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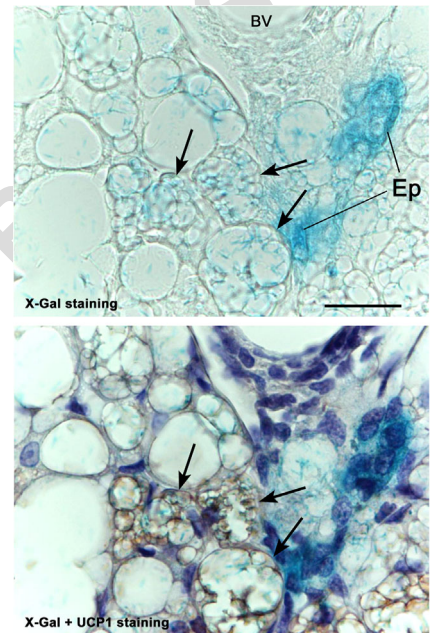
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RAPID COMMUNICATION

**Antonio Giordano | Jessica Perugini | David M. Kristensen | Loris Sartini |
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In this study, we demonstrate by transmission electron microscopy that in the post-lactating mammary gland interscapular multilocular adipocytes found close to the mammary alveoli contain milk protein granules. Use of the Cre-loxP recombination system allowed showing that the involuting mammary gland of whey acidic protein-Cre/R26R mice, whose secretory alveolar cells express the lacZ gene during pregnancy, contains some X-Gal-stained and uncoupling protein 1-positive interscapular multilocular adipocytes. These data suggest that during mammary gland involution some milk-secreting epithelial cells in the anterior subcutaneous depot may transdifferentiate to brown adipocytes.



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RAPID COMMUNICATION

Mammary alveolar epithelial cells convert to brown adipocytes in post-lactating mice

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During pregnancy and lactation, subcutaneous white adipocytes in the mouse mammary gland transdifferentiate reversibly to milk-secreting epithelial cells. In this study, we demonstrate by transmission electron microscopy that in the post-lactating mammary gland interscapular multilocular adipocytes found close to the mammary alveoli contain milk protein granules. Use of the Cre-loxP recombination system allowed showing that the involuting mammary gland of whey acidic protein-Cre/R26R mice, whose secretory alveolar cells express the *lacZ* gene during pregnancy, contains some X-Gal-stained and uncoupling protein 1-positive interscapular multilocular adipocytes. These data suggest that during mammary gland involution some milk-secreting epithelial cells in the anterior subcutaneous depot may transdifferentiate to brown adipocytes, highlighting a hitherto unappreciated feature of mouse adipose organ plasticity.

KEYWORDS

adipose organ, Cre-loxP recombination system, electron microscopy, mammary gland, pregnancy

INTRODUCTION

In all mammals, adipocytes are located in fat depots that are distributed both in subcutaneous and visceral locations and are characterized by distinctive gross and histological morphological features (Cinti, 1999, 2005). In mice subcutaneous, fat is organized in two depots, anterior and posterior, close to the limbs. The anterior subcutaneous depot has a complex shape. Its larger portion occupies the interscapular region and is the origin of several smaller depots that reach the anterior part of the body (where they give rise to subclavicular and axillary fat) and the deep dorsal region (where they give rise to subscapular and deep cervical fat). The posterior depot, at the base of the hind legs, has a simpler anatomy,

where a single strip of fat that begins in the back at the lumbar level (dorso-lumbar portion) extends to the inguino-crural region (inguinal portion) and to the pubic region (gluteal portion), where it joins the contralateral, symmetric depot.

In adult, non-pregnant female mice, the subcutaneous depots contain the mammary gland “anlagen” (Hennighausen & Robinson, 2005; Imagawa, Bandyopadhyay, & Nandi, 1990; Richert, Schwertfeger, Ryder, & Anderson, 2000; Silberstein, 2001). In the parenchyma of these depots, branched mammary epithelial ducts are connected to three bilateral nipples in the anterior subcutaneous depot and to two bilateral nipples in the posterior subcutaneous depot. During pregnancy, adipose tissue is progressively substituted by epithelial structures that soon will

1 give rise to mammary gland alveoli. In rodents, mammary gland
2 development is so massive that during lactation the subcutaneous
3 depots are almost completely occupied by large, milk-producing alveoli,
4 with a very small number of adipocytes scattered among them. Soon
5 after the end of lactation, the alveoli disappear and the subcutaneous
6 depots are again filled with adipocytes.

7 Previous ultrastructural, lineage-tracing, and explant experi-
8 ments have suggested that the plastic changes occurring in the
9 subcutaneous depots of pregnant mice are to some extent due to
10 the reversible transdifferentiation of white adipocytes to epithelial
11 milk-secreting cells (De et al., 2009; Morroni et al., 2004; Prokesch
12 et al., 2014). Importantly, epithelial milk-secreting cells are rich in
13 cytoplasmic lipid vacuoles, meeting the definition of adipocytes, that
14 is, parenchymal adipose organ cells rich in cytoplasmic lipids,
15 regardless of their function. We have proposed calling the epithelial
16 milk-secreting cells derived from adipocyte transdifferentiation
17 "pink adipocytes" (Giordano, Smorlesi, Frontini, Barbatelli, & Cinti,
18 2014).

19 Subcutaneous fat is a mixed tissue composed both of white and
20 thermogenic brown adipocytes (Cinti, 1999, 2005; Vitali et al., 2012).
21 The former are more abundant in the posterior depot and the latter in
22 the anterior depot. Notably, external cues, such as cold exposure or
23 beta-adrenergic stimuli, promote the transdifferentiation of some
24 white adipocytes to brown adipocytes, which re-convert to white
25 adipocytes soon after the stimuli are withdrawn (Altshuler-Keylin
26 et al., 2016; Barbatelli et al., 2010; Rosenwald, Perdikari, Rulicke, &
27 Wolfrum, 2013). Histological examination demonstrates that in the
28 anterior depot of lactating mice the mammary alveoli lie close to
29 interscapular brown fat. To assess whether, during mammary gland
30 involution, alveolar epithelial cells transdifferentiate not only to white
31 adipocytes, but also to brown adipocytes, we conducted an
32 ultrastructural and lineage-tracing study.

33 MATERIALS AND METHODS

34 Animals and tissues

35 Adult wild type C57BL/6 mice were purchased from Charles River
36 Laboratories (Calco, Italy). ROSA26R (R26R) mice were purchased
37 from and genotyped as described by The Jackson Laboratory (Bar
38 Harbor, ME). Whey acidic protein (WAP)-Cre mice (Wagner et al.,
39 1997) were kindly provided by C. Dickson (London Research Institute,
40 London, UK). All animals were housed in plastic cages in constant
41 environmental conditions (12 hr light/dark cycle at 22°C) with ad
42 libitum access to food and water. Handling was limited to cage
43 cleaning. All efforts were made to minimize animal suffering and to
44 reduce the number of animals used. Experiments were approved by
45 the Animal Ethics Committee of University of Ancona (Politecnica
46 delle Marche) and were carried out in accordance with EC Council
47 Directive 86/609/EEC of 24 November 1986.

48 Female double transgenic mice were obtained from crosses
49 between R26R hemizygous mice and WAP-Cre hemizygous mice.
50 They were genotyped for the presence of Cre by PCR analysis using

51 primers Cre1 (5'-ATGTCCAATTTACTGACC-3') and Cre2 (5'-CGCC
52 GCATAACCAGTGAAAC-3'), which yielded a 356 bp product. WAP-
53 Cre/R26R mice were killed 10 days into lactation ($n = 3$) and on days 1,
54 3, 5, or 10 of mammary gland involution ($n = 3$ for each time point).
55 Cre-Mediated Recombination Analysis was performed as described
56 previously (Morroni et al., 2004).

57 For morphological analyses, mice were anesthetized with 100 mg/kg
58 ketamine (Ketavet, Farmaceutici Gellini, Aprilia, Italy) in combination with
59 10 mg/kg xylazine (Rompum, Bayer AG, Leverkusen, Germany) and
60 perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate
61 buffer (PB), pH 7.4. Specimens for light microscopy and immunohis-
62 tochemistry were paraffin-embedded.

63 Transmission electron microscopy

64 Small subcutaneous fat and mammary gland specimens from perfused
65 animals were further fixed in 2% glutaraldehyde/2% formaldehyde in
66 0.1 M PB for 4 hr at room temperature, postfixed in 1% osmium
67 tetroxide, dehydrated in acetone, and embedded in epoxy resin.
68 Semithin sections were stained with toluidine blue. Thin sections were
69 obtained with an MTX ultramicrotome (RMC, Tucson, AZ), stained
70 with lead citrate and examined with a transmission electron
71 microscope (CM10, Philips, Eindhoven, the Netherlands).

72 β -galactosidase histochemistry

73 For β -galactosidase (Gal) detection, double transgenic animals were
74 perfused intracardially with 2% paraformaldehyde/0.25% glutaralde-
75 hyde in phosphate buffered saline (PBS), pH 7.3. Mammary glands
76 were dissected out and fixed in the same fixative (for up to 90 min).
77 After an overnight wash in PBS, tissues were preincubated in 2 mM
78 MgCl₂/0.01% sodium deoxycholate/0.02% Nonidet P-40 in PBS, pH
79 8.5, for 2 hr at room temperature, then incubated in X-Gal solution:
80 5 mM potassium ferrocyanide containing 1 mg/ml X-Gal (Sigma-
81 Aldrich, St Louis, MO) in the form of 40 mg/ml solution in
82 dimethylformamide, at 30°C for 24 hr (WAP-Cre/R26R) or 5 hr
83 (aP2-Cre/R26R). PBS at pH 8.5 was used to avoid detection of
84 endogenous β -Gal, which is active at pH values <5. After X-Gal
85 incubation, tissues were postfixed in 4% paraformaldehyde in 0.1 M
86 PB, dehydrated, and embedded in paraffin.

87 Peroxidase immunohistochemistry

88 Immunohistochemistry was performed on 3 μ m-thick paraffin-
89 embedded sections of interscapular brown fat and mammary gland.
90 After dewaxing, sections were thoroughly rinsed in PBS, reacted with
91 0.3% H₂O₂ (in PBS; 30 min) to block endogenous peroxidase, rinsed
92 again with PBS and incubated in a 3% blocking solution (in PBS;
93 60 min). Then, they were incubated with the primary antibody against
94 the uncoupling protein 1 (UCP1; in PBS; overnight at 4°C). UCP1 was
95 localized using a rabbit polyclonal antibody against a synthetic peptide
96 conjugated to KLH, corresponding to the amino acids 145-159 of
97 human UCP1, with N-terminal cysteine added (Abcam, Cambridge,
98 UK, Cat# ab10983 RRID:AB_2241462). After a thorough rinse in PBS,
99 sections were incubated in a 1:200 v/v biotinylated secondary

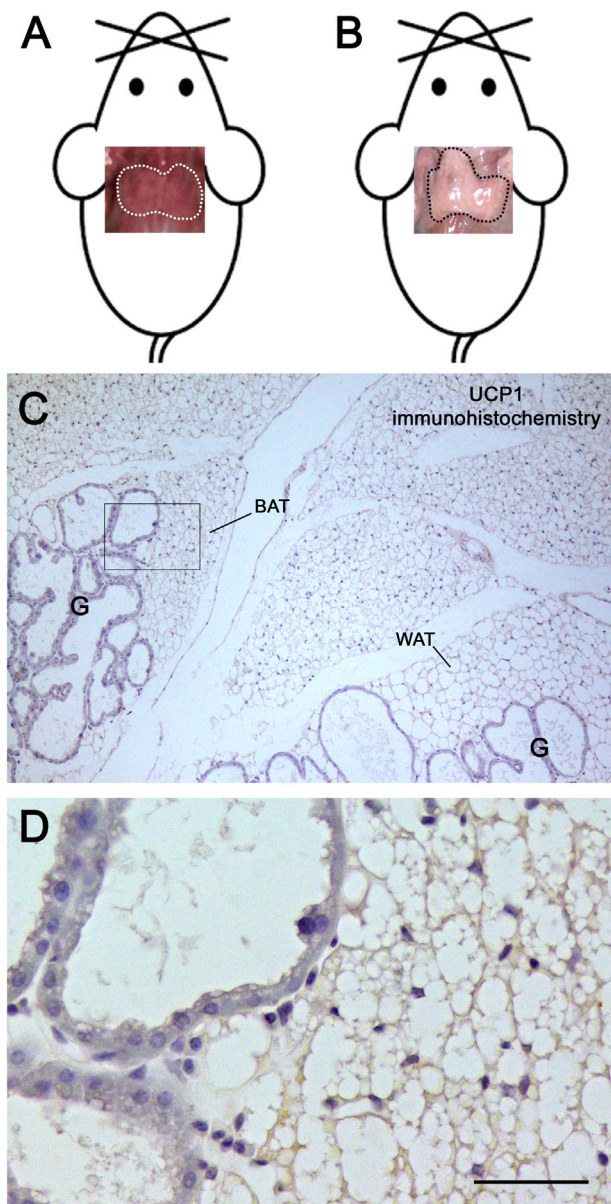


FIGURE 1 The mouse anterior subcutaneous depot during lactation. Upper panels, pictures of the gross anatomy of the interscapular depot fitted on mouse templates. In non-pregnant female mice (A), the interscapular fat (dotted area) is easily recognized by its brownish color; after 10 days of lactation (B) it turns white and the depot acquires a white fat-like macroscopic appearance. In sections processed for immunohistochemistry (C), interscapular multilocular adipocytes containing large lipid droplets exhibit very weak UCP1 expression. Mammary gland alveoli (G) are found close to both brown (BAT) and white (WAT) adipose tissue lobules. At higher magnification (D), no clear boundaries are visible between the secretory epithelium and the adipose parenchyma; note the weak and homogenous UCP1 staining in multilocular adipocytes. D is the enlargement of the area framed in C. Bar: 3 cm in (A), 250 μm in (B), 40 μm in (C)

antibody solution (in PBS; 30 min). The biotinylated HRP-conjugated secondary antibody (Vector Laboratories, Burlingame, CA) was goat anti-rabbit IgG. Histochemical reactions were performed using Vectastain ABC Kit (Vector Laboratories) and Sigma Fast

3,3'-diaminobenzidine (Sigma-Aldrich) as the substrate. Sections were finally counterstained with hematoxylin, dehydrated and mounted in Entellan. Staining was never observed when the primary antibody was omitted.

RESULTS

Light microscopy

By gross anatomical examination, interscapular brown adipose tissue (BAT) from non-pregnant mice was clearly visible in the anterior adipose depot due to its brownish color (Fig. 1A). In lactating mice, it was barely recognizable due to its white fat-like macroscopic appearance (Fig. 1B). Light microscopy disclosed that it contained multilocular adipocytes with large lipid droplets (a sign of weak thermogenic activity) exhibiting scarce or no expression of UCP1, the thermogenic protein (Ricquier, 2005) (Fig. 1C and D). These findings agree with the reduced BAT activity that is seen during pregnancy and lactation (Abelenda & Puerta, 1987; Imai-Matsumura, Matsumura, Morimoto, & Nakayama, 1990; Trayhurn & Wusteman, 1987). The BAT lobules were surrounded and infiltrated by well-differentiated mammary alveoli without clear boundaries between the adipose and the epithelial component (Fig. 1D). A progressive reduction in the number of the alveoli and a progressive expansion of BAT areas were detected in the first few days of mammary gland involution (data not shown). Quantitative data from posterior subcutaneous fat of B6 and Sv129 mice indicate that in warm-acclimated animals 5–10% of the parenchyma consists of BAT-like tissue (Vitali et al., 2012). Interestingly, serial sections of the posterior glands showed that there was no BAT-like tissue in the glands during lactation (data not shown).

Transmission electron microscopy

To assess the fate of alveolar epithelial cells during mammary gland involution and their relationships with the interscapular BAT parenchyma, the peripheral interscapular BAT, which contains both brown adipocytes and alveoli (Fig. 2A and B), was examined by transmission electron microscopy. Several multilocular adipocytes containing numerous typical "brown" mitochondria as well as milk protein granules were detected on days 1, 3, 5, and 10 of gland involution (Fig. 2C and D). These ultrastructural features could represent an intermediate phenotype of epithelial to brown cell transdifferentiation.

Lineage-tracing analysis

To obtain further evidence of the transdifferentiation of epithelial glandular cells into brown adipocytes, the Cre-loxP system was used to drive β -gal expression in the secretory epithelium under the control of cell-type specific promoters (Birling, Gofflot, & Warot, 2009; Soriano, 1999). β -gal expression was induced in WAP-Cre/R26R double transgenic mice by the promoter of WAP, a protein specific of milk-producing cells (15). The alveoli of the lactating gland stained for X-Gal, the β -gal substrate, whereas the brown adipocytes were negative

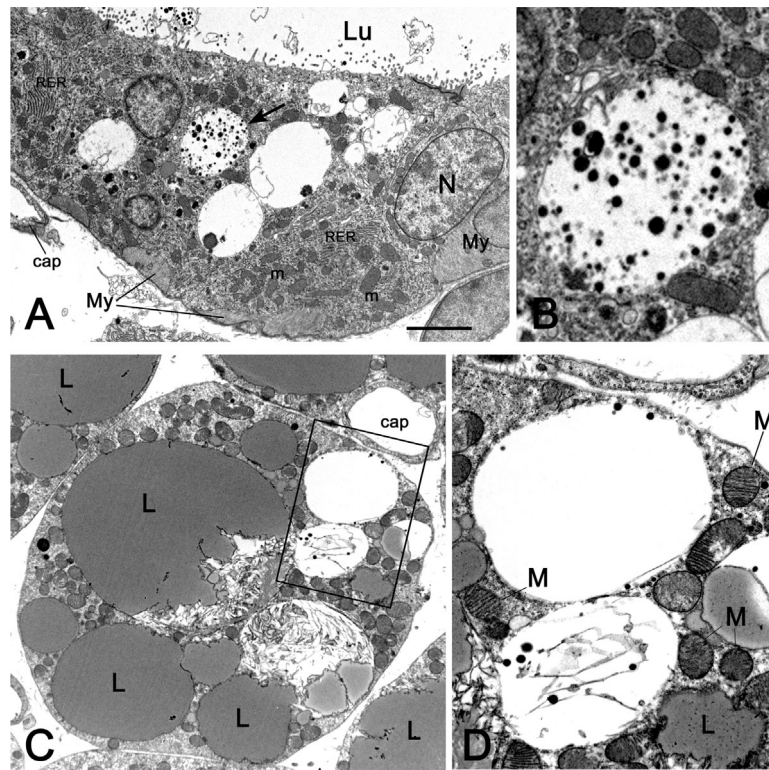


FIGURE 2 Transmission electron microscopy images from specimens of peripheral anterior mammary gland in contact with interscapular BAT on day 1 of mammary gland involution. In (A), the epithelial cells bordering the alveolar lumen (Lu) contain numerous mitochondria (m), stacked rough endoplasmic reticulum (RER), and large vacuoles containing milk protein secretory granules; the vacuole indicated by the arrow is enlarged in (B). (C) a multilocular adipocyte containing several “brown-like” mitochondria (M) packed with lamellar cristae and two milk protein secretory vacuoles (enlarged in D). Cap: capillary, My: myoepithelial cells, N: nucleus, L: lipid droplet. Bar: 1 μm in (A) and (C), 0.3 μm in (B) and (D)

(data not shown). However, in the post-lactation period, several X-Gal-positive multilocular cells were detected between BAT and the involuting glands in the mixed areas (Fig. 3A). To confirm that these multilocular adipocytes were indeed brown adipocytes, their UCP1 expression was evaluated by immunohistochemistry in the sections previously reacted for X-Gal. The results showed that the X-Gal-positive multilocular cells were also positive for UCP1 (Fig. 3B), thus confirming their brown phenotype. Brown adipocytes of the central part of interscapular fat and located at a distance from the alveoli, were UCP1-positive, but always X-Gal-negative (data not shown).

DISCUSSION

Our data suggest that, after lactation, some milk-producing alveolar epithelial cells in the anterior subcutaneous depot mammary gland convert to UCP1-positive multilocular adipocytes. Importantly, in WAP-Cre/R26R double transgenic mice only the interscapular brown adipocytes found in close proximity to the involuting glands contained milk granules and stained for X-Gal, suggesting that a subpopulation of brown adipocytes is involved in the process. These data also raise the intriguing question whether during mammary gland development some interscapular brown adipocytes transdifferentiate to alveolar

epithelial cells, as already documented in white adipocytes (De et al., 2009; Morroni et al., 2004; Prokesch et al., 2014). If this were the case, the present findings would demonstrate a further, distinctive type of cell plasticity of the mouse adipose organ, reversible brown to “pink” transdifferentiation (Giordano et al., 2014). The evidence that clusters of brown adipocytes are also found in the posterior subcutaneous depot in non-pregnant animals, and that no residual BAT-like tissue is detected at these sites during lactation, suggests that reversible brown to pink transdifferentiation may also take place in this depot. Brown to pink transdifferentiation could provide a novel perspective on the BAT inactivation and reduced non-shivering thermogenesis that are seen in rodents during pregnancy and lactation (Abelenda & Puerta, 1987; Imai-Matsumara et al., 1990; Trayhurn & Wusteman, 1987).

Human isolated mature adipocytes has been recently shown to undergo a spontaneous process of transdifferentiation to fibroblast-like cells that can subsequently be committed to different phenotypes (Maurizi et al., 2016). The distinctive in vitro and in vivo plastic abilities of mammalian adipocytes can be exploited for therapeutic purposes. White to brown adipocyte conversion, especially in visceral depots, holds considerable potential for the treatment of obesity and metabolic syndrome (Giordano, Frontini, & Cinti, 2016). In parallel, white and/or brown adipocyte to epithelial cell transdifferentiation in the mammary gland may be of interest in mammary gland pathology,

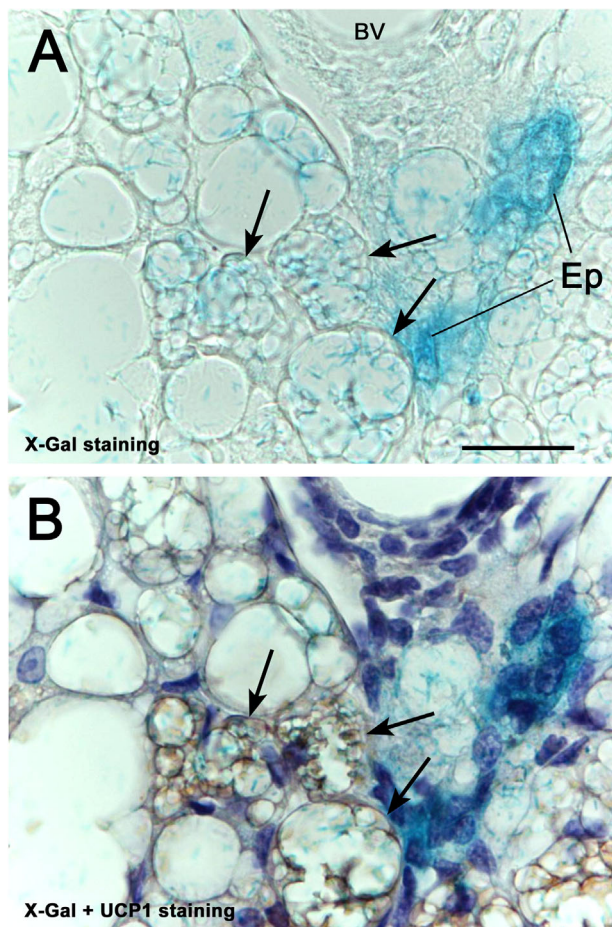


FIGURE 3 X-Gal staining of the peripheral anterior mammary gland in contact with interscapular BAT of a WAP-Cre/R26R mouse 10 days post-lactation. In (A), not only mammary epithelial structures (Ep), but also some multilocular adipocytes (arrows) show the blue X-Gal precipitate. UCP1 immunohistochemistry and hematoxylin counterstaining, performed in the same section (B), show that the X-Gal-positive multilocular adipocytes also exhibit UCP1 immunoreactivity (arrows). BV: blood vessel. Bar: 25 μ m

especially neoplastic diseases. Interestingly, loss of PPAR γ —a transcription factor involved both in adipogenesis (Hallenborg et al., 2015) and alveologenesis (Jain et al., 1998)—in mammary secretory epithelial cells creates a pro-breast tumorigenic environment during mammary gland involution (Apostoli et al., 2014); the process may to some extent be related to impaired epithelial cell to white adipocyte transdifferentiation. MiR-27a, an inhibitor of brown adipogenesis (Sun & Trajkovski, 2014), has been reported to promote breast cancer growth and metastasis in humans (Tang et al., 2012) and could be a factor involved in the still unknown relationship between brown fat and breast cancer progression that has been observed in some experimental models (Jones, Buelto, Tago, & Owusu-Boaitey, 2011).

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CONFLICTS OF INTEREST

The authors do not have conflict of interest to declare.

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