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Behavior of adipocytes in the mammary niche during pregnancy and lactation

Abstract

Primarily, the adipose tissue is known for its function storing excess energy as fat. This tissue is also an endocrine organ and communicates with several tissues, such as muscle, liver and brain. In addition, the adipose tissue is an accessible source of adult stem cells. Autologous adipose tissue transplant used for plastic surgery purposes take advantage of this feature. These pluripotent cells are also targets for gene therapy and autologous transplant in regenerative medicine. A recent study using state-of-art technology explores the plasticity within mature adipocyte into stroma of the mammary gland. Strikingly, adipocyte-derived preadipocytes de-differentiate and re-differentiate repeatedly, during pregnancy to accommodate the new imposed body metabolic demand. The emerging knowledge from this study is essential to understand the cellular processes occurring into the mammary gland during pregnancy, lactation, and involution. This idea opens new perspectives to study the involvement of the adipose tissue in systemic regulatory processes during these transitional states and has implication on conditions as cancer and inflammation-related diseases.

Keywords: adipocyte, mammary gland, de-differentiation, pregnancy, breast

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Introduction

The adipose tissue stores excess energy as lipid droplets on the adipocytes. During times of negative energy balance, these lipids are metabolized to provide energy to the body.[1] It also secrets adipokines, growth factors and proteins related to immunological and vascular function, such as adiponectin, insulin-like growth factor 1 (IGF-1) and vascular endothelial growth factor alpha (VEGF-α), respectively.[1] This organ is widely distributed through the body, and its cellularity reflects its anatomical location and function. Generally, there are three types of adipocytes: white, beige/brite and brown adipocytes.[2]

The white adipose tissue (WAT) is mainly composed of white adipocytes, and it divides into subcutaneous and visceral. In mice, the subcutaneous WAT concentrates in the inguinal region; and in non-pregnant females, the mammary glands are also associated with this depot.[2] The mammary gland starts to develop during embryonic life. After fully differentiated, it forms a duct system composed of epithelial and mesenchymal cells surrounded by mesenchymal tissue.[3] Fibroblasts, immune cells, endothelial cells and adipocytes are part of the surrounding mesenchyme.[4] During pregnancy, the mammary glands hypertrophies and takes the space once fulfilled by the adipose tissue. During the end stage of the pregnancy, alveolar epithelial cells in the mammary gland accumulate cytoplasmic lipid droplets to be used for milk production. Hence its coloration, these cells were called pink adipocytes.[3,4] After lactation, the mammary gland regress, and the adipocytes return to its original location.

The exact adaptative mechanism happening in the mammary adipose tissue during pregnancy, lactation and involution are actively under investigation. Recent insights into this topic will be discussed in this commentary.

Signaling pathways regulating adipogenic differentiation

The process of adipocyte formation or adipogenesis starts from mesenchymal stem cells (MSCs) from the embryonic mesoderm.[5] MSCs are undifferentiated cells with the ability to give rise to different cell lines, such adipocytes[6] and myoblasts. Adipocytes committed MSCs undergoes a process of cellular differentiation, involving activation and inactivation of different signaling pathways.[7] (Figure 1)

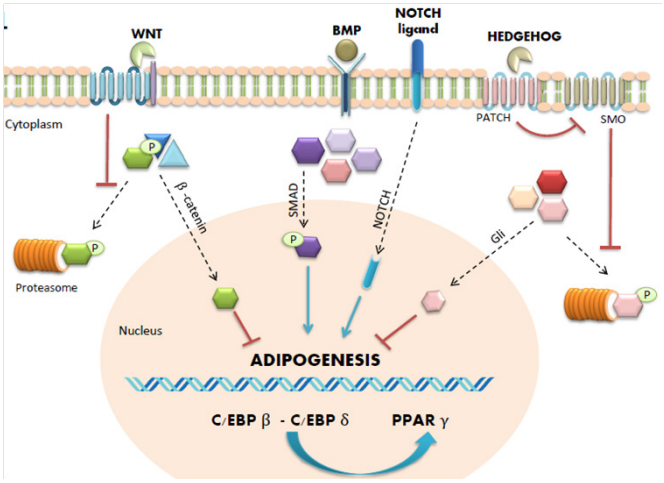

Figure 1 Signaling pathways regulating adipogenic differentiation.

Canonically, the binding of WNT proteins to the frizzled receptor and co-receptor LRP inhibits the phosphorylation of β-catenin in the cytoplasm, allowing it to migrate to the nucleus to activate genes from the osteogenic program, thus inhibiting adipogenesis. The binding of BMPs to their serine threonine kinase receptor generates phosphorylation of SMAD proteins and its migration to the nucleus to induce transcription of adipogenic program genes. Binding of the ligands to the NOTCH receptor leads to proteolytic cleavage of the last, followed by the release of its intracellular domain and its entrance into the nucleus to stimulate transcription of its target genes. Hedgehog proteins bind to PATCH receptors and inhibit their suppressive activity

on SMO, which in turn prevents the phosphorylation and degradation of Gli proteins, allowing its activation and migration into the nucleus where it inhibits adipogenesis.

The Wnt/β-catenin signaling pathway is classically inhibitory to adipogenesis.[8] The binding of Wnt proteins to Frizzled receptors and low density lipoprotein receptor-related protein (LRP) co-receptors inhibits the phosphorylation of cytoplasmic β-catenin and induces nuclear translocation.[9] There, it activates transcriptional factors related to osteogenic differentiation.[10] Although, the role of Wnt proteins in adipogenesis inhibition has been attributed mainly to the canonical pathway related to β-catenin.[11] Moreover, Wnt5b via non-canonical route has been shown to favor the adipogenic differentiation by inhibiting the migration of β-catenin to the nucleus.[12]

In the cytoplasm, β-catenin interacts with molecules such as yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ). YAP is a negatively regulated factor in the Hippo signaling pathway that is related to organ development, controlling cell growth and proliferation.[13] YAP has been shown to inhibit adipogenic differentiation by regulating β-catenin activity. For instance, YAP deletion increases the number of adipocytes in rat bone marrow and upregulates adipogenic genes, *in vitro*.[14] Although β-catenin inhibition seems to trigger adipogenic differentiation,[15] Inoue et al.[16] have shown that during adipocyte differentiation there is an increase of expression of the transcription factor BCL11, a Wnt/β-catenin pathway suppressor.[17]

Our group and others demonstrated the importance of bone morphogenetic proteins (BMPs) for the osteogenic process.[18–20] However, BMPs not only participate in osteogenesis, but they are also involved in adipogenic differentiation.[16,21] BMPs belong to the transforming growth factor-β (TGFβ) family of proteins, and on the cell membrane, they bind and activate TGFβ receptor, a serine-threonine kinase receptor, which phosphorylates Smad proteins in the cytoplasm.[22] Once phosphorylated, Smad proteins migrate to the nucleus to activate downstream targets involved in adipogenic differentiation.[23] Several studies have shown that BMP/Smad signaling pathway promotes adipogenesis through the activation of the peroxisome proliferator-activated receptor γ (PPARγ).[24–26] PPARγ and CCAAT/enhancer-binding proteins (C/EBPs) that induce adipogenic gene expression.[27,28] C/EBPβ and C/EBPδ expression is increased at the onset of adipogenesis to induce the adipocyte phenotype.[29]

Recently, Nueda et al.[13] demonstrated that over-expressing the Notch receptors 1, 2, 3 and 4 stimulates adipogenic differentiation. Moreover, non-canonical Delta-like homolog (DLK) 1 and 2 ligands promote inhibition of these receptors, consequently impairing adipogenesis. The authors also showed the relevance of Notch 1 for the adipogenesis of brown adipocytes, while the other receptors were associated with white adipocytes.[13]

The Hedgehog signaling pathway is adipogenesis inhibitor[30] and involves the ligands Indian, Deserth and Sonic Hedgehog.[31] By binding to patched homolog receptors (PATCHs) on the cell membrane they generate activation of the smoothened protein (SMO).[17] This prevents the phosphorylation and proteasomal degradation of the transcriptional mediators Gli,[12] allowing them to migrate to the nucleus to inhibit adipogenesis.[32] Adipose tissue derived stem cells (ASCs) normally express Gli1 in the absence of adipogenic stimulus. Inhibition of the hedgehog pathway decreases Gli1 expression, and significantly increases the transcription of genes related to metabolism and energy pathways, such as acetyl-CoA acetyltransferase 2 (ACAT2), cytochrome P450, ATP citrate lyase (ACLY), hydroxysteroid (17-beta) dehydrogenase 12 (HSD17B12), among others.[15]

MicroRNAs have been demonstrated to participate in the generation of adipocytes.[33–35] The pro-adipogenic miR-148a promotes Wnt1 suppression, reduction in the nuclear β-catenin, increases the expression of PPARγ2 and C/EBP-α.[36] Upregulation in C/EBP-β expression was also observed in preadipocytes transfected with miR30e. This microRNA favors adipogenesis by targeting the LRP6 co-receptor, involved with Wnt/β-catenin signaling.[5] C/EBP-α activation promotes preadipocytes differentiation into mature adipocytes via expression of miR-140-5p, which targets TGFβ receptor I.[37] Recently, another class of non-coding RNAs, the long non-coding RNAs, have also been demonstrated to participate in adipogenesis regulation.[38]

Adipocyte de-differentiation and trans-differentiation

De-differentiation, trans-differentiation, and reprogramming are important for tissue remodeling, such as the process occurring in the mammary tissue during gestation and lactation. The adipose tissue is long known for its plasticity. In 1992, brown adipocytes were identified within the white adipose niche.[39] Many years later, the white adipocytes were demonstrated to differentiate into brown/beige adipocytes when exposed to temperature variation (cold/warm) or under pharmacological stimuli.[14,26,40,41] After the stimuli removal, trans-differentiated adipocytes return to its original white form.[42] Additionally, adipocytes in the subcutaneous WAT are more prone to trans-differentiation than other depots.[43,44] The adipose tissue provides structural support during the mammary gland development, but it also signals to the developing tissue promoting the growth of the glandular ducts ramifications, and later, it participates of the epithelial gland differentiation during pregnancy and lactation.[45] As other adipose depots, this niche is subjected to endocrine and paracrine factors. For instance, adiponectin could be a good candidate mediating this interaction. Adiponectin has anti-inflammatory, anti-apoptotic, anti-fibrotic and pro-angiogenic functions; and its expression is reduced in obesity and cancer.[46] The differentiation process that mammary adipocytes undergo during pregnancy-lactation-involution promotes a great change in the stroma tissue, altering the extracellular matrix and immune cell population, which could potentially impact cancer development.[6,47,48] However, more studies are still needed to understand the molecular mechanism of the mammary tissue remodeling during the pregnancy-lactation-involution cycle. Additionally, a recent study,[21] demonstrated that invasive breast cancer cells could convert into functional adipocytes, with MEK inhibitors and anti-diabetic drug Rosiglitazone, thus inhibits cancers metastasis. However, more studies are necessary to understand how pathological states can affect breast tissue, such as cancer cells, in de-differentiation and trans-differentiation during pregnancy and lactation.

Matsumoto et al.[49] demonstrated *in vitro* the ability of mature adipocytes to de-differentiate in fibroblast-like cells with multipotent potential or de-differentiation fat (DFAT) cells. In 1986, Sugihara et al.[50] were the pioneers of DFAT generation using ceiling cell culture technique. Nowadays, DFATs can be generated from subcutaneous WAT from several species.[3,49,51] In spite of being a known technique, the changes in the cellular epigenetic landscape and the exact factors implicated in the de-differentiation process are not fully known.

During de-differentiation, the cells regress to a less specialized state, becoming a multipotent undifferentiated cell. Distinct from that, during trans-differentiation, differentiated somatic cells transition

straight to be another type of mature somatic cell, without passing through a de-differentiation process. Additionally, the process of cellular reprogramming artificially induces a mature somatic cell into a pluripotent state.[52] In 2007, the transfection of the human adult skin fibroblasts with the transcription factors: Oct-3/4, Sox-2, Klf-4 e c-Myc, also known as Yamaka factors, generated the first inducible pluripotent stem cells (iPSCs).[53] These factors reprogrammed the fibroblasts, altering its morphology, proliferative capacity, gene expression, surface antigens and telomerase activity.[52]

During gestation, the epithelial cells of the breast proliferate forming branches of the ducts lobuloalveolar and developing for milk production.[54] Adipose tissue interferes at various stages of mammary gland development as in the initial growth and branching of the ducts as well as in its maintenance and in epithelial differentiation prior to pregnancy for lactation.[45] Meanwhile, the adipose tissue of the breast decreases in volume, allowing the growth of the glandular epithelium.[55] After lactation, this epithelium suffers apoptosis, returning to the previous state, and the adipocytes again occupy a large area of the breast again, a process called involution.[55,56] Despite knowing these processes, the molecular mechanisms by which adipocytes adapt during these cycles are still under investigation.

Zwick et al.[45] demonstrated that trans-differentiation of epithelial cells to adipocyte was not the mechanism responsible for the increase in the adipocyte mass in breast tissue during the involution. Thus, the authors reported that during this phase, adipocyte hypertrophy is the main adaptive mechanism in the mammary adipose tissue. Different from Zwick et al.,[45] Wang et al.[57] demonstrated the mature adipocytes plasticity during pregnancy/lactation. The authors investigated the mechanism taking place in the mammary glands during pregnancy-lactation-involution phases, using state of art techniques, such as next-generation single-cell sequencing, Cre/loxP recombination system to manipulate gene expression and refined *in vivo* approaches. They described a new mechanism of de-differentiation of the resident stroma cell surrounding the alveolar structures of the mammary gland.[57]

In summary, it was recently identified how adipocytes in the breast tissue undergo a process of de-differentiation followed by re-differentiation as part of the adaptations suffered by the breast during the lactation and involution phases. As we mentioned, these findings open doors for many questions about the breast tissue physiology and how this translates to pathological states.

Perspectives in de-differentiation and re-differentiation of adipocytes in mammary niche

Experiments using the AdipoChaser-LacZ mouse model demonstrated that during gestation and lactation, adipocytes located in the mammary gland de-differentiate into preadipocytes, similar to fibroblasts positive for platelet-derived growth factor receptor alpha (Pdgfrα+).[57] Interestingly, Wang et al.,[57] revealed the ability of trans-differentiation and proliferation of these Pdgfrα+ cells in adipocytes that repopulate the breast after weaning (Figure 2).

Wang et al.[58] suggested a new mechanism of de- and re-differentiation of resident adipose cells between the alveolar structures of the mammary gland in mice. (Figure 2A) When fully developed, the mammary gland is surrounded by several mature white adipocytes (virgin and pregnancy). As the pregnancy progresses, the mammary gland grow occupying the space of the subcutaneous adipose tissue. During lactation, the adipocytes are de-differentiated with fibroblast-like morphology (lactation). After the lactation period, the breast involute, the pre-adipocytes differentiate into adipocytes and the adipose tissue returns to its space (involution) (Figure 2B).

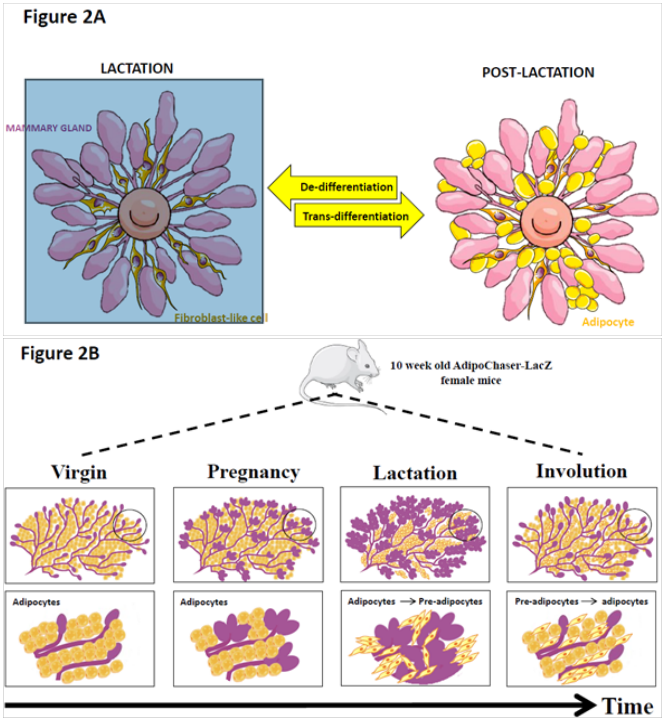

Figure 2 Behavior of adipocytes during the development of mammary gland in pregnancy and lactation.

In AdipoChaser-LacZ mouse model,[59] mature adipocytes after doxycycline treatment express the active LacZ enzyme and became permanently marked. Hence, past this treatment, all new cells that arise from adipogenesis will be LacZ negative. This elegant model was used by Wang et al.[58] to elucidate the mechanisms of de-differentiation and re-differentiation of adipocytes during breast tissue remodeling during pregnancy and lactation.[57] It is noted that the resident adipocytes at the breast area are not responsible for the mammary structures developed during pregnancy, given that these cells are LacZ negative. Granting the adipocytes have a distinct spherical morphology, filled with lipids and a positive label for LacZ. It was observed that, at the peak of lactation, to give space to the mammary alveoli, adipocytes change their characteristic morphology and are morphologically similar to fibroblasts, free of lipid droplets, but still LacZ positive. After lactation, fibroblast-like adipocytes give rise to lacZ positive adipocytes, expand and resume the space that was previously occupied by the mammary gland.[57]

It has already been proven that the three-dimensional environment directly influences tissue structure and morphogenesis during development, since, in addition to structural support, ECM provides contextual information for the cells to their environment.[60] Studies have shown that the expression of modified proteins as a non-functional and truncated form of E-cadherin and metalloproteinases were able to cause increased branching and early development of alveoli in virgin rats.[61] Information on the composition of the ECM of the mammary gland during the pregnancy–lactation-involution cycle would be of great value for the continuation of the studies of Wang et al.[57] Therefore, *in vitro* studies using the mammary ECM components present at different stages of breast development should also identify

the molecular mechanism coordinating the de-differentiation of the adipocytes in this process.

With the evolution of single-cell analysis, it became clear that tissue heterogeneity is an important component to be considered. It allows us to distinguish cellular populations within tissues that correlate to different functions and prognosis, as explored in cancer. Hormonal changes occurring during pregnancy are primordial to control the development of the mammary gland.[62] However, these changes have effects beyond the mammary glands. Additional in-depth studies are extremely important to clarify the role of hormonal factors in adipocyte de-differentiation. Consequently, considering the specific endocrine and paracrine functions of the mammary niche hormones during pregnancy and lactation and how they induce chromatin remodeling to generate cellular responses.[20,63] It would be very interesting, for example, to look at the estrogen interaction with its DNA responsive elements; to study the nuclear organization and the three-dimensional chromatin structure using techniques based on chromatin digestion and reconnection of fixed chromatin, such as chromatin conformation capture (known by the acronym 3C).[64]

These findings lead us to imagine the level of similarity between these de-differentiated adipocytes and adipose tissue-derived mesenchymal stem cells.[65] In their studies, they isolated many cells with preadipocyte characteristics from the mammary tissue of lactating females that differentiated in mature adipocytes upon adipogenic stimuli. They reinforce the idea of a possible differentiation of de-differentiated adipocytes into other cell types upon appropriate conditions. Thus, what are the changes in the epigenetic of these cells? Do they develop an epigenetic memory? Which signaling pathways are involved in the trans-differentiation and de-differentiation? Omics analysis is useful to overlap the many regulatory layers involved in cellular processes, although still being cost-limiting factors. Ultimately, the combination of techniques such as genomic, transcriptomic and metabolomic offer a global untargeted approach to the matter.[66–80]

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Conflicts of interest

Authors declare that there is no conflicts of interest.

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