RESEARCH PAPER

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The glycosyltransferase LARGE2 is repressed by Snail and ZEB1 in prostate cancer

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Abbreviations: β3GnT1, β3-*N*-acetylglucosaminyltransferase; BM, basement membrane; DG, dystroglycan; EMT, epithelial-to-mesenchymal transition.

Reductions in both expression of the dystroglycan core protein and functional glycosylation of the α-dystroglycan (αDG) subunit have been reported in a number of cancers and may contribute to disease progression. In the case of prostate cancer, one mechanism that contributes to αDG hypoglycosylation is transcriptional down-regulation of LARGE2 (GYLTY1B), a glycosyltransferase that produces the functional (laminin-binding) glycan on αDG, but the mechanism(s) underlying reduction of LARGE2 mRNA remain unclear. Here, we show that αDG hypoglycosylation is associated with epithelial-to-mesenchymal transition (EMT)-like status. We examined immunoreactivity for both functionally-glycosylated αDG and E-cadherin by flow cytometry and the relative expression of ZEB1 mRNA and the αDG glycosyltransferase LARGE2 mRNA in prostate and other cancer cell lines by quantitative RT-PCR. To study the role of ZEB1 and other transcription factors in the regulation of LARGE2, we employed overexpression and knockdown approaches. Snail- or ZEB1-driven EMT caused αDG hypoglycosylation by repressing expression of the LARGE2 mRNA, with both ZEB1-dependent and -independent mechanisms contributing to Snail-mediated LARGE2 repression. To examine the direct regulation of LARGE2 by Snail and ZEB1 we employed luciferase reporter and chromatin immunoprecipitation assays. Snail and ZEB1 were found to bind directly to the LARGE2 promoter, specifically to E/Z-box clusters. Furthermore, analysis of gene expression profiles of clinical samples in The Cancer Genome Atlas reveals negative correlation of LARGE2 and ZEB1 expression in various cancers. Collectively, our results suggest that LARGE2 is negatively regulated by Snail and/or ZEB1, revealing a mechanistic basis for αDG hypoglycosylation during prostate cancer progression and metastasis.

Introduction

Dystroglycan (DG) was discovered in skeletal muscle as a laminin-binding component of the dystrophin-associated glycoprotein complex, which links the extracellular matrix to the actin cytoskeleton.[1] DG is expressed in a variety of tissue types, including muscle, neural, adipose and epithelial, the last including prostate. Encoded by a single gene, *DAG1*, DG is post-translationally cleaved into 2 non-covalently associated subunits.[2,3] The β-subunit is a transmembrane protein that interacts with cytoskeletal linker proteins such as dystrophin and utrophin, and the α-subunit is an extracellular protein that is extensively glycosylated and binds to the laminin G-like domains of basement membrane (BM)-localized matrix proteins including laminins, perlecan and agrin.[2,4-9] A unique phosphorylated O-mannosyl glycan structure in the mucin-like domain of αDG is required for its binding to ligands within the extracellular matrix[10,11]; hence, this modification is referred to as functional glycosylation. Functional glycosylation of αDG is assessed by immunoreactivity with the IIH6 monoclonal antibody which detects a 3-xylose-α1,3-glucuronic acid-β1 disaccharide moiety that is necessary for extracellular matrix ligand binding.[11] Numerous known or putative glycosyltransferases (including ISPD, POMT1, POMT2, POMGnT1, Fukutin, FKRP and LARGE) are involved in producing this laminin-binding glycan, and mutations in these genes lead to various forms of muscular dystrophy.[12-19]

Both DG expression and αDG glycosylation are frequently reduced in breast cancer,[20,21] prostate cancer,[20,22-24] kidney cancer[25,26] and others. These changes are associated with cancer progression and predict poor outcome.[21,24,27] Consistent with these observations, dysregulation of DG impairs interactions between the cells and the BM, and thereby facilitates tumor invasion.[28,29] However, the mechanism(s) underlying impaired DG function in cancer are not yet clear. As is the case for muscular dystrophy, aberrations in any of the glycosylation-regulating genes mentioned above may, in principle, be involved in cancer. To date, however, no mutations in either these genes or in *DAG1* itself have been reported in any type of cancer. Nevertheless,

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certain other lines of evidence support their involvement in cancer. Firstly, the laminin-binding glycan on αDG is produced by the xylosyL-glucuronyltransferases LARGE and LARGE2,[11,30-32] and in breast cancer, LARGE downregulation, which appears to be under epigenetic control, results in a decrease in αDG glycosylation.[33,34] Secondly, LARGE acts in cooperation with β3-*N*-acetylglucosaminyltransferase-1 (β3GnT1), an enzyme that indirectly regulates synthesis of the laminin-binding moiety on αDG, and in cancer cells a reduction in the function of this protein was associated with both a decrease in αDG glycosylation and an increase in cell migration.[29] Thirdly, we previously reported that LARGE2 (GYLTL1B) mediates the formation of laminin-binding glycans on αDG in prostate epithelial cells,that this enzyme is down-regulated during prostate-cancer progression in humans, and functionally contributes to both invasive and proliferative phenotypes in prostate cancer cells.[24] However, the mechanism underlying LARGE2 down-regulation in this context remains unclear.

Tissue invasion by carcinomas and the eventual metastasis of carcinoma cells are associated with epithelial-mesenchymal transition (EMT), a developmental process that involves changes in both cell-cell and cell-matrix adhesion, as well as an increase in cell motility.[35,36] Induction of an EMT-like process, to distinguish it from a true developmental EMT, is considered an important and early step in cancer progression that empowers cells to metastasize from a primary tumor, and a hallmark of this event is the loss of E-cadherin expression. EMT-like changes can be triggered by various signaling pathways, including those initiated by TGF-β,[37] Wnt[38] and Notch ligands.[39] These signals activate master regulators of the EMT program, including transcription factors of the Snail, ZEB and Twist families.[35,40] The induction of a mesenchymal-like state by the overexpression of these proteins can confer a metastatic phenotype to carcinoma cells.[41,42] In part, this is carried out by direct transcriptional repression of E-cadherin.[41,43-46] Overall, the EMT transcription factors orchestrate the entire EMT program, by regulating a larger set of both epithelial and mesenchymal identity genes.[47-49] To some extent, the EMT transcription factors are functionally redundant. For example, Snail initiates EMT, and then ZEB1 and Twist act to maintain it, suggesting that these factors act in a hierarchical manner.[50,51] In the present study, we show that both Snail and ZEB1 regulate LARGE2 expression, binding directly to the LARGE2 promoter to repress the activity of its promoter. This represents one mechanism that underlies αDG hypoglycosylation during prostate cancer progression.

Results

The functional glycosylation of αDG and expression of E-cadherin are down−regulated in a subset of cancer cell lines

In a previous study we had found that a derivative of the PC-3 cell line, TEM4-18, lacks functional glycosylation of αDG, as determined by IIH6 immunoreactivity, due to a loss of LARGE2 expression ([24] and **Fig. 1A**). TEM4-18 is an aggressive subpopulation of the PC-3 line and exhibits a ZEB1-dependent mesenchymal-like morphology and complete loss of cell surface E-cadherin expression.[52] In contrast, the E-cadherin-positive PC-3E+ line, also derived from the PC-3 line,[52] is $IIH6^+$ (**Fig. 1A**). We found a similar correlation between cell surface anti-E-cadherin and IIH6 immunoreactivity in the PC-3 $IIH6^+$ and $IIH6^-$ lines, which were derived by sorting wild-type PC-3 cells based on their IIH6 staining status (**Fig. 1A**). In the case of the PC-3 prostate cancer cell line, 2 populations were identified, with one double-positive, and the other double-negative, for staining with anti-E-cadherin and IIH6 (**Fig. 1A**). To test whether functional glycosylation of αDG is more broadly associated with E-cadherin expression, we performed flow cytometry using anti-E-cadherin and IIH6 antibodies on another 11 cancer cell lines originating from various tissues (Supplementary **Fig. 1**). Five of these cell lines (22Rv1, derived from a prostate tumor; ZR-75-1, derived from a breast tumor; A431, derived from an epidermoid carcinoma; HT-29, derived from a colon tumor; and HCT116, derived from a colon tumor) were double-positive for anti-E-cadherin and IIH6 immunoreactivity. Another 4 were positive for either anti-E-cadherin (BxPC-3, derived from a pancreatic tumor; and LNCaP, derived from a prostate tumor) or IIH6 (786-O, derived from a renal tumor; and DU145, derived from a prostate tumor) immunoreactivity. Two of the lines were negative for both markers (A498, derived from a renal tumor; and MDA-MB-231, derived from a breast tumor). To determine if the mechanisms underlying the loss of both anti-E-cadherin and IIH6 immunoreactivity in these double-negative cancer cell lines might be similar to the TEM4-18 cells, we analyzed the expression of ZEB1, E-cadherin and LARGE2 mRNAs by qRT-PCR (**Fig. 1B**). This analysis showed that for all 3 double-negative cell lines (TEM4-18, A498, and MDA-MB-231) ZEB1 mRNA levels are relatively high, and E-cadherin and LARGE2 mRNA levels are relatively low compared to double-positive PC-3E+ cells.

We previously showed that the above-mentioned lack of IIH6 immunoreactivity in TEM4-18 cells is caused by downregulated LARGE2 expression and rescuing LARGE2 expression restores DG function and diminishes invasion and cell proliferation potential.[24] In light of that finding and the fact that TEM4-18 cells exist in an mesenchymal-like state, we hypothesized that loss of LARGE2 expression and the resulting loss of αDG functional glycosylation might be related to an EMT-like transcriptional program, which we had previously shown to occur in these cells.[52] We thus used qRT-PCR to assess the relative expression levels of EMT transcription factors of the Snail, ZEB and Twist families in the PC-3-derived PC-3E+ and TEM4-18 cell lines (**Fig. 1C**). Both ZEB1 and Twist2 mRNAs were greatly increased (one hundred-fold or more) in the mesenchymal-like TEM4-18 cells compared to the PC-3E+ cells, as noted previously, whereas Snail, Slug, Twist1 and ZEB2 mRNAs were expressed at similar or moderately elevated levels in the 2 cell lines. This prompted us to investigate whether LARGE2 is regulated by ZEB1 and other EMT transcription factors.

Overexpression of ZEB1 and Snail leads to αDG hypoglycosylation

To clarify the role of EMT factors in regulating LARGE2 expression and functional αDG glycosylation, we introduced a ZEB1 expression constructs into PC-3E+ cells, which express high level of glycosylated αDG and E-cadherin. As expected, Western blotting revealed ZEB1 protein in the stably transduced cell population (**Fig. 2A**). We found that ectopic ZEB1 expression led to reduced E-cadherin expression and αDG glycosylation (**Fig. 2B**). In contrast, the empty control vector, or one expressing the EMT-transcription factor Twist 2 did not (Supplementary **Fig. 2**). Notably, ZEB1-overexpressing cells stained with anti-E-cadherin or IIH6, separated into 2 populations (**Fig. 2B**): one with the same anti-E-cadherin and IIH6 immunoreactivity as the vector control cells, and the other with greatly reduced anti-E-cadherin and IIH6 immunoreactivity. We attempted to sort the latter population by flow cytometry, but the sorted cells were not stable, regaining E-cadherin expression and αDG glycosylation after a few passages (data not shown). This suggests that overexpressing ZEB1 in PC-3E+ cells is not sufficient for the cells to maintain the mesenchymal-like state, or that it is toxic. As Snail is known to be an EMT initiator and to act upstream of ZEB1,[51] it might have a broader and more general role in driving EMT, and in establishing a cellular context that facilitates maintenance of the mesenchymal-like state.[53] We tested this possibility by transducing PC-3E+ with a retroviral Snail expression vector and analyzing them by flow cytometry. Western blotting of total cell lysates from the Snail-expressing and vector control cell lines revealed that ZEB1 was up-regulated, and E-cadherin and αDG glycosylation were down-regulated (**Fig. 2C**). βDG levels also decreased marginally (~30%), but to a lesser extent than αDG glycosylation (60–70%). The Snail-expressing derivative showed decreases in cell surface E-cadherin and αDG glycosylation (**Fig. 2D**). To elucidate the molecular mechanism that underlies DG hypoglycosylation in Snail-overexpressing cells, we evaluated expression (at the mRNA level) of 9 genes that are involved in αDG glycosylation (**Fig. 2E**). Among these, only LARGE2 exhibited a significant reduction (80%) Snail-expressing cells compared to the respective vector control-transfected cells. We made similar findings by transducing a second prostate cancer cell line, 22Rv1, with Snail (Supplementary **Fig. 3**). These results indicated that loss of αDG glycosylation in Snail-expressing cells likely resulted from the decrease in LARGE2 expression.

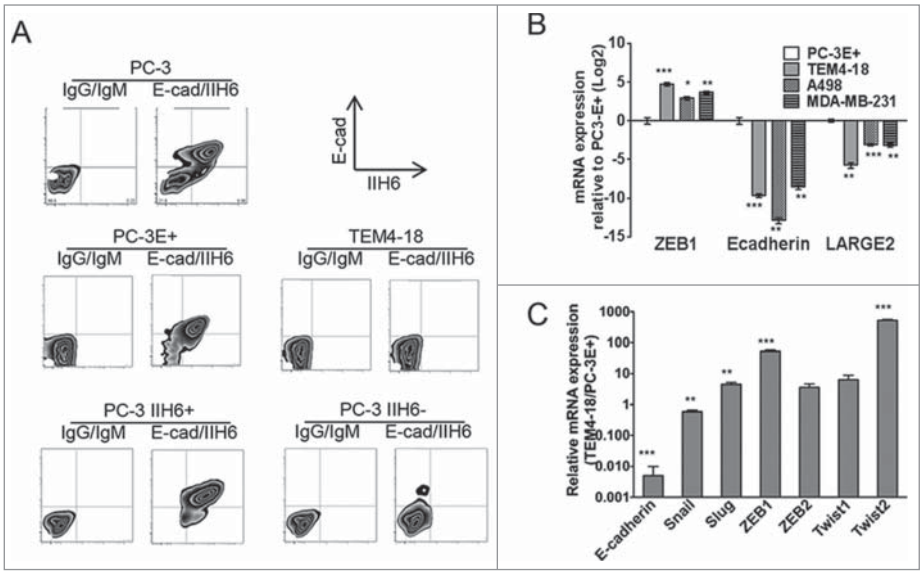


Figure 1. Correlation of E-cadherin and functional glycosylation of αDG in cancer cell lines. (**A**): Flow cytometry analysis of PC-3 and its derivatives stained with anti-E-cadherin and IIH6. (**B**): Levels of ZEB1, E-cadherin and LARGE2 mRNA in E-cadherin/IIH6 double negative lines, relative to those in the PC-3E+ line. mRNA levels were measured by qRT-PCR. (ANOVA; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (**C**): Relative expression of the E-cadherin and EMT transcription factor mRNAs in TEM4-18 compared to PC-3E+ cells, as assessed by qRT-PCR. E-cad, E-cadherin. (Student's t-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

To further investigate the regulation of LARGE2 by EMT factors, we applied small interfering RNAs (siRNAs) to silence Snail and/or ZEB1 (generating a single knockdown of each, as well as a double knockdown) in TEM4-18 cells, and used qPCR to evaluate the effect on the E-cadherin and LARGE2 mRNAs (**Fig. 3**). While Snail mRNA expression in TEM4-18 cells is slightly reduced compared to PC-3E cells by qRT-PCR (**Fig. 1C**), protein levels are low even in PC-3E cells (**Fig. 2C**). Snail knockdown (~60%, **Fig. 3B**) led to a ~4-fold increase in the expression of both E-cadherin and LARGE2 mRNAs. Consistent with our previous findings, ZEB1 knockdown (~80%, **Fig. 3B**) led to an approximately 94-fold increase in E-cadherin mRNA expression[52] and a more modest 2-fold increase of LARGE2 mRNA (**Fig. 3A**). ZEB1 knockdown, but not Snail knockdown, increased cell surface expression of E-cadherin indicating that ZEB1 exerts more potent regulation of E-cadherin in these cells (**Fig. 3C**). However, we did not observe a significant increase of IIH6 immunoreactivity in Snail or ZEB1 single-knockdown or Snail/ZEB1 double-knockdown cells (**Fig. 3C**). We also evaluated IIH6 expression in stable ZEB1 shRNA knockdown TEM 4-18 cell lines generated previously,[52] and observed a similar finding, E-cadherin expression was partially restored but IIH6 immunoreactivity was not (data not shown). These results suggest that restoration of LARGE2 mRNA expression by Snail/ZEB1 knockdown at the levels achieved here may not be sufficient to restore αDG glycosylation.

Snail-mediated repression of LARGE2 occurs through both ZEB1-dependent and -independent mechanisms

Given that ZEB1 functions downstream of Snail, we considered the possibility that the Snail-induced repression of LARGE2

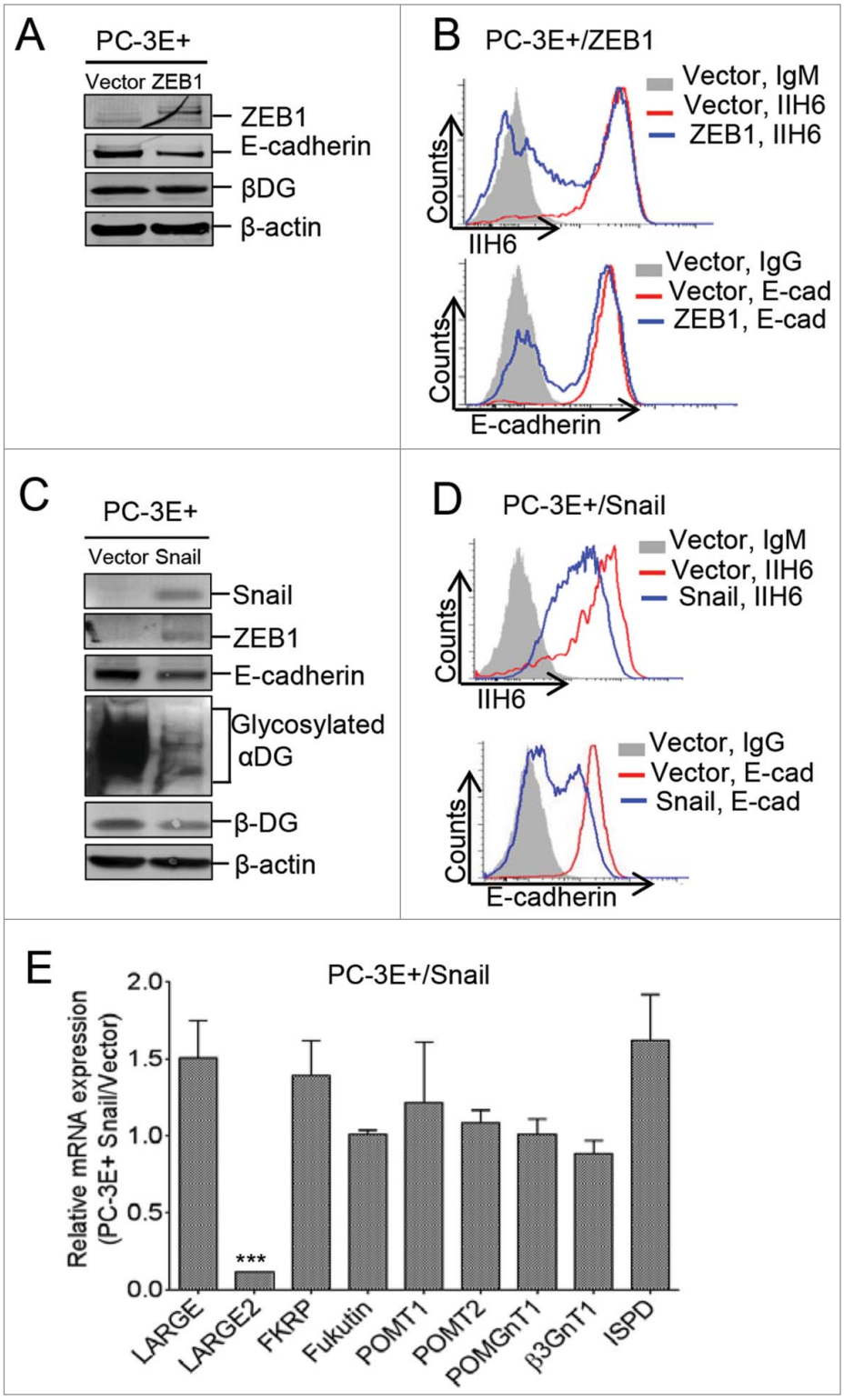


Figure 2. Overexpression of Snail and ZEB1 repress E-cadherin expression and αDG glycosylation in prostate cancer cells. (**A-B**): E-cadherin expression and αDG glycosylation of PC-3E+ cells transduced with *ZEB1* as assessed by Western blotting and flow cytometry, respectively. (**C-D**): αDG glycosylation and E-cadherin expression in PC-3E+ cells transduced with *Snail* as assessed by Western blotting and flow cytometry, respectively. β-actin served as loading control for Western blotting. (**E**): mRNA expression of genes involved in αDG glycosylation in PC-3E+ Snail cells, as analyzed by qRT-PCR. E-cad, E-cadherin. (Student's t-test; *, $P < 0.05$; ***, $P < 0.001$).

in the PC-3E+ line involves ZEB1. We developed an inducible EMT model in PC-3E+ cells by expressing Snail-ER (a fusion between Snail and the estrogen receptor)[54] and applying the estrogen agonist 4-hydroxytamoxifen (4-OHT). As anticipated, Snail-ER-expressing PC-3E+ cells underwent an EMT-like change in morphology in response to the addition of 4-OHT (data not shown). The effects on E-cadherin expression and αDG glycosylation were assessed by flow cytometry and qRT-PCR, following various periods of 4-OHT exposure. Both E-cadherin and functionally glycosylated αDG on the cell surface decreased in response to continuous exposure to 4-OHT from 3 to 9d. Interestingly, IIH6 immunoreactivity was more profoundly reduced than anti-E-cadherin immunoreactivity in 4-OHT treated PC-3E+ Snail-ER cells (**Fig. 4A**). This was not observed in either 4-OHT treated PC-3E+ Twist-ER cells or in cells of the parent PC-3E+ line treated with 4-OHT at a similar exposure, indicating that 4-OHT treatment itself was not responsible for the reduction of cell-surface E-cadherin and IIH6 immunoreactivity (data not shown). qRT-PCR of the PC-3E+ Snail-ER cells revealed that the ZEB1 mRNA was upregulated whereas the LARGE2 mRNA was downregulated during Snail-ER mediated EMT at 3 and 6d (**Fig. 4B**). The role of ZEB1 in regulating LARGE2 was tested by silencing its expression in the PC-3E+ Snail-ER cells prior to treatment with 4-OHT (**Fig. 4C**). As expected, PC-3E+ Snail-ER cells transfected with a control siRNA demonstrated an increase in the levels of ZEB1 mRNA, and decreases in levels of the E-cadherin and LARGE2 mRNAs, after 6 d of treatment with 4-OHT. In contrast, their counterparts transfected with the siZEB1 exhibited a less pronounced downregulation of LARGE2 mRNA, and E-cadherin mRNA expression did not change (**Fig. 4C**). Flow cytometry analysis showed that cell surface E-cadherin, but not IIH6 immunoreactivity, was restored in the ZEB-1 knockdown 4-OHT treated Snail-ER cells (**Fig. 4D**). These findings indicate that

the repression of LARGE2 mRNA by Snail occurs through both ZEB1-dependent and -independent pathways. Thus, as above, partially restoring LARGE2 mRNA expression may not be sufficient to achieve functional glycosylation of αDG.

Snail and ZEB1 repress activity of the LARGE2 promoter by binding to E/Z-box clusters

We next investigated the molecular mechanisms by which Snail and ZEB1 inhibit LARGE2 mRNA expression. Inspection of the LARGE2 promoter (from +2000 bp to −2000 bp of the transcription start site) revealed 5 Z-boxes (specifically TACCTG or CAGGTA) and 9 E-boxes (specifically CAGGTG, CACCTG or CATGTG). In paired configurations, Z-boxes are typically restricted to ZEB1 factors, and E-boxes are putative binding sites for ZEB1 as well as other EMT transcription factors, including Snail (**Fig. 5A**). Sequence alignment of the LARGE2 promoter was performed for human, mouse, rat and dog. We found that 2 of 14 E/Z-boxes were conserved in all 4 species, and that 4 were conserved between human, mouse and rat, or human and dog. This conservation of E/Z-boxes suggested that they are recognized by EMT transcription factors. We thus carried out a reporter assay to assess whether Snail and ZEB1 can repress the activity of the LARGE2 promoter. To this end, HEK293 cells were co-transfected with pRL-TK and an pSGG luciferase reporter plasmid containing a 849 bp fragment that encompasses nucleotides −893 to −45 bp relative to the transcription start site (+1) of the human LARGE2 promoter (GYLTL1B-prom). When co-transfected with this LARGE2 reporter, Snail and ZEB1 each led to a significant, but relatively modest (20%), reduction of LARGE2 promoter activity (**Fig. 5B**). Importantly, we observed a similar degree of repression in parallel with the E-cadherin promoter construct. Thus, in this experimental system, Snail and ZEB1 repress LARGE2 promoter activity to a similar extent as E-cadherin, a known target of Snail and ZEB1 repression.

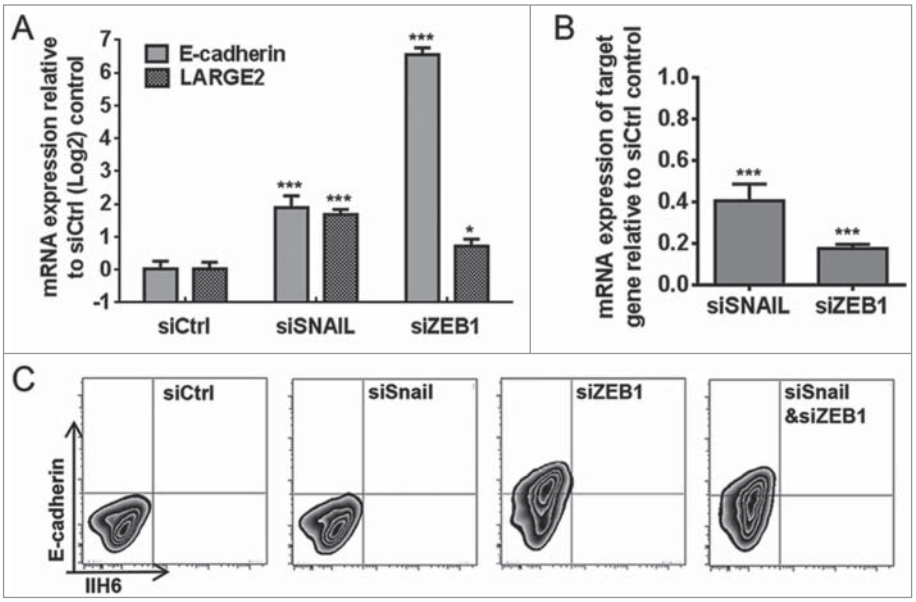


Figure 3. Silencing ZEB1 rescues expression of E-cadherin and results in increased LARGE2 mRNA expression. (**A**): Relative expression of the E-cadherin mRNA and LARGE2 genes in Snail- and ZEB1- silenced TEM4-18 cells. qRT-PCR was performed 5 days post transfection of the indicated siRNA, and mRNA levels are expressed relative to those in cells transfected with a control siRNA (ANOVA; *, $P < 0.05$, ***, $P < 0.001$). (**B**) Efficiency of knockdown in **A** as measured by qRT-PCR. (Student's t-test; ***, $P < 0.001$) (**C**): Expression of E-cadherin and functional glycosylation of αDG in Snail, ZEB1 and both-silenced TEM4-18 cells, as assessed by flow cytometry 5 days post transfection.

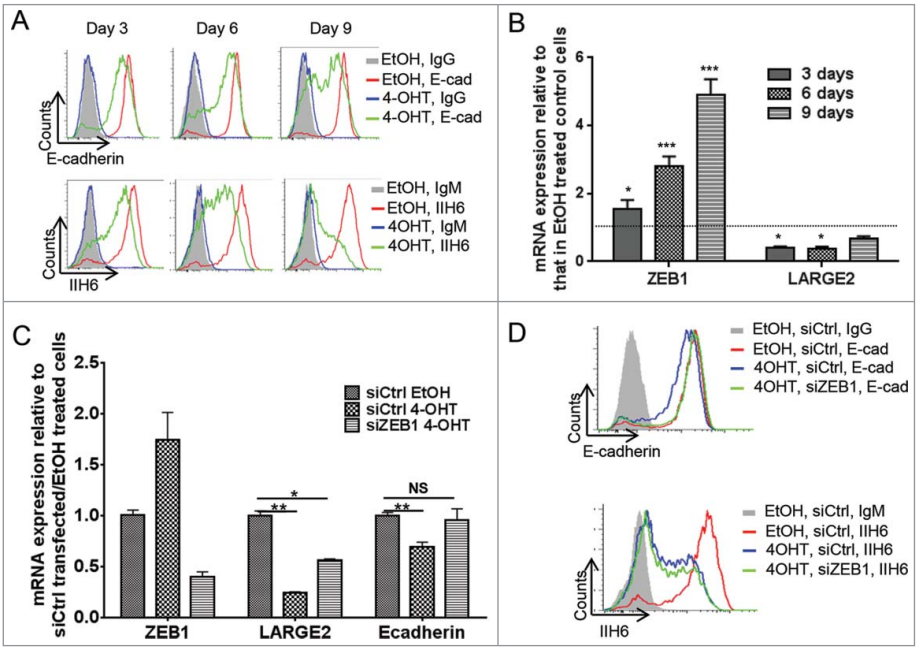


Figure 4. ZEB1 contributes to Snail-mediated reppression of LARGE2. (**A**): Cell surface expression of E-cadherin and functionally glycosylated αDG in PC-3E+ Snail-ER cells, as analyzed by flow cytometry on the indicated day after treatment with 20 nM 4-OHT. (**B**): Expression of the ZEB1 and LARGE2 mRNAs in 4-OHT treated PC-3E+ Snail-ER cells, as analyzed by qRT-PCR. (**C**): Expression of ZEB1, LARGE2 and E-cadherin mRNAs in PC-3E+ Snail-ER cells following a block of ZEB1 up-regulation during Snail-induced EMT, as assessed by qRT-PCR (transfection of siZEB1 prior to treatment with 4-OHT). (**D**): Flow cytometry analysis of cells in (**C**), using E-cadherin or IIH6 antibodies. E-cad, E-cadherin (Repeated measures ANOVA; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, non-significant).

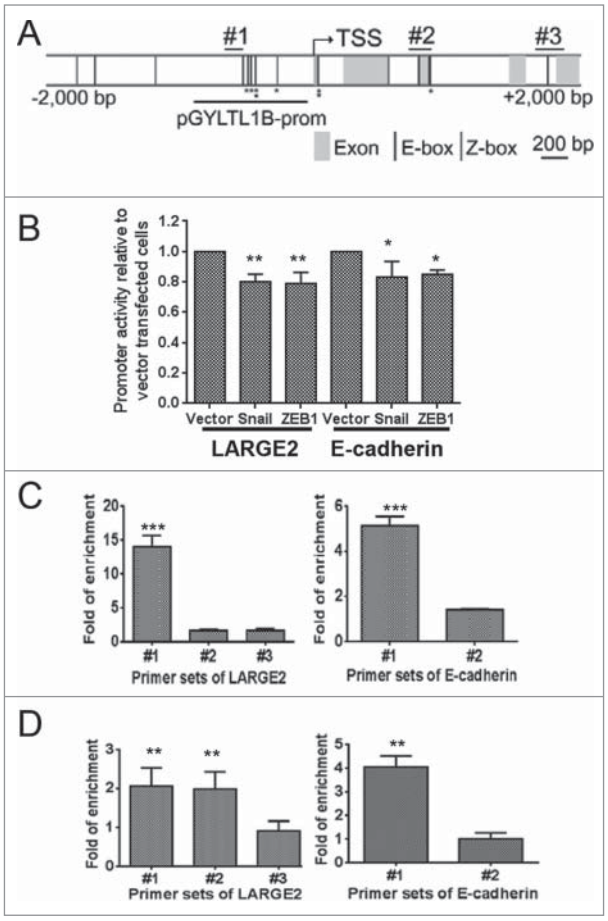


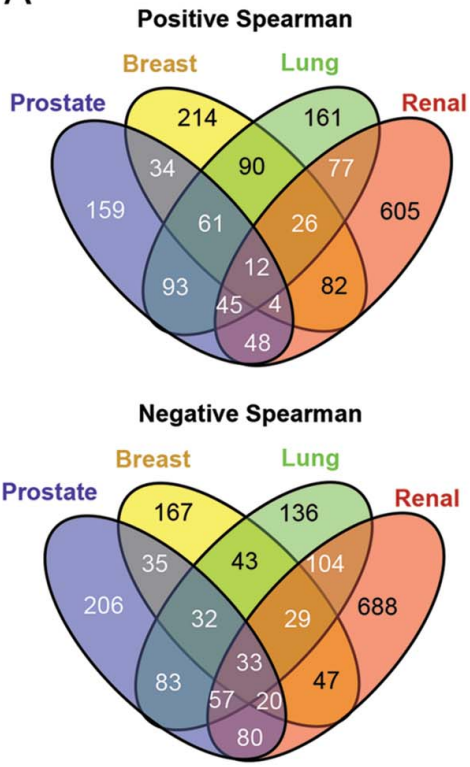
Figure 5. Snail and ZEB1 bind to the LARGE2 promoter directly and regulate its transcription. (**A**): The LARGE2 gene, as depicted from −2000 bp to +2000 bp of the transcription start site (TSS). Light gray boxes indicate exons and black bars and dark gray bars represent E-boxes and Z-boxes, respectively. Primers used for ChIP-qPCR analysis are identified by numbers, and the position of promoter construct is indicated. Asterisks indicate the level of conservation among human, mouse, rat and dog sequences. * = conservation between the human and at least one other sequence; ** = conservation among all 4 sequences. (**B**): Activity of the and E-cadherin promoters in the context of Snail and ZEB1, as assessed by reporter assays (ANOVA; *, $P < 0.05$; **, $P < 0.01$). (**C**): Sites of Snail binding to the LARGE2 and E-cadherin promoters, as assessed by chromatin immunoprecipitation in PC-3E+ Snail-ER cells 10 days after induction of the EMT (Student's t-test; ***, $P < 0.001$). Anti-Snail was used for immunoprecipitation. (**D**): sites of ZEB1 binding to the LARGE2 and E-cadherin promoters, by an assay like that described for **C** but using anti-ZEB1 for immunoprecipitation (Student's t-test; **, $P < 0.01$).

To determine whether Snail and ZEB1 interact with the LARGE2 promoter directly, we carried out chromatin immunoprecipitation (ChIP) assays of PC-3E+ cells stably expressing Snail-ER. The co-immunoprecipitated DNA was identified using qPCR and primer sets that are specific for the LARGE2 promoter (#1 and #2) and span E/Z-box clusters (**Fig. 5A**). Goat IgG was utilized as a negative control for anti-Snail and pre-immune serum served as a negative control anti-ZEB1 in the immunoprecipitation. As an additional control, ChIP-qPCR was performed using primers specific to a LARGE2 intron (#3). We found that in PC-3E+ Snail-ER cells, the Snail signal at the LARGE2 promoter highly enriched (~13-fold increase over IgG control) at E/Z-box cluster 1, which lies in the proximal promoter region (~500 bp upstream of the TSS), but not at E/Z-box cluster 2 (~800 bp downstream of the TSS) or in the intron (**Fig. 5C**, left panel). ZEB1 signal, however, was mildly enriched (~2-fold relative to intron control) at both E/Z-box clusters 1 and 2, over signal from the intron control (**Fig. 5D**, left panel). Thus, Snail and ZEB1 bind to a similar region on the LARGE2 promoter that is conserved in human, mouse, rat and dog. Our findings also indicate that ZEB1 binds to a second E/Z-box cluster, downstream of the TSS. The E-cadherin promoter serves as a positive control (#1 is specific for E-cadherin promoter corresponding to −282 to −205 bp of E-cadherin gene and #2 corresponds to −4,092 to −4,017 bp of E-cadherin gene and serves negative control), and as expected both Snail[45] and ZEB1[55] were detected here as well (**Fig. 5C, D**, right panel). Overall, these findings support the notion that Snail and ZEB1 repress LARGE2 by binding directly to the LARGE2 promoter, thereby promoting αDG hypoglycosylation.

Expression of LARGE2 mRNA is inversely correlated with that of ZEB1 mRNA in clinical samples from various tumor types

Because both αDG hypoglycosylation and EMT-like changes are observed in many tumor types, we sought to probe the generality of our findings in prostate cancer cells by analyzing the The Cancer Genome Atlas (TCGA) database derived from clinical cancer specimens. Specifically, we used the RNA-Seq data in TCGA to identify genes whose mRNA expression profiles correlate with that of *GYLTL1B*, the LARGE2-encoding gene, in 4 cancer types in which DG expression and/or glycosylation is known to be down-regulated: prostate adenocarcinoma (PRAD), clear cell renal cell carcinoma (KIRC), invasive breast carcinoma (BRCA) and lung adenocarcinoma (LUAD). The analysis of gene expression associations was carried out using the Spearman correlation method, and identified those genes whose expression correlated either positively or negatively with that of *GYLTL1B*. We then selected those genes whose expression patterns were consistent across all 4 of the cancer types analyzed (**Fig. 6A**) for further analysis. 12 genes are positively correlated and 33 genes are negatively correlated with *GYLTL1B*. Notably, the expression of *ZEB1* is inversely associated with LARGE2 expression across all data sets, consistent with our findings above that LARGE2 mRNA expression may be regulated ZEB1 (**Fig. 6A**). Moreover, it is notable that ZEB1 is well above the significance cutoff indicating that it is among the most highly negatively correlated genes with LARGE2 (**Fig. 6B**). In the set of 33 negatively correlated genes, ZEB1 is one of only 4 transcription factors including TSHZ3, FLI1 and ZCCHC24. Notably absent in this analysis is Snail or any other genes obviously associated with EMT-like phenotypes. To investigate this further, we examined the correlation of LARGE2 with a core set EMT-related genes in each of the individual tumor types. The core set of EMT-related genes was derived from a meta-analysis of 18 separate gene expression

studies focused on EMT-like phenotypes in multiple cancers, including prostate, breast, kidney and lung and is comprised of 130 genes (67 upregulated and 63 downregulated) but does not include Snail.[56] Here we found an average of 10% of this gene signature correlated with LARGE2 in prostate, breast, kidney and lung with a high degree of concordance (15/16 genes in PRAD, 11/12 genes in BRCA, 10/12 genes in LUAD, and 15/18 genes in KIRC) with respect to the hypothesis that LARGE2 is downregulated in association with an EMT-like state (**Fig. 6B** and Supplementary **Table 1A–D**). Taken together, these results support the idea that ZEB1 is an important negative regulator of LARGE2, and that within individual tumor types, LARGE2 expression is correlated with other markers of EMT. Thus, our findings in cell lines may be applicable to clinical cancers.



Gene List-Positive Spearman	
FOXRED2	RBM4
MRPS25	C1orf172
FAM71E1	FH
CLPB	APOA1BP
PACSIN3	LYPLA2
RAC3	THAP4

Gene List- Negative Spearman	
FSTL3	CRTAP
LGALS1	PLEKHO2
STS	DSE
CYP3A5	NDN
TSHZ3	GLDN
CD93	MYADM
LILRA2	FLI1
CCND2	TPSAB1
CD33	SH2B3
MGLL	MSRB3
HSPB8	MPEG1
TLR3	MNDA
CEP120	ELTD1
SGK269	ZCCHC24
SSH1	ALOX5AP
ZEB1	RBMS3
PECAM1	

B

Tumor Source (N)	Spearman ZEB1	Spearman Range (Neg-Pos)	Significance Cutoff (Absolute value)	% Changed EMT signature genes
Prostate (358)	-0.4117	-0.5131 - 0.5377	0.2286	12.31%
Breast (956)	-0.3182	-0.353 - 0.4365	0.1408	9.23%
Lung (412)	-0.4559	-0.5718 - 0.587	0.2134	9.23%
Renal (1102)	-0.2398	-0.6678 - 0.704	0.1312	13.85%

Figure 6. Expression of the LARGE2 mRNA is negatively associated with ZEB1 in cancers from a variety of tumor types. (**A**): Venn diagrams illustrate the significant positive and negative Spearman correlations between ZEB1 defects and prostate adenocarcinoma, invasive breast carcinoma, clear cell renal cell carcinoma and lung adenocarcinoma, and the associated tables list all genes that are up- or down-regulated by all of these cancer types. (**B**): Absolute Spearman correlation coefficients are presented for *ZEB1* in reference to *GYLTL1B* in each of the tumor types with associated cutoffs listed for significance and the range of Spearman coefficients found in each tumor set. % changed EMT signature genes in each tumor data set are derived by evaluating Spearman correlation of *GYLTL1B* in a core set of EMT signature genes defined in Ref. 56. Individual genes identified are listed in Supplementary **Table 1A–D**.

Discussion

In the study reported here, we show that the EMT transcription factors Snail and ZEB1 cooperatively regulate the expression of LARGE2 in prostate cancer cells. This is the first analysis to link αDG hypoglycosylation to an EMT-like process in cancer. EMT was first described in metazoan development, and involves a phenotypic switch whereby cells exchange epithelial characteristics for mesenchymal qualities that facilitate their movement in the embryo.[57] This process is reversible and may occur within a particular cell lineage a number of times over the course of development. For example, it may occur during gastrulation and later in the development of the neural crest, to achieve the organization of the mature tissue. DG has been implicated in gastrulation-associated EMT in the chick embryo.[58] In this context, DG expression (mRNA and βDG protein) is reduced in the epiblast cells as they enter the primitive streak, coincident with the loss of basement membrane proteins, including laminin, in this region. However, it remains unclear whether the functional glycosylation of αDG is modulated during chick gastrulation. In our study of cancer cells, both the expression of βDG protein and the functional glycosylation of αDG were decreased in response to forced expression of Snail. It is not yet clear whether down-regulation of DG protein (as evidenced by reduced βDG) is due to the transcriptional regulation by Snail, but the *DAG1* gene contains clusters of E/Z-boxes consisting of 5 E-boxes and 1 Z-box between −1645 bp and −1197 bp of the TSS consistent with this possibility. Taken together, this suggests that EMT may regulate DG function at multiple levels.

The expression of DG and the functional glycosylation of its αDG subunit are often dysregulated in cancer. In prostate cancer,

both are downregulated, and this feature correlates with disease progression.[22-24] αDG hypoglycosylation in prostate cancer is caused in part by the loss of LARGE2 expression.[24] The resulting loss of DG function may contribute to an invasive cancer phenotype by reducing adherence to matrix proteins within the BM, thereby possibly affecting the structural organization of those proteins.[59] Indeed, restoration of LARGE2 expression rescues functional glycosylation of αDG and results in diminished ability of prostate cancer cells to migrate in response to chemotactic stimulus, invade through Matrigel and reduces cell proliferation.[24] Similarly, EMT-like changes in cancer cells are associated with invasive phenotypes.[60-62] Although this study focuses primarily on prostate cancer cells, we note that certain breast- and renal-cancer cell lines also show simultaneous loss of E-cadherin expression and functional αDG glycosylation. Moreover, increased ZEB1 mRNA expression is correlated with decreased LARGE2 mRNA expression in these tumor types in the TCGA dataset. It is therefore possible that the mechanisms discovered here contribute to the progression of other cancers as well.

Many genes, including Snail and ZEB1, have been implicated in EMT-like changes in the context of cancer. In the case of prostate cancer, Snail expression increases during disease progression,[63,64] and ZEB1 is highly expressed in particularly aggressive prostate cancers, its levels correlating with Gleason score in human prostate tumors.[65] These patterns are consistent with the reported reductions of DG expression and functional αDG glycosylation in prostate cancer.[23,24] Moreover, our unbiased analysis of the TCGA database revealed that the expression of ZEB1 correlates negatively with LARGE2 expression across 4 tumor types. Interestingly, Snail or other core EMT-related genes were not detected in this genome-wide cross-tumor analysis, although some core EMT-related genes were correlated with LARGE2 in individual tumor types. It is possible that this results from transient expression of Snail not easily captured in whole-tumor gene expression profiles or that the nature of EMT-related changes in tumors are highly variable. However, this does support the idea that ZEB1 is an important repressor of LARGE2 mRNA.

A variety of factors, both extrinsic and intrinsic to the cancer cell, have been implicated in producing EMT-like changes in prostate and other cancers.[66] Given that the EMT may be a response to stimuli in the local tumor microenvironment or to clonal genetic variation, it has been associated with intratumoral heterogeneity. Indeed, heterogeneity of this type may be present in cancer cell lines, as supported by our finding that the PC-3 prostate cancer line contains populations that are double-positive and double-negative for E-cadherin and functionally glycosylated DG. In PC-3E+ cells, overexpression of ZEB1 did not drive EMT throughout the population, as demonstrated by the presence of distinct double-positive and double-negative populations. The fact that the population negative for both anti-E-cadherin and IIH6 immunoreactivity was unstable and disappeared over time suggested that the overexpression of ZEB1 by itself could either be toxic or be overridden by other mechanisms. In contrast, tamoxifen-inducible expression of Snail, which acts upstream of ZEB1,[51] more robustly repressed both E-cadherin and functionally-glycosylated αDG. Additionally, Snail up-regulated ZEB1 in PC-3E+ and other prostate cancer cell lines. The fact that blocking ZEB1 expression in this context partially restored E-cadherin and LARGE2 mRNA expression indicated that Snail-induced repression of E-cadherin and LARGE2 is at least partly ZEB1-dependent. These findings are consistent with the cooperative nature of interactions between EMT transcription factors, and suggest that whereas Snail has an important role in establishing an EMT-like state, ZEB1 may be more involved in maintaining it. Our discovery that Snail binds only to the E/Z-box cluster upstream of the TSS of the LARGE2 gene whereas ZEB1 additionally binds to a TSS downstream E/Z-box cluster indicates that Snail and ZEB1 can bind to the LARGE2 promoter simultaneously, or perhaps sequentially. Moreover, our data suggest that Snail may down-regulate LARGE2 transcription both by directly repressing its promoter and simultaneously inducing ZEB1, bound to a distinct E/Z-box cluster of the LARGE2 promoter, to enhance this repressive effect. We showed that when ZEB1 up-regulation was blocked during Snail-induced EMT in prostate cancer cells, the repression of LARGE2 was reduced. Thus, Snail appears to control LARGE2 expression both directly and indirectly. In contrast, E-cadherin expression was rescued when ZEB1 upregulation was blocked, indicating that Snail-mediated E-cadherin repression in these prostate cancer cells occurs primarily via ZEB1.

In TEM4-18 cells, whose mesenchymal-like characteristics are stable, siRNA knockdown of Snail, ZEB1 or both was insufficient to rescue IIH6 immunoreactivity, despite resulting in increased LARGE2 mRNA expression. Whereas we did observe an increase in cell surface E-cadherin in both Snail and ZEB1 knockdowns. At least 2 possibilities exist to explain this discrepancy: (1) We did not achieve sufficient Snail or ZEB1 (or combined) knockdown to sufficiently increase LARGE2 mRNA expression. Consistent with this possibility siRNA-mediated knockdown of Snail and ZEB1 only increased LARGE 2 mRNA levels by 4- and 2-fold respectively (**Fig. 3A**), whereas $IIH6^{+}$ PC-3E cells show greater than 32-fold higher levels of LARGE2 mRNA compared to $IIH6^{-}$ TEM4-18 cells (**Fig. 1C**). Thus, there may be a quantitative explanation for the failure of siRNA-mediated knockdown of Snail and ZEB1 to restore IIH6 reactivity in spite of increasing LARGE2 mRNA levels. (2) Alternatively, there may be additional Snail- or ZEB1-independent factors that affect LARGE2 mRNA expression/activity to promote functional glycosylation of αDG in these cells. In this regard it is worth noting that 4 of the cell lines we examined for anti-E-cadherin and IIH6 immunoreactivity (BxPC-3 LNCaP, 786-O and Du145) were single-positive for one of these markers indicating that E-cadherin and αDG glycosylation can be independently, instead of coordinately, regulated.

One possible alternative regulatory mechanism is that LARGE2 is epigenetically silenced in these cells. Consistent with this possibility, ZEB1 recruits histone deacetylases and SWI/SNF chromatin-remodeling complexes to repress E-cadherin in cancer.[67-69] Moreover, the LARGE2 homolog LARGE is likely to be epigenetically silenced in breast cancer.[33] Thus, it is possible that the LARGE2 gene is stably repressed by an epigenetic mechanism. The idea that LARGE2 is stably repressed in cells that have

passed through an EMT is consistent with previous findings that, in distant metastases of prostate tumors, functionally glycosylated αDG is nearly absent[24] but E-cadherin remains detectable.[70-72] The fact that E-cadherin is expressed in metastatic tumors has led to the concept that, like cells participating in embryonic development, cancer cells may revert from an mesenchymal-like state in a process called the mesenchymal-to-epithelial transition.[73,74] Collectively, these considerations suggest the interesting possibility that some genes associated with an EMT-like changes may not revert during mesenchymal-to-epithelial transition, and that dysfunctional αDG glycosylation might be an indicator of prior EMT.

Finally, the mechanisms elucidated here regarding the regulation of LARGE2 in cancer cells may have implications for the treatment of other diseases. For example, in one type of congenital muscular dystrophy, Walker Warburg Syndrome, the LARGE gene itself is mutated[75,76] and not only muscle but also neural abnormalities are present. Unlike LARGE, LARGE2 is expressed at relatively low levels in both of these tissue types, perhaps because it is repressed by mechanisms similar to those described here.[77] If this is the case and the repression of LARGE2 is enforced by epigenetic mechanisms, it may be possible to reactivate LARGE2 expression using chromatin-modifying drugs. Such an approach could potentially be used to compensate for mutant LARGE in these patients, and is similar to the concept of inducing the dystrophin homolog utrophin in Duchenne Muscular Dystrophy patients.[78]

Methods

Cell culture

PC-3, 22Rv1, HT-29, HCT-116, A431, ZR-75-1, A498, MDA-MB-231, BxPC-3, LNCaP, 786-O, DU145 and HEK293 cells were obtained from the American Type Culture Collection (ATCC). Lines derived from PC-3 (PC-3E+, TEM4-18, PC-3 IIH6+ and PC-3 IIH6-) had previously been generated by the Henry laboratory.[52] 293FT and GP2-293 packaging cells were obtained from BD Biosciences. All lines were grown in medium containing 10% fetal bovine serum (HyClone) and 1% non-essential amino acids (GIBCO, #11140-050). Cultures were maintained at 5% CO_2 and 37 °C.

Antibodies

The primary antibodies used in this study are the following: anti-E-cadherin (R&D System); anti-DG: IIH6 (Santa Cruz Biotechnology, sc-53987), 8D5 (Novocastra/Leica, ab49515); anti-Twist2 (Sigma, clone 3C8, SAB1401972); anti-Snail (Cell Signaling, clone C15D3, #3879 for Western blotting; R&D Systems, AF3639 for ChIP assay); anti-ZEB1 (a kind gift from Dr. Douglas Darling) and anti-β-actin (Sigma A1978, clone AC-15, SAB1401972). The secondary antibodies are: Odyssey IRDye 800CW goat anti-mouse (Li-Cor Biosciences, P/N 827-08364) and Odyssey IRDye 680 goat anti-rabbit (Li-Cor Biosciences, P/N 926-68071); donkey anti-mouse IgM HRP; donkey anti-mouse IgM Dylight 488 and goat anti-mouse IgM Dylight 549 (Jackson Immunoresearch); and goat anti-mouse IgG FITC (Millipore). Controls for flow cytometery were IgG and IgM isotype controls (Sigma, M5284 and M5909) and a control for the ChIP assay was goat IgG control (Santa Cruz, sc-2028).

Plasmid constructs

For overexpression in cell lines, the coding sequences of Snail, Twist2 (amplified from PC-3-cell cDNA), and ZEB1 (Origene) were subcloned into the pQCXIP or pLEX vector. The sequences of all plasmids generated using PCR were confirmed by DNA sequencing. pWZL-blast-Snail-ER, pWZL-blast-Twist-ER[54] and proE-cad178-Luc[79] were purchased from Addgene. pSGG-GYLTL1B and pRL-TK were purchased from SwitchGear Genomics.

Retroviral and lentiviral transfection and transduction

Retroviruses containing Twist2, Snail and Snail-ER were generated by transient co-transfection of the packaging cell line GP2-293 with retroviral vector and the packaging plasmid pVSV-G. Lentiviruses containing ZEB1 and empty vector were produced in packaging cell line 293FT. The supernatant containing retroviruses or lentiviruses was collected, passed through a 0.45-μM filter and used to infect recipient cells. After infection, cells were selected in medium containing puromycin (1 μg/ml) or blasticidin (20 μg/ml) and passaged in culture.

siRNA transfection

All siRNAs were obtained from Dharmacon. These included the: ON-TARGETplus SMARTpool siRNA for the human homologs of ZEB1; ON-TARGETplus siRNA for Snail (AAUCGGAAGCCUAACUACA); and ON-TARGETplus Non-targeting Control siRNA #1 (negative control). Transfections were performed using Lipofectamine 2000 (Invitrogen).

Western blotting

Western blot analysis was performed as previously described.[24]

Quantitative RT-PCR

RNA and cDNA were prepared using the RNeasy mini kit (Qiagen, #74104) and the iScript™ cDNA synthesis Kit (Bio-Rad, #170-8891). Real-time PCR was performed using iQ SYBR green supermix (Bio-Rad, #170-8880). qPCR analysis was performed as described previously.[80] Relative expression values were calculated using the comparative Ct method.[81] GAPDH served as an internal control.

Flow cytometry analysis

Flow cytometry was performed as described previously.[24]

Reporter assay

HEK293 cells were plated at a density of 2.0×10^4 cells per well in 96-well dishes. Twenty-four hours later, the cells were transiently co-transfected with 20 ng of promoter, 20 ng pRL-TK Renilla and 20 ng of Snail- or ZEB1-expressing construct, plus 0.25 μl of lipofectamine 2000 (Invitrogen, #11668-027).

Forty-eight hours after transfection, the dual-luciferase assay was performed using the Dual-Glo Luciferase Assay System (Promega, #E2920) according to the manufacturer's instructions. The activity of firefly luciferase was normalized to that of Renilla luciferase.

ChIP assay

The ChIP assay was performed according to Millipore's EZ ChIP (#17-371) protocol, with the following specifications: the truChIP High Cell Chromatin Shearing Kit (Covaris) was used for crosslinking and to isolate nuclei; and DNA-protein complexes were sheared for 5 minutes in a Covaris S2 sonicator, as per the manufacturer's instruction. Promoter regions were detected by qPCR, using the following primer pairs: LARGE2 #1, 5′- GAGTGAGGCGAAGGCTTCAG -3′ and 5′- ACTG CGGGAGCTCGACACGA-3′; #2, 5′- CAGCTCTTGCAT GTGGCCATC-3′ and 5′- GAGGAAGCTGAAGCTCTGA-GACCAG -3′; and #3, 5′-TGGAGAAGAGAATCATCAG TCC-3′ and 5′-AGFTTCATTAGCCCATAGAGGC-3′. E-cadherin primers were described previously.[55]

Generation of spearman correlation matrix

RNASeqV2 data from the Cancer Genome Atlas (http://cancergenome.nih.gov/) was accessed via this site's Open-Access HTTP Directory. Level-3 data from prostate adenocarcinoma (PRAD [3.1.2.0]), lung adenocarcinoma (LUAD [3.1.7.0]), breast invasive carcinoma (BRCA [3.1.2.0]), and kidney renal clear cell carcinoma (KIRC [3.1.2.0]) were downloaded and Spearman correlation coefficients for all genes were generated using tumor and normal samples. Values for all genes are currently hosted by the University of Iowa Institute for Clinical and Translational Science (https://research.icts.uiowa.edu/tcga/index.html). Correlations with a calculated $p < 1.25 \times 10^{-5}$ were marked as significant, and overlay analysis of those genes meeting significance criteria was performed using Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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