MINI-REVIEW

Key-genes regulating the liposecretion process of mature adipocytes†

Running title: molecular mechanisms of liposecretion

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Abstract

White mature adipocytes (MAs) are plastic cells able to reversibly transdifferentiate toward fibroblast-like cells maintaining stem cell gene signatures. The main morphologic aspect of this transdifferentiation process, called liposecretion, is the secretion of large lipid droplets and the development of organelles necessary for exocrine secretion.

There is a considerable interest in the adipocyte plastic properties involving liposecretion process, but the molecular details are incompletely explored.

This review analyzes the gene expression of MAs isolated from human subcutaneous fat tissue with respect to bone marrow (BM)-derived mesenchymal stem cells (MSC) focusing on gene regulatory pathways involved into cellular morphology changes, cellular proliferation and transports of molecules through the membrane, suggesting potential ways to guide liposecretion.

In particular, Wnt, MAPK/ERK and AKT pathways were accurately described, studying up- and down-stream molecules involved. Moreover, adipogenic extra- and intra-cellular interactions were analyzed studying the role of CDH2, CDH11, ITGA5, E-Syt1, PAI-1, IGF1 and INHBB genes. Additionally, PLIN1 and PLIN2 could be key-genes of liposecretion process regulating molecules transport through the membrane.

All together data demonstrated that liposecretion is regulated through a complex molecular networks that are able to respond to microenvironment signals, cytokines and growth factors. Autocrine as well as external signaling molecules might activate liposecretion affecting adipocytes physiology.

Key-words: adipocytes, mesenchymal stem cells, liposecretion, microarray analysis, molecular pathways.

Introduction

Adipose tissue is being increasingly recognized as an important endocrine organ that produces and releases a variety of factors and adipokines regulating different physiological processes (Ali et al., 2013). In particular, white adipose tissue is an organ highly specialized in storing and releasing lipids in response to a variety of signals controlling energy balance (Bou et al., 2014) and MAs are functionally the most important cell type in adipose tissue (Gimble et al., 2003). They are characterized by spherical shape with a single large lipid droplet formed by triglycerides that accounts for >90 % of the cell's volume (Cinti, 2009). The thin cytoplasm contains the nucleus, characteristically squeezed by the large lipid vacuole, usually an under-developed Golgi apparatus, rough endoplasmic reticulum (ER) made up of short, isolated cisternae and rare lysosomes (Cinti, 2009). They have a variable size that depends mainly on the size of the lipid droplet stored in them. A non-membranous electron-dense barrier, containing functionally important proteins such as perilipin, seems to separate the lipid droplet from the thin rim of cytoplasm without distinct structures (Blanchette-Mackie et al., 1996).

Despite the committed status of white adipocytes, their high plasticity has been demonstrated in vivo (Morroni et al.,2004; Barbatelli et al.,2010) and in vitro (Poloni et al. 2015 (a), (b), (c)), showing their extraordinary ability to modify, under physiological stimuli, their transcriptional programs and to directly transdifferentiate. A growing body of evidence supports the idea that the direct transformation of an adipocyte mature phenotype into a different phenotype happens without the transition through a dedifferentiation step. Evidence for transdifferentiation relies mainly on the ultrastructural data showing a gradual transition from one phenotype into another with no signs of proliferation or development of tissue precursors (Barbatelli et al., 2010; Frontini et al., 2013). More in detail adipocytes from mammary glands, for example, are able to reversibly convert into epithelial glandular cells able to produce milk (De Matteis et al., 2009). Cold exposure or appropriate beta-adrenergic stimuli are responsible for browning of white adipose tissue both in murine and human adipose organ (Giordano et al., 2014).

All these data together suggest that the process of cellular differentiation in terminally differentiated mammalian cells is not irreversible and MAs are able to reversible change their phenotype (Poloni et al., 2012). In particular, white adipocytes have recently attracted attention because, under physiological stimuli, they can directly transdifferentiate by a specific mechanism called liposecretion (Poloni et al., 2012; Maurizi et al., 2016), losing their characteristic lipid droplet (de-lipidation) and transforming into fibroblast-like cells (Poloni et al., 2012). This phenomenon has been described for the first time as a dedifferentiation process (Matsumoto et al., 2008) but then

time-lapse documentation and the ultrastructural details demonstrated that de-lipidation occurs via a previously undescribed phenomenon of liposecretion (Maurizi et al., 2016; Maurizi et al., 2017).

The main morphologic aspect of this transdifferentiation process was the secretion of large lipid droplets and the development of organelles necessary for exocrine secretion. This liposecretion process encompasses the rapid secretion of large lipid droplets, with the lipid surface changing its ultrastructure through the formation of a trilaminar plasma membrane (PM).

Notably, this process differs from the physiological delipidation of adipocytes occurring in fasting, where a progressive reduction of the lipid content is characterized by the appearance of characteristic villous-like cytoplasmic projections that are likely involved in the fatty acid extrusion from the cell (Blanchette-Mackie et al., 1981; Cinti, 1999).

Microenvironment, more than cellular commitment, can be essential to maintain, or adapt, a specific cellular phenotype to physiological challenges (Giordano et al., 2014). According to this hypothesis, it is presumable that the in vitro culture may represent the environment condition that triggers the reprogramming of human MAs and leads to the massive liposecretion in order to acquire the most convenient phenotype in the new environment. Additionally, in line with the in vitro results, liposecretion-compatible electron microscopic aspects are also observed in explants from human fat in matrigel and, rarely, in vivo, in the adipose tissue from obese patients (Maurizi et al., 2016). Collectively, these data support a new plastic process of transdifferentiation of adipocytes into a stem cell-like cells, but the molecular details that regulate this process are still largely unknown.

In this respect, the purpose of this review is to accurately describe the potential molecular mechanisms involved in the liposecretion process, underlining the prevalent gene regulatory pathways observed by studying the gene expression profile of human MAs isolated from subcutaneous adipose tissue. Previous results demonstrated that, post liposecretion, adipocytes show similar functional and molecular properties to MSCs isolated from donors age-related bone marrow. Accordingly to these data, in this review comparison between MA gene expression profile and MSC was analyzed.

In particular, this work focus on three gene clusters, specifically, the genes involved into the cellular morphology changes (Table 1), genes able to regulate the transports of molecules through the membrane (Table 2) and genes involved into the cellular proliferation (Table 3).

Potential ways to guide liposecretion process were suggested looking to the possible structural changes of adipocytes. Notably, previous data demonstrated that during the liposecretion process a well-developed set of organelles implicated in secretory processes such as mitochondria, stacked rough ER and hypertrophic Golgi complex were present in these “liposecreting” adipocytes. In line

with these observations expression analysis of the genes involved into the cellular structural rearrangement and transport through the membrane were studied.

In particular, in agreement with the completely committed status of adipocytes, results demonstrated that among the 16 genes involved in cell morphogenesis only three were upregulated in MAs respect to BM-MSC, suggesting the idea that MAs without physiological stimuli were not subjected to shape changes.

Moreover, previous data showed that 81 genes, involved in the transport through the membrane, were differentially expressed between MAs and BM-MSC, with 39 genes up-regulated in MAs and 42 in BM-MSC. More in detail, 10 pathways could regulate liposecretion through the key-genes ABCA1, LDLR, PLIN1 and PLIN2 (Maurizi et al., 2016) and in this review these specific molecular pathways involved were analyzed.

Additionally, molecular pathways involved into cell proliferation were upregulated after the liposecretion process suggesting the functional changes observed in post-liposecretion cells respect to MAs. Indeed, the analysis of these pathways could suggest models in which adipocytes changes their cellular relationship into the microenvironment, regulating, for example, their surface antigens and the secretion of chemokines, adipokines and chemoattractive factors.

Defining more clearly the molecular signals involved into the adipose tissue liposecretion process has the potential to better understand the cell biology of adipose organ. Moreover, the identification of dysregulated pathways in metabolic diseases could characterize potential therapeutic targets, such as for the treatment of obesity and T2 diabetes to improve the development and maintenance of healthy adipose tissue.

Adipocytes' morphology changes during liposecretion

The role of intercellular adhesion and extracellular-matrix (ECM) molecules are critical in stem cell lineage specification, and therefore, they could be used to develop technologies to control stem cell differentiation by exploiting cell-cell interactions (Alimperti et al., 2015). Cell adhesion molecules (CAMs), including cadherins, integrins, selectins and immunoglobulin-like CAMs, mediate cell-cell or cell-matrix interactions and regulate multiple aspects of cellular behavior such as proliferation, differentiation, apoptosis, cell polarity (Cavallaro and Dejana, 2011), embryonic stem cell self-renewal and differentiation (Li et al., 2012) and, most importantly, the maintenance of tissue integrity (Harris and Tepass, 2010). One class of CAMs is represented by cadherins, encoded by CDH2 and CDH11 genes, which mediate Ca2+ dependent homophilic interactions between cells through the formation of intercellular connections. The expression levels of cadherins may vary during different cellular processes, especially involving transition from one cellular state to another.

According to the recent studies, cadherin expression and cell-cell adhesion may also be critical in lineage specification of stem cells or reprogramming of adult cells to a pluripotent state (Redmer et al., 2011, Alimperti et al., 2014). In fact, interaction of cadherin-cadherin may lead to intercellular activation of cellular pathways, initiated through lamellipodial protrusions and followed by the cadherin-catenin-actin cluster formation. Moreover, cadherins play important roles in MSC differentiation, migration and homing to the site of damaged tissue by CDH2 and CDH11 (Xu et al., 2012, Theisen et al., 2007) proteins. Their expression levels are regulated differently in osteogenic, chondrogenic or myogenic lineages and notably, during adipogenesis, CDH2 and CDH11 were downregulated and MAs did not express either of cadherins (Shin et al., 2000). In addition, CDH11 knockdown induced adipogenic gene expression and differentiation, suggesting that CDH11 might inhibit adipogenesis (Alimperti et al., 2015). In line with these results, CDH11 was downregulated in MA and up-regulated in post-liposecretion cells.

Molecules that might modulate the microenvironment and network of ECM surrounding adipocytes, by affecting cell-ECM interactions and transduction of extracellular signals to intracellular components could be involved into the liposecretion process.

The fate of human adipose tissue stem cells is largely determined by biochemical and mechanical cues from the ECM, which are sensed and transmitted by integrins. In vitro studies (Morandi et al., 2016) showed that integrins, such as ITGA2, ITGA416, ITGA5 and ITGA638 are differentially expressed in MSCs isolated from adipose tissue and MA. The Hippo pathway, that is an evolutionarily conserved pathway that controls tissue growth regulating cell proliferation, differentiation and cell death, has recently been connected to integrin-dependent adhesion (Chen et al., 1997; Folkman and Moscona, 1978). It is controlled by extracellular mechanical cues such as ECM rigidity or cell-cell contacts and signals from those ECM constituents are mainly recognized by ITGA5 and ITGAV. ITGA5 was strongly expressed in undifferentiated cells, while downregulated in MAs. The strongest effect of ITGA5/ITGAV knockdown was an increase in differentiation and, on the contrary, transgenic ITGA5/ITGAV expression determined adipogenesis inhibiting effects (Morandi et al., 2016).

Other genes involved into the cellular morphology changes pathways were analysed in this review suggesting their possible role into the liposecretion mechanism. Data demonstrated that Serpine-1, gene that encodes the plasminogen activator inhibitor-1 (PAI-1), is a down-regulated gene during the liposecretion process. Obesity and insulin resistance are correlated with increased PAI-1 levels, and circulating PAI-1 levels are elevated at an early stage of impaired glucose tolerance and continued to be elevated while diabetes and the metabolic syndrome develop (Juhan-Vague and Alessi, 1997; Lyon and Hsueh, 2003).

Moreover, PAI-1 is overexpressed in the adipose tissue of obese mice and humans (Alessi et al., 1997; Sawdey and Loskutoff, 1991) and results from literature showed that PAI-1 deficiency promotes adipocyte differentiation. Despite that the functional role of PAI-1 in adipocytes is still unknown, it was shown that PAI-1 plays an important role in modulation of adipocyte differentiation. Indeed, besides the key regulatory roles of two well-characterized adipogenic transcription factors, CCAAT enhancer-binding protein α (C/EBPα) and peroxisome proliferator-activated receptor-γ (PPARγ) (Rosen et al., 2002), multiple events occur during adipocyte differentiation, including dynamic changes of cell matrix interactions and extensive ECM remodeling (Chavey et al., 2003).

Extra- and intra-cellular signalling could be involved into the liposecretion process, playing a role in cellular adhesion, differentiation and proliferation (Binderet al., 2007). Adipocytes' gene expression data demonstrated the lower expression respect to MSC of PLAUR, a gene that encodes the receptor for urokinase plasminogen (uPAR). More in detail, uPAR inhibits the PI3K/AKT pathway, which, downstream of insulin signaling, has previously been shown to regulate adipocyte differentiation and to increase the expression of PPARγ. Moreover, the complete absence of uPAR attenuated the increase of body weight and the adipose tissue mass by normal or high fat diet feeding.

It is presumable that the molecular pathways involving intra-cellular interactions can trigger the reprogramming of MA and leads to the massive liposecretion process. Microarray data analysis, in line with literature, demonstrated that MA seems to have the distinctive ability to reversibly change its morphology and consequentially its function during liposecretion. In this respect, also genes involved into the intra-cellular changes were analyzed in this review, such as E-Syt, IGF1 and INHBB. In particular, molecules that are able to regulate cytoskeletal re-organization, such as the ER, were analysed. ER performs key cellular functions, including lipid synthesis, Ca^{2+} regulation, and protein secretion (Friedman and Voeltz, 2011). To coordinate cellular functions and maintain cell homeostasis, the ER forms membrane junctions with other organelles, including the PM. At ER-PM junctions, the two heterologous membranes are in close apposition with a gap distance ranging from 10 to 30 nm (Toulmay and Prinz, 2011).

The Extended-Synaptotagmin (E-Syt) membrane proteins were recently discovered and data showed that E-Syt mediates the ER-to-PM tethering (Giordano et al. 2013; Manford et al. 2012) implicating a range of interrelated cellular functions, including calcium and receptor signalling, and membrane lipid transport (Herdman et al., 2016). Literature showed only few ideas of how and when these proteins are required in cellular and organism physiology and in this review the potential role of E-Syt1 in adipocytes during liposecretion is speculated. In particular, three E-Syts

(E-Syt1, 2 and 3) were identified in human like integral ER proteins (Herdman et al., 2016) implicated in ER-PM tethering and the formation of membrane contact sites, and in lipid transport and in Ca^{2+} signalling (Herdman et al., 2016). E-Syt1 is located in intracellular ER but translocated to ER-PM junctions only after an increase in intracellular Ca^{2+} concentration. Indeed, at basal Ca^{2+} levels E-Syt1 is not associated with the PM, but, as the intracellular Ca^{2+} level increases, the C2C domain of E-Syt1 will bind Ca^{2+} resulting in binding to $PI(4,5)P_2$ (Phosphatidylinositolo 4,5-bisphosphate, an inositol-containing phospholipid that is enriched at the inner leaflet of the PM regulating membrane trafficking, ion channel and transporter activity, and cytoskeleton-PM interaction) (Chang et al., 2015), in the PM and trapping of E-Syt1 at the ER-PM junction. A remarkable function of $PI(4,5)P_2$ is its ability to mediate Ca^{2+} signalling following the stimulation of cell surface receptors that is then transduced from the PM to the ER (Chang et al., 2015). Analyzing the gene expression profile of MA, it was shown an up-regulation of E-Syt1 respect to MSCs that decreased during liposecretion. Because ER-PM junctions are sites for Ca^{2+} regulation and lipid transport, changes in density, size, and gap distance of ER-PM junctions are likely to exert profound effects on these important cellular functions.

Potentially, many molecules regulate cytoskeletal organization and signal transduction, controlling cell size changes of adipocyte. Out of all the up-regulated genes in MAs respect to BM-MSC, the insulin-like growth factor-1 (IGF1) has been implicated in the differentiation and metabolic regulation of adipocytes. The role of IGF1 in adipose tissue is still under investigation, but data from literature demonstrated that it promotes the differentiation and survival of adipocytes, along with the accumulation of lipids, playing a critical role into the obesity development. Moreover, IGF1 can regulate adipocyte metabolism and suppress lipolysis (LeRoith et al., 2007).

The INHBB gene encodes for the activin B, hormone that belongs to the TGF-b superfamily, which affects a wide range of biological processes. Activins consist of two inhibin-b subunits that are held tight by a single disulfide bridge (Antenos et al., 2008). Four mammalian inhibin-b subunits (bA, bB, bC, and bE) have been identified so far. Since dimer formation occurs intracellularly, subunits must be co-expressed in the same cells in order to form the mature hormone. Whereas the bC and bE subunits are predominantly expressed in liver, the inhibin bA subunit is widely expressed. The inhibin bB subunit, encoded by the INHBB gene, is highly expressed in adipocytes (Sjöholm et al., 2006). The other inhibin-b subunits are expressed at very low levels in the adipose tissue suggesting that adipocytes produce activin B by dimerization of two Inhibin bB subunits. The inhibin bB subunit and activin B receptors are highly expressed in the adipose tissue suggesting that they are adipose tissue molecules with a specific key role (Sjöholm et al., 2006; Carlsson et al., 2009). In particular, previous data showed that locally produced activin B regulates adipocyte lipid

metabolism and adipocyte function as a negative regulator of lipolysis. Indeed, activin B significantly down regulates key genes involved in lipolysis such as ATGL and HSL (Kershaw et al., 2006; Schweiger et al., 2006). Accordantly, INHBB expression in adipose tissue is down regulated by diet-induced weight loss in humans (Sjöholm et al., 2006), a situation when lipolysis in markedly increased.

All together these data showed that molecular pathways regulating extra- and intra-cellular interactions are involved into the liposecretion process. This analysis wants to underline potential regulatory mechanisms that could be studied more in detail in future.

Transport of lipid droplets through the cell membrane during the liposecretion process

Lipid droplets (LDs) are dynamic organelles, constantly forming, growing or decreasing into the cytoplasm of adipocytes. They are mostly formed in the ER, where enzymes catalyse the lipid synthesis (Krahmer et al., 2013). Regulated processes, required for energy generation or membrane lipid synthesis determinate the LDs degradation and the mobilization of stored lipids (Ahmadian et al., 2009). Moreover, LDs are involved into multiple cellular processes, such as protein storage and degradation, and regulation of enzymes activities (Murphy et al., 2012). Notably, some proteins, among purified LDs proteins fractions of different cell types, are able to bind LDs surface regulating their size and number, such as Perilipin 1 and 2 (PLIN1 and PLIN2).

In particular, PLIN1 is expressed on the lipid droplet surface of white and brown mature adipocytes and its presence could be essential for the liposecretion process (Maurizi et al., 2016). Data from literature demonstrated that PLIN1 is an important regulator of lipolysis, protecting LDs from basal lipolysis (Zhai et al., 2010). However, when lipolysis is stimulated, PLIN1 regulates access of lipases to LDs (Zhai et al., 2010). Accordingly, PLIN1 knockout mice are lean and protected from diet-induced obesity (Krahmer et al., 2013). Moreover, PLIN1 and FSP27/CIDEC expression in adipose tissue inversely correlates with insulin resistance (Puri et al., 2008). Insulin-sensitive obese individuals showed higher levels of PLIN1 and CIDEC in adipose tissue than insulin-resistant subjects of the same weight, suggesting that higher expression of these proteins promotes triglycerides storage in adipose tissue and protects from lipotoxicity.

Results showed that TNFα reduces cellular PLIN1 level, likely leading to increased basal lipolysis and free fatty acid levels in the blood. These insulin resistance promoting TNFα-effect can be antagonized by anti-diabetic agents, such as thiazolidinediones (Souza et al., 1998).

Importantly, PLIN1 ablation causes impaired differentiation and development of adipose cells with dysregulation of adipogenic signalling (Lyu et al., 2015). Indeed, precursor's proliferation and differentiation can be modulated by the adipose tissue microenvironment (Maumus et al., 2008).

PLIN1-/- adipose tissue showed low expression of precursor's markers implicating the aberrant progenitor pool growth and their low differentiation capability. Accordingly, PLIN1-/- mice showed adipogenic transcription factors, such as C/EBPs and SREBP1c, and lipogenic enzymes, such as FAS and ACC1, downregulated (Lyu et al., 2015). More in detail, C/EBPs, SREBP-1c, PPARγ, and the lineage-adipogenic genes, such as aP2, FAS, HSL and ATGL, were typically downregulated at transcriptional level during PLIN1-/- cell differentiation (Lyu et al., 2015). However, PPARγ and SREBP-1c were not altered in PLIN1-/- and PLIN1+/+ cells, implicating that PLIN1 is a downstream target of PPARγ (Arimura et al., 2004). In line with these results, PLIN1+/- cells can be partially differentiated expressing higher number of LDs with bigger size respect to PLIN1-/- cells and markedly less than PLIN1+/+ cells. Finally, LDs growth in PLIN1-/- cells can be ameliorated by PPARγ activators and by prolonged exposure to insulin or fatty-acid loading (Lyu et al., 2015).

All together these data showed the key-role of PLIN1 in adipogenesis and LDs formation and suggest its potential involvement in the liposecretion process of mature adipocytes (Ganondra et al., 2011; Maurizi et al., 2016).

Differently to PLIN1, PLIN2 was found to be mainly expressed in mammary alveolar cells respect to adipocytes, surrounding LDs destined to be secreted during the transdifferentiation process of white adipocytes into milk-producing epithelial alveolar cells (transdifferentiation process called "pinking") (De Matteis et al., 2009; Prokesh et al., 2014). Moreover, previous results demonstrated that during the early adipogenic differentiation, LDs smaller than 1μm diameter were associate with PLIN2 protein while medium-size droplets (2μm) were associate with both PLIN2 and PLIN1. In late differentiation, the droplets larger than 3μm were coated only with PLIN1 (Lyu et al., 2015; Itabe et al., 2017).

Overexpression of PLIN2 induced LDs accumulation in fibroblasts (Imamura et al., 2002) and PLIN2-/- mice are resistant to diet-induced obesity, fatty liver disease and alcohol-induced steatosis, suggesting its potential role in lipid accumulation (McManaman et al., 2013; Carr et al., 2014). Like PLIN1, also PLIN2 expression is regulated by PPARs and RXR and treatment with a synthetic RXR agonist increased PLIN2 mRNA expression (Itabe et al., 2017).

These results suggest that PLIN2 promotes LDs formation in early stage also contributing to protect fatty acids from lipolysis. Whereas PLIN2 stabilizes LDs in the presence of a high intracellular lipid content, it is likely degraded by the ubiquitin-proteasome pathway under lipid-poor conditions (Tomaru et al., 2012). These results demonstrated the role played by PLIN2 into the adipogenic differentiation and LDs regulation. In line with that, the gene expression level of PLIN2, along with LDLR and ABCA, is higher in mature adipocytes and decreases progressively during the secretion

of LDs achieving similar value to MSCs after the liposecretion process, suggesting their role into the LDs exocrine secretion.

Adipocytes' proliferative regulation during liposecretion

MAs are committed cells differentiated from pre-adipocytes, mesenchymal origin precursor cells. Differentiation of preadipocytes starts with the proliferation arrest following the expression of adipogenic lineage genes. MAs do not proliferate but the growth of adipose tissue occurs through the formation of new adipocytes or by increasing the volume of adipocytes (Rosen et al., 2014). Even if some aspects of in vitro adipocytes differentiation are controversial, it is now clear that some cell cycle checkpoint proteins, such as the retinoblastoma gene family, influence adipogenesis (Capasso et al., 2014). The classic role for the retinoblastoma family genes RB1, RB2/P130, and P107 is the regulation of the cell cycle G_1/S transition through the negative modulation of the E2F family of transcription factors. Data demonstrated that lack of RB1 and RB2/P130 increased the percentage of BM-MSC committed toward adipocyte phenotype (Capasso et al., 2014). In particular, the adipocytes appeared not to reach a terminal differentiation or, alternatively, showed dysregulated functions. Indeed, in adipocytes lacking RB1 or RB2, the mean cellular level of early differentiation markers persisted at a high level. Moreover, adipocytes lacking RB2 showed a higher propensity in lipid uptake compared with control cells, while adipocytes lacking RB1 showed a higher capacity in lipid release (Capasso et al., 2014). All together results showed that commitment of BM-MSC to adipocyte lineage was facilitated by a lack of RB1 and RB2. They promoted differentiation of pre-adipocyte to mature cells by interacting with adipogenesis-related transcription factor (Capasso et al., 2014). Notably, BM-MSC failed to differentiated into adipocytes after C/EBP knockdown and, contrarily, overexpression of exogenous C/EBP induced adipogenesis, resulting in cellular fat droplet accumulation (Qian et al., 2010). Similarly, PPARγ without the presence of inducers, such as indomethacin (a PPARγ agonist), initiated adipocytes differentiation but the cells failed to differentiate fully (Qian et al., 2010). These data demonstrated the ability of the transcription factors to stimulate adipogenesis helping to elucidate the molecular mechanism of adipocytes differentiation and suggesting in the same time their potential role in liposecretion process.

Data from literature showed that MAs acquire their proliferative state by liposecretion process involving multiple molecular pathways. More in detail, 56 genes, among the 131 analyzed genes involved into the proliferation process, were differentially regulated in MAs and bone marrow-MSCs (Maurizi et al., 2016). Most of these genes were upregulated in MSC state and downregulated in MA, implicating their decreased proliferation capacity. Some of the pathways

involved in the regulation of proliferation and inhibition of differentiation are discussed in more detail in the following sections as well as the potential contribution of these pathways to liposecretion is hypothesized.

Canonical Wnt signaling is based on the regulation of cytoplasmic transcriptional factor β-catenin, which is degraded in the absence of WNT molecules. WNT binding to its frizzled receptors leads to the hypophosphorylation of β-catenin and relocation in the nucleus where it activates the transcription of WNT target genes. Contrarily, non-canonical, β-catenin independent, pathway is not very well understood. Wnt signaling is a well-known regulator of tissue development and crucial regulator of the preadipocyte proliferation by inhibiting the expression of two main adipogenic transcription factors PPARγ and C/EBPα (Christodoulides et al., 2009) and retaining the adipocytes' precursor cells in their proliferative state. Canonical Wnt-signaling activates genes related to the proliferation while suppressing the adipogenic genes, that suggests the involvement of this pathway in the regulation of liposecretion process.

Hippo signaling, regulator of adipocyte proliferation and differentiation, has been proposed to crosstalk with Wnt signaling (An et al., 2013) and the involvement of this pathway in the regulation of MSC proliferation and differentiation is also supported by the microarray data showing the upregulation of YAP1, Hippo downstream gene, in MAs (Maurizi et al., 2016).

Moreover, MAPK/ERK pathway is involved in the regulation of adipogenesis at different stages. Activation of ERK is required in the proliferation of preadipocytes but ERK must be dephosphorylated when the differentiation proceeds and PPARγ is activated (Camp et al., 1997; Kim et al. 2001; Prusty et al., 2002). AE binding protein (AEBP1) regulates ERK pathway by protecting ERK from phosphatases (Kim et al., 2001), that explains the higher AEB1 expression in BM-MSCs compared to MAs (Maurizi et al., 2016). In addition, Fibroblast growth factor 2 (FGF-2) has been shown to regulate many cellular processes mainly by activating two pathways: MAPK/ERK and Akt signaling pathways. Data (Maurizi et al., 2016.) supports the involvement of ERK pathway in the regulation of MSC proliferation and differentiation by modulating the expression levels of FGF-2 or fibronectin. ERK pathway seems to be important in adipocyte differentiation which is directly associated with the regulation of preadipocyte proliferation.

More in detail, FGF-2 has been shown to enhance adipogenesis in human adipose derived stem cells while in other studies it is predicted to inhibit mouse BM-MSC differentiation into adipocytes (Le Blanc et al., 2015). These controversial effects of FGF-2 in adipogenesis are claimed to be dependent on the FGF-2 concentration used in the *in vitro* studies; low concentrations of FGF-2 enhance the adipogenesis while high concentrations inhibit adipogenesis through ERK activation (Kim et al., 2001). Microarray data (Maurizi et al., 2016) would suggest the adipogenic functions of

FGF-2, with higher expression in MA compared to BM-MSC. Altogether, the capability of FGF-2 to regulate ERK pathway makes it one of the potential key regulators of post-liposecretion cells proliferation.

Additionally, ERK pathway is also connected with Wnt pathway through several genes, such as preadipocyte factor (Pref-1), building a complex signaling network that regulates proliferation but also liposecretion. Pref-1 is transcriptional factor that keeps 3T3-L1 cells in undifferentiated state (Sul et al., 2000). Soluble form of Pref-1 inhibits preadipocyte differentiation also by activating ERK pathway. The inhibition was shown to require Pref-1 binding to fibronectin that activates the downstream cascade of ERK pathway (Wang et al., 2010). Interestingly, fibronectin is upregulated in BM-MSC cells compared to MAs (Maurizi et al., 2016).

Moreover, ERK pathway can be regulated by Akt signaling (Manning et al., 2007) and data from literature showed that during preadipocyte proliferation Akt is silenced allowing ERK activity. In line with this result, microarray data showed low expression of Akt1 in BM-MSCs (Maurizi et al., 2016) suggesting the importance of shutting down this pathway during the liposecretion process.

More in detail, Akt is a serine/threonine kinase which can be activated by various growth factors via its upstream regulator, PI3K. Akt signaling has been intensively studied in various tissues and cancer types in order to determine its role in cell metabolism, survival and proliferation where it has been shown have multiple roles depending on its downstream substrates (Whiteman et al., 2002, Manning et al., 2007). Even though Akt is best known as the regulator of cell growth, survival and various metabolic processes, it has been reported to have functions also in cell proliferation.

Akt signaling can be activated or inhibited by various external or internal signaling molecules by which liposecretion of adipocytes can be regulated. As a multifunctional pathway, Akt works as a potential regulator of adipocyte proliferation where growth factors, such as FGF-2, IGF-1 and PDGFs might have key roles.

Interaction of FGF-2 and IGF-1 stimulates Akt signaling pathway in hippocampal cell cultures (Johnson-Farley et al., 2007); thus, low expression levels of FGF-2 and IGF1 in BM-MSC could potentially function as negative regulators of Akt signaling to keep the cells in the proliferative status and prevent preadipocyte differentiation. In MAs, IGF-1 and FGF2 expression levels could modulate the activity of Akt signaling and activate liposecretion process by altering cells towards proliferative state.

More in detail, data showed that IGF-1 stimulates preadipocyte differentiation into adipocytes (Scavo et al., 2004; Hu et al., 2015) and, accordingly, IGF-1 is upregulated in MA compared to BM-MSCs (Maurizi et al., 2016). Considering the importance of IGF-1 and its receptor IGF-1R in

metabolic homeostasis and their capacity to activate ERK and Akt pathway, IGF-1 represents one of the modulators of liposecretion process as a response to the metabolic changes.

Moreover, platelet-derived-growth factor receptors (PDGFR) can activate the Akt pathway by binding ligands and phosphorylating the downstream signaling molecules (Rodrigues et al., 2010). It was reported that MSC proliferation was enhanced by Akt phosphorylation through PDGF binding to PDGFRs, which simultaneously increased also VEGF expression. (Ding et al., 2010). In line with these data, array showed that PDGFRα/β were upregulated in BM-MSCs (Maurizi et al., 2016) affecting Akt activity and proliferation capacity through PDFG signaling. On the contrary, in MA the PDGFRs are downregulated implicating the non-proliferative status of these cells. Additionally, PDGFRs are reported to be able to activate, directly or through other pathways, also other signaling pathways, such as MAPK/ERK pathway (Tamama et al., 2006; Ding et al., 2010).

All together these data confirm the importance of Akt signaling in the regulation of adipocyte proliferation not excluding the involvement of the pathway in many other cellular processes, such as glucose metabolism, where adipose tissue plays a central role (Manning et al., 2007).

Molecular data analysis (Maurizi et al., 2016) suggests that liposecretion and proliferation may be regulated through same pathways, such as key roles pathways Wnt, Akt and Erk. Additionally, these pathways regulate many cellular processes and are cross linked with several molecular mechanisms, such as transforming growth factor beta (TGFβ) signaling. In particular, TGFβ is mediated through various SMAD proteins which have specific activities either as differentiation or proliferation modulators (Rodrigues et al., 2010). Data showed that Prrx1 and 2 can activate TGFβ signaling which leads to the inhibition of adipogenesis (Du et al., 2013). In line with this result, TGFβ1, Prrx1 and Prrx2 were found to be upregulated in the BM-MSCs while expressed in low levels in MAs, supporting the proliferative and anti-adipogenic functions of TGFβ signaling (Maurizi et al., 2016).

Moreover, bone morphogenetic proteins (BMPs), that belong to the TGFβ superfamily and have multiple functions, participate in the regulation of bone formation and MSC features. For example, BMP-3 is shown to enhance MSC proliferation by activating TGFβ signaling mediated by SMAD 2 and SMAD 3 (Rodrigues et al., 2010; Stewart et al., 2010). Interestingly, the BMP-3b is expressed in adipose tissues and it is shown to have functions in adipogenesis with lower expression in preadipocytes compared to MA (Hino et al., 2012). Accordingly, gene expression profile data showed that BMP-3 is expressed in higher levels MAs than in BM-MSCs (Maurizi et al., 2016). Despite the high similarity of BMP-3 and BMP-3b, these molecules may have different functions in adipocytes and precommitted cells. However, the function of BMP-3b in adipose tissue remains

unknown and an autocrine function modulating the proliferative capacity of adipocytes could be suggested.

Taken together, data showed that proliferation is regulated through multiple pathways forming a complex signaling network which can efficiently respond to the changes in the microenvironment and external signals. Many of the regulatory pathways, modulated by adipokines and signaling molecules, seem to inhibit adipogenesis, consequently allowing proliferation. Alterations in the metabolic processes can affect the microenvironment of MAs which can respond to the changes by altering their signal molecule secretion. Additionally, various cell types, such as immune cells, can secrete various cytokines and other mediators that can initiate signaling cascades affecting adipocyte physiology. Autocrine as well as external signaling molecules might activate the liposecretion in MAs by altering the MSCs profile and increasing their proliferation capacity.

Conflict of interest

Authors of the manuscript have no conflict of interest.

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Genes	MA	MSC
VCL	D	U
TNC	D	U
THBS1	D	U
SERPINE1	D	U
PLAUR	D	U
ITGA5	D	U
INHBB	U	D
IGF1	U	D
FN1	D	U
FBN1	D	U
ESYT1	U	D
COL1A1	D	U
CNN2	D	U
CDH11	D	U
CD44	D	U
ANPEP	D	U

Table 1. Expression profile of genes involved into cell morphogenesis in MA and MSC. In agreement with the completely committed status of adipocytes, gene expression analysis revealed that among the 16 genes involved in cell morphogenesis only three were upregulated in MA respect to BM-MSC. These data suggested the idea that MA without physiological stimuli were not subjected to shape changes. U=upregulated gene; D= downregulated gene.

Genes	MA	MSC	Genes	MA	MSC
WARS	D	U	LDHA	D	U
VEGFC	D	U	KIF11	D	U
UCHL1	D	U	ITGA5	D	U
TYMS	D	U	INHBA	D	U
TPX2	D	U	IGF1R	D	U
TNFRSF12A	D	U	IGF1	U	D
TNC	D	U	IFITM1	D	U
TIMP1	D	U	IFI30	D	U
THBS1	D	U	ID4	U	D
TGFB1I1	D	U	ICAM1	U	D
TFRC	D	U	HMOX1	U	D
TES	D	U	GJA1	D	U
SPP1	D	U	GATA6	D	U
SLC7A11	D	U	FN1	D	U
SERPINE2	D	U	EMP3	D	U
SERPINE1	D	U	CYP1B1	D	U
PTPRF	U	D	CTGF	D	U
PRRX2	D	U	COL1A1	D	U
PRRX1	D	U	CNN2	D	U
PLAUR	D	U	CNN1	D	U
PLAUR	D	U	CDH2	D	U
PDGFRB	D	U	CD44	D	U
PDGFRA	D	U	CCND2	U	D
PAPPA	D	U	BGN	D	U
NPY1R	U	D	BCAT1	D	U
MYOF	D	U	AXL	D	U
LTBP1	D	U	AEBP1	D	U
LOX	D	U			

Table 2. Expression profile of genes involved into proliferation in MA and MSC. Among 131 gene involved in cell proliferation pathway, 56 were differentially expressed. Notably, 49 of them were expressed majorly in BM-MSC respect to MA and only 7 were up-regulated in MA respect to BM-MSC. Data suggested the lineage commited status of MA and their low ability to proliferate. U=upregulated gene; D= downregulated gene.

Pathways	Genes U in MA and D in MSC
Transport of lipid	ABCA1, ANO3,ATP11B, LBP, LDLR, PLIN2
Transport of phospholipid	ABCA1, ANO3, ATP11B, LDLR
Clearance of lipid	ABCA1, LDLR,PLIN2,SLC2A4,VLDLR
Translocation of phospholipid	ABCA1
Efflux of lipid	ABCA1, ABCA5, ABCC1,LDLR, VLDLR
Secretion of phospholipid	ABCA1, LDLR,PLIN2,SLC2A4,VLDLR
Excretion of sterol	ABCA1, LDLR,PLIN2,SLC2A4,VLDLR
Transport of fatty acid	ABCA1, PLIN2
Transmission of lipid	ABCA1, LBP
Efflux of phospholipid	ABCA1

Table 3. Expression profile of pathways involved into lipid transportation through the membrane in MA and MSC. Gene expression profile data in MA and BM-MSC showed that 81 genes involved in transport through the cell membrane were differentially expressed with 39 genes up-regulated in MA and 42 in BM-MSC. In table 3, 10 pathways that could regulate liposecretion through the key-genes ABCA1, LDLR, PLIN1, and PLIN2 are shown. Data showed that PLIN1, PLIN2, LDLR, and ABCA1 were up-regulated in MA, and that their expression decreased during liposecretion, reaching level similar to BM-MSC. U=upregulated gene; D= downregulated gene.