

Bone marrow mesenchymal stem cells can differentiate into type II alveolar epithelial cells *in vitro*

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Abstract

In this study, we demonstrate that BMSCs (bone marrow mesenchymal stem cells) can be successfully differentiated into type II alveolar epithelial cells *in vitro* under mimic pulmonary microenvironment. BMSCs were co-cultured with MRC-5 cells in modified SAGM (small airway growth medium). The BMSC-derived type II alveolar epithelial cells morphologically resemble human lung epithelial cells. They began to appear after 10 days in co-culture and became morphologically dominant after day 15. Correspondingly, SPC (surfactant protein C), a specific functional marker of human type II alveolar epithelial cells, was detected in differentiated cells by RT-PCR (reverse transcription-PCR) analysis after day 15. Immunostaining analysis revealed the present of scattered SPC-positive cells with a differentiation efficiency of 2.43–4.21%. Our study further showed that the SPC gene expression level in differentiated cells was related to the ratio of BMSCs to MRC-5 cells and the components of modified SAGM.

Keywords: alveolar epithelial cells; bone marrow mesenchymal stem cell (BMSC); differentiation; type II alveolar cells; surfactant protein C (SPC)

1. Introduction

Evidence has suggested that bone marrow contains progenitors that can differentiate into type II alveolar epithelial cells *in vivo*. Mattson et al. (2004) reported the detection of male donor-originated type II alveolar epithelial cells in the lung of female recipients after transplantation of male bone marrow cells. Another study further revealed that, among all the different cell types in transplanted bone marrow, it is the stem cells that specifically participate in lung tissue regeneration (Kotton et al., 2001).

There are two main subpopulations of stem cells in bone marrow: MSCs (mesenchymal stem cells) and HSCs (haemopoietic stem cells). Although HSCs have been widely used in clinical situations, their ability of transdifferentiation is questioned by several groups. However, MSCs, a group of plastic adherent cells, have been reported with the potential to differentiate into a variety of cell types both *in vivo* and *in vitro*. First, several *in vivo* studies have shown that MSCs could be efficiently integrated into the hosts' organs and multi-lineage differentiation characteristics in accordance with respective organs was obtained. Secondly, *in vitro* studies have confirmed MSCs' multi-lineage differentiation ability into different kinds of cells under proper culture conditions.

In this study, we explored the possibility that MSCs can be differentiated into type II alveolar epithelial cells *in vitro* in a system mimicking pulmonary microenvironment. Pulmonary mesenchymal cells secrete soluble factors and contact directly with type II alveolar epithelial cells *in vivo*, therefore we used MRC-5 cells that were derived from normal fetal lung mesenchymal tissue, as feeder cells to co-culture with BMSCs (bone marrow mesenchymal stem cells). Modified SAGM (small airway growth medium)

was used for the induction and maintenance of type II alveolar epithelial cells differentiated from BMSCs.

2. Materials and methods

2.1. Derivation and isolation of BMSCs from human bone marrow

Fresh human bone marrow biopsies were obtained from sternum during cardiac surgery. The research was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association, and was approved by the Ethics Committee of Xinhua Hospital, Shanghai Jiaotong University School of Medicine, where the work was performed. Consent was obtained from each patient after full explanation of the purpose, nature and risk of all the procedures used. Approx. 0.5–2 ml of bone marrow sample was collected from each patient using a 23-gauge needle, which was immediately transferred into a sterile tube containing 1% heparin solution. To isolate bone marrow mononuclear cells, the samples were centrifuged in a 1.077 g/ml Ficoll (Sigma, St Louis, MO, U.S.A.) density gradient for 30 min. The cells in the white middle layer were isolated and resuspended in culture medium containing DMEM (Dulbecco's modified Eagle's medium; low glucose), 20% FBS (fetal bovine serum) and 100 I.U. (international units)/ml of penicillin-100 µg/ml of streptomycin (all from Invitrogen, Carlsbad, CA, U.S.A.). Then 1×10^6 cells were seeded in a 100 mm dish that was pre-coated with 0.1% gelatin, and incubated at 37°C with 5% CO₂. The culture medium was not changed until 72 h after the primary culture, when BMSCs had already adhered to the

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Abbreviations: BMSC, bone marrow mesenchymal stem cell; FGF, fibroblast growth factor; HSC, haemopoietic stem cell; MSC, mesenchymal stem cell; PFA, paraformaldehyde; RT-PCR, reverse transcription-PCR; SAGM, small airway growth medium; SPC, surfactant protein C.

bottom of the culture flasks while HSCs still remained suspended in the medium. Non-adherent cells were continuously eliminated by a half medium change every 3 days. After 12–16 days, when reaching approx. 90% confluence, the cells were split by 0.25% trypsin/EDTA (Sigma). From there on, BMSCs were passaged every 3–4 days and continuously cultured up to passage 30.

2.2. Flow cytometry analysis

Cells at passage 4 were digested into single cells by 0.25% trypsin/EDTA, washed twice in PBS. Then 1×10^6 cells were fixed in ice-cold 4% (w/v) PFA (paraformaldehyde) for 10 min, incubated with antibodies for 30 min on ice, and then washed twice. The cell suspension was fixed and stored at -20°C . The following monoclonal antibodies were used to detect the phenotype of BMSCs: anti-CD45-FITC, anti-CD34-FITC anti-CD10-FITC, anti-CD49b-FITC, isotype control IgG1-FITC, anti-CD117-PE, isotype control IgG1-PE, anti-CD44-PE, isotype control IgG2 β -PE, HLA-DR-PERCP, isotype control IgG2-PERCP, anti-CD13-PE and isotype control IgG1-PE (all from Becton Dickinson, Eastlake, OH, U.S.A.). Flow cytometry analysis was used to phenotypically characterize adherent cells of passage 4 in the culture. Samples were analysed using an Epics XL flow cytometer (Beckman-Coulter, Brea, CA, U.S.A.).

2.3. BMSCs co-cultured with fetal lung mesenchymal cells

The MRC-5 line was purchased from the Shanghai Institute of Biochemistry and Cell Biology. The cells were treated with $1 \mu\text{g/ml}$ mitomycin C (Sigma) in growth medium for 2 h at 37°C to serve as feeder cells. Then 0.1×10^6 BMSCs of passage 4 were directly seeded into a 100-mm dish that already contained MRC-5 feeder cells. The proportion of BMSCs to MRC-5 cells was at three ratios, 1:5, 1:10 and 1:20 respectively. In addition, 0.01×10^6 BMSCs were seeded into each well of a 24-well plate that already had coverslips on the bottom and had been pre-coated with 0.1% gelatin. On the next day, each coverslip with BMSCs grown on top was gently placed into a 60-mm dish with a feeder layer of 0.1×10^6 MRC-5 cells. Coverslips were taken out on day 15 for immunostaining of differentiated BMSCs only. SAGM or modified SAGM was applied as culture medium. SAGM is a medium specially designed for the growth and maintenance of mature distal lung epithelium, which consists of basal medium plus the following factors: 0.5 mg/ml BSA, 5 mg/ml insulin, 10 mg/ml transferrin, 30 mg/ml bovine pituitary extract, 0.5 mg/ml adrenaline (epinephrine), 6.5 ng/ml tri-iodothyronine, 0.1 ng/ml retinoic acid, 0.5 mg/ml cortisol, 0.5 ng/ml human EGF (epidermal growth factor) and antibiotics (gentamycin sulfate, 0.05 mg/ml; amphotericin-B, 0.05 mg/ml). In modified SAGM, tri-iodothyronine and retinoic acid were replaced by FGF (fibroblast growth factor) 10 at 10 ng/ml. Culture medium was changed every other day.

2.4. RT-PCR (reverse transcription-PCR)

Total RNA was extracted from cultured cells using TRIzol[®] reagent (Invitrogen). RNA samples were converted into cDNA

with Superscript III RNase H-reverse transcriptase (Invitrogen) in 20 μl of reaction mixture. The sequences of PCR primers for SPC (surfactant protein C) were as follows: 5'-TGGTGGTCCTCATCGT-CGT-3' (forward), 5'-CCTCAAGACTGGGGATGCT-3' (reverse). The product size of SPC is 250 bp. The sequences of β -actin primer were 5'-CGGGAATCGTGCGTGAC-3' (forward) and 5'-GAAGCATTGCGGTGG-3' (reverse). The product size of β -actin is 510 bp. Both primers were synthesized at the Shanghai Shenggong Company (Shanghai, China). Samples without reverse transcriptase were used as the control for genomic DNA contamination, and RNA from human lung tissue was used as positive control. The PCR products were size-fractionated by 1.2% agarose gel electrophoresis.

2.5. Immunocytochemistry

BMSCs on coverslips were fixed in 4% PFA, permeabilized with 0.1% Triton X-100, blocked with 5% BSA (Sigma) and incubated with SPC rabbit antibody (Santa Cruz Biotech, Santa Cruz, CA, U.S.A.) diluted in 1% BSA at 4°C overnight. After washing with PBS three times, rhodamine-conjugated anti-rabbit antibody (Millipore, Billerica, MA, U.S.A.) was added to the cells for 60 min at room temperature. Nuclei were stained with Hoechst (Sigma) diluted with 10% glycerol. Signals were visualized under an Olympus BX 60 fluorescent microscope (Olympus). SPC-positive cells and total cells (indicated by nuclear staining) were counted in ten random visual fields on each coverslip. The percentage of SPC-positive cells was calculated by number of positive-expressing cells divided by total number of cells. Signals on five coverslips were analysed in total and the range of the percentages of SPC-positive cells in the culture was determined.

2.6. Statistical analysis

All data are presented as means \pm S.D. Data analysis was performed using SAS6.12. Statistical significance between groups was determined by using a one-way ANOVA. A $P < 0.05$ was considered significant.

3. Result

3.1. Derivation and characterization of BMSCs

BMSCs were first observed adhering to the bottom of flasks after 3 days in primary culture. They appeared as single cells or small clusters with 2–3 cells together. These cells proliferated quickly, with small cell bodies and large nuclei. In the following 7–10 days, the majority of cells were elongated with typical spindle shape, while some cells showed polygonal morphology (Figure 1A). Cells at this stage were labelled as passage 1 and they reached confluency at day 14–16. After that, these cells were split every 3–4 days regularly with a ratio of 1:2–1:3, and they proliferated in a density-dependent manner. Figure 1(B) shows the typical morphology of BMSCs at passage 4 that had reached 100%

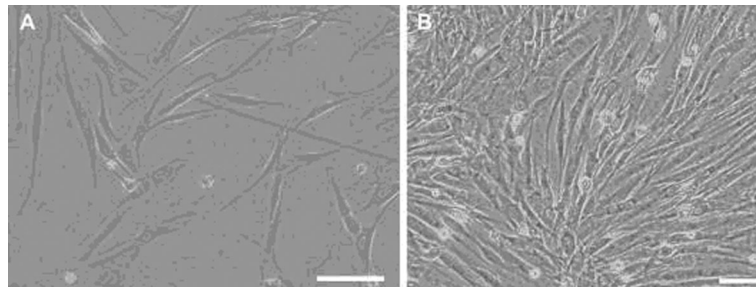


Figure 1 Morphology of BMSCs

(A) Morphology of BMSCs at passage 1. (B) Morphology of BMSCs at passage 4. Magnification: $\times 100$, scale bar=100 μm .

confluence. The BMSCs in our culture system can be expanded for over 30 passages while the growth rate continuously decreased with each passage. The population doubling time was gradually prolonged to approx. 72 h at passage 15 from approx. 48 h at passage 4 (data not shown).

The surface marker expression profile of BMSCs was examined by flow cytometry. As shown in Figure 2, the phenotype of the BMSCs was CD45, CD34, CD117 and HLA-DR negative, whereas it was CD10, CD13, CD44 and CD49b positive, which resembles the typical marker expression pattern of BMSCs as reported. These data indicate the successful derivation of BMSCs from human bone marrow biopsies in our study.

3.2. Differentiation of BMSCs into type II alveolar epithelial cells *in vitro*

To further test MRC-5 cells' inductive ability on type II alveolar epithelial differentiation of BMSCs, we cultured BMSCs on mitomycin-C-treated MRC-5 cells at a ratio of 1:10 (number of BMSCs to MRC-5 cells). In addition, modified SAGM was used for inducing type II alveolar epithelial differentiation of BMSCs. Figure 4 shows the morphology of differentiated BMSCs at days 5, 10, 15 and 20 respectively after co-culture with MRC-5 cells at ratio of 1:10 in modified SAGM. At day 5 of differentiation, BMSCs were still fibroblast-like and there was no obvious change in

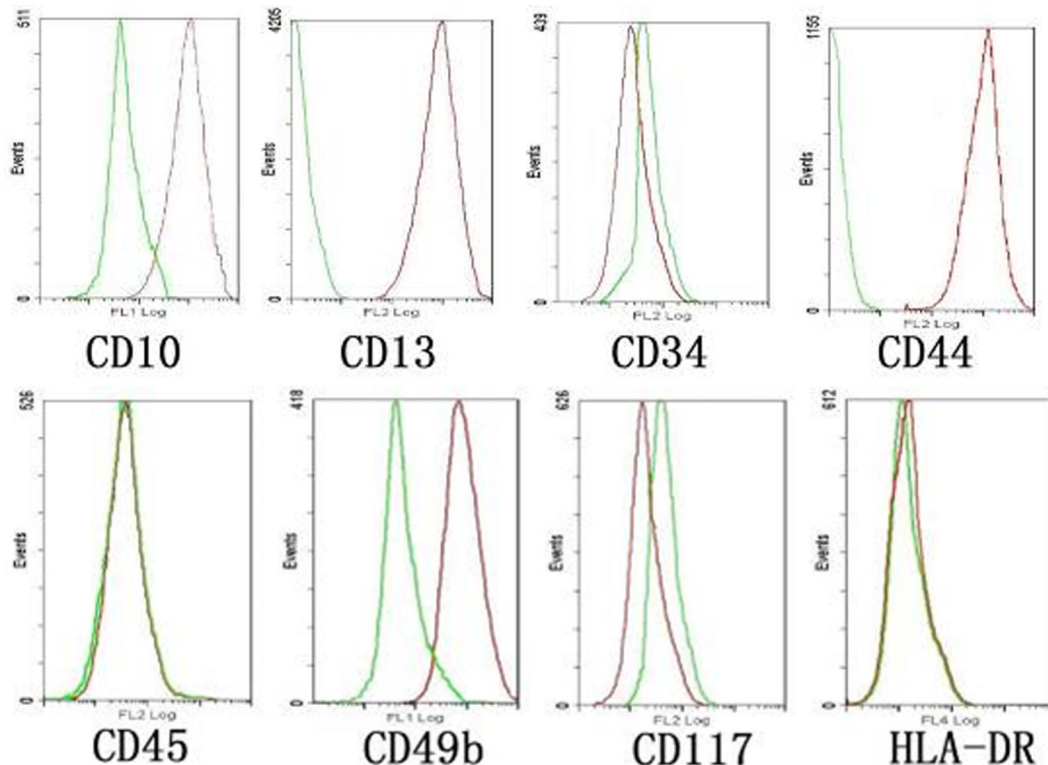


Figure 2 Phenotypes of BMSCs

Cells were stained with fluorochrome-conjugated antibodies against CD10, CD13, CD34, CD44, CD45, CD49b, CD117, HLA-DR and control IgG. Plots show the isotype control IgG staining profile against a specific antibody staining profile (green=isotype; red=specific antibody).

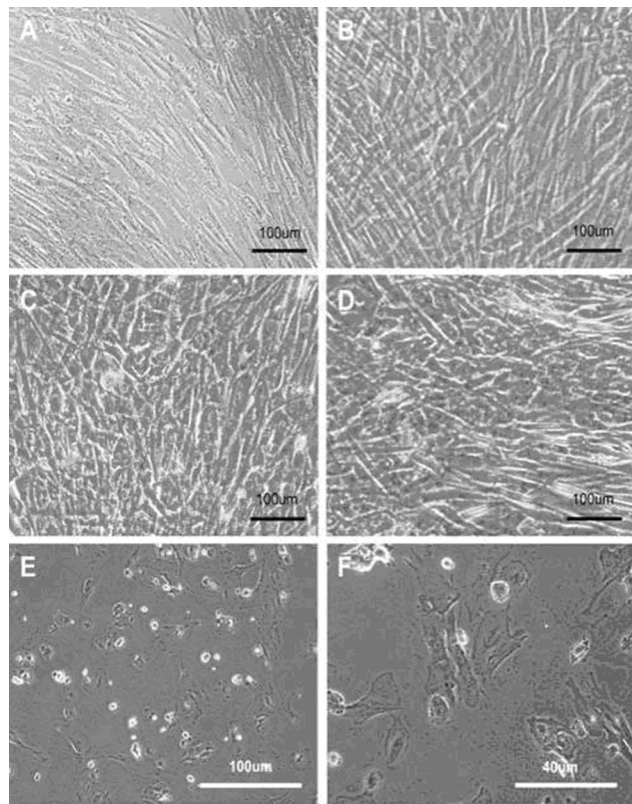


Figure 3 Morphology of BMSCs after co-culture with MRC-5 cells at different time points (A–D) Day 5, 10, 15 and 20 respectively ($\times 100$). (E, F) BMSCs grown on coverslips after 15 days' co-culture at the ratio of 1:10 in modified SAGM (E, $\times 100$; F, $\times 200$).

morphology (Figure 3A). Epithelial-like cells gradually appeared in culture at approx. day 10 (Figure 3B) and became dominant from day 15 to day 20 (Figures 3C and 3D), which indicates the successful differentiation of BMSCs towards an epithelial lineage.

In order to observe the morphological change of BMSCs without the interference of co-cultured MRC-5 cells, we plated BMSCs only on coverslips which were put on the top of mitomycin-C treated MRC-5 cells in 60 mm dishes. The same culture condition was applied that was in modified SAGM and with a ratio of BMSCs to MRC-5 cells of 1:10. At day 15 of differentiation, as shown in Figures 3(E) and 3(F), the majority of the differentiated cells were epithelial-like in morphology, which further confirmed the morphological transition of BMSCs in our differentiation system.

3.3. SPC mRNA expression

SPC gene expression was characterized in differentiated cells under various conditions, which were (i) at day 5, 10, 15, 20 and 25 of differentiation and with a ratio of BMSCs to MRC-5 cells of 1:10 in modified SAGM; (ii) with the co-culture ratio of 1:5, 1:10 and 1:20 (ratio of BMSCs to MRC-5 cells) at day 15 of differentiation in modified SAGM; and (iii) in regular and modified SAGM at day 15 of differentiation and with a ratio of BMSCs to MRC-5 cells at 1:10.

As shown in Figure 4, no SPC amplicons could be detected in the samples from non-co-cultured group or co-cultured group

at the day 5 or day 10 sampling time (Figure 4A). SPC mRNA was detected only in the samples from the co-cultured group at day 15, day 20 and day 25 sampling time (Figure 4B), which is consistent with the morphological changes of BMSCs at different time points. Furthermore, there is no significant difference on the level of SPC mRNA expression between day 15, day 20 and day 25 samples ($P>0.05$) (Figure 4C).

At day 15 of differentiation, SPC mRNA was detected in all three groups with ratio of BMSCs to MRC-5 cells of 1:5, 1:10 and 1:20 (Figure 4D). However, the expression level was shown to be lower in the group with a ratio of 1:5 than the other two groups ($P<0.05$) (Figure 4E). No significant difference was detected between the samples at ratio of 1:10 and 1:20 ($P>0.05$) (Figure 4E).

We also observed a higher expression of SPC mRNA in modified SAGM group compared with regular SAGM group at day 15 of differentiation ($P<0.05$) (Figures 4F and 4G), which indicates that modified SAGM has a more positive stimulation effect on the expression of SPC mRNA in our differentiation system.

3.4. SPC protein expression

Our previous PCR test results showed that BMSCs differentiated at a ratio of 1:10 (density of BMSCs to MRC-5 cells) expressed the highest level of SPC mRNA after 15 days of co-culture in modified SAGM. Based on this data, the same culture condition was picked to further test SPC protein expression level in differentiated BMSCs. Again, in order to avoid the interference of co-cultured MRC-5 cells in the immunostaining result, we differentiated BMSCs that were grown on coverslips and placed on the top of MRC-5 feeder cells. As shown in Figure 5, scattered SPC-immunoreactive cells were clearly observed in the culture with positive signals located in cytoplasm. Signals on 5 coverslips were analysed in total and the range of the percentages of SPC-positive cells in the whole culture was 2.43–4.21%.

4. Discussion

The fate of stem cells *in vivo* is mainly regulated by microenvironment. Fernandes et al. (2006) have demonstrated that an epithelial–mesenchymal interaction is critical in normal lung development. Fibroblasts are the major type of mesenchymal cells present in the microenvironment of lung that can induce type II alveolar epithelial cells to mature and express the SPC gene (Aliotta et al., 2005). In this study, MRC-5 cells were used to induce the differentiation of BMSCs. Our results showed that MRC-5 cell line was crucial to the differentiation of BMSCs into type II alveolar epithelial cells and the expression level of SPC gene in differentiated cells can be affected by the ratio of BMSCs to MRC-5 cells. More MRC-5 cells seemed to enhance the expression of the SPC gene.

SPC is an important functional protein (Na Nakorn et al., 2007) for type II alveolar epithelial cells. Proper growth factors in culture medium are essential for the expression of SPC in differentiated cells. In this study, we used modified SAGM medium, which contains FGF10 and regular SAGM without retinoic acid and tri-iodothyronine. Regular SAGM medium has been demonstrated to

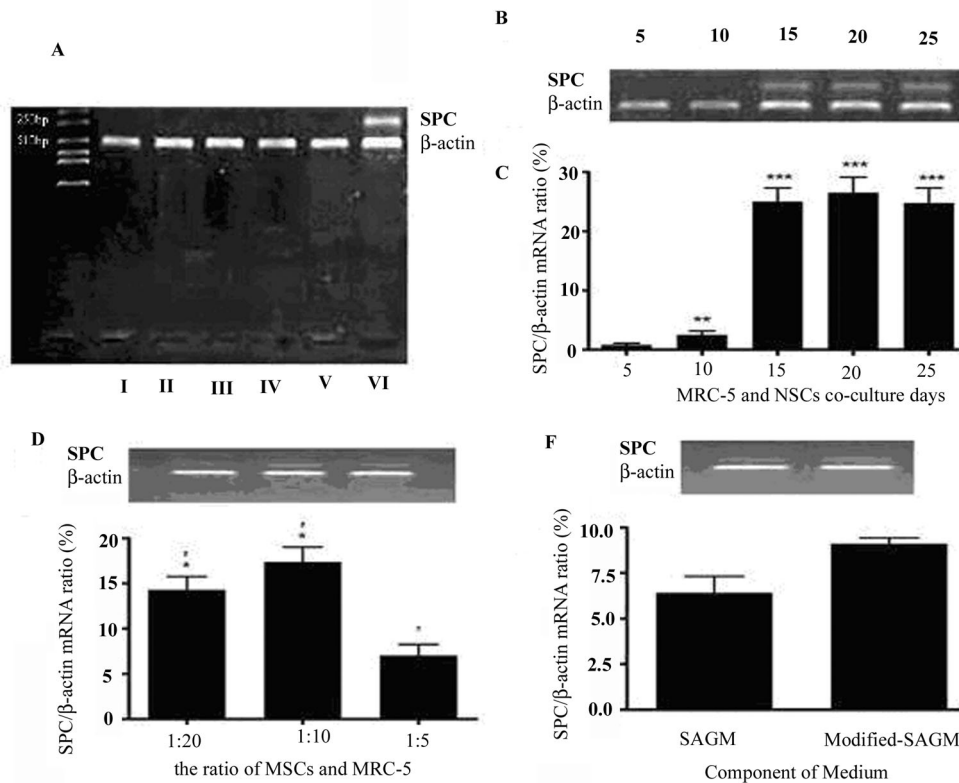


Figure 4 RT-PCR analysis of SPC transcripts

(A) Samples from left to right are: BMSCs in growth medium, MRC-5 in growth medium, BMSCs in SAGM, MRC-5 in SAGM, BMSCs co-cultured with MRC-5 in growth medium and lung tissue. (B) SPC gene expression in BMSCs co-cultured with MRC-5 at day 5, 10, 15, 20 and 25 respectively. (C) Relative amounts of SPC transcripts from (B), which was calculated as the ratio of SPC mRNA to β -actin mRNA. ** $P < 0.01$, *** $P > 0.05$. (D) Upper panel, SPC gene expression in BMSCs co-cultured with MRC-5 on day 15 at different ratio. Lower panel, relative amounts of SPC transcripts, which was calculated as the ratio of SPC mRNA to β -actin mRNA. # $P < 0.05$, * $P > 0.05$. (E) Upper panel, SPC gene expression in BMSCs co-cultured with MRC-5 on day 15 in SAGM and modified SAGM. Lower panel, relative amounts of SPC transcripts, which was calculated as the ratio of SPC mRNA to β -actin mRNA. $P < 0.05$.

be efficient in the differentiation of mouse embryonic stem cells into SPC-expressing type II alveolar epithelial cells (Ali et al., 2002). However, in other experiments, retinoic acid

and tri-iodothyronine contained in regular SAGM were reported to inhibit differentiation of type II cells and decrease the expression of the SPC gene (Rippon et al., 2004). Retinoic acid has been shown with stage-specific effects on lung development (Mollard et al., 2000) and markedly down-regulates the maturation of lung (Wongtrakool et al., 2003). In addition, retinoic acid can directly inhibit the expression of the SPC gene (George and Snyder, 1997). Tri-iodothyronine was reported with similar decreasing effect on the SPC gene-expressing population (Archavachotikul et al., 2002). Therefore we avoided these two components in modified SAGM medium, and FGF10 was utilized alternatively to facilitate the differentiation of SPC-expressing type II alveolar epithelial cells (Weaver et al., 2000). Our PCR result further confirmed that modified SAGM culture medium significantly enhanced SPC gene expression in differentiated cells.

In previous studies, alveolar epithelial cells or medium containing cell extract from alveolar epithelial cells were utilized as feeders or conditional medium in co-culture system, which are SPC-positive and interfere with the characterization for SPC expression (Sekine et al., 1999). We used MRC-5 cells as feeders, which are mesenchymal fibroblast cells and negative for SPC expression; therefore this issue is avoided in our culture system. Similar to other reports (Wang et al., 2005), the percentage of SPC-positive expression cells generated in our culture system

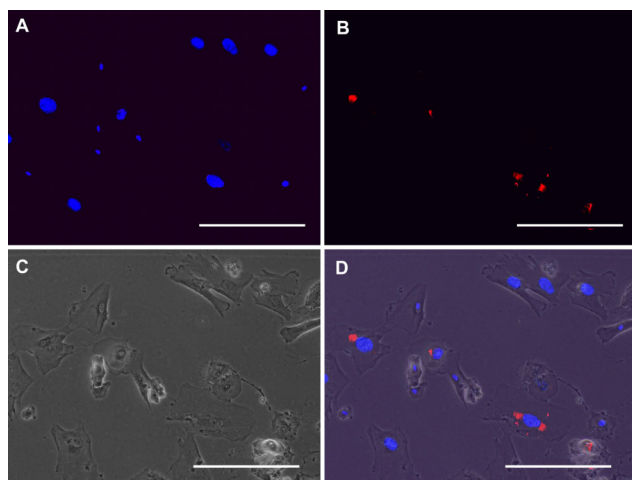


Figure 5 Immunofluorescence staining of SPC in differentiated cells

(A) Nucleus stained with Hoechst. (B) Positive expression of SPC in the cytoplasm. (C) Phase-contrast image. (D) Image merged of (A–C). Magnification: $\times 200$, scale bar = 40 μm .

was less than 5%. This may indicate that only one subset of BMSCs, which expresses receptors that match the signalling of the alveolar development, could differentiate successfully into SPC-positive type II alveolar epithelial cells. Ratajczak et al. (2004) believed that BMSCs consisted of committed tissue-specific stem cells for various organs. This theory seems helpful to explain our results: only the lung-specific/committed BMSCs can be driven to differentiate into type II alveolar epithelial cells. Further studies to probe into molecular events throughout BMSCs' differentiation process are needed to better identify the different subtypes of BMSCs, increase the differentiation efficiency with the suitable subtype and make BMSCs a reasonable source to generate type II alveolar epithelial cells for future cell therapy applications.

Author contribution

Hong Zhong and Nan Ma designed the research; Nan Ma, Ju Mei, Fang-Bao Ding and Chun-Rong Bao performed the research; Hong Zhong and Nan Ma analysed the data, Hong Zhong, Nan Ma and Hui Gai wrote the paper; Hui Gai and David M. Nguyen revised the paper; Ju Mei and Hong Zhong funded the research.

Acknowledgements

This work was completed in the Key Laboratory of Neurobiology, School of Medicine, Shanghai Jiaotong University. We would like to thank Dr Peihua Lu, Jian Zou and Min Wang for their excellent technical support.

Funding

This work was supported by National Natural Science Funds [grant number 30571843 (to H.Z.)].

References

- Ali N, Edgar J, Samadikucharsaraei A, Timson M, Romanska M, Polak M et al. Derivation of type II alveolar epithelial cells from murine embryonic stem cells. *Tissue Eng* 2002;8:541–50.
- Aliotta M, Passero M, Meharg J, Klinger J, Dooner S, Pimentel J et al. Stem cells and pulmonary metamorphosis: new concepts in repair and regeneration. *J Cell Physiol* 2005;204:725–41.
- Archavachotikul K, Ciccone J, Chinoy R, Nielsen C, Volpe V. Thyroid hormone affects embryonic mouse lung branching morphogenesis and cellular differentiation. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L359–69.
- Fernandes J, Bonacci V, Stewart G. Extracellular matrix, integrins, and mesenchymal cell function in the airways. *Curr Drug Targets* 2006;7:567–77.
- George N, Snyder M. Regulation of surfactant protein gene expression by retinoic acid metabolites. *Pediatr Res* 1997;41:692–701.
- Kotton N, Ma Y, Cardoso V, Sanderson A, Summer S, Williams C. Bone marrow-derived cells as progenitors of lung alveolar epithelium. *Development* 2001;5181–8.
- Mattson J, Jansson M, Wernerson A, Hassan M. Lung epithelial cells and type II pneumocytes of donor origin after allogeneic hematopoietic stem cell transplantation. *Transplantation* 2004;78:154–7.
- Mollard R, Ghyselinck B, Wendling O, Chambon P, Mark M. Stage-dependent responses of the developing lung to retinoic acid signaling. *Int J Dev Biol* 2000;44:457–62.
- Na Nakorn P, Meyer C, Flach R, Mendelsohn R, Galla J. Surfactant protein C and lung function: new insights into the role of alpha-helical length and palmitoylation. *Eur Biophys J* 2007;36:477–89.
- Ratajczak Z, Kucia M, Majka M, Reza R, Ratajczak J. Heterogeneous populations of bone marrow stem cells—are we spotting on the same cells from the different angles? *Folia Histochem Cytobiol* 2004;42:139–46.
- Rippon J, Ali N, Polak M, Bishop E. Initial observations on the effect of medium composition on the differentiation of murine embryonic stem cells to alveolar type II cells. *Cloning Stem Cells* 2004;6:49–56.
- Sekine K, Ohuchi H, Fujiwara M, Yamasaki M, Yoshizawa T, Sato T et al. Fgf10 is essential for limb and lung formation. *Nat Genet* 1999;21:138–41.
- Wang G, Bunnell A, Painter G, Quiniones C, Tom S, Lanson A et al. Adult stem cells from bone marrow stroma differentiate into airway epithelial cells: potential therapy for cystic fibrosis. *Proc Natl Acad Sci USA* 2005;102:186–91.
- Weaver M, Dunn R, Hogan L. Bmp4 and Fgf10 play opposing roles during lung bud morphogenesis. *Development* 2000;127:2695–704.
- Wongtrakool C, Malpel S, Gorenstein J, Sedita J, Ramirez I, Underhill M et al. Down-regulation of retinoic acid receptor alpha signaling is required for sacculation and type I cell formation in the developing lung. *J Biol Chem* 2003;278:46911–8.

Received 15 January 2011/14 April 2011; accepted 4 May 2011

Published as Immediate Publication 4 May 2011, doi 10.1042/CBI20110026