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Abbreviations: PGC-1, peroxisome proliferator-activated receptor gamma coactivator 1; UCP1, uncoupling protein 1; TFAM, mitochondrial transcription factor A; mmtv, mouse mammary tumor virus.

Abstract

The PPARγ coactivator 1α (PGC-1α) is a transcriptional regulator of mitochondrial biogenesis and oxidative metabolism. Recent studies have highlighted a fundamental role of PGC-1α in promoting breast cancer progression and metastasis, but the physiological role of this coactivator in the development of mammary glands is still unknown. First, we show that PGC-1α is highly expressed during puberty and involution, but nearly disappeared in pregnancy and lactation. Then, taking advantage of a newly generated transgenic mouse model with a stable and specific overexpression of PGC-1α in mammary glands, we demonstrate that the re-expression of this coactivator during the lactation stage leads to a precocious regression of the mammary glands. Thus, we propose that PGC-1α action is non-essential during pregnancy and lactation, whereas it is indispensable during involution. The rapid preadipocyte-adipocyte transition, together with an increased rate of apoptosis promotes a premature mammary glands involution that cause lactation defects and pup growth retardation. Overall, we provide new insights in the comprehension of female reproductive cycles and lactation deficiency, thus opening new roads for mothers that cannot breastfeed.

Keywords: Mammary glands, Nuclear Receptor, Coactivator, Development, Adipocytes, Involution.

1. Introduction

Development of the mammary gland is initiated during embryogenesis, although it's not until the onset of puberty that ductal elongation takes place and fat becomes filled with a system of epithelial ducts. The mammary glands undergo substantial changes in morphology during reproduction, being one of the most active metabolic tissues. In response to pregnancy, mammary epithelial cells develop into mature alveoli in concert with de-differentiation of the adipocytes of the fat-pad [1]. Parturition induces copious milk production in response to diminished level of progesterone resulting from placenta loss and increased secretion of prolactin by pituitary gland [2]. During lactation, the mammary glands display a high synthesis of triglycerides, utilizing both fatty acids obtained from bloodstream, as well as by *de novo* lipogenesis [3], thus providing sufficient energy to fulfil the developmental demands of the offspring. In humans, the lactating mammary glands secrete 800mL of milk per day containing almost 32g of fat [4]. The mammary glands of a lactating mouse secrete 5mL of milk per day containing 30% of lipids, accounting for approximately 30g of milk lipids over the course of 20 days, equivalent to the entire body weight of the mother [5].

Upon weaning, milk accumulation induces apoptosis of the secretory epithelial cells and their shedding in the lumen. This first phase of involution is reversible and lactation can recommence if suckling is resumed within 48 hours. If the gland remains unemptied for an extended period of time, the mammary gland undergoes changes to regenerate the fat pad, characterized by tissue remodelling and re-differentiation of adipocytes [1;6].

Until recently, the leading hypothesis of mammary gland development during pregnancy involved transdifferentiating of subcutaneous white adipocytes into "pink adipocytes", the mammary gland alveolar epithelial cells responsible for the milk production and secretion [7]. Upon interruption of lactation, pink adipocytes transdifferentiate into brown adipocytes, establishing a pregnancy-lactation adipocyte-to-epithelium-to-adipocyte circle [7;8]. Notably, this hypothesis has

been recently challenged by the new evidence showing that over multiple pregnancies, a cycle of adipocytes de-differentiation into preadipocyte and fibroblast-like cells during pregnancy and lactation occurred, and de-differentiated fibroblasts proliferated and re-differentiated into adipocytes upon weaning [9].

The peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC-1α) is a transcriptional coactivator, playing a central role in metabolism. Together with the other family members, PGC-1β and PRC, PGC-1α promotes mitochondrial biogenesis and respiration [10;11]. Typically expressed at low levels under normal conditions, PGC-1α is highly expressed in tissues with high oxidative capacity, such as brown adipose tissue during thermogenesis [12], skeletal muscle in fiber type switching [13] and in liver during fasting to promote fatty acids β-oxidation and gluconeogenesis [14;15]. Despite recent progress in understanding PGC-1α contribution to mammalian development [13;16-18], the role of this coactivator in mammary glands is still not well defined. Indeed, although different studies have pointed out a crucial role for PGC-1α in promoting breast cancer tumor growth and metastasis [19-21], its physiological role in mammary glands development remains fairly unexplored.

In this study, we investigate the contribution of PGC-1α coactivator in mammary gland development, taking advantage of a newly generated *in vivo* mouse model in which PGC-1α is selectively and specifically overexpressed in mammary tissue. The constitutive PGC-1α overexpression throughout all the stages of mammary glands evolution resulted in a precocious involution, characterized by re-differentiation into adipocytes and promotion of apoptosis, which ultimately mediated lactation and growth retardation defects.

2. Material and Methods

2.1 Animals

The mmtvPGC1α transgenic mice were generated by injecting the mmtv-SV40-PGC1α plasmid digested with HpaI into the pronuclei of the fertilized eggs of the FVB/N mice. To generate the mmtv-SV40-PGC1α, first hPGC1α (2.4 kb) fragment was generated by PCR from pcDNA4-His-PGC1α plasmid (Addgene, USA). Then the fragment was subcloned at the HindIII and EcoRI restriction sites downstream from the mmtv promoter region of the mmtv-SV40-Bssk plasmid (Addgene, USA). Mice carrying the transgene were identified by PCR of genomic DNA to confirm the presence of an hPGC1α coding sequence. Kidney, uterus, ovary, salivary glands, hypophysis and heart of transgenic mice were dissected to evaluate the specific mammary glands expression of transgene under the mmtv promoter control. For mammary gland analyses during pregnancy, lactation and involution, 8- to 16-wk-old nulliparous female mmtvPGC-1α and littermates' mice were bred to wild-type FVB/N male mice. For weight gaining-pups experiments, on day 1 from birth litter size was normalized to seven pups. For foster mother experiments, two pairs of mmtvPGC1α and control wild-type female mice were bred with wild-type FVB/N male mice and delivered pups on the same day were used. On P1, the litter size was normalized to seven pups. Five pups were switched between the mmtvPGC1α mother and the wild-type mother, and two pups were left with the original mother as controls. The fostered pups and original pups were distinguished by ear punching. For milk analysis, milk was isolated from mammary tissue at day 10 of lactation after cervical dislocation of the mice. While pressure was applied to the tissue, the milk was harvested using a Pasteur pipette put onto the nipple. Milk was then transferred into a microcentrifuge tube and stored at −80°C.

All mice were housed with a standard diet provided *ad libitum* and examined daily. Genotyping was performed using DNA extracted from tail biopsies of 4 weeks-old pups, and new breeding harems of 8 weeks-old mice were established to expand the population. The ethics

committee of the University of Bari approved this experimental setup, which was also certified by the Italian Ministry of Health in accordance with internationally accepted guidelines for animal care.

2.2 Whole Mount and histological analysis

For whole mount analysis the first inguinal glands were dissected at the indicated ages and were spread on a glass slide. After fixation with Carnoy's fixative for 2-4 hours, the tissues were hydrated and stained in Carmine alum overnight as previously described [22]. Samples were then dehydrated, cleared with xylene and mounted.

For histology and immunohistochemistry, inguinal mammary glands were dissected and fixed in 10% (vol/vol) formalin for 24 h, dehydrated and paraffin embedded and then 5µm-thick sections were obtained. Standard histology (H&E staining) and immunohistochemical procedures were performed. Briefly, samples were treated with 3% (vol/vol) hydrogen peroxide for 5 min to quench endogenous peroxidase and were subjected to antigen retrieval by boiling the slides in an antigen-unmasking solution (Vector Laboratories, California, USA) for 20 min according to the manufacturer's instructions. Sections were sequentially incubated for 1 h at room temperature in 50% (vol/vol) non immune serum (from the host animal in which the secondary antibody was raised) in PBS (to avoid unspecific signal) and overnight at 4 °C with primary antibodies rabbit peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (in-house antibody), uncoupling protein 1 (UCP1, ab10983, Abcam, Cambridge, UK) and cytochrome c oxidase 1 (COX1, LS-C343872, LifeSpan Biosciences, Washington, USA). Sections were washed for 10 min in PBS and incubated for 30 min at room temperature with the secondary biotinylated antibody (Vector Laboratories, California, USA). After three 5-min washing steps with PBS, sections were incubated with the avidin-biotin complex (Vector Laboratories, California, USA) for 30 min at room temperature. After washing in PBS, the peroxidase reaction was developed by incubation with 3,3-diaminobenzidine (Sigma-

Aldrich, Missouri, USA). Counterstaining was carried out with methylene blue (Sigma-Aldrich, Missouri, USA). For negative controls, the primary antibodies were replaced by 1% non-immune serum in PBS.

For immunofluorescence, 4µm-thick mammary gland sections were double stained with primary antibody for Wheat Germ Agglutinin, Alexa Fluor 488 Conjugate (WGA, W11261, Thermo Fisher Scientific, Massachusetts, USA) and Adipophilin, Alexa Fluor 594 conjugate (ADFP, ab206356, Abcam, Cambridge, UK). Specifically, the slides were incubated whit 10% goat serum for 1 hour at room temperature. Slides were incubated overnight at 4°C with WGA 5µl/ml and ADFP 1:50 mix. All sections were counterstained with TO-PRO-3 (Thermo Fisher Scientific, Massachusetts, USA). Negative controls were prepared with irrelevant antibody. The sections were analysed using the Leica TCS SP2 (Leica, Wetzlar, Germany) confocal laser-scanning microscope.

Image processing was performed using ImageScope software (Leica, Wetzlar, Germany). For each sample, 15 representative images were taken and the percentage of stained area/total area was measured. Values from all consecutive images for each sample were averaged and displayed as mean±SEM.

2.3 Apoptotic Assay

For the TUNEL assay in vivo, tissue specimens were fixed in 10% formalin for 12–24 h, dehydrated, and paraffin embedded. Detection of apoptosis at the single-cell level based on the labelling of DNA strand breaks was performed using the In-Situ Cell Detection kit (Roche, Basel, Switzerland) following the manufacturer's instructions.

2.4 RNA Extraction and Quantitative Real-Time PCR Analysis

Inguinal mammary glands tissues were snap frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated by RNeasy lipid tissue kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. To avoid possible DNA contamination, RNA was treated with DNase (Thermo Fisher Scientific, Massachusetts, USA). RNA purity also was checked by spectrophotometer, and RNA integrity was checked by examination on agarose gel electrophoresis. For RT-qPCR analysis of pregnancy and lactation stages, cDNA was synthesized by retro-transcribing 1µg of total RNA in a total volume of 100µL utilizing the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions. PCR assays were performed in 96-well optical reaction plates using a Quantum5 machine (Thermo Fisher Scientific, Massachusetts, USA). PCR assays were conducted in triplicate wells for each sample. The following reaction mixture was used in each well: 10µL Power SYBR Green (Thermo Fisher Scientific, Massachusetts, USA), 2.4µL of primers at a final concentration of 150nM each, 1.6µL RNase-free water, and 3µL cDNA. The following PCR conditions were used: denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and then at 60 °C for 60 s. For RT-qPCR analysis of different developmental stages, cDNA was synthesized by utilizing the High-Capacity RNA to cDNA Kit (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions. PCR assays were performed in triplicate wells for each sample, in 96-well optical reaction plates using a Quantum5 machine (Thermo Fisher Scientific, Massachusetts, USA). PCR assays were conducted utilizing TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix with UNG following the manufacturer's instructions. The following PCR conditions were used: incubation at 50˚C for 2 min, denaturation at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 s, and then at 60˚C for 60 s. For all the experiments, quantitative normalization of cDNA in each sample was performed using TBP mRNA as an internal control. Relative quantification was done using the ΔΔCT method

2.5 Gel Electrophoresis

For immunoblot analysis, tissue samples were homogenized in RIPA buffer supplemented with protease and phosphatase inhibitors. Equal amount of protein lysates (50µg), quantified using Bradford-based assay (BioRad Laboratories, Hercules, CA), were denaturated with Laemmli sample buffer, separated by 10% or 12.5% SDS-PAGE and then transferred onto a nitrocellulose membrane (Protran, Whatman). Membranes were blocked with 5% bovine serum albumin in Tris-buffered saline/0.01% Tween 20 and probed with specific antibodies against PGC-1α (in-house antibody) or UCP1 (ab10983, Abcam, Cambridge, UK). Nuclear encoded β-actin (ab8229, Abcam, Cambridge, UK) were used as loading control where possible. Membranes were finally incubated with horseradish peroxidase–conjugated secondary antibodies and developed with a chemiluminescent reagent (Bio-Rad Laboratories, Hercules, CA).

For milk quality analyses, equivalent amounts of protein from milk collected on day 10 of lactation from each mouse genotype were analysed by 12% SDS-PAGE. Post electrophoresis, the gel was fixed, stained with Coomassie Brilliant Blue, destained and kept in preservation solution.

2.6 Fatty acids analysis

Fatty acids were analysed as fatty acid methyl esters (FAMEs). The milk was transmethylated with 1 mL of BF_3 in methanol (1:20, v/v) for 60 min at 80 °C, evaporated to dryness, and the FAMEs extracted with hexane/water (3:1). The organic phase was evaporated to dryness and dissolved in 50 µL ethyl acetate. One microliter of FAMEs was analysed by gas-liquid chromatography on a 5890 Hewlett-Packard system (Hewlett-Packard, Palo Alto, CA) using a Famewax fused-silica capillary column (30 m, 0.32 mm internal diameter, 0.25 mm film thickness; Restek, Belfast, UK). The oven temperature was programmed from 110 to 220 °C at a rate of 2 °C/min, with hydrogen as the carrier

gas (0.5 bar). The injector and detector were at 225 and 245 °C, respectively. Heptadecanoic acid (Sigma) was used as an external standard for fatty acid methyl esters.

2.7 Statistical analysis

All results are expressed as mean ±SEM. Data distribution and gene expression statistical analysis were performed with GraphPad Prism software (v5.0; GraphPad Software Inc., La Jolla, CA). Comparisons of two groups were performed using Mann-Whitney U test. Comparison of four groups were performed using Kruskal-Wallis test. A value of $P<0.05$ was considered as statistically significant.

3. Results

3.1 PGC-1α is highly expressed in mammary gland during puberty and involution.

As the expression and localization of coactivator PGC-1α in the mammary gland is still unknown, we first investigated the expression of endogenous PGC-1α throughout the stages of mammary glands development. Immunohistochemistry analysis on wild-type female mice revealed that PGC-1α is expressed in normal mammary epithelium, being upregulated during puberty and within the first 12 days of pregnancy. By contrast, PGC-1α expression starts to decrease by the 18th day of pregnancy and is wholly absent during lactation (Figure 1A).

At weaning, involution of mammary gland occurs. This process takes place just after suckling interruption and it is associated with programmed cell death of secretory epithelium cells, redevelopment of mammary adipose tissue together with a remodelling of the lobular-alveolar structure [23]. Histological analysis on mammary gland on the first days after weaning displayed ductal structure surrounded by adipocytes. Moreover, as involution proceeded, we observed an increase in PGC-1α expression, which becomes extremely evident on the 3rd day (Figure 1B). The

involuted mammary glands displayed PGC-1α expression similar to that of virgin mammary glands (i.e. puberty), with prominent nuclear PGC-1α staining, surrounded by increasing accumulation of adipocytes. Concomitantly to the PGC-1α gain expression, TUNEL analysis showed a high apoptosis rate on day 3, thus suggesting that the high levels of PGC-1α actively correlates with the induction of apoptosis (Figure 1B).

Intriguingly, the re-expression of PGC-1α during the involution stage is associated with browning markers induction. In particular, the expression of Ucp1 and PR domain containing 16 (Prdm16) nearly disappeared during pregnancy and lactation, but they are promptly induced during involution (Figures 1A-C). Differently, cell death-inducing DNA fragmentation factor alpha-like effector A (Cidea) levels are low during puberty and pregnancy, but importantly increased throughout the lactation stage. At involution, Cidea expression is turned down, but still significantly induced compare to puberty and pregnancy (Figure 2C). Indeed, beside its expression in brown and beige adipocytes in mouse, Cidea is also an essential transcriptional coactivator capable of regulating mammary gland secretion of milk lipids [24-26].

The dynamic changes observed in PGC-1α expression during the mammary gland development suggest that this coactivator is dispensable during lactation, but its induction is crucial during involution.

3.2 Maternal PGC-1α overexpression in mammary glands results in growth retardation defects

To dissect the role of PGC-1α in mammary glands development, we generated a transgenic mouse model with mammary specific gain of function of PGC-1α (mmtvPGC-1α). To this end, the sequence coding for human PGC-1α was subcloned to the mmtv promoter, thereby allowing a tissue-specific expression of hPGC-1α in the mammary gland epithelium (Figure 3A). Successful overexpression was confirmed by mRNA and protein analysis of 8 weeks-old virgin mice mammary glands (Figure

3B-3C). The exclusive overexpression of hPGC-1α in mammary glands was subsequently confirmed by comparison with other tissues examined by real time qPCR (Supplementary Figure 1A).

Despite similar body weight between wild type and mmtvPGC-1α mice (Figure 3D), mammary glands harvested from 8 weeks-old transgenic virgin mice were significantly smaller compared to wild type ones as indicated by inguinal mammary glands weight to body weight ratio (MGW/BW) (Figure 3E), thus suggesting that high levels of PGC-1α may disrupt a proper mammary gland development.

Examination of wild type and mmtvPGC-1α mammary glands from 8 weeks-old virgin mice by paraffin section (Figure 4A) and whole mount (Figure 4B) revealed a difference in terminal end bud formation, ductal growth and ductal branching. Indeed, transgenic-derived mammary glands showed thinner ductal branches, lower alveolar bud-like structures and a less branched ductal network than WT mice (Figure 4B).

Then, we crossed mmtvPGC-1α female with male of same genotype and we did the same for WT mice. We monitored pups' weight from birth until the time of weaning (21 days). On the 2nd day, the litter size was normalized to seven pups. Daily measurements of pup body weight from female mmtv-PGC-1α revealed growth retardation (Figure 4C-4E, red closed square) and, in rare cases (5%), hair cycling defects and gout, compared to WT littermates (blue closed circle). At the time of weaning (postnatal day 21), these pups exhibited low dimensions and body weights (Supplementary Figure 1B). Interestingly, weight gain resumed immediately when the pups were placed on a standard chow diet (data not shown). Afterwards, pups maintained a normal phenotype that was completely dependent on maternal genotype (mmtv-PGC-1α).

To further confirm that PGC-1α expression in lactating mammary glands are indeed responsible for the observed pups' phenotype, we subjected the mice to a cross-fostering experiment. Fostering is the movement of pups from the birth (donor) dam to a recipient (foster)

dam. We fostered pups within 48 hours of birth to recipient dams that had delivered age matched litters. We completely replaced the recipient dam's litter with fostered neonates, in order to maintain a controlled number of mice (7 mice per litter) and to fully characterize the mutant newborns. As expected, mmtv-PGC-1α pups nursed by WT female completely lost their phenotype (Figure 4D-4E, red open circle) and did not show any growth retardation. On postnatal day 19, WT-fostered mmtv-PGC-1α pups reached a weight gain similar to the one observed for WT pups nursed by WT mother (8.4g±0.13 versus 8.2g±0.38) (Figure 4D-4E). On the other side, WT pups nursed by mmtv-PGC-1α dam (blue open square) showed a considerable delay on body growth, that became less consistent around weaning time when switched from maternal milk to cage food. notably, on postnatal day 19th, WT-fostered mmtv-PGC-1α pups weighted over one gram more than the WT pups nursed by mmtv-PGC-1α foster mother (8.4g±0.13 versus 7.2g±0.16) (Figure 4D-4E – Supplementary Figure 1C).

Collectively, these data suggest that PGC-1α is physiologically turned off during lactation in order to totally fulfil the metabolic needs for offspring development and its re-expression during lactation – as we observed with our transgenic model – may interfere o delay a complete development of newborns. However, as the transgenic phenotype was rescued at weaning and during cross-fostering experiments, it is not improbable to hypothesize that nutritional defects due to milk quality or quantity from mmtvPGC-1α mothers may mediate the growth retardation observed in offspring.

3.3 PGC-1α overexpression during lactation does not affect milk quality

To explore whether pup's growth defects could be ascribable to the ectopic PGC-1α overexpression, we analysed if stable induction of PGC-1α during lactation stage could affect milk production. As shown by immunohistochemical (Figure 5A) and gene expression data (Figure 5B), the

overexpression of ectopic hPGC-1α remained unchanged during lactation. Moreover, mmtvPGC-1α displayed a net increase in COX-1 staining compared to WT mice, indicating that the overexpression of PGC-1α is closely related with the activation of mitochondria metabolism (Figure 5C). In line with this data, mice overexpressing PGC-1α in the mammary glands exhibited the induction of mitochondrial transcription factor A (Tfam) and phosphoenolpyruvate carboxykinase (Pepck), two PGC-1α target genes involved in mitochondrial biogenesis and gluconeogenesis, respectively (Figure 5D). To further determine the consequences of PGC-1α expression during lactation stage, we quantified the milk composition to find out if any anomalies occurred. The proteins (Figure 5E) and fatty acids (Figure 5F) profiles were comparable in the milk harvested from both wild type and mmtvPGC-1α mice. Overall, these data indicate that the stable PGC-1α expression during lactation stage is able to boost mitochondria respiration without overt effects on milk quality.

3.4 Mammary PGC-1α overexpression during lactation leads to a rapid mammary glands involution

Since we did not observe any substantial differences between WT and PGC-1α mice regarding milk composition, we wondered if the ectopic expression of PGC-1α could have any consequences on milk quantity. At 10 days of lactation, mammary glands of transgenic mice displayed smaller cytoplasmic lipids droplets and, consequently, reduced lumina, as compared with WT (Figure 6A). However, the analysis of lipid droplets accumulation using a specific antibody against adipophilin (ADPH), a protein found in cytoplasmic and secreted-milk lipid droplets coat [27;28], did not reveal eminent differences between the two groups (Figure 6A-6B). Surprisingly, H&E staining of mammary glands sections revealed the presence of small cells, containing multilocular cytoplasmic lipid droplets, resembling brown adipocytes (Figure 6A). To confirm our hypothesis, we tested mammary glands immunoreactivity for uncoupling protein 1 (UCP1), a mitochondrial thermogenic protein uniquely expressed in brown adipose tissue [29] and we found marked areas of UCP1 staining in

mmtvPGC-1α mammary glands compared to wild type (Figure 6A-6B). At the same time, gene expression analysis revealed a trend toward induction of fat-browning related genes, such as Ucp1, Cidea and Prdm16 (Figure 6C). Interestingly, we observed high concentration of UCP1 also in alveolar epithelial cells, thus pointing out that the expression of PGC-1α during lactation promotes an early changing in mammary gland architecture, becoming repopulated with adipocytes that rapidly differentiate into brown ones. Brown adipose cells are usually densely packed with small lipid droplets together and abundant mitochondria. Indeed, we observed that mmtvPGC-1α mammary glands displayed a higher induction of mitochondrial metabolism compared to wild type ones (Figure 5C-5D). Since fatty acid β-oxidation is critically required for the thermogenic function of BAT, to further confirm our results, we examined the expression of genes involved in fatty acid β-oxidation (Peroxisome proliferator-activated receptor α, Pyruvate dehydrogenase kinase 4, and Carnitine Palmitoyltransferase 1B) and we found a trend towards increase of those genes in mammary gland harvested from transgenic mice compared to wild type ones (Figure 6D).

To understand whether the overexpression of PGC-1α is involved in the modulation of signals mediating the regression of mammary glands development, we determined the ratio between inguinal mammary gland and total mice weight on day 10 of lactation, revealing a marked decrease in mmtvPGC-1α mice compared to WT mice (Figure 6E). Concurrently, analysis on paraffin section and whole mount confirmed that the overexpression of PGC-1α in mammary glands was highly correlated with a hypoplastic phenotype and reduced alveolar structures (Figure 6A-6F). Moreover, we observed a marked increment of apoptosis rate, as indicated by TUNEL assay and the shedding of epithelial cells into the alveolar lumen (Figure 6A).

Taken together, our results depict a unique scenario in which high levels of PGC-1α during lactation prevent mammary glands development and maturation by both inducing an early apoptosis and promoting a rapid modification of mammary gland architecture, characterized by

differentiation into brown adipocytes. Taken together, all these transformations collectively accelerate the involution of tissue, and finally lead to a decrease of milk production, hence affecting normal pups' growth and development.

4. Discussion

The fundamental function of the mammary gland is to provide nourishment to newborns. Milk represents the food for young mammals, being one essential constituents of mammalian life. Human breastfeeding conveys established benefits for both maternal and child health, as maternal milk supplies calories from lipids and essential fatty acids together with signalling molecules. However, one out of two mothers stop breastfeeding before the recommended timespan, mostly due to the perception that milk quantity is insufficient to address the need of infants [30;31].

In our *in vivo* study, we used integrated approaches of molecular genetics, biochemistry and metabolomic to demonstrate that PGC-1α is dispensable during pregnancy and lactation, but specifically required during involution, where the coactivator causes regression and apoptosis of mammary epithelial cells. The timing of the observed phenotype is clearly related to the temporal expression of PGC-1α, as highlighted by immunohistochemical analysis (Figure 1A).

By using a transgenic mouse model, we showed that the stable and specific expression of PGC-1α in mammary gland is associated with lactation defects and retarded pups' growth. Indeed, the specific overexpression of PGC-1α resulted in an altered mammary gland morphogenesis, as well as a precocious mammary gland regression. Surprisingly, this regression occurred during the lactation stage, through the development of abnormal alveolar structures that contributed to lactation deficiency. In line with our experiments, it has been reported that several transgenic and knockout mouse models exhibit lactation defects as a result of insufficient development of the

alveolar structures during pregnancy [32-34] and it would be interesting if those genes could be related to the PGC-1α coactivator activity.

Normally, mammary gland involution is a two-step process which encompasses the apoptosis of secretory epithelial cells followed byre-differentiation of the adipocytes [6;9]. In the present study, we show that PGC-1α is physiologically involved in the promotion of apoptosis during involution, and its expression throughout the lactation stage contributes to the regression of mammary glands, thus limiting the availability of milk.

Recently, it has been observed that mammary adipocytes fully de-differentiates into preadipocytes during lactation and promptly re-differentiate during involution [7;9]. Interestingly, several lines of evidence indicate that white adipocytes may transdifferentiate into brown adipocytes (a process known as “browning” or “brightening” of WAT), characterized by UCP1 expression coupled with a multilocular lipid droplets morphology [35;36]. Whether this process occurs as a transdifferentiation of pre-existing white adipocytes or by *de novo* adipogenesis from a subgroup of precursor cells is still unknown [35;37;38]. Our study revealed that re-expression of PGC-1α during lactation promotes an early adipocytes transdifferentiation of mammary glands, consequently affecting milk production. Indeed, mmtvPGC-1α lactating mammary gland display large area of resembling brown adipocytes, with increased mitochondrial UCP1 expression associated with the reduction of cytoplasmic lipid droplets. It is not unfair to hypothesize that the uncoupling of the oxidative phosphorylation would release the feedback control exerted by the mitochondrial membrane potential on the respiratory NADH oxidation, thus allowing the cells to reach maximum capacity of fatty acids β-oxidation. The indication of that the re-expression of PGC-1α during lactation is sufficient to induce UCP1 expression in the mammary gland is consistent with the described regulation of the brown fat in the interscapular fat pad, as well as the induction of brown-fat-like regions within white adipose depots [39;40]. Interestingly, it has been recently

described that during mammary gland involution some milk secreting epithelial cells in the anterior subcutaneous depot (pink adipocytes) may transdifferentiate to brown adipocytes [8;41]. Although this data may contradict the “cycle of adipocyte-pre-adipocyte transition” postulated by Wang and colleagues [9], our results depict the possibility that re-expression of PGC-1α during mammary gland involution may contribute to browning of de-differentiated mammary adipocytes.

Intriguingly, several studies have indicated that brown adipocytes are a component of the mammary fatty stroma during postnatal development or in virgin mice exposed to cold, and that PGC-1α and genes involved in fatty acids β-oxidation are coordinately upregulated in this tissue [42;43]. Moreover, multilocular brown adipocytes have been detected in the adult mammary gland of Brca1 mutant mice, which develop high grade undifferentiated adenocarcinoma in a similar way to human BRCA-1 mutated breast cancer [44]. Noteworthy, a high expression of PGC-1α in cell culture and in tissue specimens isolated from established human breast cancer correlates with low survival and metastasis [45-47]. It would therefore, be intriguing to explore whether the expression of this coactivator is essential to promote tumor onset and progression. Recent observations regarding the increased risk of breast cancer in women who do not breastfeed [48-50] highlight once more the importance of PGC-1α in the pathophysiology of mammary glands, suggesting that turning off the expression of this coactivator during lactation could exert protective actions against cancer development.

All together, these data infer that PGC-1α is indeed necessary in the post-lactation stage, as it is involved in both steps of mammary glands involution by promoting apoptosis and remodelling of mammary gland architecture. Therefore, its expression is physiologically turned off during late pregnancy and lactation in order to allow the full development of secretory epithelial cells and milk production. The overall effects exerted by enhanced PGC-1α expression, including the precocious regression of the glands suggest a possible role of PGC-1α in mastitis and, consequently, milk loss.

Premature mammary glands involution promoted by PGC-1α could be mediated through NF-κB, a transcription factor that has been involved in mastitis, where it sustains the rapid loss of milk and secretory structures [51]. However, due to its fundamental role in host defence, the therapeutic intervention to block NF-κB is not recommended. Alternatively, a strategy aimed to inhibit PGC-1α could be designed to provide beneficial effects for nursing mothers.

The limitation of the present study is the lack of a loss of function model. However, it is rather possible that the effects of specific PGC-1α ablation from the mammary glands would not be easy to identify, since different coping mechanisms may occur in order to counteract the loss of this coactivator. PGC-1β expression could represent one of the possible compensatory strategies. Indeed, PGC-1β and PGC-1α exert similar functions in many tissues, and are both considered as master regulators of oxidative metabolism due to their capacity to promote mitochondrial biogenesis [52]. In mammary glands, both coactivators are significantly downregulated during pregnancy and lactation, and are target of nutri-regulated miRNA [53;54]. At the same time, oestrogens are able to induce only PGC-1β expression in the breast, thereby supporting mitochondrial biogenesis and oxidative phosphorylation in this tissue [55]. On the other hand, at av variance of PGC-1α that do not affect milk composition, PGC-1β modulation interferes with lipogenic process, thus altering the quantity and the quality of fatty acids incorporated into milk. Precisely, the negative regulation exerted by miR-25 on PGC-1β activity results in a reduced triglyceride synthesis and lipid droplets accumulation [56]. Moreover, fat-dietary type and physiological stimuli, such as the increased amount of polyunsaturated fatty acids (PUFAs) in lactating mammary glands, diminish PGC-1β expression and promotion of lipogenesis in mammary glands [57]. Thereby, it is plausible that a compensatory adaptive increase of PGC-1β under suitable stimuli would result in aberrant milk production which may impact in normal growth and development of the newborns.

Overall, taking advantage of this newly generated transgenic mouse model, we depicted a direct involvement of PGC-1α in the physiological homeostasis of mammary glands, where it actively promotes involution. Our model provides a hypothetical underlying mechanism controlling the process of adipocyte de-differentiation and re-differentiation during a female reproductive cycle. However, high coactivator levels during lactation lead to a precocious re-differentiation into adipocytes together with increased apoptosis rates consequently hasten tissue involution, thus preventing a complete development of mammary glands. Our results provide new insights in the comprehension of lactation deficiency, opening new possibilities for mothers that, for several reasons, have to face an early stop in breastfeeding and offering future perspective for prevention of breast cancer onset.

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Figures Legend

Figure 1

Dynamic expression of PGC-1α protein levels during mammary glands development and involution. Highly expressed during puberty and in the first 12 days of pregnancy, the PGC-1α levels slightly disappear in the last days of pregnancy and during lactation. On the first days after cessation of lactation, PGC-1α starts to be re-expressed, reaching its steady state on day 3 of involution. (A) Staining of mammary glands sections from wild type mice at different development stages with H&E (upper panel) and PGC-1α immunohistochemistry (lower panel) (n=3 mice per stage; Magnification 200X). (B) Mammary glands prepared from wild type mice on the indicated days of involution and stained for H&E, PGC-1α immunohistochemistry, and TUNEL (n=3 mice per stage; Magnification 100X)

Figure 2

PGC-1α expression in mammary glands is associated with UCP1 induction.

(A) Staining of mammary glands sections from wild type mice at different development stages with H&E (upper panel) and PGC-1α (middle panel) and UCP1 immunohistochemistry (lower panel) (n=5 mice per stage; Magnification 200X). (B) Western blot analysis of PGC-1α and UCP1 on mammary glands samples throughout different developmental stages. Ponceau stain was used as loading control. (C) Relative expression of PGC-1α and fat-browning related genes, Ucp1, Cidea and Prdm16, at different stages of mammary glands development evaluated by Real Time qPCR. TBP was used as housekeeping gene. Comparison of different groups (n=6,7) was performed using Kruskal-Wallis test. Data are expressed as mean ±SEM (*$p<0.05$; **$p<0.01$).

Figure 3

Generation of mouse model with stable specific overexpression of human PGC-1α in mammary glands. A transgenic mouse model, mmtvPGC-1α, was generated by cloning the human sequence of PGC-1α downstream the MMTV promoter. Analysis of 8 weeks-old wild type and mmtvPGC-1α virgin mice shows a specific PGC-1α overexpression in the mammary glands of transgenic mice. (A) Scheme of the mmtvPGC-1α transgenic mouse model generated: the hPGC-1α coding sequence was cloned downstream of the MMTV promoter of MMTV-SV40-Bssk plasmid and then injected into the pronuclei of the fertilized eggs of the FVB/N mice. (B) Relative mRNA expression of hPGC-1α in mammary glands isolated from mmtvPGC-1α and WT control mice measured by real time qPCR, using TBP as housekeeping gene and WT mice as calibrator. (C) Western blot analysis of PGC-1α on mammary glands samples isolated from transgenic and WT mice. (D) Body weight and (E) inguinal mammary glands weight to body weight ratio (MGW/BW) of wild type and mmtvPGC-1α mice. Results are expressed as mean ±SEM (***$p<0.001$, *$p<0.05$). Comparison of wild type and transgenic mice (n=6,7) was performed using Mann-Whitney U test.

Figure 4

Maternal PGC-1α overexpression decreases lobulogenesis in mammary glands during early development and results in growth retardation in the nursing neonates. Examination of wild type and mmtvPGC-1α mammary glands from 8 weeks-old virgin mice reveals that PGC-1α overexpression in transgenic mice is associated with an impairment of ductal growth and ductal branching. (A) H&E staining and PGC-1α immunohistochemistry of mammary glands sections from wild type and mmtvPGC-1α mice (Magnification 200X). (B) Whole mount of the inguinal mammary glands in a virgin wild type and a mmtvPGC-1α mouse. Developmental time line is depicted as the

average body weight (±SEM) of pups from each of 4 independent litters on each postnatal day, nursed by the (C) biological mother and the (D) foster one. (E) Comparison of the four different group depicted as percentage of body weight increase. Blue color indicates WT pups, whereas red one specifies mmtvPGC-1α newborns. Circle symbol is used for WT breastfeeding mother and square one for mmtvPGC-1α breastfeeding mother. Closed circle indicates that pups are fed by biological mother, while open circle is used to denote pups fed by foster mother.

Figure 5

PGC-1α overexpression during lactation does not affect milk quality.

Analysis of wild type and mmtvPGC-1α mammary glands from lactating females on day 10 of lactation shows that PGC-1α overexpression is preserved in transgenic mice during lactation. (A) H&E staining (left) and PGC-1α immunohistochemistry (right) of mammary glands sections from wild type and mmtvPGC-1α female mice on 10^{th} day of lactation. (B) Relative expression of human PGC-1α in lactating mammary glands evaluated by Real Time qPCR demonstrating that the transgene is not lost during lactation. GAPDH was used as housekeeping gene to normalize data and wild type mice was used as calibrators (C) COX1 immunohistochemistry of mammary glands sections from wild type and mmtvPGC-1α female mice on 10^{th} day of lactation. (D) Relative expression of PGC-1α target genes, Tfam and Pepck, in lactating mammary glands evaluated by Real Time qPCR. TBP was used as housekeeping gene. (E) A 12% SDS-polyacrylamide gel analysis of different types of casein in milk derived from lactating wild type and mmtvPGC-1α mice. The sizes of the protein molecular weight markers are indicated in lane 1. (F) Fatty acids composition of milk harvested from lactating wild type and mmtvPGC-1α females. Fatty acids were analyzed as fatty acid methyl esters (FAMEs) by gas-liquid chromatography. Comparison of wild type and transgenic mice (n=6,7) was performed using Mann-Whitney U test. Results are expressed as mean ±SEM (*$p<0.05$; ***$p<0.001$).

Figure 6

Stable overexpression of PGC-1α during lactation promotes apoptosis and mammary glands regression.

(A) Mammary tissue sections from 10 days lactating wild type and mmtvPGC-1α females mice stained with H&E (different magnification), with UCP1 immunohistochemistry, and ADPH immunofluorescence. In 100X H&E, white arrows indicate the shedding of epithelial cells into the alveolar lumen; and black arrows represents brown adipocytes. Immunolocalization of ADPH was performed using Alexa 594-conjugated antibodies against the N-terminus of mouse ADPH (red, arrowed). Luminal borders of mammary alveoli were identified by staining with Alexa 488-conjugated WGA (green). Nuclei were stained with TO-PRO-3 (Blue). (B) Quantification of UCP1 and ADPH immunostaining. Relative expression of (C) fat-browning related genes, Ucp1, Cidea and Prdm16, and (D) β-oxidation genes, Pparα, Pdk4 and Cpt1b, in lactating mammary glands evaluated by Real Time qPCR. TBP was used as housekeeping gene. (E) Inguinal mammary glands weight to body weight ratio (MGW/BW) of wild type and mmtvPGC-1α lactating mice. (F) Low and high magnification of whole mount staining together with TUNEL staining of mammary glands isolated from lactating mmtvPGC-1α and WT mice. Comparison of wild type and transgenic mice (n=6,7) was performed using Mann-Whitney U test. Data are expressed as mean ±SEM (*$p<0.05$; **$p<0.01$).

Supplementary Figure 1

PGC-1α overexpression affects pups development.

(A) hPGC-1α (white bars) and mPGC-1α (black bar) relative mRNA expression in different tissue specimens isolated from transgenic and wild type mice (n=6) by real time qPCR. TBP was used as housekeeping gene to normalize data and wild type mice was used as calibrators. Results are

expressed as mean ±SEM. (B) Picture of wild type and mmtvPGC-1α newborns at 21 days after birth. (C) Picture of wild type and mmtvPGC-1α newborns at 19 days after birth fostered by mmtvPGC-1α and wild type mother respectively.

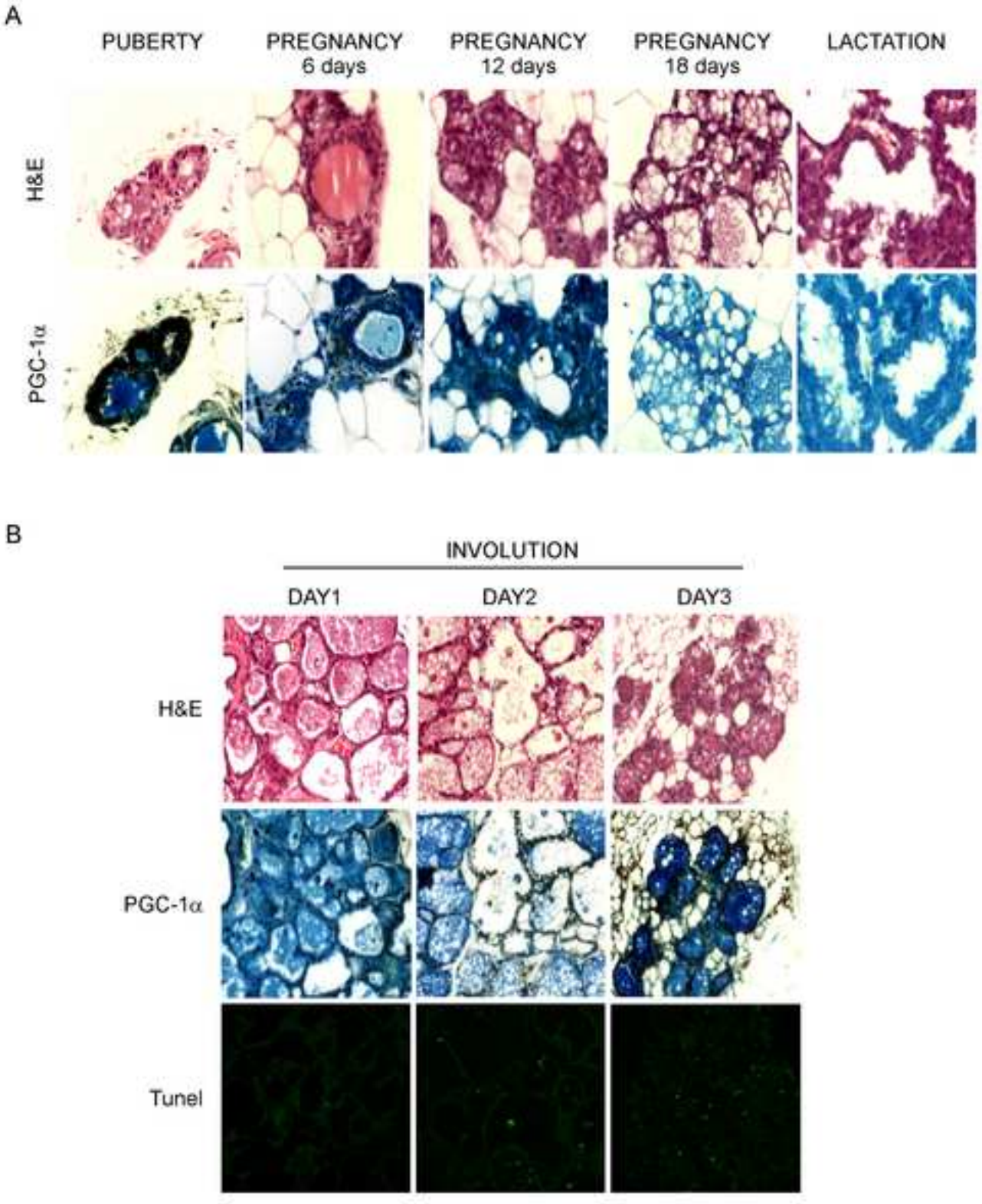

Figure 1

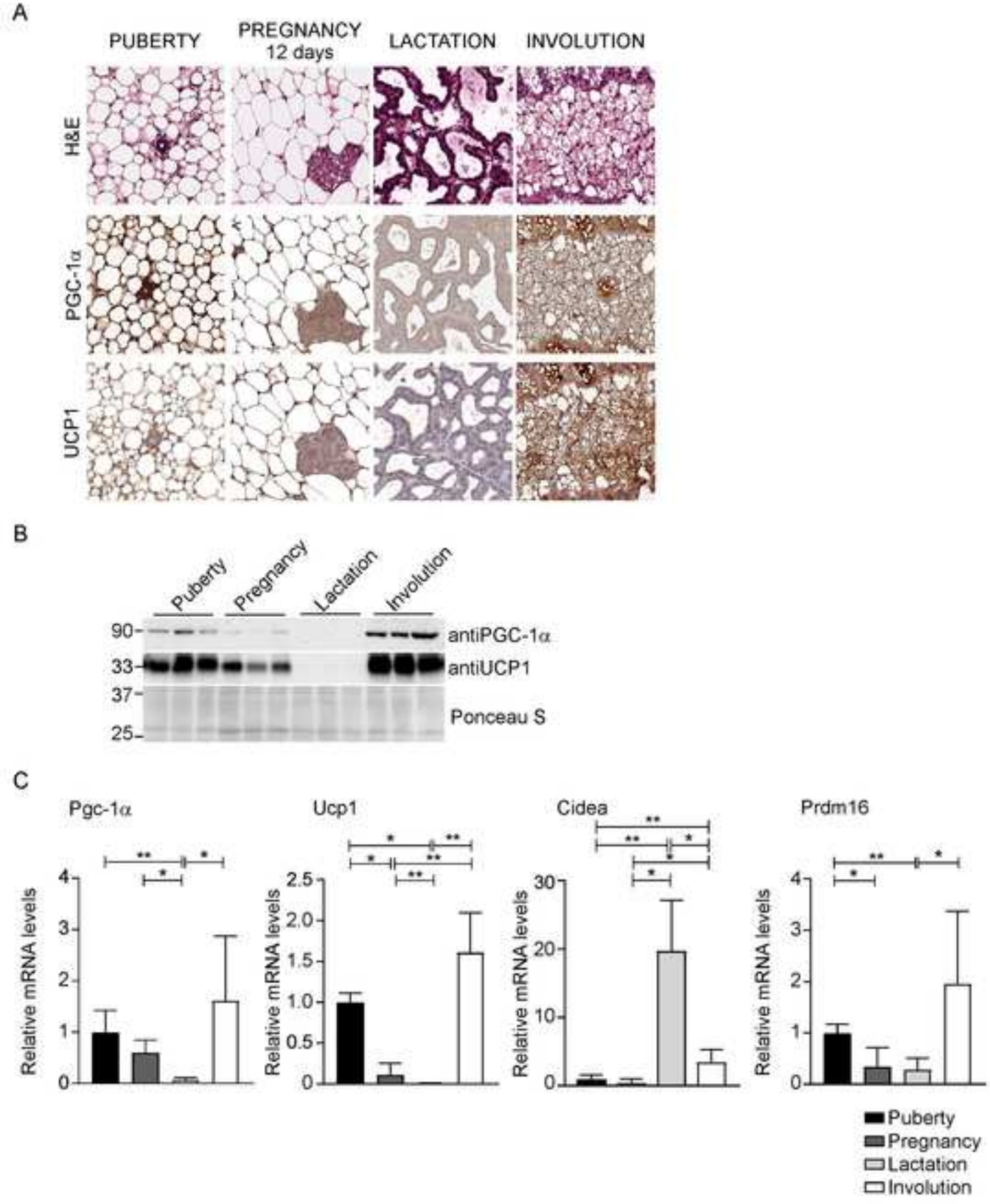

Figure 2

Figure 3 Click here to access/download;Figure;Fig3R.tif

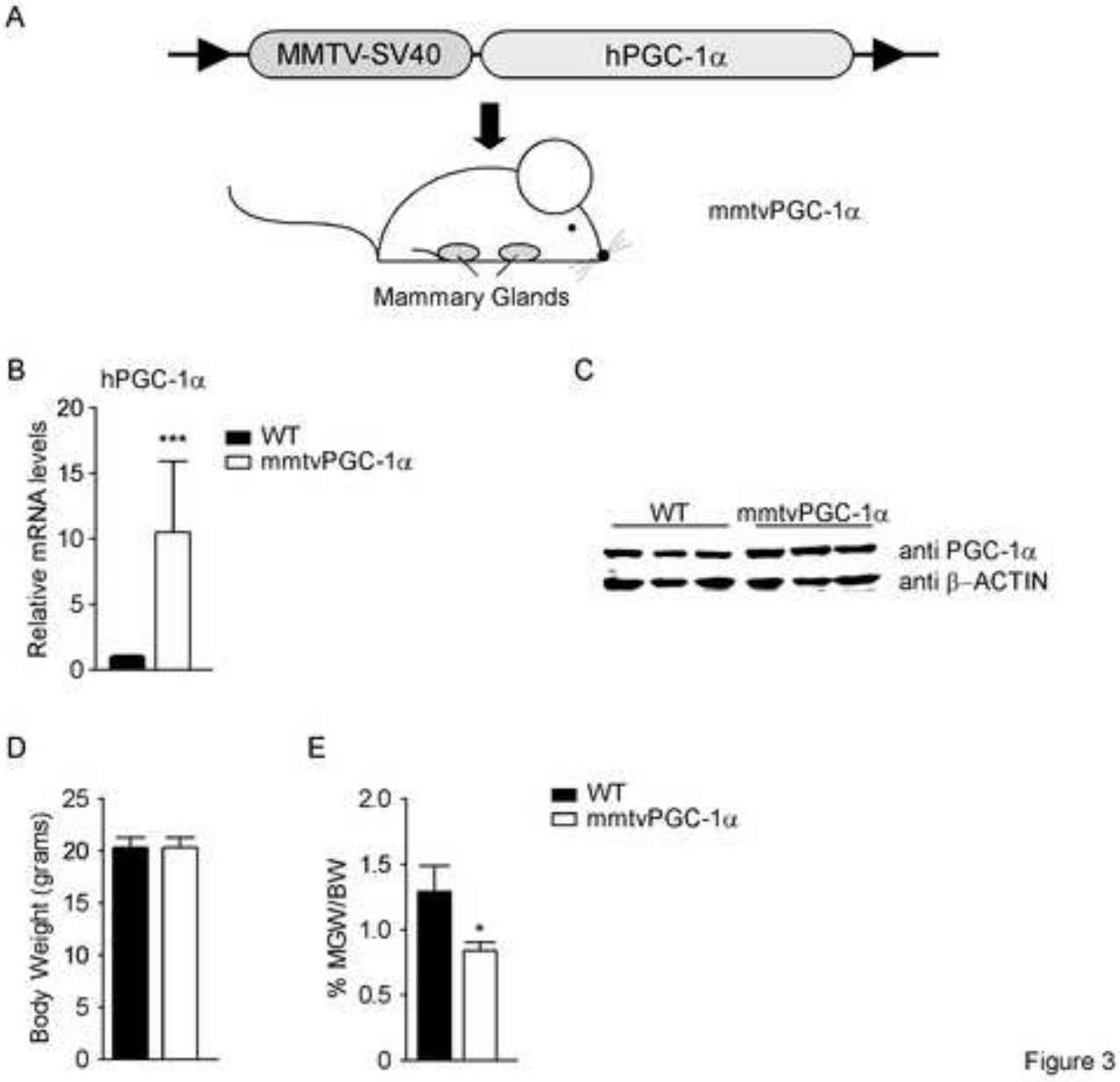

Figure 3

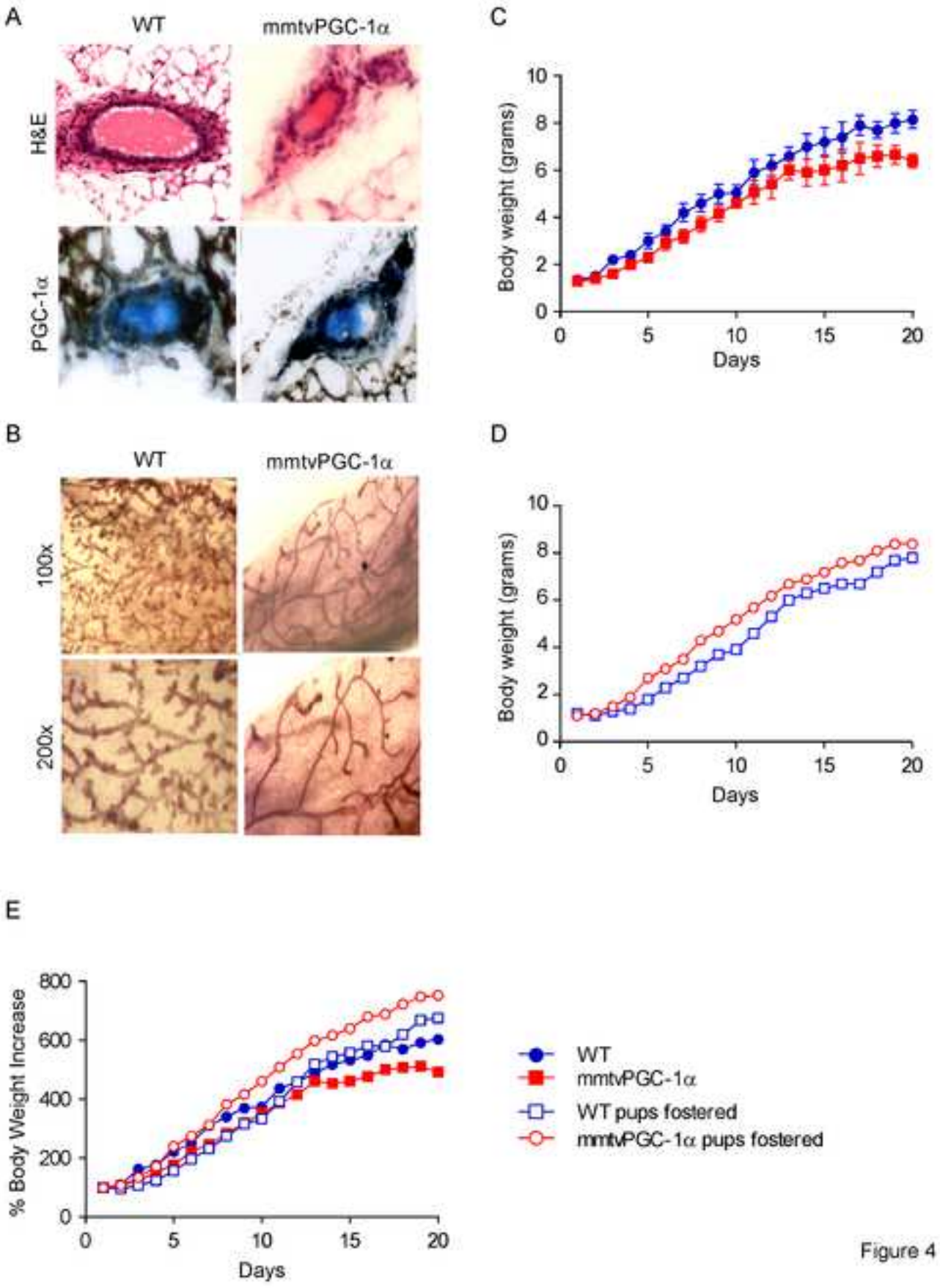

Figure 4

Figure 5

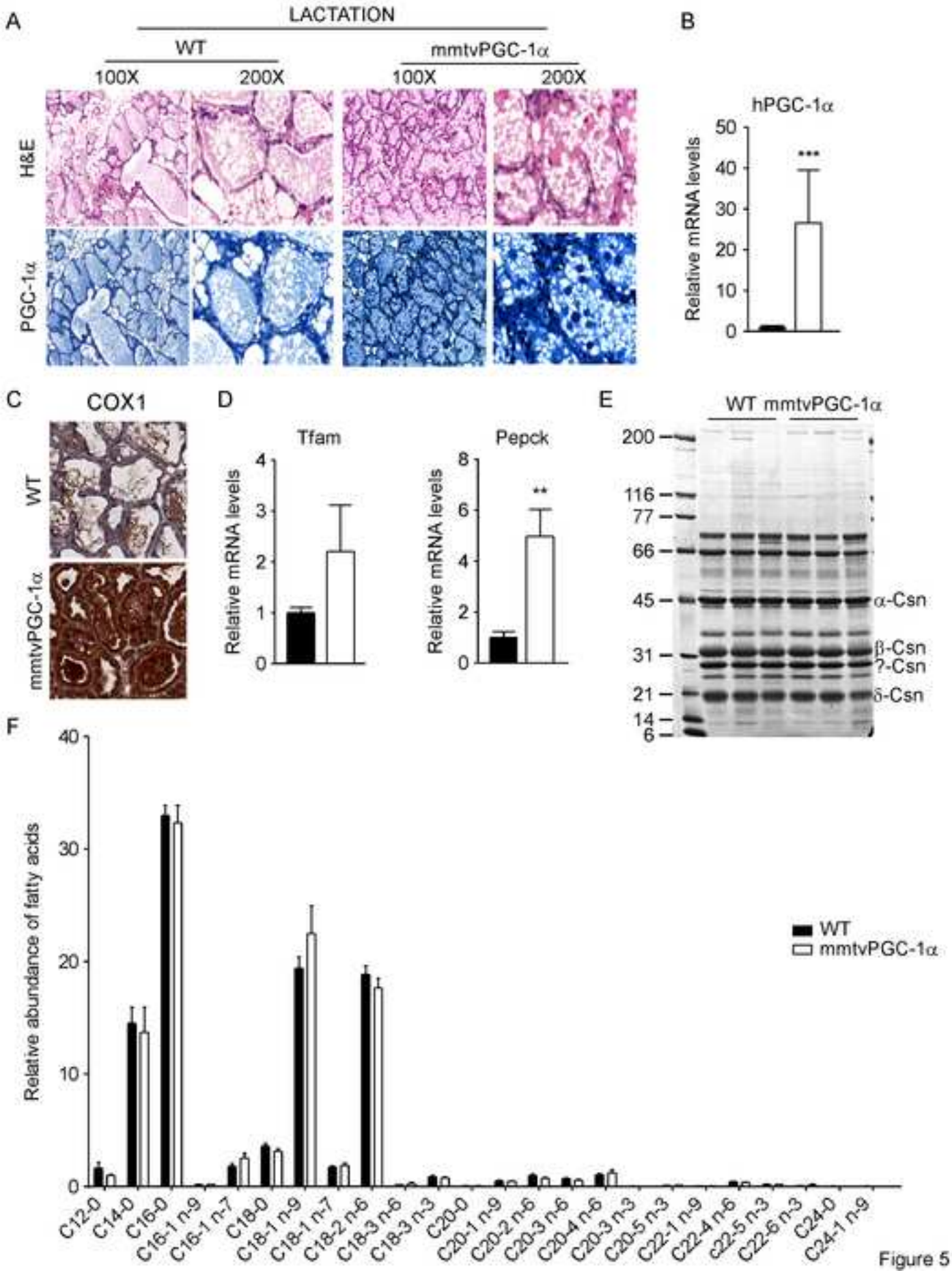

Figure 5

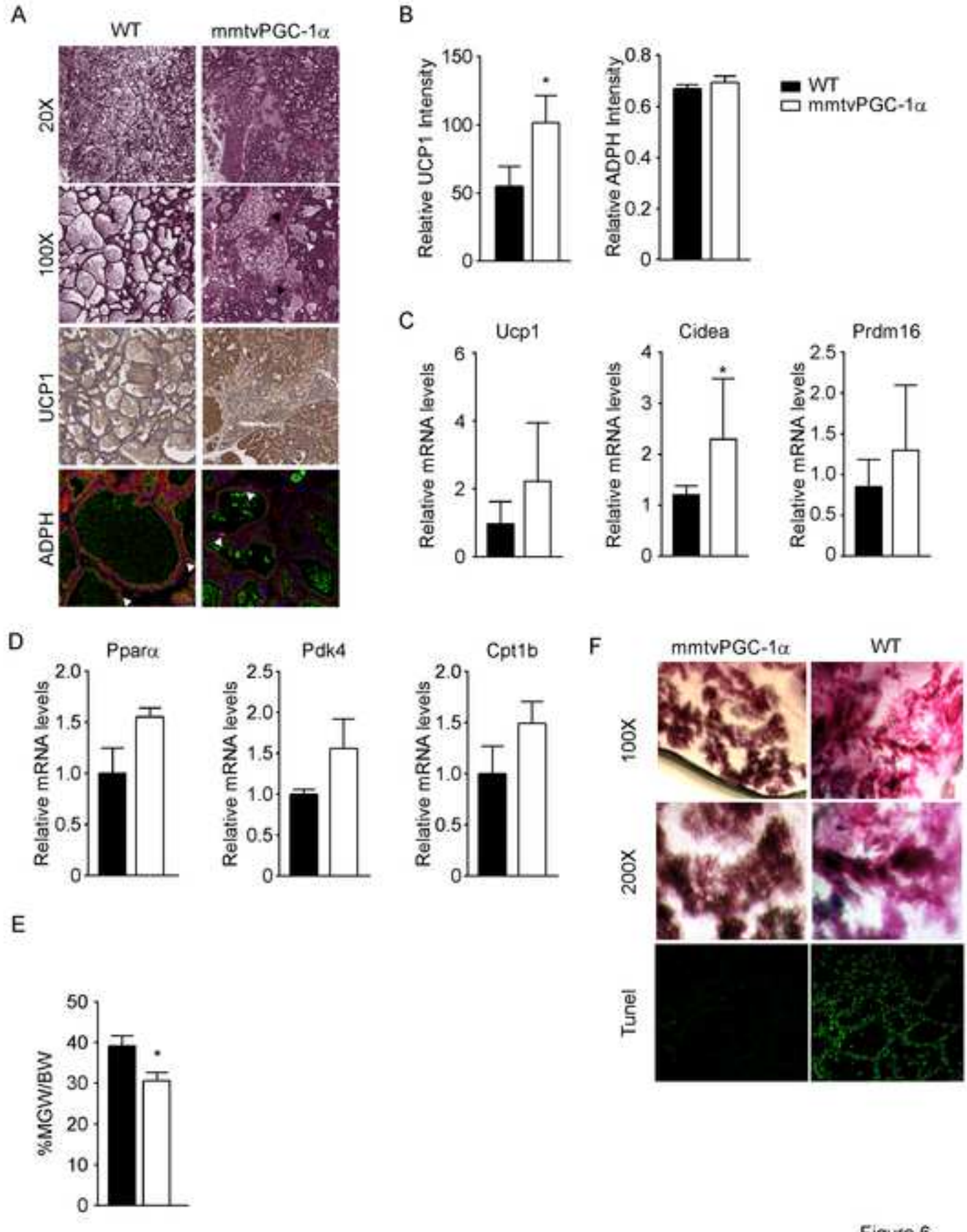

Figure 6

Supplementary Figure 1