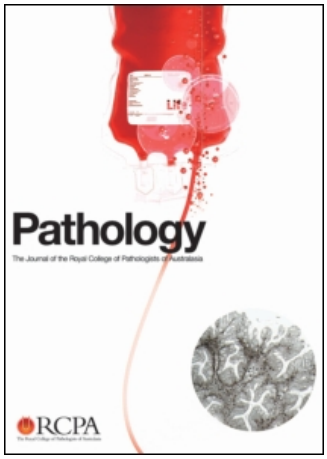

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EXPERIMENTAL PATHOLOGY

Differentiation of human mesenchymal stem cells into mesangial cells in post-glomerular injury murine model

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Summary

Aims: Adult human bone marrow contains a population of mesenchymal stem cells (MSC) that contributes to the regeneration of tissues such as bone, cartilage, muscle, tendon, and fat. In recent years, it has been shown that functional stem cells exist in the adult bone marrow, and they can contribute to renal remodelling or reconstitution of injured renal glomeruli, especially mesangial cells. The purpose of this study is to examine the ability of MSC isolated from human bone marrow to differentiate into mesangial cells in glomerular injured athymic mice.

Methods: MSC were isolated from human bone marrow mononuclear cells based on plastic adherent properties and expanded *in vitro* in the culture medium. Human mesenchymal stem cells (hMSC) were characterised using microscopy, immunophenotyping, and their ability to differentiate into adipocytes, chondrocytes, and osteocytes. hMSC were then injected into athymic mice, which had induced glomerulonephropathy (GN).

Results: Test mice (induced GN and infused hMSC) were shown to have anti-human $CD105^+$ cells present in the kidneys and were also positive to anti-human desmin, a marker for mesangial cells. Furthermore, immunofluorescence assays also demonstrated that anti-human $desmin^+$ cells in the glomeruli of these test mice were in the proliferation stage, being positive to anti-human Ki-67.

Conclusions: These findings indicate that hMSC found in renal glomeruli differentiated into mesangial cells *in vivo* after glomerular injury occurred.

Key words: Renal, histopathology, mesenchymal stem cell, mesangial cell, *in vivo* differentiation.

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INTRODUCTION

Adult human bone marrow contains a population of mesenchymal stem cells (MSC) that contribute to the regeneration of tissues such as bone, cartilage, tendon, and fat.[1] These cells when isolated display a stable phenotype and when cultured can be expanded readily without alteration of cellular characteristics.[2]

The renal glomeruli are vital components of the kidney. Irreversible changes of glomerular constitute are responsible for the initiation and progression of impaired renal function.[3] In recent years, it has been shown that functional stem cells exist in the adult bone marrow, and they can contribute to renal remodelling or reconstitution of injured renal glomeruli, especially mesangial cells.[4–6] Two bone marrow stem cells have been implicated to play a role in the reconstitution of injured kidney, namely haematopoietic stem cells (HSC) and MSC.[7] In 2005, Yokoo *et al.* showed that human MSC (hMSC) in rodent whole-embryo culture could be reprogrammed to generate kidney tissues.[8] Meanwhile, studies have shown that HSC do not contribute to reconstitution of injured renal tissues when pure HSC are used.[9,10] In this study, we investigate the ability of hMSC to differentiate into mesangial cells *in vivo* following glomerular injury in athymic mice.

MATERIALS AND METHODS

Isolation and culture of hMSC

Ten millilitres of human bone marrow aspirate, taken from the iliac crest of a patient with non-malignant blood disorder with informed consent, was diluted 1:1 with phosphate-buffered saline (PBS) (GIBCO, USA) and layered over an equal volume of 1.077 g/mL Ficoll-Paque PLUS solution (Amersham Biosciences, Sweden). After centrifugation at 900 *g* for 25 min, mononuclear cells (MNC) were recovered from the gradient interface and washed with PBS before suspension in Dulbecco's Modified Eagle's medium containing low glucose (DMEM-LG; GIBCO, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO, USA), 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine (a complete medium following this procedure). All cells were plated in culture flask at a density of 5×10^5 MNC/mL. The cultures were maintained at 37°C in 5% CO_2 in air.

After 3 days, non-adherent cells were discarded. Fresh culture medium was replaced every 4–5 days until cells reached confluence. The adherent cells were harvested after incubating with 0.25% trypsin and 1 mM EDTA (GIBCO, USA) for 5 min at 37°C and replated at 1×10^4 cells/mL, thus expanding the population of MSC.

Immunophenotyping of hMSC

The isolated and expanded hMSC were characterised at passage 3 by flow cytometric analysis of specific surface antigens. Cultured hMSC were removed from the culture flask after treatment with 0.25% trypsin and 1 mM EDTA. 10^5 cells in 100 μL harvested cells were stained for 30 min with 10 μL fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated anti-marker monoclonal antibodies. Tested markers included CD3, CD14, CD29, CD34, CD44, CD45, CD54, CD56, CD73, CD90,

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CD105, and HLA-DR. For an isotype control, non-specific mouse Ig was substituted for the primary antibody. After incubation, the cells were washed in PBS and analysed using a flow cytometer (FACScan, Becton Dickinson, USA).

Differentiation studies

Differentiation into adipocytes Cultured hMSC at passage 3 were treated in adipogenic differentiation medium consisting of complete medium supplemented with 1 μM dexamethasone and 0.2 mM indomethacin, 0.01 mg/mL insulin and 0.5 mM 3-isobutil-1-metil-xantina. The medium was changed every 3 days, and the products were subjected to Oil Red O staining after about 14 days of culture.

Differentiation into chondrocytes 3D hMSC cultures were maintained in a chemically defined basal medium consisting of complete medium supplemented with 50 μg/mL ascorbate-2-phosphate, 1.0 mM sodium piruvate, 40 μg/mL proline, 10 ng/mL transforming growth factor-β3, 6.25 μg/mL human insulin, 6.25 μg/mL transferrin, 6.25 μg/mL bovine insulin, 6.25 μg/mL selenous acid, 1.25 μg/mL linoleic acid, and 5.35 μg/mL bovine serum albumin. The 3D chondrogenic culture utilised 1.0×10^6 hMSC per pellet. hMSC were suspended in 1 mL of chondrogenic medium with medium changes every 3–4 days. Chondrogenic pellets were harvested after 5 weeks in culture. To assess chondrogenesis, Alcian Blue-PAS was used to stain cartilage matrix.

Differentiation into osteoblasts hMSC were treated in osteogenic differentiation medium consisting of complete medium supplemented with 50 μg/mL ascorbate-2-phosphate, 10 mM β-glycerophosphate, and 100 nM dexamethasone. The medium was changed every 3 days continuously for 2–3 weeks. Alizarin Red S was used to stain matrix mineralisation associated with osteoblasts.

Animal study

Eight-week-old athymic mice were maintained in a specific pathogen-free environment. All protocols and surgical procedures were approved by the Animal Research Committee of National University of Malaysia and Ministry of Health, Malaysia.

Experimental glomerulonephropathy and hMSC injection

Anti-mouse mesangial cell serum (AMMCS) was prepared by immunising a sheep with SV40-transformed mouse mesangial MES 13 cells. AMMCS when injected into mice could induce a self-limiting glomerulonephropathy (GN) characterised by mesangiolysis, mesangial cell apoptosis, followed by cellular proliferation and recovery.[11] This serum was kindly provided by Dr Jeffrey B. Kopp from the National Institute of Health, USA.

In this study, three control groups were carried out in parallel with test group. Figure 1 illustrates the experimental design of this study. On days 1, 2, and 3, sheep AMMCS was injected into test mice (with induced GN and given hMSC) and control B mice (with induced GN but without hMSC) via tail veins to induce GN. For control A mice (without GN and without hMSC) and control C mice (without GN and given hMSC), normal serum (obtained by plasmapheresis of the sheep prior to immunisation) was used instead. On day 4, 5×10^5 hMSC were injected into test mice and control mice C. No hMSC was given to control A and B mice.

Tissue processing, histological and immunofluorescence analysis

On a pre-determined day, five mice in each control group and six test mice were sacrificed. Kidneys were collected and processed in two ways. For paraffin sections, the tissues were fixed with 10% formalin before being dehydrated and embedded in paraffin wax. Sections were cut at 3 μm thickness and stained with periodic acid-Schiff reagent (Sigma, USA).

For frozen sections, the tissues were incubated in 4% paraformaldehyde overnight before being placed in 30% sucrose in PBS for 24 h. After 24 h incubation with 30% sucrose, tissues were embedded in OCT compound (Leica, Germany). Sections were cut at 4 μm thickness before being incubated with Zenon Alexa Fluor pre-labelled primary antibodies (Molecular Probes, USA) at room temperature for 1 h, and cell nuclei were counterstained with DAPI (Molecular Probes, USA). In this experiment, mouse anti-human CD105 monoclonal antibody, mouse anti-human desmin polyclonal antibody and mouse anti-human Ki-67 monoclonal antibody (Chimicon, USA) were used as primary antibodies. Zenon Alexa Fluor 488 (green florescent dye) and Zenon Alexa Fluo 594 (red florescent dye) labelling kit (Molecular Probes, USA) were pre-labelled to primary antibody before staining was carried out. After staining, tissues were washed for 5 min each in PBS before being observed under the fluorescence microscope with appropriate filters. To detect Alexa Fluor 488, Alexa Fluor 594 and DAPI, filters with excitation/emission at 490/515 nm, 594/620 nm, and 365/450 nm were used, respectively. All images were captured by a digital imaging system, and images were combined using Axio Vision software (Carl Zeiss, Germany).

RESULTS

hMSC characterisation

Isolated and expanded hMSC were spindle-shaped fibroblast-like cells. As shown in Fig. 2, these cells expressed CD29, CD44, CD54, CD56, CD73 CD90, and CD105, but were negative for CD3, CD14, CD34, CD45, and HLA-DR. In addition, MSC were shown to differentiate into adipocytes, as indicated by the accumulation of neutral lipid vacuoles, visualised by phase-contrast microscopy or stained with Oil Red O (Fig. 3B).

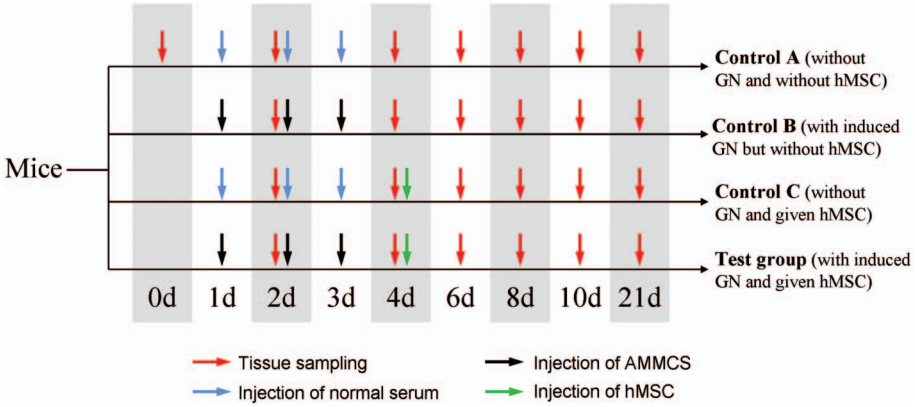

FIG. 1 Animal experimental design for this study.

While in studies involving the differentiation of hMSC into chondrocytes, the cell pellets developed chondrogenic characteristics after incubation with chondrocytes induction medium. The territorial matrix containing high negatively charged glycosaminoglycans was stained blue with Alcian Blue, whereas the interterritorial matrix containing low negatively charged glycosaminoglycans and collagen was stained pink with PAS (Fig. 3C). In osteocyte differentiation, after 2–3 weeks of culture, the cells driven to osteogenic differentiation clearly showed an increase in calcium accumulation, as revealed by Alizarin Red S staining (Fig. 3D).

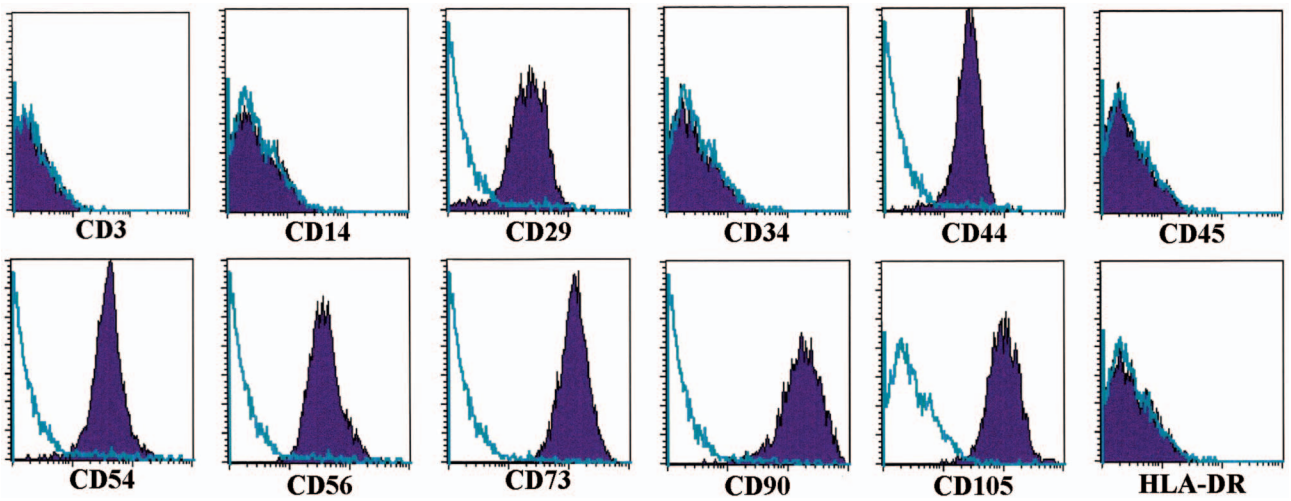

FIG. 2 Flow cytometric analysis of cell-surface antigen profile for hMSC. hMSC expressed CD29, CD44, CD54, CD56, CD73, CD90, and CD105, but were negative for CD3, CD14, CD34, CD45, and HLA-DR.

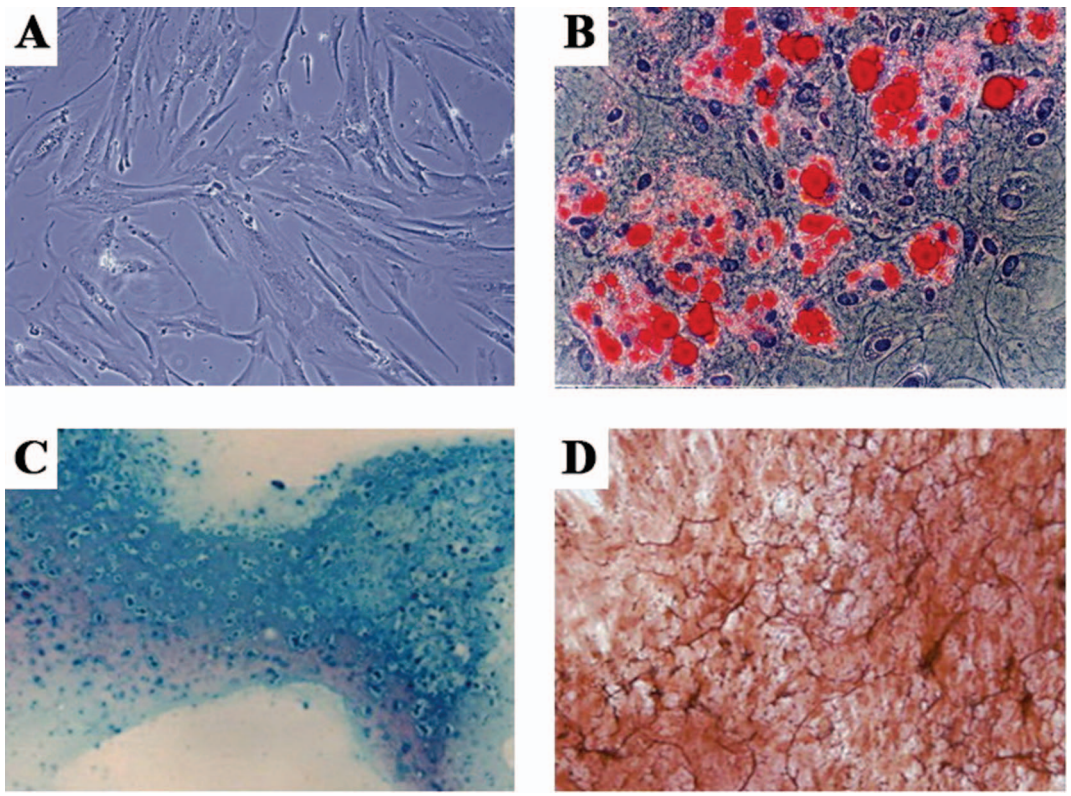

FIG. 3 (A) Isolated and expanded hMSC were spindle-shaped fibroblast-like cells (×100). (B) hMSC-derived adipocytes stained positively for Oil Red O, indicating adipocytes differentiation (×100). (C) Alcian Blue-PAS stains on MSC pellet after 5 weeks incubation in chondrogenic medium (×400). The chondrocytes were seen occupying in the lacunars surrounded by territorial matrix (stained blue). The interterritorial matrix was stained pink. (D) hMSC-derived osteocytes were stained orange red at pH 4.0 with Alizarin Red S (×400).

Induced GN in athymic mice

Injection of AMMCS caused acute mesangiolysis and mesangial cell apoptosis, followed by cellular proliferation (Fig. 4). Glomerular morphological changes in response to AMMCS were very similar to those previously reported in FVB/N mice and SCID/CB17 mice.[11] In mice that received

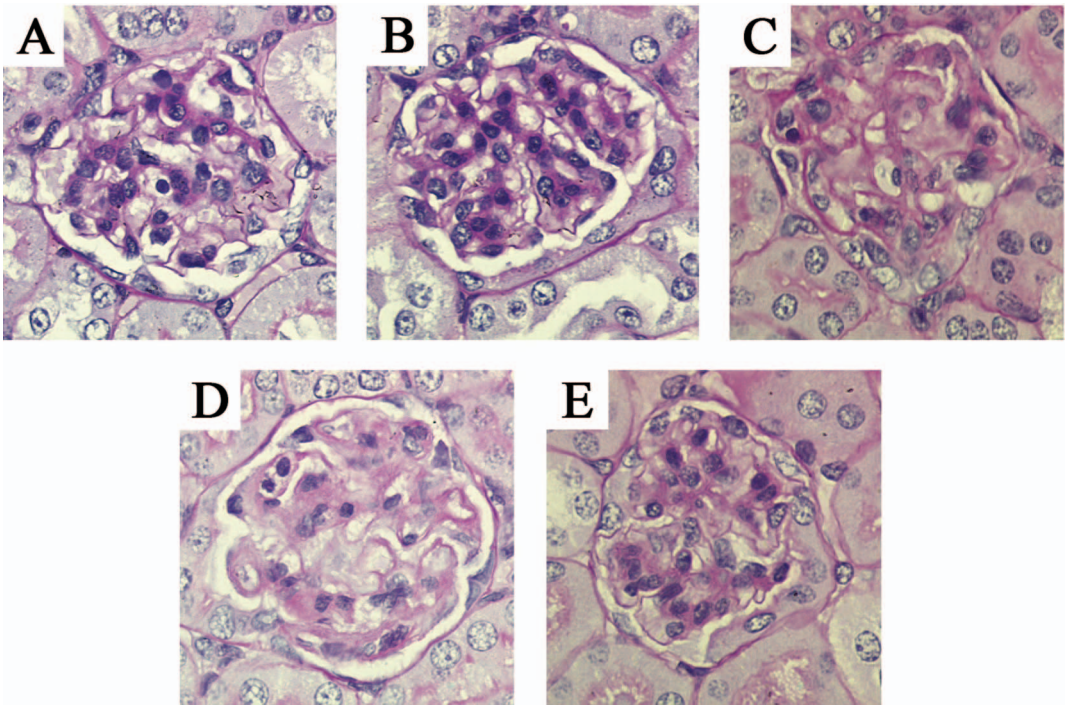

FIG. 4 Glomerular histology of acute GN by AMMCS. (A) Glomerular morphology in normal mice (Control A mice). (B) At day 2, the glomerulus from AMMCS treated mice were similar to that of control A mice. (C,D) At days 6 and 8 after injection of AMMCS, there was marked loss of mesangial cells. (E) At day 21, glomerular morphology showed partial recovery (PAS stain, ×400).

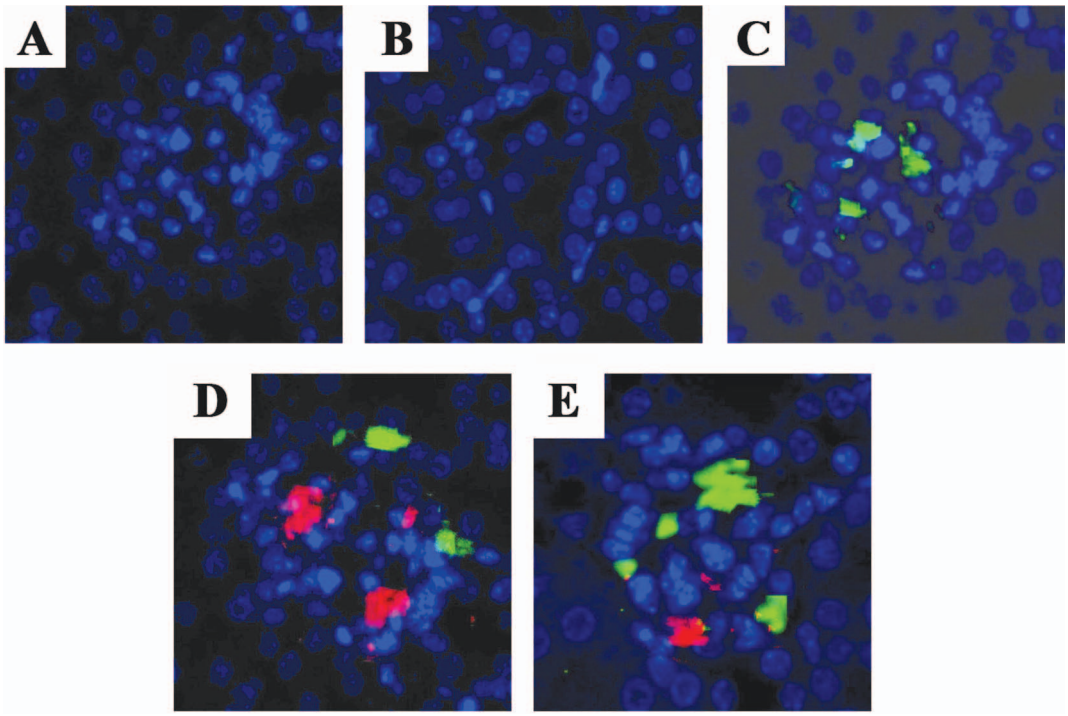

FIG. 5 IF staining for injured mice renal tissue to detect presentation of anti-human $CD105^+$ cells (green colour) and anti-human $desmin^+$ cells (red colour); cells' nucleus stained with DAPI (blue colour). (A) IF staining at day 0, as a negative control. (B) IF staining at day 4 just after injection of hMSC. (C) At day 6, anti-human $CD105^+$ cells were detected. (D,E) At days 10 and 21, anti-human $desmin^+$ cells were detected (×400).

AMMCS, abnormalities seen at days 6 and 8 included the loss of mesangial cells. On day 21, glomerular morphology showed partial recovery.

No non-specific binding to mice tissue by anti-human antibodies

Prior to IF staining, all mouse anti-human antibodies were tested to make sure they did not exhibit non-specific binding at mice tissue. Mice in control A and control B tested for anti-human CD105, anti-human desmin, and anti-human Ki-67. All results were consistently negative (indicating that there is no cross-reaction), while for the control C, as expected, cells positive for anti-human CD105 were found in the glomeruli, but not cells positive for anti-human desmin or anti-human Ki-67 (data not shown).

Reconstitution of the glomeruli after injury

To identify the characteristics of cells post-injury, we examined cells for the expression of desmin, which is a marker for mesangial cells. Figure 5 shows some hMSC (anti-human CD105$^+$ cells) stained with green fluorescence located within the mesangium 2 days after injection of hMSC. At day 10, 6 days after the injection of hMSC, some anti-human desmin$^+$ cells were observed, suggesting that some mesangial cells of the recipients were of donor origin (hMSC derived).

Proliferation of hMSC occurred in the mesangium

Proliferation hMSC in the mesangium was demonstrated using the proliferation marker Ki-67 in the test mice. At day 10, anti-human desmin$^+$ cells were shown in the proliferation stage being positive to anti-human Ki-67 (Fig. 6). In this experiment, cells positive for anti-human desmin exhibited green fluorescence, and cells positive for anti-human Ki-67 exhibited red fluorescence. As a result, cells positive for both anti-human desmin$^+$ and anti-human Ki-67$^+$ exhibited orange fluorescence.

DISCUSSION

Over the last decade, glomerular components—endothelial, mesangial, and epithelial cells—have been shown to exhibit regenerative potential. It has also been demonstrated that bone marrow mononuclear cells can give rise to endothelial as well as epithelial cells.[6,12–13] However, until now, no

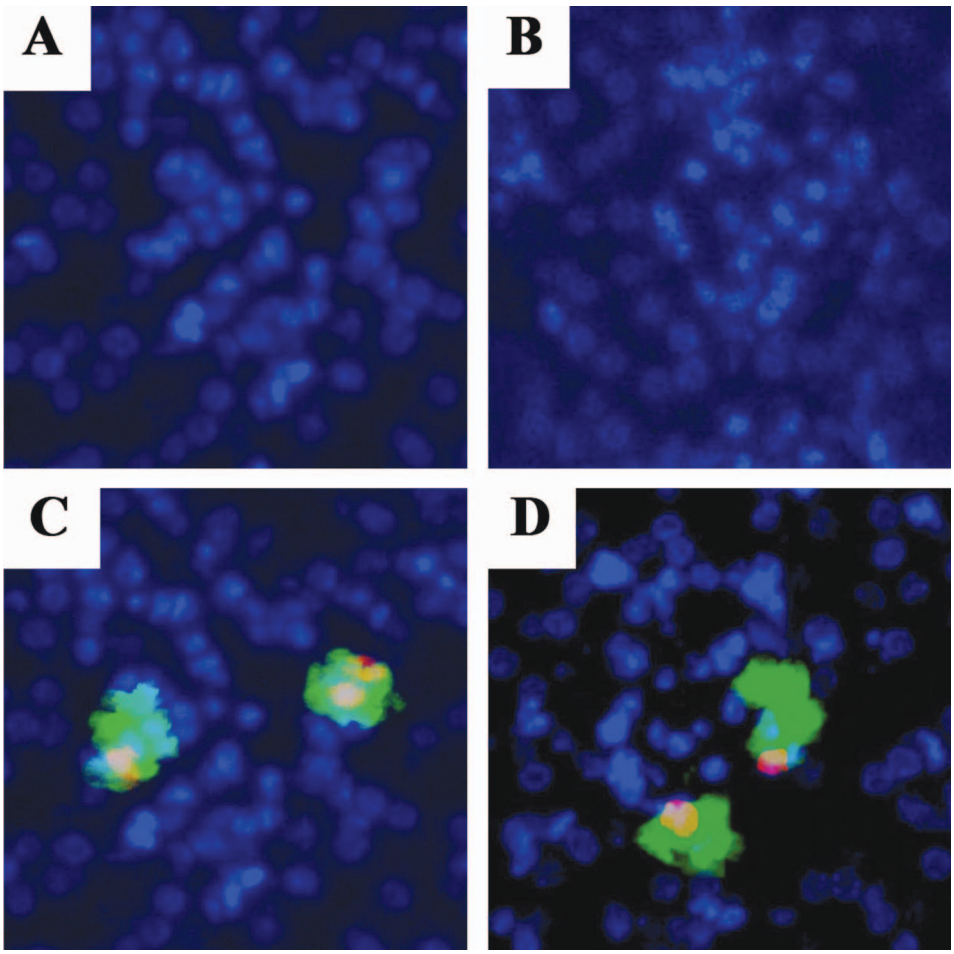

FIG. 6 IF staining for test mice to detect presentation of anti-human desmin$^+$ cells (green colour) and anti-human Ki-67$^+$ cells (red colour). A combination of green colour and red colour produced the orange colour; the cell's nucleus was stained with (DAPI) blue colour. This demonstrated that anti-human desmin$^+$ cells were in the proliferation stage. (A) IF staining at day 0, as a negative control. (B) Day 6. (C,D) At days 10 and 21, anti-human desmin$^+$ cells were shown in the proliferation stage being positive to anti-human Ki-67 ($\times$400).

report has specified which bone marrow component contributes to the regeneration of glomerular components such as mesangial cells, even though Suzuki *et al.* had shown that bone marrow mononuclear cells could be converted into glomerular mesangial-like cells *in vitro*.[14] Our study, to the best of our knowledge, is the first report to show that hMSC could migrate to injured glomeruli of athymic mice and differentiate into mesangial cells *in vivo*.

Anti-Thy-1 nephritis in the rat is a model of mesangial glomerulonephritis. Rat mesangial cells express the lymphocyte antigen Thy-1. Antibodies to Thy-1 induce mesangiolysis, mesangial proliferation, and mesangial matrix expansion before normal glomerular architecture is restored.[15] However, mice mesangial cells lack Thy-1 antigen. In this experiment, anti-mouse mesangial cell serum generated from sheep was used. This model of GN has similarities to that in the rat. Both models involve intravenous administration of antibodies directed against mesangial cells, and both models also generated a self-limited acute GN, characterised by a phase of reduced cell numbers and subsequent compensatory glomerular cell proliferation.[11]

In this experiment, hMSC were used rather than rat MSC or mouse MSC, with the intention to translate experimental findings to future clinical trial. If hMSC can be shown to differentiate into mesangial cells in athymic mice, hMSC would behave similarly when transplanted into the human body. Athymic mice were used in this experiment as a recipient of hMSC. Since athymic mice lack a thymus, which is essential for the production of T-cells, they do not reject cell transplantation from another species. When hMSC were injected into mice with injured kidneys, hMSC were differentiated into mesangial cells that were positive for anti-human desmin. In our experiment, hMSC were injected into the mice at day 4, cells positive to anti-human CD105 were detected at day 6, while cells positive for anti-human desmin were detected at day 10. These results indicate that hMSC took 1–2 days to reach injured kidneys after injection, and required about 4 days to differentiate into mesangial cells. At day 10, human desmin$^+$ cells were also positive for Ki-67, indicating that human-derived mesangial cells were proliferating to contribute to the mesangial reconstitution.

Dual labelling for both CD105$^+$ and desmin$^+$ in a single cell in the mouse glomeruli was not observed in this experiment (Fig. 5). When MSC differentiate into terminal cells or are exposed to a certain environment, they might lose certain surface properties. This observation was reported by Azizi *et al.* and Forte *et al.* Azizi *et al.* reported that after MSC infusion into the brain, MSC lost their immunoreactivity to antibodies for collagen I.[16] On the other hand, Forte *et al.* showed that MSC lost other cell markers such as CD105 and c-kit as they were exposed short-term to hepatocyte growth factor.[17]

In this study, MSC in uninjured mice were not in the proliferating stage. In control group C (without GN and given hMSC), when hMSC were injected into uninjured mice, hMSC remained in the glomeruli and did not differentiate into mesangial cells, or hMSC showed no proliferation. Only anti-human CD105$^+$ cells were detected, but not anti-human desmin or anti-human Ki-67. Niu *et al.* also reported that when MSC were injected into injured heart, MSC differentiated into cardiac muscle like-cells, but not in the normal heart.[18] Tissue injury plays an important part in inducing *in vivo* differentiation.[19–20]

Our findings provide proof that MSC have a capacity to differentiate into mesangial cells *in vivo* following glomerular injury.

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