In vitro production of differentiated endothelial cells from Mesenchymal Stem Cells collected from adult rat Adipose Tissue (AD-MSCs): preliminary results.

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INTRODUCTION

Mesenchymal Stem Cells (MSCs) are multipotent cells that reside within various tissues, including adipose tissue and bone marrow. Research concerning the potential therapeutic role of MSCs has significantly advanced over the last decade. Many studies have demonstrated that adipose tissue is an excellent source of MSCs (AD-MSCs), easily accessible in animals (1). AD-MSCs can be expanded *in vitro* over the short term and they can differentiate into different cell types of mesodermal lineage (2). Thus, they are thought an attractive tool for cell therapy applications. Selection of appropriate animal models for preclinical evaluations is crucial for optimization and validation of therapeutic protocols in regenerative medicine. Therefore, *in vitro* studies are necessary to future *in vivo* applications of MSCs. Rat is an animal model usefull for *in vitro* studies. Many authors have demonstrated that in murine model, AD-MSC can differentiate into endothelial cells and promote *in vivo* neovascularization (3, 4). Procedures for AD-MSC endothelial differentiation, usually, employ colture medium enriched with some induction factors. Aim of this work is the in vitro production of endothelial cells, differentiated from rat AD-MSCs and the study of the gene expression levels for stemness and endothelial markers.

MATERIAL AND METHODS

AD-MSCs were obtained from subcutaneous adipose tissue of 5 Wistar rats, in accordance to the European Directives (86/609/CEE and 63/2010/EU). The adipose tissue samples were processed for *in vitro* AD-MSCs isolation and culture. In brief, they ware digested in 0.2% Collagenase Type IA (GIBCO). After filtration with 70 µm filter (BD - Falcon) and centrifugation at 300 g. for 10 min, AD-MSC were cultured in D-MEM low Glucose medium (GIBCO), enriched with 20% FBS and Atibiotic – Antimycotic 100X solution (GIBCO), at 37°C and 5% CO_2. Nonadherent cells were removed after 2 days and medium was replaced (5). Adherent AD-MSC population was expanded for 5 subcultures. To assess the gene expression of stemness markers, OCT-4, SOX-2 and NANOG, total RNA was extracted from harvested cells, from 0 up to 5 *in vitro* subculture, using an *RNeasy Mini kit* (Qiagen), according to the manufacturer's instructions. cDNA was synthesized using a *Superscript Vilo cDNA synthesis* (Invitrogen). The Sybr Green Real Time amplifications were performed in a total volume of 20 µl, with 2 µl of cDNA, 10 µl of Power SYBR green PCR Master Mix (Applied Biosystems) and 1µM of each foward and reverse primer. Specific primers pairs for OCT-4, SOX-2 and NANOG were designed by the use of Primer Express software (Tab. 1). The cycling condition were: one cycle of initial denaturatuon at 95°C for 10 min; 40 cycles of denaturation at 95°C for 30 sec followed by annealing and extension at 60°C for 1 min; an additional melting curve analysis was performed at 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec (Step One Plus Real Time PCR System, Applied Biosystems). Gene expression levels were normalized according to that of GADPH housekeeping gene. (Tab. 1). In order to induce endothelial differentiation, AD-MSC at 0 and 1 *in vitro* subculture, were seeded onto 6-well plates, and differentiation was induced when cells were 60% confluent. AD-MSC were cultured at 37°C with 5% CO_2, for 15 days in endotelial grow medium, EGM-2 bullet kit (Euroclone) (4). Control cells were cultived in D-MEM low medium, in the same atmosphere and temperature conditions. To verify the gene expression of endothelial markers CD31, eNOS and vWF, Real Time Sybr Green PCR amplifications were performed as described above. The primers for endotelial markers were designed by the use of Primers Express software (Tab.2). Gene expression analysis were normalised according to that of GADPH.

Tab. 1: primers for stemness markers and housekeeping gene GADPH

Nome primer	*Sequenza*
OCT-4F	**5'-GAGGCCTTTCCCTCTGTTCCT-3'**
OCT-4R	**5'-GGCTGGTGCCTCAGTTTGAA-3'**
SOX-2F	**5'-GCGGCAACCAGAAGAACAG-3'**
SOX-2R	**5'-CCACACCATGAAGGCATTCA-3'**
NANOG-F	**5'-TCAGCGCCGGTGGAGTA-3'**
NANOG-R	**5'-TCCAGACGCGTTCATCAGATAG-3'**
GADPH-F	**5'-AAC TTTGGC ATC GTG GAA GGG-3'**
GADPH-R	**5'-AGG GAT GAT GTT CTG GGC TGC-3'**

Tab. 2: primers for endothelial markers

Nome primer	*Sequenza*
CD31-F	5'-GAGGCCTTTCCCTCTGTTCCT-3'
CD31-R	5'-GGCTGGTGCCTCAGTTTGAA-3'
eNOS-F	5'-GCGGCAACCAGAAGAACAG-3'
eNOS-R	5'-CCACACCATGAAGGCATTCA-3'
vWF-F	5'-TCAGCGCCGGTGGAGTA-3'
vWF-R	5'-TCCAGACGCGTTCATCAGATAG-3'

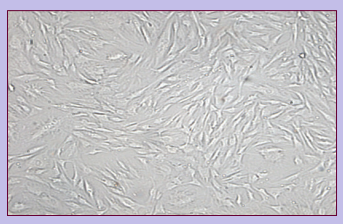

Fig. 1: AD-MSC from subcutaneous fat of rat Wistar

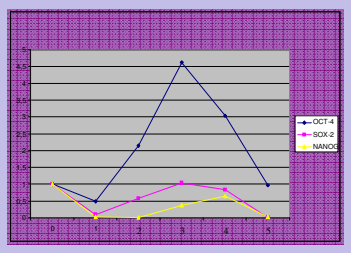

Fig. 2: Gene expression levels for stemness markers

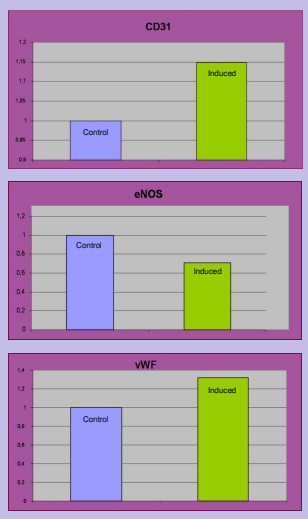

Fig. 3: Gene expression levels for endothelial markers

RESULTS AND CONCLUSIONS:

AD-MSCs were isolated from subcutaneous fat of rats. They show a fibroblast-like phenotype and grow out in culture (Fig.1). Results concerning gene expression analysis for OCT-4, SOX-2 and NANOG markers show, for all three markers considered, an initial decrease of activities, during the *in vitro* adaptation, from subculture 0 to subculture 1. These levels of activity begin to increase from *in vitro* passage 1 up to passage 3, for OCT-4 and SOX-2. The expression of NANOG, begins to increase from passage 2 up to passage 4 (Fig.2). Regarding the results of endothelial differentiation, no changes in morphology was observed after 15 days of culture. However, an increase of expression levels only for CD31 and vWF was observed, compared to control cells (Fig.3). Regenerative medicine is a new interdisciplinary sector aimed at repairing, replacing and regenerating damaged tissues and organs through the use of cells manipulated *ex vivo*. Endothelial dysfunction is a prominent feature of a wide variety of pathologies. The present study has demonstrated that MSCs can be easily isolated from adipose tissue of rat. They express different levels of mRNA for OCT-4, SOX-2 and NANOG during the subsequent subcultures, showing a progressive loss of stemness characteristics. This could be crucial to establish the right moment for induction of differentiation. The presence of medium EGM-2, in initial subcultures, was sufficient to induce an increase of the gene expression level of endothelial markers. These are preliminary results of a project aimed to the *in vitro* production of endothelial differentiated cells from AD-MSC of rat and to study their potential use for therapeutic purposes. Further experiments are required to confirm the acquisition of the endothelial phenotype and the correlation between gene and protein expression.

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