells + normal hMSC + 5-FC group did not show any significant change in the presence of 5-FC. (e) The MKN45 cells + CD-hMSC group was similar to the control groups (b and c). (f) The MKN45 cells + CD-hMSC group in the presence of 100 mmol 5-FC showed cytotoxicity towards MKN45. Culture plates were photographed at × 100 (large box) and × 400 (small box) magnifications. (g) The MTT assay was carried out to assess cytotoxicity effects after 5-FC treatment in a hMSC and MKN45 cells direct co-culture system. The CD- and 5-FC-treated group shows more pronounced changes than seen in the groups with only CD or only 5-FC. Each MTT assay bar represents the mean of three different experiments and results are means ± standard errors of the mean. The asterisk indicates $P < 0.001$ versus other groups.

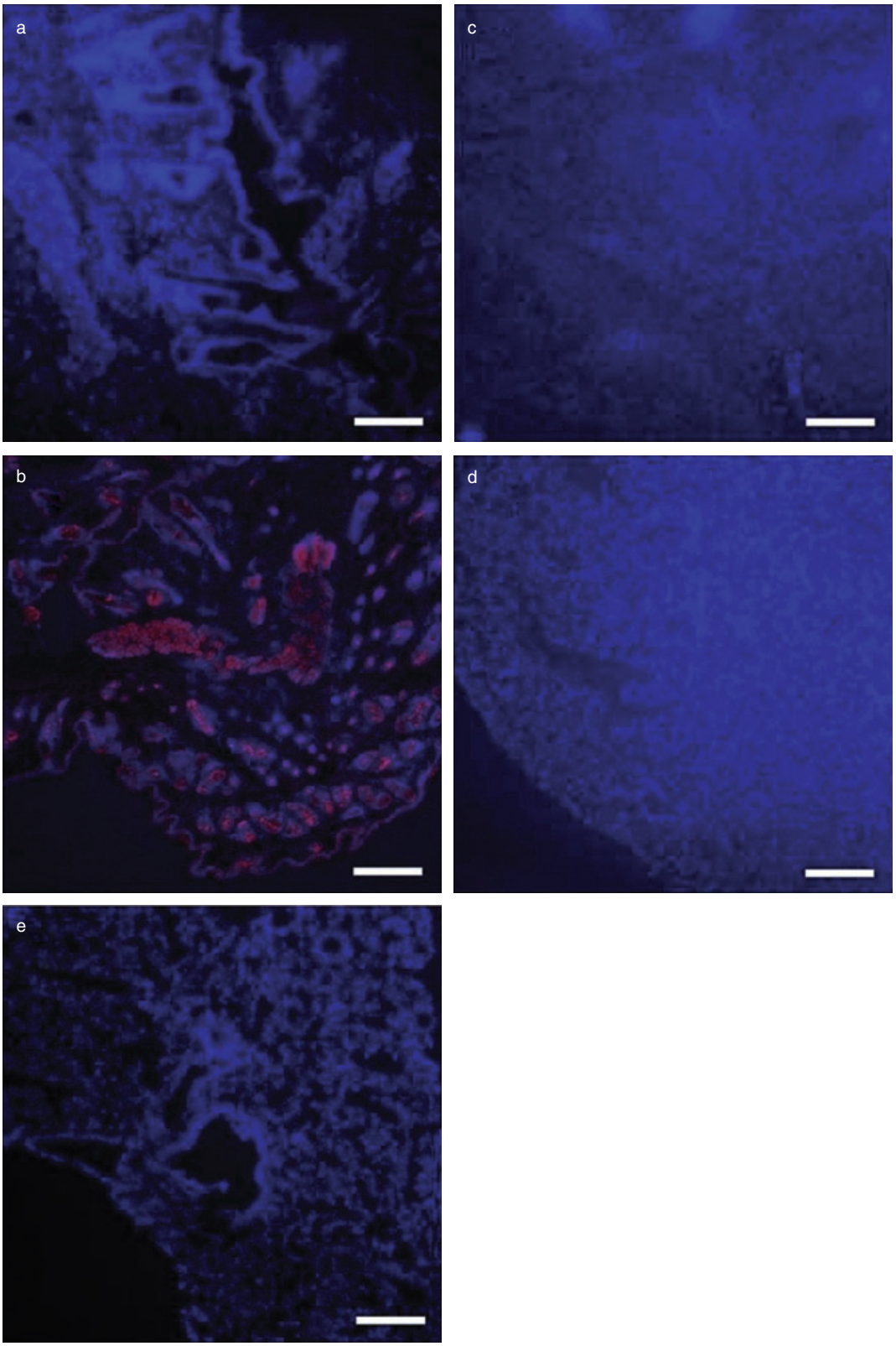

Figure 3 Intravenously injected human bone marrow-derived mesenchymal stem cells (hMSC) migrate to tumor regions of athymic mice harboring MKN45 tumors. (a) MKN45 xenograft mice, with no injected hMSC, were used as negative controls. (b) hMSC cultured with CM-DiI (red fluorescence) were detected in MKN45 tumor regions of athymic mice (bar, 50 μm). The efficacy of CM-DiI labeling is close to 100%. In liver (c), kidney (d) and lung (e), hMSC (red fluorescence) were not detected.

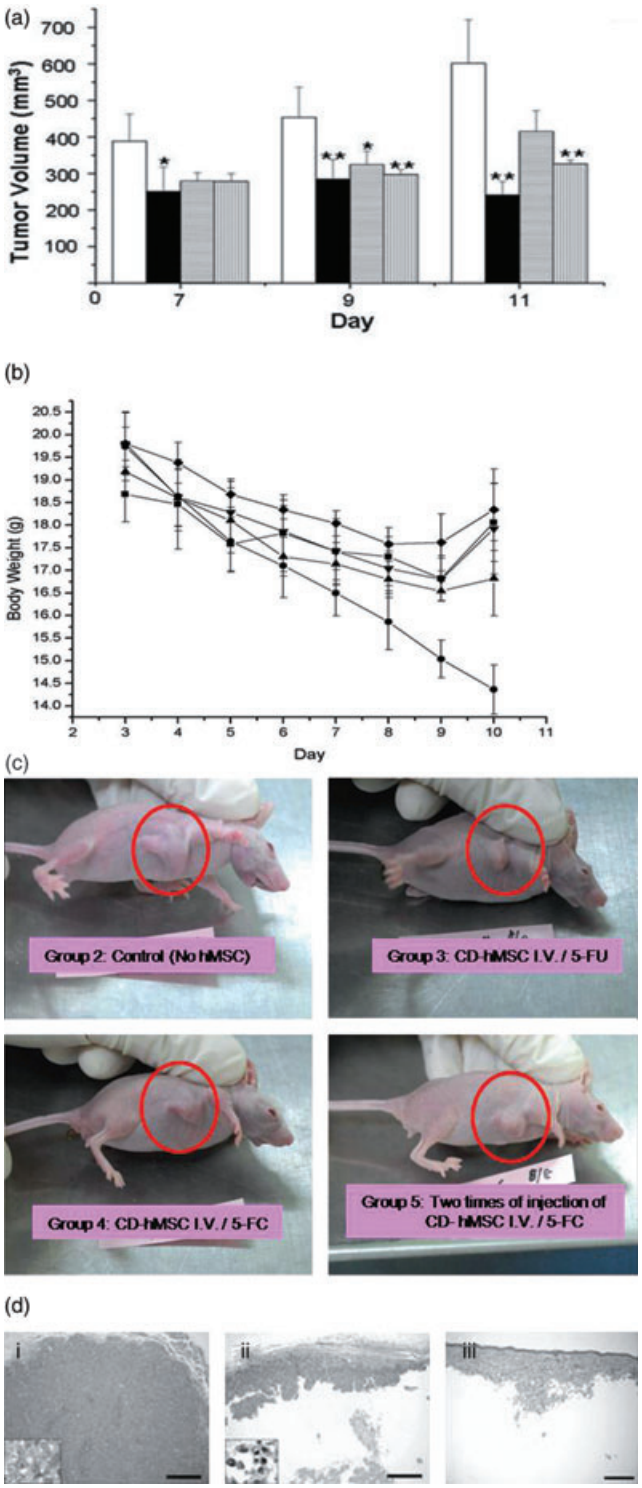

Figure 4 *In vivo* antitumor effect of injected cysosine deaminase human bone marrow-derived mesenchymal stem cells (CD-hMSC) in the presence of 5-fluorouracil (5-FU). (a) Tumor volume in group 3 (CD-hMSC i.v./5-FU) showed a statistically significant difference ($P < 0.05$) from controls on day 7. On day 9, mice in all treatment groups showed statistically significant differences (groups 3 and 5, $P < 0.01$; group 4, $P < 0.05$). Mice in groups 3 and 5 showed statistically significant differences ($P < 0.01$) on the last day after treatment. (b) Adverse reactions of anticancer drugs, as shown by effects on bodyweight. Mice in group 3 (only) showed a statistically significant difference ($P < 0.01$) from day 7 to day 10. These data show that, in groups 4 and 5, the prodrug (5-fluorocytosine [5-FC]) was activated only in CD-hMSC near tumors leading to high local concentrations of the drug (5-FU). (c) Tumors in MKN45 transplanted mice. (d) (i) Group 2 (control, no MSC): MKN45 tumor is well demarcated from dermal tissue and composed of closely packed round cells separated by a fine stroma, Inset: MKN45 cells. (ii) Group 3 (CD-hMSC treated with 5-FU) and (iii) Group 4 (CD-hMSC treated with 5-FC): the neoplastic cells remaining in the peripheral lesion are shrunken and have distinct cell borders. The hyperchromatic nuclei are centrally located with condensed eosinophilic cytoplasm, which has apoptotic bodies. The geographic necrosis is severe in both group 3 and group 4. Inset: apoptotic cells having an apoptotic body. Bar, 200 mm. (a) ▭, Group 2: Control (No hMSC), ▬, Group 3: CD-hMSCI.V./5-FU, ▤, Group 4: CD-hMSC IV./5-FC, ▥, Group 5: Two times of injection of CD-hMSCI.V./5-FC. (b) -▪-, Control (No hMSC), -•-, CD-hMSCI.V./5-FU, -▲-, CD-hMSC IV./5-FC, -▾-, Two times of injection of CD-hMSCI.V./5-FC, -◆-, hMSC.I.V./5-FC.

examined. As shown in Figure 3b, CM-Dil-labeled hMSC (red fluorescence) were localized in the tumor nodules 24 h postinjection. However, hMSC were not detected in any other organs examined (Fig. 3a–e). Similar data were obtained from mice killed at 72 h. CM-Dil-labeled hMSC were counted under the confocal microscope. Considering the tumor volume, the injected hMSC were counted as 7.2×10^5, which was comparable to 72% of injected hMSC. Microscopically, the subcutaneous MKN45 xenografts consist of densely packed plygonal cells separated by a fine stroma. However, no microscopic abnormalities were noted in other organs examined.

Antitumor effect of CD-hMSC *in vivo*

The antitumor effect of CD-hMSC/5-FC was evaluated 7, 9, and 11 days after initial MKN45 cell injection by measurement of tumor volume and bodyweight. As shown in Figure 4a, compared with group 2, significant inhibition of tumor growth was seen in group 3 ($P < 0.01$) on day 7 and this antitumor effect continued, and became more pronounced, until day 11. In group 4 ($P < 0.05$ compared with group 2), an antitumor effect was noted on day 9 ($P < 0.01$) but disappeared on day 11. Compared with group 4, a significant degree of antitumor effect was seen on both days 9 and 11. However, there was no antitumor effect in group 6. Significant weight loss ($P < 0.01$) compared with group 2 was first observed only in group 3 on day 8 (Fig. 4b), whereas groups 4, 5 and 6 showed no such weight loss. Hematology did not detect any significant abnormalities; hematopoiesis was appropriate (data not shown). Gross picture of tumors (Fig. 4c) and pathologic photographs (Fig. 4d) supported the antitumor effect of CD-hMSC treated with 5-FC. The presence of 5-FU converted from 5-FC by CD in the tumors of group 5 was confirmed by high performance liquid chromatography (Fig. S1).

Discussion

In the present study, an *in vitro* migration assay and *in vivo* administration of hMSC into tumor-bearing mice clearly showed that hMSC had the ability to track tumor cells, as has been shown in

other studies.[4,5] In the *in vitro* migration assay, MKN45 cells were able to attract hMSC, whereas fibroblast and keratinocyte cell lines failed to do so. The present *in vivo* migration study showed, 24 h later, that about 72% of injected hMSC arrived at tumor nodules, thus suggesting the presence of strong chemo-attractive activity from tumor cells for hMSC. In the system used in the present study, CM-Dil was used as a cell surface marker. CM-Dil is very useful because this particle stably attaches to the cell surface and remains for more than 30 days if the cells are alive. Transplanted MKN45 showed very rapid growth (around 4 days were enough to reach 1 cm in diameter). Tumors developed from transplanted MKN45 might have rich new vessels, which means strong angiogenesis might induce MSC arrival. It is well known that hMSC migrate to areas with various types of tissue injury, attracted to the inflammatory mediators produced at injury sites.[4,5] The hMSC can also migrate to tumor tissues after systemic administration, in a manner similar to that seen after tissue injury. Previous reports have shown that several factors produced by tumors are involved in the chemotaxis of hMSC.[21–23] We did not find any significant direct side-effects after systemic hMSC administration, at least up to the time the mice were killed. Autologous hMSC have been systemically given to stroke patients and cases with multiple system atrophy.[16,17] The data showed that hMSC therapies were able to delay disease progress and were safe at 1 year of follow-up. The present findings clearly show that hMSC are likely to serve as an effective and safe gene delivery vehicle in cancer cell therapy.

Combined CD and 5-FC treatment has been suggested as a means to overcome the systemic toxicity of 5-FU.[6,11] 5-FU can easily diffuse into cells and induce the effect obtained with CD/5-FC. The present *in vitro* conversion study showed that over 80% of 5-FC was converted to 5-FU. CD-hMSC/5-FC treatment resulted in significant inhibition of tumor growth with minimal systemic side effects, as shown by histopathology, bodyweight change and basic hematology. The conversion from 5-FC into 5-FU by CD in the tumors was confirmed by high performance liquid chromatography (Fig. S1). The CD gene was transfected with lipofectamine and this treatment is transient transfection. Therefore, the expression of CD expressing MSC will not continue for more than 3 days. We believe that 3 days are enough for the expression of the CD gene in MSC because if we use 5-FC after 24 h after the injection of the CD gene expressing MSC (which is necessary time for MSC arrival to tumor), production of 5-FU by CD will destroy the tumor, including MSC (suicidal effect). Thus, repeated injection with CD-MSC might be helpful to enhance the antitumor activity.

A Phase 1 clinical study of CD/5-FC in colon cancer patients has already shown the safety of CD/5-FC.[23] In that study, the anticancer drug 5-FU was used, and activated the drug to 5-fluorodeoxyuridine monophosphate (5-FdUMP), which is an active form of 5-FU and forms a tight ternary complex with TS which, in turn, is a key enzyme in DNA synthesis.[14] Thus, it has been suggested that this complex will inhibit the de novo pathway of DNA synthesis and induce tumor cell death.[13–15] From this point of view, cell apoptosis is indirect evidence of anticancer effects.[24]

To date, hMSC have been obtained from human neuron cultures, umbilical cord blood, embryonic endothelial progenitor cells, bone marrow and adipose tissue.[25–27] Among these several stem cell sources, bone marrow-derived MSC (BM-MSC) are safe, have no ethical problems and show easy expansion in culture systems. Furthermore, the present results showed that BM-MSC did not stimulate tumor growth *in vivo*, although several reports have suggested that MSC provide stromal support for tumor cells in different models.[28,29] Kucerova *et al.* showed that adipose tissue-derived human MSC (AT-MSC) could be used as delivery vehicles to human colon tumor regions in athymic nude mice.[4] Although surface antigen markers failed to distinguish between BM-MSC and AT-MSC, microarray data have shown that the gene expression profiling of BM-MSC differs from that of AT-MSC,[30] suggesting that such differences might have a significant impact on the relative efficiencies of these cells as gene delivery vehicles.

The present study clearly confirms and extends the results of previous studies, showing that the CD-hMSC/5-FC system is a potential molecular chemotherapeutic tool for cancer treatment.[4] Gastric cancer is the most common type of cancer in Korea. According to the national cancer registration database, about 22% of male and 17% of female cancer-related deaths are from gastric cancer. However, further studies are needed before clinical application of the technique in gastric cancer patients; necessary advances include the development of a more efficient and safe genetic transduction system, description of an hMSC boosting schedule, delineation of appropriate dosages of anticancer drugs, and, eventually, identification of a more powerful prodrug. The present study clearly shows the potential of human BM-MSC as an effective delivery system that targets tumors and minimizes the side-effects of anticancer drugs.

Acknowledgments

The present work was supported by the Daewoo Medical Foundation, 2005 grant from the Department of Medical Sciences, The Graduate School, Ajou University. The present work was also supported by the BK21 program for Ajou University and the Department of Veterinary Science, Seoul National University. We thank Ms. Hyun-Seung Kang for excellent technical assistance and Dr. Young-Bae Kim for his critical review of pathologic findings.

References

1 Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J. Clin.* 2005; **55**: 74–108.
2 Kim HJ, Chang WK, Kim MK, Lee SS, Choi BY. Dietary factors and gastric cancer in Korea: a case-control study. *Int. J. Cancer* 2002; **97**: 531–5.
3 Reiser J, Zhang XY, Hemenway CS, Mondal D, Pradhan L, La Russa VF. Potential of mesenchymal stem cells in gene therapy approaches for inherited and acquired diseases. *Expert Opin. Biol. Ther.* 2005; **5**: 1571–84.
4 Kucerova L, Altanerova V, Matuskova M, Tyciakova S, Altaner C. Adipose tissue-derived human mesenchymal stem cells mediated prodrug cancer gene therapy. *Cancer Res.* 2007; **67**: 6304–13.
5 Kucerova L, Matuskova M, Pastorakova A *et al.* Cytosine deaminase expressing human mesenchymal stem cells mediated tumour regression in melanoma bearing mice. *J. Gene. Med.* 2008; **10**: 1071–82.
6 Studeny M, Marini FC, Champlin RE, Zompetta C, Fidler IJ, Andreeff M. Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. *Cancer Res* 2002; **62**: 3603–8.

7 Studeny M, Marini FC, Dembinski JL *et al.* Mesenchymal stem cells: potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents. *J. Natl. Cancer Inst.* 2004; **96**: 1593–603.
8 Huber BE, Austin EA, Good SS, Knick VC, Tibbels S, Richards CA. In-vivo antitumor-activity of 5-Fluorocytosine on human colorectal-carcinoma cells genetically-modified to express cytosine deaminase. *Cancer Res.* 1993; **53**: 4619–26.
9 Moolten FL. Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes—paradigm for a prospective cancer control strategy. *Cancer Res.* 1986; **46**: 5276–81.
10 Gilula NB, Reeves OR, Steinbac A. Metabolic coupling, ionic coupling and cell contacts. *Nature* 1972; **235**: 262–65.
11 Kato H, Koshida K, Yokoyama K, Mizokami A, Namiki M. Potential benefits of combining cytosine deaminase/5-fluorocytosine gene therapy and irradiation for prostate cancer: experimental study. *Int. J. Urol.* 2002; **9**: 567–76.
12 Mullen CA, Coale MM, Lowe R, Blaese RM. Tumors expressing the cytosine deaminase suicide gene can be eliminated in vivo with 5-fluorocytosine and induce protective immunity to wild type tumor. *Cancer Res.* 1994; **54**: 1503–6.
13 Spears CP, Gustavsson BG, Mitchell MS *et al.* Thymidylate synthetase inhibition in malignant tumors and normal liver of patients given intravenous 5-fluorouracil. *Cancer Res.* 1984; **44**: 4144–50.
14 Spears CP, Shahinian AH, Moran RG, Heidelberger C, Corbett TH. In vivo kinetics of thymidylate synthetase inhibition of 5-fluorouracil-sensitive and -resistant murine colon adenocarcinomas. *Cancer Res.* 1982; **42**: 450–6.
15 Parker WB, Cheng YC. Metabolism and mechanism of action of 5-fluorouracil. *Pharmacol. Ther.* 1990; **48**: 381–95.
16 Bang OY, Lee JS, Lee PH, Lee G. Autologous mesenchymal stem cell transplantation in stroke patients. *Ann. Neurol.* 2005; **57**: 874–82.
17 Lee PH, Kim JW, Bang OY, Ahn YH, Joo IS, Huh K. Autologous mesenchymal stem cell therapy delays the progression of neurological deficits in patients with multiple system atrophy. *Clin. Pharmacol. Ther.* 2008; **83**: 723–30.
18 Nakamizo A, Marini F, Amano T *et al.* Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. *Cancer Res.* 2005; **65**: 3307–18.
19 Royet JP. Stereology: a method for analyzing images. *Prog. Neurobiol.* 1991; **37**: 433–74.
20 Chang J, Park K, Bang YJ *et al.* Expression of transforming growth factor beta type II receptor reduces tumorigenicity in human gastric cancer cells. *Cancer Res.* 1997: **57**: 2856–9.
21 Studeny M, Marini FC, Dembinski JL, *et al.* Mesenchymal stem cells: potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents. *J. Natl. Cancer Inst.* 2004; **96**: 1593–603.
22 Hung SC, Deng WP, Yang WK *et al.* Mesenchymal stem cell targeting of microscopic tumors and tumor stroma development monitored by noninvasive in vivo positron emission tomography imaging. *Clin. Cancer Res.* 2005; **11**: 7749–56.
23 Crystal RG, Hirschowitz E, Lieberman M *et al.* Phase I study of direct administration of a replication deficient adenovirus vector containing the E. coli cytosine deaminase gene to metastatic colon carcinoma of the liver in association with the oral administration of the pro-drug 5-fluorocytosine. *Hum. Gene. Ther.* 1997; **8**: 985–1001.
24 Sun SY, Hail N, Lotan R. Apoptosis as a novel target for cancer chemoprevention. *J. Natl. Cancer Inst.* 2004; **96**: 662–72.
25 Bieback K, Kern S, Kluter H, Eichler H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. *Stem Cells* 2004; **22**: 625–34.
26 Kim SK, Kim SU, Park IH *et al.* Human neural stem cells target experimental intracranial medulloblastoma and deliver a therapeutic gene leading to tumor regression. *Clin. Cancer Res.* 2006; **12**: 5550–6.
27 De Keyser J. Autologous mesenchymal stem cell transplantation in stroke patients. *Ann. Neurol.* 2005; **58**: 653–4.
28 Djouad F, Plence P, Bony C *et al.* Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. *Blood* 2003; **102**: 3837–44.
29 Sato T, Sakai T, Noguchi Y, Takita M, Hirakawa S, Ito A. Tumor-stromal cell contact promotes invasion of human uterine cervical carcinoma cells by augmenting the expression and activation of stromal matrix metalloproteinases. *Gynecol. Oncol.* 2004; **92**: 47–56.
30 Wagner W, Wein F, Seckinger A *et al.* Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. *Exp. Hematol.* 2005; **33**: 1402–16.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Copy number alteractions.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.