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Mesenchymal stem cells as a gene therapy carrier for treatment of fibrosarcoma

Juanjuan Xiang[1*], Jingqun Tang[1†], Chao Song[1,2], Ziquan Yang[1‡], David Graham Hirst[3], Qiu-Jian Zheng[4] and Gang Li[1,2]

[1]Centre for Cancer Research and Cell Biology, School of Medicine, Dentistry and Biomedical Sciences, Queen's University Belfast, Belfast, UK, [2]The Li Ka Shing Institute of Health Sciences, Department of Orthopaedics and Traumatology, Faculty of Medicine, The Chinese University of Hong Kong, Clinical Sciences Building, Prince of Wales Hospital, Shatin, Hong Kong, PR China, [3]School of Pharmacy, Queen's University Belfast, Belfast, UK, and [4]Department of Orthopaedics and Traumatology, The People's Hospital of Guangdong Province, Guangzhou, Guangdong Province, PR China

Background aims

Cell-based gene therapy is an alternative to viral and non-viral gene therapy. Emerging evidence suggests that mesenchymal stem cells (MSC) are able to migrate to sites of tissue injury and have immunosuppressive properties that may be useful in targeted gene therapy for sustained specific tissue engraftment.

Methods

In this study, we injected intravenously (i.v.) 1×10^6 MSC, isolated from green fluorescent protein (GFP) transgenic rats, into Rif-1 fibrosarcoma-bearing C3H/HeN mice. The MSC had been infected using a lentiviral vector to express stably the luciferase reporter gene (MSC-GFP-luci). An in vivo *imaging system (IVIS 200) and Western blotting techniques were used to detect the distribution of MSC-GFP-luci in tumor-bearing animals.*

Results

We observed that xenogenic MSC selectively migrated to the tumor site, proliferated and expressed the exogenous gene in subcutaneous fibrosarcoma transplants. No MSC distribution was detected in other organs, such as the liver, spleen, colon and kidney. We further showed that the FGF2/FGFR pathways may play a role in the directional movement of MSC to the Rif-1 fibrosarcoma. We performed in vitro *co-culture and* in vivo *tumor growth analysis, showing that MSC did not affect the proliferation of Rif-1 cells and fibrosarcoma growth compared with an untreated control group. Finally, we demonstrated that the xenogenic MSC stably expressing inducible nitric oxide synthase (iNOS) protein transferred by a lentivirus-based system had a significant inhibitory effect on the growth of Rif-1 tumors compared with MSC alone and the non-treatment control group.*

Conclusions

iNOS delivered by genetically modified iNOS-MSC showed a significant anti-tumor effect both in vitro *and* in vivo. *MSC may be used as a target gene delivery vehicle for the treatment of fibrosarcoma and other tumors.*

Keywords

Fibrosarcoma, gene therapy, inducible nitric oxide synthase, mesenchymal stem cells.

Introduction

Solid tumors comprise two distinct but interdependent compartments: neoplastic cells and the stroma that the neoplastic cells induce and in which they are dispersed. Stem cells are mainly referred to as tumor-supporting fibroblasts and they may derive from resident fibroblasts in the organ/tissue [1] or circulating mesenchymal progenitor cells [2–5].

*Present address: Cancer Research Institue, Xiangya School of Medicine, Centeral South University University, 90 Xiangya Road, Changsha, Hunan, 410078, PR China.
†Present address: Department of Cardiothoracic Surgery, Xiangya Second Hospital, Central South University, 139 Renmin Zhong Road, Changsha, Hunan, 410011, PR China.
‡Present Address: Department of Orthopadedics, The 2nd Hospital of Shangxi Medical University, Taiyuan, Shanxi 030001, PR China.
Correspondence to: Professor **Gang Li**, The Li Ka Shing Institute of Health Sciences, Department of Orthopaedics and Traumatology, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, China. E-mail: gangli@ort.cuhk.edu.hk

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Bone marrow (BM) is a major source of mesenchymal stem cells (MSC), a well-characterized population of adult stem cells. MSC can differentiate into cells of connective tissue lineages, including bone, fat, cartilage and muscle, and other types of tissue, such as hepatic, renal, cardiac and neural [6]. MSC preferentially home to bone, lung and spleen when injected intravenously (i.v.), as they favor adhering to matrix components. MSC have been used in regenerative medicine and tissue-engineering applications, as they are readily collected and mobilized and can differentiate into various tissue cells in response to wound signals [7–9].

A number of studies have demonstrated that MSC selectively home to sites of injury [10,11]. During the wound-healing process, many factors, including growth factors, hormones, cytokines and extracellular matrices, may regulate the recruitment and homing of MSC to sites of injury. Similar to the repair responses, inflammatory cytokines, growth factors and extracellular matrices also play important roles in cancer/tumor development and progression. The stroma of malignant tumors closely resembles the granulation tissue of a healing wound [11]; solid tumors generate a wound-like environment on their boundary, causing the physical and chemical stresses associated with their unrestrained growth. Tumors can, therefore, be regarded as sites of tissue damage or wounds that never heal.

Given the limitations in the efficiency and safety of most current cancer therapy, cell-based gene therapy could be the alternative to the use of currently available vectors. Developing a systemic delivery system would allow this approach to be used clinically for tumors inaccessible to direct transgene injection. The injury-homing characteristic of MSC is very attractive for this application. The direct targeting of anti-tumor agents into the tumor microenvironment might increase anti-cancer treatment efficacy and reduce the side-effects to other organs. The tumor microenvironment has been demonstrated to promote preferentially the engraftment of MSC compared with other tissues [2–4,11,12]. Therefore, the application of MSC to cancer therapy is of growing interest to medical researchers [2,3,12,13].

Genetically modified MSC producing anti-cancer molecules such as interferon (INF)-β and nitric oxide (NO*) may be used for anti-cancer treatment. Inducible nitric oxide synthase (iNOS) gene therapy, leading to targeted generation of high levels of NO*, has been identified as an anti-tumor strategy in pre-clinical models, leading to extensive apoptosis [14]. iNOS gene therapy has also been shown to cause dilation of the tumor-associated vasculature and increased tumor blood flow, which may sensitize the radiotherapy and chemotherapy of cancer [15–17]. The aim of the current study was to determine whether xenogenic MSC can be used as a delivery vehicle for iNOS gene therapy in a fibrosarcoma cell culture system and animal model.

Methods

Mononuclear cell culture and experimental animals

Green fluorescent protein (GFP)-transgenic rats were kindly provided by Professor M Okabe (Osaka University, Osaka, Japan). Rat MSC were obtained from BM aspirates of healthy young GFP-transgenic rats. The isolation and culture of MSC were performed using previously described methods [18,19]. Murine fibrosarcoma (Rif-1) cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum at 37°C under 5% CO_2 and 95% air.

Syngeneic transplanted fibrosarcomas were set up by intradermally injecting 4×10^5 Rif-1 cells into the rear dorsum of 10-week-old female C3H/HeN mice (Harlan, Huntington, UK). The animals were examined every day after the injection and treated in different groups when the tumor masses reached a volume of 100 mm^3. All animal experiments were carried out in accordance with the Animal (Scientific Procedure) Act 1986 and conformed to the current UKCCCR guidelines.

Lentiviral vectors and MSC transfection

The lentiviruses were created using the ViraPower™ Lentiviral Expression System (Invitrogen, Paisley, UK). The coding sequence of luciferase or iNOS was subcloned into pLenti6/V5-D-TOPO (Invitrogen). pLenti6/V5-D-TOPO/luciferase or pLenti6/V5-D-TOPO/iNOS vector and the ViraPower™ Packaging Mix (Invitrogen) were co-transfected using a gene carrier kit (Epoch-Biolabs, Missouri City, TX, USA.) into the 293T cell line to produce a lentiviral stock. Forty-eight hours post-transfection, virus-containing supernatant was harvested by collecting the medium. Viral particles were purified by ultracentrifugation through a 20% sucrose cushion. For infecting MSC, cells were cultured in 24-well plates and, when the culture reached 80% confluence, the concentrated lentivirus was

added to the culture dishes. After incubation for 48 h, the medium was replaced with selection medium containing 10 μg/mL blasticidin. The selection medium was replaced every 2 days until antibiotic-resistant colonies were identified and thus a stable cell line of MSC-GFP-luci or MSC-GFP-iNOS was established.

Immunocytochemistry

Cells grown on plates were fixed with 4% paraformaldehyde for 10 min at room temperature (RT) and washed with phosphate-buffered saline (PBS) (pH 7.4). After several washes with PBS (pH 7.4), cells were incubated for 30 min at RT with 10% serum in antibody (Ab) diluent plus background-reducing components (Dako, Cambridgeshire, UK), followed by primary Ab (1:100 in Ab diluent). The Ab included GFP goat polyclonal Ab (Abcam, Cambridge, UK), iNOS rabbit polyclonal Ab (Upstate Biotechnology, Lake Placid, NY, USA), CD44 mouse anti-rat Ab, CD45 mouse anti-rat Ab (Serotec, Oxford, UK), major histocompatibility complex (MHC) I, MHC II mouse anti-rat Ab (Antigenix America Inc., Huntington Station, NY, USA), VCAM, ICAM-1, endoglin rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and TFR mouse monoclonal Ab (Abcam).

The corresponding secondary fluroescein isothiocyanate (FITC)-conjugated Ab or R-Phycoerythrin (RPE)-conjugated (Dako) Ab diluted 1:100 in Ab diluent were applied for 1 h at RT in the dark. Monoclonal Ab of irrelevant specificity were used as negative controls. After the incubation period, the cells were washed extensively in PBS (pH 7.4). Cells were observed under an epi-fluorescence microscope (Nikon, Tokyo, Japan). The tumor samples were embedded in OTC compound (Sakura Finetek, Torrence, CA, USA), snap-frozen in liquid nitrogen and stored at −70°C. Tissues were sectioned (6 μm) and processed for immunofluorescence as shown above. Cells were counterstained with 4′-6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) (Sigma, Poole, UK).

Chemotaxis assays

Chemotaxis was measured using Dunn chemotaxis chambers (Hawksley Technology, Lancing, UK). Glass coverslips were plated with MSC-GFP. Cells were starved in serum-free Dulbecco's modified Eagle medium (DMEM) for 24 h before chemotaxis analysis. Gradients of recombinant human FGF2 (PeproTech EC Ltd, London, UK) were formed by placing serum-free DMEM in the inner well and different concentrations of FGF2 in DMEM in the outer well of the Dunn chamber slides. For an additional experimental group, MSC-GFP were pre-treated with neutralizing Ab to FGFR2 (1:100 dilution; Abcam) for 1 h prior to the chemotaxis assay. For a negative control, rhFGF4 (200 ng/mL; PeproTech EC Ltd) was added to the outer well of the Dunn chamber slide. The coverslip was inverted on to the assay slide and the edges sealed with wax. The assay slide was then placed on the heated (37°C) stage of an inverted Nikon microscope with a ×100 phase-contrast objective linked to a CCD camera. The process was recorded at a time-lapse interval of 5 min over a 10-h period. The data were analyzed using MATHEMATIC 3.0 (Wolfram Research, Champaign, IL, USA) and AQM 2001 software (Kinetic Imaging Ltd, Manchester, UK). A transwell system was also used to test the migration of MSC-GFP. The MSC-GFP were cultured at the insert and the bottom well was filled with 50% conditioned medium (72 h of culture) of Rif-1 cells with or without neutralizing Ab to FGF2 (100 ng/mL; Abcam). The numbers of cells migrating through the transwell membrane were compared.

Reverse transcription–polymerase chain reaction

The RNA samples and cDNA samples were prepared using Trizol reagent (Invitrogen) and a Qiagen QuantiTect reverse transcription (RT) kit (Qiagen, Crawley, West Sussex, UK). The polymerase chain reaction (PCR) was performed using PCR master mix (Promega, Southampton, UK). Briefly, a master mix was prepared by mixing 2× PCR master mix (10 μL), primer mix (2 μL), RNase-free water (6 μL) and cDNA sample (2 μL; 200 ng/μL). Amplification was performed as follows: 28 cycles of 30 s at 94°C for denaturation, 45 s at 55°C for annealing and then 45 s at 72°C for amplification. The PCR products were evaluated by electrophoresis in 1.0% agarose gels. The primers used are shown in Table I.

In vivo imaging of transplanted MSC

MSC-GFP-luci cells were trypsinized with 0.25 M EDTA and 0.05% trypsin (Invitrogen) on the day of transplantation and resuspended in PBS at 1×10^7 cells/mL; 1×10^6 of cells were injected i.v. into the tail veins of 10-week-old C3H/HeN mice bearing tumors that had grown to 100–150 mm^3. The animals were examined at different time points after the injection using the imaging system

Table I. The primers used for RT-PCR.

Rat FGFR1	Forward	5′–ACAGACAACACCAAACCAAACC–3′
Rat FGFR1	Reverse	5′–TTAATGCTCCCATACTCGTTCTC–3′
Rat FGFR2	Forward	5′–CACCAACTGCACCAATGAAC–3′
Rat FGFR2	Reverse	5′–GAATCGTCCCCTGAAGAACA–3′
Rat FGFR3	Forward	5′–GAGAAGGCTGCTTTGGACAG–3′
Rat FGFR3	Reverse	5′–CCCCCAACAGGTTAATGATG–3′
Rat FGFR4	Forward	5′–GTGGCTGTGAAGATGCTGAA–3′
Rat FGFR4	Reverse	5′–GAGGAATTCCCGAAGGTTTC–3′
Rat FGF2	Forward	5′–GGCTGCTGGCTTCTAAGTGT–3′
Rat FGF2	Reverse	5′–TATGGCCTTCTGTCCAGGTC–3′

IVIS 200. D-Luciferin was given to each mouse by intraperitoneal (i.p.) injection at a dose of 150 mg/kg and the mice left for 8 min while being anesthetized in a chamber with 3% isofluorane. Mice were then imaged using a 20-cm field of view and an exposure time of 3 min (3 min exposure; f-stop, 1; binning, 16; field of view, 15 cm). Bioluminescence values were calculated by measuring photons/s/cm^2/sr in the region of interest.

Western blot

Internal organs, including brain, heart, lung, liver, spleen, kidney as well as the tumor masses, were excised at the end of the animal experiments. MSC-GFP were also collected for Western blot analysis. Proteins were extracted using the mRIPA method (50 mM Tris, pH 7.4, 100 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS). The protein concentration was determined using a BCA protein assay kit (Pierce, Cramlington, UK). The Western blot system was set up using a Biorad Bis-Tris gel system, according to the manufacturer's instructions (Biorad, Hempstead, Hertfordshire, UK); 25 µL of sample of the desired protein concentration were loaded on to the gel. Electrophoresis was performed at 160 mV for 1.5 h, and the gel was then transferred to pre-soaked PVDF membrane (Amersham Biosciences, Amersham, UK). Transfers were carried out at 25 mV for 1.5 h. After the transfer was complete, the membrane was washed in 3% blocking buffer (3% skimmed milk, 0.1% Tween) for 1 h at RT. Goat anti-GFP and rabbit anti-FGFR-2 Ab (Abcam) were prepared in 3% blocking buffer at a dilution of 1:1000. The primary Ab was incubated with the membrane at 4°C overnight followed by a brief wash and incubation with secondary Ab for 1 h at RT. Finally, peroxide and luminol solutions 1:1 (Pierce) were added to cover the blot surface for 5 min at RT and the membrane was placed in a developing cassette.

Cell proliferation assay

Rif-1 cells were plated into 24-well plates. MSC-GFP or MSC-GFP-iNOS was plated on a polycarbonate membrane with 3.0-µM pores (Corning, New York, NY, USA). Forty-eight hours after co-culture in the transwell, the inserts were removed. Cell proliferation was then measured with a BrdU incorporation ELISA kit (Amersham Biosciences). Briefly, 10 µL BrdU solution were added to each well to a final concentration of 10 µM. The cells are incubated for another 24 h and fixed with 70% ethanol for 30 min at RT, then incubated for 30 min in blocking buffer (1% protein in 50 mM Tris-HCl and 150 mM NaCl, pH 7.4). The blocking buffer was removed and 100 µL 1/100 diluted peroxidase-labeled anti-BrdU Ab was added to each well and the plates incubated for 90 min at RT. The Ab was then removed and the wells washed three times with 300 µL/well of wash buffer. A total of 200 µL 3,3′, 5,5′-tetramethylbenzidine (TMB) in 15% (v/v) DMSO was added to each well and the plate was covered and oscillated gently in the dark for 5 min at RT. The reaction was stopped by adding 25 µL 1 M sulfuric acid to each well and the plate was read on a micro-ELISA reader at 450 nm.

For the clonogenic assay, Rif-1 cells, treated as described above, were trypsinized and seeded into six-well plates in triplicate. The plates were incubated in at 37°C under 5% CO_2/95% air for 14 days. The cells were then fixed in 70% methanol for 20 min and stained with crystal violet (0.4%). The number of colonies was counted with an Ingenius Syngene Bio Imaging System (Syngene, Frederick, MD, USA).

In vivo tumor growth delay assay

Rif-1 cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FSC) at 37°C under 5% CO_2/95% air. On the day of transplantation, Rif-1 cells were trypsinized and counted; 4×10^5 Rif-1 cells were injected

intradermally (i.d.) into the rear dorsum of 10-week-old female C3H/HeN mice (Harlan). The animals were examined every day after the injection and, when the tumor masses reached a volume of 100 mm^3, they received i.v. injection of MSC-GFP-iNOS (1×10^6 cells in 200 μL PBS, $n=7$), PBS control (200 μL, $n=4$) or GFP-MSC (1×10^6 cells in 200 μL PBS, $n=5$). Tumor diameters were measured every day using a ruler until the tumor volume reached four times its size on the day of treatment. The animals were then killed and samples collected. Tumor growth curves over time were plotted for each group.

Statistical analyzes

Statistical analyzes were performed using Excel (Microsoft) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA); *t*-test and ANOVA were used for comparison. $P<0.05$ was considered a significant difference.

Results

Identification for MSC

A previous study had confirmed that MSC from GFP-transgenic rats have the ability to differentiate into chondrocytes, osteoblasts and adipocytes [18]. The MSC from GFP-transgenic rats were positive for Thy-1 and CD44 and negative for CD34 and CD45. MSC represent a heterogeneous cell population consisting of several different cell types. In this study, the MSC from GFP-transgenic rats were positive for CD44, CD71 (TFR), CD105 (SH2, endoglin), CD54 (ICAM-1) and CD106 (VCAM-1) (Figure 1A–F) and negative for CD34, CD45, MHC I and MHC II (data not shown).

FGF2 mediates MSC migration toward Rif-1 fibrosarcoma cells

Chemotactic migration of MSC-GFP was significantly stimulated by FGF2 in a dose-dependent manner from 20 ng/mL to 1600 ng/mL (Figure 2A). MSC-GFP showed random migration when they were pre-treated with neutralizing Ab to FGFR2 (Figure 2B). MSC-GFP also showed random migration when rhFGF4 (200 ng/mL) was added to the outer well of the Dunn chamber slide (Figure 2C). FGF2 is known to bind to several related receptors (FGFR) that signal via tyrosine kinase transduction pathways. There are four isoforms of FGF receptors, FGFR1, FGFR2, FGFR3 and FGFR4, and each prototype receptor has a different ligand-binding capacity and tissue distribution [19]. To explore which receptor may be responsible for FGF2/FGFR-mediated MSC migration, we compared the localization

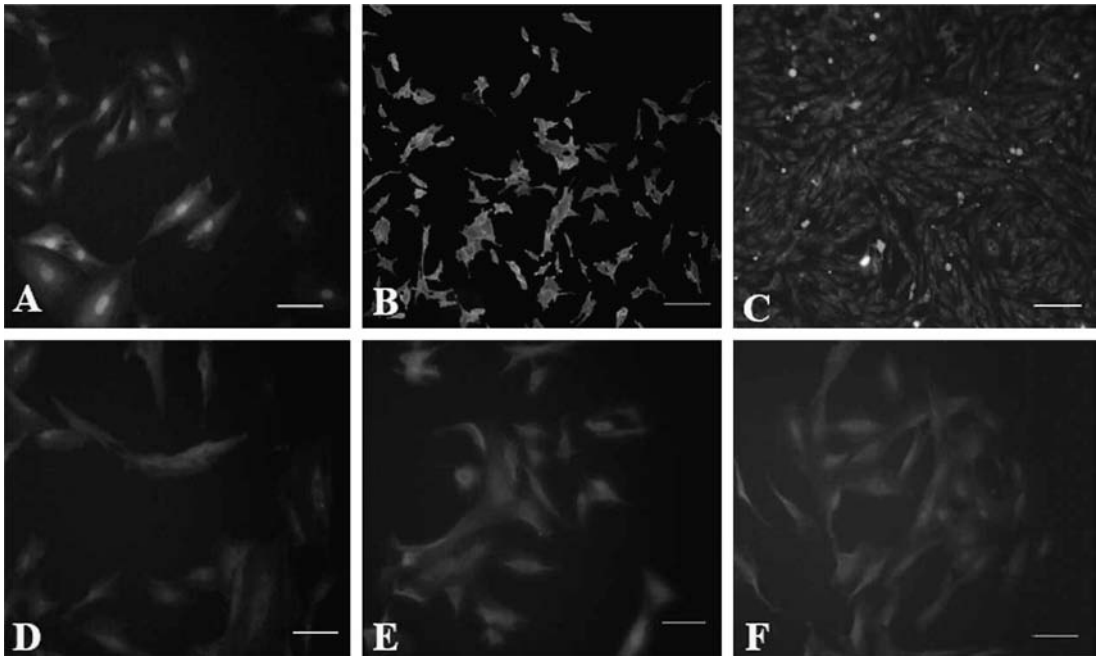

Figure 1. *Characterization of MSC-GFP. (A) Cells were observed for GFP expression directly under a fluorescent microscope. Bar = 100 μm. (B) Immunostaining of CD44. Bar = 100 μm. (C) Immunostaining of CD71 (TFR). Bar = 400 μm. (D) Immunostaining of CD105 (SH2, endoglin). Bar = 50 μm. (E) Immunostaining of CD54 (ICAM-1). Bar = 50 μm. (F) Immunostaining of CD106 (VCAM-1). Bar = 50 μm.*

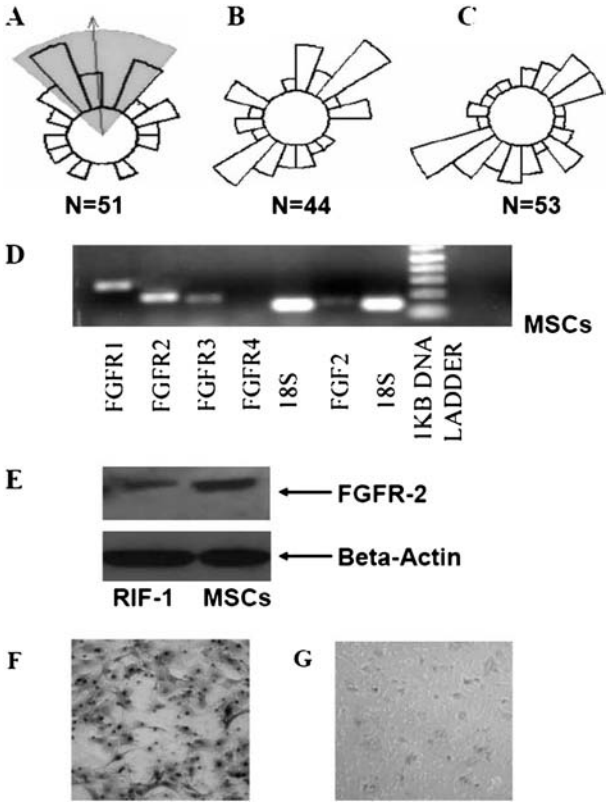

Figure 2. *(A–C) Directional migration of MSC-GFP in an FGF2 gradient was studied in a Dunn chemotaxis chamber. The direction of migration was depicted as horizon plots in which the bar length represents the proportion of cells moving in that particular direction and where the chemoattractant source was located at the top of the plot. The horizon was the distance from the starting point to a virtual horizon, which was set at 30 μm. The cells moving beyond 30 μm were valid for statistical analysis and the number of cells analyzed for each treatment is given. (A) The arrows and the green (gray) sector represent the mean significant direction of migration of MSC toward the FGF2 gradient and 95% confidence interval (*$P<0.01$*, Rayleigh test). (B) MSC showed random migration when they were pre-treated with neutralizing Ab to FGFR2. (C) MSC showed random migration when the rhFGF4 (200 ng/mL) was added to the outer well of the Dunn chamber slide. (D) Various FGF receptor genes were expressed in MSC-GFP and confirmed by RT-PCR examination. FGFR1, FGFR2 and FGFR3 were expressed in MSC-GFP but there no FGFR4 expression was found. (E) Western blotting confirmed that FGFR2 was expressed in MSC-GFP and Rif-1 cells. (F) In the transwell system, MSC-GFP migrated through the transwell membrane when the conditioned medium of Rif-1 cells was added to the bottom of the transwell. (G) When neutralizing Ab to FGF2 (100 ng/mL) was added to the conditioned medium in the bottom well, only a few MSC-GFP cells migrated through the transwell membrane.*

of mRNA of the four FGF receptor genes using RT-PCR, and found that FGFR1, FGFR2 and FGFR3 were expressed in MSC-GFP but there was no FGFR4 expression (Figure 2D). We further confirmed by Western blot that FGFR2 was expressed in MSC-GFP and Rif-1 cells (Figure 2E). In the transwell system, MSC-GFP migrated through the transwell membrane when the conditioned medium of Rif-1 cells was added to the bottom of the transwell (Figure 2F), whereas only a few MSC-GFP cells migrated through the transwell membrane when neutralizing Ab to FGF2 (100 ng/mL) was added to the conditioned medium in the bottom well (Figure 2G).

Systemically administered xenogeneic MSC selectively home to subcutaneous fibrosarcoma transplants

High luciferase activity was observed in the lung and tumor 24 h after injection. Comparatively high luciferase activity was also observed in the long bones at 24 h. Luciferase activity was mainly found in the tumor sites 2 days after the injection, while the luciferase activity in the lung decreased gradually to baseline by about 1 week after administration. No luciferase activity was detectable in any other organs, such as the liver, spleen, colon and kidney (baseline; Figure 3A, B). The luciferase activity in tumor sites gradually increased after MSC integration into the tumors, probably as a consequence of proliferation of MSC in the tumor sites. The luciferase activity was significantly increased by approximately two-fold ($P<0.05$) at the tumor sites compared with BM tissues (Figure 3C). Western blots for GFP were also performed to confirm the distribution of MSC after systemic administration. As early as 24 h after i.v. injection, GFP was detected in the lung, spleen and tumor (Figure 4A); 10 days after i.v. injection, MSC were observed only in the tumor volume (Figure 4B). Immunofluorescent staining for GFP demonstrated that GFP-expressing MSC engrafted in the tumor sites and expressed GFP (Figure 4C, D).

MSC stably expressing iNOS inhibit the growth of fibrosarcoma cells *in vitro* and *in vivo*

MSC stably expressing iNOS generated by lentivirus were confirmed and further analysis demonstrated that the multi-differentiation potentials of MSC-GFP-iNOS were maintained (data not shown). However, in the transwell co-culture system the cell proliferation of Rif-1 cells was reduced by 40% ($P<0.001$, Student's *t*-test) when the

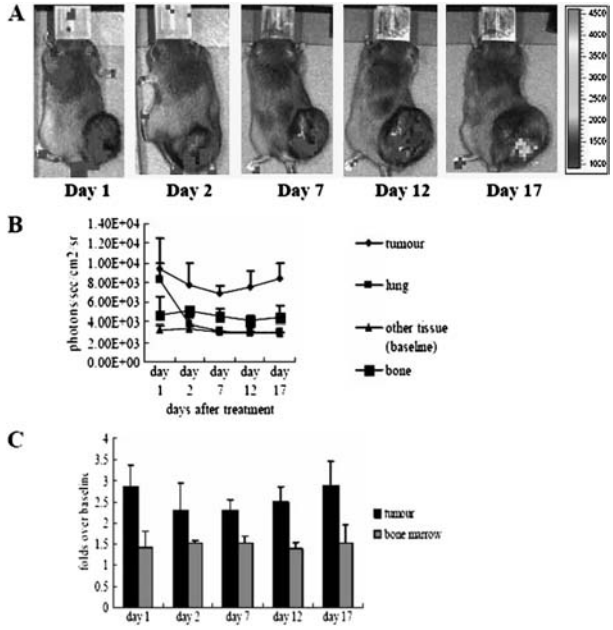

Figure 3. In vivo *distribution of i.v. administered MSC-GFP-luci. (A) Representative bioluminescent images showing MSC-GFP-luci location after i.v. administration at 1, 2, 7, 12 and 17 days. Luciferase activity was mainly seen in the tumor site 2 days after MSC injection and retained till 17 days. (B) High luciferase activity was observed in the lung and tumor 24 h after injection, with low luciferase activity observed in the long bones. Luciferase activity in the lung decreased gradually to the baseline level about 1 week after administration. The baseline level of luciferase activity was measured in the other organs, such as liver, spleen, colon and kidney. (C) The luciferase activity in the tumor was significantly higher than that in the long bone at all time points (*P*<0.01, Student's* t*-test). (B, C) All experiments were repeated three times, Mean ± SD are plotted.*

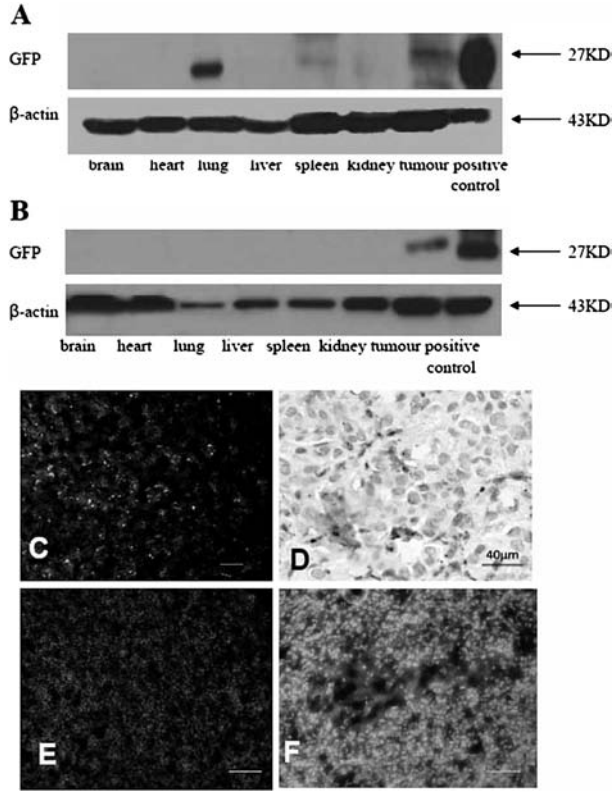

Figure 4. *Detection of MSC* in vivo *after injection into the tumor-bearing animals. (A) Western blotting demonstrated that GFP expression (27 kDa immunoblot band) was seen in lung and tumor at day 1 after injection. (B) Western blotting confirmed that GFP expression was only seen in the tumor at day 10 after injection. (C) The distribution of MSC-GFP in the tumor is shown by immunofluorescence staining. Bar = 100 μm. (D) GFP-positive cells (arrows) were determined by immunostaining of anti-GFP Ab in tumor sections. Bar = 40 μm. (E) Tumor nuclear morphology was visualized by counterstaining with propidium iodide. Bar = 100 μm. (F) A merged image of green and red fluorescence showing some MSC were inside the tumor. Bar = 50 μm.*

MSC-GFP-iNOS was present in the system, compared with the control (saline) and MSC-GFP groups (Figure 5A). Clonogenic assay showed that MSC-GFP-iNOS inhibited Rif-1 cell colony formation by 58% ($P<0.001$, Student's *t*-test), whereas the MSC-GFP had no significant effect on Rif-1 cell colony formation (Figure 5B).

The effect of MSC-GFP-iNOS after systemic administration *in vivo* was also investigated. A significant growth delay was observed between the control PBS group or MSC-GFP group and the MSC-GFP-iNOS injection group ($P<0.05$, ANOVA; Figure 6A). Tumor masses in the animals receiving PBS grew to four times larger than their original volume by 8 days after the PBS injection, whereas tumors in animals injected with MSC-GFP-iNOS were significantly smaller. Eight days after treatment, tumors in animals receiving MSC-GFP-iNOS were 1.6 times smaller than those in the PBS injection group. Immunofluorescent staining for iNOS demonstrated that iNOS-expressing MSC engrafted in the tumor sites and expressed iNOS (Figure 6B). No adverse effect was observed in the C3H/HeN mice that received a xenogenic rat MSC injection. There was no sign of immunogenic rejection or inflammatory cell infiltration in the region surrounding rat MSC inside the tumor tissues.

Discussion

It has become apparent that the antigenic phenotype of MSC is not unique and that, so far, no single marker is

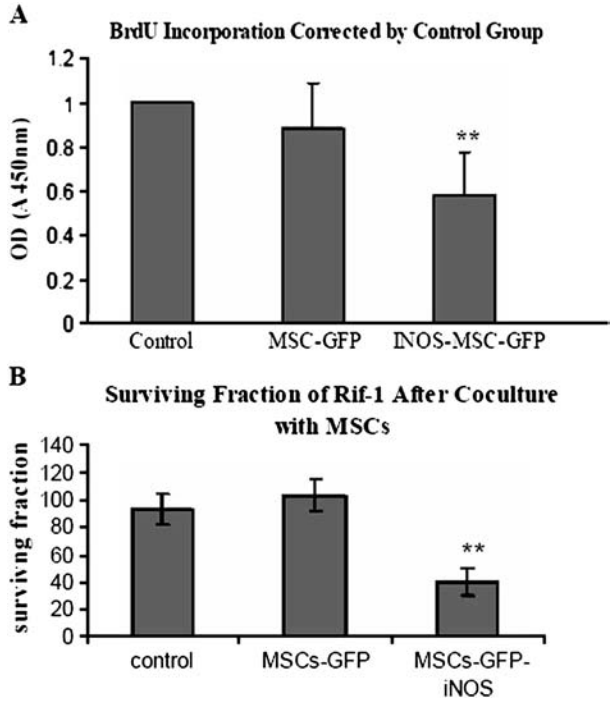

Figure 5. *(A) In the transwell mixed cell culture system, MSC-GFP-iNOS inhibited Rif-1 cell proliferation by 40% (**P<0.001, Student's* t*-test) compared with control (saline) and MSC-GFP groups (Mean±SD). (B) MSC-GFP-iNOS co-cultured with the tumor Rif-1 cells significantly inhibited Rif-1 colony formation by 58% compared with the control group (**P<0.001,* t*-test). All experiments were repeated three times, Mean±SD are plotted.*

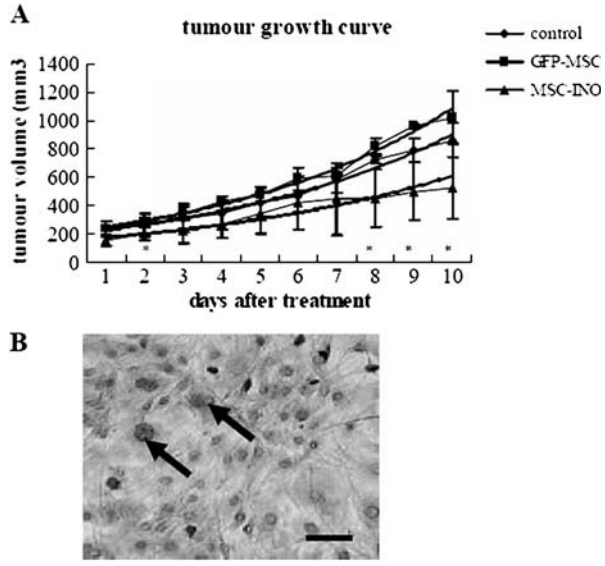

Figure 6. *(A) Growth curve of Rif-1 fibrosarcomas in C3H/HeN mice following i.v. injection of MSC-GFP or MSC-GFP-iNOS. Tumors in the animals receiving the MSC-GFP-iNOS injection* (n=7) *had significant growth inhibition (*P<0.05, ANOVA and* t*-test) compared with animals receiving MSC-GFP* (n=5) *or saline injection* (n=4), *Mean±SD are plotted. (B) MSC-GFP-iNOS were found engrafted inside the tumor. The engrafted MSC were iNOS-positive cells (arrow) immunostained by a specific Ab for iNOS. Bar=100 μm.*

known to be specific exclusively for MSC. It is not certain which markers must be expressed for that cell to be classified as an MSC. MSC represent a heterogeneous cell population consisting of several different cell types that are defined by their ability to self-replicate and differentiate towards different cell lineages. The MSC used in this study were confirmed by characteristic surface makers, multipotent potential and constitutive GFP expression.

In this study, we have demonstrated that xenogenic rat MSC can survive and function as a gene-delivery vehicle in the tumor-bearing C3H/HeN mice. We have previously demonstrated that the xenogenic GFP rat MSC have immunosuppressive properties and that undifferentiated GFP rat MSC do not trigger any immune response when mixed with human lymphocytes or dendritic cells [18]. Other studies have demonstrated that MSC are negative for MHC I and MHC II, which may reduce the incidence of graft-versus-host reaction [20,21]. Immune responses may be induced when the class I and II MHC are up-regulated after MSC differentiation *in vivo* [22,23]. However, our previous study demonstrated that GFP rat MSC survived 11 weeks after xenogeneic implantation in immunocompetent MF1 mice [24]. The unique characteristic of hypo-immunogenicity of MSC facilitates the allogenic or xenogenic MSC engraftment and survival in the cancer stoma at the tumor sites. In the current study, we have demonstrated that GFP rat MSC incorporated into the tumor architectures, proliferated and constitutively expressed the exogenous gene iNOS.

There are concerns that MSC-mediated immunosuppression may promote cancer growth. Several studies have suggested that stem and progenitor cells may contribute to sustained tumor growth and malignant progression [25]; MSC may actively promote the growth of the adjacent, transformed epithelial cells [26]. But there are also studies that show that MSC can inhibit tumor growth [13,27]. In the present study, the MSC co-cultured with Rif-1 tumor cells did not promote tumor cell proliferation and we did not find any evidence of excessive tumor growth in the tumor-bearing animals receiving unmodified MSC injection.

The effects of MSC on various tumor/cancer cells may be different. We recommend that the negative or no effect should be confirmed first *in vitro* in a co-culture system before applying MSC *in vivo*.

The recognition that MSC may lead themselves to sites of tissue repair, regeneration and tumors has been particularly exciting [28,29]. In the present study, we have demonstrated that systemically administered MSC selectively migrated to the tumor sites, preferentially survived and proliferated in the presence of malignant cells and became incorporated into the tumor architecture as stromal fibroblasts. We know that MSC are capable of homing to the BM of non-human primates following systemic infusion [30] but in the tumor-bearing animal model the MSC homed to tumor sites at a significantly higher degree than to BM sites. The underlying mechanisms could depend on growth and differentiation factors, hormones and cytokines that are released from the tumor cells, surrounding stromal fibroblasts or surrounding inflammatory cells, which have been implicated in the process of MSC recruitment to injury and tumor sites [29,31–35]. Among the factors that may act as chemoattractants for MSC, FGF2 is of particular importance for the fibrosarcoma tumor model because FGF2 is secreted by Rif-1 fibrosarcoma cells and binds to the extracellular matrix of the tumor *in vivo*, which could form chemo-attractive gradients. FGF2 has also been shown to induce migration of endothelial cells both *in vivo* and *in vitro* [36,37]. We have demonstrated that MSC migrated in a directional manner toward low concentrations of FGF2. FGF2 is known to bind to several related receptors that signal via tyrosine kinase transduction pathways, and we found that GFP rat MSC express FGFR (subtypes 1–3). Thus, the FGF2 together with other factors may be involved in regulating MSC recruitment and homing.

The development of iNOS gene therapy for cancer treatment has been described in previous studies, in which we have demonstrated that a single intratumoral injection of 25 μg iNOS-expressing construct in a cationic lipid vector can yield extensive apoptosis in the Rif-1 fibrosarcoma model [16,17,38]. In all the previous studies we delivered the iNOS constructs into the tumors by direct injection. In this study, the iNOS gene was delivered by stably inserting it into the MSC genome, which ensured an adequate expression level. MSC-mediated iNOS delivery used in the present study achieved a similar delay in tumor growth to that which we have achieved previously with a single direct injection [17]. In the present study, the majority of the MSC were restricted to inside tumor tissues 48 h after systemic injection, except for a small proportion of MSC that had moved into the BM tissues. However, MSC persisted and survived for longer periods in the tumor site and the long-lived MSC may continue to express the therapeutic transgene iNOS and generate the cytotoxic agent (NO or its reactive intermediates) inside tumors. Diffusion of NO will also result in extensive bystander cell killing of surrounding non-transfected tumor cells.

In summary, we have demonstrated in a mouse fibrosarcoma-bearing animal model that xenogenic MSC can selectively home to and engraft into the tumor stroma. MSC alone did not affect Rif-1 cell proliferation *in vitro* and *in vivo*. iNOS delivered by genetically modified iNOS-MSC showed a significant anti-tumor effect both *in vitro* and *in vivo*. MSC may be used as a target gene delivery vehicle for the treatment of fibrosarcoma and other tumors.

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