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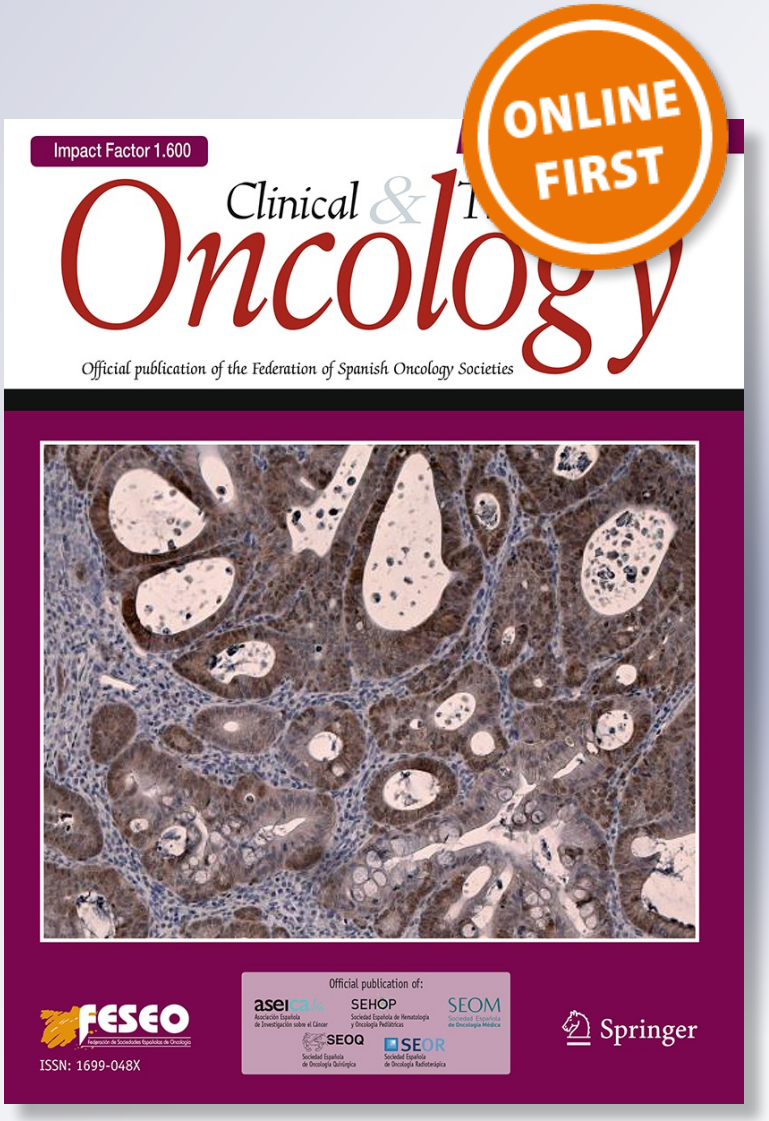

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RESEARCH ARTICLE

GAL3ST2 from mammary gland epithelial cells affects differentiation of 3T3-L1 preadipocytes

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Abstract
Introduction In the mammary gland, the involution that occurs when lactation ends is an important period for cancer development. We have previously demonstrated stromal–epithelium interactions evaluating conditioned medium of adipose tissue on breast epithelial metalloproteases activity (Creydt et al., Clin Transl Oncol 15:124–131, 2013). Here, we evaluated the effects of conditioned medium of breast epithelial mammary cells on stromal cells.
Materials and methods Conditioned medium from normal murine mammary gland cell line (NMuMG) and conditioned medium proteins were obtained. Then, they were evaluated on modulation of adipocyte differentiation, using 3T3-L1 cell line.
Results We described, for the first time, that breast epithelial mammary cells could produce the enzyme galactose 3-*O*-sulfotransferase 2 (GAL3ST2). Importantly, GAL3ST2 is present in NMMuMG and two human breast cancer cell lines, and it is more strongly expressed in more metastatic tumors. When 3T3-L1 preadipocyte differentiation was triggered in the presence of conditioned medium from NMuMG or GAL3ST2, triglyceride accumulation was decreased by 40 % and C/EBPβ expression by 80 % in adipocytes. In addition, the expression of FABP4 (aP2), another marker of adipocyte differentiation, was inhibited by 40 % in GAL3ST2-treated cells.
Conclusions Taken together, these results suggest that GAL3ST2 would interfere with normal differentiation of 3T3-L1 preadipocytes; raising the possibility that it may affect normal differentiation of stromal preadipocytes and be a link to tumor metastatic capacity.

Keywords Adipocytes · Breast mammary gland · Triglycerides · Cancer · Cell lines

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Introduction

The role of the microenvironment has become increasingly important in studies of breast cancer initiation and progression [1]. Consequently, the mammary gland can no longer be simply viewed as an organ composed of epithelial cells within a passive stromal microenvironment. Many studies have reinforced the notion that mammary epithelial cell growth, differentiation, lactation, and progression to cancer involve bidirectional interactions between epithelial cells and their surrounding stroma [2].

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Stromal and epithelial cells share positive and negative interactions, which are regulated by possibly paracrine signaling. Fibroblasts have recently been associated with tumors capable of increasing the secretion of the metallopeptidase [3]. In addition, fat cells have been associated with tumors overexpressing metalloproteinase matrix 11 and inflammatory cytokines, such as IL-6 and IL-1β [4]. These changes in protein expression are also accompanied by a decrease in adipogenic markers, inhibition of lipid accumulation, and a decrease in the expression of FABP4 (aP2), an adipocyte normal protein marker. Adipocyte dedifferentiation appears to be an important aspect of tumorigenesis [5]. In mice, expression of the adipogenic transcription factor PPARγ is attenuated during mammary tumorigenesis [6].

Adipocytes in close proximity to epithelial cells undergo a more rapid depletion of their lipid stores than those in the cleared mammary fat pad or in distal areas of the fat pad, which are separated from the mammary parenchyma [7]. Hence, both local and systemic factors influence lactation-induced lipolysis in mammary gland adipocytes. Epithelial cells are known to induce lipolysis in adjacent mammary adipocytes during lactation via lipoprotein lipase. Yet, the interactions between preadipocytes and other types of cells in the surrounding microenvironment during adipocyte differentiation remain unclear.

Differentiation of preadipocyte cell line 3T3-L1 was completely inhibited by co-culturing 3T3-L1 cells with breast cancer cell lines (T47D, MCF-7, SSC202, SSC78, and SSC30) or with their conditioned medium; in contrast, normal human primary epithelial breast cells did not produce this effect [8]. Here, we describe for the first time that we found galactose 3-*O*-sulphotransferase 2 (GAL3ST2) in normal murine mammary gland (NMuMG) cell line-conditioned medium, and describe a potential role for this protein as a contributor to adipocyte–epithelial cell interactions. Recent studies suggested that GAL3ST2 is linked to increase tumor metastatic potential by inhibiting integrin expression and decreasing selectin adhesion [9]. Considering the important role of integrins in cancer biology [10] and how a protumoral environment is established remain unknown; we also decided to evaluate GAL3ST2 expression in some cancer cell lines.

Materials and methods

Cell cultures and drugs

3T3L1 (mouse preadipocyte cell line), NIH 3T3 (mouse fibroblast cell line), NMuMG (normal mouse mammary epithelial cell line), MCF-7 and MDA-MB-231 (human breast cancer epithelial cell lines), BHK-21 (normal baby hamster fibroblast cell line) and HEK 293 (embryonic kidney) were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured at 37 °C with 5 % CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 25 mM glucose and 10 % fetal bovine serum.

Cell-conditioned medium

Conditioned medium was collected from NMuMG, NIH3T3 and 3T3-L1 cell cultures. Cells were allowed to achieve confluence, then cultures were changed to a medium without serum and this conditioned medium was collected every 4 days during 8 days. The medium was then transferred to a dialysis membrane and immediately concentrated tenfold using solid polyethylene glycol and filter sterilized (0.2 μm membrane pore). The medium was then aliquoted and kept frozen at −20 °C until used. Only to evaluate serum influence on our system, we collected NMuMG-conditioned medium from cells growing with FBS 10 %, this medium was also concentrated tenfold and sterilized as above described, then it kept frozen at −20 °C until used.

Column chromatography

NMuMG-conditioned medium from cell growth in the absence of serum was fractionated in a Sepharose-12 Fast Performance Liquid Chromatography (Pharmacia Biotech), equilibrated and eluted with 0.01 M phosphate saline buffer (pH 7). The column was calibrated using bovine serum albumin (67,000), ovalbumin (43,000), chymotrypsinogen A (25,000), and ribonuclease A (13,700) as standards for determining the apparent molecular weights of the sample proteins. Eluates were evaluated for protein content at 280 nm and stored at −20 °C. The fractions were thawed and filter sterilized for Tg adipocyte differentiation experiments.

3T3-L1 adipocyte differentiation

The differentiation protocol is developed by culturing 3T3-L1 pre-adipocytes in MDI media (FBS 10 %—DMEM supplemented with 0.5 mM 3-isobutyl-1-methyl xanthine, 0.1 μM dexamethasone, and 2 μM insulin) for 48 h, then transferred to FBS 10 %—DMEM with 2 μM insulin and incubated for 3 days. Cells were finally cultured in DMEM (10 % FBS) for the remainder of the experiment (for 12–14 days). MDI medium addition is considered day 0 of differentiation protocol. At day 10 of differentiation protocol, the cells are fully differentiated and showed 70–80 % cells with refractive lipid droplets observable microscopically [11, 12].

MDI-treated cells were considered differentiating cells (DC), and vehicle-treated cells were considered control cells (CC) or undifferentiated cells. NMuMG-conditioned medium, TgIF, protein extract from GAL3ST2-transfected cells (GAL3ST2), column protein obtained from GAL3ST2-transfected cells protein extract (GAL3ST2p), or NIH3T3 and 3T3-L1 conditioned media were added at day 0 of differentiation to the MDI medium. As indicated, cells were then washed once with 0.01 M phosphate-buffered saline (PBS) at room temperature, scraped into 0.5 mL 0.01 M phosphate-buffered saline, and frozen until triglyceride determination was performed. For Western blots and protein determination, scraped cells were lysed with a buffer containing 1 % SDS in 60 mM Tris–HCl, boiled for 10 min, and centrifuged at 15,000 rpm at 4 °C for 10 min. Then samples were resuspended in buffer supplemented with protease inhibitor mixture (Thermo protease inhibitor cocktails).

MALDI-TOF

We performed two-dimension electrophoresis gel to separate proteins from column fractions, then we used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) to identify the proteins. MALDI-TOF experiments were developed at CEQUIBIEM—Universidad de Buenos Aires.

Transfection assays

(a) The vector pSVK3 was used to express GAL3ST2 cDNA between *Eco*RI and *Sal*I sites [13]. The pSVK3–GAL3ST2 was amplified in competent cells (*E. coli*, JM strain) by heat shock transformation. Next, 100,000 BHK-21 cells were transfected with pSVK3–GAL3ST2 or pSVK3 using Lipofectin (Lipofectin Reagent 1 mg/mL, Invitrogen) in the absence of serum. 72 h after transfection, cells were trypsinized, and washed with 0.01 M PBS, and then lysed using sterile buffer containing Triton 0.1 %, which was followed by two cycles of freezing and thawing. Next, the samples were centrifuged for 5 min at 10,000*g*. The resulting supernatant was then filtered using a 0.22-μm membrane pore and used to evaluate the effects of GAL3ST2 in adipogenesis. The presence of GAL3ST2 was verified by Western blots analysis using anti-GAL3ST2 (ab 107767-Abcam).

(b) The vector pcDNA4/V5-His C (Invitrogen^{TM}) was used to express GAL3ST2 cDNA between *Eco*RI and *Xho*I. The pcDNA4/GAL3ST2-V5-His was amplified in competent cells (*E. coli*, JM strain) by heat shock transformation. Next, 100,000 HEK 293 cells were transfected with pcDNA4/GAL3ST2-V5-His (GAL3ST2p) or pcDNA4/V5-His C (MOCK) using Lipofectamine (Lipofectin Reagent 1 mg/mL, Invitrogen) in the presence of serum. 24 h after transfection, cells were trypsinized, washed with 0.01 M PBS and frozen at −70 °C until use.

Column purification system

We used Probond purification system for purification of polyhistidine-containing recombinant proteins (Invitrogen^{TM}). Briefly, we resuspended the cells in native binding buffer (26 mM NaH_2PO_4 and 0.5 mM NaCl, pH 8) containing 0.5 μg/mL Leupeptin; then, we lysed them by two freeze–thaw cycles using dry ice/ethanol bath and a 42 °C water bath; then we centrifuged the lysate at 3,000*g* for 15 min to pellet the cellular debris and used the supernatant to protein purification. We prepared Pobrond column according to manufacture instructions, and added the supernatant obtained under native conditions to prepared purification column and allowing protein binding during 60 min. After washing with native binding buffer, we eluted the proteins with native elution buffer (native binding buffer plus 0.25 M imidazole, pH 8), and collected 1 mL fractions. They were analyzed by western blot or stored at −20 °C until use. Cell extracts from HEK 293 cells transfected with pcDNA4/GAL3ST2-V5-His (GAL3ST2p) or pcDNA4/V5-His C (MOCK) were evaluated.

Tg and protein level determinations

Tg accumulation was assessed using the TG color GPO/PAP AA kit (Wiener Laboratory, Rosario, Argentina). Tg content is expressed as g Tg/g protein. Proteins were quantified by the Bradford method using crystalline bovine serum albumin as a standard [14].

MTT assay

A MTT [Bromide 3-(4,5-dimethylthiazol-2-yl)-2,5-difeniltetrazol] viability assay was performed to evaluate the toxicity of GAL3ST2 treatment during adipogenic differentiation [15]. Cells were seeded at 20×10^4cells/well; MDI-treated cells were considered differentiating cells (DC), and vehicle-treated cells were considered control cells (CC). MDI treatment in the presence or absence of GAL3ST2 was developed. At indicated times, MTT assay was performed. 300 μL of MTT was added to each well. After incubating for 1 h, the media were removed, and the cells were treated with ethanol for 10 min. The absorbance was measured at 550 nm; the absorbance of control cells

(CC) was considered 100 %, and the results are presented as percentage of CC.

Western blots

Proteins (10 μg from each sample) were separated on a 10 % SDS–polyacrylamide gel and transferred to nitrocellulose membranes (Amersham, GE Healthcare). Membranes were soaked in blocking buffer (0.1 % BSA, 0.4 % Tween, and 1 mM EDTA in 0.01 M PBS) for 1 h and incubated overnight with a primary antibody at 4 °C. Membranes were probed with horseradish-conjugated secondary antibodies (Santa Cruz Biotechnology) and then treated with an enhanced chemiluminescence (ECL) substrate kit (Amersham ECL Plus Western Blotting Detection System, GE Healthcare). The primary antibodies were anti-FABP4 (Eurogentec, Seraing, Belgium), anti-GAL3ST2 (ab 107767-Abcam), anti-C/EBPβ (Santa Cruz Biotechnology) and anti-V5 (Invitrogen). Protein expression was normalized to GAPDH expression.

Samples were obtained at days 0 (CC), 3, and 5 of the differentiation protocol in the presence and absence of conditioned medium (F), TgIF, GAL3ST2 and GAL3ST2p.

Statistical analysis

Results are expressed as mean ± SD. Statistical analysis was performed by one-way analysis of variance followed by post hoc analysis [16]. For MTT, Tg and protein, results are shown as the average of four independent experiments [mean ± standard deviation (SD)]. For western blots, results are shown as the average of three independent experiments (mean ± SD).

Results

3T3-L1, a preadipocyte cell line, is able to differentiate into adipocytes following a standard protocol with MDI, and by day 10 of differentiation protocol 70–80 % of cells are fully differentiated showing refractive lipid droplets, as previously shown [11]. We have previously showed that conditioned medium from cultured NMuMG cells, which were grown in media with 10 % of serum (F-10 %), contained specific protein fraction which inhibited triglyceride (Tg) accumulation in 3T3-L1 adipocytes [17]. With the aim to evaluate a possible effect of serum in NMuMG-conditioned medium composition responsible for the reduction in the adipogenic potential of 3T3-L1 preadipocytes, we cultured NMuMG cells in media supplemented with 0 % of serum (F) or 10 % of serum (F-10 %), and no significant difference in the inhibition of Tg content was observed [day 10: 0.59 ± 0.3 g Tg/g protein (F) vs. 0.50 ± 0.1 g Tg/g protein (F-10 %)]. Therefore, we decided to evaluate conditioned medium from cultured NMuMG cells which were grown in the absence of serum (F) on Tg accumulation during 3T3-L1 preadipocytes cell differentiation.

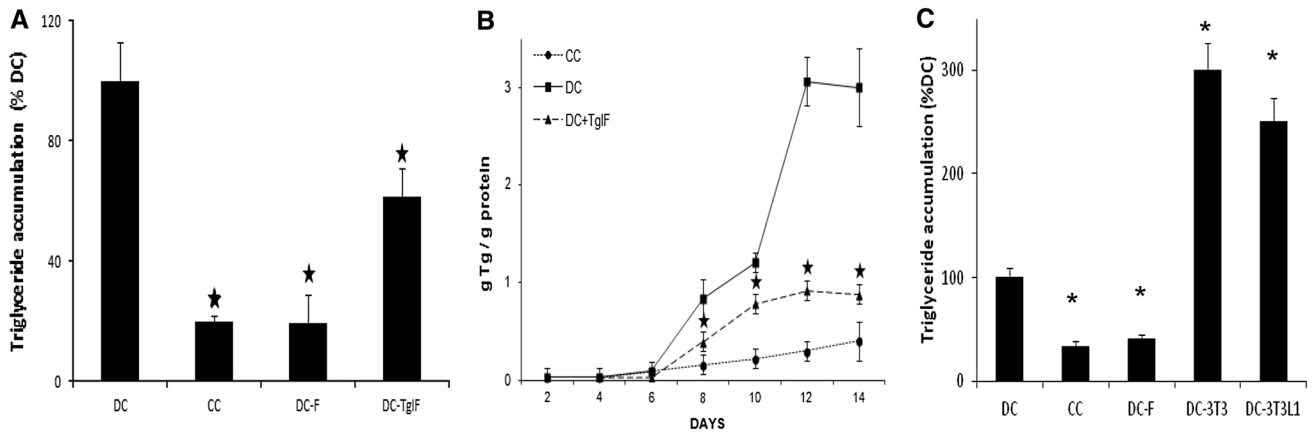


Fig. 1 Triglyceride accumulation in 3T3-L1 during adipogenic differentiation. **a** Triglyceride accumulation at day 10 of adipogenic differentiation: Tg content in control cells (CC), MDI-treated cells (DC), MDI plus NMMuG-conditioned medium-treated cells (DC-F), or MDI plus TgIF-treated cells (DC-TgIF). Both conditioned medium (M) and chromatographic protein fraction TgIF (DC-TgIF) were added at day 0 of differentiation protocol and replaced every 2 days during 10 days. The results are the average of four independent experiments (mean ± SD), $*p < 0.01$, CC, DC-F or DC-TgIF vs. DC. **b** Triglyceride accumulation during adipogenic differentiation: TgIF was added at day 0 and replaced every 2 days for 14 days (DC + TgIF). The results are the average of four independent experiments (mean ± SD), $*p < 0.01$, DC + TgIF vs. DC. **c** Triglyceride accumulation at day 10 of adipogenic differentiation: Tg content in control cells (CC), MDI-treated cells (DC), MDI plus NMMuG-conditioned medium-treated cells (DC-F), MDI plus NIH3T3-conditioned medium-treated cells (DC-3T3) or MDI plus 3T3-L1-conditioned medium-treated cells (DC-3T3L1). All conditioned media were added at day 0 of differentiation protocol and replaced every 2 days during 10 days. The results are the average of four independent experiments (mean ± SD), $*p < 0.01$, CC, DC-F, DC-3T3 or DC-3T3L1 vs. DC

We observed that optimal protein concentration in the medium F for maximum inhibition Tg accumulation was 28 μg/mL. More than 70 % decrease in Tg accumulation was observed in MDI plus NMuMG-conditioned medium-treated cells (DC-F) compared to the MDI-treated cells (DC) (Fig. 1a). After column fractionation of medium F, we obtained a fraction, which we named as Tg inhibitor factor (TgIF), able to inhibit nearly 40 % of Tg accumulation by day 10 (Fig. 1, DC-TgIF). When a kinetic study was developed, the strongest inhibitory effect was observed at day 12 (Fig. 1b). We also evaluated NIH 3T3 fibroblast-conditioned medium and 3T3-L1-conditioned medium on cell differentiation protocol, both increased Tg accumulation in the cells (Fig. 1c).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) was used to identify proteins responsible for blockade of adipogenesis, and GAL3ST2 was found to be a component of TgIF protein fraction (Fig. 2a). Then, we hypothesized that it may be responsible for inhibiting Tg accumulation. To test this possibility, GAL3ST2 was overexpressed in BHK-21 cells by transfection, and protein extracts of these cells were then used as source of GAL3ST2 (Fig. 2b). SW480 human colon cell line that endogenously expresses high levels of GAL3ST2 is shown as a positive control. We evaluated GAL3ST2 expression during differentiation protocol and observed that this enzyme is not present in any condition (Fig. 2c); therefore we could suggest that 3T3-L1 did not produce GAL3ST2.

When 3T3-L1 preadipocytes were induced to differentiate in the presence of lysates of cells that overexpressed GAL3ST2, a reduction more than 40 % of Tg accumulation was detected (Fig. 3a) [2.50 ± 0.51 protein (DC) vs. 1.05 ± 0.15 g Tg/g protein (DC-GAL3ST2), $p < 0.01$]. Importantly, the MTT assay used to evaluate possible cytotoxicity of GAL3ST2-enriched protein extract treatment did not exhibit any cytotoxic effect on 3T3-L1 cells (Fig. 3b). Further, as shown in Fig. 3c, no phenotypic differences were observed between cells differentiated in the presence or absence of GAL3ST2 (panel DC-GAL3ST2 vs. MOCK and DC). It is well known that upon induction of adipocyte differentiation of 3T3-L1

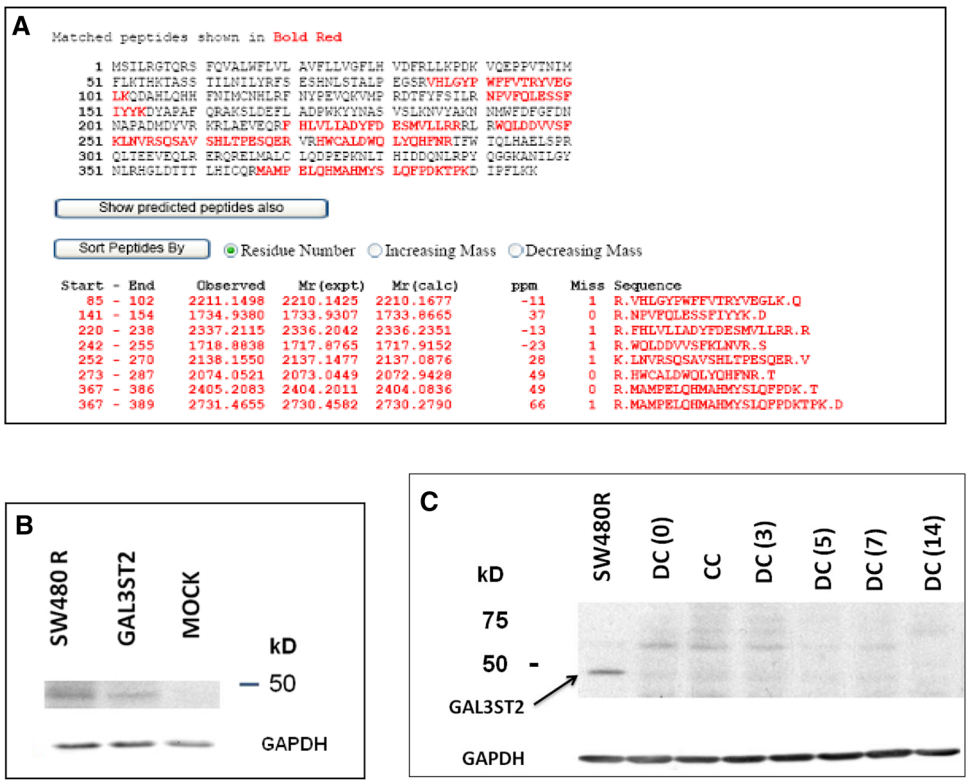
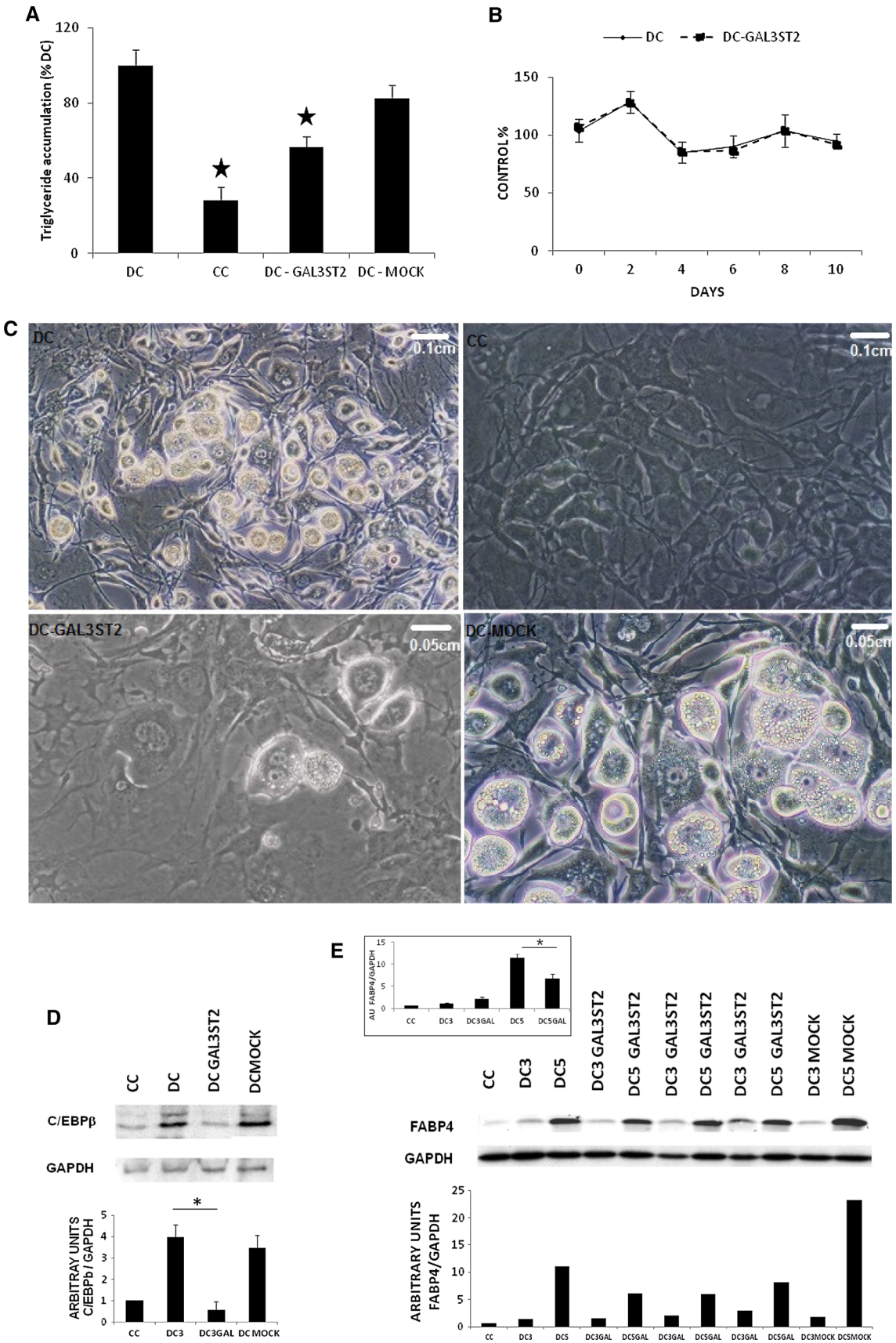


Fig. 2 Identification of GAL3ST2. **a** Tryptic peptides pattern obtained from TgIF, detected and analyzed by MALDI-TOF. Result: presence of galactose 3-*O* sulfotransferase 2. **b** GAL3ST2 expression in protein extracts: GAL3ST2 protein is detected in protein extract from: SW480R (human colon), GAL3ST2 (protein extract from BHK-21 cells, transfected with pSVK3-GAL3ST2), MOCK (protein extract from BHK-21 transfected with empty vector pSVK3). Representative results from one of the three independent western blot experiments with similar results are shown. **c** GAL3ST2 expression in 3T3-L1 protein extracts: GAL3ST2 protein is detected in protein extract from: SW480R (human colon), CC (control cells), and DC(0), DC(3), DC(5), DC(7), DC(14) (MDI-treated cells, the cells were harvested at indicated times: 0, 3rd, 5th, 7th or 14th day of differentiation protocol). Representative results from one of the three independent western blot experiments with similar results are shown



◀**Fig. 3** GAL3ST2 effect on 3T3-L1 cells during adipogenic differentiation. **a** Triglyceride accumulation in 3T3-L1 at day 10 of adipogenic differentiation in control cells (CC), MDI-treated cells (DC), MDI plus GAL3ST2-treated cells (DC-GAL3ST2), or MDI plus MOCK-treated cells (DC-MOCK). Both protein extracts, GAL3ST2 and MOCK, were added at day 0 of differentiation protocol and replaced every 2 days during 10 days. The results are the average of four independent experiments (mean ± SD), $*p < 0.01$, CC, DC-GAL3ST2 or DC-MOCK vs. DC. **b** GAL3ST2 effect on cell viability during adipogenic differentiation: Comparison of MDI-treated cells (DC) and MDI plus GAL3ST2-treated cells (DC-GAL3ST2). At indicated times, MTT assay was performed; control cells' (CC) absorbance was considered as 100 %. Data are presented as percentage of control cells. The results are the average of four different experiments (mean ± SD). **c** *Left-up* MDI-treated cells (DC), *right-up* control cells (CC), *left-below* MDI plus GAL3ST2-treated cells (DC-GAL3ST2), *right-below* MDI plus MOCK-treated cells (DC-MOCK). Representative results from one of the four independent experiments with similar results are shown. **d** Adipogenic marker expression C/EBP at day 3 of adipogenic differentiation: C/EBP and GAPDH proteins are detected in protein extract from: control cells (CC), MDI-treated cells (DC), MDI plus GAL3ST2-treated cells (DC3GAL), MDI plus MOCK-treated cells (DC MOCK). The *values* represent fold increase in protein expression compared to CC. The *bars* shown are the average of three different experiments (mean ± SD). Representative results from one of the three independent western blot experiments with similar results are shown. Results are expressed in arbitrary units, $*p < 0.01$ DC3GAL vs. DC. **e** Adipogenic marker expression FABP4 at days 3 and 5 of adipogenic differentiation: FABP4 and GAPDH proteins are detected at day 3: MDI-treated cells (DC3), MDI plus GAL3ST2-treated cells (DC3GAL3ST2), MDI plus MOCK-treated cells (DC3MOCK), and at day 5: MDI-treated cells (DC5), MDI GAL3ST2-treated cells (DC5GAL3ST2), MDI plus MOCK-treated cells (DC5MOCK). The *values* represent fold increase in protein expression compared to CC. In the *left upside,* the *bars* shown are the average of three different experiments (mean ± SD). Representative results from one of the three independent western blot experiments with similar results are shown. Results are expressed in arbitrary units, $*p < 0.01$ DC5GAL vs. DC5

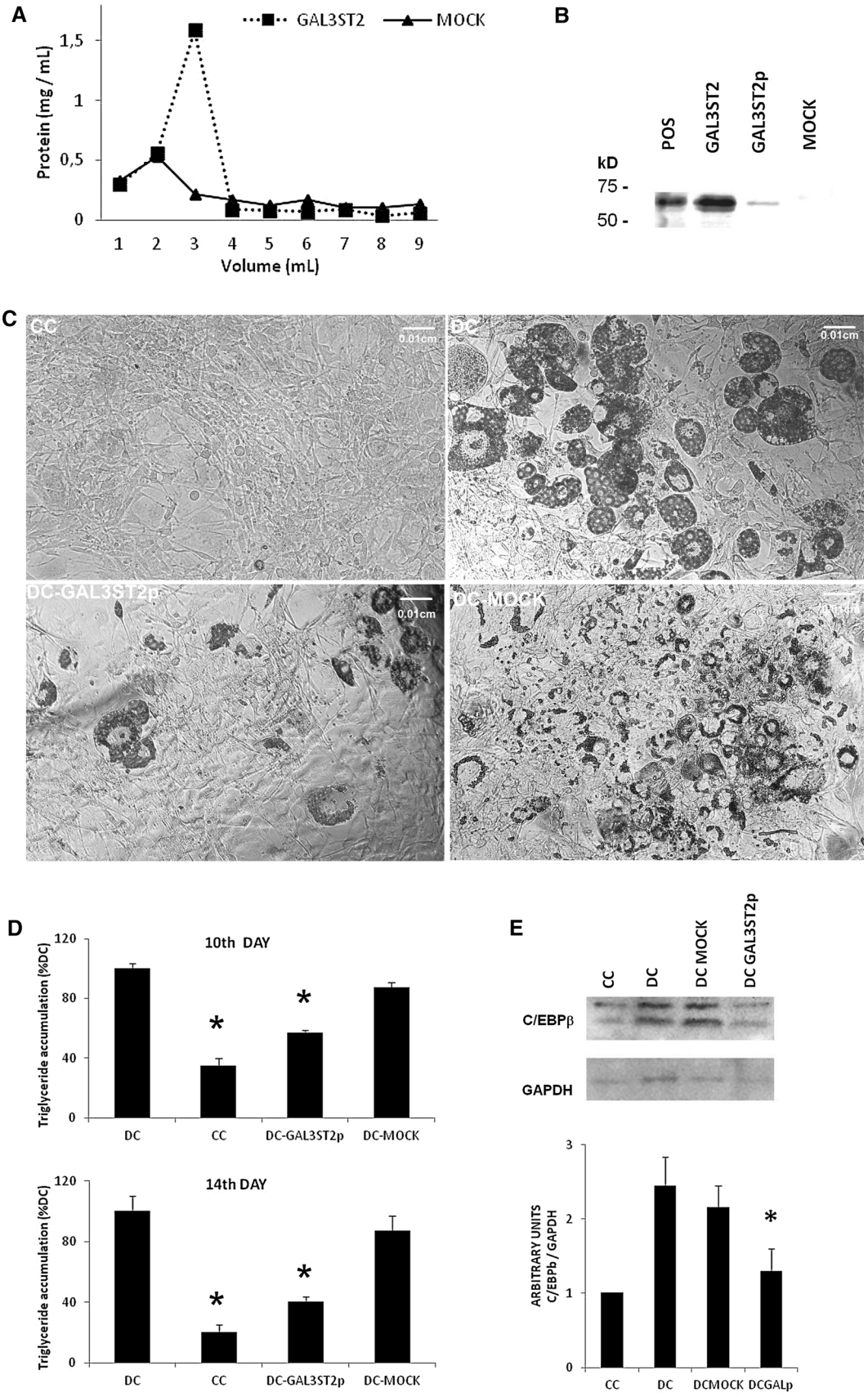
preadipocytes, a transient increase in the expression of adipogenic transcription factor CCAAT/enhancer binding protein beta (C/EBPβ) is detected between days 1 and 3 post-induction of differentiation pathway [18]. We hypothesized that GAL3ST2 could interfere with differentiation program of adipocytes by blocking the increased expression of C/EBPβ. Therefore, we evaluated C/EBPβ expression in cells at day 3 of differentiation protocol; GAL3ST2 significantly decreased the expression of C/EBPβ: 3.96 ± 0.6 AU [DC3] vs. 0.56 ± 0.39 AU [DC3GAL], $p < 0.01$ (Fig. 3d). Thus, the inhibitory effect of GAL3ST2 on Tg accumulation may be, in part, due to the blockade in the normal expression of C/EBPβ at the onset of adipogenic program. Fatty acid-binding protein 4 (FABP4, also known as Ap2) is an obesity-related protein that is a well-studied marker of adipocyte differentiation [11], whose expression significantly increased at day 5 of differentiation in MDI-treated cells. GAL3ST2 significantly inhibited FABP4 expression (Fig. 3e), its level in GAL3ST2-treated cells reached only 60 % of the levels observed in DC [11.33 ± 1.01 AU (DC5) vs. 6.77 ± 0.99 AU (DC5-GAL), $p < 0.01$]; coincident with GAL3ST2 inhibition effect on Tg accumulation. Taken together, the blockade in C/EBPβ expression GAL3ST2, are in agreement with inhibition of the adipogenic program.

We developed a partial purification of GAL3ST2 and evaluated protein effect on Tg accumulation during cell differentiation. After HEK293 cells transfection with pcDNA4/GAL3ST2-V5-His, cell extract was obtained, and GAL3ST2 with histidine tag (GAL3ST2p) was purified by a nickel column (Fig. 4a), and verified the presence of it in column fraction (Fig. 4b); we also developed a purification of mock transfection cells to evaluate possible artifact effects. Afterwards, we reproduced the experiments developed with protein extract containing GAL3ST2; 3T3-L1 preadipocytes were induced to differentiate in the presence of GAL3STp (Fig. 4c), with a reduction of nearly 40 % of Tg accumulation after 10 days of treatment (Fig. 4d) [1.80 ± 0.06 g Tg/g protein (DC) vs. 1.03 ± 0.03 g Tg/g protein (DC-GAL3ST2p), $p < 0.01$]; which reached 60 % after 14 days [2.69 ± 0.17 g Tg/g protein (DC) vs. 0.95 ± 0.06 g Tg/g protein (DC-GAL3ST2p), $p < 0.01$]. As shown in Fig. 4c, no phenotypic differences were observed between cells differentiated in the presence or absence of GAL3ST2p. Finally, we evaluated if GAL3ST2p could block the increased expression of C/EBPβ during cell differentiation and evaluated its expression at day 3 of differentiation protocol; GAL3ST2p significantly decreased the expression of C/EBPβ: 2.44 ± 0.40 AU [DC] vs. 1.30 ± 0.30 AU [DCGAL3ST2p], $p < 0.01$ (Fig. 4e). Thus, the inhibitory effect of GAL3ST2 on Tg accumulation might be, in part, due to the blockade in the normal expression of C/EBPβ at the onset of adipogenic program.

Finally, we evaluated GAL3ST2 expression in two breast cancer cell lines. We found that GAL3ST2 is expressed in MCF-7 and MDA-MB-231 cell lines (Fig. 5), being the expression stronger in more metastatic tumor cell line MDA-MB-231 (MDA-MB-231: 1.45 ± 0.03 UA vs. MCF-7: 1.23 ± 0.05 UA, $p < 0.01$).

Discussion

The interactions between mammary parenchymal and stromal compartments have been well documented. Adipocytes and epithelial cells play vital roles in normal and carcinomatous states of the breast through paracrine and endocrine signaling via secreted cytokines [19]. Mature adipocytes can affect the growth and migration of estrogen-positive carcinoma epithelial cells [20]. Previously, we have shown that adipocyte-conditioned media from patients with breast tumors increased the proliferation of mammary epithelial cells in vitro [21]. In addition, we



◀**Fig. 4** GAL3ST2p effect on 3T3-L1 cells during adipogenic differentiation. **a** Fractions' column of cell extracts from transfected HEK 293 cells with pcDNA4/GAL3ST2-V5-His (GAL3ST2) or pcDNA4/V5-His C (MOCK) was evaluated for protein content (mg/mL); the first 9 fractions from 21 are shown. After the 5th fraction, no protein was detected. **b** GAL3ST2 expression in protein extracts: GAL3ST2 protein is detected in protein extract from: POS (POSITIVE: protein extract from BHK-21 cells, transfected with pSVK3-GAL3), GAL3ST2 (protein extract from HEK293 transfected with pcDNA4/GAL3ST2-V5-His), GAL3ST2p (column fraction number 3 obtained from loading cell extract of HEK 293 transfected with pcDNA4/GAL3ST2-V5-His), MOCK (column fraction number 3 obtained from loading cell extract of HEK293 transfected with empty vector pcDNA4/V5-His C). Representative results from one of the three independent western blot experiments with similar results are shown. **c** *Left-up* control cells (CC), *right-up* MDI-treated cells (DC), *left-below* MDI plus GAL3ST2p-treated cells (DC-GAL3ST2), *right-below* MDI plus MOCK-treated cells (DC-MOCK). Representative results from one of the four independent experiments with similar results are shown. **d** Triglyceride accumulation in 3T3-L1 at days 10 or 14 of adipogenic differentiation in control cells (CC), MDI-treated cells (DC), MDI plus GAL3ST2p-treated cells (DC-GAL3ST2p), or MDI plus MOCK-treated cells (DC-MOCK). Both column protein fraction, GAL3ST2p and MOCK, were added at day 0 of differentiation protocol and replaced every 2 days during 14 days. The results are the average of four independent experiments (mean ± SD), $*p < 0.01$, CC, DC-GAL3ST2p or DC-MOCK vs. DC. **e** Adipogenic marker expression C/EBP at day 3 of adipogenic differentiation: C/EBP and GAPDH proteins are detected in protein extract from: control cells (CC), MDI-treated cells (DC), MDI plus MOCK-treated cells (DC MOCK), MDI plus GAL3ST2p-treated cells (DC3GAL). The *values* represent fold increase in protein expression compared to CC. The *bars* shown are the average of three different experiments (mean ± SD). Representative results from one of the three independent western blot experiments with similar results are shown. Results are expressed in arbitrary units, $*p < 0.01$ DC GAL3ST2p vs. DC

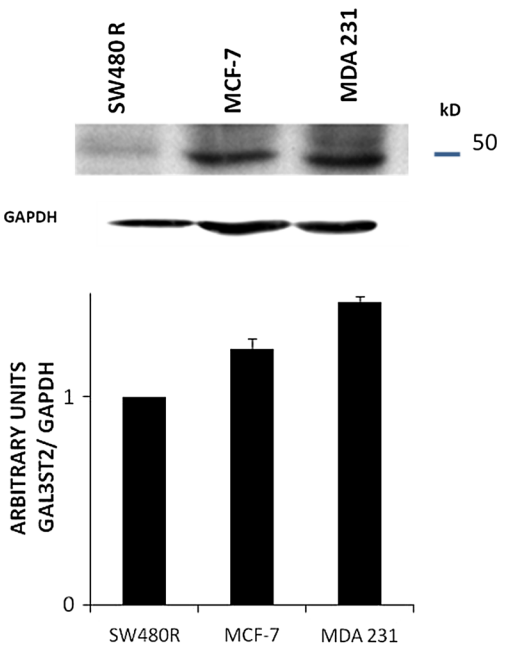


Fig. 5 GAL3ST2 expression in cancer cell lines. GAL3ST2 protein is detected in protein extracts from: SW480 (human colon cells), MCF-7 (human breast cancer cells), MDA 231 (human breast cancer cells). The *values* represent fold increase in protein expression compared to SW480. The *bars* shown are the average of three different experiments (mean ± SD); results are expressed in arbitrary units. Representative results from one of the three independent western blot experiments with similar results are shown above the graphic

found that adipocyte differentiation influenced the proliferation and migration of both normal and tumor mammary epithelial cells [22].

The interactions between adipocytes and other cells are also important for cellular differentiation. Here, we report an interrelationship between 3T3-L1 adipocytes and NMuMG mammary epithelial cells. This relationship may be mediated by extracellular components [17], including secretory proteins such as pro-inflammatory cytokines (INF gamma, TGF beta and TNF alpha). In this study, we showed that NMuMG-conditioned medium was able to decrease Tg accumulation in 3T3-L1 adipocytes by approximately 70 %.

Tg accumulation depends on the expression of adipogenic transcription factors, such as C/EBPs and PPARγ. C/EBPs and PPARγ are considered "master regulators" of the transcription of adipocyte-specific genes [23]. Consequently, they are responsible for regulating the expression of adipokines and other proteins found in mature adipocytes. We have previously shown that the TgIF obtained from breast cell-conditioned medium affected differentiation inhibiting C/EBPβ and PPARγ expression [24], which suggested that TgIF affected signaling cascades responsible for adipogenic differentiation, leading to the inhibition of Tg accumulation.

Here, we used MALDI-TOF to identify GAL3ST2 as the enzyme responsible for TgIF activity. GAL3ST2 inhibited the expression of C/EBPβ and FABP4; in addition, it inhibited Tg accumulation without any toxicity. GAL3ST2 is responsible for transferring sulfate groups to galactose. In previous studies, TGF-[α/β], which inhibit Tg accumulation during adipogenic differentiation, have also been linked to sulfotransferase activity [25]. Recently, the overexpression of estrogen sulfotransferase in pre-adipocytes has also been shown to prevent adipocyte differentiation by activating ERK1/2, thereby inhibiting insulin signaling and leading to a failure to switch from clonal expansion to differentiation [26].

Other glycosyltransferases have been described in breast cancer cell lines, including T47-D, MCF-7 and MDA-MB-231 [27]. While the presence of GAL3ST2 in tumor tissues and cancer cell lines has been previously demonstrated [28]; we show the presence of GAL3ST2 in breast cancer cell lines for the first time. Its stronger expression in more metastatic tumor cell line reinforcing the possibility that GAL3ST2 may be a factor associated with the mammary tumor cells that may interfere in the normal process of

adipogenesis of preadipocytes present in the mammary gland.

GAL3ST2 plays a role in regulating adhesion capacity and may be related to tumor metastasis in lung giant and hepatoma cancer cells where its elevated expression could correlate with higher metastatic potential [9]. GAL3ST2 siRNA also inhibited AKT and ERK phosphorylation [29]; moreover, Lewis x trisaccharide 3′-sulfate, a product of GAL3ST2, also increases AKT and ERK phosphorylation. These kinases couple integrins and growth factors with downstream signaling pathways involved in the cell cycle progression. In addition, these kinases regulate cell behavior by modulating lipid metabolism [30]. Thus, we speculate that GAL3ST2 may generate multiple signals related to integrin activation, including its effect on pre-adipocyte differentiation. Further studies of conditioned media from other metastatic and non-metastatic cancer cell lines are needed to evaluate and define this relationship. Additional studies of the interactions between NMuMG and 3T3-L1 cells using co-culture system would also be informative.

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Conflict of interest None.

References

1. Conklin MW, Keely PJ. Why the stroma matters in breast cancer: insights into breast cancer patient outcomes through the examination of stromal biomarkers. Cell Adh Migr. 2012;6:249–60.
2. Schedin P, Hovey R. The mammary stroma in normal development and function. J Mammary Gland Biol Neoplasia. 2010;15:275–7.
3. Tyan SW, Hsu CH, Peng KL, Chen CC, Kuo WH, Lee EY, et al. Breast cancer cells induce stromal fibroblasts to secrete ADAMTS1 for cancer invasion through an epigenetic change. PLoS One. 2012;7:e35128.
4. Dirat B, Bochet L, Dabek M, Daviaud D, Dauvillier S, Majed B, et al. Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion. Cancer Res. 2011;71:2455–65.
5. Martinez J, Cifuentes M. Adipose tissue and desmoplastic response in breast cancer. In: Mehmet G, editor. Breast cancer, carcinogenesis, cell growth and signalling pathways. Washington: National Academies Press; 2011. p. 447–56.
6. Skelhorne-Gross G, Reid AL, Apostoli AJ, Di Lena MA, Rubino RE, Peterson NT, et al. Stromal adipocyte PPARγ protects against breast tumorigenesis. Carcinogenesis. 2012;33:1412–20.
7. Hovey RC, Aimo L. Diverse and active roles for adipocytes during mammary gland growth and function. J Mammary Gland Biol Neoplasia. 2010;15:279–90.
8. Xu F, Gomillio C, Maxson S, Burg K. In vitro interaction between mouse breast cancer cells and mouse mesenchymal stem cells during adipocyte differentiation. J Tissue Eng Regen Med. 2009;3:338–47.
9. Shi BZ, Hu P, Geng F, He PJ, Wu XZ. Gal3ST-2 involved in tumor metastasis process by regulation of adhesion ability to selectins and expression of integrins. Biochem Biophys Res Commun. 2005;332:934–40.
10. Rathinam R, Alahari S. Important role of integrins in the cancer biology. Cancer Metastasis Rev. 2010;29:223–37.
11. Calzadilla P, Gomez-Serrano M, García-Santos E, Shiappacasse A, Abalde Y, Calvo JC, et al. N-Acetylcysteine affects obesity protein expression in 3T3-L1. Redox Report. 2013;18:210–8.
12. Tonneatto J, Guber S, Charo NL, Susperreguy S, Galigliana MD, Piwien-Pilipuk G. Dynamic mitochondrial-nuclear redistribution of the immunophilin FKBP51 is regulated by the PKA signaling pathway to control gene expression during adipocyte differentiation. J Cell Sci. 2013;126:5357–68.
13. Honke K, Tsuda M, Koyota S, Wada Y, Tanaka N, Ishizuka I, et al. Molecular cloning and characterization of a human Gal-3′-sulfotransferase that acts on both type 1 and type 2 (Gal1-3/1-4GlcNAc-R) oligosaccharides. J Biol Chem. 2001;276:267–74.
14. Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–54.
15. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65:55–63.
16. Analytical Software, Statistix 8, Tallahassee, FL, USA: McGraw–Hill/Irwin, 2003.
17. Julianelli V, Guerra LN, Calvo JC. Cell–cell communication between mouse mammary epithelial cell and 3T3-L1 preadipocytes: effect on triglyceride accumulation and cell proliferation. Biocell. 2007;31:237–45.
18. Mac Dougald O, Lane MD. Transcriptional regulation of gene expression during adipocyte differentiation. Annu Rev Biochem. 1995;64:345–73.
19. Yang CC, Ellis SE, Xu F, Burg K. In vitro regulation of adipogenesis: tunable engineered tissues. J Tissue Eng Regen Med. 2007;1:146–53.
20. Manabe Y, Toda S, Miyazaki K, Sugihara H. Mature adipocytes, but not pre-adipocytes, promote the growth of breast carcinoma cells in collagen gel matrix culture through cancer–stromal cell interactions. J Pathol. 2003;201:221–8.
21. Creydt V, Fletcher S, Giudice J, Bruzzone A, Chasseing N, González E, et al. Human adipose tissue from normal and tumoral breast regulates the behaviour of mammary epithelial cells. Clin Transl Oncol. 2013;15:124–31.
22. Creydt V, Sacca P, Tesone A, Vidal L, Calvo J. Adipocyte differentiation influences the proliferation and migration of normal and tumoral breast epithelial cells. Mol Med Rep. 2010;3:433–9.
23. Calzadilla P, Sapochnik D, Cosentino S, Diz V, Dicelio L, Calvo JC, et al. N-Acetylcystein reduces markers of differentiation in 3T3-L1 adipocytes. Int J Mol Sci. 2011;12:6936–51.
24. Sapochnik D, Piwien G, Croce M, Calvo JC, Guerra LN. Factores de inhibición lipídica secretados por células epiteliales mamarias: efecto sobre diferenciación de adipocitos y metabolismo oxidativo. Medicina. 2007;67:248.
25. Schonherr E, Jarvelainen HT, Sandell L, Wight T. Effects of platelet-derived growth factor and transforming growth factor-beta-1 on the synthesis of a large versican-like chondroitin sulfate proteoglycan by arterial smooth muscle cells. J Biol Chem. 1991;266:17640–7.
26. Wada T, Ihunnah C, Gao J, Chai X, Zeng S, Philips BJ, et al. Estrogen sulfotransferase inhibits adipocyte differentiation. Mol Endocrinol. 2011;25:1612–23.
27. Chandrasekaran EV, Xue J, Neelameghan S, Matta K. The pattern of glycosyl and sulfotransferase activities in cancer cell lines: a predictor of individual cancer-associated distinct carbohydrate structures for the structural identification of signature glycans. Carbohydr Res. 2006;341:983–94.
28. Chandrasekaran EV, Jain RK, Rhodes JM, Chawda R, Piskorz C, Matta KL. Characterization of distinct Gal: 3-*O*-sulfotransferase activities in human tumor epithelial cell lines and of calf lymph node GlcNAc: 6-*O*-sulfotransferase activity. Glycoconj J. 1999;16:523–36.
29. Zhang C, Hu P, Fu D, Wu W, Jia C, Zhu X, et al. 3′sulfo Le x is important for regulation of integrin subunit alphaV. Biochemistry. 2010;49:7811–20.
30. Donzelli E, Lucchini C, Ballarini E, Scuteri A, Carini F, Tredici G, et al. ERK1 and ERK2 are involved in recruitment and maturation of human mesenchymal stem cells induced to adipogenic differentiation. J Mol Cell Biol. 2011;3:123–31.