Survival of Bone Marrow-Derived Mesenchymal Stem Cells in a Xenotransplantation Model

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ABSTRACT: Mesenchymal stem cells (MSCs) are immunoprivileged and the allogeneic MSCs implantation has been used to facilitate tissue repairs such as bone and cartilage defect. The present study aimed to investigate the feasibility of xenogeneic MSCs implantation. Green fluorescent protein (GFP) transgenic rat bone marrow-derived MSCs were loaded into HA/TCP Skelite^{TM} blocks and implanted intramuscularly into the quadriceps of the MF1 and SCID mice. After 11 weeks, the implants were harvested and processed for further examinations. The peripheral blood mononuclear cells of each animal were also collected to measure the in vitro immune responses using mixed lymphocyte culture and cytotoxic assay. In the MF1 mice, some surviving MSCs were found in the explants after 11 weeks of implantation, but there was no sign of new bone formation as neither osteocalcin mRNA nor osteoid tissues were detected in the explants; the lymphocyte proliferation and cytotoxicity against donor MSCs were significantly increased in the animals with the xenogeneic MSCs implantation compared with the control littermates without transplantation. In the control SCID mice, osteoid tissues derived from the implanted MSCs were found in the explants; no difference of lymphocyte proliferation and cytotoxicity against the donor MSCs was detected between the SCID mice with or without MSCs implantation. The data suggested that rat MSCs survived the 11 weeks of xenotransplantation in the MF1 mice, but the increased host immune sensitization led to the impaired in vivo osteogenesis potential of MSCs. © 2007 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 25:926–932, 2007

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INTRODUCTION

Recently, mesenchymal stem cells (MSCs) have been demonstrated to be immunoprivileged and do not elicit immune responses upon co-culture with different subsets of immune cells in vitro.[1] It is further confirmed by the successful allogeneic MSCs transplantation in many animal models and human trials.[2–5] Moreover, it is suspected that MSCs are not immunogenic even under xenogeneic conditions. Subsequent efforts to test this hypothesis using different xenogeneic transplantation models produced diverse outcomes. Human MSCs engrafted and demonstrated site-specific differentiation after in utero xenotransplantation into sheep, both before and after the expected development of immune competence.[6] Other xenotransplantations using myocardial infarction model showed conflicting results with either no donor cell survival or functionally active chimera reported.[7–9] As the in vitro and in vivo studies have demonstrated that the immunogenicity of allogeneic MSCs remains unchanged even after osteogenic differentiation,[10,11] we further investigated the feasibility of xenogeneic MSCs transplantation using an ectopic bone formation model.[12]

MATERIALS AND METHODS

Experimental Animals

All the procedures were performed under the animal licenses issued by the UK Home Office according to Animals (Scientific Procedures) Act 1986. Green fluorescent protein (GFP) transgenic rats (male, 6 months old, kindly provided by Prof. M. Okabe, Osaka University, Japan) were used as donors to isolate bone

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marrow-derived MSCs. MF1 mice (male, 6 months old, Harland, UK) and severe combined immunodeficiency (SCID) mice (male, 6 months old, Harland, UK) were used as recipients for implantation. The SCID mice were kept under sterile conditions specially designed for immunocompromised animals. After surgery, all the animals were monitored regularly and special cares were given whenever signs of discomfort occurred. At the time of termination, the blood was taken from all the experimental animals and the corresponding untreated ones for later assays.

MSCs Isolation

Bone marrow was harvested from the femurs of the GFP rats. After homogenizing in PBS, the cell suspension was layered onto Lymphoprep™ (1.077 g/ml; Nycomed-Amersham, Norway) for density gradient centrifugation (1,840 rpm, 30 min). The mononuclear cells were collected from the buffy coat layers, resuspended in DMEM medium containing 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 2.5 μg/ml fungizone, and 2 mM L-glutamine (Invitrogen, Paisley, UK) and seeded into T75 flasks (Nunc, Roskilde, Denmark) at the density of $1-3 \times 10^5$ cells/cm^2. The cells were incubated at 37°C in humidified atmosphere with 5% CO_2. At confluence, the primary MSCs were detached using Trypsin-EDTA solution and passaged at 1×10^4 cells/cm^2 into new T75 flasks.

MSCs Loading and Implantation

The passages 1–2 MSCs were used for implantation. Aliquots of 1×10^6 MSCs were incubated with OsteoStim™ Skelite™ blocks (Millenium Biologix, Inc., Kingston, Canada) in 96-well plate (Nunc) for 40 min to allow attachment. The cell attachment on the blocks was confirmed by scanning electron microscopy (SEM) examination as previously described.[13]

The implantation procedure was performed under general anesthesia using a gaseous mixture of 3% isoflurane (Abbott Laboratories Ltd., Kent, UK) in a 50:50 mixture of N_2O_2:O_2 at 2 l/min in a sealed chamber. MSCs-loaded blocks were intramuscularly implanted (i.m.) into quadriceps of MF1 and SCID mice. The left side of all the animals were implanted with MSCs-loaded blocks and right side with cell-free blocks as negative controls. MF1 mice did not receive any immunosuppressive treatment. After 11 weeks, the animals were terminated and the implants and peripheral blood were collected for further analysis.

Mixed Lymphocyte Culture (MLC)

To examine the response of recipient lymphocytes to donor MSCs, the peripheral blood were taken from MF1 and SCID mice (with and without implantation) upon termination by cardiac puncture. The mononuclear cells (MNCs) were collected through Lymphoprep™ (1.077 g/ml) density gradient centrifugation (1,840 rpm, 30 min), washed in PBS, and they were used as main source for responder peripheral blood lymphocytes (PBLs). The stimulator cells were the GFP rat MSCs. Triplicates of 1×10^5 MNCs from the MF1 or SCID mice were seeded into 96-well plates together with 1×10^5 mitomycin C-treated (2.5 μg/ml, Sigma, Dorset, UK) GFP rat MSCs. Two controls were run in parallel: For negative controls, both the stimulator cells (MSCs) and the responder cells (PBLs) were from the littermate GFP rats; for positive controls, the simulator cells (MSCs) were from the GFP rats and the responder cells (PBLs) were from the MF1 mice. The cells were co-cultured in RPMI 1640 medium supplemented with HEPES (25 mM/L), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mmol/L L-Glutamine, 1 mM sodium pyruvate, 10 μM 2-mercaptoethanol, and 10% FBS (Invitrogen) and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 5 days without medium change. At day 5, cell proliferation assay of the co-cultured cells was performed using a cell proliferation detection kit (Cell Proliferation Biotrak ELISA System, Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions.

Quantitative Cytotoxic Assay

Cytotoxic assay was to detect the extent of cell lysis mediated by the stimulated cytotoxic T lymphocytes. Nonradioactive cytotoxicity assay kit (Promega, Southampton, UK) was used according to the manufacturer's instruction in this experiment. In brief, 1×10^5 MNCs from the MF1 or the SCID mice together with 5×10^4 mitomycin C-treated GFP rat MSCs were added in each well of the 96-well plates in triplicates, respectively. One hundred microliters RMPI 1640 medium containing 20 mg/ml phytohemagglutinin (PHA, Sigma-Aldrich, Dorset, England) was added to each well, which was to activate the resting lymphocytes to become cytotoxic T lymphocytes. For negative controls, both the stimulator cells (MSCs) and the effector cells (PBLs) were from the littermate GFP rats; for positive controls, the simulator cells (MSCs) were from the GFP rats and the effector cells (PBLs) from the MF1 mice. The cells were then incubated at 37°C for 4 h in a humidified atmosphere with 5% CO_2. The amount of lactate dehydrogenase (LDH) released from the lysed cells was quantified following the kit's instruction using a plate reader.

Histology Preparations

Upon termination, explants were harvested and half of them were fixed in 10% buffered formalin (HD Supplies, Aylersburg, UK) for 2 days, followed by decalcification in 20% formic acid (Vickers Laboratories Limited, West Yorkshire, UK) for 2 weeks. The samples were embedded in paraffin and 5 μm sections were cut using an HM 355S microtome (Microm, Walldorf, Germany), mounted on adhesive slides (Star Frost®, Knittelgläser, Germany), and stored at room temperature for further use. The other half of the samples were snap frozen in liquid nitrogen after dissection and stored at −80°C. Prior to sectioning, the samples were first embedded in

OCT compound (Tissue TEK, BDH, Leicestershire, UK) and 8-μm sections were cut using a cryomicrotome CM 1900 (Leica Microsystems Nussloch GmbH, Nussloch, Germany) and mounted on adhesive slides. The sections were then post-fixed with freshly prepared 4% paraformaldehyde solution and stored at −20°C for future use.

Histology and Immunohistochemistry Examinations

For hematoxylin and eosin (H&E) staining, paraffin sections were dewaxed in xylene for 5 min and rehydrated through graded alcohols, stained with H&E (HD Supplies), rehydrated and mounted in DPX mounting medium. For immunohistochemistry (IHC), the procedure was carried out as previously described[13] with slightly modification using a M.O.M.™ immunodetection kit (Vector Laboratories, Ltd., Peterborough, UK) according to the to the manufacturer's instructions. The primary monoclonal antibodies used were: mouse anti-GFP antibody (SC-9996, Santa Cruz, Insight Biotechnology Ltd., UK) and rat anti-mouse CD45 antibody (550539, Pharmingen, Oxford, UK).

In Situ RT-PCR Examination

The procedure was carried out as described previously with minor modifications.[14] QIAGEN OneStep RT-PCR kit (QIAGEN Ltd., West Sussex, UK) was used. The slides were pretreated with 2 mg/ml pepsin (Sigma) at 37°C for 90 min, washed in DEPC water for 1 min, followed in 100% ethanol for 1 min and air-dried. The slides were digested with 20 U DNase I (New England Biolabs, Hertfordshire, UK) overnight at 37°C to avoid nonspecific signals due to DNA repair and washed in DEPC water and 100% ethanol. The RT-PCR mixture consisted of 1 μl dNTP Mix (200 μM), 1 μl Fluorescein-dUTP (20 μM, Roche, East Sussex, UK), 2 μl each of OC primers (0.6 μM) as published before,[15] 4 μl QIAGEN OneStep RT-PCR Enzyme Mix, 10 μl 5 × reaction buffer, and 30 μl RNase free water. The sections were covered with 25 μl of above mixture with a thin layer of mineral oil overlay. For positive control no DNase was added to the slides; and negative control has no primers being added. The slides were then covered with coverslips, anchored with nail polish, and put on the heating block of Techne Genius thermocycler (Techne, Cambridge, UK). The RT-PCR program was as follows: hot start at 60°C for 30 min, reverse transcription (RT) at 60°C for 45 min, initial activation of HotStarTaq® DNA polymerase at 94°C for 15 min, 25 cycles of PCR amplification (denature at 94°C for 2 min, anneal at 54°C for 2 min, and extend at 72°C for 15 min), and final extension at 72°C for 10 min. The slides were washed in 0.1 × SSC containing 0.2% (w/v) BSA at 60°C for 15 min and visualized with Leica DC300 fluorescent microscope.

Statistical Analysis

The means are compared by ANOVA using software SPSS version 13.0, and $p < 0.05$ was considered statistically significant. The data are presented as the mean ± SD of triplicates.

RESULTS

General Procedures

Rat bone marrow MSCs were cultured at confluence (Fig. 1A). They were induced to differentiate into osteoblasts (Fig. 1B) and adipocytes (Fig. 1C) to confirm the primitive property of cultured MSCs. SEM examination showed that MSCs were attached to the surface of the blocks after loading (Fig. 1D). The animals were carefully monitored after surgery and no adverse effects had been observed. The blocks were examined by regular X-ray imaging throughout the experiment (Fig. 1E). Gross morphology of the explants suggested that the MSCs-loaded blocks were incorporated into muscles (Fig. 1F).

Immune Reactions In Vitro

Mixed lymphocyte culture showed that MSCs did not stimulate lymphocyte proliferation in the MF1

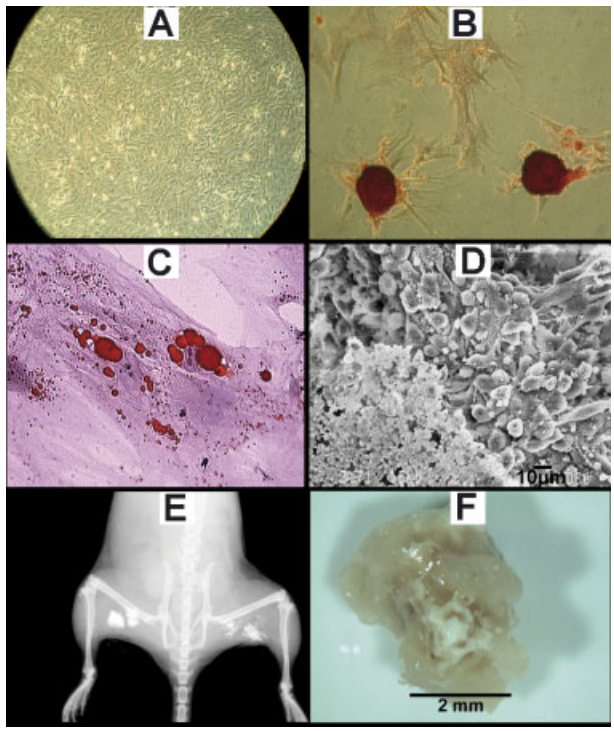

Figure 1. Experimental procedure of MSCs implantation. (A) Rat MSCs at confluence (×10). (B) The osteogenic-differentiation of passaged progeny cells. (C) The adipogenic-differentiation of passaged progeny cells. (D) SEM image showing that rat MSCs were attached on the surfaces of the delivery block (×450). (E) Radiograph showing the localization of implants in vivo. (F) The gross morphological appearance of the implant after 11 weeks of intramuscular implantation in the MF1 mice.

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mice without implantation. However, the rate of lymphocyte proliferation from the MF1 mice was significantly increased after MSCs implantation (Fig. 2A). No difference of lymphocyte proliferation was observed in the SCID mice with or without implantation (Fig. 2A). For cytotoxic assay, stimulated lymphocytes had significantly higher cytotoxic effect against MSCs in the MF1 mice with implantation than the littermates without treatment (1.48 ± 0.06 vs. 0.34 ± 0.21, $p < 0.05$, Fig. 2B), although the level of the cell lysis was not as high as the positive control (1.48 ± 0.06 vs. 2.90 ± 0.01). In the SCID mice with or without transplantation, the level of the cell lysis was weak and similar to that of the negative control (0.14 ± 0.05, 0.12 ± 0.02 vs. 0.10 ± 0.05, Fig. 2B).

Histology Examination and Immunostaining

H&E staining of the cell-free control groups (MF1, Fig. 3A and SCID, Fig. 3B) showed no sign of bone formation, with only small amount of connective tissues surrounding the delivery blocks. After MSCs implantation, cell mass were detected in both groups (SCID, Fig. 3C and MF1, Fig. 3D). IHC with monoclonal anti-GFP antibody on serial sections showed that the cell mass seen in H&E-stained sections (Fig. 3C, D) were GFP-positive, suggesting that the donor MSCs survived the 11-week experimental period (MF1, Fig. 3E and SCID, Fig. 3F). No bone formation was observed in the explants from the MF1 mice, but osteoid tissues were found in adjacent to the implants in the control SCID mice (Fig. 3G).

Active new blood vessel formation was observed in the local implantation area in the MF1 mice (Fig. 4A). The recipient leukocyte infiltrations were also detected using IHC with anti-mouse CD45

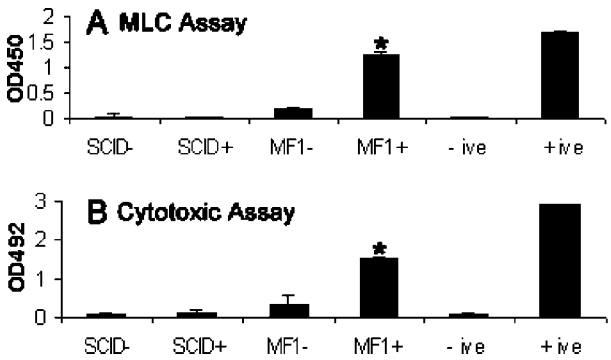

Figure 2. In vitro immune reactions of peripheral blood lymphocytes of recipients against donor-MSCs. (A) Mixed lymphocyte culture data showed that the peripheral blood lymphocytes from the MF1 mice with xenogenic MSCs implantation (MF+) exhibited significantly higher proliferation against the donor MSCs than that of the group without MSCs implantation (MF1−), but the rate of proliferation was lower than that of the positive control (+ive). No difference of lymphocyte proliferation was seen between the SCID mice with (SCID+) and without MSCs implantation (SCID−), and both were similar as the negative control (−ive). (B) Cytotoxic assay data showed that PHA-stimulated lymphocytes from the implanted MF1 mice exhibited significantly higher cytotoxic effects against donor MSCs than the ones without implantation, but less intense than that of positive control. *$p < 0.05$.

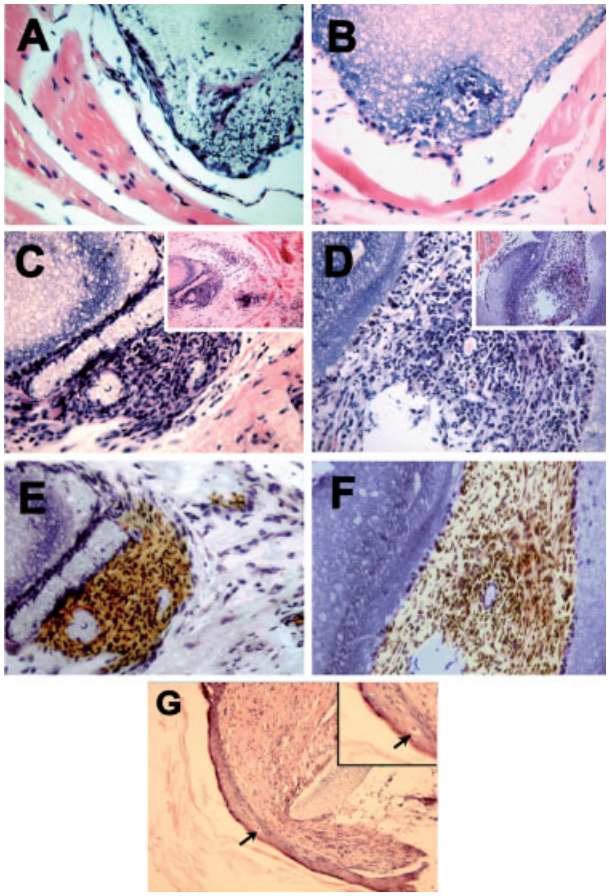

Figure 3. Histology and immunostaining examination. H&E-stained cell-free implants from the SCID mice (A, ×200) and the MF1 mice (B, ×200) showed no cells between the biomaterial and the surrounding muscle. In contrast, cell mass was seen in the areas between the biomaterial and muscle in the MSCs-loaded implants in the SCID mice (C, ×200) and the MF1 mice (D, ×200). Insets in (C) and (D): the areas at lower magnification (×100). Immunostaining using anti-GFP antibody on the corresponding serial sections confirmed that the cells were of donor origin in both groups (E, ×100) and (F, ×100). Newly formed osteoid tissues (arrow) were seen at the surface of the rat MSCs-loaded implants in the SCID mice after H&E staining (G, ×100). Inset in (G): the corresponding area in higher magnification (×200).

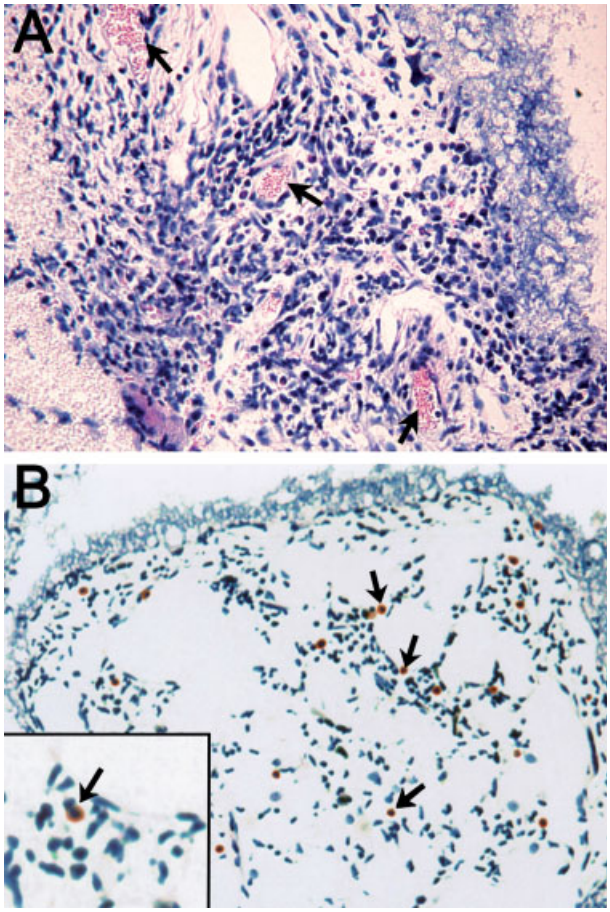

Figure 4. Angiogenesis and leukocyte-infiltration in the MF1 mice. (A) H&E staining showed new blood vessel formation in the MSC-implanted area of the MF1 mice after 11 weeks (arrows, ×100). (B) The infiltrated CD45-positive recipient leukocytes were also detected among the donor-MSCs in the porous space of the block (arrows, ×100). Inset in (B): CD45-positive cell at higher magnification (arrow, ×200).

antibody, suggesting the interaction between the donor MSCs and the immune system of recipient MF1 mice (Fig. 4B).

Osteocalcin mRNA Expression in MSCs

Fluorescent RT in situ PCR was employed to further examine the specific osteogenic marker Osteocalcin (OC) expression in the implanted MSCs. OC expression was detected only in the explants from the SCID mice (Fig. 5D), but not the MF1 mice. For SCID mice, H&E staining on frozen section showed general morphology (Fig. 5A). There was a strong nuclear signal in the positive control (Fig. 5B) due to DNA-mispriming and the negative control showed no sign of amplification,

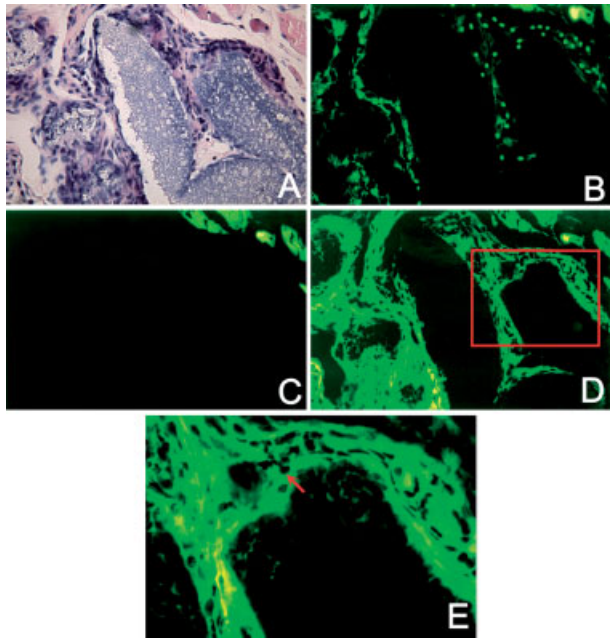

Figure 5. Direct fluorescent in situ RT-PCR detection of osteocalcin mRNA expression in the implants from the SCID mice. (A) General morphology in the H&E-stained frozen section (original magnification, ×100). (B) Positive control (no DNase): The green dots represent nuclear signals of the nonspecific DNA mis-priming (original magnification, ×100). (C) Negative control (DNase, no primers): No signal was detected in the implant material except the auto-fluorescent muscle tissues (original magnification, ×100). (D) After digestion with DNase, OC mRNA expression was visualized (original magnification, ×100). (E) Higher magnification of the boxed area in (D) showed the cytoplasmic pattern of the fluorescent signals with empty nuclei (arrow, original magnification, ×200).

with only some auto-fluorescence from the surrounding muscles (Fig. 5C). The OC mRNAs were mainly localized in the cytoplasm of MSCs (Fig. 5E).

DISCUSSION

The present study showed that xenogeneic MSCs can survive in the MF1 mice for 11 weeks without any immunosuppression. However, there was an increased host immune reaction against donor MSCs following implantation, and neither OC mRNA nor bone formation was detected in the explants, indicating immune sensitization.

Although donor xenogeneic MSCs were found in both MF1 and control SCID mice after 11 weeks of implantation, further examination suggested functional difference, i.e., osteogenesis, between them. Xenogeneic MSCs transplantation has been

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conducted previously with diverse outcomes, ranging from no survival to specific differentiation into destination cells (all the experiments were performed without any immunosuppression).[7–9] The first one reported no survival of adult human bone marrow-derived MSCs in Sprague-Dawley (SD) rats 1 week post-transplantation.[7] There are several explanations for the possible differences between this previous study and the present one. Firstly, the depletion of xenogeneic MSCs in their study may be due to the direct intramyocardium injection used in the myocardial ischemia model. More immune cells were therefore able to participate in the local immune response against foreign implants (MSCs) immediately after the surgical procedure. In contrast, MSCs in the present study were not in direct contact with the host immune cells, as they were partially protected by the loading scaffolds and thus avoided prompt confrontation with the host immune cells. After neovascularization, the implanted MSCs were exposed to recipient immune cells along with blood supply as evidenced by the CD45-positive recipient leucocytes found in paraffin sections from MF1 mice (Fig. 4). The quantity of immune cells the implanted MSCs encountered was few initially, but increased gradually. The immune responses were therefore less effective in terms of destroying the xenogeneic MSCs, but rather lead to an immune sensitization. After certain period, the sensitized immune cells of recipients were able to react against xenogeneic MSCs, leading to cell depletion. Previous data also suggested a dose-dependent fashion of the immunosuppressive function of MSCs.[16] MSCs could only exert immunosuppressive function at advantageous numbers over the immune cells. When outnumbered, the foreign MSCs will be rejected by normal immune deletion process. This was supported by the histology results wherein the survived MSCs were mainly seen in clusters while the randomly distributed MSCs were rarely seen as they had a higher chance of being eliminated.

Another explanation is that both donors and recipients are of rodent-origin in the present study (rats vs. mice), whereas the human MSCs were implanted into rats in the other study. The species variance is more obvious in the latter condition as evidenced by the numerous macrophages infiltration observed. It has been noticed that MSCs from different species tend to exhibit distinct characteristics in vitro, including surface molecule expression, immune activity, and corresponding mechanisms.[7,17,18] The species diversity may explain, at least in part, the different behavior of human-derived and rodent-derived MSCs in the in vivo conditions.

The survival of xenogeneic MSCs was also reported by another two studies using similar myocardial infarction rat models.[8,9] In one study, the xenogeneic mice MSCs engrafted into rat bone marrow shortly after systemic infusion, and were detected in the infracted myocardium after 12 weeks,[8] whereas in another study, the xenogeneic mouse MSCs were directly injected into infracted area immediately after left coronary ligation and were still present 4 weeks afterwards.[9] In addition, they observed that the donor MSCs were functionally active as seen by the positive staining for cardiomyocyte-specific proteins on MSCs in local area, indicating differentiation. However, the numbers of differentiated MSCs detected in the infarcted area were very low.[8,9]

In the present study, the osteogenesis-specific OC mRNA was not detected in MSCs implanted into MF1 mice (also cbfa-1/RUNX2, data not shown), suggesting that these MSCs did not differentiate to regenerate bone. The implanted xenogeneic MSCs might experience gradual host immune rejection, and their functionalities were therefore compromised despite the survival. It is known that approximately 90% of mice genomes are homologous to human ones, and mice are capable of mounting sophisticated immune reaction against non-self antigens. The lack of functional activities of implanted MSCs in the present study was the direct result of this powerful host immune response.

In conclusion, xenogeneic rat MSCs transplanted into immunocompetent MF1 mice have survived for a prolonged period, but without any differentiation activity. This may be due to the initial immune sensitization of recipients after exposure to the xenogeneic MSCs, and later moderate host anti-graft immune response. Although xenogeneic MSCs per se are not capable of regenerating in vivo, whether xenogeneic MSCs can be used as a vehicle to deliver target genes to host tissues remains to be tested.

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