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INFLUENCE OF THE ANATOMICAL LOCATION OF THE HARVEST ON THE OSTEOGENIC DIFFERENTIATION OF ADIPOSE-DERIVED STROMAL CELLS

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INFLUENCE OF THE ANATOMICAL LOCATION OF THE HARVEST ON THE OSTEOGENIC DIFFERENTIATION OF ADIPOSE-DERIVED STROMAL CELLS

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ABSTRACT

Stromal cells derived from the adipose tissue (ADSCs) have self-renewal capabilities and the capacity to differentiate along different mesenchymal and epithelial lineages, including the osteoblast phenotype. Additionally, these cells can be easily harvested trough a minimal invasive surgical procedure, as comparing to the harvest of stromal cells from other sources, such as the bone marrow. This characteristic has made their application potentially useful in regenerative medicine approaches, particularly within the bone regenerative process. While widespread data is available on the impact of fat tissue harvest and cell isolation procedures for adequate ADSCs functionality, little attention has been given to the anatomical differences verified within the fat tissue, as well as the impact of these differences on the functional activity of ADSCs.

Accordingly, this experimental work aimed the isolation and characterization of ADSCs from rat's adipose tissue, harvested from different anatomical locations, i.e., subcutaneous inguinal, abdominal inguinal, flank, neck and omentum. Following, grown cells were characterized, following specific induction, regarding osteogenic differentiation capability through biochemical, histochemical, gene expression and microscopical techniques, in two-dimensional (monolayer) and three-dimensional (spheroids) culture systems.

Established ADSC cultures from distinct anatomical locations revealed a distinct biological behavior. In regards to the proliferative capacity, abdominal inguinal revealed the highest potential, while the osteogenic capability was highest in cultures established from flank. ADSCs derived from all anatomical locations formed efficiently three-dimensional aggregates that maintained their viability during the culture period.

Broadly, and given the verified functional differences of ADSCs grown from distinct anatomical locations, it might be adequately to address the fat tissue anatomical location in order to adequately select the populations most relevant for the specific targeted regenerative application.

RESUMO

As células estromais derivadas do tecido adiposo (ADSCs) têm a capacidade de se auto-renovarem e de se diferenciarem em linhagens mesenquimais, incluindo a linhagem osteoblástica. Além disso, estas células podem ser facilmente obtidas a partir de um procedimento cirúrgico minimamente invasivo, quando comparado com a recolha das células estromais de outros locais, nomeadamente da medula óssea. Todas estas características, fazem destas células estromais potencialmente úteis quando aplicadas num contexto de medicina regenerativa. Embora existem estudos acerca do impacto dos procedimentos da recolha do tecido adiposo e do isolamento das células estromais na funcionalidade das ADSCs, pouca atenção tem sido dada às diferenças anatómicas verificadas dentro do tecido adiposo, bem como o impacto dessas diferenças na atividade funcional das ADSCs.

Deste modo, o trabalho experimental aqui presente tem como objetivo o isolamento e a caracterização das ADSCs derivadas de tecido adiposo de rato recolhido de diferentes localizações anatómicas, nomeadamente, inguinal subcutânea, abdominal inguinal, flanco, pescoço e omento. De seguida, as células cultivadas e submetidas a indução apropriada, foram caracterizadas tendo em conta a capacidade de diferenciação osteogénica, através de técnicas bioquímicas, histoquímicas, microscópicas e expressão génica em culturas a bidimensionais (monocamada) e a tridimensionais (esferóides).

As culturas estabelecidas a partir das diferentes localizações anatómicas, revelaram um comportamento biológico distinto. Em relação à capacidade proliferativa, a zona abdominal inguinal revelou maior potencial, enquanto a capacidade osteogénica foi maior nas culturas estabelecidas do flanco. ADSCs derivadas de todas as localizações anatómicas formaram eficientemente agregados tridimensionais que mantiveram a sua viabilidade durante o período de cultura.

Em termos gerais, e dadas as diferenças funcionais verificadas nas ADSCs cultivadas a partir das diferentes localizações anatómicas, poderá ser adequado ter em consideração o local anatómico de recolha do tecido adiposo a fim de selecionar as

populações celulares mais relevantes para uma aplicação direcionada em medicina regenerativa.

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ABREVIATIONS

ASCs – Adipose Mesenchymal Stem Cells

ADSCs - Adipose-derived Stromal Cells

AA - Ascorbic acid

BMI – Body Mass Index

BMSCs – Bone Marrow Stromal Cells

CFU-F – Colony Forming Unit-Fibroblast

CM – Control Medium

DMEM - Dulbecco's Modified Eagle Medium

DMEM-HG - Dulbecco's Modified Eagle Medium-High Glucose

DMEM-LG - Dulbecco's Modified Eagle Media-Low Glucose

HDT- Hanging-Drop Technique

hEGF - human Epidermal Growth Factor

bFGF- basic Fibroblasts Growth Factor

FDAL – Fat from differentiated anatomical locals

FUAL - Fat from undifferentiated anatomical locals

FBS - Fetal Bovine Serum

Fungi - Fungizone

LOT- Liquid Overlay Technique

MDSCs – Muscle-Derived Stromal Cells

MEM - Modified Eagle Media

α-MEM - α-Modified Eagle Media

MLR - Mixed Lymphocyte Reaction

MSCs – Mesenchymal Stromal Cells

OM – Osteogenic Medium

OM1 – Osteogenic Medium 1

OM2 – Osteogenic Medium 2

Pest – Penicillin/streptomycin

PBS - Phosphate-Buffered Saline

RT-PCR – Reverse-Transcription Polymerase Chain Reaction

SA-βgal - Senescence-associated beta-galactosidade

SM-MSCs – Synovium Membrane-derived Stromal Cells

SVF - Stromal Vascular Fraction

TGF - Transforming Growth Factor

TGFβ - Transforming Growth Factor-β

T-MSCs – Teeth-derived Mesenchymal Stromal Cells

UCB – Umbilical Cord Blood

UCM-MSCs – Umbilical Cord Blood-derived Mesenchymal Stromal Cell

I. LITERATURE OVERVIEW

Elisabete Gonçalves

MESENCHYMAL STROMAL CELLS (MSCs)

Mesenchymal stromal cells (MSCs) are adult stromal cells traditionally found in the bone marrow and originating from the mesenchyma, which is an embryonic tissue derived from the mesoderm [1]. But, MSCs can be found in other tissues, including adipose tissue, umbilical cord, muscle, periosteum, synovial membranes, trabecular bone, skin, tendons, dental pulp of deciduous teeth [1]–[4], and also, in organs such as the liver, spleen and lung [3].

These cells have the ability to differentiate into multiple lineages including adypocites, chondrocytes and osteocytes [2] (see Figure 1). Moreover, MSCs play an important role as a support system for hematopoietic stem cells maturation and development, within the marrow [2], [5].
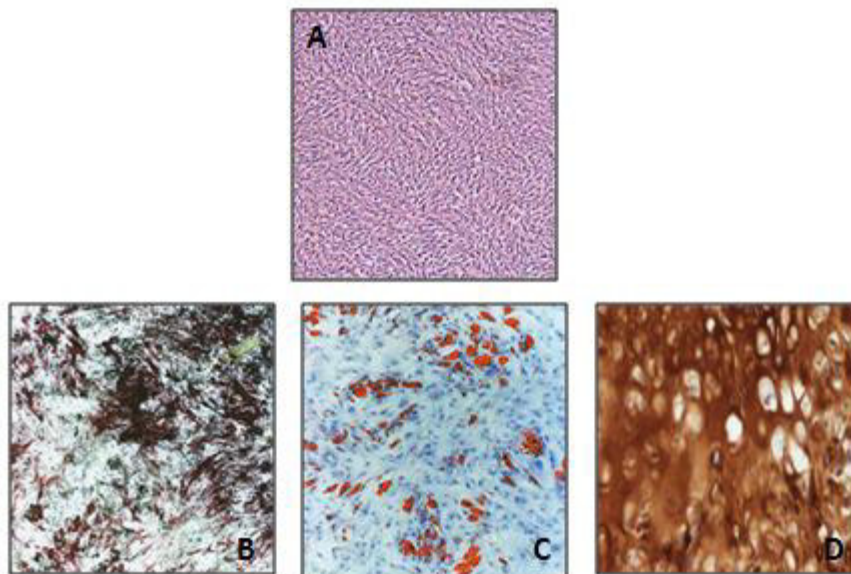
Figure 1 Histological images from MSCs in monolayer culture (A) and following differentiation into three lineages: osteogenic (B) staining with von Kossa, adipogenic (C) staining with Nile Red O and chondrogenic (D), immunostaining with an antibody specific for type II collagen (adapted from (2)).

CHARACTERIZATION OF MESENCHYMAL STROMAL CELLS

Over the time, there has been a controversy regarding the nomenclature used to described plastic-adherent cells isolated from bone marrow and other sources [4], [5]. Some authors used the term "mesenchymal stem cells" and others sustain the term "mesenchymal stromal cells". The International Society for Cellular Therapy propose that plastic-adherent cells, regardless of the tissue from which they are isolated, normally designated as mesenchymal stem cells can be, more adequately regarded as "multipotent stromal cells", while the term "mesenchymal stem cells" is used for the cells that meet specified stem cell criteria. The acronym "MSC" can be used in both situations [4].

The discussion about the nomenclature to be adopted relates to the term "stem", since it presupposes "the long-term self renewing cell that is capable of differentiation into specific multiple cell types *in vivo*" [4]. Nevertheless, this criterion is not always analyzed or observed in isolated cell populations and, moreover, the plastic-adherent cells isolated are quite heterogeneous, so, in these situations, it is better to use the term multipotent or mesenchymal stromal cells without ascribing homogeneity or stem cell activity [4].

Throughout this work, the term mesenchymal stromal cells, as well as the acronym MSCs, to define this cell population, will be used.

Immunologically, mesenchymal stromal cells (MSCs) are characterized by using specific markers, however that characterization is hampered by a lack of a determining and specific single cell marker for MSCs populations [6]. The International Society for Cellular Therapy proposed three minimal criteria for the identification and characterization of cultured human MSCs: (i) plastic adherence when maintained in standard culture conditions, (ii) expression of CD73, CD90 e CD105 and lack of CD11b or CD14, CD19 or CD79α, CD45 and HLA-DR expression, and (iii) their trilineage differentiation potential into adipocytes, chondrocytes and osteoblasts under standard *in vitro* differentiating conditions [4], [6].

Phenotypically these cells express a number of surface markers that are not phenotype specific. However, it is known that adult human MSCs do not express the hematopoietic markers CD45, CD34, CD14 or CD11a [7], [8]. These cells also do not express the costimulatory molecules CD80, CD86 and CD40, or the adhesion molecules CD31, CD33 and CD56, but they can express CD105, CD73, CD44, CD90 and Stro-1 [9] and exhibit high expression of integrin α1, α5 and β1, low expression of α1, α3, α6,αV, β2 and β4, and no expression of α4, αL and β2 [2], [8]. The adhesion molecules CD106 (vascular cell adhesion molecule), CD166 (activated leukocyte cell adhesion molecule), intercellular adhesion molecule (ICAM)-1 and CD29 are also broadly expressed [2], [8], [9]. In other species, the molecules expressed by MSCs may not be particularly the same and even within the same species, a variable expression pattern, due to variations in tissue source, the method of isolation and culture, may be verified [9].

The gold standard assay used to identify MSCs is the colony forming unit-fibroblast (CFU-F) assay, which can be used to identify the adherence and the morphology (characteristic spindle-shaped) that form colonies [10].

In a general way, MSCs isolation is based on their ability to adhere to the tissue culture plate and they are expanded by keeping their multipotency during the culture [3].

The MSCs isolated from fetal blood, liver, spleen and bone marrow, although phenotypically similar, showed heterogeneity in differentiation potential, which is directly related to the tissue source [9]. These examples illustrated that mesenchymal cells are phenotypically heterogeneous, and the relationship between traditional bone-marrow-derived MSCs and these other MSC-like populations remains to be fully clarified [9].

In vitro, MSCs' have the capability to differentiate into distinct lineages, giving rise to cell populations generally found in bone, fat and cartilage. To achieve this, it is necessary to supplement the culture medium with specific molecules and growth factors that modulate genetic events, thus involving transcription factors [11]. The differentiation to a particular phenotype pathway can be controlled by some regulatory genes that can induce progenitor cell differentiation to a specific lineage [11]. Moreover, basal nutrients, cell density, spatial organization, mechanical forces,

growth factors and cytokines, influence, in a large part, the MSCs differentiation process [10]. Other factors, broadly related to the donor characteristics (like age, gender, presence of trauma or systemic disease), may also influence the yield and expansion of MSCs [8].

SOURCES OF MESENCHYMAL STROMAL CELLS

As already mentioned, the MSCs can be found in several tissues. In the literature, the tissues most studied are bone marrow, adipose tissue, umbilical cord blood, synovium membrane, muscle and teeth.

Bone marrow derived mesenchymal stromal cells (BMSC)

Alexander Friedenstein and their co-workers were the first to isolate bone marrow stem cells [12]. They placed bone marrow in plastic culture dishes and removed the non-adherent cells after 4 hours, thus discarding most of the heterogeneous cells. They concluded that the adherent cells were heterogeneous in appearance, but the most adherent cells were spindle-shaped and formed foci of two to four cells, which remained inactive for 2-4 days and then began to proliferate rapidly [9]. After some time in culture, the adherent cells became more homogeneously fibroblastic in appearance [9].

The main characteristics of BMSCs are the ability to form fibroblastic-like colonies, extensive proliferative capacity, the ability to express several common cell surface antigens and, also, the ability to differentiate into several mesodermal lineages [13]. Because of these characteristics, bone marrow stem cells may potentially be useful in the field of regenerative medicine. However, they show some disadvantages compared to other sources. The percentage of MSCs in bone marrow was found to be very low, about 0.001% to 0.01% [14]. Further, it has been demonstrated that the potential of differentiation and the yeld of cells decreases with age [15]. Further, the harvest procedure is invasive and can cause pain and morbidity to the donor site [16], [17]. Thus, alternative sources of mesenchymal stem cells have been investigated.

In humans, the MSCs are generally isolated from an aspiration of bone marrow, harvested from the superior iliac crest of the pelvis, from the tibial and femoral marrow compartments and thoracic and lumbar spine. In rodents it is harvested from the mid-diaphysis of the tibia or femur [18].

Numerous studies show that adult bone-marrow stem cells can regenerate non-hematopoietic tissues, like brain, skeletal muscle, liver and epithelium, endothelium and heart [18], but the clinical validity of these approaches originated a lot of controversy and discussion within the scientific community.

The selective differentiation of precursor cell populations, *in vitro*, depends on the specific environmental cues combined with growth factors and cytokines supplied into the culture medium [19]. Many studies have evaluated the expansion and the differentiation of BMSCs using a variety of basal media, including α-Modified Eagle Media (α-MEM), Dulbecco's Modified Eagle Medium-High Glucose (DMEM-HG) and Dulbecco's Modified Eagle Media-Low Glucose (DMEM-LG) [20], but have broadly demonstrated that media based on α-MEM were more suitable for the expansion of human BMSCs [20].

Adipose-derived mesenchymal stromal cells (ADSCs)

Adipose tissue has gained a lot of interest in the last years since the validation of the possible to isolated mesenchymal stromal cells from this tissue. Isolated cells reported to ability to self-renew and differentiated into the classical lineages: adipogenic, osteogenic and chondrogenic [21]–[23].

Due to the importance of the adipose tissue as a source of stromal cells to the present work, this issue will be further developed in detail.

Umbilical cord blood derived mesenchymal stromal cells (UCB-MSCs)

The umbilical cord blood (UCB) can be a source for MSCs. However, the studies are controversy regarding the ability of UCB-derived cells to be capable to differentiate into cells of different lineages like bone, cartilage and adipose tissues [15], [16], [24].

For instance, in a study from Wexler et al. [25] it was found that UCB does not yield MSCs, in the culture systems that are usually performed, despite that authors could not exclude the possibility that MSCs could be present in UBC. Another study showed the failure to obtain MSCs from UCB, concluding that in comparison to BMSCs, the stem cells from UCB do not express the general characteristics of MSCs [26].

On the other hand, Erices et al. [27] demonstrated that cells from UCB are able to generate adherent cells in culture and they express the phenotype similar to the one attained by a mesenchymal-like populations.

Goodwin et al. [28] were able to isolated mesenchymal stem cells from UCB and differentiated them in multiples lineages, like osteogenic, adipogenic, neurogenic and chondrogenic, and they concluded that UCB-MSCs can differentiate into osteoblasts and adypocites, but not into chondrocytes, still remaining the ability to express neuronal markers.

In a study that compared MSC from bone marrow, umbilical cord blood and adipose tissue [16] in terms of success rate of isolation, expansion potential, multiple differentiation and immune phenotype, the conclusion for UBC-MSCs was that they didn't show ability to differentiate into adipogenic lineage but they showed chondrogenic and osteogenic differentiation capabilities.

In general, there are some described advantages to the usage of cells from UCB, since the UCB can be easily obtained by a non-invasive method, given that after the delivery the umbilical cord and placenta are discarded [15].

Synovium membrane-derived stromal cells (SM-MSCs)

There are studies that report the ability to isolate MSCs from the synovium membrane [29], [30], and they show a capacity to proliferate in culture, maintaining multilienage differentiation potential *in vitro*. These cells may play an important role in regenerative applications for joint problems [29].

The SM-MSCs show a phenotype similar to that of MSCs form other tissues, since they express the typical markers such as CD44, CD73,CD90 e CD105, and do not express CD34, CD45, CD31 and CD14 [1].

Bari et al [29] isolated the synovium membrane-derived cells from the knee joint and differentiated them into multiples lineages (chondrocytes, osteocytes and adipocytes) and concluded that cells have the potential to properly differentiate in these lineages. In a study from *Sakaguchi et al.* [30] synovium cells demonstrated good ability for chondrogenesis, adipogenesis (the red O oil staining was highest in comparison to other analyzed sources) and osteogenesis (the rate of positive alizarin red staining was the highest too). *Yoshimura et al.* [31] even claimed that SM-MSCs are superior to bone marrow, adipose tissue, muscle derived cells in terms of colony number per nucleated cell and colony number per adherent cells; and comparing the different tissues, synoviuem-derived cells have the highest chondrogenic potential which can be used in cartilage-related tissue engineering approaches.

Muscle-derived stromal cells (MDSC)

Skeletal muscle tissue contains two different types of stem cells: satellite cells and a population of multipotent stem cells [1], [32]. Satellite cells are unipotent cells that are originated from a population of muscle progenitors during embryogenesis [1]. The population of multipotent stem cells, so called muscle derived stem cells (MDSC), seem to form a stable, self-renewing pool of stem cells in adult muscle exhibiting the ability to differentiate into a variety of cell types [32].

MDSC have high proliferation and self-renewable capacity, express markers CD34, Sca1 and don't express c-Kit [1].

Teeth-derived stromal cells (T-MSCs)

Stem cells can be found in either the dental pulp of deciduous teeth [33], [34], as within the periodontal ligament [1], [34]. Stem cells derived from human deciduous teeth are a readily accessible tissue source since they are routinely exfoliated in childhood, with little or no morbidity to the patient [34], [35].

According to Miura et al. [33] and Seo et al. [35] stem cells from pulp of human deciduous teeth and periodontal ligament seems be a ideal source for repair bone defects in pediatric patients.

Periodontal ligament-derived stem cells express the common markers from MSCs, like CD13, CD29, CD44, CD59, DC105 e STRO-1 [1]. Stem cells isolated from deciduous teeth also express MSC markers like CC9, MUUC18 and CD146.

T-MSCs show osteogenic, adipogenic and chondrogenic differentiation. The induction into these lineages is similar the other MSCs [1], [34], [35].

ADIPOSE-DERIVED STROMAL CELLS (ADSCs)

The adipose tissue, like bone marrow, is derived from the embryonic mesoderm and contains a heterogeneous stromal cell population [22]. This stromal cell population is called stromal vascular fraction (SVF) and include adipose stromal cells, hematopoietic stem cells, progenitor cells, circulating blood cells, fibroblasts, pericytes, endothelial cells, lymphocites, monocyt and macrophages [36], [37].

These cells, *in vitro*, were found to differentiate towards the osteogenic, adipogenic, myogenic, and chondrogenic lineages [37]. A lot of researchers claim the ADSCs differentiation into non-mesenchymal lineages [3], [17], [38], [39], however this plasticity is not accepted by the entire research community [6], [40].

The SVF cells in adipose tissue usually sum up to 3% of the total cell count, and this is 2,500-fold more than the frequency of stromal cells in bone marrow [41]; in other words, adipose tissue provides larger numbers of stromal cells compared to bone marrow. A bone marrow transplant contains about $6x10^6$ nucleated cells per mL, of which only 0.001-0.01% are stromal cells [14], [42], whereas the number of SFV cells that can be isolated from subcutaneous liposuction aspiration is approximately 0.5-$2.0x10^6$ cells per gram of adipose tissue [22], [42]–[44]. Other features distinguish ADSCs and BMSc: ADSCs seems to have an increased tendency for muscular differentiation than BMSc, but less robust un chondrogenic and osteogenic differentiation [37]. Further, and the adipose tissue is more easy of harvest than bone marrow.

The Internation Federation for Adipose Therapeutics and Science (IFATS) and the Internation Society for Cellular Therapy (ISCT) proposed a combination of negative and positive markers to identify the SVF and the adherent stromal cells population [37]. The markers CD13, CD29, CD44, CD73, CD105, CD90 (>40%) and CD34 (>20%) must be positive on SFV and the markers CD31 (<20%) and CD45 (<50%) must be negative on SFV [37].

ADSCs should be further be negative for hematopoietic markers, including CD11b, CD45, CD34, CD19 and positive for CD13, CD73 and CD90 [6], [37]. CD36 and CD 106 can be used to distinguish ASCs from MSCs from bone marrow [37].

The localization of stromal cell population within adipose tissue is complicated because there is not a single and specific marker that clearly identifies undifferentiated ADSCs. Recent studies suggest that a stem cell population resides in a perivascular location [3], [45], [46] and MSCs in general are a subset of pericytes or vascular stem/stromal precursor cells located near the vasculature [3], [47].

The pericytes are perivascular cells which appear in all tissues in contact with the intimal surface of small vessels [40]. Some studies show that cultured pericytes have the ability to differentiate, *in vitro*, into osteoblasts, adipocytes, chondrocytes and myocites, supporting the idea of a perivascular niche for MSCs [48]. The cell population from this niche seems to express the markers CD34, CD90 and the absence of CD31, CD45 and CD146 expression [3].

Despite all of these studies, the definite identification of the ASDC population *in situ* has not been found yet [3]. The niche (physiologically microenvironment that supports stem cells) is very important to control de self-renewal and differentiation, as well as function and phenotype of the ADSCs [49], [50].

ISOLATION OF ADIPOSE-DERIVED STROMAL CELLS

There is no consensus in isolation protocol of ADSCs leading to distinct results and conclusion. The protocols used by different laboratories and work groups around the world differing in the type of tissue digestion (enzymatic or non-enzymatic), within the enzymatic digestion, in the type and concentration of the enzyme used, time and conditions of incubation for tissue digestion, time and parameters of centrifugation, number of filtration used and methods of cell culture [37].

The first study that was aimed to isolate cells from adipose tissue was performed by Rodbell [51]. In that study, epididimal fat pads of rats were used, that were washed

and incubated with collagenase and centrifuged to separate the floating population of mature adypocites from the pelleted stromal vascular fraction (SVF) [36].

The most commonly used procedure to isolate the SVF from adipose tissue samples, obtained by biopsy or liposuction, is the enzymatic method (see Figure 3). Firstly, it is necessary to wash the tissue repeatedly with sterile PBS or culture medium. After, the tissue sample is minced using scalpels and a collagenase solution is used to incubate the sample at 37ºC, 5% CO_2 for 30 minutes to one hour, with agitation. After that time, the collagenase is neutralized with culture medium, and the sample is pipetteed up and down to facilitate the disintegration of the tissue. The digested tissue is following centrifuged so that the separation of the stromal cells from the primary adipocytes is conducted [22], [36], [37], [52]–[54].

The centrifugation results in the separation of the sample from adipose tissue into three distinct layers (Figure 2): infranatant (bottom layer), middle portion and supranatant (upper layer). The infranatant contains blood, the SVF, fluid tissue and local anesthetic (in case of liposuction from human adipose tissue); the middle portion is composed primarily by fatty tissue; and the supranatant consist in lipids from fat cells [55].
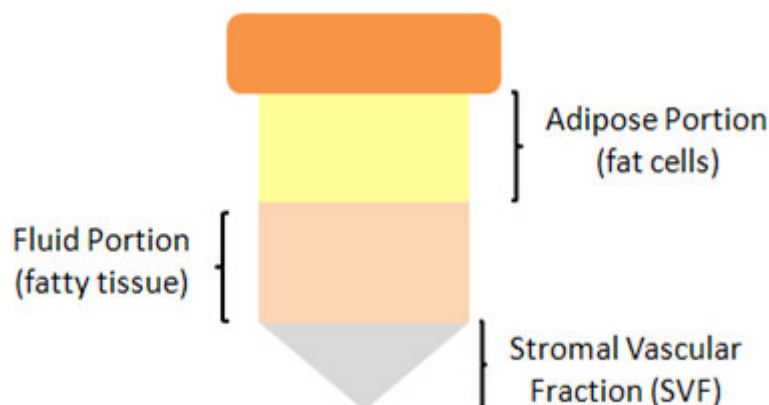
Figure 2 Schematic of tube appearance after centrifugation, conducted to obtain the stromal vascular fraction which contains the mesenchymal stromal cells

Then, the attained cell pellet within the infranatant can be resuspended in lyses buffer, incubated on ice, washed and centrifuged. The attained single-cell cell suspension can be resuspended in culture medium and filtered through strainer (Figure 3). The adipose derived stromal cells are isolated and ready to be cultured in a suitable medium and substrate [22], [36], [56].
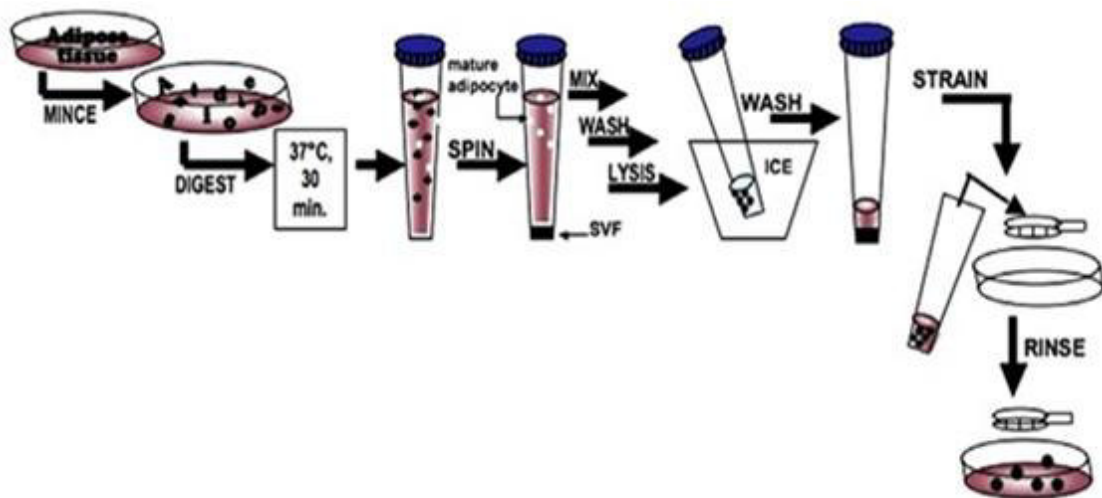
Figure 3 Procedure for isolation of ASCs from adipose tissue (adapted from (21))

Although, the enzymatic digestion is the most used isolation technique, there are some disadvantages reported in literature [57]–[61]. Enzimatic digestion is time consuming (between 30 minutes to 1 hour), expensive due to the cost collagenase, decreases cell viability due to lytic activity and ineffective when applied to large volumes of tissue [57]–[61].

Another problem in the traditional methods of isolation is the contamination of adipose tissue samples with other types of cells, due to the inefficient isolation procedure [58]. To solve this, new methods (enzymatic, non-enzimatic and mechanical techniques) are being developed to improve isolation [57]–[60].

Another factors that can influence the cell yield, growth and frequency, and which are described in the literature, include: the type of surgery used for adipose tissue harvesting (resection, tumescent liposuction and ultrasound-assisted liposuction) [62]; the harvest site [63]–[70]; donor age [3], [69], [71]; body mass index [3], [72]; isolation processes (like decantation, washing and centrifugation) [73]; the instruments used to collect the adipose tissue during the liposuction (for instance the size of liposuction cannula [56]); culture procedure (initial plating density and confluence, composition of cultured basal media, cell culture supplements, addition of antibiotics, oxygen supply and method of subculturing [3]).

While literature reports seem to converge to an agreement on some of these parameters, conflicting data is reported on the evaluation of other parameters. Some examples are following given.

In a study that compares biopsies of adipose tissue from different anatomical sites in rats, it is suggested that ASCs from the neck region seem to be the best source for regeneration of nervous tissue [64]. The study from *Jurgens et al.*, intended to understand whether the yield of ASCs are affected by the anatomical sites, reported that the abdomen seems to be preferable to the hip/thigh region for harvesting adipose tissue [65].

Schipper et al. [63] evaluated the age of donors and concluded that results are dependent on donor age and the younger age range tended to have the highest activity in each subcutaneous depot [63]. *Mojallal et al.* [71] examined adipose tissue samples from 42 women divided into 2 groups according to BMI (body mass index), and concluded that there is no significant correlation between ASC yield and proliferation capacity, and age and BMI [71]. The same result was obtained by *Buschmann et al.*[69]*,* which concluded that BMI do not influence cell yield and proliferation rate. However, *Harmelen et al*. concluded that the capacity of stromal cells appears to decrease with increasing BMI [72]. *Frazier et al.* [74] also concluded that a elevated BMI resulted in reduced ASC proliferation and osteogenic differentiation *in vitro.*

Relative to the size of liposuction cannula, it is generally accepted by researchers to have influence in cell viability, since an increase in the diameter, corresponds to an increase in the cell viability [55], [56].

The basal medium for ASCs cultures also seems to influence the proliferation and differentiation capacity [3]. Most laboratories use the Dulbecco's modified Eagle medium (DMEM) as a basal medium to culture ASCs, but there are different concentrations and components commercially available. ASCs cultivated in DMEM with a higher glucose concentration seems to present the best proliferation rate [3], despite that a low calcium concentration together with antioxidants has also been show to accelerate the proliferation of ASCs [75]. Another study that compared different types and concentrations of basal media concluded that Minimum Essential Medium Eagle with the alpha modification (α-MEM) allowed a significantly faster cell expansion than the other media used in researcher [76].

Another issue that has been discussed is the use of autologous serum, serum-free cultures supplemented with platelet-derived supplements [77] or growth factors, instead of the routine use of fetal bovine serum (FBS) [78]–[81]. The culture medium for ASC proliferation and expansion requires supplementation with serum. The main function of serum in culture is to provide hormonal compounds which stimulate cell growth and proliferation providing transport proteins, minerals, lipids, attachment and spreading factors [81]. The most used is fetal bovine serum, however the use of xenogeneic serum can cause several problems, namely induction of immune reactions and viral or bacterial infections [78], [79], [81]. The conclusions of studies that analyzed the behavior of ASC cultured in autologous serum, showed that ASCs can be efficiently propagated without the loss of specific markers and multipotency [79]. Autologous serum was found to be used as a culture medium supplement that adequately supported the expansion of ASCs [77], [82]. Within the translation of the clinical scenario, the human platelet lysate has also been study to substitute FBS, and shown to be effective in growth and expansion of ASCs, minimizing the risk of microbial infections and reducing the immunological reactions [77], [82].

DIFFERENTIATION OF ADSCS

Adipose stromal cells show multipotency, that is, they retain the ability to differentiate into cell types of multiple different lineages. They can differentiate into classical mesenchymal lineages (adipogenic, chondrogenic, osteogenic and myogenic). The differentiation of these cells can be directed by the addition of specific combinations of chemical compounds or cytokines to the culture medium [36].

Adipogenic differentiation can be induced using culture medium supplemented with isobutylmethylxanthine, indomethacin and dexamethasone [36]. The accumulation of neutral lipids can be detected by staining the cells in a solution of Oil Red [36] and the ASCs in culture expresses several adipocytic genes including lipoprotein lipase, aP2, PPARγ, leptin, Glut4, and lipid-laden intracellular vacuoles [17], [38].

The induction of osteogenic differentiation is promoted by supplementation of the medium with dexamethanose, beta-glycerol-phosphate and ascorbic acid [22], [36]. The concentrations of these inductors is not consensual, because the traditional osteogenic medium used was optimized for differentiation of bone marrow stromal cells and may not be optimal for differentiation of ASCs

To address the osteogenic differentiation, extracellular matrix calcification can be confirmed by von Kossa or Alizarin red staining, and alkaline phosphatase activity may be measured biochemically or following histochemical staining [22]. Cells can also be examined by PCR techniques for the expression of several osteogenic-specifc genes, including osteocalcin, run-related transcriptional factor 2 (Runx-2), osteopontin, ostenectin, bone morphogenetic protein (BMP-2) and, also, for the receptors involved in osteogenesis (parathyroid hormone receptor/PTHR, RXRα and vitamin D receptor/VDR) [17]. ASCs in culture, following osteogenic differentiation, express genes and proteins associated with an osteoblasts phenotype, including alkaline phosphatase, type-I collagen, osteopontin, osteonectin, osteocalcin, bone sialo protein, RunX-1, BMP-2, BMP-4, BMP receptors I and II, PTH-receptor (37).

For chondrogenic differentiation, micromass culture techniques and cell pellets are cultured with bone morphogenic proteins, dexamethasone, ascorbate-2-phosphate

and insulin [22], [36]. Chondrogenesis can be confirmed by using the histological stain Alcian Blue at acidic pH [22]. Following specific induction, ASCs produce large amounts of cartilage-related extracellular matrix molecules, including sulfated proteoglycans, collagen II and IV and aggrecan [38].

Myogenic differentiation can be induced by dexamethasone, hydrocortisone and azacytidine   [22], [38], and confirmed by immune-histochemical staining for the muscle-specific transcription factor (MyoD1) and the myosin heavy chain [22]. Following induction, cells show a pattern of expression of muscle-related genes that is consistent with normal myogenesis, with the expression of regulatory factors MyoD1, myf5, myf6 and myogenin. Morphologically, they are similar to the muscle cells, becoming long and multinucleate [38].

THREE-DIMENSIONAL CULTURE OF ADSCs

Three-dimensional (3D) cultures systems allow an increased cell-to-cell interactions and mimics more closely the physiological conditions between the cells and the extracellular matrix, when compared with two-dimensional cell culture techniques (monolayer cultures) [83]–[88].

Traditionally, 3D cell aggregates or spheroids have been used to study the behavior of tumor cells but also study biological mechanisms, angiogenesis, immune response, mimic embryogenesis in stem cell culture and induce clonal propagation and spontaneous differentiation [89]–[93].

In the literature, there are several techniques used to generate spheroids, but the common principle of all methods is preventing the cellular attachment to a culture substrate [83]–[88].

The hanging-drop technique (HDT) is based on the deposition of a droplet of cells onto the underside of the lid of a tissue culture dish. After, the lid is inverted on the lower half of the dish, and the drops are maintained on the lid by surface tension, since the cells cannot adhere on the air-liquid interface occurring, the spheroids form spntaneously [83], [86]. The HDT is simple to perform, inexpensive and allow the generation of spheroids with defined sizes, cell numbers and composition. But it's difficult at large scale production [86] and long term culture [94].

The liquid overlay technique (LOT) uses a non-adhesive surface to prevent the cellular adhesion [83], [86], [89]. These non-adhesive surface can be achieved by coating the bottom of the plate with agarose or hydrophobic polymers [83], [86], [89]. The disadvantage of this technique is the variation in size, cell number and shape; however it's simple to perform, inexpensive and easy to scale up [86].

Another method to generated spheroid is to use spinner flasks and roller bottles. If the cells are maintained in motion using a gyratory rotation incubator, they cannot adhere to surface and they will form spheroids [83], [86], [89]. With this technique it's possible generated spheroids in a large scale, in a simple way and with a dynamic control of

culture condition [86]. But, it's necessary specialized equipment and the spheroids generated have variations in size and cell number [86].

Spheroids can also be generated using conical plastic tubes. After isolation, the cells are subject to a centrifugation and the resulting cell pellet is left in the conical plastic tubes [83], [95], [96]. The tubes should be shaken in certain periods of time to avoid the pellet sedimentation [96].

Like another type of cells, mesenchymal stromal cells and namely adipose-derived stromal cells (ADSC's) have the ability to form multicellular spheroids without a scaffold [85], [88], [91], [94].

Apart from the scaffold-free techniques previously described, several 3D scaffolds can be used to provide physical support for cell self assembly and any external forces that enhance rapid cell aggregation, like low-speed centrifugation, magnetic fields and ultrasound standing wave traps, can be used in association [86].

The microenvironment generated in 3D culture systems mimic the stromal cell niche *in vitro*, improving the intercellular adhesion, the extracellular matrix interaction, proliferation and differentiation [94].

Hildebrandt el al. [94] compared three different techniques to generated multicellular aggregates of human bone marrow mesenchymal stromal (hBMSCs) cells considering the differentiation into osteogenic lineage. The techniques used were the hanging drop method combined with suspension culture, the plastic tube culture and liquid overlay technique (LOT). They concluded that the cultivation of hBMSCs in 96 well non adhesive plates was the best method since the formed aggregates were uniform, the efficiency was good and the osteogenic capability was induced [94].

In the work of Baraniak et al. [91], mice BMSCs spheroids were formed using a forced aggregation technique. The goal was understand the behavior of the spheroids in long periods of time taking into account differentiation potential and the multipotency of the cells [91]. They demonstrated that the mesenspheres formed exhibited the ability to differentiation into osteogenic and adipogenic lineage and maintained under

growth conditions for a long time (16 days). Moreover, the development of 3D culture allow control several critical parameters that can used to generate an *in vitro* model for the study of cell-cell and cell-matrix interactions.

Kapur et al. [88] using a hanging-drop technique formed human ADSCs spheroids with different cell concentrations and in different types of culture medium, to optimize the method and establish the best conditions. The spheroids formed showed ability to proliferate, renewal and differentiate in different culture medium used and the techniques applied in spheroids formation demonstrate reproducibly and efficacy [88].

Shen et al. [87] made an *in vitro* and *in vivo* study to characterize the osteogenic differentiation of human ADSCs aggregates. The technique used to generate the spheroids was the hanging drop and the differentiation was proven using histology and gene expressing. The *in vivo* assay consisted in implantation of human ADSC spheroids alone, Matrigel with human ADSCs and Matrigel alone (control) in a rat muscle pouch. Furthermore, they reseed the ASC spheroids onto monolayer culture to investigate the potential of proliferation and differentiation. The results showed that spheroids maintained the renewal potential and multilineage differentiation capabilities [87].

Lashke et al. [85] using the LOT technique generated ADSC spheroids to analyze the angiogenesis mechanism. The ADSC spheroids were incorporated into polyurethane scaffolds and a dorsal skinfold chamber model was used, as an *in vivo* assay. They conclude that ADSCs spheroids enhanced the blood vessel formation being suitable for tissue engineering applications [85].

ADSCs IN REGENERATIVE MEDICINE APPLICATIONS

Tissue engineering and regenerative medicine are two fields of science that combine tools of medicine and engineering, by acting on cells, growth factors and biomaterials, in order to enhance the tissue formation and regeneration, repair or maintenance of the physiological function of a tissue or organ [2], [97], [98].

Mesenchymal stromal cells (MSCs) in general, show a huge potential therapeutic activity that may be used in a wide range of diseases. The main reasons for the use of MSCs in tissue engineering and regenerative medicine are: the ease of isolation and expansion, high expansion potential, ability to differentiate into several mesodermal lineages, paracrine effects that modulate the host cells, multipotency, immunomodulatory properties, migratory behavior and ethical considerations [5], [7], [37], [43], [85]. Particularly, the adipose stromal cells (ADSCs) have generated a great deal of interest since they are relatively accessible [2]; the enzyme-based isolation procedures are uncomplicated [99] and they can be isolated in large quantities, and hold no ethical concerns [3].

Once ADSCs have the ability to differentiate into bone, cartilage, skeletal muscle and fat, the tissue engineering of mesenchymal organs is the main purpose [99]. The clinical application may include, for example, repair of soft tissue defects (owing to traumatic injury, tumor resection and congenital defects) [100], cartilage repair in the joints, skeletal regeneration after tumor or trauma, repair of bone defects, etc [99]. Further, other applications include the use of ASC sthrough a paracrine effect. For instance, improve the myocardium function after infraction, possibility peripheral nerve regeneration, hepatic regeneration, insulin-producing islet cell regeneration and recovery of renal function, have been considered [55]. Accordingly, the potential of ADSCs for regenerative medicine is related to their differentiation ability, but also due to their paracrine and thropic effects [40], [101] and their immunomodulatory properties [97], [102].

Unlike the BMSCS, the immunosuppressive properties of ADSCs have not been studied adequately [97]. However, several studies have shown that ASCs exert

immnomodulatory and protective effects on graft-versus-host response, inhibiting the production of inflammatory cytokines, stimulating the production of anti-inflamatory/suppressive cytokines, such as IL-10, by monocytes and T cells. Further, they have been shown to induce the generation of antigen-specific regulatory T cells and to inhibit the lymphocyte proliferation [97], [98]. Moreover, ADSCs seem to be immunopriviledged due to lack of HLA expression (major histocompatibility complex class II antigens) [97].

Another feature of ADSCs is the capacity to act as "secretome" [97], [98], [102]. In this context, ADSCs secrete proteins and growth factors into the extracellular milieu that is able to influence and modulate other cells and tissues [98], [102]. The molecules which can be related to this modulatory activity of ADSCs are, for instance, adiponectin, angiotensin, cathepsin D, penetraxin, pregnancy zone protein, retinol binding protein, hepatocyte growth factor, granulocyte and macrophage colony stimulating factors, tumor necrosis factor-alpha, vascular endothelial growth factor, brain derived neurothopic factor, nerve growth factor, adipokines, among others [98]. Many studies have tried to understand the beneficial effect of ADSC in non-mesodermal tissues based on "secretome" effect and immunomodulatory activity [102]. These studies included applications on tissue regeneration and repair on the central nervous system, immune system, heart and muscle [102].

Despite all the investigation and research carried out in the field of adipose mesenchymal stromal cells, an optimized procedure for cell harvest and expansion is still lacking, as well the definition of several parameters to optimize the isolation of ADSCs and, consequently, the cell culture and their use in a regenerative therapy.

One of the factors than is expected to significantly influence the cell proliferation and differentiation is the location of tissue harvest. Accordingly, in order to disclose this effect for prospective translational use of ADSCs in regenerative medicine applications, particularly focusing on the bone tissue, this work aims the evaluation of the cell behavior of osteogenic-induced ADSCs, harvested from different anatomical locations within the rat.

Elisabete Gonçalves

II. RESEARCH HYPOTHESIS AND OBJECTIVES

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RESEARCH HYPOTHESIS

It was hypothesized that adipose-derived stromal cells harvest from different anatomical locations showed a distinct functional activity, particular regarding cell proliferation and ostegenic differentiation capacity.

OBJECTIVES

This work aimed the isolation of ADSCs from distinct anatomical locations and the characterization of the osteogenic differentiation capability of these cells.

Specific objectives included:

- isolation of stromal cells from the adipose tissue harvested from different anatomical locations (subcutaneous inguinal, abdominal inguinal, flank, neck and omentum) within the rat;

- establishment of a two-dimensional culture (monolayer) of osteogenic-induced ADSCs;

- establishment of a three-dimensional culture (spheroids) of osteogenic-induced ADSCs;

- characterization of the established cultures through biochemical, histochemical, gene expression and microscopical techniques.

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III. MATERIALS AND METHODS

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MATERIALS

All cell culture chemicals and supplies were acquired from Merck and Sigma Aldrich (St. Louis, MO) unless otherwise noted. All tissue culture flasks and plates were obtained from Corning (Corning, NY).

METHODS

ANIMALS

In this study, twelve female Wistar rats (Charles River, Wilmington, MA), 7-8 weeks old, with a body weight of around 250-300g, were used. Animals were housed in type II plastic cages, in a monitored environment of temperature and humidity, in a 12h light/dark cycle. Dry feed and water was supplied *ad libitum*.

ISOLATION OF ADIPOSE-DERIVED STROMAL CELLS (ADSCs)

After the euthanasia of the animals, the adipose tissue was harvested in two different ways: removing fat from undifferentiated anatomical locals (FUAL) and fat from differentiated anatomical locals (FDAL). The sites were chosen taking into account previously literature reports and these included: abdominal inguinal area, flank, neck, subcutaneous inguinal area and omentum (see Figure 4).
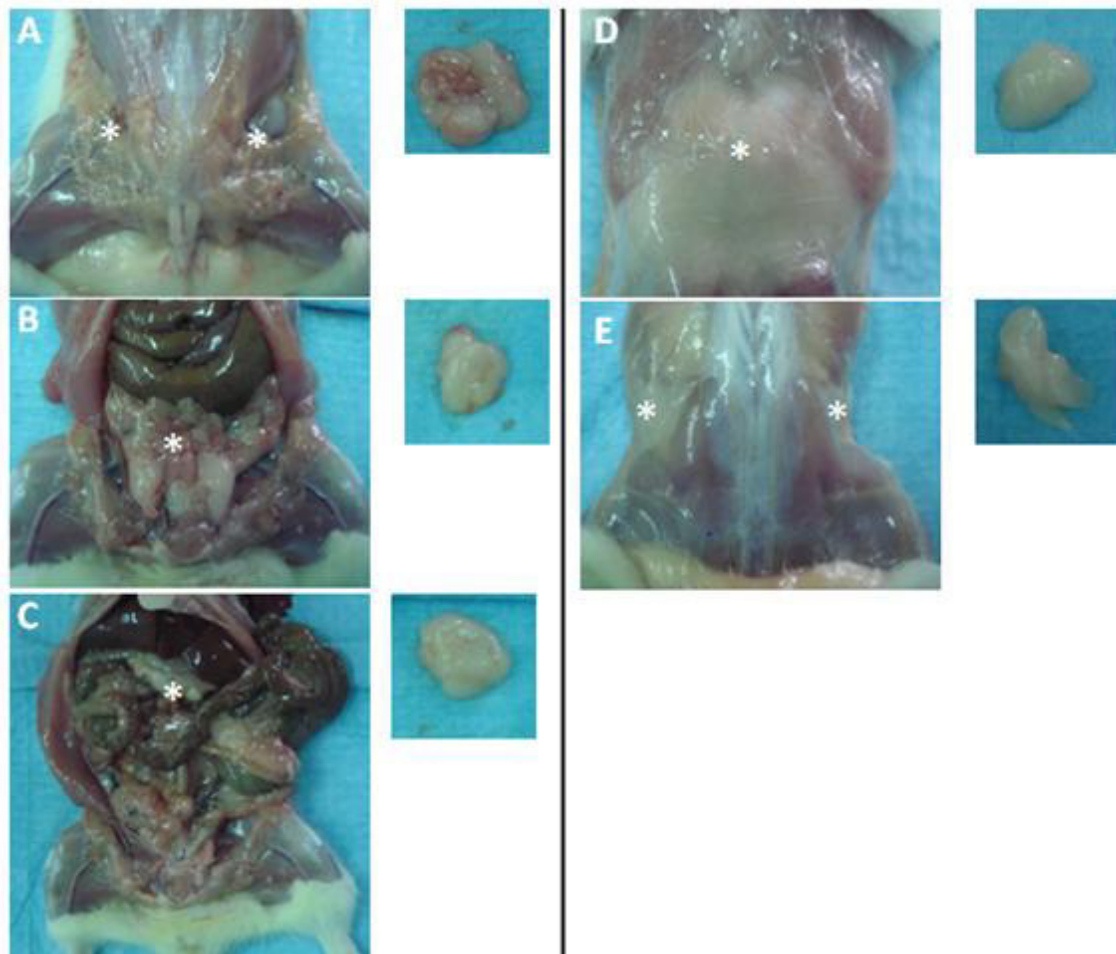
Figure 4 Different anatomical locals of fat harvest with respective harvest tissue aside. A – subcutaneous inguinal; B – abdominal inguinal: C – omentum; D – neck; E – Flank. * marks the specific site of harvest.

In these experimental procedures, large pieces of harvested fat were cut into very small pieces (around 1mm^3) and placed in a decontamination solution consisting of α-Minimal Essential Medium (α-MEM) supplemented with 2.5µg/ml fungizone (Fungi.) and 100 IU/ml penicillin-2.5µg/ml streptomycin (Pest.) for about one hour.

After this time, the adipose tissue sample was placed in a propylene tube with 0.075 mg/ml collagenase type IA in α-MEM for tissue digestion, which was incubated for one hour at 37ºC, 5% CO_2 in air, with periodic stirring to facilitate the digestion. Afterwards, the collagenase activity was neutralized by adding of 10% of fetal bovine serum (FBS) and the cell suspension was filtered and transferred to another propylene

tube. Then, the samples were centrifuged at 1200 rpm for 10 minutes and a separation into distinct layers was obtained, with the last layer (the pellet) corresponding to stromal vascular fraction (SVF) (see Figure 5).
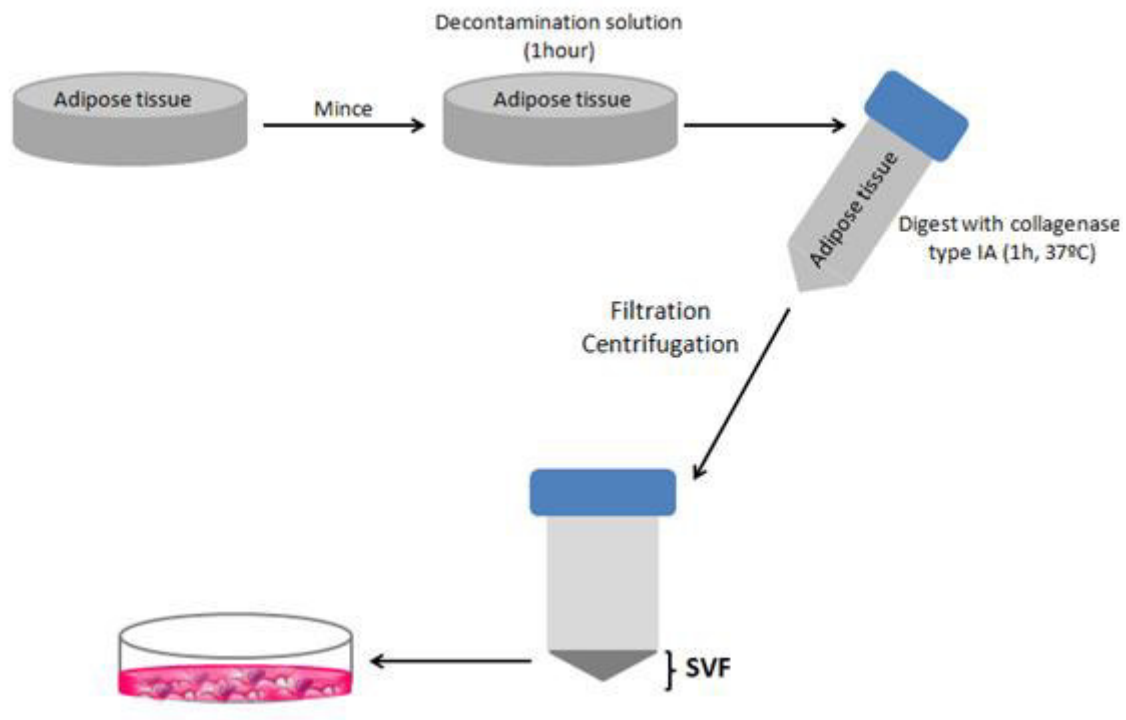
Figure 5 Schematic representation of the isolation method used to obtain the adipose-derived stromal cells from SVF

The cell pellet was resuspended in culture medium (α-MEM supplemented with 10% of FBS, Fungi, Pest and 5 mg/ml of ascorbic acid (AA)) and seeded in a 6-well plates (9 cm^2 area). The culture medium was changed the next day, and following, every 2/3 days, and the cultures were regularly observed by contrast phase microscopy.

MONOLAYER CULTURE (2D)

ADSCs from fat from undifferentiated anatomical locals (FUAL)

The primary culture took seven days to achieve a state of around 80% confluence. On this day the first sub-culture was established, as follows. Cells from primary cultures

were obtained by tripsinization (trypsin 0.04%) , the cellular viability was assessed by the exclusion method of blue trypan and following, cells were counted in a Neubauer Improved Chamber. The cells were seeded in 96-well plates (0.32 cm^2 area) at a density of 10^4 cells/cm^2.

Cells were cultured in three different conditions: the control medium (CM) – α-MEM with 10% FBS, 2.5μg/ml of Fungi, 100 IU/ml penicillin-2.5μg/ml streptomycin and 5 mg/ml of AA - osteogenic medium 1 (OM1) - α-MEM with 10% FBS, 2.5μg/ml of Fungi, 100 IU/ml penicillin-2.5μg/ml streptomycin and 5 mg/ml of AA and dexamethasone 10^{-6} M– and osteogenic medium 2 (OM2) - α-MEM with 10% FBS, 2.5μg/ml of Fungi, 100 IU/ml penicillin-2.5μg/ml streptomycin and 5 mg/ml of AA and dexamethasone 10^{-6} M and β-glicerol-phosphate 1M. Cultures were maintained during 15 days. The culture medium was renewed 2-3 times per week and cultures were analysed at adequate time points.

ADSCs from fat from differentiated anatomical locals (FDAL)

The primary culture took eight to ten days to achieve a state of around 80% confluence. On this day, cells from primary cultures were obtained by tripsinization (trypsin 0.04%), the cellular viability was assessed by the exclusion method of blue trypan and following cells were counted in a Neubauer Improved Chamber and seeded in 96-well plates (0.32 cm^2 area), at a density of $5x10^3$ cells/cm^2.

Each anatomical local was subject two different experimental conditions: control medium (CM) and osteogenic medium 2 (OM2) with the previously described compositions. The cultures were maintained during 15 days, the culture medium was renewed 2-3 times per week and cultures were analyzed at adequate time points.

Characterization of cell cultures

To characterize the established cell cultures biochemical methods were used, including, quantification of DNA, MTT assay, quantification of alkaline phosphatase activity, histochemical methods for the staining of alkaline phosphatase activity,

collagen and senescence-associated beta-galactosidade activity, microscopic techniques and gene expression analysis.

Optical microscopy

Monitorization of cell cultures was conducted periodically through observation by phase contrast microscopy to adress the morphology and proliferation.

Assessment of the cell proliferation (DNA quantification)

The DNA quantification was performed using PicoGreen®. PicoGreen® is an ultra-sensitive fluorescent nucleic acid stain for double-stranded DNA quantification. When this dye was added to the samples it blended with the double-stranded DNA and the fluorescence emitted could be measured: it had a maximum excitation at 480 nm and an emission peak at 520 nm [103]–[105].

On the day of the experiment, after removing the culture medium, the cells were washed twice with PBS and 100µL of Triton X-100 0,1% were added to each well. The plates were incubated during 45 minutes at room temperature.

During this time, the PicoGreen® solution was prepared. For this purpose, PicoGreen® was diluted in 1X-TE buffer. Posteriorly, diluted PicoGreen was added to the samples which were in a black 96-well plate. The fluorescence was measured in an ELISA plate reader (Synergy HT BioTek) with excitation at 480nm and emission at 528 nm.

Assessment of the metabolic activity (MTT assay)

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is a colorimetric assay to assess the metabolic activity of viable cells, which can be useful in the indirect evaluation of cytotoxicity, cell viability and proliferation [106]. When added to the cells, MTT was reduced by the activity of mitochondrial dehydrogenases and the reducing agents present in metabolically active cells, originate dark colored formazan salts that are insoluble in aqueous solutions (see Figure 6) [106]. The lipid soluble formazan crystals can be dissolved in an organic solvent, like dimethyl sulphoxide (DMSO) and the optical density measured on a multiwall

spectrophotometer [106], [107]. The amount of MTT formazan measured is directly proportional to the number of metabolically active cells [108], [109].
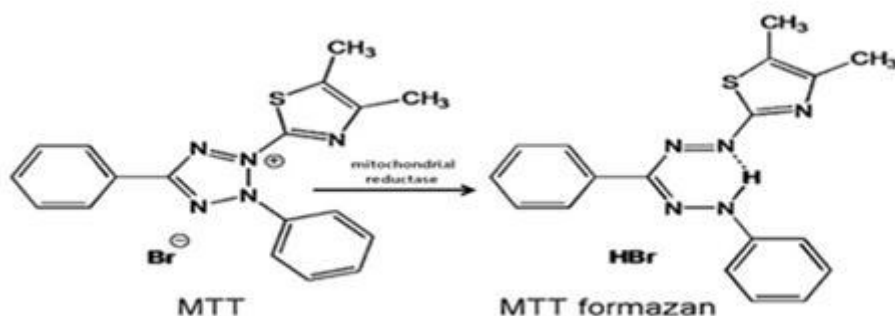
Figure 6 Chemical structure of MTT and reduction to formazan product (adapted from (105)).

The MTT analyses were conducted at specific time points. On each of these days, 10μL of MTT were added to each well and incubated for 2 hour at 37ºC with 5% CO_2. Then, the medium was removed and 100μL of DMSO were added. The absorbance was measured at 550 nm in an ELISA plate reader (Synergy HT BioTek).

Assessment of Cell Morphology

Established cell cultures were characterized by confocal laser scanning microscopy (CLSM) following staining of cytoskeleton and nucleus counterstaining. For CLSM assessment, cell cultures were fixed in 3.7% paraformaldehyde during 15 minutes and permeabilized with Triton X-100 (0.1%). Then cells were incubated with albumin (10 mg/ml) to reduce non-specific staining. Cell cytoskeleton filamentous actin (F-actin) was visualized treating the cells with Alexa Fluor 488®-conjugated phalloidin (1:20 dilution in PBS for 20 minutes) and counterstained with propidium iodide (1 μg/ml for 10 min) for cell nuclei labeling. Labeled cultures were mounted in Vectashield® and examined with a Leica SP2 AOBS (Leica Microsystems) microscopy.

Assessment of the alkaline phosphatase activity

Alkaline phosphatase (ALP) is a metalloenzyme present in various tissues, including bone. In bone, ALP is produced by osteoblasts and serves as an indicator of their activity, being considered a marker of earlier osteogenic differentiation *in vitro* [110], [111].

The presence of this enzyme is detected by using a specific substrate, the p-nitrophenylphosphate (pNPP). In a buffered solution, and at alkaline pH, the hydrolysis of pNPP occurs mediated by ALP, resulting in p-Nitrophenol (yellowish compound) and a phosphate group, according to the following reaction [112]–[114]:

Figure 7 Reaction of pNPP hydrolysis to form p-nitrophenol and phosphate

The amount of p-Nitrophenol formed is determined by the absorbance at a specific wavelength (the value is around 400nm). The quantity generated is proportional to the concentration of ALP present in sample, according to Beer's law [115].

After removing the culture medium, the cells were washed twice with PBS (Phosphatase Buffered Saline). Then, 100µL of Triton X-100 0,1% were added to each well and the plates were incubated at room temperature during 45 minutes. After that time, the buffered substrate was prepared (this is, p-NPP in alkaline buffer solution), added to the samples and incubated for 1 hour at 37ºC with 5% CO_2 in air. To stop the reaction, 20 µL of NaOH 5M were added and the absorbance was measured in an ELISA plate reader (Synergy HT BioTek) at 400nm.

Histochemical methods

The cell cultures were fixed on specific time points for subsequent realization of histochemical assays, namely the staining of alkaline phosphatase, staining of collagen and senescence staining. The culture medium was removed and all wells were washed twice with PBS. The cells were treated with glutaraldehyde at 1.5% during 10 minutes and, thereafter, the cells were maintained in sodium cacodylate buffer 0,14M and conserved at 4ºC.

Staining of alkaline phosphatase activity

The staining of alkaline phosphatase is based in hydrolysis of sodium phosphate naphthyl (Na-α-naphtyl). The phosphate formed reacts with a diazononium salt ("Fast Blue RR Salt") and forms a colored precipitate [116], [117]. The staining intensity depends on the activity of the alkaline phosphatase enzymes present on cells, and varies between light brown to black.

In the present work it was prepared a Tris buffer solution 0,1M with 2mg/mL of Na-α-naphtyl phosphatase and 2mg/mL of Fast Blue RR Salt complex. This solution was added to the culture that was previously fixed. After spending an hour in the dark, samples were washed with distilled water and air-dried. Stained cultures were photographed in a Nikon TMS Inverted Phase Contrast microscope with magnifications of 40x and 100x.

Staining of total collagen content

The staining of collagen is based in the interaction of a strong anionic dye, called Sirus Red, with collagenous fibrils. This dye stains collagen through a reaction of its sulphonic acid groups with basic groups present in the collagen molecule [118].

After removing the buffer, 100µL of Sirus Red dye were added to each well. An hour was waited for the dye to react and, after that, the samples were cleaned with hydrochloric acid 0.01N, in order to clean all the excess dye. Stained cells cultures were photographed in a Nikon TMS Inverted Phase Contrast microscope with magnifications of 40x and 100x.

Staining of senescence-associated beta-galactosidade activity

Senescence-associated beta-galactosidade (SA-βgal) is an hydrolase enzyme that is only present in senescent cells [119]–[121].

The principle of the assay is based on cleavage of the chromogenic substrate 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-gal) by β-galactosidade, that following reaction yealds blue precipitate [119], [121].

This staining was made using the "Senescence Cells Histochemical Staining Kit" from Sigma Aldrich. After removing the fixation fluid, the cells culture were washed twice with warm PBS and 50µl of staining mixture (that contains, among others, the X-gal solution) was added to each well. Then, the culture was incubated overnight at 37ºC without CO_2 (120). Stained cultures were photographed in a Nikon TMS Inverted Phase Contrast microscope with magnifications of 40x and 100x.

Gene Expression Analysis

Reverse-transcription polymerase chain reaction (RT-PCR) analysis was done in the ADSCs from FUAL and FDAL at specific time points. All were evaluated for the expression of Run-related transcription factor-2 (RUNX-2), Peroxisome proliferator-actived receptor gamma (PPAR-γ), Sox9, MyoD and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used are listed on Table 1. The total RNA was extracted using the NucleoSpin® RNA II Kit (Macherey-Nagel) according to the manufacturer's instructions. The concentration and purity of total RNA in each sample were assessed by UV spectrophotometry at 260 nm and by calculating the A260nm/A280nm ratio, respectively. RT-PCR was done using the Titan One Tube RT-PCR system (Roche® Applied Science), according to the manufacturer's instructions, for 30 cycles. RT reaction mixtures consisted of extracted RNA, Titan RT-PCR buffer, dithiothreitol (DTT), deoxynucleoside triphosphate (dNTP), primers for each tested gene and water, in a total volume of 25 µl. Total RNA was reverse transcribed with cDNA (30 minutes at 50ºC), which was then amplified with recombinant Taq- DNA polymerase at different annealing temperatures. For all the genes, the annealing temperature was 55ºC. The PCR products were electrophoresed in a 1% agarose gel,

stained with ethidium bromide and semi-quantitatively assessed by densitometry with Image J® software (US National Institutes of Health) version 1.37. The data were expressed as normalized ratios by comparing te integrated density values for all tested genes with those for GAPDH.

Table 1 Primers used on RT-PCR analysis

Gene	5' Primer sequence	3' Primer sequence
GAPDH	CTGAGTATGTCGTGGAGTCTACTG	CATACTTGGCAGGTTTCTCCAG
RUNX-9	CTATCCAGCCACCTTCACTTAC	CAGAGTCCACCTCTCTGAATTG
MyoD	CGACACGCGGATGACTTCTAT	CTCTCAGAGCACCTGGTAAATC
Sox-9	CATCTCTCCTAACGCCATCTTC	ACTCTGTCACCATTGCTCTTC
PPAR-γ	TCCCCAGCATTTCTGCTCCACACT	ATACAAATGCTTTGCCAGGGCTCG

SPHEROID FORMATION AND 3D CULTURES

The technique used to formed the ADSCs spheroids was the liquid overlay [83], [84]. First, an 1% of agarose solution (Seakem LE Agarose, Lonza) was prepared in destiled water and sterilized by autoclaving. After melt in microwave, 50 µl of liquid agarose was placed in the bottoms of 96-well plates. This coating avoids the cell adhesion allowing the spontaneous spheroid formation.

ADSCs from fat from undifferentiated anatomical locals (FUAL)

For this experiment, the ASCs isolated were seeded in two plates with 58 cm^2 of area. After eight days, the cells reached the adequate confluence (around 80%) and were trypsinized (trypsin 0.04%). The cellular viability was assessed by the exclusion method of blue trypan followed by cell quantification in a Neubauer Imporved Chamber.

The cells were seeded in 96-well plates (0.32 cm^2 area) previously coated with 1% of agarose solution, at two different densities: 5×10^4 cells/cm^2 and 20×10^4 cells/cm^2. For

each seeding density 16 wells were prepared and the spheroids were subjected two different experimental conditions: control medium (CM) and osteogenic medium (OM2) with the compositions previously described.

After 24 hours of incubation at standard conditions, the formation of spheroids was verified by optical microscopy and formation efficiency (ratio between obtained aggregates and initial number of seeded aggregates) was determinate. Cultures were maintained during 17 days and, twice a week, half of the culture medium was renewed. The maintenance rate (M.R.), consisting in the number of spheroids during the days of culture in relation to the number of formed aggregates at 24 hours, was measured.

ADSCs from fat from differentiated anatomical locals (FDAL)

Isolation of adipose tissue was conducted as previously described. Following, first subcultured cells were seeded in 96-well plates (0.32 cm^2 area) previously coated with 1% of agarose solution at a density of 20×10^4 cells/cm^2. For each anatomical location 32 wells were prepared and the spheroids were subjected two different experimental conditions: control medium (CM) and osteogenic medium (OM2) with the compositions previously described.

After 24 hours of incubation at standard conditions, the formation of spheroids was verified by optical microscopy and the formation efficiency was determined (ratio between obtained aggregates and initial number of seeded aggregates). On day 6, the maintenance rate (M.R.) of spheroids was measured in different anatomical locals. Cultures were maintained during 9 days and twice a week, half of the culture medium was renewed.

Image analysis

At specific time points, spheroids from FUAL and FDAL were photographed in a Nikon TMS Inverted Phase Contrast microscope with magnifications of 40x and 100x.

The microscopic images were analyzed to quantify the size of the spheroid in terms of cross-sectional area using Image J® software (US National Institutes of Health) version

1.37. The cross-sectional area was measured automatically using the appropriate tools from the software used.

STATISTICAL ANALYSIS

The data presented within this work is the result of 3 separate experiments. Multivariate analysis of variance (MANOVA) was conducted and no significant differences were verified between experimental settings. Accordingly, the shown results, are the ones from a representative experiment. Further, within quantitative data (MTT assay, ALP activity, DNA and PCR gene expression) each point represents the mean values ± standard error and for cross-section area of evaluated spheroids, each point represent mean values ± standard deviation. For quantitative assays, 5 replicates except where otherwise noted were conducted. Qualitative analysis and microscopical evaluation were conducted in triplicates. Statistical analysis was done by *t-student* test in the software Excel. P values ≤0.05 were considered significant.

IV. RESULTS

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Characterization of the two-dimensional ADSC cultures (monolayer)

ADSCs FROM FUAL

Cells were cultured in control medium during the primary culture (Figure 8) and the first subcultures were evaluated for cell morphology, proliferation, viability and osteoblastic differentiation, following specific induction, during 15 days.

Cultures established in the absence of osteogenic inducers were referred as "Control" and cultures grown in osteogenic medium (OM) with dexamethasone as "OM1" and osteogenic medium with dexamethasone and β-glicerol-phosphate medium as "OM2".

Figure 8 ADSCS in primary culture (40x)

Cell proliferation

Total DNA content was assayed and results presented in Graphic 1. Control cell cultures of ADSCs derived from FUAL increased the proliferation from day 1 to day 11 of culture, diminishing afterwards. Cell proliferation increased in both osteogenic medium (OM1 and OM2) during the days of culture. Nonetheless, comparatively, OM media induced a reduced proliferation at late culture time points (i.e., at day 8 and 11).
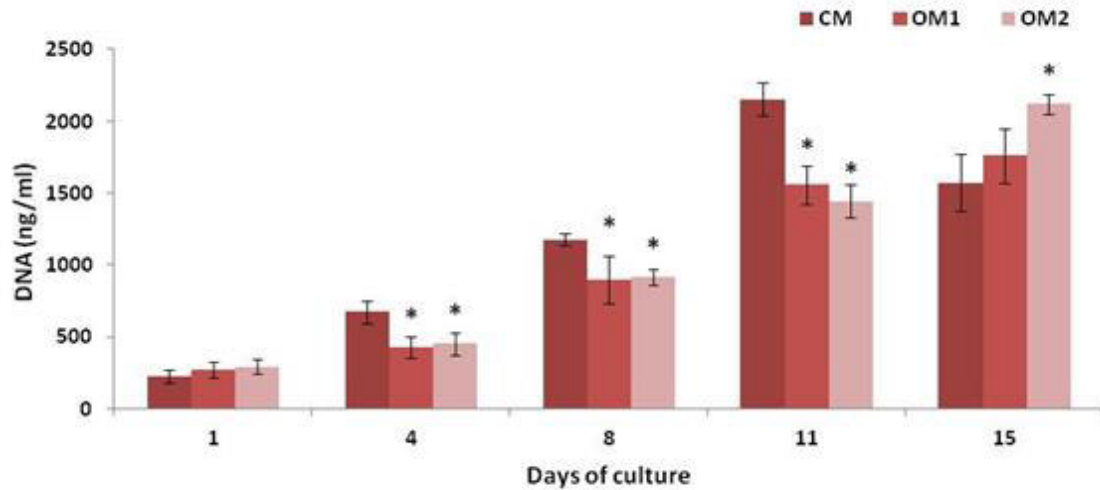
Graphic 1 Cell proliferation of ADSCs from FUAL during the fifteen days of culture. CM: control medium; OM1: osteogenic medium with dexamethasone; OM2: osteogenic medium with β-glicerol-phosphate; * - significantly different from control (p < 0.05).

Metabolic activity

Metabolic activity was evaluated by the MTT assay (Graphic 2). The cells from all groups gradually increased the metabolic activity during the 11 days of culture, diminishing afterwards. Significant differences were only verified at day 1, with osteogenic medium reducing the metabolic activity of the cultures.
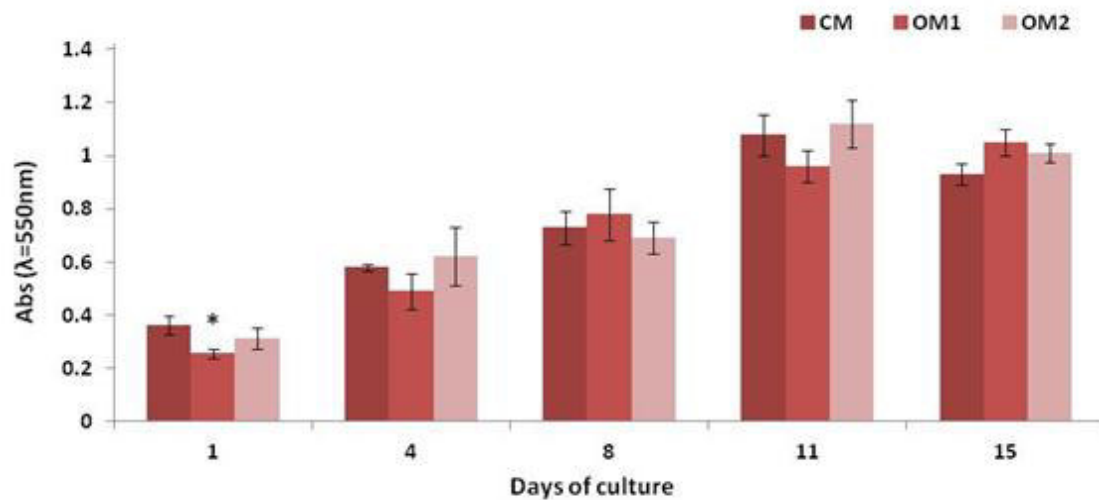
Graphic 2 Metabolic activity of FUAL subjected to three different conditions. CM: control medium; OM1: osteogenic medium with dexamethasone; OM2: osteogenic medium with β-glicerol-phosphate. phosphate . * - significantly different from control (p < 0.05).

Alkaline phosphatase activity

The Graphic 3 shows the values of the normalized alkaline phosphatase activity that was performed on days on days 1, 4, 8, 11 and 15 of culture. The values of ALP activity of the three groups, was found to increase until day 11, and then, decreased with the culture time. Osteogenic medium, both OM1 and OM2, were found to increase ALP activity at day 11 of the culture, as comparing to control.
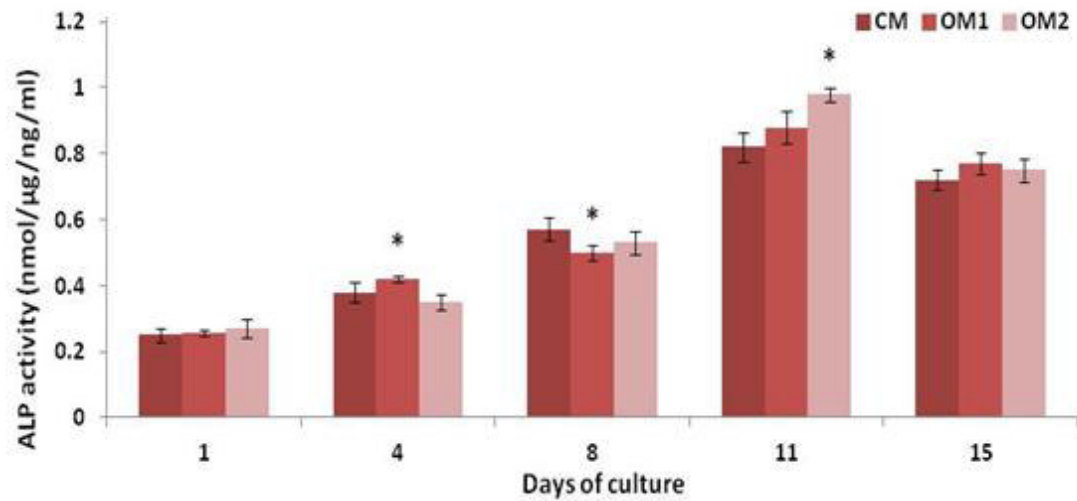
Graphic 3 Alkaline phosphatase activity (normalized by the amount of total protein) quantification during the fourteen days of culture. CM: control medium; OM1: osteogenic medium with dexamethasone; OM2: osteogenic medium with β-glicerol-phosphate. * - significantly different from control ($p < 0.05$).

Staining of alkaline phosphatase

Optical microscopy images from alkaline phosphatase activity, evaluated by histochemical staining, are presented in Figure 9. On control, a dense monolayer organization can be observed from cells grown up to day 11. With the addition of osteogenic inducers, a change in the culture morphology occurs, with the formation of nodular structures in which grown cells converge to. Comparatively, OM2 seems to induce a greater nodular organization (with darker and better organized nodules) than OM1, as observed by the histological staining at day 11.
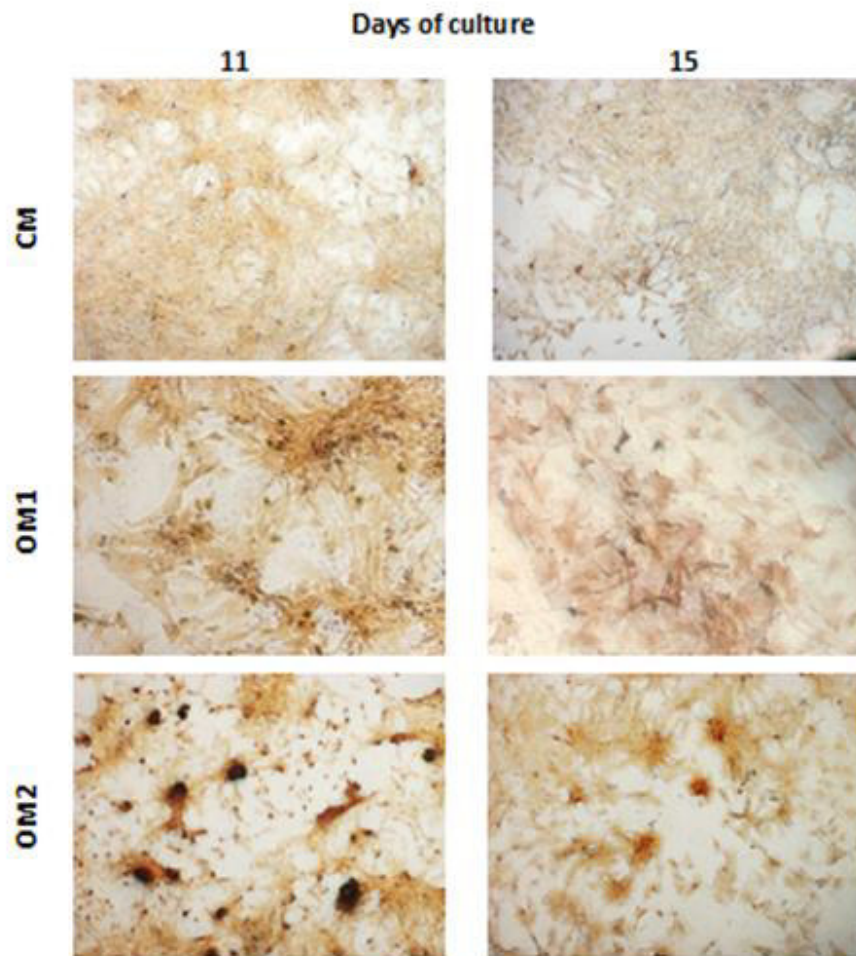
Figure 9 Microcopy images from alkaline phosphatase histochemical on day 11 and 15. CM: control medium; OM1: osteogenic medium with dexamethasone; OM2: osteogenic medium with dexamethasone and β-glicerol-phosphate. Magnification of 40x for images from day 11 and magnification of 100x for images from day 15.

Staining of collagen

Optical microscopy images from collagen staining as assessed histochemical are presented in Figure 10. In control, an homogenous cell layer is observed at day 11, staining positive for total collagen. Osteogenic medium induced a nodular organization of the culture, with an increased stain at the nodular structures. While a distinct morphologic organization is verified in osteogenic medium cultures, in comparison to

control, no significant differences are readily identified between the two osteogenic medium conditions.

Days of culture
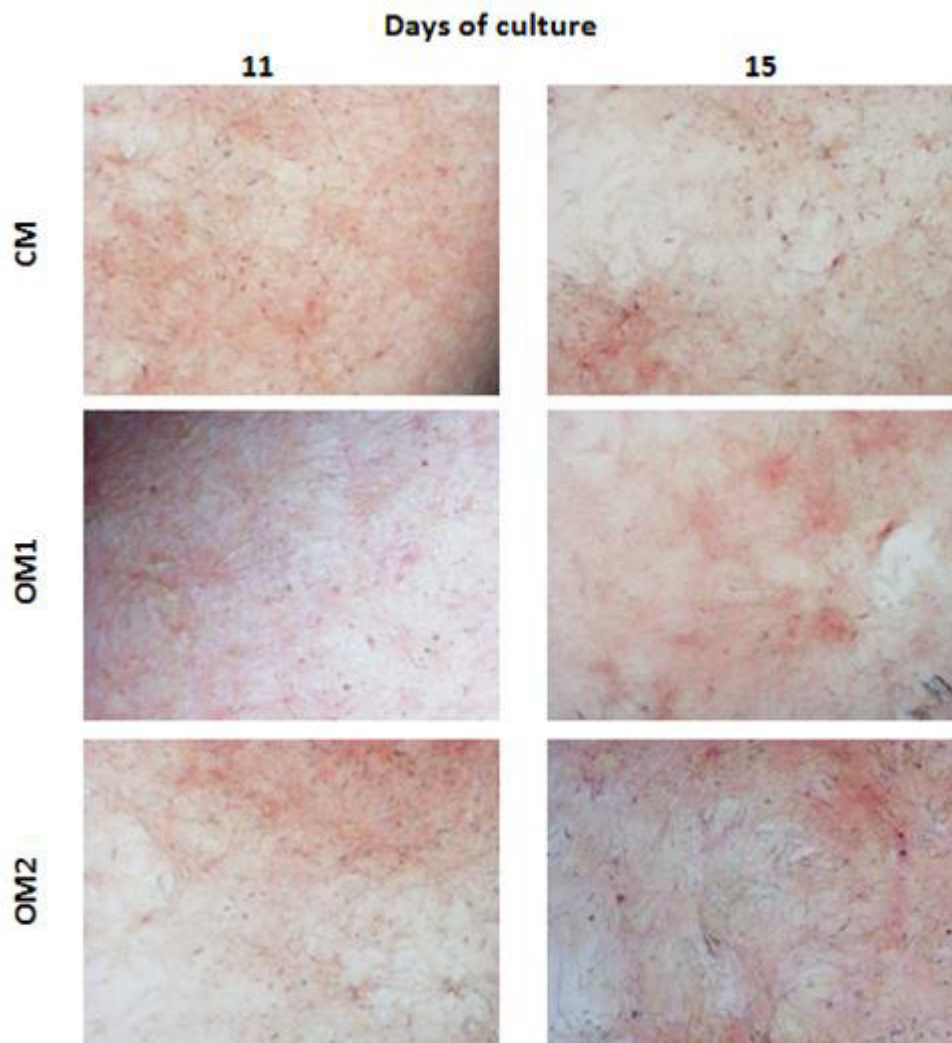
Figure 10 Microcopy images from collagen (Sirus Red) histochemical on day 11 and 15. CM - control medium; OM1- osteogenic medium with dexamethasone; OM2 - osteogenic medium with β-glicerol-phosphate. Magnification of 40x for images from day 11 and magnification of 100x.

Gene expression analysis

ADSCs cultures derived from FUAL were analyzed by RT-PCR at day 8. The genes analyzed were the housekeeping GAPDH, the osteogenic transcription factor Runx-2, the adipogenic transcription factor PPAR-γ, the muscular transcription factor MyoD and the chondrogenic transcription factor Sox9. The results shown in Graphic 4 were

normalized by the value obtained for GAPDH on each experimental condition. Significant differences were only attained regarding the increased expression of PPAR-γ with the OM2.
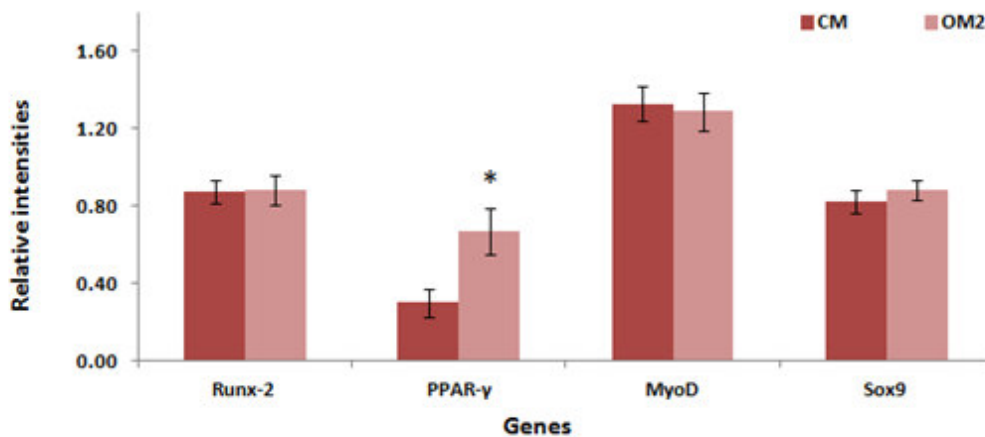
Graphic 4 RT-PCR gene expression of Runx-2, PPAR-γ, MyoD and Sox-9 in ADSCs derived from FUAL in the absence (CM) and presence of ostegenic inductors (OM2) on day 8 of culture. * - significantly different from control (p < 0.05).

ASCs FROM FDAL

The cells harvested and processed from each anatomical local were cultured in control medium (see Figure 11) as previously described. Following, the first subcultures were evaluated for cell morphology, proliferation, viability and osteoblastic differentiation, following specific induction, during 15 days.

Cultures established in control medium were referred as "CM" and cultures grown with dexamethasone and β-glycerophosphate as "OM2".
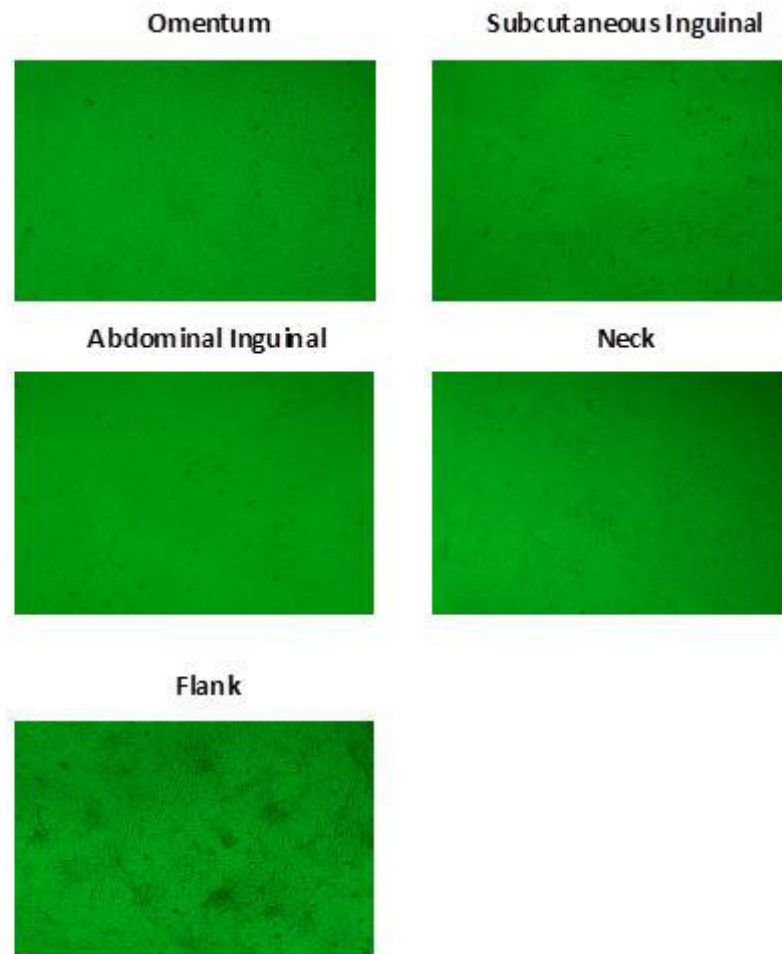
Figure 11 ADSCs from different anatomical sites in primary culture at day 15 (40x).

Cell proliferation

Graphic 5 shows the results of cell proliferation that was assessed by DNA quantification during the time of culture. It is possible verify that ADSCs from distinct anatomical locations reveal different proliferation rates. For instance, ADSCs from abdominal inguinal report an increased initial proliferation attain the highest peak at day 8 of culture. Cultures grown from subcutaneous inguinal and flank, achieved the highest peak at day 11, while cultures from the neck and omentum increased proliferation continuously, until day 15. Despite the different proliferative behaviours, the presence of the OM within the culture microenvironment was broadly found to reduce cell proliferation, particularly from day 8 onwards. The cell proliferation of

cultures established from the neck was the one to be the least affected by OM, as no significant differences were found between experimental conditions.
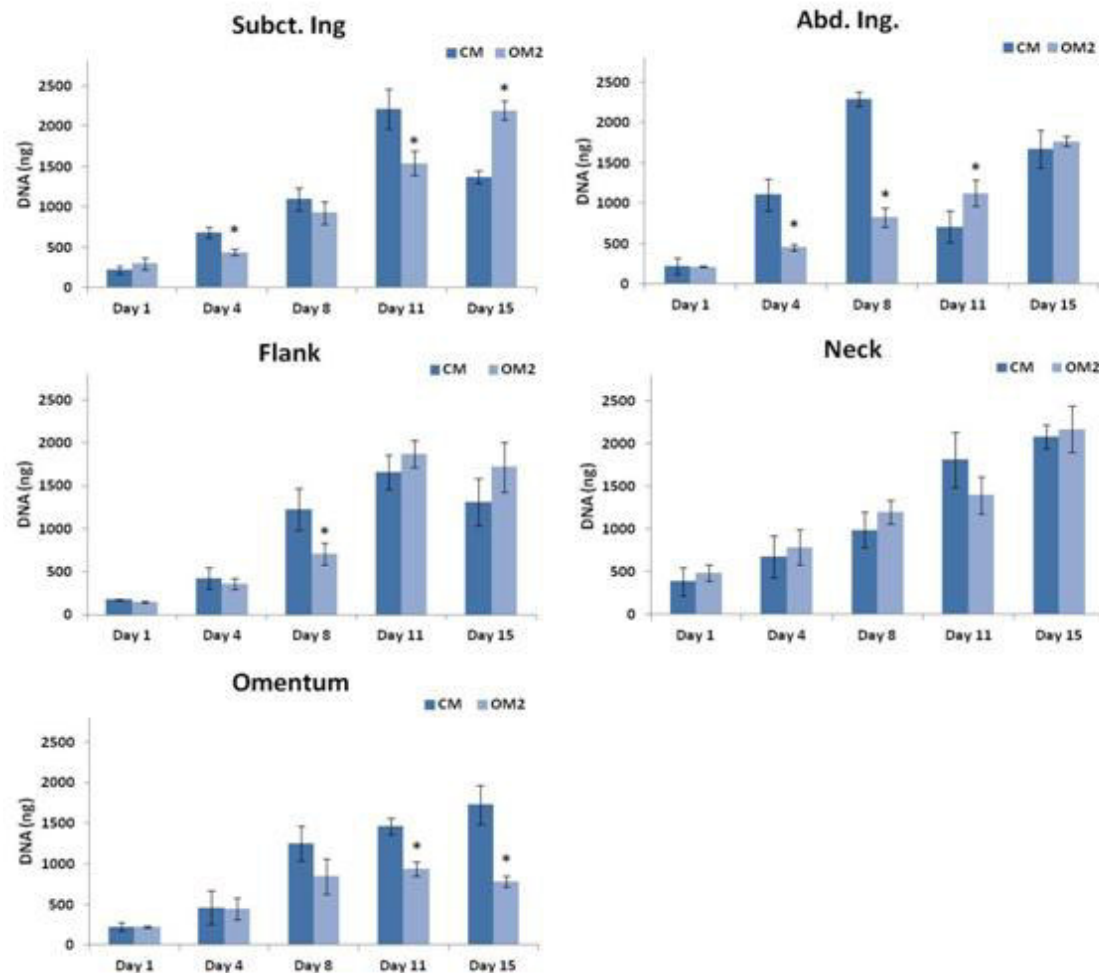
Graphic 5 Cell proliferation of ADSCs from FDAL in the absence (CM) and in presence of osteogenic medium (OM2). * - significantly different from control for each individual anatomical local (p < 0.05).

Metabolic activity

Metabolic activity was evaluated through MTT assay (Graphic 6). In control conditions, the metabolic activity was broadly found to increase until day 11, diminishing afterwards. Cultures established from abdominal inguinal fat achieved the highest MTT reduction value at day 8. Generally, MTT reduction values were diminished when

cultures were grown in the presence of OM, with the exception of those established from neck, in which the metabolic activity of the cultures was induced.

In all anatomical locals, except in abdominal inguinal control, flank and neck with osteogenic medium, the metabolic activity increase gradually from day 1 to day 11 and then decrease. In abdominal inguinal control, the higher value of MTT was observed on day 8, occurring a decrease to day 11 and then, to day 15, the value increased. In flank and neck the metabolic activity of ADSCs culture increased during the all time of culture.
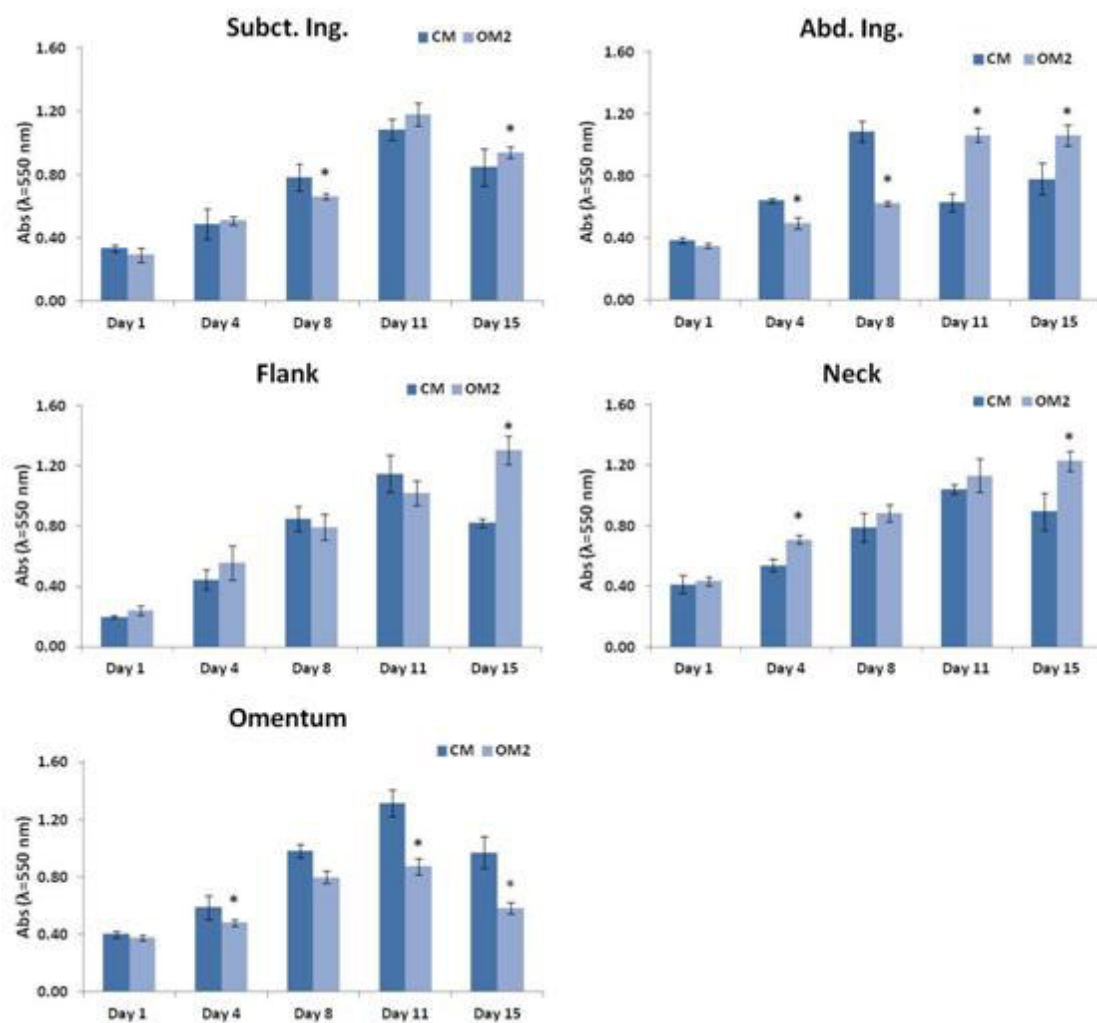
Graphic 6 Metabolic activity of ADSCs from FDAL in absence (CM) and presence of osteogenic medium (OM2). * - significantly different from control for each individual anatomical local (p < 0.05).

Cell morphology

CLSM was the method used for the assessment of cell morphology and the acquired images are showed in Figure 12. There were no significant differences between anatomical locals. Broadly, cells grown presented a characteristic fibroblastic morphology with several cytoplastic processes and an intense actin staining at the cell border. No significant differences were found on themorphology between ADSCs from different locations, despite the variability of the cell number.
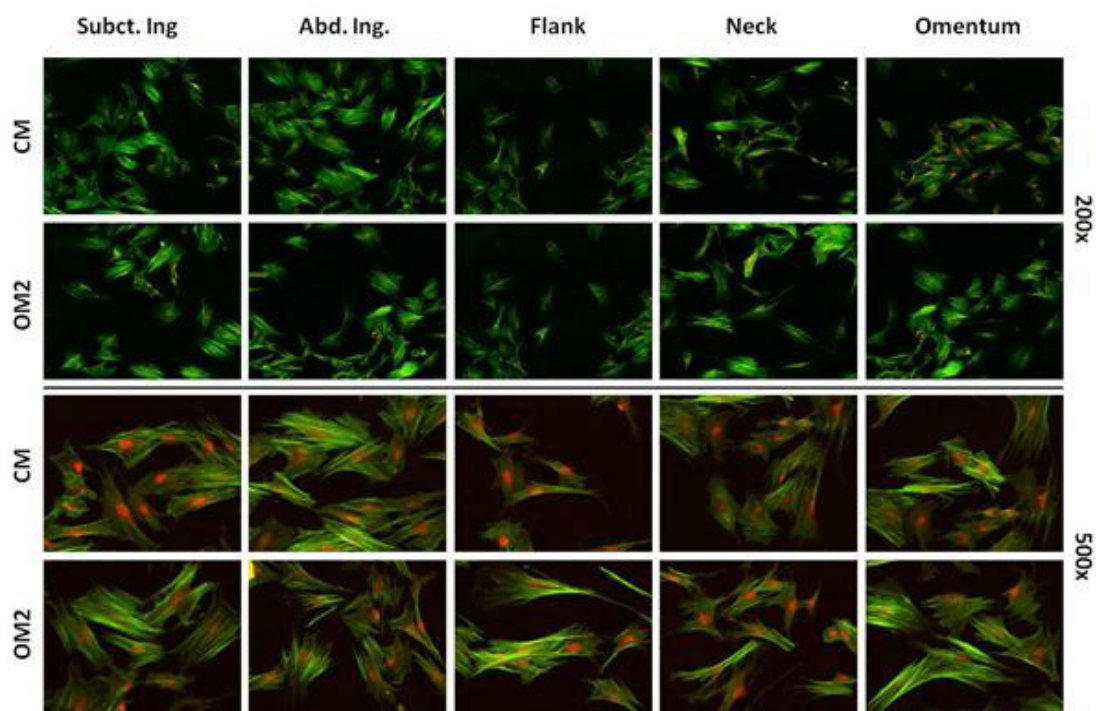
Figure 12 CLSM imaging of ADSCs derived from FDAL on day 3 in the absence (CM) and presence of osteogenic inductors (OM2). Cytoskeleton was stained green and nucleus counterstained in red.

Alkaline phosphatase activity

The values of alkaline phosphatase (ALP) activity presented in Graphic 7, were normalized to the amount of DNA. Broadly, ALP activity increased throughout the culture period, with the exception of cultures grown from abdominal inguinal fat, in which the peack of activity was attained at day 8. Broadly, in cultures established from all the anatomic locations, the presence of OM2 was found to significantly increase the

ALP activity. Comparatively, higher ALP activity was attained for cultures grown from flank and omentum.
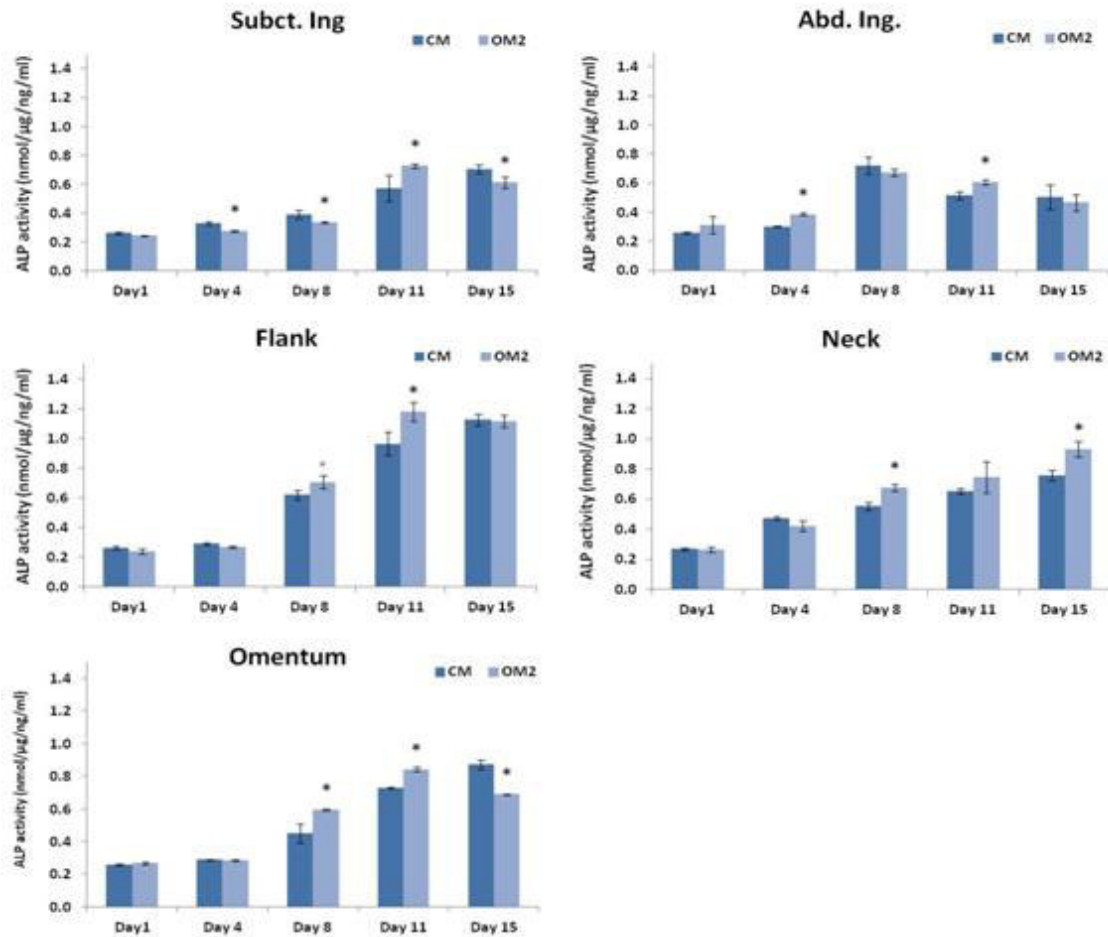
Graphic 7 ALP activity of ADSCs from FDAL in absence (CM) and presence of osteogenic medium (OM2). * - significantly different from control for each individual anatomical local (p < 0.05).

Staining of alkaline phosphatase

Optical microscopy images from alkaline phosphatase activity, evaluated by histochemical staining, are presented in Figure 13. Broadly, the staining intensity is verified to increase throughout the culture time for control cultures, established from the different anatomical locations, with the exception of cultures grown from abdominal inguinal region, that reveal a higher cell density and staining intensity at day 8. Cultures grown in osteogenic-inducing conditions (OM medium) present an increased staining intensity and a modification in the morphological organization of the culture. From day 8 onwards, cells converge to the organization of nodular structures that stain intensively. With the increase in culture time, the nodular structures seem to become densely organized with an increased staining intensity. This beahviour is not broadly verified on cultures established on control conditions and remnants of nodular organization can only be initially disclosed on subcutaneous inguinal, flank and neck ADSCs-derived cultures, at day 15.
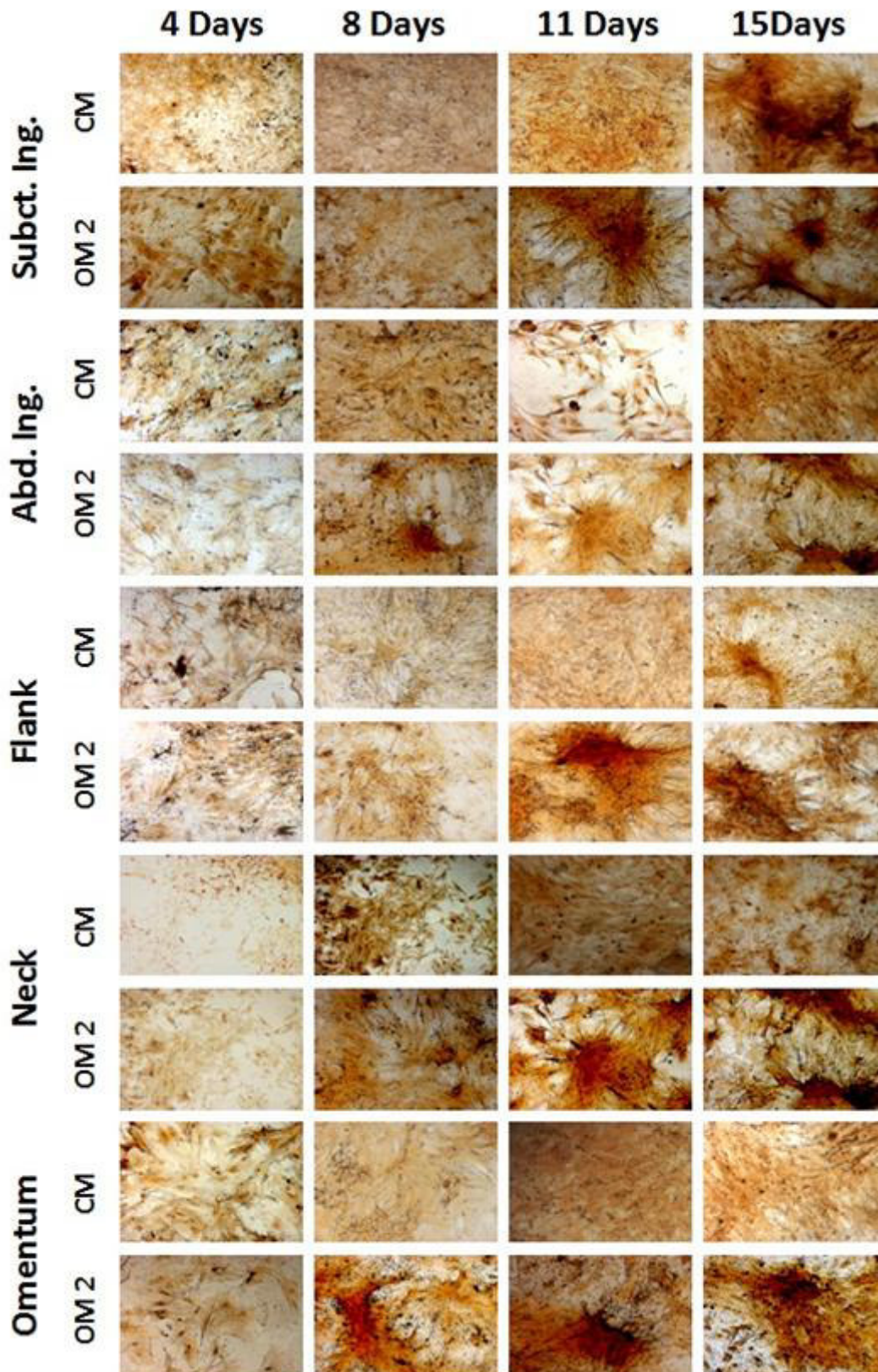
Figure 13 Alp staining of different ADSC's from FDAL, in the absence and in the presence os osteogenic inducers. CM – Control Medium; OM2 - ostegenic medium with dexamethasone and β-glycerophosphate. Magnification 100x.

Staining of collagen

Optical microscopy images from collagen staining as assessed histochemically are presented in Figure 14. The collagen staining seems to increase, in control cultures, throughout the culture period. Remarkably, ADSCs grown from omentum seem to display the highest collagen staining.

With the induction with OM, cultures were found to organize in nodular structures, in a similar fashion to that previously described following the assessment of the staining of ALP activity. The staining intensity was also found to increase within the nodular structures throughout the culture period, being the highest at day 15. The nodular organization of control cultures, as verified with the ALP staining, is less evident with this histochemical staining.

It is possible to observe differences between the cells from anatomical sites in study and between the days of culture. The ADSCs from all locals were initially dispersed, but over days they aggregated giving rise to nodular structures on day the eleventh and in osteogenic medium (OM2). The color intensity increased during the culture.
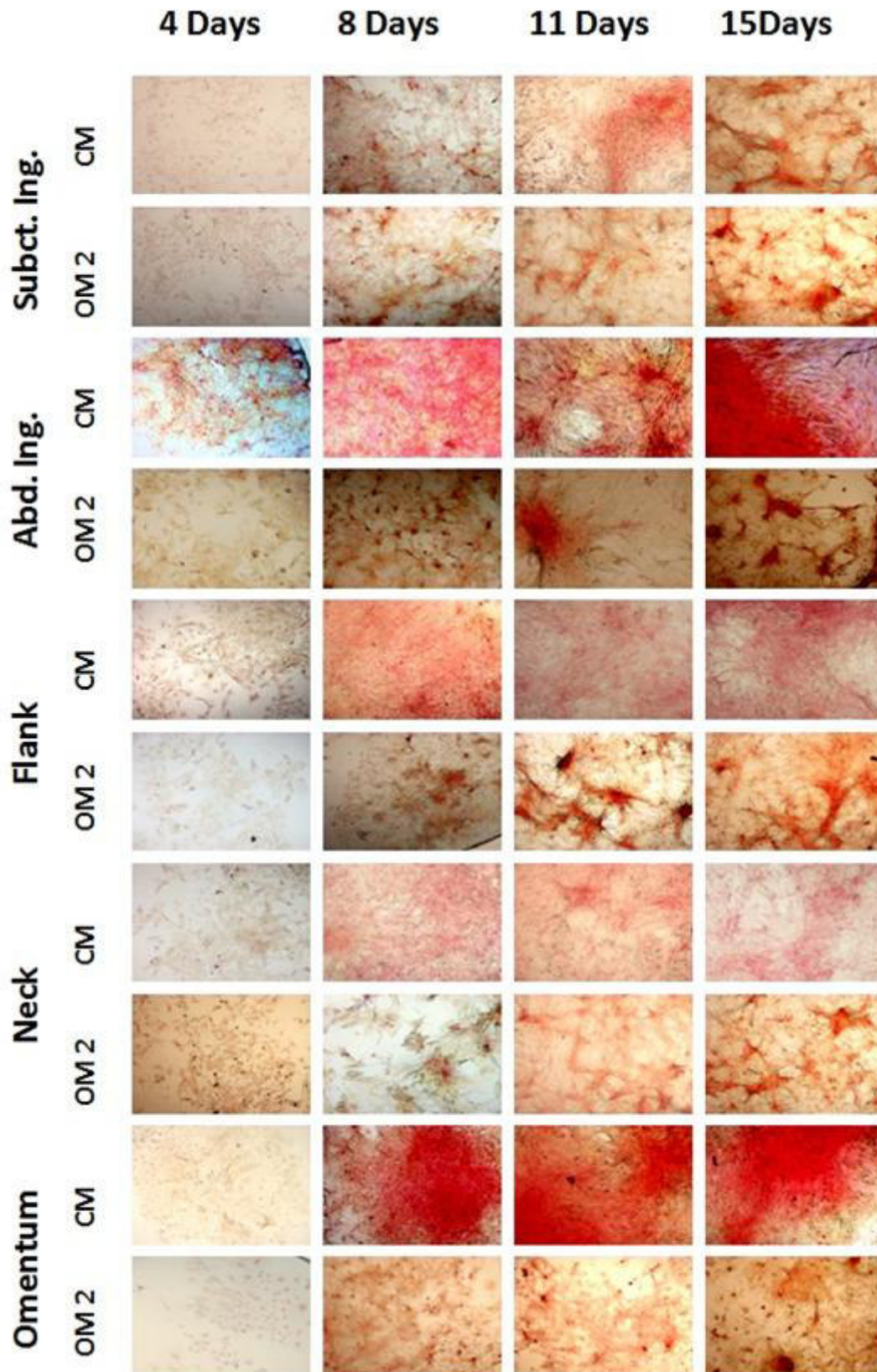
Figure 14 Colagen staining (Sirus Red) of different ADSC's from FDAL, in the absence and in the presence os osteogenic inducers. CM – Control Medium; OM2 - ostegenic medium with dexamethasone and β-glycerophosphate. Magnification 40x.

60

Staining of SA-ϐgal

Senescent cells in FDAL cultures were detected by staining the cells on days 1 and 4. However, after observation by optical microscopy, it was possible to verify that blue-stained cells (quiescent cells) were very reduced, and to low to be quantified. In the vast majority of observed microscopic fields no stained cells were identified witch precluded the calculation of the percentage of cells expressing β-galactosidade. In Figure 15, a rare example of senescent cell, found in ADSCs control culture, from inguinal subcutaneous local, on day 4 of culture, is presented. Stained cells were only identified in subcutaneous inguinal, abdominal inguinal and neck locations, at day 4, but in a very reduced proportion.
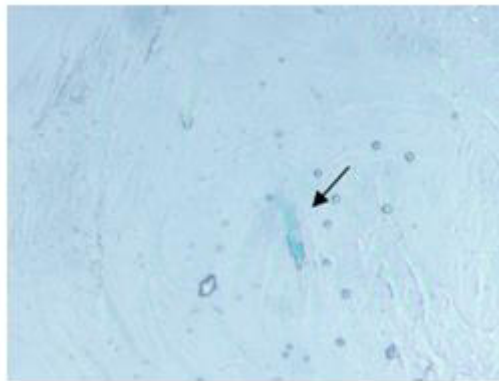
Figure 15 Reprentative sstaining of senescence-associated beta-galactosidade activity (arrow) from ADSCs control culture from inguinal subcutaneous local. Magnification 200x.

Gene expression analysis

ADSCs cultures derived from FDAL were analyzed by RT-PCR at day 8. The genes analyzed were the housekeeping GAPDH, the osteogenic transcription factor Runx-2, the adipogenic transcription factor PPAR-γ, the muscular transcription factor MyoD and the chondrogenic transcription factor Sox9. The results shown in Graphic 8 were normalized by the value obtained for GAPDH on each experimental condition. It is possible verify that in cultures established from all anatomical locations, the osteogenic medium, was found to induce the expression of PPAR-γ. Broadly, MyoD expression was also enhanced in subcutaneous inguinal, abdominal inguinal and flank,

while in ADSCs from omentum, the OM was found to induce the expression of RUNX2 and SOX9.
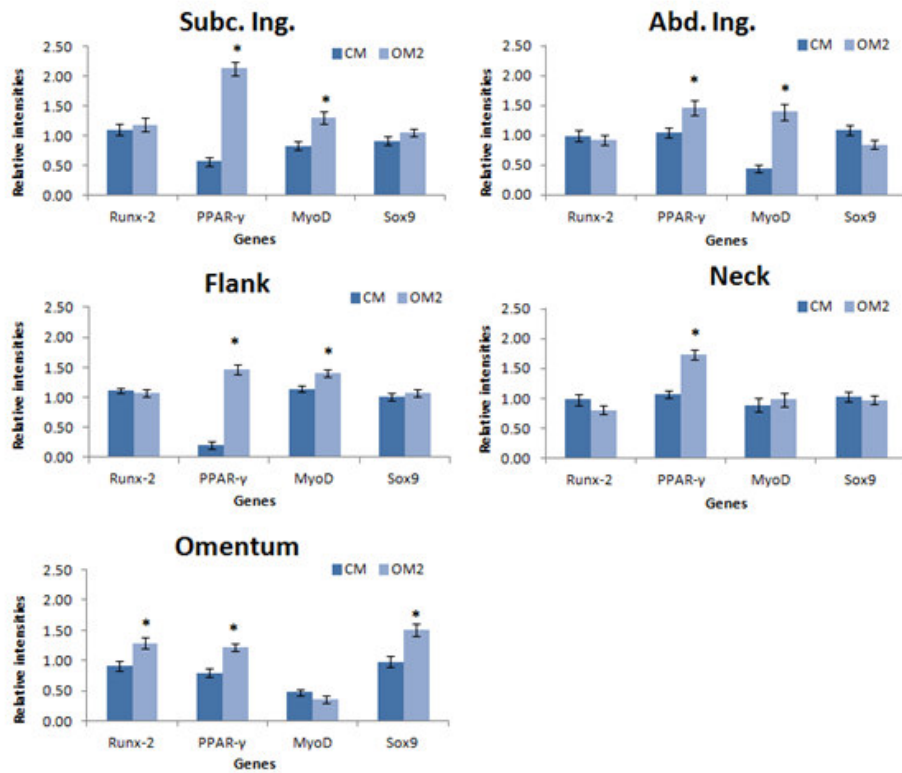
Graphic 8 RT-PCR gene expression of Runx-2, PPAR-γ, MyoD and Sox-9 in ADSCs derived from FDAL in the absence (CM) and presence of ostegenic inductors (OM2) on day 8 of culture.

* - significantly different from control (p < 0.05);

Characterization of the three-dimension ADSC cultures (spheroids)

The technique used to form the spheroids proved to be effective and after 24 hours of culture, the cells were found to aggregate and showed a spheroidal aspect. The spheroids from two experimental groups were cultured in control medium (OM) and osteogenic medium (CM1).

ASCs FROM FUAL

Spheroids from ADSCs from FUAL were maintained during 17 days and the culture was ended at this time point since the vast majority of spheroids grown from $C=5x10^4$ cells/cm^2 spontaneously disaggregated. Overall, the spheroid formation efficiency after 24 hours was 75% in the $C=20x10^4$ cells/cm^2 and 100% in the $C=5x10^4$ cells/cm^2 (see Table 2).
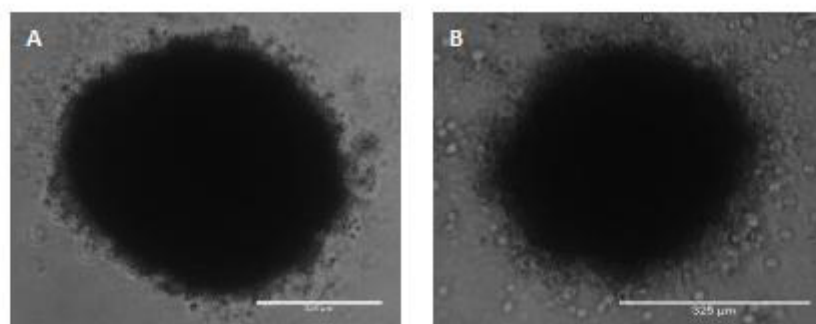
Figure 16 Photographs (phase-contrast) on day 1; A- cellular concentration of 20x104 cells/cm^2 ; B - cellular concentration of 5x104 cells/cm^2. Scale bar of 325 µm.

Table 2 The table show the number efficiency of spheroid formation and area after 24 hours. The spheroid formation efficiency was as percentages of the number of formed aggregates in relation to the number of seeded aggregates (n). * - significantly different from C=20x10^{4}cell/ml (p < 0.05).

Concentration of the seeded cell per cm^2	n	Number of spheroids formed after 24h	Spheroid formation efficiency	Area after 24h (µm^2)
20x10^4	16	12	75%	441.9
5x10^4		16	100%	185.0*

The Graphic 9 shows the cross-sectional area of spheroids during the 17 days of culture in the two concentrations. It is possible to verify that, comparatively, the spheroids were smaller when the initial cellular concentration was 5x10^4 cel/cm^2 . The spheroids cultured in osteogenic medium were always smaller than those cultured in control medium, for both concentrations. Further, either in control or osteogenic conditions, spheroids were found to decrease in size throughout the assayed experimental period.
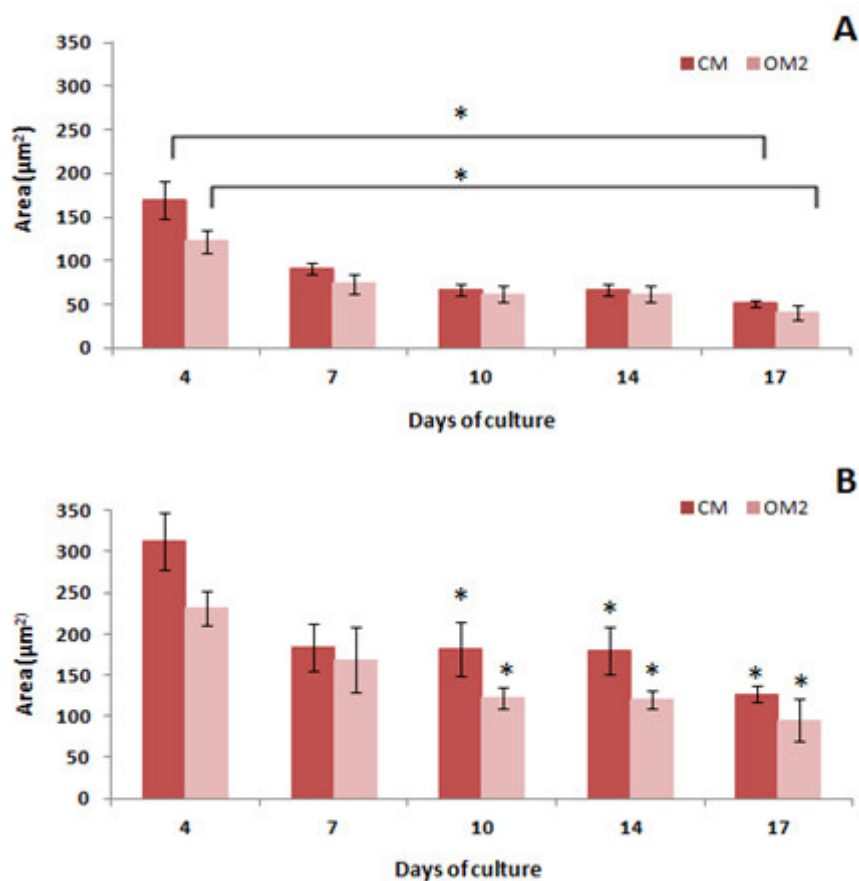
Graphic 9 Cross-sectional area of ADSCs spheroids seeded of two different concentrations - C= 5x10^4 cel/cm^2 (A) and C=20x10^4 cel/cm^2 (B) – in the absence (CM) and presence of osteogenic inductors (OM2) during 17 days of culture. * - significantly different from day 4 (p < 0.05).

During the days of culture, some spheroids were lost, due to, mainly, the change of culture medium. The Table 3 showed the number of spheroids (N.S.) and the maintenance rate (M.R.) during the 17 days of culture. The spheroids formed with 20x10^4 cells/cm^2 kept the highest value of M.R. during the whole culture

Table 3 The table show the number of spheroids (N.S.) and the maintenance rate (M.R.) of spheroids in relation to the number of formed aggregates at 24 hours during the 17 days of culture.

Cellular Concentration (cells/ml)	Day 4		Day 7		Day 10		Day 14		Day 17	
	N.S.	M.R.	N.S.	M.R.	N.S.	M.R.	N.S.	M.R.	N.S.	M.R.
20×10^4	12	100%	8	66.67%	8	66.67%	8	66.67%	8	66.67%
5×10^4	16	75%	10	62.50%	8	50%	8	50%	7	43.75%

ADSCs FROM FDAL

Spheroids generated from ADSCs from FUAL were cultured during 6 days. After 24 hours, the cells derived from all locals formed the spheroids, but the number of spheroid formed varied greatly among the assessed anatomic locations (see Table 4). Spheroids formed by ADSC's derived from abdominal inguinal area showed a formation efficiency higher than 96%, but in the omentum the formation efficiency was only 59.4%.

After 24 hours, the flank spheroids showed the highest surface area, whereas the subcutaneous inguinal the lowest (see Table 4).

The Figure 17 show the photographs (phase-contrast) of spheroids at 24 hours after culture and on day 6. It's possible verify the different morphology between the anatomical locals and during the days of culture.

Table 4 The table show the efficiency of spheroid formation and the mean area after 24 hours in the five anatomical locals. The spheroid formation efficiency was as percentages of the number of formed aggregates in relation to the number of seeded.

Anatomical Locals	n	Number of spheroids formed after 24h	Spheroid formation efficiency	Area after 24h (μm)
Subct. Ing.		28	87.5%	117.6
Abd. Ing.		31	96.8%	202.7
Flank	32	25	78.1%	320.5
Neck		28	87.5%	209.2
Omentum		19	59.4%	201.7

Over the days, the number of spheroids in culture decreased, manly, due to the medium changes. The maintenance rate (M.R.), ration between spheroids at day 6 in relation to the number of formed aggregates after 24h, was showed in Table 5. The flank spheroids had the highest M.R. and the subcutaneous inguinal spheroids the lowest M.R..

Table 5 The table show the number of spheroids at day 6 and the maintenance rate (M.R.) of spheroids at day 6 in relation to the number of formed aggregates at 24 hours in different anatomical locals.

Anatomical Locals	Number of spheroids	M.R.
Subct. Ing.	20	71.4%
Abd. Ing.	23	74.2%
Flank	24	96.0%
Neck	26	92.9%
Omentum	14	73.7%
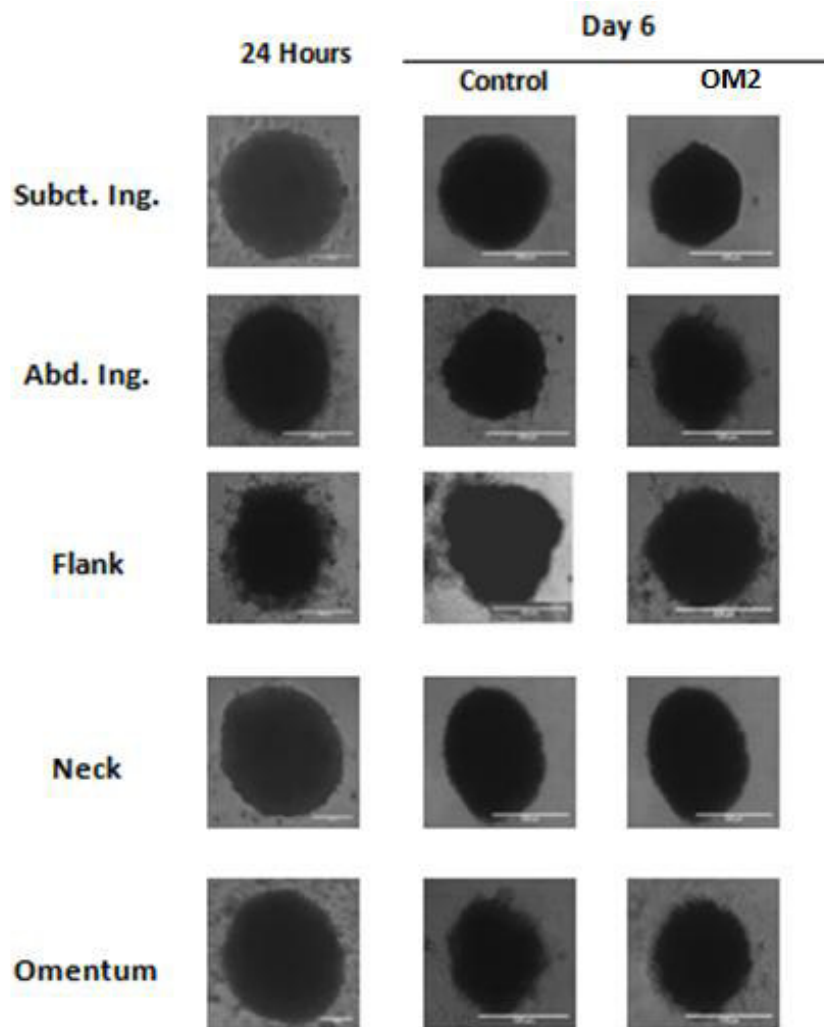
Figure 17 Photographs (phase-contrast) of spheroids at 24 hours after culture and on day 6. Scale bar of 325 μm.

On day 6, spheroids cultured in the presence of osteogenic medium (OM2) presented a reduced cross-sectional area, as comparing to control (Graphic 10).
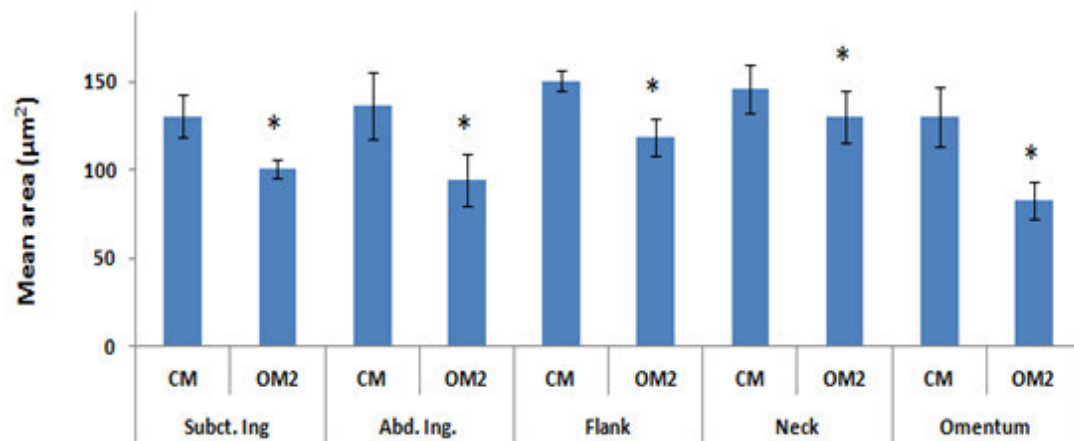
Graphic 10 Cross sectional area of ADSCs spheroids from different anatomical locals at day 6 in the absence (CM) and presence of osteogenic inductors (OM2). * - significantly different from control (p < 0.05).

Elisabete Gonçalves

V. DISCUSSION

Elisabete Gonçalves

The adipose-derived stromal cells (ADSCs) are quite promising in regenerative medicine, namely in orthopedic tissue engineering. These cells can be obtained in large quantity and through a minimally invasive method. Moreover, they have the ability to differentiate themselves into multiple lineages allowing an application in various fields of regenerative medicine [23], [122].

From among all the factors which may influence the proliferation and the differentiation capacity of adipose-derived stromal cells isolated from stromal vascular fraction (SVF), the harvest site of adipose tissue has gained great importance [65], [67], [69], [70], [123], [124].

The main goal of this work was established a ADSCs culture from different rat anatomical sites to verify if there were differences in yield, proliferation and osteogenic capability of expanded and differentiated of that cells.

Thus, in a first part of this study, we established a culture of adipose-derived stromal cell (ADSCs) from undifferentiated anatomical locals (FUAL). The rat adipose tissue was harvested randomly from the places where more fat was available. After isolation of ADSCs from SVF through collagenase digestion, cells were seeded given rise to a primary culture that was established for seven days – around 70-80% confluence. Afterwards, a first-subculture was established and the cells were cultured in three different conditions – control medium (CM), osteogenic medium with dexamethasone (OM1) and osteogenic medium with dexamethosone and β-glicerol-phosphate medium (OM2) – during 15 days. The dexamethasone is synthetic glucocorticoid and is known as an osteogenic inductor in MSCs [36], [125]–[127] and β-glicerol-phosphate, which is also an osteogenic inducer, capable of induce calcification and mineralization of the extracellular matrix [54], [127], [128]. The monolayer culture from FUAL was then characterized assessing the cell morphology, proliferation, metabolic activity, and osteogenic capability, by histochemical staining and gene expression analysis. Further, in order to disclose the differentiation capability of the grown cells, the expression of significant transcription factors of the osteogenic, adipogenic, myogenic and chrondogenic lineages were also evaluated.

Cell proliferation was evaluated by DNA quantification and the results showed that, in control, cells proliferated actively until day 11, diminishing afterwards. With OM induction, cells proliferated actively throughout the assayed culture period but a reduced cell proliferation was verified, from day 8 onward, as comparing to control. The metabolic activity of the cultures followed a similar trend: increasing until day 11 on control, and throughout the assayed culture period in OM conditions. Comparatively, OM induction was not found to significantly influence the metabolic activity of the cultures. Accordingly, osteogenic induction with dexamethasone and beta-glicerophosphate has been previously found to alter the proliferation of precursor populations. While known to induce the osteogenic differentiation, cell proliferation is broadly hampered by osteogenic inducers given the enhanced culture commitment to differentiation activities [22], [127], [129].

The alkaline phosphatase activity is an indicator of the osteogenic differentiation *in vivo* and in vitro. Within established differentiating osteogenic cultures ALP is normally increased at early culture time points and peaks just before the initiation of the mineralization process, diminishing afterwards [111], [114], [130]. Histochemical staining for ALP can be correlated the biochemical determination being possible to verify the presence of nodular structures, on days 11 and 15 on OM-induced cultures (more evident on OM2), which is characteristic of the osteogenic differentiation process [130], [131]. Histochemical staining correlated well with biomechanical determination of ALP activity, in which is verified the maximum activity of cultures grown for 11 days on OM2 conditions. Regarding collagen expression, by observing the established cultures, an increase staining is verified with the increase in culture time. In OM, it is possible observe that cells tend to form clusters; which is associated with an increased staining of collagen. This pattern of expression is common during the osteoblastic differentiation, as previously verified by other reports [53], [127], [131], [132].

The RT-PCR analysis was performed only in two groups – control (CM) and osteogenic medium 2 (OM2), given the increased osteogenic induction verified with this condition. Broadly, the expression of transcription factors of distinct differentiation

lineages were assessed: Runx-2 (osteogenic), Sox-9 (condrogenic), PPAR- γ (adipogenic) and MyoD (myogenic).

Runx-2 is the central control gene for osteoblast phenotype. This gene provides instructions for making a protein that is involved in bone development and maintenance. It is essential for osteoblastic differentiation and acts as a scaffold for nucleic acid and regulatory factors involved in skeletal gene expression [133]. Sox-9 is a master transcription factor for chondrogenic differentiation and it is expressed in all chondro-progenitor cells and in differentiated chondrocytes. Sox-9 is essential during the embryonic chondrogenesis but also expressed in adult tissues, being crucial for chondrogenic differentiation of mesenchymal stromal cells [134]. PPAR-γ is a member of the nuclear hormone receptor family and it is predominantly expressed in adipose tissue and is required for adipogenesis *in vivo* and *in vitro* [135], [136]. MyoD is a protein that belong to the family of myogenic regulatory factors and are expressed earlier during muscle development and are involved in the determination of the myogenic lineage [137]. Within the induction with OM2, no significant differences were found regarding the expression of the assayed transcription factors, except for PPAR-γ, which expression was found to increase. This was unexpected, given the previously verified changes in the culture morphology and increased in ALP and collagen expression, during OM-induction. Accordingly, it might be expected to verify an increase in RUNX2 expression. Despite the absence of RUNX-2 up-regulation, one cannot preclude the effective and increased osteogenic commitment of grown ADSCs from FUAL, with OM induction, particularly given the increased markers of osteogenic commitment – increased ALP activity and collagen expression, and characteristic organization of the culture in nodular structures.

Despite the attained results, some factors could converge to assist on the verified results. Gene expression analysis was conducted at day 8, in which the nodular arrangement of the culture was already verified following OM treatment, supporting the effective osteogenic commitment. As so, RUNX2 activity could have been upregulated earlier than day 8, contribute to an effective osteogenic commitment, and being following downregulate to basal levels [133]. In order to verify this hypothesis, gene expression analysis could be further conducted at earlier time points to disclose

the effective activation of RUNX2, and also, at later time points, in order to address the expression of later markers of osteogenic development, such as osteocalcin and bone sialoprotein [133].

One may not also neglect that dexamethasone, used within the OM composition, is also broadly employed in adipogenic differentiation of MSCs [127] and even within the osteogenic differentiation, spontaneous adipogenic differentiation may also occur, as previously reported [126], [136]. This might be related to the wide variability of cell populations present in the SVF. In fact, these were shown to include potential ADSCs, endothelial (progenitor) cells, pericytes, blood-derived cells, fibroblasts, vascular smooth muscle cells, and preadipocyte [138].

The differentiation potential of ADSCs is also a matter of debate. The *in vitro* differentiation of ADSCs into multiple cell types of mesodermal origin has been shown in a wide variety of studies and experimental conditions, with ADSCs being cultured by serial passaging, without losing their multipotent properties. Different studies described ADSCs plasticity towards chondrocytes, osteoblasts, adipocytes, and myocytes, as well as to differentiate into lineages of nonmesodermal origin [139], [140]. However, at the single cell level, ADSCs demonstrated that not every cell possesses the characteristic trilineage mesenchymal differentiation potential for adipogenesis, osteogenesis, chondrogenesis, using lineage-specific differentiation media. Cell clones from human ADSCs were found to differentiate into at least one of the lineages in 81% of the situations, while only 52% of the clones differentiated into two or more of the lineages [141], sustaining the high variability of the differentiation potential of ADSCs.

Furthermore, and apart from the established compositional variability, one may not also forget that phenotype expression is related to the plastic adherent capabilities of the cells and that cultured population dramatically changes the phenotype very early during cell culture [3].

Some authors sustain that the heterogeneity of cultured ADSCs can presumably be reduced by a washing procedure at early culture time points, or by applying flow cytometric cell sorting methodologies, either of positive or negative discrimination, in

order to include or exclude specific populations [142]. Nonetheless, the usage of such techniques for the reduction of heterogeneity is arguably beneficial since it leads to a very small cell yield, which may significant hamper the usability of the fat tissue as a source of MSCs-like populations for regenerative approaches.

Following, and set on data of ADSC cultures established from FUAL, ADSC cultures established fat, harvested from different anatomical sites was conducted. Current evidence shows a distinct biological behavior, with numerous biological and genetic differences between the adipose tissue of distinct anatomical locations [143]. For instance, in humans, the upper body/visceral fat tissue is broadly associated with metabolic complications, in antagonism to the fat accumulation in the lower body [144]. In fact, visceral fat has been associated with an increased inflammatory milieu, through the production of pro-inflammatory cytokines, at the expense of adiponectin production – which endeavors an insulin resistance profile and favors the development of atherosclerosis [145]. Correlating with the distinct biological behavior of the fat tissue in distinct anatomic locations, the secretome analysis of visceral, subcutaneous, and gonadal fat revealed differentially secreted proteins among the three fat depots, showing that that the type of proteins and their role in different biological processes diverged significantly, thus emphasizing and supporting the differential role of adipose tissue in accordance to its anatomical localization [146].

In this work, it was verified a distinct biological behavior of grown cultures of ADSCs isolated from distinct anatomic locations. In which regards the proliferative capacity, abdominal inguinal revealed the highest potential (with the highest value being attained at day 8 of culture), with the cells from omentum and neck showing the lowest proliferative potential (proliferation increased throughout the 15 days of the culture), and subcutaneous inguinal and neck, with intermediate proliferative capacity. Cultured ADSCs were previously shown to have an extensive proliferative ability, broadly found to be superior to that verified in other MSCs from distinct locations, such as the bone marrow [147]. Furthermore, established cell cultures, at passage 1, also revealed a very high viability and broadly low senescence (as assessed histochemically in this work), which is in accordance with previously reported data [22].

In regards to the osteogenic capability, broadly assessed by the quantitative determination of ALP activity, this was found to be the highest in ADSC cultures from flank, and then decrease within those from omentum, neck, subcutaneous inguinal and abdominal inguinal, respectively. OM induction broadly decreased cell proliferation but increased ALP activity, thus supporting the effective osteogenic differentiation of the grown ADSCs [21], [22], [127]. Attained biochemical data correlated well with histochemical analysis. This further revealed the nodular organization of the cultures grown within OM-induction, which is characteristic of the osteogenic phenotype [127], [130], [131]. Cultures grown in the presence of OM were also found to increase the collagen expression, which is in accordance with their effective osteogenic commitment [118], [127].

In terms of the assessment of the expression of transcription factors, in a similar fashion to the observed data in FUAL, OM-induction broadly increased PPAR-γ expression in cultures from all anatomical locations. While MyoD expression was also increased in subcutaneous inguinal, abdominal inguinal and flank, within cultures derived from omentum, Runx2 and Sox9 were significantly increased. While gene expression analysis should further be validated by specific lineage-induction differentiation and confirmation of cell differentiation by specific protein analysis, attained data may suffice to validate a distinct biological behavior of ADSCs isolated from distinct anatomical locations. In which regards to osteo/chondral-related applications, cells isolated from omentum, reporting high ALP activity and enhanced RUNX2 and SOX9 activation following OM-induction, show the most promising potential for application in regenerative medicine applications targeting bone and cartilage.

While no other study particularly addresses the biological characterization of ADSCs from so many distinct anatomical regions, there are some data on literature that converge to sustain a differenced behavior of cell populations isolated from different fat depots. Of relevance within osteo/chondral-related applications, the study of Jurgens et al. [65] addresses the evaluation of human subcutaneous adipose tissue from abdomen and hip/thigh region, in terms of cell yield and the functional characteristics of harvested ADSCs, following chondrogenic and osteogenic

differentiation. The results obtained showed that the lineage-induced ADSCs from hip/thigh region tended to show higher levels of alkaline phosphatase activity than those from the abdomen region, but in chondrogenic differentiation, no difference were detected between the cells grown from the two anatomical regions. However, the yield of ADSCs, but not the total amount of nucleated cells per volume, were found to be higher within the abdomen-derived fat, rather than within the fat of the hip/thigh region [65].

Emre et al. addressed the osteogenic behavior of lineage-induced human ADSCs from superficial and deep adipose layers of abdominoplasty specimens [148]. The authors found that the osteogenic differentiation began as early as 1 week and while there was no significant difference in the degree of osteogenic differentiation between the ADSCs from both depots in female donors, in assessed male-derived ADSCs, those from the superficial depot differentiated faster and more efficiently than those from the deep depot. Furthermore, male cells from both depots differentiated more effectively than female ADSCs from both depots, which support the existence of differences in the biological behavior of cells isolated from distinct anatomical locations, but also gender-specific differences.

Following to the characterization of the established ADSC cultures in two dimensions, three dimensional cultures were also developed following induction of cell aggregation, and were characterized in order to disclose the cell behavior in 3D conditions, in the absence of any given substrate.

The use of a non-adhesive surface to prevent the cellular adhesion is widely applied to mimic the *in vivo* three-dimensional environment [83]. The liquid overlay technique (LOT) is a technique based on this principle and proved to be efficient for the formation of ADSC spheroids. In the present work, 96-wells plates were coated with 1% of agarose solution and isolated cells were seeded at two different concentrations, 5×10^4 cel/cm^2 and 20×10^4 cel/cm^2.

After 24 hours of incubation under standard cell culture conditions, cell aggregates were visible in the assayed cell concentrations. However, the spheroid formation efficiency was not the same for both concentration, being 100% on C=5×10^4 cel/cm^2

and 75% on $C=20x10^4$ cel/cm^2. Further, the mean area of the attained spheroids was higher with the cellular concentration of $C=20x10^4$ cel/ cm^2, which is in accordance with the higher cell density used. Throughout the culture period, larger spheroids (those from $C=20x10^4$ cel/ cm^2) were more easily identified and the handling during medium change was easier, which is proven by higher maintenance rate. Spheroids grown in the presence of OM revealed a reduced size, as comparing to those grown in control conditions. Osteogenic induction is expected to induce changes in the functional activity of the cells, and thus affect cell-to-cell interactions, as well as the extracellular matrix formation and composition, which may further contribute to sustain the verified reduced spheroid diameter [94]. Furthermore, along culture time, and in all experimental conditions, the area of spheroids decrease progressively. This can be explained by the inefficient mass transport and the accumulation of metabolic waste inside the spheroids, which leads to the formation of necrotic core [86]. Moreover, cell aggregation is expected to increase with culture time due to hemophilic cadherin-cadherin interactions [86].

In the last days of the culture, the vast majority of the spheroids of $C=5x10^4$ cel/cm^2 spontaneously disaggregated, while those from $C=20x10^4$ cel/cm^2 remained viable. Accordingly, this concentration was selected for the following experimental setting that aimed the characterization of spheroids from ADSCs derived from FDAL.

The ADSCs from FDAL were cultivated at density of $20x10^4$ cells/cm^2 and for each anatomical local 32 wells were prepared (coated with 1% of agarose solution).

Three-dimensional aggregates were formed after 24 hours of seeding. The number of formed aggregates and, consequently, the spheroid formation efficiency differed from local to local. The cells derived from abdominal inguinal area formed more aggregates, 31 in total, with a spheroid formation efficiency of 96.86% and the cells from omentum formed only 19 spheroids (spheroid formation efficiency of 59.38%). The low value for spheroids formed with ADSCs from omentum, may be related to the fact of ADSCs from this anatomical local showed the formation of multiple small spheroid per well, broadly two or three small spheroids were identified. These multi-spheroids weren't formed in other anatomical locals in study. Some of these multi-spheroids converged

and aggregated over the culture period. After 24 hours, the ADSCs from flank formed the largest spheroids with a mean area of 320.5 μm^2 and the subcutaneous inguinal allowed the formation of spheroids with the smallest mean area, of 117.6 μm^2.

Throughout the culture period, spheroids became more compact and reduced in size – either in diameter and area. These date are consistent with the results of Metzger el al. [83] and Hildebrandt et al. [94] who observed a decrease in the spheroids diameter during the time of culture, an issue probably related to the inefficient mass transport and metabolic waste accumulation inside the spheroids which gives arise a necrotic core inside and a small proliferation zone in the periphery [86].

As previously reported for the spheroids organized with FUAL, osteogenic medium (OM) induction significantly reduced the size of the spheroids, in all anatomical locations. This may be related to the osteogenic commitment of the growing cells, which reveal a hampered proliferation at the expense of the functional activity being committed to the osteogenic differentiation. In order to further validate these observations, biochemical assays and gene expression analysis should be conducted.

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VI. CONCLUSION

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Adipose-Derived Stromal Cells (ADSCs) have a self-renewing capability, as well as the ability to differentiate themselves into multiple lineages allowing the application in regenerative applications. Of relevance, their capacity to be inducted into the osteogenic lineage has found great interest in orthopedic tissue engineering applications.

In this work, a two-dimensional and a three-dimensional culture system of osteogenic-induced rat ADSCs, was established. Initially, fat from undifferentiated anatomical locals (FUAL) was isolated and established first subcultures were characterized. Following, fat from differentiated anatomical locals (FDAL), i.e., subcutaneous inguinal, abdominal inguinal, flank, neck and omentum, were adequately characterized.

Cell cultures established from FUAL showed a distinct behavior from control, when subjected to osteogenic medium induction. The cell proliferation was hampered by osteogenic inducers while, the osteogenic differentiation was enhanced. This was verified by the alkaline phosphatase activity and by histochemical methods, verifying the presence of nodular structures within the induced cultures.

Cultures established from FDAL showed a distinct biological behavior. In regards to the proliferative capacity, abdominal inguinal revealed the highest potential, while the osteogenic capability was highest in cultures established from flank, following specific osteogenic induction.

Spheroids from FUAL and FDAL were efficiently formed after 24 hours of incubation. Over the days, spheroids decreased in terms of area and compared to the control medium, spheroids in OM were significantly reduced the size. These were found to maintain their viability throughout the culture period.

Broadly, and given the verified functional differences of ADSCs grown from distinct anatomical locations, it might be adequately to address the fat tissue anatomical location in order to adequately select the populations most relevant for the specific targeted regenerative application, as significant differences in terms of cell proliferation and osteogenic differentiation capability were verified in the assayed experimental model.

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VII. REFERENCES

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