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RESEARCH ARTICLE

Biological safety of human skin-derived stem cells after long-term *in vitro* culture

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Abstract

The aim of this study was to investigate the biological safety of human skin-derived mesenchymal stem cells (HSMSCs) cultured *in vitro* by detecting changes in karyotype, major histocompatibility complex expression and tumorigenicity. Before the 21st passage of the *in vitro* culture, cell surface markers were analysed by flow cytometry; major histocompatibility complex expression was detected by RT–PCR and flow cytometry. The tumorigenicity of HSMSCs was tested using SCID mice and observing changes in the injection site and pathological sections. Flow cytometry demonstrated that HSMSCs express CD73, CD105 and vimentin, but haematopoietic markers CD34, CD45 and CD19 were not expressed. Human leukocyte antigen (*HLA-I* and *HLA-DR*) mRNA was detected by RT–PCR; the protein expression of HLA-I was 29.5–31.7%, but HLA-DR protein expression was not detected in HSMSCs. The result of karyotype analysis before the 21st passage was normal and tumour formation was not detected in the mice. Taken as a whole, our results suggest that HSMSCs cultured *in vitro* may be safely transplanted *in vivo*, due to moderate expression of HLA-I and low expression of HLA-DR, non-tumorigenicity and normal karyotype. Copyright © 2010 John Wiley & Sons, Ltd.

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Keywords **human skin mesenchymal stem cells; major histocompatibility complex; karyotype; biological safety**

Introduction

Mesenchymal stromal cells (MSCs) derived from early mesoderm and ectoderm have the capacity for self-renewal and are able to differentiate into multiple lineages. Studies have shown that MSCs also have immunomodulating properties and are essential for the growth and differentiation of primitive haematopoietic cells in the bone marrow microenvironment (Wulf *et al*., 2006; Devine *et al*., 2000). Furthermore, human skin-derived MSCs are easily isolated and have the ability to autotransplant.

The potential clinical applications of MSCs have generated a great deal of interest; thus, it is crucial to assess the efficacy and safety of MSCs in long-term *ex vivo* expansion. Many experiments (Kim *et al*., 2009; Bernardo *et al*., 2007; Choumerianou *et al*., 2008) have demonstrated that bone mesenchymal stromal stem cells (BMSCs) in long-term expansion retained normal telomerase activity. In addition, karyotypes were unaltered and tumour formation did not occur. However, almost no studies have assessed the efficacy and safety of transplantation with human skin-derived mesenchymal stem cells (HSMSCs). The objective of this study was to investigate the biological safety of HSMSCs cultured long-term *in vitro*.

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Materials and method

Cell culture

Primary isolation, the method used for cell isolation, is related to the issue of a patent, which has not yet been published. Thus, we have not described it here.

HSMSCs were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) with 4.58 g/L glucose supplemented with 18% fetal bovine serum (FBS) (Sijiqing, Hangzhou, China), 1% penicillin/streptomycin (Gibco), 1% glutamine (AMRESCO, Cochran, Solon, USA), non-essential amino acids (Gibco-BRL), 10^{-5} g/L human basic fibroblast growth factor (hbFGF; Pepro Tech, Wessex Barn, UK) and 2×10^{-5} g/L human stem cell factor (hSCF; Pepro Tech). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. The medium was changed every 2–3 days and, upon reaching confluence, the cells were subcultured (split ratio, 1 : 1) using trypsin–EDTA for detachment.

Fluorescence-activated cell sorting (FACS) analysis

For cell-surface marker studies, MSCs that reached 80% confluency were detached and washed three times with phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA, USA), then 5.5×10^6 cells were resuspended in microfuge tubes. Cells were stained with the following antibodies for 30 min at room temperature: CD73-FITC, HLA-DR-FITC, IgG2a-FITC, CD34-RPE, IgG1-Percp, CD45-PerCP (Bioscience (BD), San Jose, CA, USA). CD105-PE, CD19-FITC, vimentin-RPE, HLA-I-FITC, IgG1-FITC, IgG2a-RPE (Invitrogen). Cytolysis agents were also purchased from Invitrogen and used according to the manufacturer's instructions. The cells were then washed again and analysed using the FACScan flow cytometer (Becton-Dickinson (BD), San Jose, CA, USA).

Karyotype analysis

HSMSCs were cultured to passage (P)10, P11 or P21. The cells were then subjected to metaphase mitotic arrest with colcemide treatment (0.2 g/ml) for 2–2.5 h. Cells were then subjected to hypotonic shock with 0.075 M KCl, followed by fixation with methanol : acetic acid (3 : 1). Chromosomes were visualized after a standard G-banding procedure and karyotyped according to the International System for Human Cytogenetic Nomenclature (1987).

Tumorigenicity test

The 20 severe combined immunodeficiency (SCID) mice (half male and half female; weight, 16–18 g; age, 4–6 weeks old) used in the study were purchased from the Laboratory Animal Center of Sun Yat-Sen University. Mice were cared for in accordance with the requirements of Sun Yat-Sen University's animal care and use committee and administrative panel on laboratory animal care. The mice received an intraperitoneal injection (intraperitoneal group, seven mice) or subcutaneous injection (subcutaneous group, seven mice) of cell suspension (0.2 ml, 5×10^7 cells/ml). The mice were further divided into groups injected with P11 or P21 cells. The mice were fed in a specific-pathogen-free (SPF) environment with a 12 h light–dark cycle (23–25 °C, humidity 50–60%). The injection sites of all animals were monitored throughout the study, as well as the animals' mental status, diet and defecation. All animals were sacrificed 3 months after the initiation of the experiment. Skins, organs, nodules and thoracic and abdominal cavities were examined macroscopically. Skins and organs were removed for pathological examination; haematoxylin and eosin (H&E) staining was carried out to determine histological structure.

Major histocompatibility complex analysis

HSMSCs (P11 and P21) were stained with conjugated mouse anti-human antibodies (FITC-conjugated HLA-I, HLA-DR and corresponding isotype controls) for 30 min at room temperature and analysed with a BD FACS Calibur flow cytometer (Becton Dickinson) according to the method detailed above (FACS analysis).

The expression of *HLA-I* and *HLA-DR* lineage-specific mRNA was assessed using reverse transcription polymerase chain reaction (RT–PCR). Total RNA was extracted from HSMSCs using TRIzol (Invitrogen). First-strand cDNA was synthesized from total RNA using a reverse transcriptase kit (Fermentas, Burlington, Canada) according to the manufacturer's protocol, using β-actin and lymphocytes as positive controls and template-free reaction as the negative control. The 25 l RT–PCR reaction was performed according to the manufacturer's protocol, using 1 l cDNA (0.5 ng) and 0.25 mM forward and reverse primers (see Table 1). The reaction was carried out under standard RT–PCR cycling conditions: (95 °C for 10 min, 94 °C for 30 s, 60 °C for 40 s, 72 °C for 10 s) for 30 cycles, followed by extension at 72 °C for 10 min. PCR products were separated on a 2% agarose gel.

Results

HSMSCs were cultured at a density of 5×10^5 in a 25 cm^2 flask. Once the culture reached confluence, 2×10^6–3×10^6 cells were harvested for further experiments. Subcultured cells reached 90–100% confluence in 2–3 days. Most cells were star-shaped or short spindle-shaped, similar to fibroblast morphology. This morphology was maintained through the 21 passages (Figure 1)

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Table 1. Primers used in gene amplification

Gene	Sequence		PCR product size (bp)
	Forward	Reverse	
β-Actin	5′-GTGGGGCGCCCCAGGCACCA-3′	5′-CTCCTTAATGTCAGGCACGATTT-3′	550
HLA-I	5′-CCTTGGATCTGTGGTCTCTGGA-3′	5′-ACAAGGCAGCTGTGCATCTCA-3′	185
HLA-DR (Souwer *et al*., 2009)	5′-CATGGGCTATCAAAGAAGAAC-3′	5′-CTTGAGCCTCAAAGCTGGC-3′	180

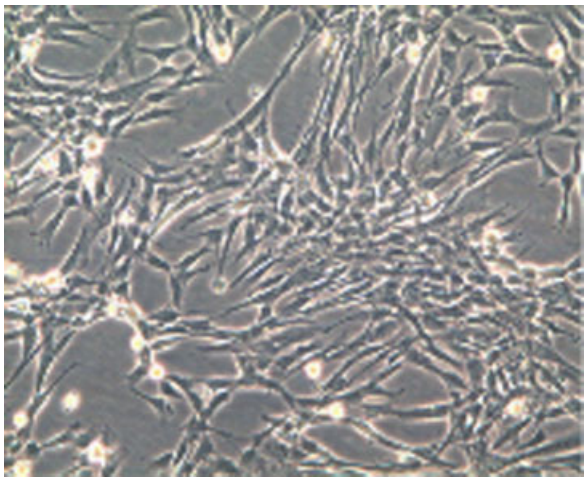

Figure 1. Human skin mesenchymal stem cells cultured *in vitro* to P21 (magnification, ×10). The cells exhibited a spindle-shaped morphology, similar to that of a fibroblast, and were dispersed or colony-like, Scale bar = 100 μm

Analysis of cell surface markers on P10–P21 cells by FACS demonstrated that the HSMSCs were positive for CD73 and CD105 and negative for monocyte–macrophage and haematopoiesis antigens, such as CD45, CD34 and CD19. Cells expressing vimentin accounted for 29.8–37.5% of the total cells. The set of each figure was based on the blank controls. The data presented in the figure had been subtracted of the background colour (homogeneous control) (Figure 2). These results were consistent with data already reported for human HBMSCs.

HSMSCs from various donors were analysed before the 21st passage and found to have a normal human karyotype, 46 XY (Figure 3 shows the karyotypes of the 11th and 21st passages), with no inversions, deletions, duplications, interfusions or ring chromosomes.

The mice appeared well following inoculation with P11 and P21 HSMSCs, with normal energy and appetite and no problems at the inoculation sites. The mice were sacrificed 3 months after inoculation. No macroscopic pathological finding or mass was identified in either subcutaneous or introperitoneal groups, and no nodules were detected in the peritoneal cavity or related organs of mice in the intraperitoneal group. However, a small nodule was found in each pathological section of two mice in the subcutaneous group (Figure 4C). No significant difference was detected between the nodules and the surrounding connective tissue. Histopathological examination of one nodule showed that it was wrapped in fibrous connective tissue and exhibited many new capillaries and infiltration of fibroblasts and fat cells; however, no mitotic pathology was found (Figure 4C). The diagnoses were made by pathologists at Sun Yat-Sen University: (a) subcutaneous fibre capillary section; and (b) malignant change could be excluded. The other nodule was normal (Figure 4D)

HLA-I and *HLA-DR* expression was examined in HSMSCs from P11–P21, using RT–PCR and flow cytometry. *HLA-I* and *HLA-DR* mRNA were found in both P11 and P21 (Figure 5). By flow cytometry, HLA-I protein expression was shown to be downregulated from P11 to P21. HLA-DR protein was not detected in P11 or P21 (Figure 6).

Discussion

MSCs have became an attractive adult stem cell source, due to their capacity for differentiation and autotransplantation (Krause, 2002; Bianco *et al*., 2001), providing hope for the treatment of many diseases. To achieve stage-specific differentiation and sufficient numbers of cells, stem cells must undergo *ex vivo* expansion prior to transplantation. Because the potential for genetic mutation, tumorigenicity and immunogenicity exists during *ex vivo* long-term culture, it is essential to determine safety before transplantation. In this study, we cultured skin-derived MSCs *ex vivo* from P10–P21. The results of FACS analysis, using positive surface markers CD73, CD105 and vimentin and negative markers CD34, CD45 and CD19, as well as major histocompatibility complex analysis, karyotyping and tumorigenicity analysis, confirmed the safety of HSMSCs *ex vivo* long-term culture before P21 under the study conditions.

Abnormal karyotypes are closely associated with all types of genetic diseases, including cancer. In the event of chromosome inversions, deletions, duplications, interfusions or ring chromosomes, mutations can alter the structure and regulation of oncogenes, potentially causing cancer (Cheng *et al*., 2003; Geisler *et al*., 2003). In this study, we found normal karyotypes in HSMSCs in P10, P11 and P21 cultured *ex vivo* long-term.

MSCs are pluripotent stem cells (Woodbury *et al*., 2000) that, under appropriate conditions, can easily be differentiated into neurocyte, fibroblast and cartilage cells. Because immortality and excessive proliferation can induce cancer in animals, we assessed the tumorigenicity of HSMSCs cultured *ex vivo* long-term, using SCID mice. Subcutaneous and abdominal cavity injections of HSMSCs

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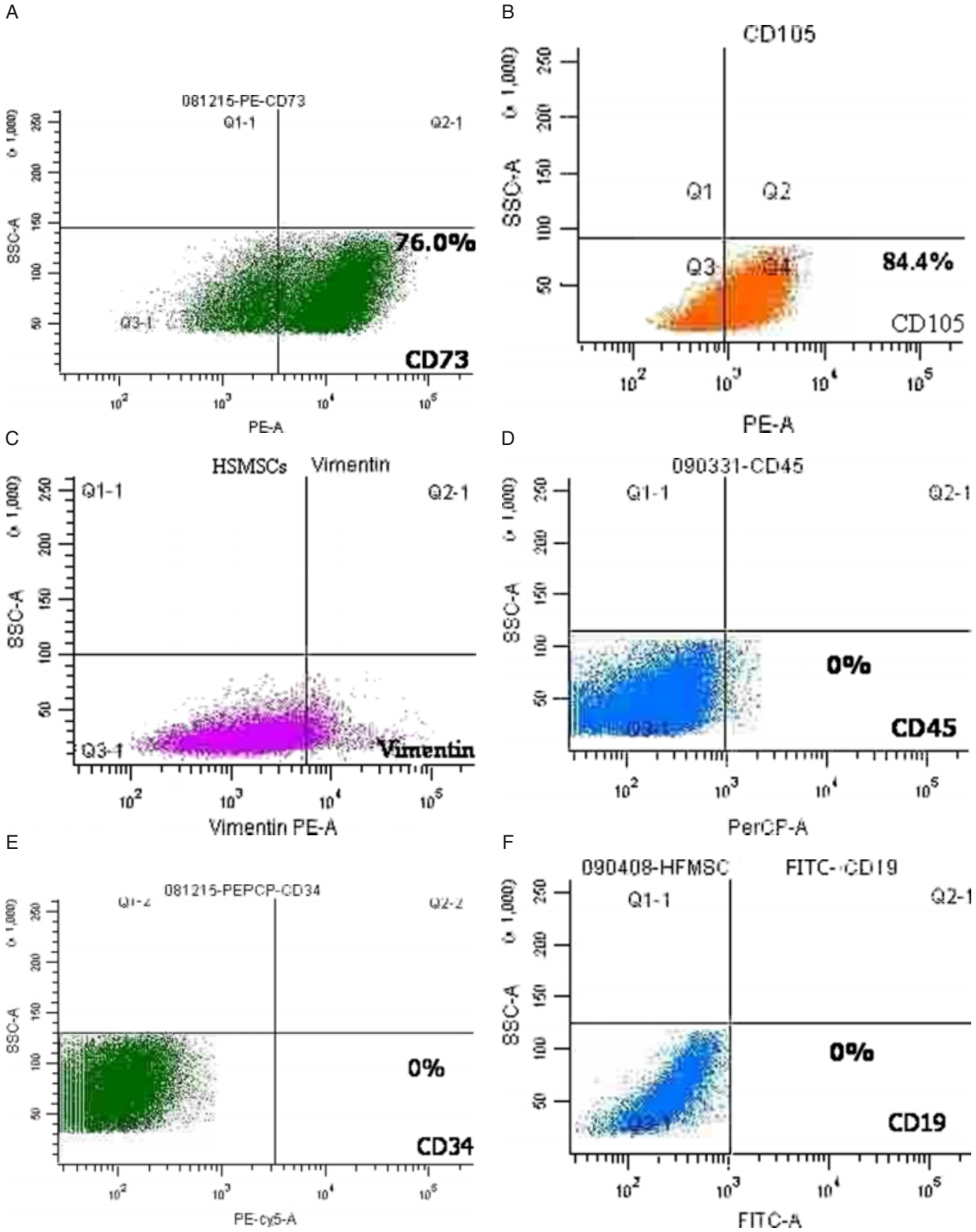

Figure 2. Flow cytometry analysis of HSMSCs. (A) CD73 (74.6–77.7%); (B) CD105 (60.4–84.4%) and (C) vimentin (29.0–37.5%) are positive markers. HSMSCs did not express monocyte, macrophage and haematopoiesis antigens, such as (D) CD45, (E) CD34 or (F) CD19

were administered to determine the risk of host-vs.-graft reaction. The SCID mice appeared well 3 months after injection, and no tumour formation was observed. Only one inflammatory nodule was identified in a pathological section, which was considered to be the result of heterogeneous rejection. Therefore, we assumed that autologous skin-derived cells could avoid transplantation rejection.

Rejection is the paramount consideration for transplantation. Major histocompatibility complex (MHC) proteins, including the HLA proteins, are associated with rejection. In the present study, HLA-I antigen was 29.5–31.7%

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Figure 3. Karyotype analysis of HSMSCs in (A) P11 and (B) P21, showing no chromosomal abnormalities

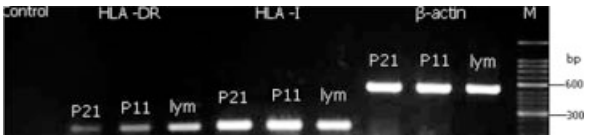

Figure 5. Agarose gel electrophoresis of RT–PCR amplification products *HLA-I* and *HLA-DR*. M, marker; *β-actin*, 550 bp; *HLA-I*, 185 bp; *HLA-DR*, 180 bp

positive in P11 and negative in P21; HLA-DR antigen was negative in every cell passage in our study. However, *HLA-I* and *HLA-DR* mRNA were found in P11 and P21. However, RT–PCR amplification of *HLA-DR* from different passages was lower compared with controls. While the RT–PCR results showed a clear band on the electrophoresis gel if <2% cells expressed *HLA-DR*, <2% of cells expressing the proteins was defined as negative expression. Thus, expressions of mRNA and protein were presumed to be consistent. HLA-I antigen can be found in all karyocytes and HLA-DR antigen is only expressed in antigen-presenting cells. Previous studies (Pittenger *et al*., 1999; Basham *et al*., 1983; Pujol-Borell *et al*., 1987) have demonstrated that interferon-γ (IFNγ) and tumour necrosis factor-α (TNFα) can increase HLA-I and HLA-DR expression and induce HLA-DR expression in cells that do not normally express the antigen; however, the mechanism responsible for this effect is unclear. Our results demonstrate that HSMSCs exhibit low immunogenicity and moderate expression of HLA-I, and low or non-expression of HLA-DR after *ex vivo* long-term culture.

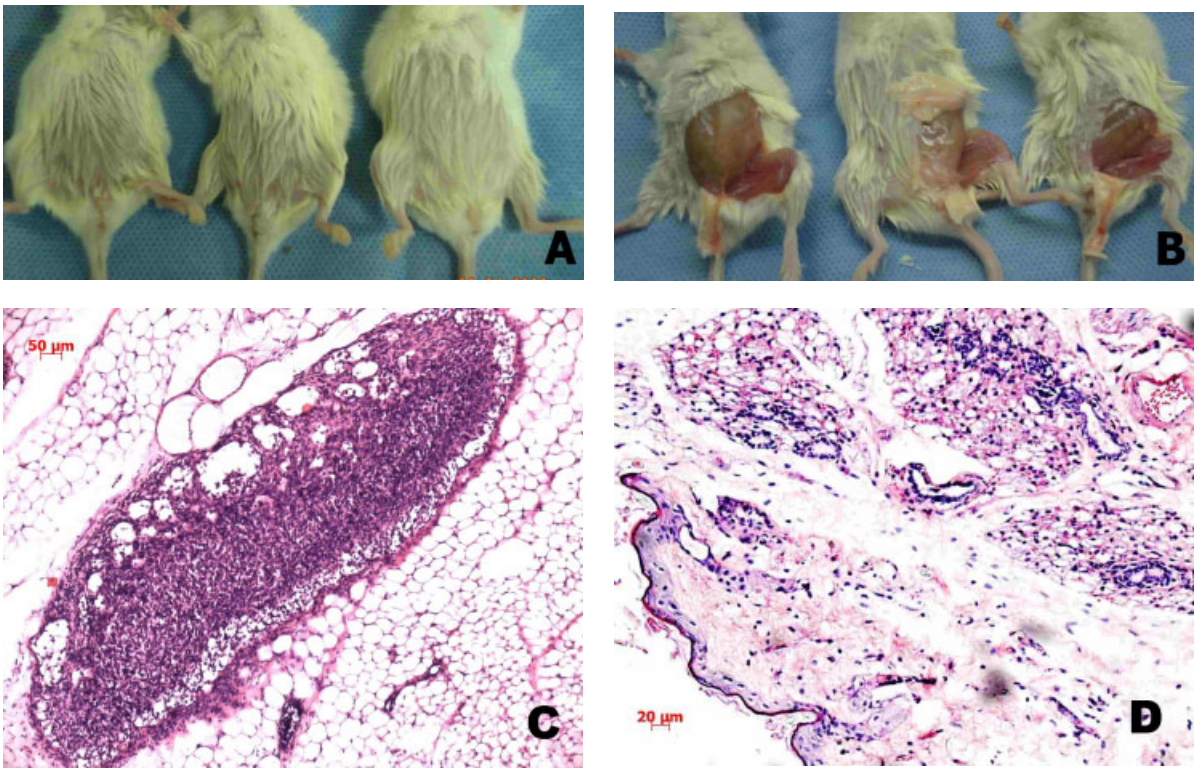

Figure 4. (A, B) *In vivo* transplantation of long-term cultured HSMSCs for the tumorigenesis assay. No changes were detected macroscopically. (C) Subcutaneous fibre capillary tubercule (magnification, ×20; H&E stain; scale bar = 50 μm). (D) Normal subcutaneous connective tissue (magnification, ×20; H&E stain; scale bar = 20 μm)

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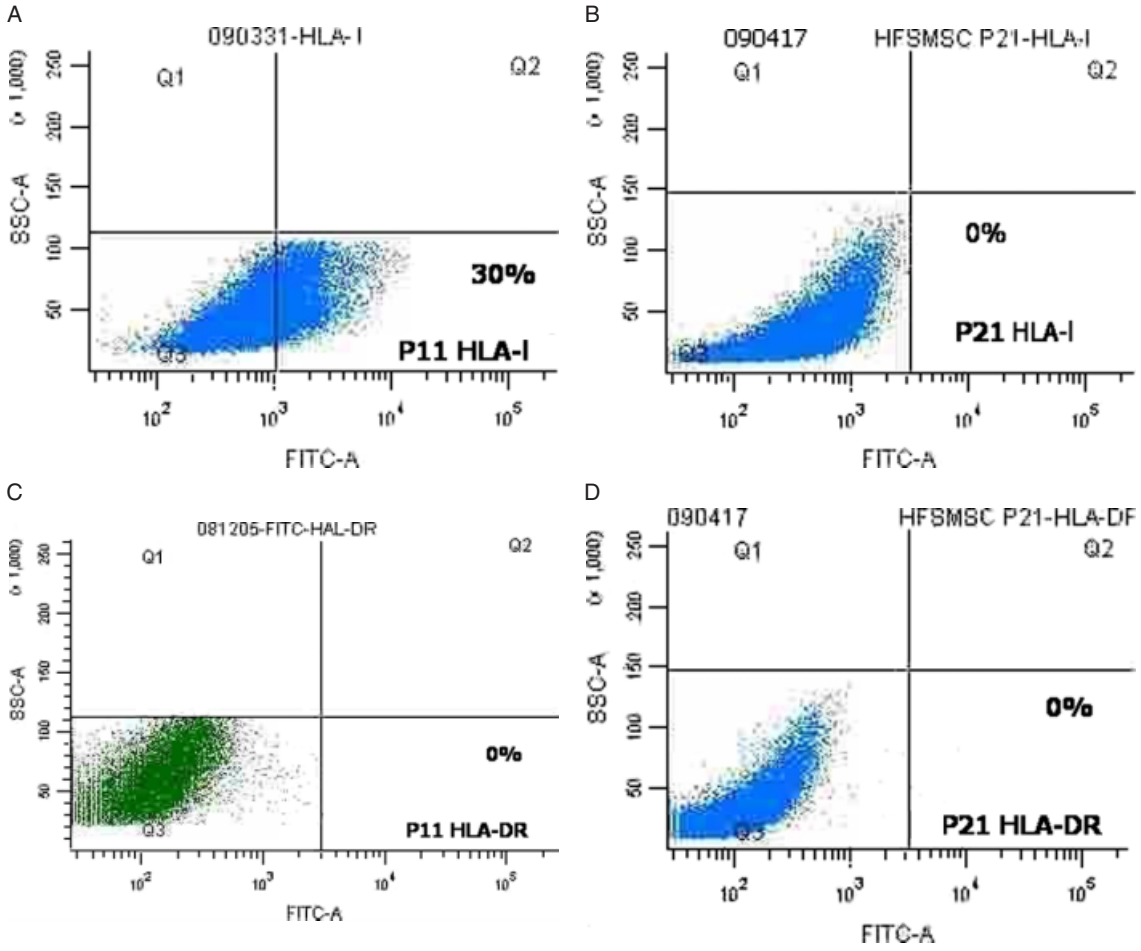

Figure 6. Flow cytometry analysis of HSMSCs. HLA-I was 29.5–31.7% positive in (A) P11 and was negative in (B) P21. HLA-DR was negative in both (C) P11 and (D) P21 cells

Numerous studies have demonstrated that MSCs with low expression of MHC-I and low or no expression of MHC-II were characterized by low immunogenicity (Deans *et al*., 2000; Kircher *et al*., 2002; Jiang *et al*., 2002).

In conclusion, the human-skin derived HSMSCs can be amplified in large quantities within 21 passages while maintaining normal karyotype. They are not carcinogenetic *in vivo* and have low immunogenicity, which makes them suitable for clinical use. However, the present study is a preliminary study of biological safety and the study of immunogenicity was based primarily on the major histocompatibility complex. Thus, the roles of antigen molecules, such as the surface co-stimulating molecules (CD40, CD40L, CD80 and CD86) in immunogenicity require further investigation. Additionally, issues such as the risk of mycoplasma and viral contamination, *in vivo* differences in immunogenicity, migration and differentiation of the cells, the degree of the stem cell differentiation, and the number of cells and treatment times required should be considered in the clinical setting.

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