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Isolation, culture and characterization of mesenchymal stem cells from adipose tissue

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Abstract

Recent reports strongly support the idea that the majority of adult tissues contain stem cells with the potential to replicate and differentiate beyond the limits imposed by the parental tissue. This statement can be extended to organs that apparently do not posses an evident regenerative capacity, such as the brain, heart and pancreas. Thus, the challenge is to identify and isolate these adult stem cells and try to decipher the mechanisms that activate the differentiation events. As with embryonic stem cells, adult stem cells can be expanded in vitro and differentiated towards certain lineages, although the latter still requires substantial improvements. Tissues bearing adult stem cells with special scientific interest are bone marrow, blood, liver, brain, retina, adipose tissue and skin. Among these tissues, adipose-derived mesenchymal stem cells have awakened a special interest in several scientific sectors, since they are easy to isolate and have the ability to differentiate to a large variety of cell fates, which include mesenchymal and non-mesenchymal related cells. In this context, adipose mesenchymal stem cells offer the possibility to repair damaged tissues, opening new cell therapy-based strategies for the treatment of several pathologies, including diabetes.

Keywords: adult stem cells, adipose mesenchymal stem cells, cell culture, adipose tissue.

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Abbreviations:
AMSCs: adipose mesenchymal stem cells; ASCs: adult stem cells; b-FGF: basic fibroblast growth factor; BMMSCs: bone marrow mesenchymal stem cells; BMP: bone morphogenetic protein; DMEM: Dulbecco's modified eagle medium; DMSO: dimethyl sulfoxide; EGF: epidermal growth factor; ESCs: embryonic stem cells; FBS: fetal bovine serum; HGF: hepatic growth factor; IBMX: isobutyl methylxanthine; PBS: phosphate buffered saline; TGF: transforming growth factor.

Introduction

Stem cells possess two main properties, i.e. self-renewal and pluripotentiality. These two main characteristics establish stem cells as an unlimited source of biomaterial with an enormous plasticity for future clinical cell therapy assays. According to their origin, they can be classified as embryonic stem cells (ESCs), isolated from the inner cell mass of the blastocyst, and adult stem cells (ASCs), present in adult tissues. ASCs have the mission of renewing damaged cells due to trauma, disease or programmed cell death (cell turnover). Recent evidence strongly supports the concept that the ASCs of a certain tissue can overpass the limits imposed by this tissue and display a broader differentiating capability, giving rise to cells present in other tissues. In addition, ASCs can be expanded in culture and differentiated in vitro towards specific lineages.

Multipotent ASCs have been identified in various organs, including bone marrow, blood, muscle, liver, brain, retina, gut, pancreas, adipose tissue and skin. However, many adult organs present a particular tissue called the mesenchyme. This tissue can be formed in organs derived from the three embryonic layers and possesses connective tissue characteristics with an abundant and viscous extracellular matrix that is rich in collagen and fibroblasts. The mesenchyme gives rise during tissue differentiation to blood vessels and the cardiovascular system, as well as smooth muscle, mesothelium, lymphatic tissue and connective tissue. Normally, the term mesenchymal cells refers to stem cells isolated from any mesenchymal tissue of the organism, although bone marrow mesenchymal stem cells (BMMSCs) and adipose mesenchymal stem cells (AMSCs) have recently become the centre of attention of many studies. Mesenchymal cells derived from these tissues seem to share certain morphological and phenotypical characteristics. Both cell types present the ability to differentiate into many cell types

Table 1. Different growth factors added to the culture media to differentiate human AMSCs to specific lineages

Desired final cell/ tissue product	Growth factors used
Bone[8]	Dexamethasone, ascorbic acid, β-glycerophosphate, BMP2, vitamin D
Adipose tissue[8]	Indometacin, dexamethasone, L-glutamine, IBMX
Cartilage[9]	TGF, BMP7, dexamethasone
Skeletal muscle[10]	Hydrocortisone, L-glutamine, ITS (insulin, transferrin, selenium)
Nerve cells[1]	Mercaptoethanol
Hepatic cells[7]	EGF, HGH, b-FGF, oncostatin M, nicotinamide, dexamethasone, ITS+premix

but, conversely to BMMSCs, AMSCs are easy to isolate. Whereas BMMSCs are isolated from millilitres of marrow aspirates, AMSCs are purified from litres of lipoaspirates. This implies a much greater initial cell number, which facilitates their in vitro expansion as well as the design of differentiation protocols.

Interestingly, several studies have demonstrated the potential of AMSCs to differentiate and express markers that are associated with classical mesenchymal tissues such as cartilage, bone and adipose tissue.[1, 2] Other tissue markers may be also expressed under specific culture conditions, including those corresponding to skeletal muscle, vascular system, nervous system, cardiac muscle and more recently hepatic tissue.[3-10] In order to achieve this differentiation, it is necessary to use various growth factors in the culture medium (table 1). This manuscript will intend to summarize all the aspects to take into account during the isolation, culture and characterization of AMSCs. This is the first step in order to obtain pure populations that can be reprogrammed into insulin-secreting cells and re-implanted in diabetic patients.

Isolation of human adult mesenchymal stem cells from adipose tissue

AMSCs are isolated from the subcutaneous adipose tissue of patients undergoing minor surgery or taken directly from a lipoaspirate. The isolation procedure has been established by Zuk and collaborators.[11] Briefly, the adipose tissue is washed several times with phosphate buffer saline (PBS) in order to eliminate blood and other fluids. Then, the tissue is treated with 0.075% collagenase for 30 min at 37 ºC in gentle agitation in order to disrupt the extracellular matrix. It is necessary afterwards to inactivate the enzymatic activity in order to avoid excessive damage to the cells during the collagenase treatment. This is achieved by diluting the enzyme in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) at a 1:1 rate. This mix is quickly centrifuged (172 × g for 10 min) and the supernatant containing the collagenase is discarded. The pellet that contains the AMSCs is filtered through a sterile gauze to eliminate tissue debris. The percentage of living cells is determined by the trypan blue test. Afterwards, the cells are seeded in adherent dishes at a density of 12000 cells/cm^2 and incubated in 5% CO_2 atmosphere at 37 ºC. It is important to wash the cells several times with PBS after the 24 h incubation. This manipulation allows the elimination of non-adherent cells that are mainly dead cells, red blood cells and adipocytes. Culture is maintained over several passages under the same incubation conditions.

Maintenance of adipose mesenchymal stem cells

The maintenance of AMSC requires biweekly medium changes and to be regularly detached using trypsin and re-seeded when they reach a confluence of 80-90%. As soon as they are detached, the trypsin must be quickly diluted with the same volume of DMEM. After seeding, the medium must be changed often in order to completely eliminate any trace of the trypsin and floating cells. Investigators must pay attention and be very careful in these routine manipulations in order to maintain a good culture quality. This will later allow more reproducible results when applying differentiation protocols.

In this context, the differentiation protocols include modifications of the culture medium as well as the culture conditions. For instance, the initial number of cells to be seeded can be lowered to 1000-6000 cells/cm^2. Due to their extraordinary adherent properties, it is important to mention that the cells can be mechanically, instead of enzymatically, detached by scraping. In these cases, a sterile scraper is used in order to gently detach the cells from the culture surface. Afterwards, the cells can be recov-

ered by centrifugation and the corresponding protocol can be later applied.

Clonal selection of adipose mesenchymal stem cells

As previously mentioned, the isolation protocol of AMSCs results in a heterogeneous cell population. The adherent properties of these cells allow their purification from other cell types through subsequent passages. However, the AMSC population is in itself a heterogeneous population in which different mesenchymal cells display different degrees of differentiation. For this reason, the most rigorous publications as well as our own experience recommend to purify homogeneous mesenchymal cell lines from the original population.[12] This can be achieved by single cell cloning, giving rise to a cell population derived only from one cell. The advantage is that all derived cells display similar characteristics. This long and patient manipulation avoids the "background noise" and the variability of the results that usually appear when working with a heterogeneous population of AMSCs. It is important to perform this single cell cloning with early passages of AMSCs.

Two methods can be used to obtain the different cell clones:[13]

A) Seed the cells at a dilution such that single cells can be easily identified on the culture surface. The culture progresses to the formation of cell colonies from these single cells. When they reach a number of 30-50 cells/colony, they are trypsinized using cloning rings that allow the isolation of the selected clone. They are then expanded under the afore-mentioned culture conditions.[1,12]

B) Alternatively, a single cell can be seeded per well in a 96 multiwell plate. Although the culture progression is slower than in the first method described, each well will contain clones derived from a single cell.[10]

Freezing and thawing of adipose mesenchymal stem cells

Although the freezing procedure is similar to others described for different cell types, we briefly summarize several recommendations that can increase the viability of these cells when they are re-thawed. Freezing can be performed in standard culture medium, but it is important to supplement with 10% DMSO and an additional 10% of FBS. According to our experience, the optimal cell number is 1-2 $\times$ 10^6 cells/cryovial. Freezing must be performed gradually. We recommend the use of Mr Frosty® (Nalgene, Barcelona), a special tube container, filled with isopropanol. The container is placed in a -80 ºC freezer, where it will assure a slow temperature decent of 1 ºC/min. This procedure minimizes the formation of ice crystals in the cells, reducing the risk of massive cell death. After 24 h, the cells can be stored in liquid N_2 at -196 ºC.

On the other hand, cell thawing must be performed at 37 ºC in a water bath, although it is possible to allow its thawing at room temperature. It is important afterwards to eliminate all traces of DMSO. To this end, cells from the cryovial are transferred into a sterile tube containing culture medium, centrifuged (70 $\times$ g for 5 min) and the pellet is re-suspended in the corresponding volume before seeding in the culture plates. Medium should be changed after 24 h in order to eliminate the dead cells.

Characterization of adipose mesenchymal stem cells

AMSCs are long, fibroblast-like cells (figure 1) that can change during the differentiation protocols (figure 2). AMSCs and BMMSCs present similar phenotypic and functional traits. The most favourable characterization of the different mesenchymal stem cell populations is ac-

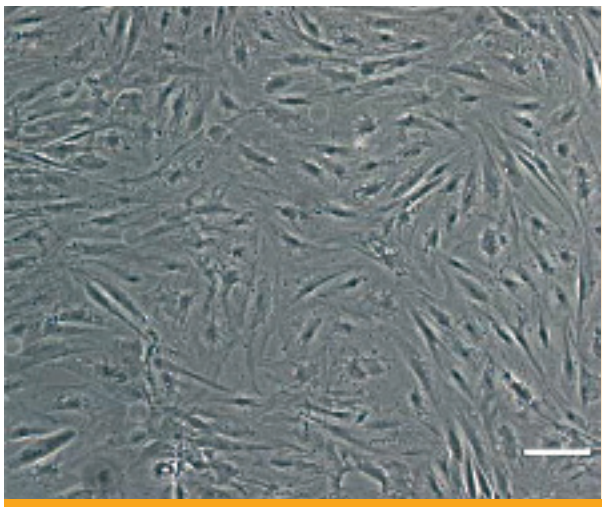

Figure 1. Phase-contrast images of human AMSCs after 17 days in culture with control medium. Cells present a high confluence but maintain their original size and morphology. Nucleus is small and cytoplasm large and elongated. Bar: 100 µm

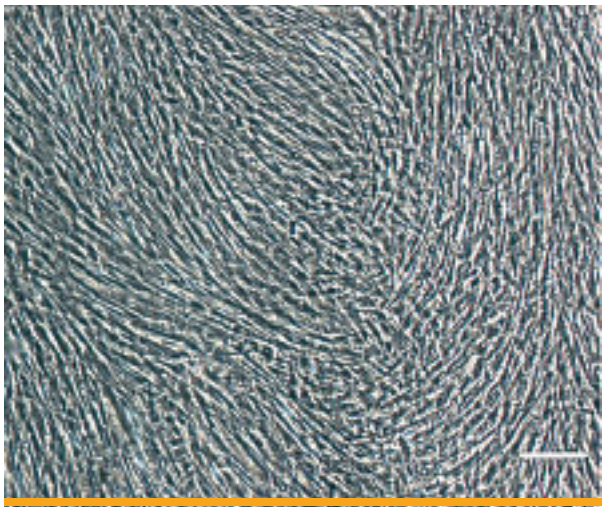

Figure 2. Phase-contrast images of human AMSCs after 17 days in culture in the presence of osteogenic medium. The composition of the medium used to induce the osteogenic differentiation is control medium + 0.2 mM ascorbic acid-2-phosphate, 10 mM β-glycerophosphate and 0.1 µM dexamethasone. Morphological changes are evident when compared to the cells in figure 1: confluence has increased, cells display an elongated morphology and tight junctions between them can be observed. Bar 100 µm

cording to the expression of cell surface antigens. In this fashion, BMMSCs and AMSCs are positive for CD29, CD44, CD71, CD90, CD105 and CD166.[1,12] However, CD49d is a specific antigen for human AMSCs which is not expressed in human BMMSCs.[1] In addition, AMSCs are $CD13^+$ and express low levels of CD106.[11] The characterization process of these markers is performed by flow cytometry.

Conclusions

Altogether, ASCs currently represent an important alternative to ESCs in future cell therapy protocols. First of all, these cells do not display immune rejection, provided that donor and recipient is the same person. Second, the use of ASCs is not subject to the ethical barriers that constrain the use of ESCs. The major challenge that scientists must face in this case concerns the limited differentiation potential presented by these cells. However, recent reports have opened a window of hope proving that ASCs can be reprogrammed to cell lineages that greatly differ from their original niche.

However, the use of ASCs, and in particular mesenchymal stem cells, is not free of problems. In this context, long term culture of AMSCs, as well as in ESCs, favours cell transformation, leading to the activation of oncogenes.[14-16] This finding is not negative in essence, as these transformed cells could represent an interesting model for the study of several types of human cancers, although further investigations must be performed before raising this conclusion.

Altogether, the study of ASCs and in particular AMSCs will help to understand the biological mechanisms that govern the differentiation processes and thereby evaluate their potential in future clinical trials. If reprogramming could be successfully achieved, AMSCs could represent an interesting source of cells to be used in replacement protocols to obtain insulin-secreting cells in order to treat diabetic patients.

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Key points

- Adult stem cells (ASCs) are cells from adult tissues with the potential to replicate and differentiate beyond the limits imposed by the parental tissue.
- ASCs represent currently an alternative to embryonic stem cells for future cell therapy protocols. ASCs can be expanded in vitro and differentiated with some limitation towards certain lineages.
- Among other tissues, adipose mesenchymal stem cells offer the possibility to repair damaged tissues. In particular, these ASCs could represent an interesting source of cells to be used in replacement protocols to obtain insulin-secreting cells to be used in diabetes.

References

1. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002; 13:4279-95.
2. Lin Y, Liu L, Li Z, Qiao J, Wu L, Tang W, et al. Pluripotency potential of human adipose-derived stem cells marked with exogenous green fluorescent protein. Mol Cell Biochem. 2006; 291:1-10.

3. Safford KM, Hicok KC, Safford SD, Halvorsen YD, Wilkison WO, Gimble JM, et al. Neurogenic differentiation of murine and human adipose-derived stromal cells. Biochem Biophys Res Commun. 2002;294:371-9.
4. Rangappa S, Fen C, Lee EH, Bongso A, Wei ES. Transformation of adult mesenchymal stem cells isolated from the fatty tissue into cardiomyocytes. Ann Thorac Surg. 2003;75:775-9.
5. Planat-Benard V, Silvestre JS, Cousin B, Andre M, Nibbelink M, Tamarat M, et al. Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation. 2004;109:656-63.
6. Kokai LE, Rubin JP, Marra KG. The potential of adipose-derived adult stem cells as a source of neuronal progenitor cells. Plast Reconstr Surg. 2005; 116:1453-60.
7. Taléns-Visconti R, Bonora A, Jover R, Mirabet V, Carbonell F, Castell JV, et al. Hepatogenic differentiation of human mesenchymal stem cells from adipose tissue in comparison with bone marrow mesenchymal stem cells. World J Gastroenterol. 2006;12:5834-45.
8. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage Potential of Adult Human Mesenchymal Stem Cells. Science. 1999;284:143-7.
9. Knippenberg M, Helder MN, Zandieh Doulabi B, Wuisman PIJM, Klein-Nulend J. Osteogenesis versus chondrogenesis by BMP-2 and BMP-7 in adipose stem cells. Biochem Biophys Res Commun. 2006;342:902-8.
10. De la Fuente R, Abad JL, García-Castro J, Fernández-Miguel G, Petriz J, Rubio D, et al. Dedifferentiated adult articular chondrocytes: a population of human multipotent primitive cells. Exp Cell Res. 2004;297:313-28.
11. Zuk PA, Zhu M, Mizuno H, Huang JI, Futrell WJ, Katz AJ, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 2001;7:211-28.
12. Guilak F, Lott KE, Awad HA, Cao Q, Hicok KC, Fermor B, et al. Clonal analysis of the differentiation potential of human adipose-derived adult stem cells. J Cell Physiol. 2006;206:229-37.
13. Boquest AC, Shahdadfar A, Brinchmann JE, Collas P. Isolation of stromal stem cells from human adipose tissue. Methods Mol Biol. 2006;325: 35-46.
14. Wakitani S, Takaoka K, Hattori T, Miyazawa N, Iwanaga T, Takeda S, et al. Embryonic stem cells injected into the mouse knee joint form teratomas and subsequently destroy the joint. Rheumatology. 2003;42:162-5.
15. Enseñat-Waser R, Santana A, Vicente-Salar N, Cigudosa J, Roche E, Soria B, et al. Isolation and characterization of residual undifferentiated mouse embryonic stem cells from embryoid body cultures by fluorescence tracking. In Vitro Cell Dev Biol Anim. 2006;42:115-23.
16. Rubio D, García-Castro J, Martín MC, De la Fuente R, Cigudosa JC, Lloyd AC, et al. Spontaneous human adult stem cell transformation. Cancer Res. 2005;65:3035-9.

FE DE ERRATAS

En el número 24(1) de *Avances en Diabetología* se produjo un error en la formulación de las preguntas 8 y 9 del cuestionario de evaluación de la sección «Seminarios en diabetes» (página 50 de la revista), lo que ha producido cierta confusión entre los alumnos que realizan el test para la obtención de créditos.

Los enunciados correctos deberían ser:

8. Acerca del tratamiento del edema macular diabético
9. Acerca de la fotocoagulación retiniana en el tratamiento de la retinopatía diabética