Original Article

MOLECULAR METABOLISM

Brown adipocytes can display a mammary basal myoepithelial cell phenotype *in vivo*

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ABSTRACT

Objective: Previous work has suggested that white adipocytes may also show a mammary luminal secretory cell phenotype during lactation. The capacity of brown and beige/brite adipocytes to display a mammary cell phenotype and the levels at which they demonstrate such phenotypes *in vivo* is currently unknown.

Methods: To investigate the putative adipocyte origin of mammary gland cells, we performed genetic lineage-labeling experiments in BAT and the mammary glands.

Results: These studies indicated that the classic brown adipocytes (Ucp1^{+}) and subcutaneous beige/brite adipocytes (Ucp1$^{-/+}$) were found in the mammary gland during lactation, when they exhibited a mammary myoepithelial phenotype. Up to 2.5% of the anterior dorsal interscapular mammary myoepithelial cell population had a brown adipocyte origin with an adipose and myoepithelial gene signature during lactation. Eliminating these cells, along with all the brown adipocytes, significantly slowed offspring growth, potentially demonstrating their functional importance. Additionally, we showed mammary epithelial lineage Mmtv^{+} and Krt14^{+} cells expressed brown adipocyte markers after weaning, demonstrating that mammary gland cells can display an adipose phenotype.

Conclusions: The identification of a brown adipocyte origin of mammary myoepithelial cells provides a novel perspective on the interrelationships between adipocytes and mammary cells with implications for our understanding of obesity and breast cancer.

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Keywords Brown adipocytes; Mammary gland; Lactation; Basal myoepithelial cells; Beige/brite adipocytes

1. INTRODUCTION

Early research suggested that there are two types of adipocytes: white and brown [1]. White adipocytes store excess energy intake as a large unilocular lipid droplet. In contrast, brown adipocytes have much smaller multilocular lipid droplets, and abundant mitochondria. Brown adipocytes generate heat via activation of uncoupling protein 1 (UCP1) that resides on the inner mitochondrial membrane, and acts by decoupling proton transfer across the membrane from synthesis of ATP [1]. More recently, it has been demonstrated that a unique lineage of cells, called 'brown in white (brite)' [2] or 'beige' [3] adipocytes, have the capacity to show both brown and white phenotypes under different conditions. These beige/brite cells originate from special adipocyte precursor cells, which are distinct from white adipocyte precursor cells [3]. Furthermore, classic brown adipocytes to arise from a Myf5^{+}/Pax7^{+} skeletal muscle stem cell origin [4–7], but a recent study suggested that Myf5^{+} is location marker rather than a marker of a specific cell lineage [8–10]. Hence, the current classification of 'fat' cells includes three main types: white adipocytes with an exclusively white phenotype, brown (Ucp1^{+}) adipocytes from the muscle lineage that can only exhibit the brown phenotype, and 'beige/brite' (Ucp1$^{-/+}$) adipocytes that can show both phenotypes [11].

The mammary gland is a milk producing exocrine gland. The mammary duct is composed of several cell lineages including an inner layer of secretory- or ductal-like luminal epithelial cells (Krt8^{+}/Krt18^{+}) that secrete milk and an outer layer of smooth muscle-like basal myoepithelial cells (Krt14^{+}/Krt5^{+}), which contract the inner mammary ducts in response to the hormone oxytocin, facilitating milk letdown [12–14]. In virgin female mice, the mammary gland already has rudimentary ductal structures in the anterior and posterior subcutaneous white adipose tissues. During pregnancy, the proliferation of mammary myoepithelial (Lin^{-}:CD24^{+}:CD29^{hi}) and luminal (Lin^{-}:CD24^{+}:CD29^{low}) stem cells located in the mammary ductal terminal end buds (TEB) facilitates the branching and development of the mammary duct, These ducts gradually penetrate and substitute almost all of the subcutaneous fat pads [15–17].

An often neglected aspect of adipocyte biology is the suggestion that, in female mice, white adipocytes might be capable of

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transdifferentiation into mammary luminal secretory epithelium cells [18,19], sometimes called 'pink' adipocytes [20]. As these 'pink' adipocyte studies were performed prior to the discovery of the beige/brite lineage, it is unclear whether these transformed mammary gland cells were truly white or if they were beige/brite adipocytes presenting a white and then pink phenotype. Moreover, whether classic brown adipocytes might also be able to transform into mammary gland cells is unknown. This may be important because it was recently demonstrated that there is increased expression of brown and beige/brite adipocyte markers (MYF5 and UCP1) among breast cancer cells, that influences the formation of xenografts in mice [21]. Critically, depletion of Ucp1^{+} or Myf5^{+} cells significantly reduced tumor development. Thus, conversion of brown and beige/brite adipocytes into cells displaying a mammary gland phenotype may be an important contributor to the risk of developing breast cancer. It is currently unclear, however, whether brown and/or beige/brite adipocytes are able to transform into mammary gland cells. To answer this question, we performed multiple lineage labeling experiments in four different transgenic mouse models. We demonstrate that classic brown adipocytes, and probably beige/brite adipocytes, are capable of showing a mammary basal myoepithelium phenotype *in vivo*, but not a luminal secretory phenotype. Additionally, we show that if cells that express UCP1 are killed during lactation then the growth of their offspring is reduced, suggesting the conversion of brown/beige adipocytes to mammary cells is functionally significant, even though numerically small.

2. MATERIALS AND METHODS

2.1. Animals

All animal experiments were approved by the Institute of Genetics and Developmental Biology Chinese Academy of Sciences (IGDB-CAS) Institutional Animal Care and Use Committee (IACUC). All animals were housed in rooms kept at 23 $\pm$ 1 °C with a dark—light cycle of 12 h—12 h (lights on at 0730 h) and fed *ad libitum* with a standard low fat chow diet. *Myf5-Cre* mice were kindly donated by Dr Kuang and Dr Zhu. Ucp1-HBEGF/eGFP (Ucp1-DTR) and Ucp1-CreER mice were kindly donated by Dr Wolfrum. Tamoxifen induction of Cre activity was performed by gavaging 3 $\times$ daily 200 µL tamoxifen (10 mg/mL, Sigma—Aldrich) in sunflower oil when the animals were kept at 5 °C. *Ucp1-iCre* mice were built by Biocytogen. To avoid disrupting *Ucp1* expression, *IRES-Cre* was introduced between the coding sequence of exon 6 and the 3′UTR. The internal ribosome entry site (IRES) was used to allow *Ucp1* and *iCre* expression at the same time with lower levels. To avoid disrupting the polyA signal of *Ucp1* expression, a Neo cassette flanked by frt sites was inserted 300 bp downstream of the 3′UTR. Heterozygous mice were healthy and fertile. We then crossed *Ucp1-iCre* mice with *Rosa-mTmG* reporter mice. Transgenic mice *Mmtv-Cre* (Stock #003553), *Krt14-Cre* (Stock #004782) and *Rosa-mTmG* reporter mice (Stock #007676) were purchased from the Nanjing Biomedical Research Institute of Nanjing University (NBRI), and the SCID-beige mice were purchased from Charles River.

2.2. Immunohistochemistry

Animals were perfused with 4% paraformaldehyde (PFA), and mammary gland or BAT were post-fixed by 4% PFA at 4 °C overnight and embedded with OCT after dehydration by 30% sucrose solution for 48 h. Twenty micrometer sections were cut using a Leica cryostat (CM3050S). Frozen sections were fixed in cold PFA for 20 min then rinsed in PBS three times. Then sections were incubated in blocking buffer (5% BSA/0.1% Triton in PBS) at room temperature for 1 h, primary antibodies were added in appropriate concentrations in staining buffer (1% BSA/0.1% Triton in PBS) at 4 °C overnight, followed by a wash and incubation with a secondary antibody for 1 h at room temperature. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole). Fluorescence images of frozen sections were acquired on a FV1000 confocal microscope (Olympus) and cultured cell images were taken on a LSM780 confocal microscope (Zeiss).

2.3. Antibodies

The following primary antibodies were used: anti-GFP (rat, 1:2000, MBL), anti-GFP (rabbit, 1:1000, Abcam), anti-beta-Casein (goat, 1:200, Santa Cruz), anti-KRT14 (rabbit, 1:1000, Covance), anti-KRT14 (mouse, 1:1000, Thermo), anti-KRT5 (rabbit, 1:1000, Covance), anti-KRT5 (mouse, 1:1000, Thermo), anti-KRT8 (rabbit, 1:1000, Abcam), anti-E-cadherin (mouse, 1:1000, BD), anti-Perilipin1 (goat, 1:1000, Abcam), anti-PPAR-γ (rabbit, 1:1000, Cell Signaling Technology), anti-UCP1 (rabbit, 1:1000, Abcam), Deep red LipidTOX neutral lipid stain (1:500, Invitrogen). All secondary antibodies were Alexa Fluor-conjugated from Invitrogen: anti-mouse Alexa 647, anti-rabbit Alexa 647, anti-goat Alexa 647, anti-rat Alexa 488, anti-rabbit Alexa 488, anti-mouse Alexa 594, anti-rabbit Alexa 594, anti-goat Alexa 594, anti-rabbit Alexa 405.

2.4. Flow cytometry

Mammary cells were obtained as performed in earlier studies [15,22]. In brief, inguinal mammary gland or interscapular BAT samples were dissociated by scissors and then incubated with 5% fetal bovine serum containing collagenase (300 IU/mL, Sigma) and hyaluronidase (100 IU/mL, Sigma) for 60 min at 37°C. Samples were then centrifuged at 500 g for 5 min, and the cell fractions were incubated with 0.25% trypsin-EGTA for 3 min, then resuspended in Dispase (5 mg/mL, Sigma) and DNaseI (50 IU/mL, Takara) for 5 min, and red blood cell lysis (0.64% NH_4Cl) for 3 min before filtration through a 40 µm cell mesh. Antibodies were incubated in PBS with 5% FBS for 20 min. The following primary antibodies were used: Percp-cy5.5 conjugated anti-CD24 (eBioscience, Clone M1/69), APC conjugated anti-CD29 (eBioscience, Clone HMB1-1), PE-cy7 conjugated anti-CD31 (eBioscience, Clone 390), PE-cy7 conjugated anti-CD45 (eBioscience, Clone 30-F11). The positive antibody signals were gated based on fluorescence minus one (FMO) control every time. Cell sorting was performed on FACSAria, and the data were read using Flowjo7.6.1 software.

2.5. Administration of AAV vectors

The AAV2/9-CAG-DIO-mCherry (1.2 $\times$ 10^{12} vg/mL) was purchased from the HanBio company. The *Ucp1-iCre* mice were anesthetized with isoflurane. For interscapular BAT administration, a longitudinal skin incision at the interscapular region was performed, and each side of the BAT received three injections of 5 µL AAV solution.

2.6. Ucp1-GFP cell preparation and cell transplantation

Six-week-old virgin *Ucp1-iCre-ROSA*^{mTmG} female mice were anesthetized with isoflurane. Interscapular BAT was removed and minced into small pieces then incubated with 5% fetal bovine serum containing collagenase (300 IU/mL, Sigma) and hyaluronidase (100 IU/mL, Sigma) for 30 min at 37 °C before filtration through a 100 µm cell mesh. Floating adipocytes were collected by a syringe, and mature brown adipocytes (DAPI negative) were sorted by 130 mm diameter nozzle to exclude the debris and other unrelated cells using BD Bioscience Aria sorter according to a recent study [11]. The Ucp1-GFP positive suspension was mixed with 0.9% germ-free sodium chloride and injected into one side of the inguinal fat pad of recipient 8-week-old female SCID-beige mice. After the surgery, mice were injected with

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