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Molecular Aspects of Adipoepithelial Transdifferentiation in Mouse Mammary Gland

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Abstract

The circular, reversible conversion of the mammary gland during pregnancy and involution is a paradigm of physiological tissue plasticity. The two most prominent cell types in mammary gland, adipocytes and epithelial cells, interact in an orchestrated way to coordinate this process.

Previously, we showed that this conversion is at least partly achieved by reciprocal transdifferentiation between mammary adipocytes and lobulo-alveolar epithelial cells. Here, we aim to shed more light on the regulators of mammary transdifferentiation. Using immunohistochemistry with cell type-specific lipid droplet-coating markers (Perilipin1 and 2), we show that cells with an intermediate adipoepithelial phenotype exist during and after pregnancy. Nuclei of cells with similar transitional structural characteristics are highly positive for Elf5, a master regulator of alveologenesis. In cultured adipocytes, we could show that transient and stable ectopic expression of *Elf5* induces expression of the milk component *whcy acidic protein*,

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Author Contributions

A.P.: experimental design, performance of cell culture experiments and gene expression measurements, analysis of the microarray data set and manuscript writing; A.S.: performance and design of morphological analysis, data analysis and interpretation, manuscript writing; J.P.: collection and assembly of data, performance of experiments; M.M.: performance of animal experiments; P.C.: data analysis; E.M.: performance of morphology experiments; K.K.: conception and design, interpretation of data; Z.T.: financial support, conception and design; A.G.: morphological data analysis and interpretation; critical review of manuscript; J.G.B.-S.: financial support and revision of manuscript; S.C.: conception and design, financial support, data analysis and interpretation, manuscript writing, performance of experiments, final approval of manuscript. A.P. and A.S. contributed equally to this article.

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although the general adipocyte phenotype is not affected suggesting that additional pioneering factors are necessary. Furthermore, the lack of transdifferentiation of adipocytes during pregnancy after clearing of the epithelial compartment indicates that transdifferentiation signals must emanate from the epithelial part. To explore candidate genes potentially involved in the transdifferentiation process, we devised a high-throughput gene expression study to compare cleared mammary fat pads with developing, contralateral controls at several time points during pregnancy. Incorporation of bioinformatic predictions of secretory proteins provides new insights into possible paracrine signaling pathways and downstream transdifferentiation factors. We discuss a potential role for osteopontin (secreted phosphoprotein 1 [Spp1]) signaling through integrins to induce adipopoietic transdifferentiation.

Keywords

Cell transdifferentiation; Adipocytes; Mouse mammary gland; Elf5 protein; Spp1 protein

Introduction

The intriguing concept of adipose tissue plasticity has emerged from numerous studies on adipocyte origin and properties that revealed the complex nature of this tissue initially regarded as a connective tissue devoid of physiological functions other than storing of fat and providing cushioning and cold insulation. On the contrary, adipose tissue is distributed over many body depots that are organized to form a large organ with discrete anatomy, specific vascular and nerve supplies, complex cytology, and high physiological plasticity [1–3]. Deeper knowledge of the nature of adipose tissue revealed its involvement in the regulation of many essential functions of the organism and highlighted its versatility [4]. Clinical and experimental studies ascribed the plasticity of adipose tissue to the presence of progenitor cells by showing the capacity of these multipotent cells to differentiate into adipocytes as well as in other mesodermal or even nonmesodermal cell types [5, 6]. At the same time, some studies suggested that the versatility of adipose tissue could also be attributed to the plasticity inherent in mature adipocytes. Mature fat cells are able to dedifferentiate generating lipid-free fibroblast-like cells with reestablished, active proliferation ability, and multipotent capacities [7, 8]. However, dedifferentiation is only one aspect of the plasticity of mature adipocytes, as they also seem capable of directly modifying their specialized phenotype to respond to different physiological needs of the organism [4]. The phenomenon of “browning” provides an example of adipose tissue adaptability that manifests in the emergence of brown adipocytes within classical white adipose tissue depot following adrenergic stimulus, cold exposure, or genetic manipulation [9, 10]. Our own data, in agreement with the results of other laboratories, suggest that most of the brown adipocytes responsible for “browning” derive from a direct transformation of white into brown adipocytes [9, 11–14] to fulfill the thermogenic needs of the organism.

The mammary fat pad, which is commonly considered a simple scaffold for the development of mammary epithelium, offers a further special example of adipose organ plasticity. The mammary gland is a unique organ that undergoes plastic and cyclic changes during the reproductive life of female mammals, especially during pregnancy and lactation when the

mammary subcutaneous depots transform contributing to the formation of milk-secreting glands. This phenomenon, regulated by specific signals like hormones, is characterized by a progressive expansion and specialization of the mammary alveolar epithelium, and a concomitant reduction in the adipose tissue [15–17]. A major working model states that the expansion and maturation of the epithelial portion in the complex mammary microenvironment is ascribed to the proliferation and differentiation of mammary epithelial stem cells generating alveolar structures [18], whereas adipocytes, while playing an active and important role for epithelial differentiation [17, 19], would lose their lipid content during lactation, become depleted, and remain dormant among the epithelial structures. After lactation, the epithelial component of the gland would regress through apoptosis, while a rapid refilling of the slimmed adipocytes would restore the adipose component [15]. However, an alternative/additive model is the pregnancy-induced transdifferentiation of mammary adipocytes into mammary epithelial cells. This model is supported by our results from ultrastructural analyses, highlighting the presence of adipocytes with intermediate aspects between adipocytes and alveolar cells that suggest the occurrence of a process of adipoepithelial transdifferentiation [20]. These data are supported by in vivo lineage tracing studies and ex vivo transplantation experiments [20–22]. Underlying mechanisms of transdifferentiation could be the complex cell–cell and paracrine interactions between variously differentiated epithelial cells and the mammary stroma. These molecular communications affect mammary gland development during pregnancy and define a distinctive extracellular microenvironment [23] that can drastically affect cell behavior [24, 25].

In this study, we provide additional evidence for adipoepithelial transdifferentiation by identifying cells with an intermediate phenotype that are positive for the master regulator of alveologenesis, Elf5 transcription factor [26]. Elf5 is further shown to be able to induce expression of the mammary marker gene *whey acidic protein (Wap)* [26] in cultured adipocytes and therefore established as one of the pioneering factors in adipoepithelial transdifferentiation. To shed more light on the molecular players responsible for this process occurring in the mammary gland during pregnancy, we finally designed a microarray study comparing cleared fat pads (mammary glands without epithelial component) and contralateral mammary tissue in virgin mice and at different stages of pregnancy. This yields a list of potential (paracrine) mediators of adipoepithelial transdifferentiation opening pathways to new discoveries.

Materials and Methods

Mouse Experiments

Animal experimentation was performed using CD1 mice housed in the Animal Facility (University of Ancona). Animal care, handling, and experimentation were performed in accordance with the Italian Institutional Guidelines and the local Ethical Committee. For microarray study, 60 female CD1 mice (3 weeks old) from in-house breeding were used. In each animal, the epithelial component of the fourth right mammary gland was removed to obtain a cleared fat pad, and the fourth left gland was subjected to a simple skin excision for control evaluations. At 10–15 weeks of age, mice that underwent appropriate mammary

epithelial clearing, as revealed by whole-mount analysis [27] were mated and killed on pregnancy days 0, 10, 15, 17, 19 (6 mice/group) to collect tissue samples from cleared and control glands. Collected specimens were sampled for RNA extraction, microarray analysis, and morphological examination.

Immunohistochemistry

Tissue samples harvested from mice after transcardial perfusion with 4% paraformaldehyde/0.1% phosphate buffer, pH 7.4 were fixed at 4°C overnight in the same buffer, dehydrated, cleared, paraffin-embedded, and sectioned. Histological evaluation was performed on hematoxylin/eosin-stained sections. Immunohistochemistry was performed with the avidin–biotin–peroxidase complex (ABC) method [28] using goat-anti-rabbit IgG and rabbit-anti-goat IgG secondary antibodies and Vector's Vectastain ABC Kit (Vector Laboratories, Peterborough, UK) and Sigma Fast 3,3'-diaminobenzidine substrate (Sigma-Aldrich S.r.l. Milan, Italy). Sections were hematoxylin counterstained. Tested primary antibodies: polyclonal rabbit anti-perilipin1, 1:300 dilution, and polyclonal rabbit anti-Adrp (Plin2), 1:150 dilution (both kindly provided by Prof. A. Greenberg, Tufts University, Boston); polyclonal rabbit anti-Wap (R-131)/(sc-25526), dilution 1:400, and polyclonal goat anti-Elf5 (N-20)/(sc-9645), dilution 1:300 (both from SantaCruz Biotechnology, Heidelberg, Germany).

Electron Microscopy

Mammary gland specimens were fixed in 2% glutaraldehyde–2% paraformaldehyde in 0.1 M phosphate buffer, postfixated in osmium tetroxide, dehydrated in acetone, and embedded in epoxy resin. Thin sections were obtained with an MTX ultramicrotome (RMC) and examined under a transmission electron microscope (CM10, Philips).

Microarray Experiments

RNA samples from cleared mammary fat pads and contralateral control tissue harvested on the indicated time points during pregnancy were pooled to yield 2–3 biological replicates and processed for microarray analysis [29]. Briefly, cleared and contralateral samples for each time point were indirectly labeled during reverse transcription and cohybridized on a cDNA microarray in a dye-swap configuration. Hybridization was performed in a 42°C water bath for >16h. Slides were washed and scanned using an Axon scanner. After pin-tip loess normalization [30] replicates were averaged and the data were submitted to NCBI GEO (GEO accession: GSE50447). K-means cluster analysis and generation of heatmaps was performed using Genesis [31].

Functional Annotations and Mappings

For DAVID functional annotation, Gene IDs of differentially regulated gene lists (at least 1.5-fold in one or more time points) were submitted to the DAVID Web site [32]. GO_FAT terms and KEGG pathways were selected for mapping and the top 10 functional clusters were plotted against their enrichment scores (negative logarithm of geometric mean of *p* values of entities in each cluster). Gene-set enrichment analysis [33] was performed with a preranked data set containing all measured transcript on d19 of pregnancy mapped against

the c2_CP gene sets containing pathways from several databases (e.g., KEGG, Biocarta, and Reactome). Pathways with a false discovery rate <0.1 were considered significant. For identification of potentially secreted factors, RefSeq transcript IDs of genes from the upregulated cluster were converted into RefSeq protein IDs using EnsemblBiomart (<http://www.ensembl.org/biomart/>). Those were intersected with entries for *Mus musculus* from the secreted protein database (SPD; [34]) and their FASTA sequences were submitted as batch to SignalP 4.1 [35] to identify signal peptides.

cDNA Cloning

The *Elf5* (NM_001145813) coding sequence was PCR-amplified with coding sequence-flanking primers from mouse mammary tissue cDNA and cloned into a pMSCV mammalian expression vector (Life Technologies, Vienne, Austria) between BglII and XhoI restriction sites using standard procedures. Correct cloning was verified by sequencing of the entire insert.

Cell Culture Experiments

Stably *Elf5* overexpressing 3T3-L1 cells were generated by incubation with supernatant of Phoenix viral packaging cells that were transfected with a pMSCV vector containing the *Elf5* coding sequence. This incubation was followed by 1 week of puromycin selection (3 µg/µl). Control cells were derived in the same way, only empty pMSCV vector was used. All 3T3-L1 cells were propagated and maintained in Dulbecco's modified Eagle's medium (DMEM; high-glucose DMEM, Life Technologies, Vienne, Austria) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Life Technologies, Vienne, Austria). Two days postconfluency cells were induced to undergo adipogenesis by addition of a standard dexamethasone/IBMX/insulin cocktail (DMI) mix (1 µM dexamethasone, 500 µM 3-isobutyl-1-methylxanthine, and 5 µg/ml insulin; Sigma, Vienne, Austria). From day 3 onward growth medium was supplemented with only 1 µg/ml insulin. Where indicated mature adipocytes were treated with a cocktail containing pregnancy hormones (10 µg/ml insulin, 5 µg/ml apotransferrin, progesterone 1 µg/ml, 1 mg/ml hydrocortisone, 1 µg/ml prolactin, 0.88 µg/ml ascorbic acid, and 10 ng/µl EGF as described in [36]) from day 7 to 14. For transient transfection mature 3T3-L1 adipocytes were detached and electroporated with either empty vector (pMSCV) or *Elf5* overexpression construct as described previously [37].

qPCR Analyses

For cell culture samples total RNA was isolated with the GeneElute Mammalian Total RNA kit (Sigma, Vienne, Austria), reverse transcribed with a Qiagen QuantiTect RT kit, and cDNA was then amplified using Sybr QPCR supermix (Life Technologies, Vienne, Austria) on an ABI7000 sequence detection system. Primers used are listed in Supporting Information File 1. Expression values were calculated with an in-house tool [38] using the AnalyzerMiner algorithm [39].

Statistical Analyses

For comparative quantitative polymerase chain reaction (qPCR) measurements upon Elf5 overexpression a two-tailed, unpaired Student's *t*-test was used. A $p < 0.05$ was considered as statistically significant (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). For time series qPCR measurements, a two-way analysis of variance was used to determine significant differences between cleared and contralateral pads (p value given in figure). A posthoc test was not performed due to low number of replicates in some time points. qPCR time series measurements and bar graphs are shown as average $\pm$ SEM from independent experiments (sample size as indicated in figure legends).

Results

Cells With Both Adipocyte and Epithelial Hallmarks Exist in the Mammary Gland During Pregnancy

Perialveolar adipocytes at late stage of pregnancy exhibit light microscopy appearance and ultrastructural features consistent with transdifferentiation of white adipocytes into mammary alveolar epithelial cells [4, 20]. To confirm that these cells were indeed transdifferentiating, we evaluated by immunohistochemistry whether the perialveolar adipocytes express alveolar epithelial markers. Perilipin1 (Plin1) is a lipid droplet associated protein, highly expressed in mature adipocytes, where it coats the large lipid droplet (LD) [40] and possibly regulates basal and stimulated lipolysis [41]. Perilipin2 (Plin2 or ADRP) is a ubiquitously expressed LD-associated protein playing an essential role in LD formation and maintenance in nonadipose cells, including mammary alveolar milk-secreting cells [42, 43]. Studies on LD maturation in the 3T3-L1 cells have shown that the composition of LD-associated proteins changes as LDs enlarge and mature. While Plin2 is found on small LDs in 3T3-L1 preadipocytes and in early differentiating adipocytes, it is absent in fully differentiated adipocytes, where the large and centrally located LD is coated by Plin1 [44]. Thus, in the mammary gland Plin2 is typically found in the alveolar milk-secreting cells [43], whereas Plin1 is expressed by white adipocytes [45]. Immunohistochemical analysis performed on paraffin-embedded serial sections of mammary gland (pregnancy day 18) confirmed that only adipocytes are Plin1 positive (Fig. 1A), while only alveolar epithelial cells are immunoreactive to Plin2 (Fig. 1B). Nevertheless, in some areas where adipocytes showed morphologic aspect of adipoepithelial transdifferentiation (Fig. 1C, 1D) [4, 20], we found isolated perialveolar adipocytes, still retaining positivity for Plin1, but at the same time, clearly immunostained by Plin2 antibodies (Fig. 1A, 1B). This observation suggests that the adipoepithelial transdifferentiation process requires intermediate stages where adipocyte and epithelial markers coexist in the same cell. On the other hand, we found isolated adipocyte-like cells expressing both Plin1 and Plin2 in the early stage of postlactation (Fig. 1E, 1F). Furthermore, in this early stage of postlactation, we found about 10–15% of adipocyte-like cells immunoreactive for Wap (Supporting Information Fig. 1), a marker of milk-producing alveolar cells [46], in line with previous electron microscopic studies showing milk-protein-like granules in about 15% of adipocytes in this postlactation stage [20].

Cells With Intermediate Phenotype Are Elf5 Positive

To confirm that the perialveolar adipocytes are indeed committed to the epithelial phenotype, we evaluated whether they also express the transcription factor *Elf5*, highly specific for secreting epithelia and regarded as a master regulator of mammary alveolar differentiation [47, 48]. As described elsewhere [49], *Elf5* was detected by immunohistochemistry in most ductal and alveolar epithelial cells of late stage pregnancy mammary gland, while the white adipocytes were negative (Fig. 1G). Notably, in some areas in which adipocytes showed transdifferentiation aspects [4, 20] the perialveolar adipose cells exhibited specific *Elf5* nuclear staining (Fig. 1G, asterisks).

Elf5 Is a Pioneering Factor for Milk Gene Expression in Cultured Adipocytes

Our finding that, in late pregnancy, cells with an adipoe epithelial phenotype show strong *Elf5* signals in their nuclei prompted us to ask whether *Elf5* is key in adipoe epithelial transdifferentiation. To address this question, we ectopically expressed *Elf5* in cultured, mature adipocytes, and subsequently treated the cells with a cocktail resembling pregnancy hormone status [36]. Upon successful overexpression of *Elf5* (Fig. 2A), we measured a significant increase in the expression of the canonical *Elf5* target *Wap* as well as the early epithelial marker *Keratin18* (*Krt18*) (Fig. 2B). However, cell morphology (as judged by microscopy, Supporting Information Fig. 2) and adipocyte marker genes (Fig. 2B) were unchanged in comparison with empty vector controls. Considering that in mature adipocytes an interlocked feed-forward loop of transcription factors stabilizes their phenotype [50, 51], the lack of dedifferentiation upon transient *Elf5* expression in adipocytes is not surprising. To assess whether constitutive *Elf5* expression throughout the differentiation process of 3T3-L1 cells has an impact on the development of the phenotype, we used a retroviral system to stably overexpress *Elf5* (Fig. 2C). Similar to the results from transient *Elf5* expression, we saw no changes in cell morphology (Fig. 2D) and adipocyte marker gene expression (Fig. 2E) no matter if the cells were treated with a standard adipogenic cocktail (DMI) or seven additional days with pregnancy cocktail (PC). However, with both protocols, we again observed a robust increase of *Wap* mRNA (Fig. 2E). These results suggest that, although *Elf5* alone is not sufficient to change the adipocyte phenotype in culture, it is one pioneering factor for the expression of mammary epithelial genes.

Epithelial Compartment Is Necessary for Transdifferentiation of Mammary Adipocytes During Pregnancy

Next, we wanted to clarify whether the epithelial part of the mammary gland or the massive change in hormones during pregnancy per se is responsible for the observed adipoe epithelial transdifferentiation. We surgically removed the epithelial part (including the nipple and the rudimentary ductal tree) in 3 weeks old female mice, where the ductal anlage is confined to the proximal part of the mammary fat pad close to the nipple [52, 53]. This leaves a cleared mammary fat pad in its endogenous environment. Whole mount analysis confirmed that the removal of epithelial tissue was complete (not shown). At the age of 10–15 weeks, the tissues were harvested before and 17 days after the start of pregnancy (Fig. 3). Comparing the cleared fat pads to sham-operated contralateral controls at late pregnancy, we note a complete lack of epithelial development in the cleared pad (Fig. 3D). Also electron

microscopy and gene expression data confirmed lack of epithelial differentiation in cleared fat pad (not shown). This supports the notion that the existence of the epithelial ducts is prerequisite for the initiation of the transdifferentiation process and that the hormonal milieu during pregnancy alone is not sufficient to elicit changes in the phenotype of mammary adipocytes.

Microarray Study to Define Adipoepithelial Transdifferentiation Factors

The possibility that Elf5 is present in nuclei of cells undergoing adipoepithelial transdifferentiation and the fact that it is sufficient to induce Wap in cultured adipocytes, lead us to ask which signaling pathways might stimulate the expression of pioneer factors in mammary adipocytes. Additionally, the lack of transdifferentiation of a cleared mammary fat pad suggests that transdifferentiation cues originate from the epithelial part of the mammary gland, possibly signaling via paracrine pathways as reported in other studies [36]. Hence, we used two-color microarrays comparing cleared and contralateral mammary glands before and at 10, 15, 17, and 19 days of pregnancy (Fig. 4A). The use of two-color microarrays should yield a list of potential candidates expressed in the developing normal gland, because the hormone-induced gene expression changes in the fat pad compartment are directly corrected through the competitive hybridization inherent in the technology. To verify the RNA material and the microarray measurements, we used qPCR to determine mRNA levels of the epithelial markers Elf5 and Wap at all time points (Fig. 4B). The expression level of another five genes (*Xdh1*, *Fabp3*, *Mfge8*, *Klf4*, and *Endod1*) were measured with qPCR on day 17 and 19, showing a high correlation between microarray and qPCR data ($R^2 = 0.8$; Supporting Information Fig. 3). K-means clustering of genes from the microarray data set that are at least twofold differentially expressed at one or more time points delivered one cluster with genes with increasingly higher expression levels in the contralateral, developing gland during pregnancy, whereas the other cluster contains genes that show gradually higher expression in the cleared fat pad (possibly due to downregulation in contralateral gland). Figure 5A shows that cluster 1 (upregulated) and cluster 2 (downregulated) contain 619 and 893 unique, differentially regulated transcripts, respectively. Apart from a few exceptions, gene expression is very similar between contralateral and cleared pads in virgin mice (day 0) pointing to the fact that virgin mammary tissue is mostly comprised of adipocytes. The top 20 genes in each cluster are shown as heatmap in Figure 5B. Among the transcripts in the upregulated cluster, we find several epithelial marker genes (e.g., *Mfge8*, *Expi*, *Muc1*, *Keratins*, and *Claudins*) as well as *Plin2* (*Adrp*), which are consistent with our immunohistochemical findings in Figure 1. In cluster 2, a number of transcription factors such as nuclear receptors (*Car*, *Ear-2*, *TR2*, *Lxra*, *Rev-Erba*, and *Pparg*), fibroblast growth factors (*Fgf2* and *Fgf10*), *Stat5a*, *Cebpd*, and *Klf4* are noticeable. Most of these are known adipogenic transcription factors and reduction in their mRNA profiles is plausible because of the massive decrease of adipocyte numbers in the developing contralateral pad. To systematically characterize the data set, we used functional annotation with DAVID [32] and GSEA [33]. Gene ontology (GO; [54]) and pathway (KEGG; [55]) mapping of upregulated transcripts with DAVID functional clustering (Fig. 5C, left) revealed that ribosome-related terms and pathways are by far the strongest enriched categories. This functional cluster contains the KEGG pathway “ribosome”, GO cellular component (CC) “ribosome” as well as the GO biological process (BP) “translation,” all of which hint to the increased demand of

milk protein production in prelactation mammary tissue. Other clusters containing nucleolus- and vesicle-related terms also relate to functional epithelial tissue, because ribosomal RNA is mainly transcribed in nucleoli, whereas vesicle formation is a prerequisite for the secretion of milk protein and lipids by epithelial cells [56]. Ribosomal/translational activation is corroborated by GSEA analysis, which shows a strong enrichment of genes expressed in the contralateral rather than in the cleared gland in the reactome [57] pathway “translation” (Fig. 5D, left). Mapping downregulated genes with DAVID and GSEA reveals the “extracellular matrix (ECM)” as most prominent category emerging from both analyses (Fig. 5C, 5D, both right). It is known that intricate remodeling of the ECM is necessary for mammary alveologenesis [58] and our data suggest that ECM components need to be downregulated for alveologenesis to take place. Furthermore, “cytoskeleton organization,” “vasculature development,” and “cell motility/locomotion” are found when mapping the downregulated genes, indicating that these essential pregnancy processes [59] are continually downregulated during late pregnancy (>day 10). The full lists from DAVID clustering and GSEA analysis are given in Supporting Information File 2. Taken together, the microarray data correctly recapitulate processes occurring during mammary alveologenesis. By comparing developing (contralateral) mammary epithelium with nondeveloping (cleared) mammary fat pad, these data constitute a useful tool to identify factors regulating the transdifferentiation process during pregnancy.

Identification of Candidate Paracrine Transdifferentiation Mediators

Next, we wanted to narrow down the list of transdifferentiation factors to putative paracrine mediators. Therefore, we focused on the transcripts in the upregulated cluster arguing that, during pregnancy, a paracrine factor would signal from the epithelial to the adipose compartment of the mammary gland in order to induce adipoepithelial transdifferentiation. Supporting this, the highest ranking GSEA pathway highly enriched in upregulated genes pertains to “Signal recognition particle (SRP)-dependant cotranslational protein targeting to the membrane” (Fig. 6A, Supporting Information File 2). This reactome pathway describes the first step in the classical secretory pathway where signal peptides of nascent amino acid chains are processed via the signal recognition particle (SPR) for targeting to the membrane or for secretion [60]. Furthermore, the GSEA analysis produced “protein export” as the second ranking KEGG pathway enriched for upregulated genes (Fig. 6B, Supporting Information File 2). Transcripts mapping to both of these pathways (Fig. 6A, 6B) are strongly enriched in the contralateral gland, suggesting an activation of the classical secretory pathway in the epithelial compartment during pregnancy. To more comprehensively assess the secretome of the developing mammary gland, we utilized the SPD [34], a precomputed collection of putative secreted proteins in the mouse genome, and defined the overlap between gene products predicted to be secreted and genes that are upregulated in contralateral versus cleared mammary gland. Additionally, we used SignalP 4.1 [35] to find upregulated genes housing an *N*-terminal signal peptide, which is essential for targeting proteins to the secretory pathway. This combined approach delivered 79 proteins mapped from 63 transcripts whose profiles are shown in Supporting Information Table 1. This list of genes likely contains plausible candidates for factors determining adipoepithelial transdifferentiation. As an example, a known secreted protein whose gene shows the highest expression difference between contralateral and cleared mammary gland

is secreted phosphoprotein 1 (Spp1; also known as osteopontin). Spp1 has been described as necessary for mammary gland alveologenesis in mice [61] and its mRNA levels are reduced in the mammary gland of heterozygous Elf5 knock-out mice with impaired pregnancy-associated mammary gland development [62]. To verify whether the upregulation of Spp1 comes from higher expression in the epithelial compartment or from downregulation in fat pad, we measured the expression profile of *Spp1* along with its potential receptor $\beta 3$ -*integrin* (*Itgb3*) [63] with qPCR (Fig. 6C, 6D). Figure 6C shows a strong upregulation of *Spp1* mRNA in late pregnancy, while the expression levels of *Itgb3* are consistently higher in the cleared mammary fat pad, both indicating that this signaling pathway might present a valuable hypothesis as discussed below in more detail. Therefore, combining our unique microarray data set with bioinformatic predictions of secreted proteins, we derive a highly relevant list of candidates that can be further experimentally scrutinized to reveal paracrine transdifferentiation factors in the mammary gland during pregnancy.

Discussion

During pregnancy and lactation milk-producing epithelial cells develop in the mammary glands [52]. We previously showed that mammary gland adipocytes are able to reversibly transform into milk-secreting epithelial cells [20, 21]. Still, a wide literature supports the predominating model of ductal stem cells as source for milk-producing cells [26, 64, 65]. These potentially conflicting notions could be explained by a bimodal nature of alveologenesis during pregnancy in mice. In the first alveologenic period around days 10–15 most of alveolar cells are devoid of lipid droplets. In later pregnancy (days 15–20), many alveoli are characterized by lipid-rich epithelial cells (Supporting Information Fig. 4) [66]. We propose that these late lipid-rich alveolar cells derive from direct conversion of adipocytes. Hence, it is conceivable that at first alveologenesis is initiated by recruitment of stem cells, while during later pregnancy an adipoepithelial transdifferentiation takes place. This hypothesis is in agreement with our lineage tracing data showing that 70% of the milk-secreting epithelial cells of alveoli derive from conversion of adipocytes, thus leaving the possibility that 30% of alveolar cells derive from ductal stem cells [20].

In this study, we present further evidence for reversible adipoepithelial conversion because adipocytes with morphologic signs of transdifferentiation (i.e., for example cytoplasmic compartmentalization, [20]), expressed proteins that are deemed epithelial-specific. On the other hand, presence of an epithelial marker protein was demonstrated in developing adipocytes in early postlactation stage. The molecular mechanisms underlying this remarkable phenomenon are completely unknown.

The transcription factor Elf5 is known to be crucial for mammary, lobulo-alveolar development during pregnancy [48, 49]. There are several indications suggesting Elf5 as a pioneering factor (as defined in [67]) in the process of adipoepithelial transdifferentiation: (a) Elf5 has been shown to control cell fate decisions of luminal progenitor cells during pregnancy [49] and could therefore also contribute to direct the fate of mammary adipocytes towards an epithelial phenotype. (b) As member of the epithelial-specific (ETS) transcription factor family, Elf5 harbors a pointed domain that is specialized for protein–protein interaction [26] making it likely that Elf5 can bind and recruit chromatin remodelers

to specific genomic loci that need to be activated for transdifferentiation. (c) Even in virgin mice, forced *Elf5* expression in mammary glands induces alveologenesis and milk protein production [49]. Our data showing that *Elf5* is detectable in nuclei of many lipid-rich alveolar cells (immunohistochemistry and electron microscopy of such structures suggested that they are indeed early formed alveoli (see Supporting Information Fig. 4) [4, 66] and that ectopic expression in cultured adipocytes induces transcription of the milk component *Wap* strongly suggest *Elf5* as a pioneering factor. However, upon stable overexpression of *Elf5* throughout the differentiation of 3T3-L1 cells the cell phenotype is not changed as evidenced by lipid staining and marker gene expression. This is still true if mature *Elf5*-expressing adipocytes are incubated with a cocktail mimicking the hormone status during pregnancy. Therefore, it is likely that in addition to *Elf5* other factors are necessary to induce adipoepithelial transdifferentiation. One interesting example is *GATA3* which is among the upregulated genes in our data set. *GATA3* is responsible for the development and maintenance of luminal epithelial cells in the mammary gland [68], and forced expression of *GATA3* suppresses adipocyte differentiation [69]. Also *GATA3*, along with other proteins of the GATA family, is known as pioneering factor able to bind condensed chromatin and thereby determine cell fate [67]. Hence, a combinatorial overexpression of *GATA3*, *Elf5*, and possible other factors in adipocytes might be a valuable experimental approach to define the minimal set of transdifferentiation factors, as performed in other studies [70, 71].

The roles of prolactin and progesterone for alveologenesis in pregnancy are well-studied and mammary adipocytes express receptors for both of these hormones [72, 73]. Still, the lack of lobuloalveolar development during pregnancy in mammary fat pads with removed ducts clearly points to the importance of signaling through paracrine factors eventually produced by the ductal epithelium and suggests that these signals are pivotal steps for adipoepithelial transdifferentiation. Although the effects of the hormones prolactin and progesterone are shown to be relayed to the transcriptional level via *Elf5* in luminal progenitors [26], the signaling mechanisms by which these hormones and/or unknown paracrine factors activate *Elf5* remain elusive.

Our microarray studies comparing gene expressions between cleared fat pad and normal gland at different time points of pregnancy reveal possible candidates for paracrine factors acting from epithelial cells towards adipocytes. *Spp1* (also known as osteopontin) seems to be of particular interest for several reasons. It is progressively expressed during pregnancy and is produced and secreted by epithelial cells [74]; its knockdown in mammary epithelial cells results in a failure to undergo alveologenesis [61], while its targeted transgenic expression drives alveologenesis [75]. Furthermore, *Spp1* is a sibling protein acting on the *Itgb3* receptor (*Itgb3* or CD61 [63]), which is widely considered as a marker of alveolar cell progenitors [76, 77]. We found that *Itgb3* is highly expressed in mammary adipocytes throughout pregnancy making this signaling pathway an intriguing candidate in the search for molecular mechanisms of transdifferentiation.

Conclusion

Collectively, this work adds further experimental evidence for in vivo adipoepithelial transdifferentiation in the mouse mammary gland, pointing to *Elf5* as one pioneering factor

for the induction of milk proteins in adipocytes. Additionally, it provides a list of potential paracrine factors signaling from epithelial cells of the transforming mammary gland during pregnancy towards mammary adipocytes.

Cell lineage commitment and differentiation have traditionally been regarded to be unidirectional and irreversible. Transdifferentiation, however, allows differentiated cells to switch to another cell type. First observed in the retinal pigment epithelium of several species of amphibians to replace extirpated lens cells [78], transdifferentiation has subsequently been reported in several in vitro settings, in pathological conditions (notably cancer), and during development [79–81]. The reversible transdifferentiation of white adipocytes into mammary epithelial alveolar cells in the mouse subcutaneous adipose organ during pregnancy is a remarkable example of transdifferentiation occurring in the normal, adult animal and under physiological stimuli. In this context, transdifferentiation is emerging as a distinctive aspect of the adipocyte biology, possibly representing a fast, safe and/or less expensive mechanism through which the mammalian adipose organ plastically changes structure and function to fulfill a range of metabolic and environmental challenges. This is exemplified by the white into brown adipocyte transdifferentiation observed in fat in response to cold exposure [9–11, 14]. Dissecting the molecular pathways involved in adipocyte transdifferentiation and reprogramming is expected to offer important insights for biomedical applications, such as regenerative medicine and cell-based disease modeling. Furthermore, the reversible transdifferentiation of adipocytes into mammary secretory alveolar cells also have the potential to shed light on breast cancer biology, as suggested by recent data showing that loss of PPARc, the master regulator of adipocyte differentiation [82], by mammary secretory epithelial cells creates a pro-breast tumorigenic environment [83].

Supplementary Material

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Figure 1.

Mouse mammary gland histology in serial sections, pregnancy day 17 (A–D) and day 1 postlactation (E, F). Plin1 (perilipin1) is adipocyte-specific (A) and Plin2 (perilipin 2 also called ADRP) is epithelial specific (B). Some structures with intermediate features between adipocytes and alveoli (compartmentalized adipocytes or early alveoli) are also present and marked for both proteins (squared areas and enlarged in insets), see also (C) as an example of early alveolus and (D) as an example of compartmentalized adipocyte (stage of adipoeithelial conversion, see also ref. 20). (C, D): High magnification of a resin embedded

gland (allowing more detailed morphology than paraffin embedded tissue) comparable to that shown in (A) and (B). (E, F): Mouse mammary gland histology in serial sections on first postlactation day. A rare adipocyte (enlarged in insets) is marked by both proteins. **(G):** Mammary gland at 17th day of pregnancy: anti-Elf5 antibody revealed positive nuclei in alveolar–glandular epithelial cells (lu in the lumen of glands) and in a group of small adipocytes nearby (some indicated by asterisks); larger adipocytes (a) were negative as well as all other cell types in the tissue (see negative blue nuclei of capillaries). Transcription factor Elf5 is considered a master regulator of alveologenesis. Scale bar = (A, B, E, F) 70 μm (inset 23 μm); (C, D) 15 μm ; (G): 12 μm . Abbreviations: Elf5, ETS transcription factor 5; lu, lumen of gland; Plin2, perilipin 2.

Figure 2.

Forced expression of *Elf5* in 3T3-L1 adipocytes increases whey acidic protein (*Wap*) expression, while adipogenic markers stay unchanged. (**A**, **B**): 3T3-L1 cells were differentiated into adipocytes for 7 days. Cells were detached and electroporated in the presence of either empty vector (pMSCV) or *Elf5* overexpression vector (pElf5). Cells were then kept on pregnancy hormones for 48 hours and harvested for RNA isolation to determine expression of *Elf5* (**A**) and epithelial and adipogenic marker genes (**B**) ($n = 4$). (**C**): *Elf5* mRNA expression. 3T3-L1 cells stably expressing *Elf5* were differentiated for 7 days with

standard dexamethasone/IBMX/insulin cocktail (DMI). Where indicated, cells were treated an additional 7 days with a cocktail containing pregnancy hormones (PC) ($n = 3$). **(D):** Phase contrast microscopy of Elf5 overexpressing and control cells after 7 days of DMI cocktail or additional 7 days with pregnancy cocktail. Magnification: $\times 40$. **(E):** Expression of epithelial and adipogenic marker genes ($n = 3$). All quantitative polymerase chain reaction measurements were normalized to Uxt expression and related to empty vector control and a two-tailed, unpaired Student's t -test was used to determine statistical significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Abbreviations: DMI, dexamethasone/IBMX/insulin cocktail; Elf5, ETS transcription factor 5; PC, pregnancy cocktail; pMSCV, empty vector.

Figure 3. Control gland and cleared mammary fat pad comparative hematoxylin/eosin histology. (A, C): Normal glands in virgin (A) and 17 days pregnant (C) animals. (B, D): Cleared fat pad in virgin (B) and 17 days pregnant (D) animals. Scale bar = 80 μ m in all panels. Abbreviation: WAT, white adipose tissue

Figure 4.

(A): Sampling and experimental setup for the microarray study. “Surgical intervention” refers to the removal of the ductal anlage in the fourth mammary gland from 3 weeks old CD1 female mice. Before and on four time points during pregnancy, 5–6 mice for each time point were killed and tissues were harvested and prepared for gene expression analysis. (B): *Elf5* and whey acidic protein (*Wap*) expression was measured from the same samples used for microarray analysis. Quantitative polymerase chain reaction measurements ($n = 2-3$) were normalized to *Uxt* expression and a two-way analysis of variance was used to determine significant differences between cleared and contralateral over all time points (p value is given in figure). Abbreviations: *Elf5*, ETS transcription factor 5; GSEA, Gene Set Enrichment Analysis; SPD, secreted protein database; *Wap*, whey acidic protein.

Figure 5.

Bioinformatic analyses of the microarray data set. **(A)**: Two clusters resulting from k-means clustering depicting genes that are either upregulated (cluster 1) or downregulated (cluster 2) for more than twofold on at least one time point during pregnancy. y -Axis shows $\log_2$ expression values. **(B)**: Heat maps indicating expression levels ($\log_2$), RefSeqIDs and NCBI gene symbols of top 20 regulated genes. **(C)**: DAVID functional clustering was performed with genes upregulated (left panel) or downregulated (right panel) focusing on GO terms and KEGG pathways. Shown are the top 10 enriched clusters (left of bars with contributing

DAVID domains in parentheses). The x -axis shows the cluster enrichment scores representing a geometric mean ($-\log$) of p values of entities in each cluster. **(D)**: GSEA enrichment plots are generated by preranking all genes from d19 of pregnancy by expression values, mapping it against the c2_CP gene sets containing pathways from several databases (e.g., KEGG, Biocarta, and Reactome), and calculating a cumulative enrichment score (green line). Pathways with a false discovery rate (FDR) <0.1 were considered significant. Abbreviations: BP, biological processes; CC, cellular components ECM, extracellular matrix; FDR, false discovery rate; GSEA, Gene Set Enrichment Analysis; MF, molecular functions; NES, normalized enrichment score.

Figure 6.

Enrichment of upregulated genes in pathways involved in protein secretion. **(A, B):** Gene set enrichment analysis as in Fig. 5D. **(C):** Secreted phosphoprotein 1 (*Spp1*) and **(D)** β -intergrin (*Itgb3*) mRNA expression was measured from the same samples used for microarray analysis. Quantitative polymerase chain reaction measurements ($n = 2-3$) were normalized to Uxt expression and a two-way analysis of variance was used to determine significant differences between cleared and contralateral over all time points (p value is given in figure). Abbreviations: FDR, false discovery rate; *Itgb3*, β -intergrin; KEGG, Kyoto Encyclopedia of Genes and Genomes pathway; NES, normalized enrichment score; *Spp1*, secreted phosphoprotein 1; SRP, signal recognition particle.