

Adipose-derived stem cells from the breast

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Background: The adipose tissue is deemed as an ideal source of adipose-derived stem cells (ADSCs). Previous studies have reported that ADSCs can be isolated from several organs and locations; however, slight attention has been paid to the breast. We would like to report our experiences in isolating breast ADSCs (bADSCs). **Materials and Methods:** Adipose tissues were harvested from the breasts of seven hypertrophic breast patients. Collagenase I was used to isolate the primary ADSCs. Surface markers were analyzed by flow cytometry. Cellular morphologies were observed. Proliferations of different passages were compared. Viabilities after the cryopreservation were evaluated. Adipogenic and osteogenic differentiation was induced. **Results:** Primary cultured cells showed morphologies similar to fibroblasts, and expressed surface markers including CD13, CD44, CD90, and CD105. There was no statistical difference of proliferation between different passages ($P > 0.05$) and between with and without cryopreservation ($P > 0.05$). Additionally, isolated cells were differentiated into adipocytes and osteoblasts. **Conclusion:** bADSCs may represent an alternative candidate for tissue engineering. Further studies are needed to obtain more comprehensive understanding on bADSCs.

Key words: Adipose-derived stem cells, breast, mammary gland

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INTRODUCTION

Since the first report of adipose-derived stem cells (ADSCs) by Zuk *et al.*^[1,2] in 2001, these stem cells have been continually researched in the past decade^[3-8] and deemed as an alternative stem cell to bone marrow-derived mesenchymal stem cells. Tissue engineering using ADSCs has shown great promises for soft tissue reconstruction.^[9-11]

ADSCs can be harvested from many locations, such as beneath the skin and around the internal organs.^[12,13] In breasts, clear boundaries can be identified between the adipose tissue and the mammary gland, which allows the harvesting of adipose tissue and the subsequent obtainment of ADSCs.

It has been reported that the characteristics of ADSCs from different locations are different.^[14] Characteristics of breast ADSCs (bADSCs) are not very clear yet, which limits their potential applications in tissue engineering. Additionally, it has been reported that bADSCs promote breast cancer progression.^[15] bADSCs would be potentially meaningful in stem cell therapy of breast cancer.

However, only slight attention has been paid to bADSCs so far and more understanding on them is needed. In the

present study, bADSCs were isolated from hypertrophic breasts and their multiple differentiation potentials were evaluated. Aim of this study was to provide some preliminary evidences of bADSCs for further comprehensive studies.

MATERIALS AND METHODS

The experiment was approved by the ethics board of Union Hospital (Wuhan, China) and conducted in accordance with the Declaration of Helsinki.^[16] Adipose tissues were harvested from the breasts of seven hypertrophic breast patients between July 2011 and April 2012 [Figure 1]. All patients had been informed preoperatively. Scalpels were used to avoid potential heat damages. Patients' ages ranged from 23 to 53 years (mean = 35.2) and their body mass indexes ranged from 20.3 to 23.8 kg/m² (mean = 22.9).

Isolation of bADSCs

In a super clean bench, isolation was performed according to our previous experiences.^[17] After digestion with collagenase I (Invitrogen, Carlsbad, USA), centrifugation, and filtration, the obtained cell fractions were cultured with Dulbecco's Modified Eagle Medium (DMEM)/F12 (Boster Biotech, Wuhan, China) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, USA), 100 U/ml of penicillin, and 100 mg/ml of streptomycin (Boster Biotech, Wuhan, China) at 37°C

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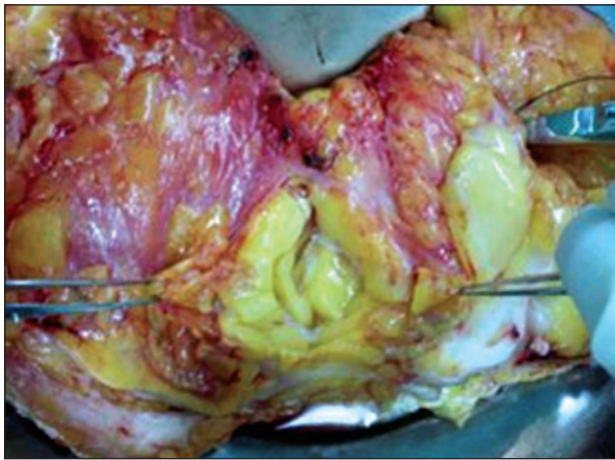


Figure 1: Resected hypertrophic mammary gland

in the presence of 5% CO₂ for 48 h. Then, the non-adherent fractions were removed and the remaining cells were washed twice with phosphate-buffered saline (PBS; Boster Biotech, Wuhan, China). The medium was replaced every 3 days thereafter. When 80% confluence was reached, the cells were digested and reseeded into T25 flasks (Boster Biotech, Wuhan, China) at ratio of 1:2.

Flow cytometry of surface markers

Cells were treated with phycoerythrin (PE)-, fluorescein isothiocyanate (FITC)-, allophycocyanin (APC)- or peridinin chlorophyll protein complex (PerCP)-conjugated rabbit anti-human monoclonal antibodies against CD13, CD14, CD34, CD44, CD45, CD71, CD90, and CD105 (Beckton Dickinson, Franklin Lakes, USA), respectively. All samples were then analyzed by flow cytometry (Beckton Dickinson, Franklin Lakes, USA) with CellQuest Pro software (Beckton Dickinson, Franklin Lakes, USA).

Immunofluorescence analysis

Cells were seeded into 6-well plates (Boster Biotech, Wuhan, China) at a density of 1.5×10^4 cells/well and then incubated at 37°C in the presence of 5% CO₂. Seventy-two hours later, the cells were fixed with 4% paraformaldehyde (Boster Biotech, Wuhan, China) for 2 min and processed with 0.5% Triton X-100 for 10 min. After washing with PBS, the cells were blocked in 5% bovine serum albumin (Boster Biotech, Wuhan, China) for 1 h at 24°C. Then the cells were washed and incubated at 4°C with antibodies in a ratio of 1:100 (CD34, CD44, CD45, CD105, and vimentin; Gene Tex, Alton Pkwy, USA) for 12 h. And then, the cells were incubated with fluorescence labeling rabbit anti-mouse antibodies in a ratio of 1:100 (AP-, PE-, and PerCP-conjugated; Beckton Dickinson, Franklin Lakes, USA) at 24°C for 10 min. After washing, the cells were mounted in PBS-glycerol (Boster Biotech, Wuhan, China). The images were acquired with an inverted fluorescence microscope (Olympus, Tokyo, Japan).

In vitro proliferation assessment

Cells from the 3rd passage (P3), 5th passage (P5), and 10th passage (P10) were seeded into 96-well plates (Boster Biotech, Wuhan, China) with a density of 4×10^3 /ml and a volume of 100 µl/well, respectively. Proliferation was measured with Cell Counting Kit (CCK)-8 Kit (Dojindo, Kumamoto, Japan) every day at 450 nm using Multiskan GO reader (Thermo Scientific, Waltham, USA). The optical densities were analyzed.

Cryopreservation and resuscitation

Cells in P3 were harvested and the survival rates were evaluated by trypan blue staining. Cells were then stored in liquid nitrogen tank (MVE-Chart, Garfield Heights, USA) at -80°C for 3 months. Survival rates were evaluated again after 3 months of cryopreservation. Then the cells were cultured at 37°C in the presence of 5% CO₂, the proliferations were measured with CCK-8 Kit, and the growth curves were made.

Ultrastructural observation

Cells in P3 were harvested and centrifuged, and then were fixed in 2.5% glutaric dialdehyde (Boster Biotech, Wuhan, China). After incubation at 4°C for 12 h, the cells were fixed in 1% osmic acid (Boster Biotech, Wuhan, China) at 4°C for 1 h, dehydrated in graded acetone series, and embedded in Epon 812 (Boster Biotech, Wuhan, China). Ultrastructural identification was performed under Tecnai G220TWIN transmission electron microscope (Fei, Hillsboro, USA).

In vitro differentiation

Cells in P3 were seeded into 6-well plates at a density of 1.5×10^4 cells/well. Experimental groups were cultured, respectively, with the adipogenic differentiation medium, which was DMEM/F12 supplemented with 10% FBS, 100 U/ml of penicillin, 100 mg/ml of streptomycin, 400 µl insulin (Sigma, St. Louis, USA), 200 µl indomethacin (Sigma, St. Louis, USA), 200 µl 3-Isobutyl-1-methylxanthine (IBMX; Sigma, St. Louis, USA), 2 ml glutamine (Boster Biotech, Wuhan, China), and 200 µl dexamethasone (Boster Biotech, Wuhan, China); and with the osteogenic differentiation medium, which was DMEM/F12 supplemented with 10% FBS, 100 U/ml of penicillin, 100 mg/ml of streptomycin, 400 µl ascorbic acid (Boster Biotech, Wuhan, China), 2 ml β-sodium glycerophosphate (Boster Biotech, Wuhan, China), and 20 µl dexamethasone (Boster Biotech, Wuhan, China). The control group was cultured with DMEM/F12 supplemented with 10% FBS, 100 U/ml of penicillin, and 100 mg/ml of streptomycin. The medium was replaced every 3 days. Three weeks later, the cells were stained with Oil Red O (Boster Biotech, Wuhan, China) and Alizarin Red S (Boster Biotech, Wuhan, China), respectively.

Statistical analyses

Statistical analyses were performed with SPSS 13.0 (SPSS Inc., Chicago, USA). Growth curves of P3, P5, and P10 cells were compared with one-way analysis of variance (ANOVA). Growth curves of cells before and after the resuscitation were compared with paired *t*-test. *P* values lower than 0.05 were considered significant.

RESULTS

Isolation and characterization

Twenty-four hours after isolation, the cells started to attach. Their morphologies were observed [Figure 2].

The immunofluorescence analysis showed that CD44 and CD105 were significantly expressed; on the other hand, the expression of CD34 and CD45 was not significant. Vimentin was expressed in more than 95% of cells [Figure 3].

The cell cycle analysis showed that 90% of cells in P3 were in G0/G1 phase. Obvious expression of CD13, CD14, CD90,

and CD105 was observed. Minimal expression of CD14, CD34, and CD45 was detected [Figure 4].

It was observed with the transmission electron microscope that the cultured cells had prominent nucleoli and cytoplasm, containing lysosomes, rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER), and mitochondria [Figure 5].

In vitro analysis of proliferation

Proliferations of cells in P3, P5, and P10 were in accordance with the description of Zuk *et al.*^[1,2] No significant difference ($P > 0.05$) among P3, P5, and P10 was observed [Table 1].

P3 cells were resuscitated and cultured after 3 months of storage in the liquid nitrogen tank. The survival rate was 85.7%. These cells started to attach 4 h after seeding and reached 80% confluent after 6 days. Growth curves of cells after resuscitation and of normal P3 cells were analyzed;

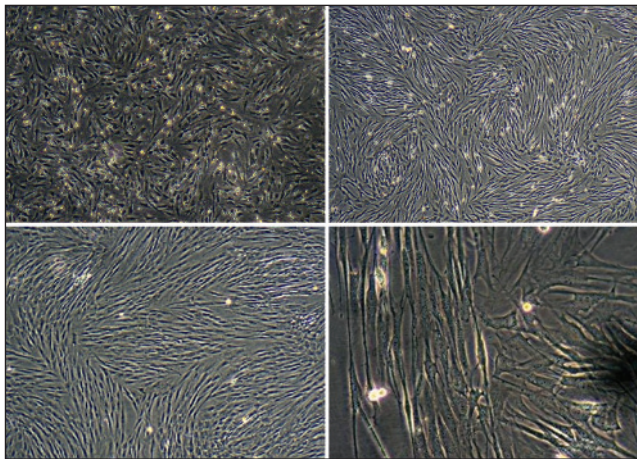


Figure 2: Morphological characteristics of bADSCs: Passage 0 at 48 h (×40, above left); passage 0 at 6 days (×40, above right); passage 3 (×40, below left; ×200, below right)

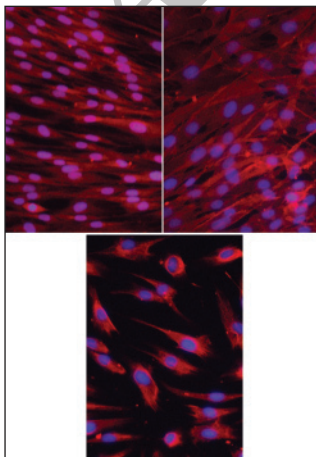


Figure 4: Immunofluorescence staining of bADSCs (×200): CD105+ (left), CD44+ (center), vimentin+ (right)

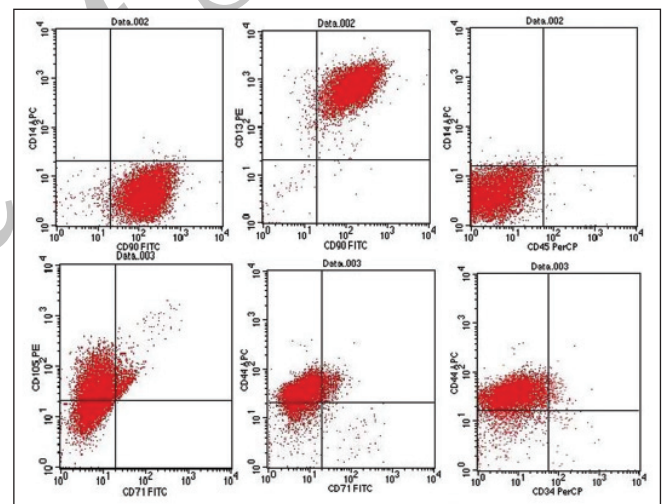


Figure 3: Surface markers of bADSCs determined by flow cytometry

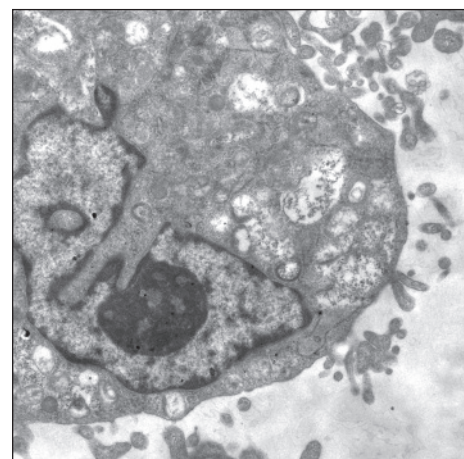


Figure 5: bADSCs under transmission electron microscopy (×2500)

no significant difference was observed between these two groups [Figure 6] ($P > 0.05$).

Adipogenic and osteogenic differentiation

In the adipogenic differentiation group, the morphology was similar to adipocytes. All specimens were stained positive by Oil Red O. In the osteogenic differentiation group, brown mineralized nodules formed in the induced cells, as visualized on Alizarin Red S staining. All samples from the control group were negative [Figure 7].

DISCUSSION

ADSCs have been widely used in tissue engineering because of their remarkable advantages: (1) abundant quantity, (2) multi-differentiation potential, and (3) minimally invasive harvesting.^[18] In the present study is reported our experiences in the isolation, characterization, and differentiation of bADSCs.

Proliferation of bADSCs from P3, P5, and P10 showed no statistical difference. Cell viability and proliferation capability were maintained after cryopreservation. bADSCs significantly expressed mesenchymal cell markers (CD13, CD44, CD90, and CD105). On the other hand, expression of hematopoietic and endothelial markers (CD14, CD34, CD45, and CD71) was not obvious; vimentin was expressed in more than 95% of cells.^[19] When cultured in specific differentiation medium, the cells differentiated into adipocytes and osteocytes. All the above results indicate that the property of these cells is similar to that of mesenchymal stem cells.^[20-27]

To our knowledge, a few studies have focused on bADSCs so far. Zhao *et al.*^[28] have reported the isolation of bADSCs and their differentiation. Results of the present study are in agreement with their results. Additionally, we evaluated the surface marker, ultrastructure, and the proliferation of bADSCs. We believe these together would be important evidences for further bADSCs studies.

The breast adipose tissue and the mammary gland closely exist together in the breast. The mammary gland is composed of myoepithelial cells and luminal epithelial cells, both of which originate from mammary stem cells (MaSCs).^[29,30] MaSCs are theoretically the most suitable seed cells for breast tissue engineering. However, it is difficult to harvest MaSCs abundantly and regulate their differentiation direction. On the other hand, bADSCs can be easily obtained. If adult stem cells from the same microenvironment share similar differentiation capabilities, bADSCs could possibly be potential alternatives of MaSCs in future breast tissue engineering.

The present study has limitations. Only bADSCs from hypertrophic breasts were analyzed and evaluated; however, no bADSCs from normal breasts were isolated or evaluated. There may be significant differences between these two kinds of bADSCs, such as the adipogenic differentiation potential. Additionally, the comparison between bADSCs and ADSCs from other sources was not carried out, which may lead to ignoring the special characteristics of bADSCs. Further studies are needed to obtain more comprehensive understanding on bADSCs.

Table 1: Comparison of bADSCs' optical density from 3rd, 5th, and 10th passages ($n = 5$)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
3 rd passage	0.023±0.009	0.029±0.011	0.063±0.021	0.122±0.026	0.163±0.015	0.190±0.046	0.201±0.059	0.209±0.050
5 th passage	0.016±0.003	0.018±0.009	0.057±0.016	0.137±0.030	0.176±0.034	0.187±0.021	0.197±0.047	0.198±0.072
10 th passage	0.024±0.010	0.029±0.007	0.055±0.002	0.130±0.033	0.165±0.026	0.179±0.038	0.189±0.067	0.190±0.077
P value	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

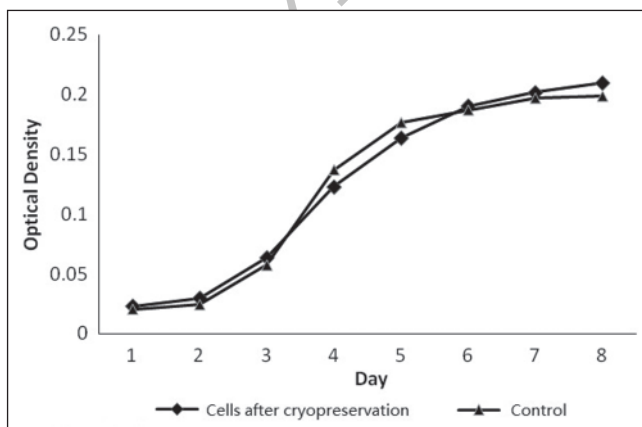


Figure 6: Proliferation of cells with and without cryopreservation ($n = 5$, $P > 0.05$)

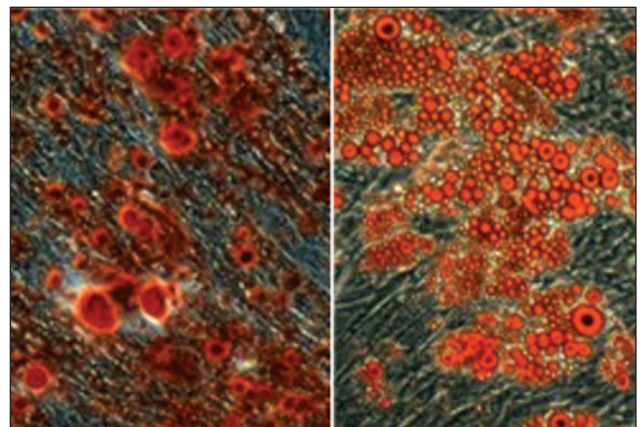


Figure 7: Osteogenic and adipogenic differentiation of cells: Alizarin Red S staining on 21st day ($\times 100$, left), Oil Red O staining on 21st day ($\times 100$, right)

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