ADIPOCYTES 3D SCAFFOLD FREE MICROTISSUES FOR PRECLINICAL APPLICATIONS

Barbara de Servi, Elisa Caviola and Marisa Meloni | VitroScreen, in Vitro Research Laboratory | P.le Giulio Cesare, 17, 20145 Milan (Italy) | Corresponding author: marisa.meloni@vitroscreen.com

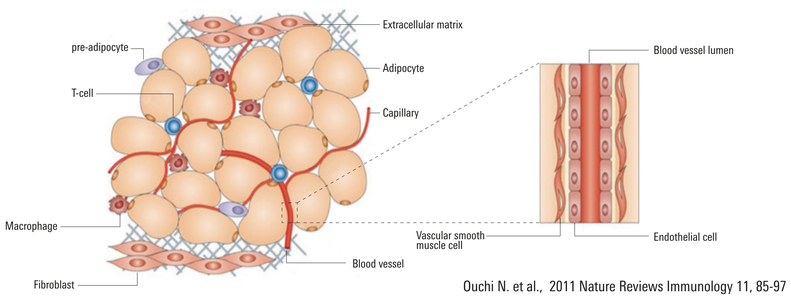
VitroScreen
excellence in *in vitro* science

INTRODUCTION

The white adipose tissue (WAT) is a complex organ composed mainly of differentiated adipocytes responsible for the body energy homeostasis (storage and mobilization of energy in the form of triglycerides), the metabolism of sex steroids and glucocorticoids and the modulation of important biological processes through adipokines acting at both local and systemic level.
Adipocyte differentiation is an ordered multistep process requiring the sequential activation of several groups of transcription factors, including CCAAT/enhancer-binding protein (C/EBP) gene family and peroxisome proliferatoractivated receptor-γ (PPAR-γ) which drive the expression of genes of great importance in WAT, such as genes of glucose and lipid metabolism and adipokines, establishing the differentiated state: preadipocytes acquire the characteristics of mature adipocytes, such as accumulation of lipid droplets and the ability to respond to hormones such as insulin.
Lipolysis consists of the sequential hydrolysis of TAG to its constituent molecules glycerol and three fatty acids, catalyzed by three different enzymes:

- TAG is hydrolyzed to diacylglycerol (DAG) and one molecule of fatty acid by the enzyme adipose TG lipase (ATGL)
- DAG is converted to monoacylglycerol (MAG) and a second fatty acid by the action of the hormone-sensitive lipase (HSL)
- Monoglyceride lipase (MGL) hydrolyses MAG, producing glycerol and a last fatty acid
- Regulatory mechanisms of lypolysis have been identified on multiple levels of the lipolytic pathway, including gene transcription and translation, post-translational modifications, intracellular localization, protein–protein interactions, and protein stability/degradation. (Fig.1)
- Many evidences have supported the primary interaction of the adipose tissue with the skin and its appendages as potential target for different types of treatments (topical, chirurgical) in particular for slimming and re-pulping efficacy.

Fig.1 ADIPOSE TISSUE COMPOSITION



Ouchi N. et al., 2011 Nature Reviews Immunology 11, 85-97

- Adipocytes are the main cellular component of adipose tissue.
- Other cell types within adipose tissue are precursor cells (including pre-adipocytes), fibroblasts, vascular cells (endothelial cells and vascular smooth muscle cells) and immune cells (macrophages and T cells).
- These cells constitute the stromal vascular fraction of adipose tissue.
- Factors that are secreted by these different cellular components are critical for maintaining homeostasis in adipose tissue and throughout the body.

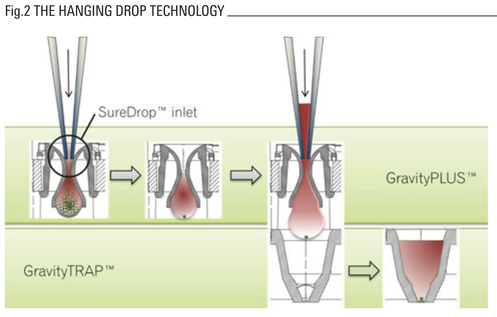
AIM OF THE STUDY

The hanging drop technology is one of the culture techniques that allow to produce highly functional cellular models that more closely mimic the native tissues and organ: microtissue. Single cells are suspended in a hanging drop of culture medium and subsequently form a miniature tissue under the pull of gravity. Cells are in close contact and produce their own ECM and the derived spheroids retain most of the cell-cell and cell-extracellular matrix (ECM) contacts.
Compared to 2D monolayers it is possible to underline many advantages in culturing cells in a 3D environment compared to 2D monolayers:

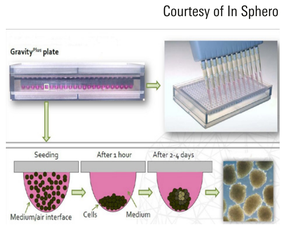
- Scaffold free: no interference with exogenous matrix, physiological 3D constructs
- High degree of flexibility and spheroid standardization: cell number per drop defines the spheroid size and guarantees uniformity in size and cellular composition
- Production of either single cell type or co-culture models
- Possibile to use single/pool donors with different BMI.
- Convenient 96-well plate format, highly reproducible
- Simple transfer of spheroids to other platforms for downstream applications
- Long term experimental models (up to 28 days)
- Dose closer to in vivo ranges

This work presents the results of the production, development and caracterization of 3D human scaffold free mature adipocytes microtissues (**3D-MA-MTS**) by using the hanging drop technology and by investigating the tissue response to lipolitic treatment (Forskolin) at transcriptional level.
In parallel a modifed differentiation process has been applied to better understand the type and morphology of the adipocyte in the microtissue and to explore new potential applications.

Fig.2 THE HANGING DROP TECHNOLOGY



Courtesy of In Sphero

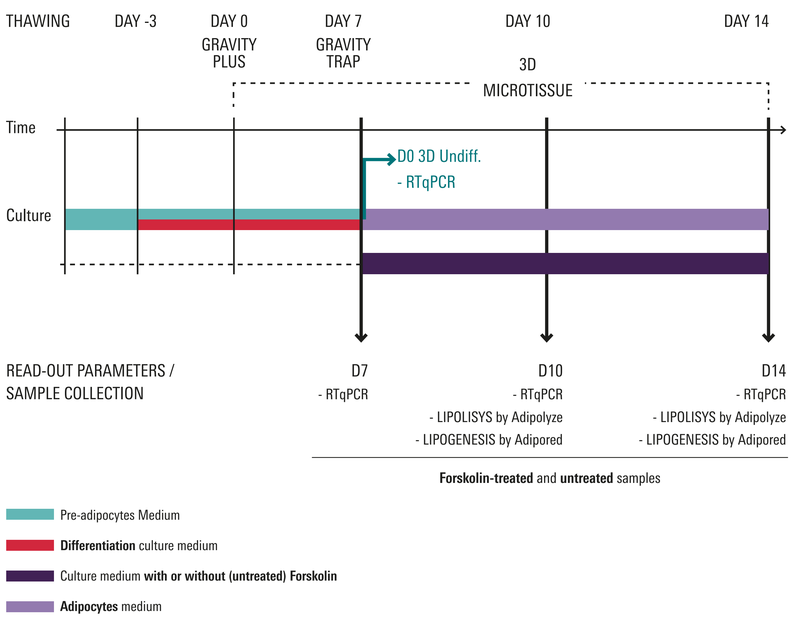


EXPERIMENTAL DESIGN

- CELL TYPE: Subcutaneous human preadipocytes, BMI= 26,6 (Tebu-Bio, F)
- MEDIA: Preadipocyte Medium (cat. PM-1) / Adipocyte Differentiation Medium (cat. DM-2) / Adipocyte Medium (cat. AM-1)
- PLATES: Gravity^{PLUS} / Gravity^{TRAP} (In Sphero)
- PARAMETERS:

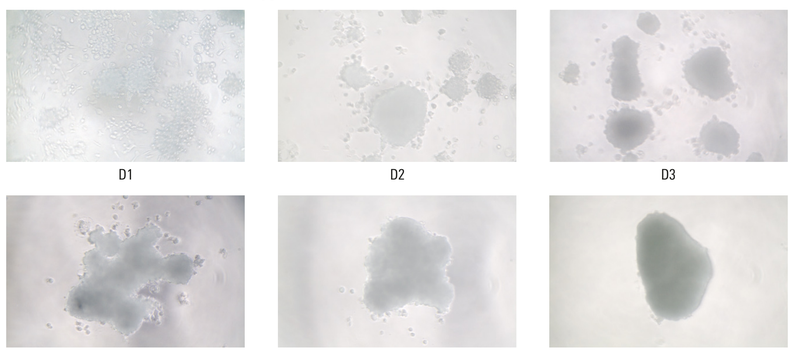
A multiple endpoint approach has been adopted to characterize the established "microadipe model" at biochemical, morphological, gene and protein expression levels compared to a 2D monolayer: the 2 models were compared in term of intracellular lipid droplets (adipogenesis), amount of glicerol release (lipolysis) and for the transcriptional activity of specific markers (TABLE I) of mature adipocytes (e.g. PPARγ2, FABP4, ADIPOQ, LEPTIN, GLUT4, ADIPOQ, PNPL2A, UCP1) by qRT-PCR*
*Gene expression: total RNA has been extracted from duplicate HCE samples (RNAqueous kit, Life-technologies) and retro transcribed in cDNA (RNAqueous kit, Life-technologies). RTqPCR analysis by ΔΔCt methods to quantify expression level of target genes and GAPDH (as reference gene) TaqMan Assay and TaqMan Master mix in ABI7500 system Life-technologies.
• IMMUNOHISTOCHEMISTRY: performed on FFPE sections using ANTIBODY PERILIPINE rabbit polyclonal (Abcam, ab3526). All the images have been captured using Leica Instruments (DM2500 microscope or SP2 confocal microscope) and LASX software.

EXPERIMENTAL DESIGN: FORSKOLIN STUDY — Fig. 3



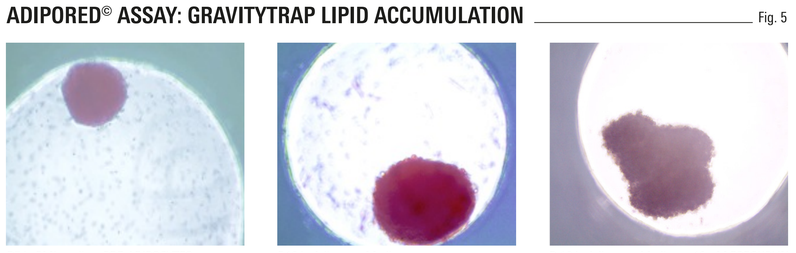
RESULTS

MA-MTS formation in Gravityplus — Fig. 4

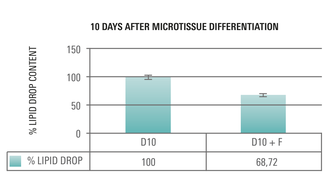


Starting from 5000 cells/droplet the round shaped microtissues (spheroide) is formed after 7days

ADIPORED© ASSAY: GRAVITYTRAP LIPID ACCUMULATION — Fig. 5



D7 in PMI medium PRE-ADIPOCYTES | D10 in Adipocyte Medium | D 10 in Adipocyte Medium +10 μM FORSKOLIN



The ADIPORED color is visible at inverted microscope observation and reach a maximum after 10 days; the color intensity is reduced in presence of forskolin

TABLE 1: TARGET GENES

GENE	MATURE ADIPOCYTES BIOMARKERS
PPARγ2:	Peroxisome proliferator-activated receptor, is a master regulator of adipocyte differentiation
FABP4	Fatty acid transporter, the gene of which is a PPARγ2 target
CEBP- α	CCAAT/enhancer binding protein (C/EBP) α expressed at high levels in adipose tissue
GLUT4 (SLC2A4)	Insulin-regulated glucose transporter found primarily in adipose tissue
ADIPOQ	Adiponectin is an adipokine expressed by mature adipocyte, anti-inflammatory and improving sensitivity to insulin
LEPTIN	Hormone produced by adipose cells that helps to regulate energy balance by inhibiting hunger.
PNPL2A (ATGL, desnutrine)	The gene encodes an enzyme which catalyzes the first step in the hydrolysis of triglycerides in adipose tissue.
UCP1	UCPs separate oxidative phosphorylation from ATP synthesis with energy dissipated as heat, also referred to as the mitochondrial proton leak. This gene is expressed only in brown adipose tissue, a specialized tissue which functions to produce heat.

mRNA transcriptional activity — Fig. 6

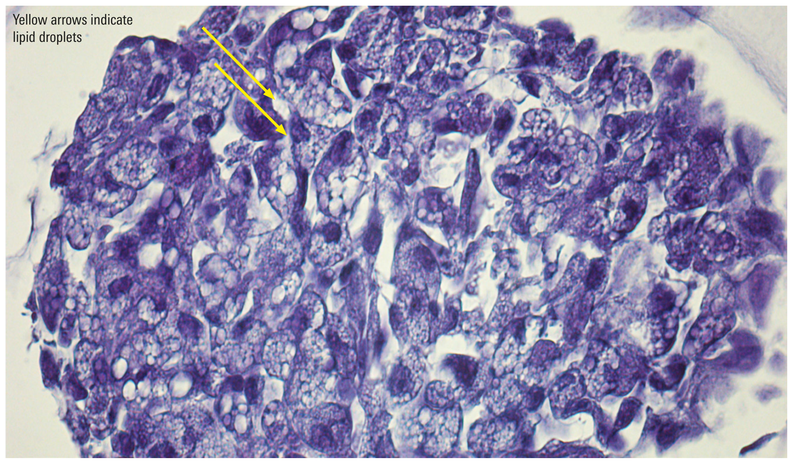
	D7-3D UNDIFF	D7 -3D	D10-3D	D14-3D
ADIPOQ	1	18,89	1,65	8,36
CEBPA	1	5,07	1,28	6,71
FABP4	1	21,75	4,62	21,43
LEP	1	0,03	3,79	1,07
PPARG	1	3,93	0,94	3,63
SLC2A4=GLUT4	1	10,40	3,59	14,37

Compared to undifferentiated model the genes associated to adipogenesis are over-expressed during preadipocytes differentiation in 3D microtissues

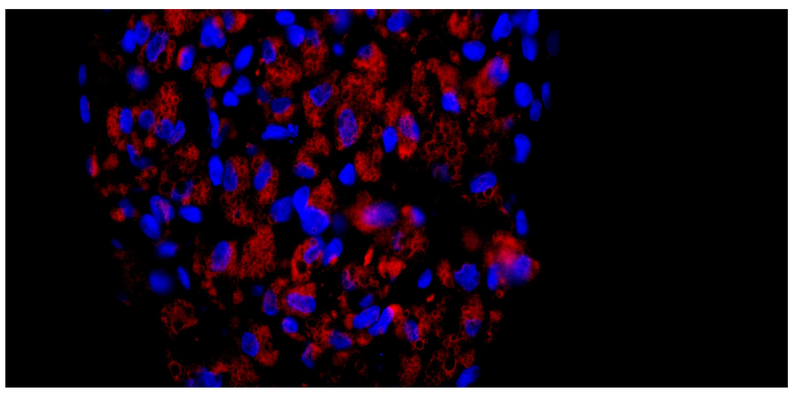
RQ VALUE =1 D10/D14 FORSKOLIN TREATMENT	D10	D10-3D
ADIPOQ	0,72	0,71
CEBPA	1,01	0,34
FABP4	0,96	0,34
LEP	0,0	0,34
PPARG	1,11	0,34
SLC2A4=GLUT4	0,13	0,26

Forskolin treatment has determined the down regulation of all the target genes

D7- H&E STAINING. — Fig. 7



D7-PERILIPINE: lipid droplets are stainined in red — Fig. 8



CONCLUSION

The established "microadipe model" represents a promising tool because it has been demonstrated to be more biologically relevant and predictive compared to 2 D monolayer : it can be applied to early screening of active ingredients for cosmetic and nutritional purpose allowing to establish the mecanism of action in a 3D environment. Last but not least it could be applied to perform a toxicological screening of lipophilic actives metabolism and accumulation.
The 3D-MA-MTS seems to better recapitulate the adipose tissue dynamic evolution compared to 2D monolayers and can be used to investigate the morphology and phenotype of adipocyte. and morphology of the adipocyte in the microtissue and to explore new potential applications.

1_ Higher biological relevance
2_ Extracellular matrix produced by the adipocyte itself
3_ Early and efficient differentiation
4_ Early polarisation of the 3D construct
5_ Low cell number compared to 2D
6_ Viable for 10-14 days
7_ Efficient (96 plate) and robust screening : multiple end points approach
8_ Histology and IF easy to be performed
9_ Higher doses of actives compared to 2D models