

CHARACTERISATION OF ADIPOSE-DERIVED STEM CELLS IN THE STROMA OF BREAST TISSUE FROM NORMAL AND CANCER PATIENTS

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It has been postulated that stem cells derived from malignant breast tumours may play a role in the pathogenesis of breast cancer. The role of the mesenchymal stem cell (MSC) in the adipose tissue and stroma of the breast remains to be defined. The aim of this initial research is to optimize the methods for isolation and characterization of human adipose derived adult progenitor (stem) cells from the adipose tissue and stroma of breast specimens.

This initial research will provide seed cells for further research into the potential role(s) of mesenchymal stem cells within the breast stroma and aid in further understanding the etiology of breast disease.

Methods

Excised adipose tissue was finely minced with a scalpel and collagenase-digested to liberate cells from connective tissue. Following centrifugation and filtration, the resulting cell pellet was seeded into cell culture flasks.

Cells were cultured in media comprising DMEM/F-12 containing 10%FCS, 10ng/ml bFGF and 1% pen/strep. Cells were trypsinised with 0.5mM/0.05% trypsin when subconfluent.

Cells counts were performed at each passage and Population Doublings (PD) and Times (PDTs) were calculated using the following formulae:

$$PD = \frac{\log Y - \log X}{0.301} \quad PDT = \frac{T \times \log 2}{\log Y - \log X}$$

Y = cell nos at end of passage
X = cell nos seeded at start
T = days in culture

Adipose-derived MSCs (Ad-MSCs) between passages 1 to 6 were used for all experiments. Phenotypic characterisation was performed by flow cytometry with a panel of fluorescent-labelled specific antibodies.

Lineage	Induction Media	Lineage markers
Adipogenic	DMEM/F12, 20ng/ml HEPES, 10ng/ml TGF- α (Transferrin), 35ng/ml Biotin, 18 μ M Putrescine, 50ng/ml Insulin, 25ng/ml IGF-1, 10ng/ml Dexamethasone, 10ng/ml TGF- β , 1% Pen/Strep, 5ng/ml FGF	Lipid accumulation (Oil-Red-O), RT-PCR of adipocyte binding protein-2 (ABP2), PPAR- α , C/EBP- α
Myogenic (skeletal)	DMEM/F12, 10% FBS, 5% horse serum and 10ng/ml hydrocortisone, 1% Pen/Strep	RT-PCR of MyoD, myogenin, myosin heavy chain (MHC)
Osteogenic	DMEM, 5.1mM Dexamethasone, 0.05ng/ml Ascorbic Acid Phosphate, 10ng/ml Glycerol phosphate, 10ng/ml 1,25-dihydroxy-Vit. D3, 10% FBS	Calcitonin Receptor Related Receptor (Alkaline Phosphatase)
Chondrogenic	DMEM, 10.1M Dexamethasone, 10ng/ml TGF- β , 50ng/ml ascorbic acid, 2.5ng/ml Vit. B12, 5ng/ml sodium butyrate, 1% Pen/Strep	Glycosaminoglycans (Alcian Blue), Collagen Type 2

Ad-MSCs were induced to differentiate towards the adipogenic, osteogenic, chondrogenic and myogenic lineages in appropriate induction media and at the end, lineage-specific markers were evaluated (Table 1).

Real-time PCR was carried out on the ABI system, using the TaqMan probe from the manufacturer.

48-hour conditioned culture media were collected from samples and layered onto the cytokine arrays (RayBio® Human Cytokine Antibody Array, C series 1000) and detection performed according to manufacturer's instructions.

Results and Discussion

Growth and Expansion Characteristics

Ad-MSCs displayed a fibroblast-like morphology (Figure 1A) at early passages, with a tendency to exhibit larger and flatter morphologies when progressively passaged (greater than 12 passages) (Figure 1B).

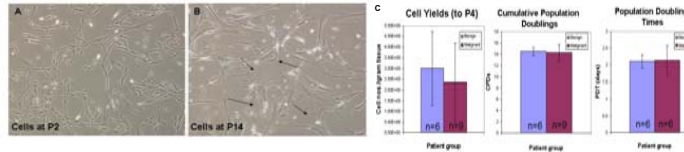


Figure 1

Phase contrast microscopic images of Ad-MSCs at two different stages in culture. A) Ad-MSCs at passage 2 exhibited fibroblast-like morphology. B) Ad-MSCs at passage 14 are larger, with a wide and flat morphology (arrows). All images are at $\times 100$ mag. C) Growth characteristics of Ad-MSCs. Samples were divided into Benign and Malignant groups. All parameters were calculated for cultures expanded to the fourth passage, in order to avoid potential effects of prolonged culture. Results are expressed as Mean \pm Std Dev.

To facilitate comparisons between groups, we computed the expandable cell yields, cumulative population doublings (CPDs) and population doubling times (PDTs) up to the fourth passage. No significant differences between the two patient groups were found for all parameters (Figure 1C), indicating that Ad-MSCs derived from both patient groups exhibited very similar growth characteristics.

Antigen	Benign group, n# (range)	Malignant group, n# (range)
HLA Class I	87.50% (70) (80-97.50%)	87.41% (70) (80-97.50%)
HLA-DR	1.25% (1) (0-2.50%)	13.56% (10) (0-44.30%)
CD29	28.54% (23) (0-74.30%)	38.86% (27) (0-45.00%)
CD44	22.84% (18) (0-53.30%)	1.29% (1) (0-4.30%)
CD73	78.75% (63) (45.00-90.00%)	87.86% (63) (87.50-97.50%)
CD90	0.00%	0.00%
CD105	1.66% (1) (0-5.00%)	2.29% (2) (0-4.30%)
CD166	87.50% (70) (80-97.50%)	87.29% (63) (80-97.50%)
CD182	0.00%	0.00%
CD184	0.00%	0.00%
CD186	0.00%	0.00%
CD188	0.00%	0.00%
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Table 2

Summary of Ad-MSC immunophenotype as determined by single-colour flow cytometric analysis. The table shows percentage of positive cells for the antigen of interest and are expressed as Mean \pm Std Dev.

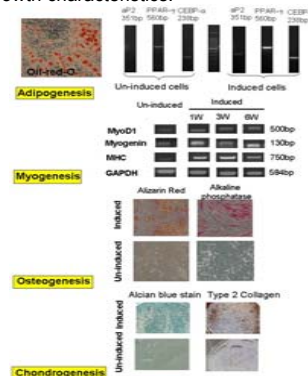


Figure 2

Lineage differentiation of Ad-MSCs. Ad-MSCs were induced to differentiate along 4 lineages in induction media. Differentiation was then confirmed by assessing appropriate markers of differentiation.

Flow cytometric analyses revealed the immunophenotype of Ad-MSCs to be similar for that described for MSCs, i.e. positive for HLA Class I, CD29, CD44, CD73, CD90, CD105 and CD166 (Table 2, Mean $>$ 80%). Reactivities of antibodies against several antigens were significant (Mean \leq 80%) but showed some degree of variation. These included CD9, CD49d and CD271. In some samples, we also detected small populations of cells expressing the Stro-1 antigen, which has been identified as a multipotential marker in bone-marrow MSCs (Dennis et al, 2002). Ad-MSCs were negative for haematopoietic lineage markers CD14, CD34 and CD45. Interestingly, significant percentages of HLA-DR positive Ad-MSCs were found in our samples, notably samples belonging to the Malignant group. MSCs are not known to express HLA-DR. The significance of this observation warrants further investigation.

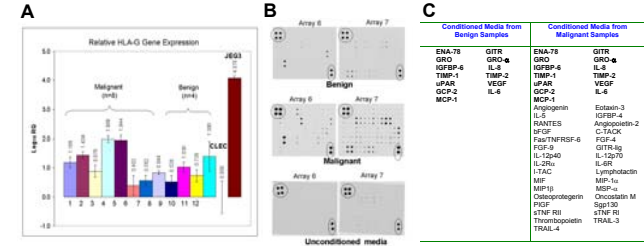


Figure 3

(A) Relative HLA-G expression in Ad-MSCs determined by real-time PCR. The calibrator used for analysis was CLEC (cord lining epithelial cells); JEG3 cells (placental human choriocarcinoma line) were included for comparison. (B) Cytokine array results of conditioned media collected from Ad-MSCs (Benign and Malignant). Representative results from each patient group shown. Circled spots are array's positive control. (C) List of cytokines detected in conditioned media from Ad-MSCs (Benign & Malignant). Cytokines in bold type are found in both sample groups, whilst those in normal type are found only in the Malignant group. Cytokines in bold type (except for IL-6) were significantly up-regulated in Malignant samples ($p \leq 0.05$).

Relative expression levels of HLA-G transcripts were shown to be variable in Ad-MSCs (Figure 3A). However, no significant differences were found between the 2 patient groups.

Detection of cytokines by antibody arrays gave a global profile of secreted cytokines in the conditioned media (Figure 3B&C). The majority of secreted cytokines identified by array analysis possessed mitogenic, chemotactic, metastatic or pro-angiogenic activities. Hence these cytokines are potentially able to enhance tumour progression, through complex interactions between Ad-MSCs, tumour cells, infiltrating immune cells and other resident cell types in the breast stroma.