Preparation of AD-MSCs

AD-MSCs cell line used in this pilot study was obtained from abdominal adipose tissues of healthy donors in Department of Plastic surgery, Peking Union Medical College Hospital. Orthopedic patients were invited to donate their adipose tissues if they met the following inclusion criteria: 1) Orthopedic patients who were in the age of 18-35 for females and 18-40 for males; 2) Orthopedic patients without infectious agents (human immunodeficiency virus, hepatitis B virus, hepatitis C virus, syphilis); 3) Orthopedic patients whose Electrocardiogram, X-rays of chest, bone marrow morphology and routine blood test were all normal. 4) Healthy donors who willing to sign informed consent.

The detailed procedures and steps are as follows.

1. Isolation and passage of AD-MSCs (adipose tissue derived mesenchymal stem cells,AD-MSCs）

Liposuction were operated on the superficial abdominal fat tissue layer in healthy persons aged 18-60 years after obtaining informed consent from the individuals according to procedures approved by the Ethics Committee at Chinese Academy of Medical Sciences and Peking Union Medical College.

The raw lipoaspirate were digested with 0.2% collagenase II (Sigma) at 37℃for 30 min and cells were plated in T-75 flasks at a density of 2×10^6 cells/mL. The culture medium used was DF12 containing 40% MCDB medium (Sigma), 2% fetal calf serum (Gibco Life Technologies), 1 × insulin transferrin selenium (Gibco Life Technologies), 10^{-9} M dexamethasone (Sigma), 10^{-4} M ascorbic acid 2-phosphate (Sigma), 10 ng/mL epidermal growth factor (R&D Systems), 10 ng/mL platelet-derived growth factor BB(R&D Systems), 100 U/mL penicillin, and 1,000 U/mL streptomycin (Gibco).Cultures were placed into 37℃, 5% CO_2 incubator.

Once adherent cells were more than 80% confluent, they were detached with 0.125% trypsin and 0.01% EDTA and reseeded at a 1:3 dilution under the same culture conditions.

Reference:

Sun Z, Han Q, Zhu Y, Li Z, Chen B, Liao L, et al. NANOG has a role in mesenchymal stem cells' immunomodulatory effect. Stem Cells Dev. 2011 Sep;20(9):1521-8.

2. Biological safety and biological activity evaluation

Cultured AD-MSCs were spindle-shaped with a fibroblast-like morphology (Figure 1).

The tested items are listed in Table 1. Analysis of the clinical-grade cells[$] recognized by the National Institutes for Food and Drug Control (NIFDC) included the absence of cell cross-contamination between species or contamination by pathogenic microorganisms (Bacteria and fungi, Mycoplasma, Human papilloma virus, Human immunodeficiency virus 1, Epstein-barr virus, Human hepatitis C virus, Human cytomegalovirus, Human hepatitis B virus, Human herpesvirus 6, Human herpesvirus 7, Human papillomavirus, Reverse transcriptase activity, Bovine virus and Porcine virus) or endotoxin (≤0.5 EU/mL), Bovine serum albumin residuals (＜5 ng/mL), being negative for Hemagglutination test of 9 to 11-day-old chick embryo allantoic fluid, with a cell viability ≥ 90% and an identity and purity pattern characterized by positivity (≥ 95%) of CD73, CD90, CD44 and CD105 and negative expression (≤ 2%) of CD45, CD34, CD19,CD11b and HLA-DR, and an inhibitive property of lymphocyte proliferation **(**≥ 40%**)**

3. Cryopreservation of AD-MSCs

AD-MSCs were digested as described in passage of AD-MSCs. The DF12 cryomedia containing 20%FBS and 10%DMSO, was added to resuspend and subpackage cells into frozen vials. After freezing in programmed freezing containers (Corning Cool Cell LX) overnight in a freezer at 80 ℃, the vials were transported in the gas phase of a liquid nitrogen tank.

4. AD-MSCs Thawing

Cryopreserved cells were thawed in 37℃ heating block, and then quickly transferred into 15-ml centrifuge tubes containing pre-warmed MSC culture medium . Cell pellets were resuspended by fresh MSC culture medium after centrifugation, counted and then seeded into 75-cm^2 cell culture flask at 1×10^6 cells/mL. Cultures were placed into 37 ℃, 5% CO_2 incubator.

5. Adipogenic, osteogenic and chondrogenic differentiation

For adipogenic differentiation, AD-MSCs were digested by 0.125% trypsin as described before, resuspended by fresh MSC culture medium and seeded into 24-well plates containing 6×10^4 cells/well. MSC culture medium was replaced by adipogenic differentiation medium（BI, 05-330-1B，prepared as instruction manual told）at 90% confluence. AD-MSCs should be changed adipogenic differentiation medium once every 3 days. Identification by Oil-red O (VivaCell, C37A00150) staining were carried out, after differentiation of 21 days. For osteogenic differentiation, AD-MSCs

were digested by 0.125% trypsin as described before, resuspended by fresh MSC culture medium and seeded into 24-well plates containing 6× 10^4 cells/well. MSC culture medium was replaced by osteogenic differentiation medium （BI, 05-440-1B, prepared as instruction manual told). AD-MSCs should be changed osteogenic differentiation medium once every 3 days. Identification by Alizarin Red (VivaCell, C37C00150) staining were carried out, after differentiation of 21 days. For chondrogenic differentiation, AD-MSCs were digested by 0.125% trypsin as described before, resuspended by fresh MSC culture medium and seeded into 96-well plates containing 1×10^5cells/well. MSC culture medium was replaced by osteogenic differentiation medium（BI，05-220-1B, prepared as instruction manual told）at 90% confluence.AD-MSCs should be changed osteogenic differentiation medium once every 3 days. Identification by Alcian Blue (VivaCell, C37B00150) staining were carried out, after differentiation of 21 days.

The staining results showed in Figure 2.

6. Flow cytometry

Cells in flasks ready for the fifth passage were detached from the flask by 0.125% trypsin , and cells suspension were washed with PBS , then adjusting the cells density of 1×10^6 cells/mL. The cells were incubated with hMSC Positive Cocktail : anti-CD90 (conjugated to FITC,Clone:5E10),anti-CD105(conjugated to PerCP-Cy5.5,Clone:266), anti-CD73(conjugated to APC,Clone:AD2), anti-CD44(conjugated to PE,Clone:G44-26), hMSC Negative Cocktail(conjugated to PE, CD34, Clone:581, CD11b, Clone:ICRF44, CD19, Clone:HIB19, CD45, Clone:HI30, HLA-DR,Clone:G46-6), hMSC Positive Isotype Control Cocktail, hMSC Negative Isotype Control Cocktail for 30 minutes. All antibodies were purchased from BD Biosciences (Human MSC Analysis Kit, 562245). Data was acquired and analyzed by flow cytometry (BD AriaII)

The expression of immunophenotyping showed in Figure 3.

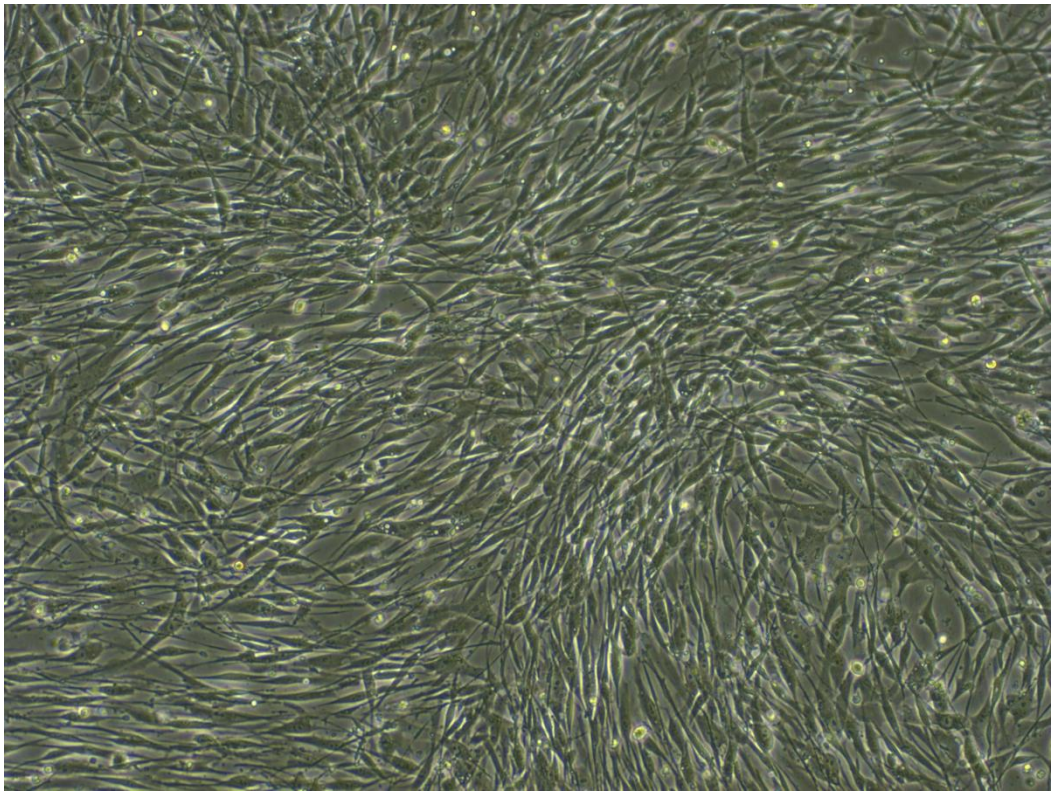


Figure 1 Images of AD-MSCs. Cultured AD-MSCs were spindle-shaped with a fibroblast-like morphology under microscope.

Table 1 Biological safety and biological activity analysis

	Contents	ADMSCs
Cell Characteristics	Cell morphology	Adherent growth, Spindle-shape morphology
	Short tandem repeats (STRs)	Each STR locus has 1-2 alleles
	Cell viability	96%
	Cell cycle analysis	G0/G1 41.3% S 31.2% G2/M 20.9%
	Isozyme analysis	B-type of human origin
Biological safety analysis	Species identification and cell cross-contamination between species	Negative
	Bacteria and fungi	Negative
	Mycoplasma	Negative
	Human papilloma virus (HPV)	Negative
	Human immuno deficiency virus I (HIV- I)	Negative
	Epstein-barr virus (EBV)	Negative
	Human hepatitis C virus (HCV)	Negative
	Human cytomegalovirus (HCMV)	Negative
	Human hepatitis B virus (HBV)	Negative
	Human herpesvirus 6 (qPCR)	Negative
	Human herpesvirus 7 (qPCR)	Negative
	Human papillomavirus	Negative
	Reverse transcriptase activity	Negative
	Bovine virus	Negative
	Porcine virus	Negative
	Bovine serum albumin residuals	＜5 ng/mL
	Endotoxin level	＜0.5 EU/mL
	Hemagglutination test of 9- to 11-day-old chick embryo allantoic fluid	Negative
	Survival rate of 5- to 6-day-old chick embryos	＞90%
Biological activity analysis	Cell surface antigen analysis	CD73 96.6.%, CD90 99.8%, CD105 97.3%, CD44 99.1% CD11b, CD19, CD34, CD 45, HLA-DR ≤1%
	Cell differentiation ability	Differentiated into adipocytes, osteoblasts and chondrocytes in vitro
	immunophenotypic properties	Lymphocyte proliferation inhibition ration 45.6%

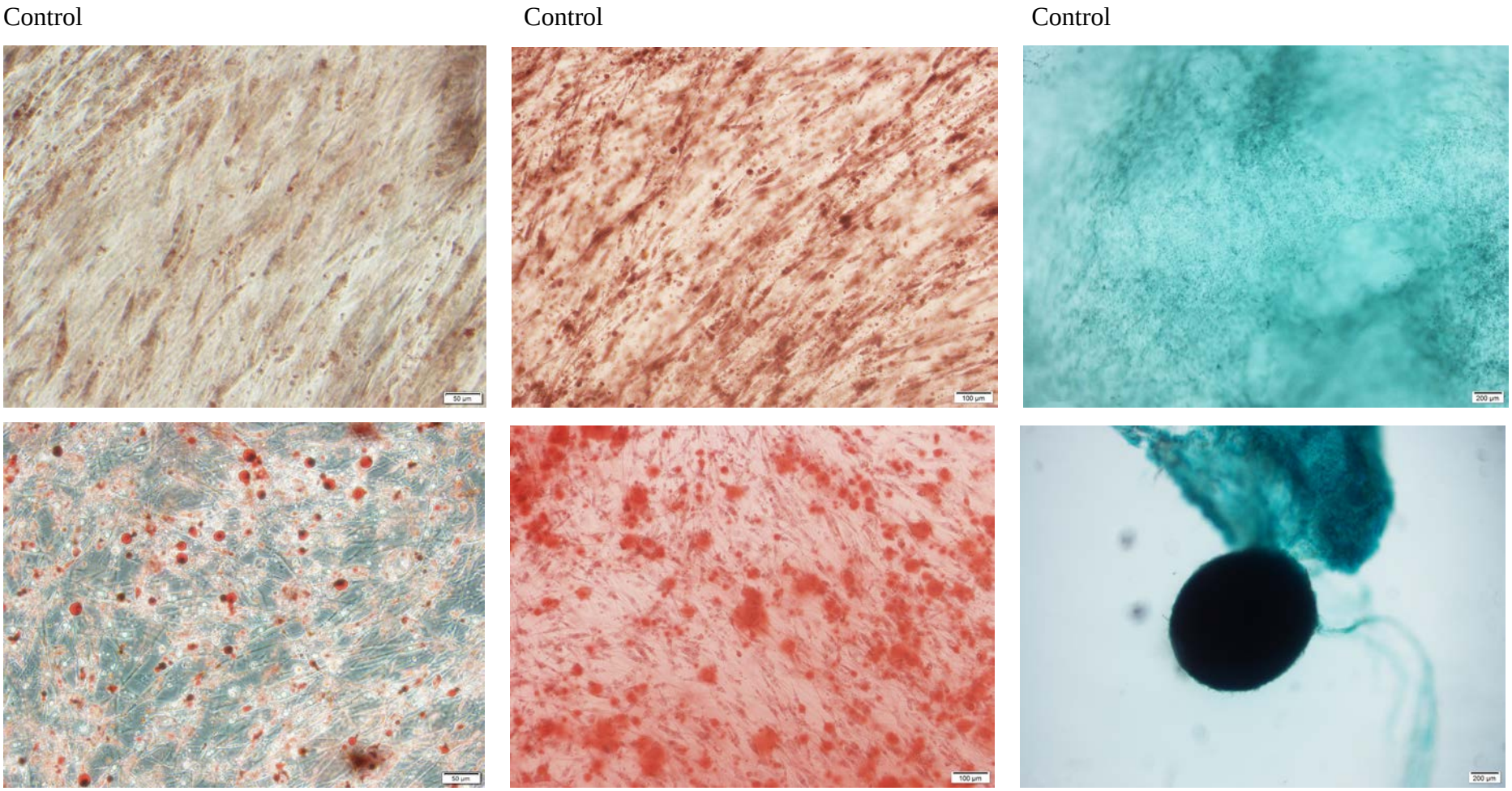
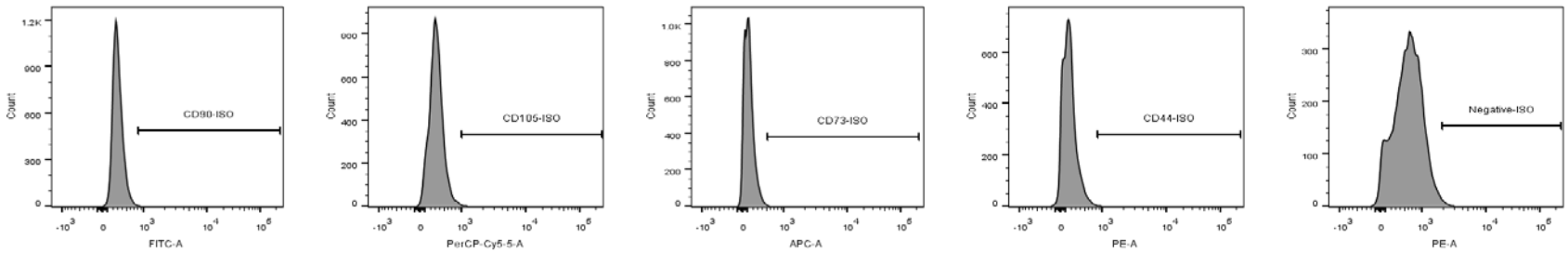


Figure 2 The differentiation capacity of adipose tissue-derived mesenchymal stem cells (AD-MSCs). Oil-red O staining (VivaCell, C37A00150)

was performed to detect the presence of lipids, thereby confirming adipogenic differentiation. Likewise, osteogenic and chondrogenic differentiation were confirmed by the presence of mineralized plaques and cartilaginous substrates, respectively, as shown by Alizarin Red (VivaCell, C37C00150) and Alcian Blue (VivaCell, C37B00150) staining ,respectively.



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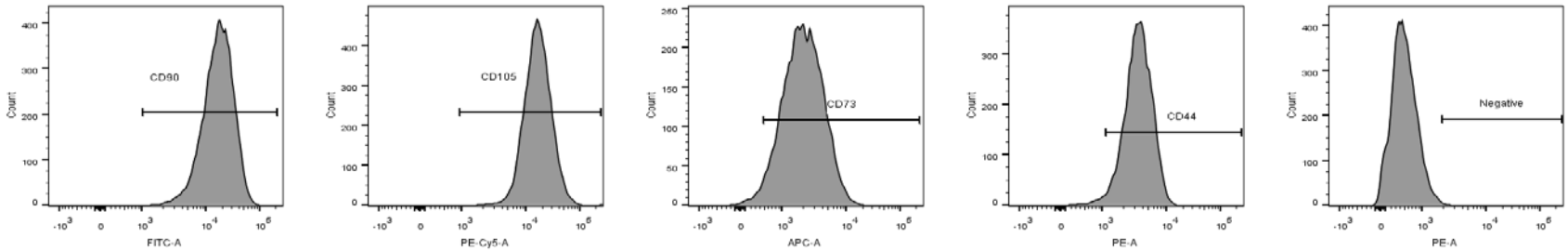

CD90:99.8% CD105:97.3% CD73:96.6% CD44:99.1% Negative:0.5%

Figure 3 Cytometry analysis of hAD-MSCs. Cells express CD73, CD44, CD105 and CD90, but not CD34, CD45, CD11b, CD19 and HLA-DR (negative cocktail).