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New Insights on the Role of Adipose Tissue Using 3d Scaffold-Free Organoids

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Keywords: Adipogenesis, 3D scaffold-free spheroids, lipid metabolism, forskolin, adipose tissue

This was the best poster at the 25th IFSCC Conference in Milan, Italy, September 30 - October 2, 2019.

Abstract

Human adipose tissue is the master regulator of human body homeostasis. It is composed of a complex network of different cellular compartments that allows the regulation of thermogenesis, lipids storage, hormone secretion and metabolism. Secretion of hormones and cytokines contributes to activation of the metabolic activity of other tissues such as liver and skeletal muscles. 3D scaffold-free spheroids developed using the hanging drops system represent the new generation of in vitro tissue models able to reproduce all the main features of native human tissue in terms of organization, architecture and biological functions without the need of a scaffold. Our previous results on gene expression analysis demonstrated that genes associated with adipogenesis were overexpressed during preadipocyte differentiation in 3D microtissues after 7, 10 and 14 days from spheroids formation compared with undifferentiated microtissues; the downregulation of all target genes reported for microtissues treated with forskolin confirm the potential of the 3D scaffold-free model as a biologically relevant and predictive tissue system compared with the conventional monolayer [1].

3D adipose microtissue is proposed as an innovative screening platform responsive to adipogenic induction and to active ingredients with a known role in the lipid metabolism, such as forskolin.

INTRODUCTION

White adipose tissue is a complex organ that functions as the major energy storage site in the human body [2]. It originates from the mesodermal germ layer, very close to the bone marrow features, and is based on a supportive stromal compartment of heterogeneous cellular populations, including smooth muscle cells, pericytes, fibroblasts, mast cells, pre-adipocytes [3, 4], immune cells [5] and mesenchymal stem cells [4]. In particular, this stem cell population, also called processed lipoaspirate (PLA) cells, can be isolated from human lipoaspirates and, like bone marrow mesenchymal stem cells, are able to differentiate into adipocytes [3, 4, 6], chondrocytes [3, 4, 7, 8], osteoblasts [9, 10], myocytes, cardiomyocytes [11, 12] and neuronal cells.

Molecular and biochemical approaches confirmed that PLA cells express multiple CD marker antigens similar to those observed in mesenchymal stem cells and that PLA cells and clones are able to differentiate into putative neurogenic cells, exhibiting a neuronal-like morphology and expressing several proteins consistent with the neuronal phenotype [6].

Finally, in addition to all these cellular components, a microvascular network embedded in an extracellular matrix formed by several collagen types (collagen III, IV, V, and VI) gives complexity and functionality to adipose tissue [13]. Factors and soluble molecules secreted by these different cellular components are critical for maintaining homeostasis in adipose tissue and throughout the body [5]. In recent years an important contribution has been made to better characterization of human adipose tissue and its main functions in the human body [14]: Leptin was identified as a key regulator of hormonal function, leading to adipose tissue inclusion in the field of endocrine organs. Besides leptin, indeed, white adipose tissue secretes a number of additional peptide hormones, including several cytokines, adipsin and acylation-stimulating protein, angiotensinogen, plasminogen activator inhibitor-1, adiponectin, resistin and steroid hormones. Thanks to its complex structure and multiple functions, adipose tissue is considered the core of a complex network influencing energy and vascular homeostasis, glucose and lipid metabolism, immune response, and reproduction. Taken together, these findings have driven interest in the role of human adipose tissue in medical research and cosmetology.

The prevalence of overweight individuals and discovery of metabolic disorders (obesity, type-2 diabetes, hypertension) and a variety of medical approaches in esthetic

and plastic surgery require the characterization of adipose tissue and a new generation of 3D *in vitro* models in order to investigate molecular pathways in adipogenic differentiation and lipid metabolism processes [15, 16]. Among all *in vitro* models, none of the current 2D monolayer models represent native human tissue, including adipose tissue, due to the absence of the stromal compartments essential for mimicking the native counterpart [15, 17, 18]. Advances in tissue engineering methods has allowed generation of a 3D adipose tissue model thanks to employment of a variety of biocompatible biomaterials, such as polycaprolactone (PCL), chitosan, polyester, hyaluronic acid, collagen, polyethylene glycol, alginate chemically modified, porous poly lactic-co-glycolic acid, ensuring the tridimensional architecture [15, 17, 18]. Despite tridimensional tissue support, these models showed a few limitations, such as the inadequate scaffold stiffness increase, impairing adipocyte functionalities [15].

3D scaffold-free spheroids represent a valid alternative to *in vitro* tissue models, aiming to reproduce the native architecture of human tissue without the need of an exogenous scaffold. 3D scaffold-free spheroids are compact cellular aggregates which allow single cells to interact with each other thanks to the self-assembly approach, with development of structural organization across scales [15, 19]. As a result of their 3D structure, spheroids exhibit the following improved biological properties compared with 2D cellular systems: enhanced cell viability, high biological relevance, stable morphology, polarization, increase in proliferative activity and physiological metabolic function [20]. Several studies revealed that spheroid properties are ideal for tissue engineering applications for the following reasons:

- generation of a large amount of tissues thanks to high-throughput generation;
- improved regenerative features compared with 2D cell culture;
- reproduction of more complex hierarchical structure thanks to the combination of different cell populations seeded in co-culture (vascular system, stromal compartment, subcutaneous bacteria);
- a useful screening platform to test new actives and molecules; and v) tissue generation of different sizes and shapes keeping in contact pooled microtissues as a bio-Ink in a bottom-up approach [19 - 21].

3D scaffold-free adipose spheroids (adMTs – admipose microtissues) scould be produced from the stromal cellular component containing the multipotent adipose tissue-derived mesenchymal stem cells (ASCs) [3, 4, 6]. Due to their extreme versatility and wide pluripotency, ASC proliferation and differentiation capabilities are limited by the donors' sex, BMI and age. In order to go beyond these limitations, mesenchymal stem cells derived from induced pluripotent stem cells (iPSC-derived MSCs) were employed for 3D adipose tissue model generation, avoiding all difficulties related to patient-derived ASC applications [22].

In this work a novel adipose 3D scaffold-free model based on the scaffold-free technology of a hanging-drop culture system was developed using induced pluripotent stem cells (iPSCs). Previous internal results have shown how more interesting these cells are than preadipocytes of single/pool donors with different BMIs for active ingredient screening. The model relevance and responsiveness were evaluated by stimulation with forskolin, a lipolysis inductor that acts as a key regulator of lipid storage mobilization, increasing the cAMP levels [23 - 25]. Development of adMTs and their metabolic activity were evaluated by qRT-PCR monitoring of the activation of key genes of adipogenic differentiation (PPARG) and the lipolysis mechanism (PNPLA2) and by quantification of glycerol release in the culture media as an indicator of adipogenic differentiation.

EXPERIMENTAL

Cells

Human 3D scaffold free spheroids were developed starting from PCi-mesecnchymal stem cells (PCi-MSCs) developed from human iPSC (Phenocell, Grasse, France) and cultured in the MSC Expansion Culture Media Kit XF (StemMACS, Miltenyi Biotec, Bergisch Gladbach, Germany), supplemented with Penicillin 100 units/mL/Streptomicin 100 μg/mL (Sigma Adrich, Darmstadt, Germany), during cellular expansion in order to preserve the undifferentiated cellular phenotype. Three days before starting spheroid seeding, cells were treated for adipogenic differentiation with Adipodiff culture medium (StemMACS, Miltenyi). During cellular amplification, cells were cultured in a monolayer for 7 days at an initial density of 5000 cells/cm^2 and maintained at 37 °C with 5 % CO_2. When cells reached a confluence of 90 %, they were washed with DPBS 1X without Ca^{2+}/Mg^{2+} and detached for spheroid formation.

3D scaffold-free spheroids production

After treatment with 0,05 % Trypsin/EDTA solution (Sigma Aldrich, Dramstadt, Germany), cells were counted, and 1x106 were seeded into each plate in order to obtain 3D scaffold-free spheroids of 10000 cells each. Briefly, the method employed for spheroid generation was the Hanging Drop Technology in which specific culture plates (GravityPLUS and GravityTRAP plate, In Sphero Zurich, Switzerland, ***Figure 1A***) were used, allowing development of scaffold-free spheroids with the self-assembly strategy and avoiding use of scaffolds or exogenous support: the cells, thanks to a special geometry of the plates, aggregate by themselves, generating compact spheroids in 3 - 4 days within the hanging drops; the small volumes of cellular suspension and the gravity on the hanging drops allow the optimal cellular aggregation for generation of homogeneous and compact 3D adipose scaffold-free microtissues (adMTs). Once assembled, the adMTs were transferred to a multiwell plate with V-shaped wells and cultured for an additional 14 days in adipogenic medium to allow complete adipogenic differentiation ***(Figure 1B)***. After 7 days of differentiation, the adMTs were treated for 7 more days with Forskolin 10 μM (Sigma Aldrich), a metabolic stimulator of lipolysis and inibitor of lipid accumulation during adipogenic differentiation. Lipolysis stimulator was prepared and added to the fresh culture medium every day thereafter. At the end of adipogenic induction, adMTs prepared under different experimental conditions showed a perfectly round-shaped structure that was stable, well defined and reproducibile ***(Figure 1C)***.

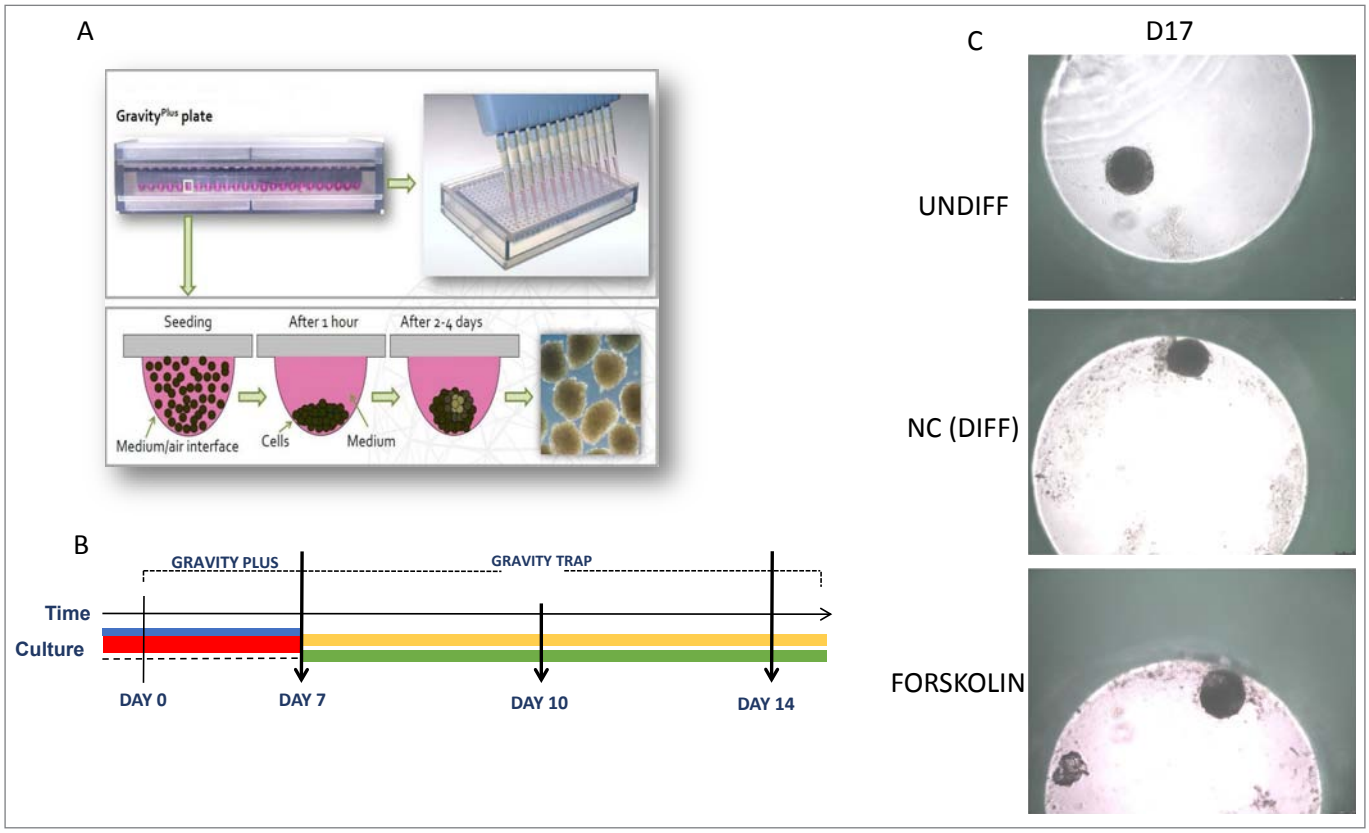

Figure 1 Adipose microtissues (adMTs) development using inSphero 3D tissue plate systems.
A) Schematic representation of the hanging drops culture systems; B) Scheme of the experimental design for adMTs development; and C) images of adipose 3D spheroids in different experimental conditions after 14 days of culture.

RNA isolation, reverse transcription and real-time PCR (qRT-PCR)

Total RNA was extracted from pooled adMTs (12 adMTs/each) in biological triplicates and retro-transcribed in cDNA with a RNAqueous kit (Life-technologies, Carlsbad, California, USA). qRT-PCR analysis performed with ΔΔCt methods was used to quantify the expression levels of target genes of adipogenic differentiation (PPARγ2, FABP4, ADIPOQ, CEBPA, PNPL2A, SLC2A4, ***Table I***) by means of the TaqMan Assay and TaqMan Master mix (Life-technologies) in the ABI7500 system (Life-technologies). Data were normalized against the internal control glucose-6-phosphate dehydrogenase (GPDH).

Glycerol release colorimetric assay

Glycerol is the main product derived from lipid and triglyceride metabolism and its release in the culture medium is a key indicator of an active lipid metabolism. Glycerol released by adipose spheroids was quantified in the culture medium during adipogenic stimulation in three different types of samples: undifferentiated, differentiated, and treated with forskolin 10 μM. The colorimetric assay (Sigma Aldrich) was performed on culture media collected at different time points of differentiation. Glycerol reference standards were prepared in different, known concentrations (0 mM, 0,3 mM, 0.6 mM, and 1 mM) according to the manufacturing procedure in order to calculate the amount of glycerol released. For each sample, a defined volume per well of assay buffer (200 μL), enzyme mix (2 μL), ATP and dye reagent (1 μL for both) was added and the sample then incubated for 20 min in the dark at RT. After incubation 50 μL of the stop solution were added to each well and the colorimetric signal was read at 570 nm.

Perilipin Immunolabeling

To evaluate the adipogenic differentiation in terms of lipids accumulation during the culture period, immunostaining for perilipin was performed. Perilipin is a key biomarker expressed on the lipid droplet

Table I Scheme of Gene Key Biomarkers

PPARγ2: Peroxisome proliferator-activated receptor, it is a master regulator of adipocyte differentiation.
FABP4: Fatty acid binding protein 4, a PPARγ2 target.
CEBPA: CCAAT/enhancer binding protein-α is expressed at high levels in adipose tissue.
SLC2A4: Insulin-regulated glucose transporter (GLUT4) found primarily in adipose tissue.
ADIPOQ: Adiponectin is an adipokine expressed by mature adipocyte, anti-inflammatory and improving sensitivity to insulin.
PNPL2A: ATGL is an enzyme which catalyzes the first step in the hydrolysis of triglycerides in adipose tissue.

surface, and its expression is directly related to the final differentiation in mature adipose tissue.

At different culture times adMTs were collected and fixed in formalin solution 10 % for 1 h at RT. After fixation, the samples were rinsed once with PBS and embedded in 2 % agarose-PBS solution before histological paraffin embedding. Pooled adMTs collected in agarose were dehydrated with ethanol 70 %, 95 % and 100 %, and antigen blocking was performed for 30 min at RT in blocking solution PBS-BSA 2 % on FFPE sections. Primary and secondary antibody were diluted in fresh blocking solution. Citrate-mediated, high-temperature antigen retrieval was performed and the rabbit polyclonal perilipin antibody (Abcam, ab3526) 1:100 was incubated overnight at 4 °C. Alexa Fluor 555 donkey anti-rabbit (Life Technologies) 1:400 was incubated for 1 h at RT as the secondary antibody. Nuclei counterstaining was performed with Fluoroschield+DAPI (Sigma Aldrich). Imaging was performed by confocal microscopy (Leica TCS SP2, Leica Microsystems, Mannheim, Germany) and images were processed using LASX and ImageJ softwares.

For lipid accumulation, AdipoRed Assay Reagent staining (Lonza, Basel, Switzerland), was performed on frozen tissue section of adMTs at different time points for 10 min at RT. The evaluation of lipid content (red signal) was quantified by ImageJ software.

2.6 Statistical analysis

All assays were performed in biological triplicates and the results expressed as the mean ±SD. Statistical analysis were perfoermed using the one-way ANOVA test. $P<0.05$ was considered to be statistically significant.

RESULTS

Gene expression results in adMTs during differentiation

To evaluate complete differentiation of the 3D adipose spheroids, the lipid accumulation and metabolic activity were investigated ***(Figure 2)***. Lipid accumulation was evaluated by AdipoRed reagent staining of frozen sections and the amount of lipid calculated by red signal quantification ***(Figure 2A)***. The 3D adipose scaffold-free microtissues (adMTs) differentiated dynamically during adipose induction, as shown by an increase in lipid accumulation in % ***(Figure 2A)*** and an increase in glycerol release in the culture media from day 7 to day 14 as evidence of a dynamic modification of adMTs during a progressive differentiative program ***(Figure 2B)***.

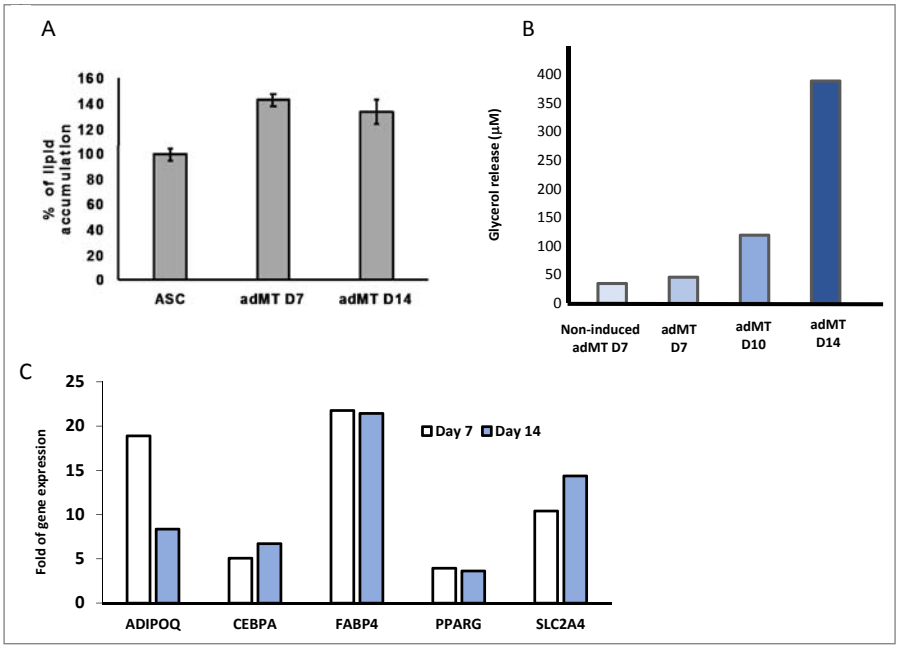

Figure 2 Dynamic evaluation of adipose differentiation in adMTs.
A) lipid accumulation in % measured by AdipoRed Signal quantification (ASCs are related adipose stem cells derived from donors); B) glycerol release quantified in the culture media during differentiation; and C) gene expression of key genes of adipogenesis (see also ***Table I***).

The gene signature of adipogenic differentiation was monitored by gene expression after 14 days of adipogenic induction. As can be seen in ***Figure 2C***, all key genes were upregulated when compared with adMTs collected at day 7. The downregulation of adipokine (ADIPOQ) expression showed a gene profile close to the physiological decrease observed in the native adipose tissue.

3D adipose microtissue metabolic capacity

The responsiveness of 3D adipose microtissues (adMTs) to molecules with a known effect on adipose metabolism was investigated by treatment of adMTs with forskolin 10 µM as an inductor of lipogenesis. As shown in ***Figure 3***, forskolin treatment reduced lipid accumulation, leading to a downregulation of key molecular biomarkers of adipose differentiation and lipid metabolism. The effect of forskolin was observed on the expression of crucial genes related to lipid accumulation, which were downregulated after treatment. The gene biomarkers affected included adiponectin (ADIPOQ), fatty acid binding protein 4 (FABP4), which is a PPARγ2 target, CCAAT/enhancer binding protein-α, which is expressed at high levels in adipose tissue (CEBPA), and the insulin-regulated glucose transporter 4 found primarily in adipose tissue (SLC2A4). The profile of gene expression was compared with that of untreated samples ***(Figure 3A)*** as the baseline condition. The effect on the metabolic activity of forskolin in adipose microtissues was confirmed by quantification of lipid accumulation by staining in the lipid droplet with AdipoRed staining reagent ***(Figure 3B)***.

For a deeper understanding of the effects of forskolin on adipose MTs and their responsiveness to this metabolic inductor, the gene expression of PPARG and PNPLA2 was investigated in differentiated treated and untreated samples ***(Figure 4)***. As seen in the ***Figure 4A***, the master regulator of adipogenic differentiation and lipid accumulation PPARG increased its expression during the differentiation period (from RQ = 1.00 of NC_D3 to RQ = 12.64 of NC_D17), indicating a dynamic behav-

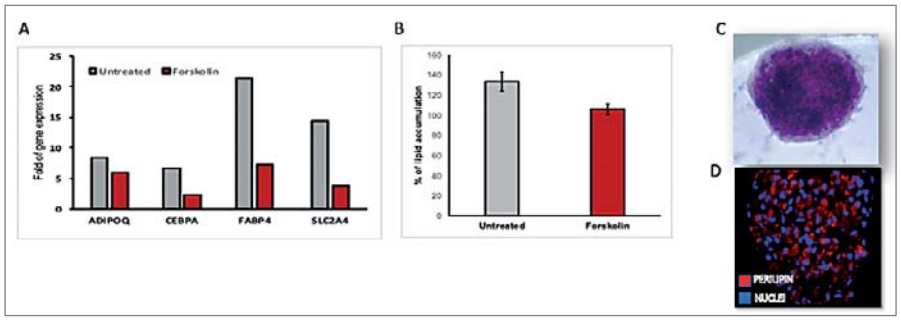

Figure 3 Effects of forskolin treatment on adMTs.
A) Gene expression of adMTs compared with untreated samples (all samples were collected at the end of differentiation on Day 17);
B) percentage of lipid accumulation quantified by AdipoRed Reagent Staining.

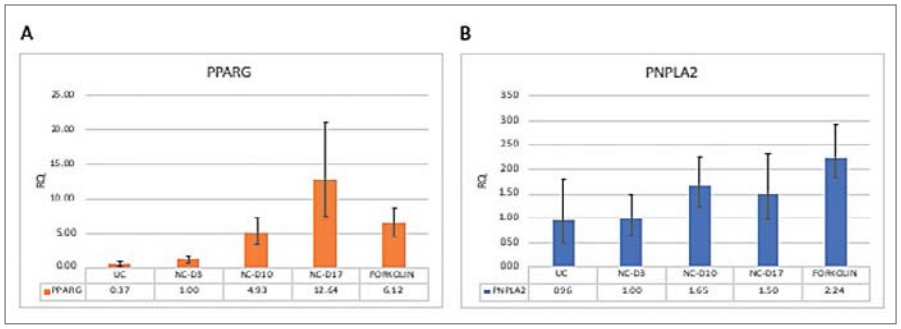

Figure 4 Effects of forskolin treatment on adMTs metabolism. Gene expression of PPARG (A) and PNPLA2 (B) in treated and untreated samples.

ior of spheroids due to their progressive lipid accumulation during the culture time. Treatment with forskolin reduced the expression of PPARG to low values (RQ = 6.12) comparable to those of the early phases of adipose differentiation ***(Figure 4A)***. In the same way, this hypothesis was confirmed also by the evaluation of PNPLA2 expression after forkolin treatment ***(Figure 4B)***. This gene regulates adipose metabolism and lipid degradation, and the forskolin treatment led to the upregulation of gene expression compared with that of the untreated controls (RQ = 1.50 of NC_D10 *versus* 2.24 of treated samples), indicating an efficient metabolic activity in adipose microtissue and an effective responsiveness of our models.

DISCUSSION

The intrinsic complexity of the human adipose tissue derives from its multiple functions related not only to lipid accumulation but also indirect effects on other tissue compartments and organs [14, 16]. The complexity of white adipose tissue derives from its origin during embryonal development and its cellular composition: derived from mesoderm, the germ layer, human adipose tissue is a supportive tissue composed of smooth muscle cells, pericytes, fibroblasts, vascular cells, mast cells and mesenchymal stem cells [22]. The synthesis and secretion of cytokines, hormones and steroids and their synergetic effect on different compartments and organs have resulted in inclusion of white adipose tissue in the field of endocrine tissues [14].

Thanks to its versatility and complexity, several researchers have focused their attention on adipose tissue for the generation of *in vitro* alternative models for clinical applications, medical investigations, and cosmetic treatments. In fact, in addition to clinical and medical requirements triggered by metabolic disorders and the high incidence of obesity, demands for *in vitro* generation of soft tissue equivalents in reconstructive and plastic surgery for cosmetic treatments are increasing, since adipose tissue represents a useful tool for breast reconstruction following mastectomies and cosmetic facial reconstructions (cheek, chin, and jaw) [26]. Techniques to increase the tissue volume for treatment of congenital deformities or post-traumatic tissue regeneration also include autografting of fat pads or injection of adipocytes isolated by liposuction, but low fat graft survival and necrosis due to the absence of vascularization are major limitations leading to a progressive resorption of the implant over time [17, 27].

In addition to these innovative applications, there is also interest in adipose tissue associated with evidence indicating metabolic effects of probiotics on adipose tissue and lipid metabolism [28]. In this work Roberts demonstrated that the cell free supernatant obtained from probiotic microorganisms is able to modify the differentiation of the murine preadipocytes 3T3-L1 in mature adipocytes. In particular, the L. acidophylus secretome was shown to be a key element in the inhibition of adipogenesis observed in the probiotic mixture. We demonstrated that the treatments with secretomes of Lactobacillus gasseri and Lactobacillus acidophilus for 7 days induced the activation of lipolysis and the metabolism in 3D scaffold-free microtissues (data not shown).

Our 3D scaffold-free spheroids developed using a hanging drops culture system starting from human iPSC-MSC derived cells reproduced the main features of native human adipose tissue in terms of architecture, structure and metabolic functions. The model is responsive to adipogenic induction, with a progressive change observed during the differentiative culture time reflecting a dynamic behavior of the model in terms of lipid accumulation, adipogenic gene expression and glycerol release.

CONCLUSION

Taken together, these results show that
- our 3D model is a powerful *in vitro* system that can directly be investigated to better understand the metabolic pathways of lipid and fatty acid accumulation thanks to its high biological relevance;
- it represents a screening platform for testing effects of actives and compounds with a known role in adipose tissue; and
- it can potentially be used to evaluate complex interactions between adipose tissue and other organs and body compart-

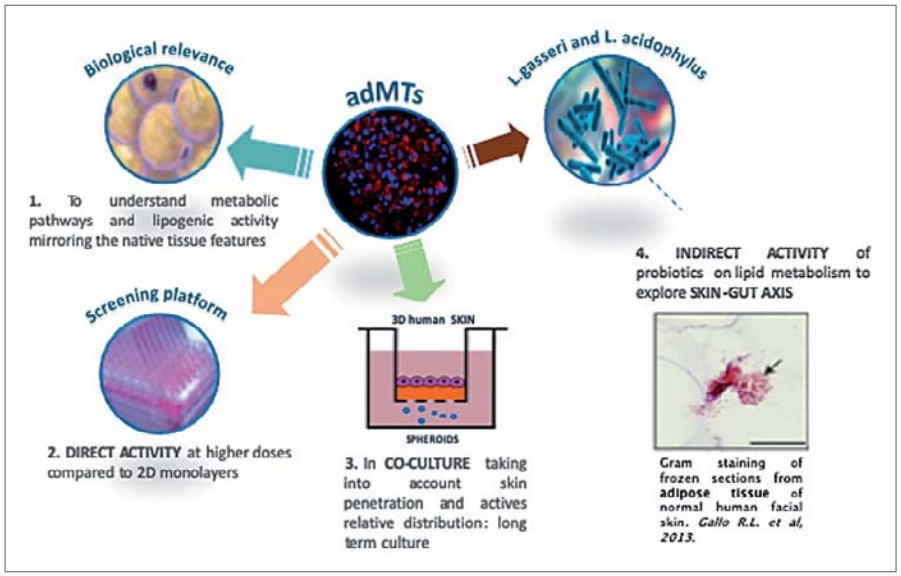

Figure 5 Summary of the potentiality and applications of 3D scaffold-free adipose microtissues (adMTs).

ments, such as the interplay between adipose tissue and skin (and the distribution of actives in the subcutaneous compartments after topical application to skin) as well as the pathway related to the gut-skin axis and the role of probiotics and their secretomes in lipid metabolism ***(Figure 5)***.

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