Zeb1 affects epithelial cell adhesion by diverting glycosphingolipid metabolism

Daniel Mathow¹, Federica Chessa¹, Mariona Rabionet², Sylvia Kaden¹, Richard Jennemann¹, Roger Sandhoff², Hermann-Josef Gröne¹,* & Alexander Feuerborn¹,3,**

Abstract

This study proposes that the transcription factor Zeb1 modulates epithelial cell adhesion by diverting glycosphingolipid metabolism. Zeb1 promotes expression of a-series glycosphingolipids via regulating expression of GM3 synthase (St3gal5), which mechanistically involves Zeb1 binding to the St3gal5 promoter as well as suppressing microRNA-mediated repression of St3gal5. Functionally, the repression of St3gal5 suffices to elevate intercellular adhesion and expression of distinct junction-associated proteins, reminiscent of repression of St3gal5–induced disruption of cell–cell interaction and partially antagonizes elevation of intercellular adhesion imposed by Zeb1 knockdown. These results highlight a direct connection of glycosphingolipid metabolism and epithelial cell adhesion via Zeb1.

Keywords adhesion; EMT; glycosphingolipids; TGF-β; Zeb1

Results and Discussion

Repression of Zeb1 decreases expression of a-series GSLs and St3gal5

To examine a putative role of selected EMT-TFs in controlling GSL metabolism, we made use of mammary epithelial NM18 cells. These cells are derived from heterogeneous NMuMG cells and represent a morphological-homogenous subpopulation that has been characterized in previous studies [6–8]. The considered TFs (FoxA2, FoxQ1, Zeb1, Zeb2, c-Jun and Bnc1) were selected based on their proven impact on cell cohesion and our previous knowledge about their constitutive expression in NM18 cells [8].

RNAi-mediated suppression of these TFs in conjunction with thin-layer chromatography of GSLs identified Zeb-TFs to exert a notable influence on the expression of GSLs (Supplementary Fig S1A and B).

We subsequently characterized changes of GSLs in Zeb1-repressed cells in more detail. Suppression of Zeb1 elevated levels of lactosylceramide (LacCer) in the neutral fraction as well as 0-series GSL GM1b in the acidic fraction. Acidic a-series GSLs

1 Department of Cellular and Molecular Pathology, German Cancer Research Center (DKFZ), Heidelberg, Germany
2 Department of Cellular and Molecular Pathology, Lipid Pathobiochemistry Group, German Cancer Research Center (DKFZ), Heidelberg, Germany
3 Sir William Dunn School of Pathology, University of Oxford, Oxford, UK
*Corresponding author. Tel: +49 6221 424350; E-mail: h.j.groene@dkfz-heidelberg.de
**Corresponding author. Tel: +44 1865 285483; E-mail: alexander.feuerborn@path.ox.ac.uk
GM1a and GM2 were reduced (Fig 1A and B, Supplementary Fig S1A). Similar results were obtained with an independent siRNA targeting Zeb1 (Supplementary Fig S1C, left panel and D).

Next, we performed gene expression profiling of Zeb1-repressed cells and specifically extracted differentially expressed genes annotated with gene-ontology terms ‘lipid metabolic process’ and ‘sialyltransferase activity’ (Supplementary Table S1). Within this panel, we noted reduced expression of St3gal5 (GM3 synthase) in Zeb1 knockdown cells, which was further confirmed via qRT–PCR (Fig 1C, Supplementary Fig S1C, right panel). GM3 synthase converts LacCer to GM3, the precursor of higher gangliosides belonging to a-, b- and c-series (Fig 1A). Reduced expression of
St3gal5 switches expression of GSLs from the a-series towards the 0-series [9], just as we observed for Zeb1 knockdown cells, implicating that Zeb1 may affect GSL expression by transcriptionally regulating the expression of St3gal5. Indeed, the repression of St3gal5 mimicked knockdown of Zeb1 with regard to changes in GSL expression (Fig 1B). Quantification of neutral GSLs via liquid chromatography–electrospray ionization tandem mass spectrometry confirmed a significant increase in overall LacCer levels with similar acyl chain saturation in Zeb1- and St3gal5-silenced cells (Supplementary Fig S1E).

Acidic GSLs were further treated with Vibrio cholera sialidase which cleaves terminal sialic acid residues (from e.g. GM1b), but not inner sialic acid residues (from e.g. GM1a). Sialidase treatment resulted in accumulation of GA1 (GM1b without sialic acid) in the neutral fraction (Fig 1D, left panel), whereas GM1a remained present in the acidic fraction (Fig 1E). Immune overlay further confirmed the accumulation of GA1 upon incubation of acidic GSLs with sialidase (Fig 1D, right panel). Hence, the acidic GSL enriched upon Zeb1 and St3gal5 knockdown is GM1b, a 0-series specific compound harbouring a terminal sialic acid residue.

These results identify Zeb1 as a novel regulator of GSL metabolism, controlling the turnover of LacCer into a-series GSLs (b- and c-series GSLs were not detected in NM18 cells). Consistently, levels of LacCer increase upon Zeb1 repression, which, in the case of NM18 cells, is partially metabolized into 0-series GSLs (exemplified by the increase in GM1b). The findings that exclusive repression of St3gal5 mimics the GSL pattern of Zeb1-impaired cells and that expression of St3gal5 is reduced upon Zeb1 knockdown strongly imply that St3gal5 is the key target gene of Zeb1 for the observed alterations in GSL expression.

Zeb1 exerts a dual-regulatory role on St3gal5 expression

Next, we addressed regulatory mechanisms underlying Zeb1-dependent control of St3gal5 expression. Zeb1 binds to E-box sequences, and in silico analysis of St3gal5 promoter regions identified a single consensus (5’-CACC(T)G-3’) and seven degenerated (5’-CANN(T)G-3’) E-boxes within ~2.8 kb upstream of the transcription start site (TSS) (Fig 2A, Supplementary Fig S2A).

Luciferase reporter assays testing St3gal5 promoter fragments of different lengths (~2.7 kb, ~2 kb and ~0.7 kb) revealed strongest expression of the reporter containing the longest promoter fragment (Fig 2A). Truncated versions (2 kb and 0.7 kb) only expressed ~50% of luciferase compared to the 2.7 kb variant reporter. Activity of the 2.7 kb construct decreased to ~66% in Zeb1-repressed cells compared to respective controls. In contrast, the repression of Zeb1 did not significantly decrease reporter activity of the intermediate (2025-LUC) or shorter (687-LUC) promoter fragments (Fig 2A). These results suggest that Zeb1 may directly influence the St3gal5 promoter within the region containing the consensus E-box sequence. Consistently, site-directed mutagenesis of this site reduced reporter activity which was not further significantly impaired by Zeb1 repression (Fig 2A).

Luciferase activities of 2025-LUC and 2668mut-LUC seemed slightly reduced upon Zeb1 knockdown. Though the differences did not reach statistical significance, we assume that degenerated E-box binding sites and/or binding sites of TFs regulated by Zeb1 may contribute to these observations.

Next, we tested a putative interaction of Zeb1 with endogenous St3gal5 promoter. Previous work suggested that low protein expression levels of Zeb1 limit applicability of ChIP experiments [10]. We thus induced Zeb1 expression by exposing NM18 cells to TGF-β1, which also increased expression levels of St3gal5 mRNA (Fig 2B). ChIP experiments resulted in reproducible PCR-based detection of St3gal5 promoter (Fig 2C) after precipitation of Zeb1 from TGF-β1-induced cells. In contrast, a control region in intron 1 was not detected. These results argue that Zeb1 is a direct regulator of St3gal5 expression. Following the above outlined observations (e.g. Fig 2A–C), the Zeb1–St3gal5 regulatory axis seems to be constitutively present in NM18 cells but only robustly traceable in ChIP experiments upon TGF-β1 stimulation and increased levels of Zeb1 in these cells.

Zeb1 also represses microRNA (miRNA) expression, including the miR-200 family. Members of the miR-200 family themselves are established inhibitors of Zeb1 expression [11]. St3gal5-3’-UTR contains two conserved binding sites for miRNAs, namely miR-19a/b and miR-141/200a, as well as a non-conserved binding site for miR-141/200a (Supplementary Fig S2B). Expression analyses of miR-19a/b and miR-141/200a in Zeb1 knockdown cells revealed increased expression of miR-200a, whereas miR-19a/b remained unaffected (Supplementary Fig S2B and C). Expression of miR-141 could not be reliably detected. Luciferase assays employing St3gal5-3’-UTR revealed reduced expression of luciferase reporter in Zeb1 knockdown cells (Fig 2D), compatible with increased expression of miR-200a. Site-directed mutagenesis of the conserved miR-141/200a binding site partially antagonized the loss of luciferase signal in Zeb1 knockdown cells as did exclusive mutation of the non-conserved miR-141/200a binding site, though to a lesser extent. Mutating both sites blocked the decrease of luciferase activity upon Zeb1 knockdown (Fig 2D). Finally, a miR-200a inhibitor impaired the decrease of luciferase activity in Zeb1 siRNA-transfected cells (Fig 2D, right side).

Figure 1. Suppression of St3gal5/GM3 synthase recapitulates changes in GSL expression of Zeb1 knockdown cells and repression of Zeb1 decreases St3gal5 expression.

A Schematic representation of enzymes (black boxes) and metabolites (grey) related to (G)SL metabolism. GM3 synthase (GM3S, red box) converts LacCer into GM3, the precursor for all higher gangliosides of a-, b- and c-series. Gangliosides of the b- and c-series were not detected in NM18 cells.
B TLC-based comparison of GSLs. Cells repressed in Zeb1 or St3gal5 (72 h) reveal similar alterations in GSL expression as indicated by elevated levels of LacCer (†) and GM1b (α) and reduced levels of GM2(−) and GM1a (●). * indicates no GSL.
C qRT–PCR-based analysis of St3gal5 mRNA expression in Zeb1- and St3gal5-repressed cells. Cells were transfected for 72 h with indicated siRNAs (n = 3). Mean ± SD, n = 3, *p ≤ 0.05, **p ≤ 0.01, paired t-test.
D Acidic GSLs after treatment with sialidase reveal elevated levels of GA1 in the neutral fraction of Zeb1- and St3gal5-repressed cells (left panel). Validation of GA1 via immune overlay (right panel).
E Acidic GSLs of Zeb1- and St3gal5-silenced cells after treatment with Vibrio cholera sialidase. Sialidase cleavage leads to conversion of GM1b to GA1, detectable in the TLC of neutral GSLs (see D). GM1a levels are decreased in Zeb1- and St3gal5-knockdown cells (●).
Together, these results imply that Zeb1 exerts a dual-regulatory impact on St3gal5 expression including St3gal5 promoter and St3gal5 3’-UTR, the latter involving miRNA-200a. Several observations independent from this study suggest that Zeb1 may control expression of St3gal5 in various additional cell types. For example, ChIP data deposited at ENCODE [12] (Hudson Alpha Institute for Biotechnology, Richard Myers) suggest Zeb1 binding to St3gal5 promoter regions in B lymphocytes. Moreover, expression profiling of renal cell carcinoma samples with reduced expression of miR141/200a reveals increased expression of St3gal5 and Zeb1 [13].

Expression of Zeb1 and St3gal5 mRNA is also increased in human lung cancer cells (H358 cells) undergoing Twist-induced EMT-like progression as implicated by gene expression profiling [14]. This pattern is in line with our previous notion that Zeb1 regulates the expression of St3gal5. However, to further experimentally assess this, we repressed Zeb1 in H358 cells and analysed the

Figure 2.
expression of St3gal5 as well as GSLs. As shown, knockdown of Zeb1 resulted in decreased expression of St3gal5 mRNA (Supplementary Fig S2D) and reduced expression levels of a-series GSLs (e.g. GM3 and GM2), similar to repression of St3gal5 (Supplementary Fig S2D and E). However, unlike in NM18 cells, the repression of St3gal5 in H358 did not fully mimic changes in GSL expression imposed by Zeb1 repression as exemplified by the stronger elevation of LacCer as well as globotriaosylceramide (Gb3Cer) in St3gal5 knockdown cells. These results imply that in H358 cells Zeb1 may affect the expression of additional enzymes besides GM3 synthase, which may continuously metabolize substrates, such as LacCer or Gb3Cer.

The differences between H358 and NM18 cells in GSL expression upon Zeb1/St3gal5 repression are consistent with the notion that overall shifts in GSL expression patterns are the result of cell type-specific enzymatic repertoires, which determine the overall metabolite accumulation and utilization.

Repression of St3gal5 fosters expression of junction-associated proteins and elevates intercellular adhesion

Zeb1 represses the expression of junction-related proteins [15], and knockdown of Zeb1 suffices to elevate intercellular adhesion [8]. We thus tested next whether repression of St3gal5, like Zeb1, may affect the expression of junction-related proteins and intercellular adhesion [15,16]. Knockdown of St3gal5 partly mimicked the effects of Zeb1 repression and resulted in increased protein expression of E-cadherin as well as plakoglobin (Fig 3A, Supplementary Fig S3A), findings also reproducible in H358 cells (Supplementary Fig S3B).

The increased expression of E-cadherin and plakoglobin was paralleled by elevated intercellular adhesion in Zeb1- and St3gal5-repressed cells as assessed by hanging-drop assays, which became especially evident upon antagonizing intercellular adhesion by TGF-β1 stimulation (Fig 3B and C).

These findings were further supported by ultra-structural analyses (Fig 3D, left panel), which revealed an increase in total junction length (Fig 3D, right panel) as well as an increase in average length per junction between interfacing cells (Supplementary Fig S3C) upon Zeb1 or St3gal5 knockdown.

These findings demonstrate that suppression of St3gal5, like knockdown of Zeb1, elevates the expression of cell junction components as well as intercellular adhesion and implies that Zeb1 may partially affect these features by controlling GSL expression.

Overexpression of enzymatically active St3gal5 partially impairs elevated cell adhesion of Zeb1-repressed cells

Our results suggested that Zeb1 may modulate cell–cell adhesion via St3gal5-dependent control of GSL metabolism. To further assess a causal relation between Zeb1, St3gal5, cell–cell adhesion and GSLs, we tested whether overexpression of enzymatically active or inactive St3gal5 antagonizes elevated cell–cell adhesion of Zeb1-knockdown cells.

NM18 cells stably overexpressing wild-type St3gal5 (St3gal5-wt) (Fig 4A, upper panel) revealed decreased expression levels of LacCer (Supplementary Fig S4A and B) as well as 0-series lipids (GM1b) and maintained expression of a-series GSLs despite Zeb1 knockdown (Supplementary Fig S4A, right panel). Also, LacCer remained decreased upon Zeb1 knockdown (Supplementary Fig S4A, left panel and b) despite the decrease of endogenously expressed St3gal5 upon Zeb1 repression (Fig 4B, upper panels). In other words, alterations in GSL expression imposed by Zeb1 repression and subsequent decrease of endogenous St3gal5 expression are overcome by exogenously overexpressed St3gal5-wt.

Phenotypically, overexpression of St3gal5-wt resulted in reduced expression of E-cadherin and plakoglobin (Fig 4C, left panel) and hanging-drop assays revealed an enhanced sensitivity towards TGF-β1-mediated disruption of cell–cell adhesion. Repression of Zeb1 partially antagonized reduced intercellular adhesion of St3gal5-overexpressing cells in the context of TGF-β1 induction (Fig 4D, left panel).

In contrast, NM18 cells overexpressing mutant St3gal5 (St3gal5-mut) (Fig 4A, lower panel), which also showed decreased expression of endogenous St3gal5 upon Zeb1 knockdown (Fig 4B, lower panel), were incapable of counteracting lipid changes imposed by the repression of Zeb1 (Supplementary Fig S4C). Neither did they reveal decreased levels of E-cadherin nor plakoglobin (Fig 4C, right panel). Additionally, these cells were not sensitized towards TGF-β1-induced loss of intercellular adhesion (Fig 4D, right panel).

These phenotypic differences between cells overexpressing wild-type versus mutant St3gal5 further support the conclusion that the altered cell–cell adhesion and expression levels of junction components are determined by Zeb1- and St3gal5-dependent expression changes of GSLs.

However, overexpression of wild-type St3gal5 only mitigates but does not block the elevation of cell–cell adhesion upon Zeb1 knockdown, stressing that the control over GSL expression forms only a part of the repertoire of Zeb1 to influence cell–cell adhesion.
The Zeb1–St3gal5 regulatory axis promotes expression of a-series GSLs during TGF-β1-induced EMT-like progression

As previously outlined, a characterizing aspect of EMT-like processes is the impairment of intercellular adhesion. Based on our earlier observations of increased expression of St3gal5 (and Zeb1) upon TGF-β1 stimulation (Fig 2B), we speculated that TGF-β1-induced EMT-like progression of NM18 cells may be accompanied by elevated expression of a-series GSLs.

As shown, NM18 cells undergoing EMT-like progression (Fig 5A) indeed expressed elevated levels of GM1a, GM2 and GM3 (Fig 5B, right panel). Additionally, however, they revealed a slight increase in GM1b (Fig 5B, right panel) and clearly accumulated LacCer as well as decreased levels of hexosylceramide (HexCer) (Fig 5B, left panel). Repression of Zeb1 or St3gal5 (± TGF-β1 stimulation) antagonized elevation of a-series GSLs GM1a as well as GM2 and expectedly accumulated LacCer as well as GM1b (Fig 5C).
Figure 4.
The alterations in GSL expression in the absence of TGF-b1 stimulation were phenotypically paralleled by a pronounced epithelial morphology, as indicated by the ‘cobblestone’-like arrangement of cells within sheets (Fig 5D). An augmented alignment of cells within sheets was also evident in H358 cells upon Zeb1 and St3gal5 knockdown (Supplementary Fig S5A), though this was less pronounced than in NM18 cells.

In the presence of TGF-b1, Zeb1- and St3gal5-repressed NM18 cells became partially elongated but maintained sheet structures (Fig 5D), in line with cells detaching from each other.

The identified changes in GSL expression in NM18 cells undergoing EMT-like progression are coherent based on the following (Fig 5D), in line with cells failing to detach from each other.

Future work will need to test our assumption that Zeb1- or St3gal5-mediated expression changes of GSLs primarily modify cell–cell adhesion. Despite the divergent changes in GSL expression patterns and their reported various implications in different cells, we assume that they are all functionally united by affecting the organization of the cellular membrane. GSLs are known to separate membranes into specialized domains. These ‘GLS microdomains’ have been implicated in diverse cellular processes, such as those evoked during EMT-like processes, including receptor signalling [5], migration [4,5] and cell–cell adhesion (this study).

With regard to intercellular adhesion, GSLs are thought to participate in the formation of ‘glycosynapses’, GSL microdomains in which carbohydrate-mediated trans-interactions of GSLs with GSLs or proteins of adjacent cells [19] mediate cell–cell adhesion.

Here, we suggest that the loss of intercellular adhesion during TGF-b1-induced EMT-like progression is partially mediated via the engagement of Zeb1-St3gal5 and subsequent fostered expression of a-series GSLs. Though we can functionally not exclude changes in lipid expression that remained undetected in our study or combinatorial effects of different lipids, the expression of a-series GSLs seems associated with elevated or reduced intercellular adhesion (Fig 5E).

Future work will need to test our assumption that Zeb1- or St3gal5-mediated expression changes of GSLs primarily modify plasma membrane organization, in particular GSL microdomains (‘glycosynapses’), accounting for altered intercellular adhesion.
Figure 5.
Materials and Methods

Cell culture, treatments and retroviral infections

NM18 cells (kindly provided by Peter ten Dijke), retroviral infections, transfection of siRNAs and TGF-β1 induction have previously been described [8]. H358 cells (kindly provided by Peter Wirthschaft) were maintained in RPMI-1640 medium (Sigma) containing 10% (v/v) FCS. 

Transfection of small-interfering RNAs (siRNAs)

All siRNAs were obtained from Qiagen (Hilden, Germany). siRNAs targeting murine Zeb2 and St3gal5 were SI00209139 and SI01418431. Zeb1-specific siRNAs were SI01476279 (Zeb1 si1) and SI01476293 (Zeb1 si3, primarily used throughout the study). All additional murine-specific siRNAs have been described earlier [8]. siRNAs targeting human Zeb1 and St3gal5 were SI04272492 (Zeb1 si2), SI04368301 (Zeb1 si4), SI00718704 (St3gal5 si4) and SI04219950 (St3gal5 si6).

Affymetrix-based gene expression profiling

Gene expression profiling and data processing were performed as previously described [8]. Gene expression data are available at Gene Expression Omnibus (GSE63164).

Giemsa staining

Cells were fixed in methanol (−20°C) for 5 min and incubated with Giemsa (Merck Millipore) for 5 min prior to washing with PBS. Details on RNA isolation, qRT–PCR, TaqMan Small RNA Assays, analyses of glycosphingolipids and LC-ESI–MS/MS, Western blot, confocal microscopy, cloning, luciferase reporter assays, ChIP, hanging-drop assays, transmission electron microscopy, miRNA inhibitors and related statistical analyses are provided in the Supplementary Material and Methods.

Supplementary information for this article is available online:
http://embor.embopress.org

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Author contributions

DM, H-JG and AF designed the project and experiments. DM, FC, MR, SK, RJ and AF performed the experiments. DM, MR, RJ, RS, H-JG and AF analysed the data. DM, H-JG and AF wrote the manuscript with input from all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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