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ETS transcription factor ELF5 induces lumen formation in a 3D model of mammary morphogenesis and its expression is inhibited by Jak2 inhibitor TG101348

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ABSTRACT

The loss of expression of a single gene can revert normal tissue to a malignant phenotype. For example, while normal breast has high lumenal expression of CEACAM1, the majority of breast cancers exhibit the early loss of this gene with the concurrent loss of their lumenal phenotype. MCF7 cells that lack CEACAM1 expression and fail to form lumena in 3D culture, regain the normal phenotype when transfected with CEACAM1. In order to probe the mechanism of this gain of function, we treated these cells with the clinically relevant Jak2 inhibitor TG101348 (TG), expecting that disruption of the prolactin receptor signaling pathway would interfere with the positive effects of transfection of MCF7 cells with CEACAM1. Indeed, lumen formation was inhibited, resulting in the down regulation of a set of genes, likely involved in the complex process of lumen formation. As expected, inhibition of the expression of many of these genes also inhibited lumen formation, confirming their involvement in a single pathway. Among the genes identified by the inhibition assay, ETS transcription factor ELF5 stood out, since it has been identified as a master regulator of mammary morphogenesis, and is associated with prolactin receptor signaling. When ELF5 was transfected into the parental MCF7 cells that lack CEACAM1, lumen formation was restored, indicating that ELF5 can replace CEACAM1 in this model system of lumenogenesis. We conclude that the event(s) that led to the loss of expression of CEACAM1 is epistatic in that multiple genes associated with a critical pathway were affected, but that restoration of the normal phenotype can be achieved with reactivation of certain genes at various nodal points in tissue morphogenesis.

1. Introduction

Normal lumenal morphology is rapidly lost during the progression of breast epithelia from hyperplasia to ductal carcinoma in situ (DCIS) to invasive cancer [1,2]. Since normal breast epithelial cells such as HMECs form acini with lumena in 3D culture [1,3,4] while breast cancer cell lines generally do not [5], the 3D culture assay is an informative phenotypic assay for studying differential gene expression in the progression from normal mammary morphogenesis to cancer. We have focused our research on the functional role of CEACAM1, a cell adhesion molecule abundantly expressed on the lumen of normal breast, but down-regulated or absent in breast cancer [6,7]. For example, MCF10A cells that express CEACAM1 and form lumena in 3D culture, fail to form lumena when CEACAM1 is down-regulated by anti-sense, anti-CEACAM1 antibodies, or CEACAM1 blocking peptide [8]. On the other hand, MCF7 cells that fail to form lumena in 3D culture, regain this function when transfected with the CEACAM1 gene [9]. Previously, we transfected MCF7 cells with either wild type or a functionally phosphorylation null version of CEACAM1 and found over 300 genes were differentially regulated in the cells that were able to form lumena [10]. Given the complexity of the lumen forming process as described in the literature [11,12], we decided to pick one prominent signaling pathway as a target for inhibition in the MCF7/CEACAM1 transfected cells to determine which subset of the 300 genes were aligned with a single critical pathway. Since the 3D culture system requires bovine pituitary extract [13], a source of prolactin, and prolactin is a key hormone for mammary expansion during pregnancy [14], the prolactin-prolactin receptor pathway was selected for this study.

Prolactin, a key hormone for lactogenesis, is produced in the pituitary during pregnancy causing an increase in aveolar cells and initiation of milk production [15–17]. Upon binding its receptor, Jak2 is activated by phosphorylation, which in turn, phosphorylates Stat5a that translocates to the nucleus, turning on key genes in the lactogenesis program. Among the genes previously studied, SOCS2 stands out as a direct inhibitor of Stat5a signaling, and together with the Ets transcription factor Elf5, mediate prolactin induced mammary gland development [18]. Importantly, STAT5a knock out mice, among other

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defects, fail to lactate during pregnancy [17]. The advent of a specific, clinically relevant Jak2 inhibitor TG101348 (TG) [19], allowed us to interrupt prolactin receptor signaling immediately downstream of the receptor in MCF7/CEACAM1 cells in a time and dose dependent manner, achieving a significant inhibition of lumen formation ($p < 0.005$ at a dose of 500 nM). The results were confirmed with RNAi inhibition of Jak2 in these cells ($p < 0.005$), demonstrating a connection between Jak2 and CEACAM1 signaling in lumen formation. When comparative RNAseq was performed on these cells before and after Jak2 inhibition with TG, the genes down-regulated and involved with CEACAM1 transfected MCF7 cells were identified. Among the top 30 genes identified, six were selected for further study by blocking their expression with RNAi. Interestingly, both SOCS2 and its related antisense gene, SOCS2-AS1, scored high in the list, but when knocked down with RNAi, were barely significant in inhibition of lumen formation ($p < 0.05$). Although ELF5 appeared near the bottom of the list in terms of fold reduction, RNAi to ELF5 almost completely blocked lumen formation, in agreement with its previous connection to the prolactin receptor signaling pathway [18]. More importantly, transfection of parental MCF7 cells with ELF5, restored lumen formation to these cells, demonstrating that ELF5 can reactivate lumen formation in the absence of CEACAM1. This result is in agreement with the designation of ELF5 as a master regulator of mammary morphogenesis [20]. In addition, several genes associated with apoptosis were identified (CASP4 and BIK), and when their expression was inhibited with RNAi, lumen formation was significantly inhibited ($p < 0.004$ and < 0.005, respectively). These results strengthen the conclusion of our previous studies that showed apoptosis is involved in clearing the central lumen during lumenogenesis [9]. Thus, this approach has the ability to identify genes involved in this important function and to segregate their functions according to distinct receptor signaling pathways.

2. Experimental procedures

2.1. Reagents

TG101348 (SAR302503), prolactin, and bovine transferin were purchased from Sigma, BSA and Cell Extraction Buffer from Life Technologies, Matrigel from Corning, and pJAK2 ELISA and Cell Lysis Buffer from Santa Cruz or Cell Signaling Technology. RNAi negative control oligos were from Qiagen, Lipofectamine RNAiMAX and Opti-MEM reduced serum medium were from Invitrogen. Anti-JAK2 antibody was from invitrogen, anti-ELF5, mouse anti-human actin, anti-STAT5, anti-pStat5a, anti-pSTAT5, and anti-BIK from Santa Cruz Biotechnology, and anti-CALML5 from Sigma.

2.2. Cell culture

The MCF7 cell line was obtained from ATCC (HTB-22™) and cultured in MEM supplemented with 10% FBS, 1× Antibiotic-Antimycotic, 1 mM sodium pyruvate, 0.15% sodium bicarbonate, and 1× non-essential amino acids. MCF7/CEACAM1 cells were previously described as well as the 3D culture method [9]. Lumen formation was scored by counting the number of lumena per acini with an inverted microscope at 40x. At least 200 acini were examined and p values determined by Fisher's Exact Test.

2.3. Cell Treatment with prolactin and JAK2 inhibitor TG101348

MCF7/CEACAM1 cells were plated on 60-mm dishes one day before the experiment at a density of 1×10^6 cells per dish and were serum starved overnight. The next day media was removed and replaced with DMEM/F-12 without phenol red supplemented with 200 μg/mL BSA and 10 μg/mL bovine transferrin, and 0.1% DMSO. Cells were treated with 100 ng/mL prolactin for 5, 15, 30, and 60 min. Five dishes were pretreated with 500 nM of the JAK2 inhibitor TG for 1 h, replaced with fresh media, and treated with100 ng/mL prolactin for 5, 15, 30, 60 min. Pervanadate (100 μM) was added to the medium 5 min before cells were lysed. Media was removed and cells washed with PBS prior to lysis of the cells for 0, 5, 15, 30, and 60 min time points for pSTAT5 and pJAK2 ELISA assays. A 24 h co-treatment of cells with JAK2 inhibitor TG and prolactin was cultured for immunoblot analysis.

2.4. pSTAT5 and pJAK2 ELISA

Cells treated with JAK2 inhibitor TG and prolactin were washed with PBS and lysed on dishes with 500 μL Cell Lysis Buffer for pSTAT5 ELISA and 500 μL Cell Extraction Buffer for pJAK2 ELISA. Lysed cells were incubated on the dishes for 5 min on ice and transferred to microcentrifuge tubes. Cell lysates were then incubated on ice for 30 min and centrifuged at 14,000 RPM at 4 °C for 10 min. After centrifugation, supernatants were probed for pSTAT5 or pJAK2.

2.5. Immunoblot analysis

Cells treated with TG and prolactin for 24 h were washed with PBS and lysed on dishes with 500 μL with RIPA Lysis and Extraction Buffer. Lysed cells were transferred to microcentrifuge tubes. Cell lysates were incubated on ice for 30 min and centrifuged at 14,000 RPM at 4 °C for 20 min. Equal amounts of protein (Bradford Assay) were loaded onto SDS-PAGE gels for electrophoresis and transferred onto PVDF membranes. The blots were probed with rabbit anti-pSTAT5 (Tyr694) and mouse anti-β-actin. Imaging was performed using IR dye labeled secondary antibodies from LI-COR on the Odyssey infrared imaging system.

2.6. RNAseq sample preparation

RNA was prepared using the Qiagen RNeasy Mini Kit from cells in 3D culture after TG treatment. Ten-cm dishes were coated with 1 mL of Matrigel and incubated at 37 °C for 30 min until the Matrigel solidified. Cells resuspended in 10 mL of mammary epithelial basal medium with bovine pituitary extract at a density of 1×10^6 cells were plated for 3 h on Matrigel. Attached cells were overlaid with 1 mL of 50% Matrigel in mammary epithelial basal medium with bovine pituitary extract and 500 nM TG in 0.1% DMSO or 0.1% DMSO alone as control. Mammary epithelial basal medium with bovine pituitary extract and TG or DMSO was added to the culture and incubated overnight, two days, and four days. Acini were recovered by dissolving Matrigel with 5 mL Cell Recovery Solution and incubation at 4 °C for 1 h with gentle shaking. Cells were harvested and RNA isolated for RNAseq.

2.7. RNAi treatment

Cells were transfected with RNAi oligos (Table 1) using the Lipofectamine RNAiMAX protocol. Briefly, 30 pmol RNAi duplex in 250 μL of Opti-MEM plus 5 μL Lipofectamine RNAiMAX in 250μl Opti-MEM were mixed gently for 10–20 min at room temperature. The mixture was added to 6 well plates containing 250,000 cells in 2 mL of OPTI MEM or complete growth medium without antibiotics. The cells were further incubated for 48 h at 37 °C in a CO_2 incubator. After 48 h incubation, the cells were trypsinized and harvested for analysis.

2.8. qRT-PCR

RNA was quantitated with qPCR using primers (Table 1) according to the BioRad iQ5 Multicolor Real-time PCR Detection System. Briefly, 0.05 μg of cDNA from the reverse transcription reaction with 20 pmol of each primer in a total volume of 25 μL using the Sense Mix Plus SYBR under the following conditions: initial denaturation step at 94 °C