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In vitro isolation, culture and identification of adipose-derived stem cells*★

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Abstract

BACKGROUND: Adipose-derived stem cells are easily collected and abundantly cultured, which can proliferate rapidly when being cultured *in vitro*. With multi-directional differentiation potential, adipose-derived stem cells are expected as seed cells for tissue engineering.

OBJECTIVE: To isolate, culture and identify of adipose-derived stem cells from Sprague-Dawley rats *in vitro*.

METHODS: The subcutaneous adipose tissue was obtained from the iliac region of rats under the aseptic condition, and then was digested with 0.075% type Ⅰ collagenase and cultured *in vitro*. The morphology and proliferation characteristics of the cells were observed under an inverted phase contrast microscope. The third passage was put into gauge for growth curve by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and the cells were also identified by CD44, a stem cell marker, with immunofluorescence staining. Adipose-derived stem cells were induced and differentiated into adipocytes in Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 containing 10% fetal bovine serum, dexamethasone and insulin, and then the cells were identified with oil red "O" staining. Adipose-derived stem cells were induced and differentiated into neural cells, and then the cells were identified with immunohistochemical staining.

RESULTS AND CONCLUSION: The growth curve of adipose-derived stem cells was opposite-like "S" shape, and it strongly expressed CD44 that can designate a stem cell. The passage cells were exposed to a defined medium for adipocyte differentiation, and then could be stained with oil red. After being induced and differentiated into nerve cells, the cells expressed neuron-specific enolase. The adipose-derived stem cells of Sprague-Dawley rats are characterized by easy isolation, culture and proliferation *in vitro*, expressing related phenotypes of mesenchymal stem cells, as well as induction and differentiation under certain conditions.

Subject headings: stem cells; adult stem cells; cell differentiation; mesenchymal stem cells; adipogenesis; collagenases

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INTRODUCTION

In 1960s, Rodbell *et al* [1] for the first time reported the method to isolate cells from adipose tissue, and then the isolation method was constantly improved. In 2000s, Zuk *et al* [2-3] were the first to isolate pluripotent stem cells from adipose tissue, called adipose-derived stem cells, using liposuction method. These adipose-derived stem cells cultured *in vitro* can be induced to differentiate into adipocytes, osteoblasts, chondrocytes, and nerve cells. A large number of adipose-derived stem cells can be harvested from human adipose tissue, and the cells cultured *in vitro* are in fibroblast morphology, characterized as a stable multiplication effect and aging decrement. In recent years, adipose-derived stem cells demonstrate the potential advantages in tissue engineering. In comparison with bone marrow mesenchymal stem cells, adipose-derived stem cells are from embryonic mesoderm, with a strong proliferation and differentiation potential. Adipose-derived

stem cells are also characterized as a wide variety of sources, easily obtained, less pain to patients, rapid proliferation and no ethical issues, which are becoming an ideal source of stem cells for bioengineering research[4-9]. This study aimed to observe the isolation and culture of adipose-derived stem cells and biological characteristics for induced differentiation to adipocytes, thereby providing experimental basis for the application of adipose-derived stem cells in tissue engineering research in the future.

MATERIALS AND METHODS

Design

In vitro cytological observation.

Time and setting

The experiment was completed at the Medical Research Center, the First Affiliated Hospital of Xinjiang Medical University from June 2011 to January 2012.

Materials

Animals: Two healthy male Sprague Dawley rats, specific pathogen-free grade, weighing 160−180 g, were provided by the Experimental Animal Center, the First Affiliated Hospital of Xinjiang Medical University. All animal procedures were in accordance with animal ethics standards[10].

Main reagents and instruments for isolation, culture and identification of adipose-derived stem cells are as follows.

Reagent and instrument	Source
Low-glucose Dulbecco's modified Eagle's medium (DMEM) medium, Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 (DMEM/F12) medium, rabbit anti-rat CD44 antibody, fluoresceine isothiocyanate (FITC) labeled rabbit anti-rat IgG, rat neuron-specific enolase antibody, rat epidermal growth factor antibody, rat basic fibroblast cell growth factor antibody	Wuhan Boster Bio-engineering Limited Company
Fetal bovine serum, 0.25% trypsin	Hyclone
Type I collagenase, green streptomycin dope	Gibco
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetr azolium bromide (MTT), insulin, dexamethasone	Sigma
Dimethyl sulfoxide	Amresco
Low temperature centrifuge machine, CO_2 incubator, and microplate reader	Thermo
Inverted phase contrast microscope and photomicrography system	Olympus
Culture flasks and culture plates	Corning

Methods

In vitro isolation, culture and passage of adipose-derived stem cells

Sprague Dawley rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (3 mL/kg) under sterile conditions, to extract the inguinal adipose tissue. After removing soft tissue and small blood vessels, the tissues were washed with PBS three times. Then, according to the method of Zuk *et al* [2], the tissues were cut into pieces by a microsurgery scissor, digested with 0.075% type Ⅰ collagenase at 37 ℃ for 40 minutes and centrifuged at 1 000 r/min (centrifugal radius, 150 mm) for 10 minutes. After the removal of supernatant and PBS resuspension, the samples were filtered using 400-mesh filter and then centrifuged at 1 000 r/min (centrifugal radius, 150mm) for 5 minutes to harvest sediment. The sediment was even mixed with low-glucose DMEM medium containing 10% fetal bovine serum, and cultured in a 37 ℃, 5% CO_2 incubator. After 24 hours, the medium was changed for the first time, and then it was changed every 3 days. When the cells reached over 90% confluence, the cells were digested with 0.25% trypsin/ethylene diamine tetraacetie acid and then passaged at a ratio of 1:2.

MTT determination of growth curve of adipose-derived stem cells

The third generation of adipose-derived stem cells were digested with trypsin to prepare single cell suspension, and then seeded in 96-well plates at a concentration of 5×10^3 per well. After cultured 1−8 days, 20 μL of 5 g/L MTT solution was added for continuous culture in the dark for 4 hours. After liquid removal, 150 μL dimethyl sulfoxide solution was added per well, and the samples were placed on the micro-oscillator at room temperature for 10 minutes. Absorbance value at 490 nm was detected using the microplate reader, and cell growth curve was drawn.

Identification of stem cell surface markers

The third generation of cells were seeded on 6-well plates with sterilized coverslip. The sample was placed in 60 mm petri dish, rinsed with PBS three times, fixed in cold acetone for 8 minutes, washed with PBS 5 minutes×3, fixed with 3% H_2O_2 for 10 minutes, washed with PBS 5 minutes×3. The diluted rabbit anti-rat CD44 antibody was added to the sample overnight at 4 ℃ wet box. After washed with PBS 5 minutes×3, the diluted FITC-labeled rabbit anti-rat IgG was added to the sample at room temperature for 1 hour. Then, the sample was washed with PBS (pH 7.4) 5 minutes×3, and cemented with

4,6-diamino-2-phenyl indole. Fluorescence microscope (Olympus) was used to take photos in the darkroom.

Adipogenic differentiation of adipose-derived stem cells and identification using oil red "O" staining

The third generation of adipose-derived stem cells were divided into experimental and control groups, three wells per group. After digestion, the cells were collected and counted. Cells were seeded into 6-well plates with sterilized coverslip at a density of 5×10^4 cells per well, and then cultured in adipogenic induction medium: DMEM/F12 medium containing 10% fetal bovine serum, 1 μmol/L dexamethasone, and 10 μmol/L insulin (experimental group), or DMEM/F12 medium containing 10% fetal bovine serum (control group). The culture medium was exchanged every 3 days. After 14 days of induction, the culture slide was rinsed with distilled water, and incubated and sealed with oil red O dilutions in the dark for 10–15 minutes. Before usage, 6 mL stock solution was taken, 4 mL distilled water was added for standing 5–10 minutes, and then, the sample was filtered twice and used within 2 hours. The sample was differentiated after addition of 60% ethanol under microscopy till the gap was clear, and then washed with water. Mayer's hematoxylin nuclear staining was done for 1 minute followed by washing. The sample was mounted with glycerogelatin glue, and observed under the phase contrast microscope. Digital camera was used to take photos. In the control group, the same procedures were done.

Neuronal differentiation of adipose-derived stem cells and identification

After centrifugation, the third generation of cells were resuspended using neural stem cell culture medium and seeded into 6-well plates for neuronal differentiation. After 6 days of directional induction, the cell culture slide was placed in 60 mm petri dish, rinsed with PBS (pH 7.4) three times, fixed in cold acetone for 8 minutes, washed with PBS (pH 7.4) 5 minutes×3, fixed with 3% H_2O_2 for 10 minutes, washed with PBS (pH 7.4) 5 minutes×3. The diluted first antibody was added to the sample overnight at 4 ℃ wet box. After washed with PBS (pH 7.4) 5 minutes×3, 50–100 μL horseradish peroxidase anti-mouse/anti-rabbit polymer was added to the sample at room temperature for 10 minutes. Then, the culture slide was washed with PBS (pH 7.4) 5 minutes×3. After removal of PBS, 100 μL fresh 3,3'-diaminobenzidine was added to the culture slide for coloration under microscope. After rinsing with tap water, the culture slide was couterstained with hematoxylin, and treated with 0.1% HCl for differentiation. After rinsing with tap water, the culture slide was dehydrated, transparent, mounted with neutral balsam, and observed under phase contrast microscope. Digital camera was used to take photos.

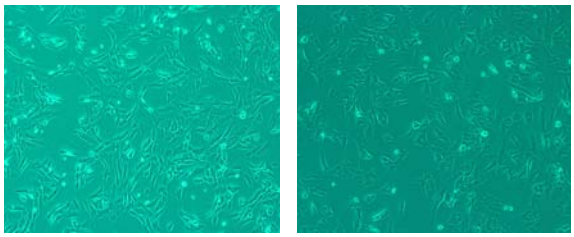
Main outcome measures

(1) *In vitro* morphology of adipose-derived stem cells and growth curve reflecting the proliferative capacity; (2) CD44 expression in adipose-derived stem cells; (3) identification results of adipogenic and neuronal differentiation of adipose-derived stem cells.

RESULTS

Morphology of cultured cells

Under the inverted phase contrast microscope, primarily cultured adipose-derived stem cells were round or oval, adherent cells were spindle-shaped and cells were proliferated to form colonies in fish-like or spiral arrangement. After primary culture for 8–10 days, more than 90% of cells were confluent. After passage, fusiform cells appeared and grew fast with the same shape and size, and no significant difference in the morphology was found between different cell generations (Figure 1).



A: Fusiform cells appeared at passage 3, showing the same shape and size

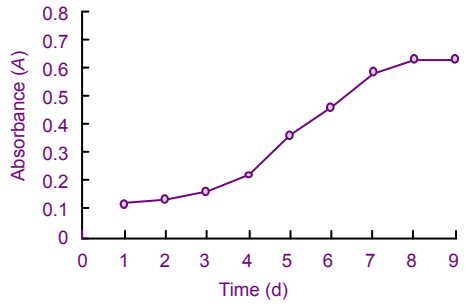
B: Fuisform cells appeared at passage 5, showing the same shape and size

Passaged cells were fusiform, and there was no difference in the cell morphology between different generations.

Figure 1 Morphology of adipose-derived stem cells in culture (Inverted phase contrast microscope, ×100)

Growth curve of cultured adipose-derived stem cells

Growth curves obtained by MTT assay showed active adipose-derived stem cells with strong proliferation ability were in logarithmic growth. At 1 or 2 days, the cells were in the incubation period; from the 3rd day, the cells began to enter the logarithmic phase, and reached a peak at day 8 followed by entering the plateau phase. The cell growth curve was in "S" shape, shown in Figure 2.

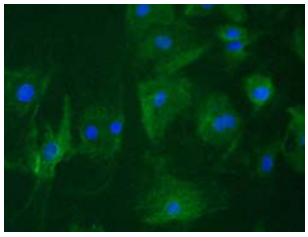


The third generation of adipose-derived stem cells showed an “S” shaped growth curve. The cells showed a logarithmic growth at 3 d, and plateau growth at 8 d.

Figure 2 Growth curves of adipose-derived stem cells at passage 3

Cultured adipose-derived mesenchymal stem cells possess stem cell properties

Immunofluorescence staining showed that adipose-derived stem cells strongly expressed CD44 that existed in the cytoplasm. Cell outline was clear shown by green fluorescence, nuclei were stained with 4,6-diamino-2-phenyl indole (blue), in line with mesenchymal stem cells properties (Figure 3).



Cytoplasmic expression of CD44 was found in adipose-derived stem cells, with blue nuclei, in line with the characteristics of mesenchymal stem cells.

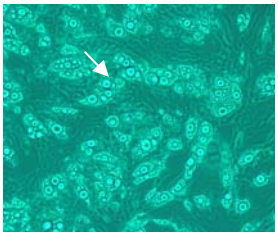
Figure 3 Immunofluorescence showed CD44 positive expression in adipose-derived stem cells (×200)

Adipogenic differentiation of cultured adipose-derived stem cells

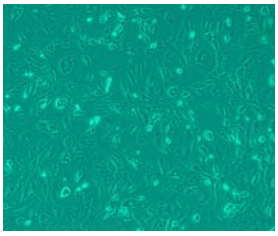
After adipogenic differentiation, the polygonal cells were seen. Small intracellular lipid droplets were visible at day 3, and became significantly larger at day 7, which are positive for oil red “O” staining. In the control group, no lipid droplets appeared (Figure 4).

Neuronal differentiation of cultured adipose-derived stem cells

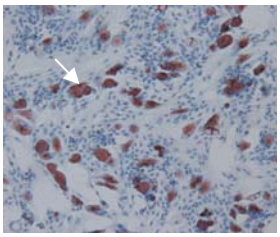
After neuronal differentiation, the cells were extended, and the longer protrusions formed and were mutually connected, all of which showed neuron-like cell morphology. Immunohistochemistry examination showed that the induced cells expressed neurons specific enolase (Figure 5).



A: In the experimental group, lipid droplets appeared obviously at 8 d after adipogenic differentiation of adipose-derived stem cells (arrow)

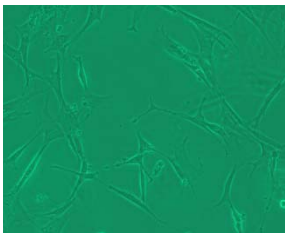


B: In the control group, there were no obvious abnormalities at 8 d during normal culture of adipose-derived stem cells

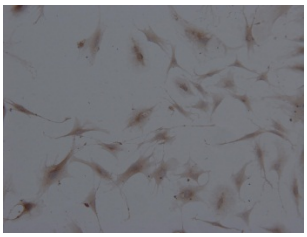


C: In the experimental group, the lipid droplets were positive for oil red "O" staining at 14 d after adipogenic differentiation of adipose-derived stem cells

Figure 4 Adipose-derived stem cells were induced into adipocytes and identified with oil red “O” staining (×200)



A: The longer protrusions were visible at 6 d after neuronal differentiation of adipose-derived stem cells



B: The induced cells expressed neuron-specific enolase at 6 d

After neuronal differentiation, the induced adipose-derived stem cells showed neuron-like morphology.

Figure 5 Adipose-derived stem cells were induced into neural cells and identified with neuron-specific enolase (×200)

DISCUSSION

Stem cells are a class of self-renewal and pluripotent cells in human and animal body. According to the developmental stage, the stem cells are divided into

embryonic stem cells and adult stem cells[11-22]. Embryonic stem cells are highly undifferentiated cell derived from the inner cell mass of a blastocyst, with developmental pluripotency, but embryonic stem cells are limited in clinical application because of its limitations in regulation of cell differentiation and ethical problem[23]. Adult stem cells, in certain conditions, have mesodermal differentiation capacity, which brings new hope for clinical treatment of various diseases[24]. Therefore, many scholars focus on “adult stem cells”. Adipose-derived stem cells as a new kind of adult stem cells are found in recent years, and have been paid more attentions because of its wide variety of sources, easily obtained, less pain to patients, rapid proliferation and no ethical issues[25-26].

There are mainly four methods for isolation and purification of adipose-derived stem cells: immunomagnetic bead method, flow cytometry method, density gradient centrifugation, enzyme digestion and adherent separation[27-28]. In this study, we successfully isolated and cultured adipose-derived stem cells from Inguinal adipose tissue of Sprague-Dawley rats using enzymatic digestion and adherent separation, and then the cultured cells were passaged and purified. It has been confirmed that these cells demonstrated strong proliferation and differentiation capacity. MTT assay showed the growth curve of adipose-derived stem cells was similar to “S” shape, in line with the growth rule of normal stem cells[29]. Adipose-derived cells are most active for proliferation at passage 3 and remain a stronger proliferative capacity at passage 10. CD44 is a marker which is expressed most widely in adult stem cells[30], and distributed in the cell membrane and cytoplasm. Presence of CD44 can further identify stem cells. In our study, adipose-derived stem cells strongly expressed CD44.

We found that adipose-derived stem cells could differentiate into adipocytes when only cultured in DMEM/F12 medium containing 10% fetal bovine serum, dexamethasone and insulin, without adding of other ingredients, which are inconsistent with national reports[31-32]. During the whole process of induced differentiation, adipose-derived stem cells gradually change from long spindle to round or polygonal at 3 days, and small lipid droplets were seen; at the end of 1 week, small lipid droplets could been found in the majority of induced cells. After 2 weeks, lipid droplets which were positive for oil red “O” staining increased in number and merged mutually, indicating that the induced cells had differentiated into adipocytes. Oil red “O” could be dissolved in lipid droplets showing orange. In this study, staining methods were also improved. Without formalin fixation, the direct dyeing effect was good only after washing with distilled water. Studies have shown that the specific mechanisms underlying adipogenic differentiation of adipose-derived stem cells may be related to the adipogenic medium that stimulate the differentiation of pluripotent stem cells to fat mother cells, and activate specific gene expression regulating adipogenic differentiation[33-34]. Another study has shown that adipose-derived stem cells can differentiate into adipocytes possibly through intracellular signaling pathways[35-36]. Neuron-specific enolase is a marker of neurons, and the expression of this marker can confirm that the adipose-derived stem cells can be induced *in vitro* into neuron-like cells under specific inducers. Ashjian *et al* [37] not only found neuronal morphology and neuron-specific expression of the signal, but also discovered electrophysiological phenomena after neuronal differentiation of adipose-derived stem cells. After induction, potassium channels were observed and delayed rectifier potassium currents were recorded, which prompts that the early development of ion channels generates after neural induction. It is similar to the function of mature nerve cells.

In conclusion, with the continuous in-depth study of functional properties and molecular mechanisms, adipose-derived stem cells, based on their own merits, can have broad application prospects in tissue engineering construction.

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脂肪源性干细胞的体外分离培养与鉴定**★

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文章亮点：

1 文章特点在于成功运用胶原酶Ⅰ消化法从脂肪组织中分离培养出数量多、纯度高、活力强的大鼠脂肪源性干细胞，此方法克服了其他方法容易导致的细胞活力低下、易分化、纯度不高等缺点。

2 对成脂诱导过程中发现，体外成脂诱导培养基 DMEM/F12 里含有体积分数 10%胎牛血清、地塞米松和胰岛素就能刺激脂肪源性干细胞向脂肪分化，无需添加其他成份，既往研究往往需要添加吲哚美辛等。

3 文章创新点在于获取的体外分离培养的脂肪源性干细胞性状稳定，易于体外扩增，可行性高。

关键词：

干细胞；脂肪干细胞；细胞培养；成脂诱导；免疫荧光法；干细胞鉴定；省级基金；干细胞图片文章

主题词：

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摘要

背景：脂肪间充质干细胞来源丰富、取材方便、体外有较强增殖能力并具有多向分化的潜能，有望成为组织工程的种子细胞。

目的：体外分离培养 SD 大鼠脂肪干细胞并进行鉴定。

方法：取 SD 大鼠腹股沟区皮下脂肪组织，0.075%Ⅰ型胶原酶消化分离、培养脂肪源性干细胞，倒置相差显微镜下观察脂肪源性干细胞的细胞形态和增殖特征。取第 3 代细胞用 MTT 比色法描绘生长曲线，免疫荧光法鉴定干细胞标志物 CD44，含有体积分数 10%胎牛血清、地塞米松和胰岛素的 DMEM/F12 定向诱导成脂分化，油红“O”染色成脂分化鉴定。免疫组化法鉴定向神经细胞诱导分化的结果。

结果与结论：分离出的大鼠脂肪源性干细胞生长曲线呈“S”形，强烈表达干细胞标志物 CD44。成脂诱导分化经油红“O”染色呈橘红色。向神经细胞诱导分化后表达神经元标志物神经元特异性烯醇化酶。说明 SD 大鼠脂肪源性干细胞在体外具有易于分离培养和扩增，表达间充质干细胞相关表型，特定条件下可诱导分化的特点。

*作者贡献：*第一作者进行实验设计，第一、三、四、五作者实施，第二作者进行评估，资料收集为第一、三、四、五，第一作者成文，通讯作者审校，第一、通讯作者对文章负责。

*利益冲突：*课题未涉及任何厂家及相关雇主或其他经济组织直接或间接的经济或利益的赞助。

*伦理要求：*实验过程中对动物的处置应符合 2009 年《Ethical issues in animal experimentation》相关动物伦理学标准的条例。

*学术术语：*脂肪抽吸术—利用负压吸引和/或超声波、高频电场等物理化学手段，通过较小的皮肤切口或穿刺，将预处理的人体局部沉积的皮下脂肪祛除，并结合脂肪颗粒注册移植等技术，以改善形体的一种外科手术，特点为封闭、钝性、非连续性切割。

*作者声明：*文章为原创作品，数据准确，内容不涉及泄密，无一稿两投，无抄袭，无内容剽窃，无作者署名争议，无与他人课题以及专利技术的争执，内容真实，文责自负。

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