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Human mesenchymal stromal cells could deliver erythropoietin and migrate to the basal layer of hair shaft when subcutaneously implanted in a murine model

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ABSTRACT

Mesenchymal stromal cells (MSC) are an attractive cell-targeting vehicle for gene delivery. MIDGE (an acronym for Minimalistic, Immunologically Defined Gene Expression) construct is relatively safer than the viral or plasmid expression system as the detrimental eukaryotic and prokaryotic gene and sequences have been eliminated. The objective of this study was to test the ability of the human MSC (hMSC) to deliver the erythropoietin (EPO) gene in a nude mice model following nucleofection using a MIDGE construct. hMSC nucleofected with MIDGE encoding the EPO gene was injected subcutaneously in Matrigel at the dorsal flank of nude mice. Subcutaneous implantation of nucleofected hMSC resulted in increased hemoglobin level with presence of human EPO in the peripheral blood of the injected nude mice in the first two weeks post-implantation compared with the control groups. The basal layer of the hair shaft in the dermal layer was found to be significantly positive for immunohistochemical staining of a human EPO antibody. However, only a few basal layers of the hair shaft were found to be positively stained for CD105. In conclusion, hMSC harboring MIDGE-EPO could deliver and transiently express the EPO gene in the nude mice model. These cells could be localized to the hair follicle and secreted EPO protein might have possible role in hair regeneration.

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1. Introduction

Erythropoietin (EPO) stimulates red blood-cell production and is produced in the fetal liver and adult kidney (Jelkmann, 1992; Fisher, 2003). Since the first successful cloning of the human EPO gene in 1985, recombinant EPO (rhuEPO) has become a therapeutic option for renal anemia in chronic renal failure (CRF) patients and several forms of non-renal anemia. In light of predicted rise in CRF in aging populations globally, the need to develop a cost-effective alternative is apparent (Tsakiris, 2000; Eckardt, 2001). Transplanting EPO-producing cells of human origin is an ideal therapeutic alternative for CRF patients because it would be more cost effective and less likely to result in the serious complications of pure red-cell aplasia due to auto-regulation (Bennett et al., 2004).

The success of producing a biologically functional EPO depends on glycosylation of the EPO protein (Krantz, 1991). The N-glycans, for example, play a major role in secretion, molecular stability, solubility, receptor binding affinity and *in vivo* elimination of EPO (Elliott et al., 2003). The choice of host cells, the culturing conditions and the purification procedures determine the composition of the glycan isoforms of a rhuEPO preparation (Jelkmann, 2008). To date, human EPO gene has also been transfected in myoblast (Hamamori et al., 1995) and skin cells (Gothelf et al., 2010) and has successfully corrected anemia in renal failure in a murine model. Mesenchymal stromal cells (MSC) have also been explored to carry and deliver functional EPO in *in vivo* studies (Bartholomew et al., 2001; Eliopoulos et al., 2003, 2006).

MSC is a type of stem cells that are easy to be isolated, robust in *ex vivo* growth and amenable to genetic modifications (Doering, 2008). Human MSC express intermediate levels of human leukocyte antigen (HLA) major histocompatibility complex (MHC) class I molecules and can be induced by interferon-γ to express HLA class II and Fas ligand; they do not express the co-stimulatory molecules B7-1, B7-2, CD40, or CD40 ligand. They should therefore be recognized by alloreactive T-cells. However, human, baboon, and murine MSC failed to elicit a proliferative response from

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allogeneic lymphocytes. When pre-cultured with interferon-γ for full HLA class II expression, MSC still escape recognition by alloreactive T-cells. Unlike other non-professional antigen-presenting cells, this failure is not reversed by provision of CD28-mediated costimulation. Furthermore, MSC are immunosuppressive and inhibit T-cell alloreactivity induced in mixed lymphocyte cultures or by non-specific mitogens (Tse et al., 2003; Singer and Caplan, 2011).

MSC also possess the capability to migrate to sites of tissue damage or inflammation. This ability to migrate to sites of acute tissue injury has been demonstrated in the setting of bone fracture, cerebral ischemia and in infarcted heart (Caplan and Bruder, 2001; Tomita et al., 2002; Parekkadan and Milwid, 2010). Local delivery of EPO by MSC is clinically useful to rescue damaged tissues from compromised local circulation such as critical hind-limb ischemia, myocardial infarction or spinal cord injury. There are ample animal studies to show that EPO could induce formation of new blood vessels in damaged tissues (Meer et al., 2005; Zhang et al., 2007).

Our previous experiments have shown that human mesenchymal stromal cells (hMSC) isolated from adult bone marrow could be used as a carrier for the EPO gene. The transfected hMSC could stably express the EPO protein as determined by enzyme-linked immunosorbent assay (ELISA) (Mok et al., 2012), leading to induction of differentiation of human hematopoietic stem cells into erythroid colony *in vitro* (Mok et al., 2008). In these studies, transfection was achieved through nucleofection of a new construct, named MIDGE (Minimalistic, Immunologically Defined Gene Expression). MIDGE is a linear, double-stranded DNA consisting solely of the expression cassette, capped with hairpin structures at the ends for protection against exonuclease degradation. The MIDGE construct offers the following advantages: (i) the construct contains only the necessary elements required to express the desired gene with no resistance markers or other unwanted genes, (ii) immune-stimulatory sequences, such as CpGs, are minimized, (iii) the construct eliminates the risk of recombination and mutagenesis of wild-type viruses, and (iv) the construct could be transferred easily as it is smaller in size compared with the plasmid system (Schakowski et al., 2001).

In the current study, we aimed to test the hMSC in carrying and delivering the EPO gene in a MIDGE construct following nucleofection in an *in vivo* system. The nucleofected cells were subcutaneously implanted in the dorsal flank of nude mice and the effects of the expressed EPO protein on the hemoglobin levels in the peripheral blood were studied for two months. The implanted cells expressing EPO protein were found to be localized in the subcutaneous layer of mice.

2. Materials and methods

2.1. Nucleofection of MIDGE-EPO into hMSC

Nucleofection of MIDGE-EPO into hMSC was performed using U-23 pulsing program as previously described (Mok et al., 2008). Generally, 2 μg of MIDGE-EPO was used to nucleofect 5×10^5 hMSC and seeded on 35 mm cell culture petri dishes containing Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin antibiotics (Gibco-Invitrogen, Grand Island, NY). After 24 h, the adherent nucleofected cells were then trypsinized for implantation into nude mice.

2.2. Western blot analysis

To detect the presence of EPO in the supernatant of nucleofected cells, Western blot analysis was performed using a rabbit polygonal anti-human EPO polyclonal antibody (Cat. No. H-162) (Santa Cruz Biotechnology; Santa Cruz, CA). The EPO immunoreactivity was visualized using the WesternBreeze Chromogenic Western Blot Immunodetection Kit (Gibco-Invitrogen). For positive control, rhuEPO (Cat. No. 02625) (Stem Cell Technologies, Vancouver, Canada) was used.

2.3. Implantation of hMSC into mice

In a preliminary study on the sustainability of hMSC in the Matrigel one week post-implantation, a Balb/c mouse was used. Approximately 5×10^5 hMSC were stained using the PKH-26 red fluorescent cell linker mini kit (Sigma–Aldrich, St. Louis, MO, USA) according to the protocol recommended by the manufacturer. These cells were then washed with phosphate buffered saline (PBS) by centrifuging the cells at $200 \times g$ for 10 min, suspended in 50 μl of PBS and mixed with 0.5 ml thawed BD Matrigel Matrix High Concentration (Cat. No. 356231) (BD Biosciences, Bedford, MA) at 4 °C. The Matrigel mixture was then injected subcutaneously into the left flank of the mouse. At body temperature, the Matrigel would rapidly acquire a semisolid form.

To study the delivery of EPO protein by the nucleofected hMSC, experiments were performed on 12-week old Nu/Nu male mice (BioLASCO, Taipei, Taiwan) with protocols approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (PP/PAT/2008/AINOON/12-AUGUST/227-JAN-2009-DEC-2011). Six mice were used for each group of experiment: mice implanted subcutaneously with Matrigel mixed with PBS, mice implanted with non-nucleofected hMSC encapsulated in Matrigel and mice implanted with nucleofected hMSC (hMSC-EPO) encapsulated in Matrigel. Approximately 5×10^5 cells were injected into the subcutaneous tissues of the nude mice according to the protocol stated above. Non-nucleofected cells were prepared by nucleofecting cells without MIDGE-EPO.

2.4. Determination of human EPO and hemoglobin levels in the blood:

Under general anesthesia, 100 μl of blood was withdrawn from the retro-orbital venous plexus of the mice using heparinized capillary tube (Hirschmann Laborgerate, Eberstadt, Germany) and transferred into a 0.5 ml ethylenediaminetetraacetic acid (EDTA) tube (Greiner Bio-One GmBH, Kremsműnter, Austria). The tube containing the blood sample was then centrifuged at $400 \times g$ for 20 min to obtain the plasma for human EPO measurement using the Human Erythropoietin ELISA Immunoassay Kit (Stem Cell Technologies). Hemoglobin measurement was performed according to the procedures described in Choudhri et al. (1997).

2.5. Immunohistochemical staining on Matrigel implants and other organs

At 2 and 8 weeks post-implantation, the mice were sacrificed by cervical dislocation to harvest the implant, together with the skin and the muscle layer. The harvested implants were fixated and sections of 3 μm were prepared for hematoxylin and eosin (H&E) (Sigma–Aldrich), and immunohistochemical staining using the LSAB+ System-Horseradish Peroxidase Kit (Dako Cytomation, Glostrup, Denmark). For the detection of human EPO and CD105, anti-human rabbit EPO (Cat. No. sc-7956; dilution 1:200) (Santa Cruz Biotechnology) and anti-human mouse CD105 (Cat. No. 555690; dilution 1:200) (BD Biosciences Pharmingen, San Diego, CA) primary antibodies were used. To localize hMSC in other major organs, the lung, heart, spleen, kidney, liver and brain were also harvested on the second and eight weeks, and stained according to the above procedure.

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2.6. Statistical analysis

Results are expressed as mean ± S.E.M. The results were analyzed with Mann–Whitney *U* test. Two-sided *p* value less than 0.05 were considered statistically significant.

3. Results

3.1. Nucleofected hMSC expressed and secreted human EPO protein into the supernatant of culture medium

Isolated hMSC were expanded as previously described (Mok et al., 2008). The hMSC were shown to exhibit CD73, CD90 and CD105, but not CD34 and CD45 phenotypes (data not shown; see Mok et al., 2003; Choong et al., 2007; Wong et al., 2008). Cultured cells from passage 3 to 5 were then nucleofected with 2 μg MIDGE-EPO using the U-23 pulsing program. A day following nucleofection and prior to implantation of the nucleofected cells into the nude mice, a Western blot analysis was performed to demonstrate successful nucleofection of MIDGE-EPO in the hMSC samples, and that these cells expressed and secreted the EPO protein (Fig. 1). Supernatants obtained from three independently nucleofected samples showed bands with a molecular mass (36 kDa) corresponding to the rhuEPO used as the positive control. Quantification of EPO protein in the supernatant using Human Erythropoietin ELISA Immunoassay Kit (Stem Cell Technologies, Vancouver, Canada) showed highest level of expressed protein on day 5 (1.70 ± 1.30 U/ml) and sustained at (0.41 ± 0.32) U/ml to (0.80 ± 1.37) U/ml from day 15 to day 50 post-nucleofection (data not shown; see Mok et al., 2012).

3.2. Nucleofected hMSC could transiently deliver human EPO into nude mice and raised the hemoglobin levels in the blood

The practical use of hMSC for the secretion of therapeutic gene products in cell-based therapy application relies on their capacity to engraft and sustain *in vivo* following transplantation (Ankrum and Karp, 2010; Karp and Teo, 2009). Hence, we chose to use Matrigel as a biomaterial substrate to embed the cells to avoid loss of cells into the empty spaces of subcutaneous layer and other tissues, and to enhance survivability in the less oxygenated subcutaneous tissue, which might eventually lead to cell death, and therefore affect our results (Toma et al., 2002; McGinley et al., 2011). The mechanical properties of the substrate to which cells adhere have been found to mediate many aspect of cellular function including proliferation, migration, differentiation, and also to improve gene delivery activities (Kong et al., 2005).

Prior to implantation of the nucleofected cells, a preliminary study was performed on a Balb/c mouse to determine the sustainability of hMSC in the Matrigel implant. hMSC was labeled with PKH-26 red fluorescent stain before mixing with cold Matrigel and the mixture was subcutaneously injected into the left flank of a Balb/c mouse. One week following the implantation, the Matrigel was harvested (Fig. 1B), fixated and sectioned to monitor migration of the implanted cells. Under the fluorescence microscope, most red fluorescent cells were found at the edge of the Matrigel implant (Fig. 1C) indicating that hMSC could survive and sustain in the Matrigel substrate in the subcutaneous tissues of immunocompetent mouse one week after injection.

The nucleofected cells were next injected into the subcutaneous tissues of nude mice. Following implantation, blood was withdrawn from the retro-orbital venous plexus every week for eight

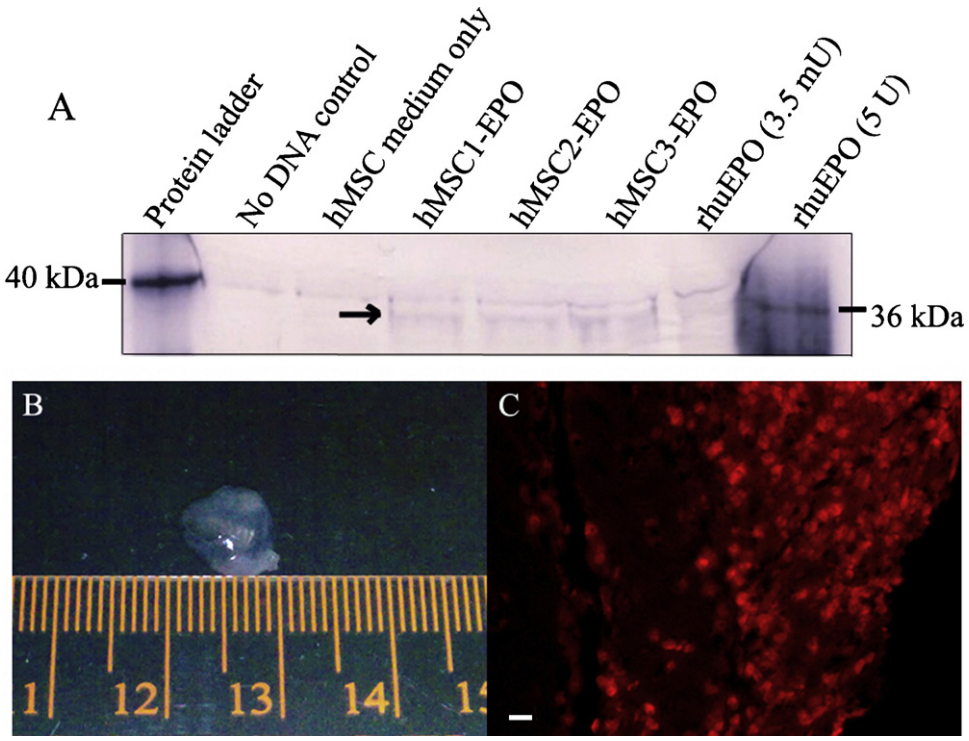

Fig. 1. Validation of EPO expression and secretion by the nucleofected hMSC and sustainability of injected hMSC in the Matrigel substrate. (A) Western blot detection of EPO (indicated with arrow) in the supernatant of three nucleofected hMSC samples (labeled as hMSC1-EPO, hMSC2-EPO and hMSC3-EPO) 24 h after nucleofection. A recombinant human EPO (rhuEPO) was used as the positive control. Supernatants harvested from a no-DNA control for nucleofection and culture medium were used as negative controls. (B) Prior to injection, hMSC was labeled with PKH-26 red fluorescent stain. The figure shows Matrigel excised from the mouse one week post-implantation. (C) The Matrigel implant was fixated in paraffin wax, sectioned and observed under a fluorescent microscope (magnification 40×).

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weeks. The blood was then analyzed for the presence of human EPO in the plasma and for the hemoglobin levels. In the first week following implantation, the EPO concentration was significantly higher in the test group of hMSC-EPO (3.94 ± 0.95 mU/ml) than the control groups of hMSC (0.60 ± 0.03 mU/ml) or Matrigel only (0.64 ± 0.04 mU/ml) (Fig. 2A). The level of EPO in the hMSC-EPO group, however, dropped to 1.01 ± 0.24 mU/ml on week 2, which was slightly higher than the controls, and to a level corresponding to the control groups on week 3 onwards (Fig. 2A).

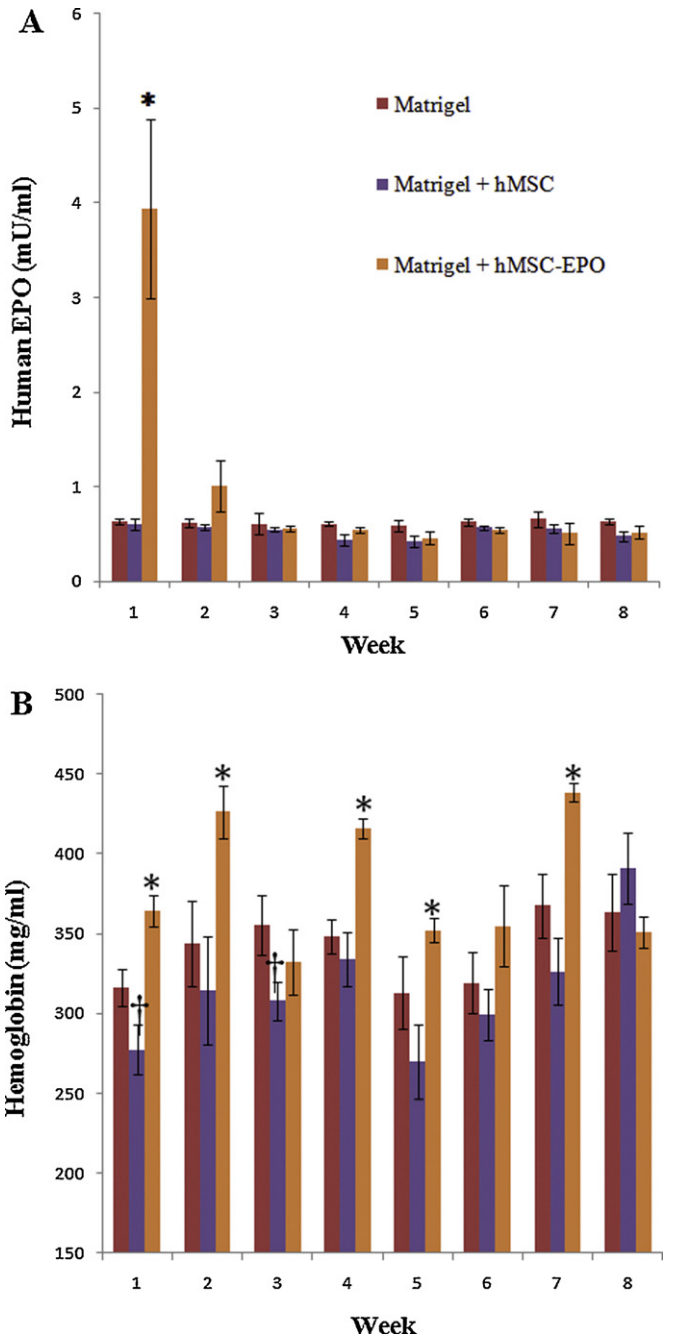

Fig. 2. Time course of circulating human EPO and hemoglobin levels after implantation of hMSC into the nude mice. Blood samples were collected at different time points for determination of (A) plasma human EPO and (B) hemoglobin levels in mice implanted with nucleofected cells (hMSC-EPO) and in comparison with the controls (mixture of Matrigel and hMSC or Matrigel alone). $N = 6$ for all groups. $^{*}p < 0.05$ between group of mice implanted with nucleofected hMSC (Matrigel+hMSC-EPO) and non-nucleofected cells (Matrigel+hMSC). $^{\dagger}p < 0.05$ between group of mice implanted with hMSC and Matrigel alone.

Our results in the injected nude mice showed that there was no significant difference in the hemoglobin levels between the two control groups of hMSC and Matrigel except in weeks 1 and 3. The hemoglobin level was significantly raised in mice implanted with hMSC-EPO on week 1 (364.34 ± 9.47 mg/ml) compared with the control group of non-nucleofected hMSC (277.48 ± 15.28 mg/ml) (Fig. 2B). The significant rise in hemoglobin continued until week 7, except in week 3 when the test group of hMSC-EPO had lower plasma Hb (332.16 ± 20.39 mg/ml) than the control group implanted with Matrigel only (355.36 ± 18.99 mg/ml). On week 6, the mice implanted with hMSC-EPO had a higher Hb level (354.95 ± 25.06 mg/ml) than in the control groups, hMSC alone (299.37 ± 16.41 mg/ml) and Matrigel alone (319.19 ± 18.99 mg/ml), but the differences were not significant (Fig. 2B).

3.3. Localization of hMSC expressing EPO protein in the subcutaneous layer of nude mice

To study the *in vivo* fate of the injected hMSC, H&E and immunohistochemical stainings were performed to locate EPO-producing hMSC in the Matrigel implant and the adjacent tissues of the skin and muscle after two and eight weeks of subcutaneous injection.

The H&E staining on the epidermal and dermal layer of the skin of implants harvested from each of the hMSC-EPO and non-nucleofected hMSC groups after 2 weeks of implantation is shown in Fig. 3A and B. In the Matrigel section from both samples, the presence of foamy macrophages, eosinophils and fibroblast-like cells was observed (Fig. 3C and D).

When the sections were stained for human EPO, it was found that the basal layer of the hair shaft was stained positively in mouse implanted with hMSC-EPO (Fig. 4A, C and E). Mice injected with non-nucleofected hMSC were stained negatively for human EPO (Fig. 4B, D and F). In the mice implanted with hMSC-EPO, only a few hair shafts, and not the fibroblast-like cells in the Matrigel section, were stained positively for human CD105 (Fig. 4G) indicating that the basal layer of the hair shaft was of hMSC origin. To determine possible migration to other organs, we have harvested the lung, heart, spleen, kidney, liver and brain and stained them with both antibodies. However, all organs were stained negatively for EPO-producing or CD105 positive cells. The same negative staining results were obtained from all the organs including Matrigel implants or hair shafts after 8 weeks post-implantation (data not shown).

4. Discussion

We have previously isolated hMSC from adult marrow and our *in vitro* studies demonstrated the potential of the MIDGE construct for extended and stable EPO protein expression up to 55 days in hMSC cultures (Mok et al., 2012). In this work, we carried out an *in vivo* study in nude mice to test the ability of hMSC to deliver EPO protein *via* the MIDGE construct.

Our results showed that hMSC could deliver EPO protein into the peripheral blood of nude mice following subcutaneous implantation. Presence of secreted human EPO could enhance the production of red blood cells and, thus, resulted in a significant rise in hemoglobin levels in the hMSC-EPO group (Fig. 2). Despite the transient expression of human EPO in the plasma, the sustenance of higher hemoglobin levels until week 7 was probably due to the long lifespan of red blood cells (Bannerman, 1983) or prolonged effect of EPO protein itself (Kato et al., 1997). The drop of concentration at week 3 in the current study corresponded to our *in vitro* data that quantified on the EPO protein level in the supernatant of nucleofected cells. The decline in transcriptional activity, which was in accord with those reported for other gene from plasmids

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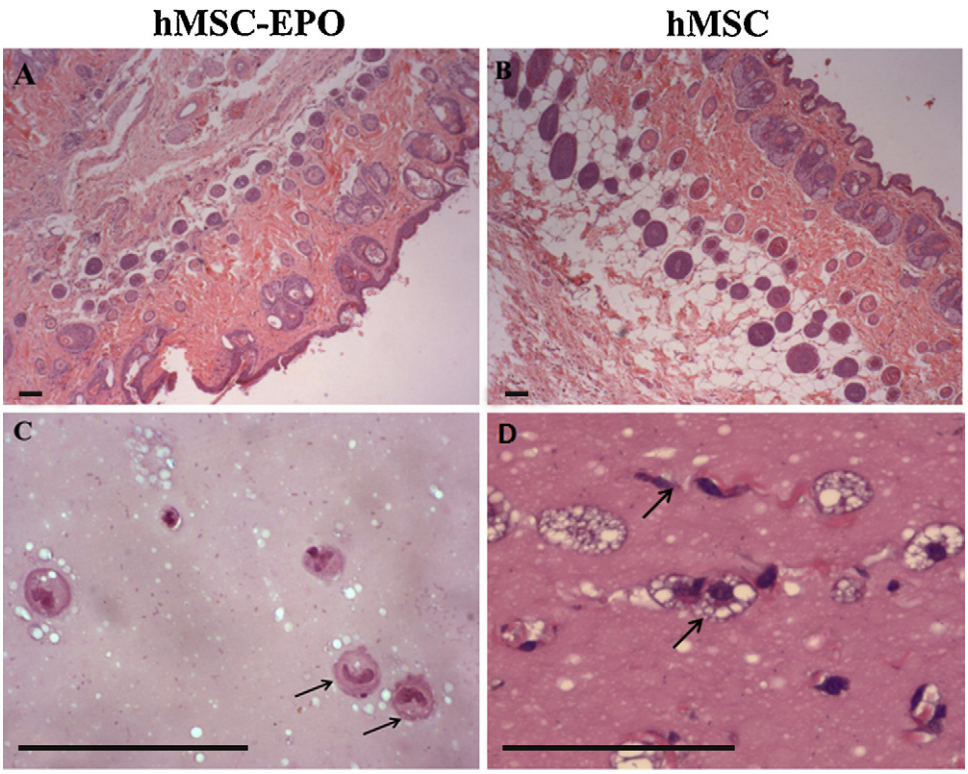

Fig. 3. Observation of injected hMSC in the Matrigel implant and tissue changes in the skin and muscle of nude mice by hematoxylin and eosin (H&E) staining. Sections of implant consisting layers of skin, Matrigel substrate and muscle harvested from mouse injected with (A) hMSC-EPO (40×) or (B) hMSC alone (40×). In the Matrigel section, (C) eosinophils (indicated with arrows) (400×) and (D) foamy macrophages and fibroblast-like cells (both indicated with arrows) (400×) were found in the mouse injected with hMSC-EPO or hMSC alone.

(Herweijer and Wolff, 2003), could be due to promoter silencing (Kim et al., 2011). Although our previous data showed sustained gene expression from day 15 to day 50 post-nucleofection (Mok et al., 2012), the secreted EPO protein might not be detected by ELISA method when diluted into the blood stream. In addition to promoter silencing, immune responses to the cells in the *in vivo* system could be triggered by the EPO gene construct (Lifshitz et al., 2009, 2010) as well as the method of gene introduction into the cells (Bell et al., 2010). In MHC unmatched allogeneic mice model, EPO auto-antibody could also form and neutralize the activity of secreted EPO protein (Eliopoulos et al., 2005; Campeau et al., 2009).

Local delivery of EPO by MSC has manifested a more potent therapeutic effect for treatment of cerebral ischemia (Esneault et al., 2008) and myocardium infarction (Copland et al., 2008) than with delivery of MSC or EPO alone in *in vivo* studies. In neurodegeneration, hMSC could serve as a vehicle to deliver EPO protein into injured tissues as rhuEPO could not pass through the blood–brain barrier. While high concentrations of rhuEPO could be administered intravenously to enable some protein to reach the injured sites, there are also risks of thrombosis and hypertension to be considered (Baskin and Lasker, 1990; Loo and Beguin, 1999; Ghezzi and Brines, 2004; Lieutaud et al., 2008). Therefore, in a tissue reparative treatment strategy that only requires short-term supply of EPO protein, delivery of hMSC harboring the MIDGE-EPO should be sufficiently beneficial.

The transient EPO expression demonstrated by our current study, however, should not hamper future effort to isolate and pool cells that carry the MIDGE-EPO gene before implantation in order to improve the outcome of the EPO protein delivery. There have been a number of reports that have used viral vectors carrying gene for transduction into hMSC and showed sustainably high EPO expression in the animal models (Bartholomew et al., 2001; Daga et al., 2002; Eliopoulos et al., 2003, 2006; Liu et al., 2000; Wang et al., 2009). Although the results are promising, we should not rule out the risk of oncogene activation and the immunotoxicity that the viral vectors might pose. Stable expression of hMSC nucleofected with MIDGE-EPO has significant benefit for correction of anemia in patients suffering from chronic kidney disease (Brines and Cerami, 2008). Current treatment for patients with chronic kidney disease suffering from anemia involves a long-term regiment of repetitive rhuEPO injections. It is not only costly, but also carries risk of infection, possible development of pure red-cell aplasia and poor compliance to treatment. Of note, tests on the safety of MIDGE construct, including study on the site of integration, should be warranted before it could be translated into clinical use.

To monitor the implanted cells, the Matrigel implants were harvested from the injected mice. Two weeks post-implantation, the presence of foamy macrophages, eosinophils and fibroblast-like cells was observed (Fig. 3). Despite evidences that MSC are transplantable across allogeneic barriers, immune reaction can still occur in a xenogenic model. In a study, injection of hMSC at the site of infarcted myocardium of Spraque-Dawley (SD) rats has triggered infiltration of immune cells as early as second day post-injection. The infiltration became massive and no presence of hMSC was found after one week. The infiltration of cells were, however, less prominent in immunosuppressed SD and athymic rats (Grinnemo et al., 2004). Meanwhile, the fibroblast-like cells could have mostly originated from the host as our results showed that they were stained negatively to anti-human CD105. It is likely that the hMSC embedded in the Matrigel was either removed by macrophages as a result of the inflammatory reaction or had migrated out of the Matrigel. This was supported by our unreported *in vitro* observation, which showed that the cells encapsulated in the same concentration of Matrigel, when incubated with complete medium, could actually migrate out from it and attach onto the surface of culture dish.

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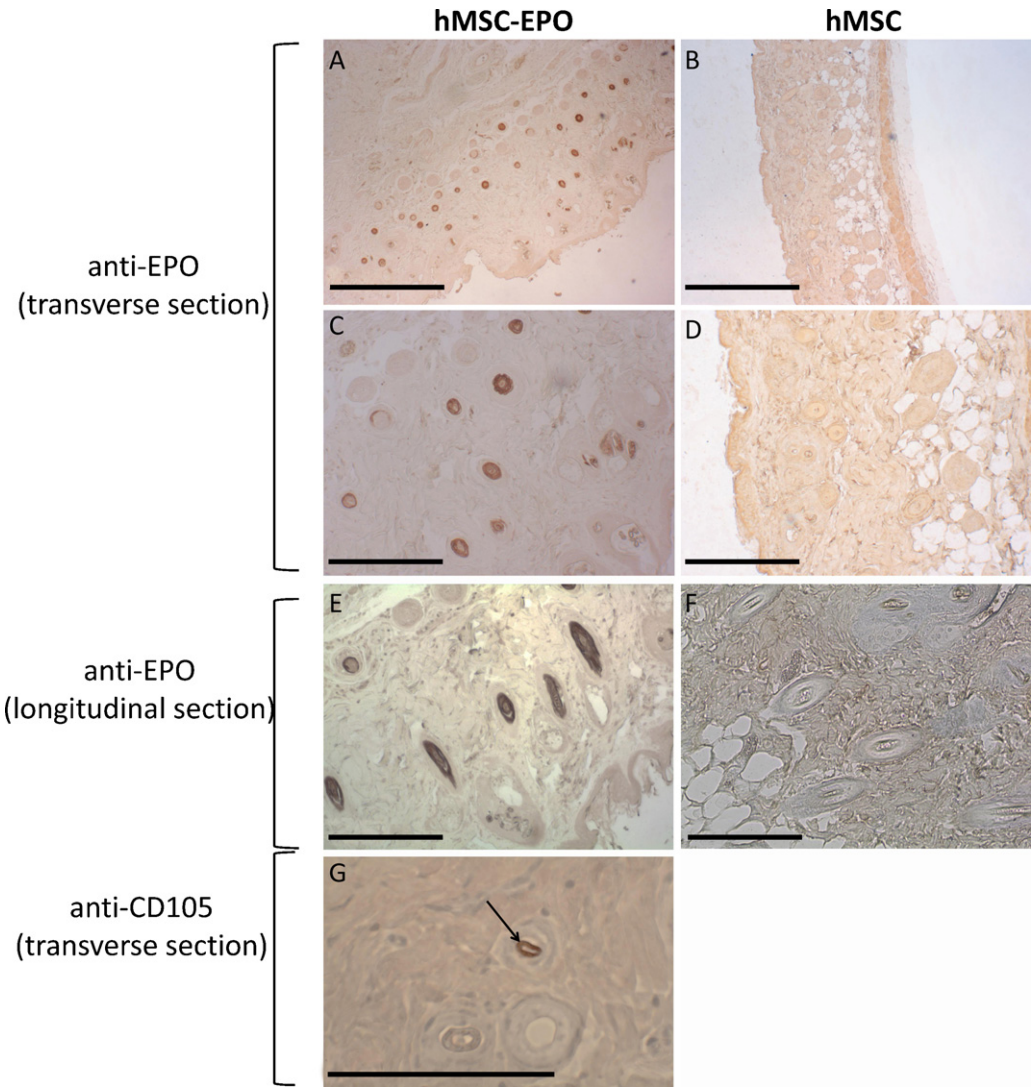

Fig. 4. Localization of EPO-producing hMSC in the basal layer of hair shaft. Immunohistochemical staining using an anti-human EPO antibody showed (A, C and E) positive staining on the basal layer of the hair shaft in the dermal of nude mouse implanted with hMSC-EPO and (B, D and F) negative staining on the basal layer of the hair shaft in the nude mouse implanted with non-nucleofected hMSC alone. There was a few hair shafts that demonstrated positive staining to anti-human CD105 antibody (G) (indicated by arrow) in the mouse injected with hMSC-EPO.

The EPO-producing cells in the Matrigel implants were significantly stained positive for EPO in the basal layer of hair shaft in the dermal layer of the injected mouse (Fig. 4A, C and E), suggesting that hMSC had migrated to the skin layer and possibly contributed to the formation of hair follicles. The hMSC could have formed the basal layer and lost the CD105 expression marker as there was only a minute number of hair follicles demonstrating positive staining for CD105 (Fig. 4G). There are evidences showing that adherent fibroblast-like cells derived from dermal papilla of hair bulbs demonstrated properties similar to MSC and that transplanted dermal papilla derived from MSC could induce new hair follicles in athymic mice (Hooduijn et al., 2006; Stenn et al., 2007; Yoo et al., 2010). Of particular interest, it remained unclear to us whether contribution to formation of hair follicles was specific to EPO-producing cells as we were unable to locate positive cells with CD105 expression on the basal layer of hair shaft of mouse transplanted with hMSC alone. Histological microscopic approach is subjected to variability and sampling errors as only a few sections (and usually only a limited number of optical fields per section) are sampled from the harvested explant and tissues. In addition to that, animals have to be biopsied or euthanized for the collection of specimens and therefore, longitudinal tracking of cell fate is difficult or impossible in the same animal. Thus, we suggest future investigation on the specificity of EPO-producing cells to migrate and engraft in the environment surrounding the hair follicles by using a more sensitive cell tracking method, for example, labeling of cells with iron particles and visualization with magnetic resonance imaging (Terrovitis et al., 2010).

Nevertheless, it seemed to us the environment surrounding the hair follicles could have formed a suitable niche for the engraftment of injected hMSC, and secreted EPO protein from the nucleofected cells might have enhanced effects in hair growth in our study. Recently, there was functional evidence of the EPO protein in hair regeneration in both *in vitro* and *in vivo* model. Kang et al. (2010) has reported that rhuEPO could significantly elongate hair shafts with increased proliferation of matrix keratinocytes in cultured human hair follicles. Additionally, they demonstrated presence of EPO receptor in human dermal papilla cells (DPC) and *in vitro* incubation with rhuEPO has significantly stimulated rate of DPC proliferation in a dose-dependent manner. EPO receptor (EPO-R) signaling pathway mediators such as EPO-R and Akt were phosphorylated by EPO protein in human DPCs. Subcutaneous injection

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of beads containing rhuEPO into the C57BL/6 mice had not only promoted anagen induction from telogen but also prolonged anagen phase. In another study, *in vitro* experiments on auditory hair cells showed a protective effect of EPO in ischemia- and gentamicin-induced hair-cell damages (Naldi et al., 2009).

5. Conclusions

In conclusion, hMSC harboring MIDGE-EPO could deliver and transiently express the EPO gene in the nude mice model. These cells could migrate and form the basal layer of the hair shaft, and secreted EPO could possibly be useful in hair regeneration strategy.

Authors' contributions

P.L. Mok: Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing. S.K. Cheong: Conception and design, financial support, data analysis and interpretation, approval of manuscript. C.F. Leong: Administrative support, provision of study materials of patients, data analysis and interpretation. K.H. Chua: Conception and design, data analysis and interpretation. O. Ainoon: Conception and design, data analysis and interpretation, approval of manuscript.

Competing interests

The authors declare that they have no competing interests.

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