Cell Isolation and expansion

Adipose tissues were collected after informed consent of all donors and in accordance with the local Ethical Commission. Human adipose derived mesenchymal stem cells (AD-MSC) were isolated from adipose tissue by enzymatic digestion. In particular, the tissue sample was extensively washed with PBS (GIBCO, ThermoFisher Scientific, Waltham, MA USA) containing penicillin/streptomycin (P/S) in order to remove debris. The tissue was placed in a sterile petri dish with collagenase type I (Roche, Basel, Switzerland) and dispase (Roche, Basel, Switzerland) prepared in α-MEM medium (GIBCO, ThermoFisher Scientific, Waltham, MA USA) with P/S (Sigma-Aldrich, St. Louis, MO, USA) and using sterile surgical blades (Paragon, UK) tissue was minced into small segments. The sample was digested for 1h at 37ºC and in order to facilitate (speed up) further the digestion the sample was pipetted up and down with a 10 or 25ml pipette several times intermittently. Single cell suspension was obtained by passing the cells through a cell strainer avoiding the solid aggregates. AD-MSCs were obtained by centrifugation at 1000rpm for 5min. The supernatant was removed and the pellet was resuspended with MSC medium (a-MEM, 15% FBS (Gibco, ThermoFisher scientific, Waltham, MA USA), Glutamine, penicillin, and streptomycin). The primary cells were cultured in T75 flask (Corning, Sigma-Aldrich, St. Louis, MO, USA) at 37ºC and 5% CO_2 for about 2 weeks until they reached confluence and were defined as passage 0. The cells had a typical fibroblast-like spindle shape (Fig1) and were passaged at a ratio 1:3. The adherent AD-MSC were expanded and either assessed for gene expression and flow cytometry, or induced to differentiate toward the chondrogenic, adipogenic and osteogenic lineages. The cells used in subsequent experiments were between passages 3 and 6.

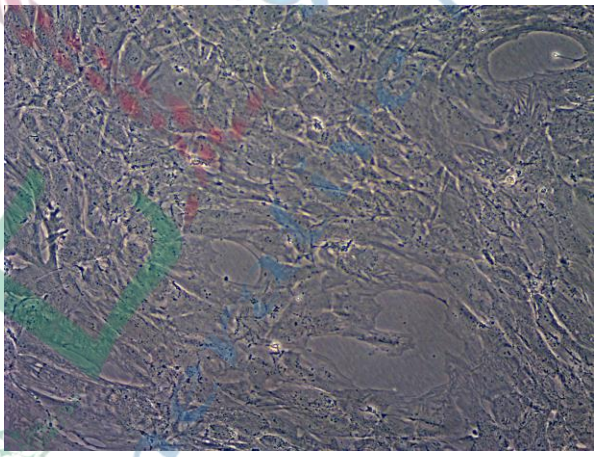


Fig. 1. Morphology of AD-MSC culture *in vitro*.

Characterization of undifferentiated AD-MSC.

Flow cytometry was performed in order to identify the presence of specific cells surface antigens that are used as markers in order to identify AD-MSC.

The cells were harvested when reaching 90-100% confluence. Sufficient volume of trypsin-EDTA was added and the cells were placed in the incubator for 5min. After verifying under the microscope that more than 90% of the attached cells have become detached from the surface, MSC medium was added in order to allow the serum contained in the medium to neutralize the trypsin reaction. The cells were centrifuged at 1000 rpm for 5min, were resuspended in MSC medium and were counted using trypan blue to determine viability. Because there is no single marker to identify AD-MSC subpopulation, a multicolor identification with a combination of fluorochrome-labeled antibodies for different surface antigens was used. For each analysis of surface epitope characterization of AD-MSC the cells were rinsed in PBS and resuspended at a density of 0.3-0.5x10^6 cells/100μl FACS buffer (0.1% sodium azide, 1% BSA in PBS) into 1.5ml microfuge tubes. Additionally, a sample containing only cell suspension as a control for autofluorescence was prepared. The cells were first blocked with 5μg of human IgG (Sigma-Aldrich, St. Louis, MO, USA) for 15min on ice and subsequently were stained with the following fluorochrome conjugated antibodies in order to characterize the cell population: CD90-FITC (all obtained from Biolegend, San Diego, CA, USA) CD73-PE-Cy7 (both expressed by AD-MSC) and CD45-APC (negative marker). For each sample a viability marker (7-AAD) was also included in order to define a region for live cells (cells excluding 7-AAD). Cells were incubated with various combinations of antibodies for 20min in the dark on ice. Stained cells were washed twice with FACS buffer (PBS, 1% BSA) and analyzed. Guava easyCyte flow cytometer (Merck-Millipore, Darmstadt, Germany) with the pre-installed software for data acquisition and analysis was used (Fig 2). In addition, quality control was performed by including the analysis of fluorescent beads to validate the function of the lasers before each analysis. A total of 10,000 events were acquired for each sample. Unstained cells were used as a control to set the gates and analysis region.

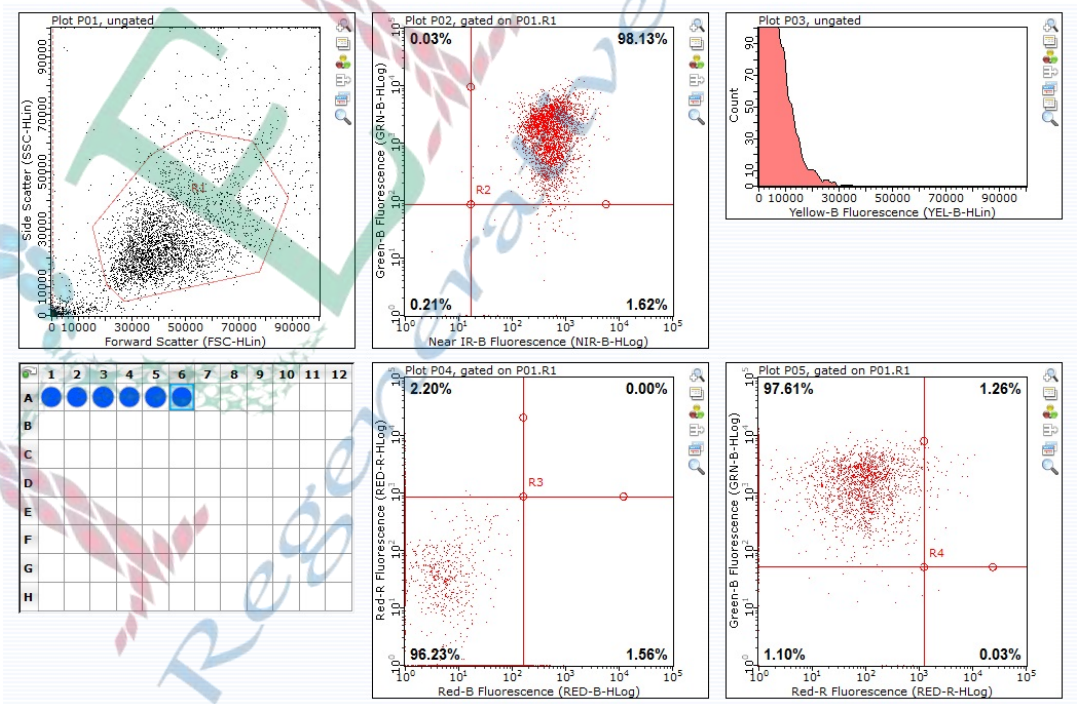


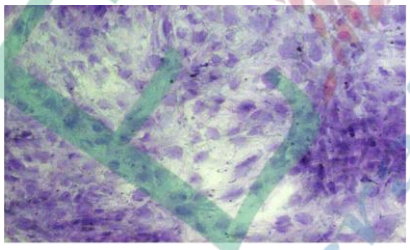
Fig.2. Characterization of AD-MSCs by flow cytometry. Representative diagrams showing the expression of positive mesenchymal marker (CD90, CD73) and negative mesenchymal marker (CD45).

Cell Differentiation Assays

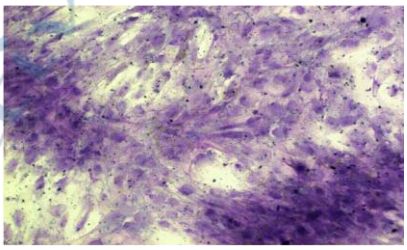
A hallmark of MSCs is their multipotency which means that by using specific culture medium it is possible to direct MSCs differentiation to specific cell types in vitro. However, not all MSCs in cultures present this ability due to cellular senescence or lack of optimal cocktail that drives differentiation. Based on those observations a trilineage differentiation potential of the AD-MSCs was performed in order to confirm cells multipotency.

Chondrogenic differentiation

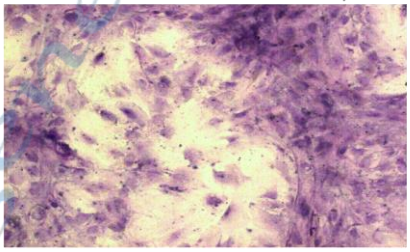
AD-MSC were seeded in 6-well plates until confluency and were differentiated into chondrocytes by culturing the cells into chondrogenic differentiation medium consisting of a-MEM supplemented with 15% FBS, Glutamine, penicillin, streptomycin, ITS, dexamethasone and transforming growth factor-β1. Cells were cultured for 7, 14 and 21 days with the differentiation medium (medium changes twice per week) and progression of the differentiation potential was monitored using the inverted microscope. In addition, evaluation of the chondrogenic differentiation was carried out by staining with Toluidine blue. Cells were fixed in 10% formalin for 30min, washed 2 times with PBS and stained with 0.1% Toluidine blue for 30min in order to highlight the presence of proteoglycans and subsequently the level of chondrogenic differentiation. Morphological changes could be observed by comparing uninduced and induced AD-MSCs (Fig 3). Gene expression analysis was also performed as described below.



Chondrocytes 7 days toluidine blue



Chondrocytes 14 days toluidine blue



Chondrocytes 21 days toluidine blue

Fig.3. *In vitro* chondrogenic differentiation. **a**) AD-MSC kept in the growth medium and used as controls. **b)** toluidine blue staining.

Osteogenic differentiation

The osteogenic differentiation capacity of AD-MSC was investigated in cultures in the presence of osteogenic medium, a-MEM supplemented with 15% FBS, Glutamine, penicillin, streptomycin, dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), glycerolphosphate (Sigma-Aldrich, St. Louis, MO, USA). After culture for 7, 14 and 21 days with the differentiation medium (medium changes twice per week) calcium accumulations were observed under the microscope. mRNA analysis was performed as described below.

Adipogenic differentiation

The adipogenic differentiation capacity of AD-MSC was investigated in the presence of adipogenic medium, a-MEM supplemented with 15% FBS, Glutamine, penicillin, streptomycin, µM dexamethasone, IBMX (Sigma-Aldrich, St. Louis, MO, USA), insulin and indomethacin (Sigma-Aldrich, St. Louis, MO, USA). After culture for 7, 14 and 21 days with the differentiation medium (medium changes twice per week) lipids droplets were observed under the microscope. mRNA analysis was performed as described below.

mRNA isolation and Real-time quantitative RT-PCR assays

Total mRNA was isolated using the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol and Epoch microplate spectrophotometer was used to quantify RNA. cDNA synthesis was performed by using 0.5 µg RNA as template and the PrimeScript RT reagent kit (Takara Shuzo Co., Kyoto, Japan). Real-Time PCR was carried out in duplicate using KAPA SYBR Fast® Mix (KK4601 Kapa Biosystems, Woburn, MA, USA) in StepOne™ (Applied Biosystems, ThermoFisher Scientific, Waltham, MA USA). The PCR specifications were as follows: 95°C for 10 minutes, 40 cycles for 5 seconds at 95°C, 60°C for 30 seconds. B2M was used as reference gene and aggrecan was used to define the stage of chondrogenic differentiation. Collagen type X was used as a marker for hypertrophic differentiation (Fig4).

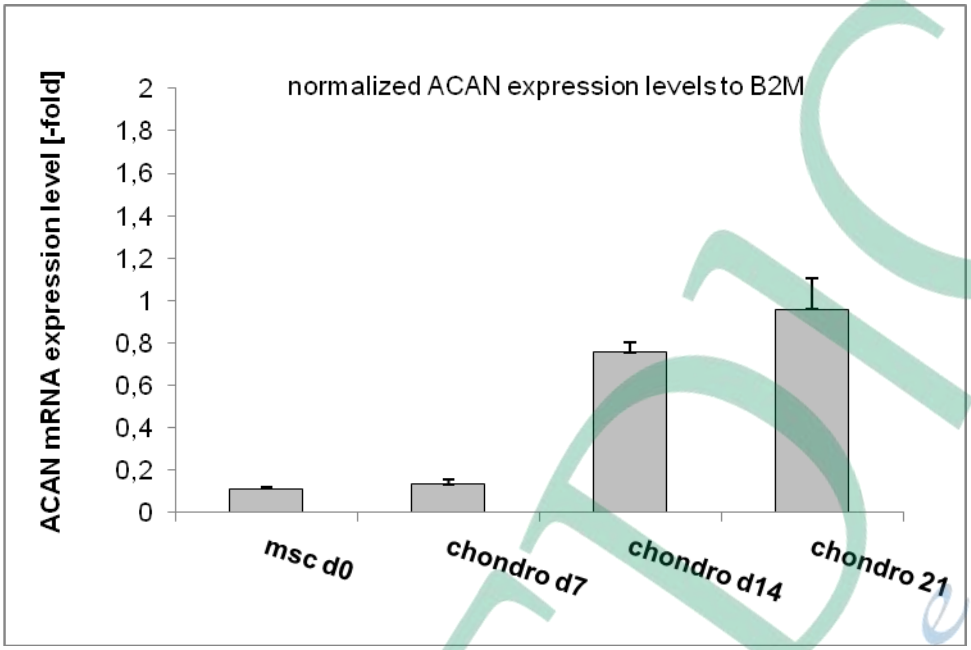


Fig.4. Real-time PCR analysis of the chondrogenic expressed gene *ACAN* at 7, 14 and 21 days post-induction. Results are normalized to the expression of the housekeeping gene B2M.