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Epigenetic regulation of galectin-3 expression by β1 integrins promotes cell adhesion and migration

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ABSTRACT

Introduction of the integrin β1- but not the β3-subunit in GE11 cells induces an epithelial-to-mesenchymal-transition (EMT)-like phenomenon that is characterized by the loss of cell-cell contacts, cell scattering, increased cell migration and RhoA activity, and fibronectin fibrillogenesis. Because galactose-binding lectins (galectins) have been implicated in these phenomena, we investigated here whether galectins are associated with the β1-induced phenotype. Intriguingly, we found that out of 9 galectins examined, the expression of galectin-3 (Gal-3) is specifically induced by β1 but not by β3. Using β1-β3 chimeric integrins, we show that the induction of Gal-3 expression requires the hypervariable region in the extracellular domain of β1, but not its cytoplasmic tail. Furthermore, Gal-3 expression does not depend on RhoA signaling, serum factors, or any of the major signal transduction pathways involving protein kinase-C (PKC), p38 mitogen-activated protein kinase (p38MAPK), extracellular signal-regulated kinase-1/-2 (ERK-1/2), phosphatidylinositol-3-OH kinase (PI3-K), or Src kinases. Instead, Gal-3 expression is controlled in an epigenetic manner. Whereas DNA methylation of the Lgals3 promoter maintains Gal-3 silencing in GE11 cells, expression of β1 causes its demethylation, leading to transcriptional activation of Gal-3. In turn, Gal-3 expression enhances β1 integrin-mediated cell adhesion to fibronectin (FN) and laminin (LN), as well as cell migration. Gal-3 also promoted β1-mediated cell adhesion to LN and Collagen-1 (Col)-1 in cells that endogenously express Gal-3 and β1 integrins. In conclusion, we identify a functional feedback-loop between β1 integrins and Gal-3, that involves the epigenetic induction of Gal-3 expression during integrin-induced EMT and cell scattering.

INTRODUCTION

Integrins are transmembrane receptors composed of an α- and a β-subunit that connect the extracellular matrix (ECM) to the cytoskeleton, thus ‘integrating’ the cell interior with its environment. In this way, integrins regulate cell adhesion and cell spreading, as well as cell migration, proliferation, differentiation, and ECM remodeling (1-2). Upon integrin-ligand binding, a variety of proteins associates with the cytoplasmic tail of the β-subunit, initiating cytoskeletal reorganization, assembly of macro-molecular adhesion complexes such as focal adhesions (FAs), and signaling through Rho GTPases and kinase-regulated pathways (3-6). However, individual integrins can trigger very distinct cellular responses, even if they bind the same ligand.

We have previously characterized the cellular phenotypes induced by the β1 versus the β3 subunit in the β1-deficient murine epithelial cell line GE11 (7,8). GE11 cells grow in well-defined epithelial islands and express relatively low levels of αvβ3. Introduction of the β1-subunit, which results primarily in cell-surface expression of the fibronectin (FN) receptor α5β1, causes dramatic morphological changes reminiscent of an epithelial-to-mesenchymal transition (EMT), including the loss of cell-cell contacts, cell scat-
tering, and a contractile, fibroblast-like phenotype with high cytoskeletal tension, large peripheral FAs, and multiple protrusions. The β1-induced phenotype is associated with high RhoA activity, fast but random cell migration, FN fibrillogenesis, and the assembly of fibrillar adhesions distributed along FN fibrils (7). In contrast, overexpression of the β3 subunit, leading to cell-surface expression of the alternate FN-receptor αvβ3, increases cell spreading but induces only a modest loss of cell-cell contacts and a ‘pancake’-like morphology with many small, randomly distributed FAs (8-9). Moreover, β3 stimulates Rac but not Rho activity and promotes directional rather than random migration. GE11 cells therefore constitute a unique model to study differential effects of integrins on Rho GTPase activation, cell migration, and other integrin-regulated processes.

Several of the β1-induced phenomena are associated with the actions of a family of matricellular proteins, the β-galactoside-binding lectins (galectins). The galectins comprise 15 highly conserved proteins that bind through a carbohydrate-recognition domain to N-glycosylated proteins at the cell surface, including integrins and several of their ligands such as laminin (LN) and FN (10-14). Galectins can thus modulate integrin-mediated events, and this has been most extensively documented for galectin-1 (Gal-1), galectin-3 (Gal-3), and galectin-8 (Gal-8). For instance, Gal-1 stimulates integrin-mediated adhesion and signaling in platelets and smooth muscle cells, and Gal-8 modulates cell adhesion and cell spreading in a number of cell types including neutrophils, fibroblasts, and several tumor cell lines (15-19).

The best-characterized family member, Gal-3, has an important role in a wide variety of physiological and pathological phenomena, which is at least in part mediated through its interactions with integrins. Gal-3 regulates the adhesion of epithelial cells to collagens and laminins, and promotes keratinocyte migration over LN-332 and wound re-epithelialization in mice (14,20-23). Furthermore, Gal-3 stimulates neutrophil adhesion and migration, as well as eosinophil adhesion and ‘rolling’ (24-25). Gal-3 also regulates adhesion and cell spreading of a number of cancer cell lines (26-28). In addition, Gal-3 promotes FA turnover and integrin α5β1-dependent assembly of fibrillar adhesions and FN fibrillogenesis in tumor cells (29-30). Finally, Gal-3 increases the adhesion of disseminating cancer cells to endothelium, thereby protecting them from anoikis (12,31).

In this study, we investigated whether galectins are involved in the β1-induced phenotype in GE11 cells. Intriguingly, we found that β1 but not β3 specifically triggers transcriptional activation of Gal-3, through a mechanism that involves the demethylation of the Lgals3 promoter. In turn, Gal-3 promotes β1-mediated cell adhesion and cell migration. Thus, we identify a functional feedback-loop between β1 integrins and Gal-3.
RESULTS

Gal-3 expression is induced by the integrin β1, but not the β3 subunit
To determine the expression of galectin family members in GE11 cells and GE11 cells stably expressing the β1-subunit (GEβ1), we performed quantitative-PCR (Q-PCR) analysis of 9 galectins. The only galectin that was expressed at a detectable level in GE11 cells was Gal-1, and its expression was increased 4-fold in GEβ1 cells. Intriguingly, Gal-3 was hardly expressed in GE11 cells but its expression increased dramatically (28-fold) in GEβ1 cells (Figure 1A). Next, we analyzed protein levels of Gal-1 and Gal-3 by Western blotting. To determine if the regulation of these galectins was specific for the β1 subunit, Gal-1 and Gal-3 expression was also analyzed in GE11 cells that overexpress the β3 subunit (GEβ3), which leads to elevated cell-surface expression of αvβ3 (8). Whereas Gal-1 protein was detected in GE11 cells and its expression was enhanced in both GEβ1 and GEβ3 cells, high expression of Gal-3 protein was only detected in GEβ1 cells but not in GE11 or GEβ3 (Figure 1B). Thus, whereas both β1 and β3 enhance Gal-1 expression, high Gal-3 expression is induced by β1 but not by β3.

The hypervariable region of the I-like domain of β1 is required for Gal-3 expression
Since β1 but not β3 induces Gal-3 expression, and these integrin subunits trigger very different morphological responses, we investigated whether the induction of Gal-3 expression correlates with cell morphology. Furthermore, we addressed the importance of the β1-cytoplasmic tail in the regulation of Gal-3 expression. We therefore used the β1-3 chimeric integrin, which consists of the extracellular and transmembrane regions of β1, fused to the cytoplasmic domain of β3.

Figure 1 β1 integrins induce expression of Gal-3. (A) Expression of the indicated galectins in GE11 and GEβ1 cells was investigated by Q-PCR analysis. Represented are the normalized averages of 3 independent experiments, and statistically significant differences (p<0.05) are denoted by an asterisk. (B) Protein expression of Gal-1 and Gal-3 was analyzed by Western blotting in GE11, GEβ1, and GEβ3 cells.
Expression of this chimera in GE11 cells induces a phenotype similar to that of wild-type β1, including the loss of cell-cell contacts, cell scattering, and a fibroblast-like morphology with multiple protrusions and peripheral FAs (Figure 2A) (8). Thus, the extracellular but not the cytoplasmic domain of β1 is required for these events, and the extracellular domain of β3 cannot substitute for it. The extracellular domains of integrin β-subunits vary widely in a region contained within the I-like domain, called the hypervariable region. This region affects ligand specificity and regulates the activity of Rho GTPases (32,33). Replacing the hypervariable region in β1 with that of β3 (creating β1-3-1) prevents RhoA-mediated contractility and FN fibrillogenesis, and induces a GEβ3-like morphology (Figure 2A) (34). Intriguingly, we detected high levels of Gal-3 expression in GEβ1 and GEβ1-3 cells, but not in GEβ3 or GEβ1-3-1 cells. In contrast, the level of Gal-1 expression was similar in GEβ1, GEβ1-3 and GEβ1-3-1 cells and was only marginally lower in GEβ3 cells (Figure 2B). Thus, these findings show that Gal-3 expression specifically correlates with the contractile, mesenchymal-like phenotype, and does not depend on the cytoplasmic tail of β1. Rather, the hypervariable region in the extracellular domain of β1 is required for both the β1-induced cell morphology, and the induction of Gal-3 expression.

β1-induced Gal-3 expression is not regulated by RhoA signaling or kinase-regulated signal transduction pathways

As the hypervariable region of β1 is required for both Gal-3 expression and RhoA activity, we reasoned that RhoA signaling might regulate Gal-3 expression downstream of β1. A major effector of RhoA is Rho kinase (ROCK). To test if ROCK is involved in Gal-3 expression, GEβ1 cells were incubated for 48 hrs with the ROCK-inhibitor Y-27632. This treatment efficiently inhibited ROCK activity, as judged by the dissolution of actin stress fibers and the induction of long, thin protrusions (Figure S1A). However, Gal-3 expression was not affected by ROCK inhibition at any concentration tested (Figure S1B). To assess whether RhoA regulates Gal-3 expression through another pathway, we depleted RhoA using RNAi. Whereas RhoA was completely absent 48 hrs after transfection, Gal-3 expression levels remained unchanged (Figure S1C). We also investigated if active RhoA can induce Gal-3 expression independently of β1, by introducing a constitutively active RhoA mutant fused to GFP (GFP-RhoA-Q63L) (35) into GEβ3 cells. Transfected cells were FACs-sorted for GFP and subsequently grown overnight, after which Gal-3 protein levels were determined by Western blotting. Expression of GFP-RhoA-Q63L did not induce a GEβ1-like phenotype in GEβ3 cells, indicating that the activation of Rho signaling alone is not sufficient for the induction of EMT-like changes, and that β1 is absolutely required for this morphology (data not shown). In line with the previous results, expression of GFP-RhoA-Q63L did not induce Gal-3 expression in the absence of β1 (Figure S1D). Taken together, these results suggest that RhoA signaling is not involved in the induction of Gal-3 expression by β1.

Besides Rho GTPases, β1-integrins activate a variety of signal transduction
pathways (4,5). In an attempt to identify a \( \beta 1 \)-induced signaling pathway that regulates Gal-3 expression, we treated GE \( \beta 1 \) cells with standard concentrations of a variety of compounds to inhibit signal transduction pathways that are commonly activated by \( \beta 1 \)-integrins, including PD98059 and UO126 to inhibit the extracellular signal-regulated kinase-1/2 (ERK-1/-2) cascade, SB203580 to inhibit p38 mitogen-activated protein kinase (p38MAPK), PI-103 and LY-294002 to inhibit phosphatidylinositol-3-OH kinase (PI3-K), PP1 to inhibit the Fyn/Src/Yes family of kinases, and Gö6983 to inhibit all isoforms of protein kinase-C (PKC). In addition, we used the broad-spectrum tyrosine kinase inhibitor genistein, and the
broad-spectrum serine/threonine kinase inhibitor staurosporine. None of the used compounds prevented Gal-3 expression in GEβ1 cells although genistein did reduce Gal-3 expression to some 70% of control levels, which is possibly due to cytotoxic side-effects (Figure S2A). Notably, whereas some inhibitors seemed to enhance Gal-3 expression in GEβ1 cells, they failed to do so in GE11, further underlining that expression of Gal-3 cannot be activated in the absence of β1. We then analyzed whether Gal-3 expression in GEβ1 cells depends on soluble factors that can trigger signaling pathways at the cell-surface, by culturing GE11 and GEβ1 cells in the absence or the presence of serum for 24 to 48 hrs. Gal-3 expression was only slightly elevated by serum but was not prevented in its absence, suggesting that although serum-components may potentiate β1-induced Gal-3 expression, they are not absolutely required (Figure S2B).

β1 integrins induce Gal-3 expression by demethylation of the Lgals3 promoter

The previous sections indicate that Gal-3 expression in GEβ1 cells is not maintained by RhoA signaling, serum factors, or kinase-regulated signaling. In addition, the β1-cytoplasmic tail, which contains docking sites for numerous signaling proteins, is dispensable for Gal-3 expression. It therefore seems unlikely that Gal-3 expression in GEβ1 depends on a ‘classic’ integrin signaling event. The expression of Gal-3 and other galectins is often silenced epigenetically by DNA methylation, but can be reversed to induce de novo expression, for example during tumor progression (36-38). We therefore investigated the DNA methylation status of the promoter region of Lgals3, the gene encoding Gal-3, by bisulfite sequencing. The murine Lgals3 gene contains a CpG island in the promoter region, which overlaps the start of exon 1 (Figure 3A). Following bisulfite-conversion of genomic DNA, a 268 bp Lgals3 promoter fragment containing 21 CpG sites was amplified using a bisulfite treatment-specific primer set (Figure 3A). The purified products were cloned into a TOPO vector, and sequencing of multiple individual clones (n > 9) revealed that virtually all of the CpG sites were methylated in GE11 and GEβ3 cells, corresponding with the lack of Gal-3 expression (Figure 3B). In contrast, the Lgals3 promoter was predominantly demethylated in GEβ1 cells (Figure 3B).

We subsequently employed an alternative strategy to confirm the differences in methylation status of the Lgals3 promoter, making use of the selective digestive properties of the HpaII/MspI restriction enzyme pair. Whereas both enzymes recognize the CCGG sequence, digestion by HpaII but not MspI is prevented by DNA methylation. The Lgals3 promoter contains 3 HpaII/MspI sites. When fully methylated, digestion of genomic DNA with HpaII, but not MspI, should generate a PCR fragment of 466 bp with primers overlapping the restriction sites (Figure 3C). Therefore, genomic DNA was treated with HpaII or MspI, and the 466 bp fragment was amplified by PCR and analyzed on gel. The expected fragment was generated from HpaII-digested genomic DNA isolated from GE11 and GEβ3, but not from GEβ1 cells (Figure 3C). In contrast, digestion of genomic DNA with MspI never resulted in an amplified product, thus confirming that
the Lgals3 promoter is indeed methylated in GE11 and GEβ3 cells, but not in GEβ1. These findings strongly suggest that the induction of Gal-3 expression in GEβ1 cells is caused by demethylation of the Lgals3 promoter. DNA methylation is maintained by DNA methyltransferases (Dnmts). To investigate whether the demethylation of the Lgals3 promoter in GEβ1 cells was the result of decreased overall expression or activity of Dnmts, we tested Dnmt expression by Western blotting.

Figure 3 Transcriptional activation of Gal-3 by β1-integrins is caused by demethylation of the Lgals3 promoter. (A) Relative distribution of 21 CpG sites located in the promoter region of the Lgals3 gene. Shown is the region from -164 bp upstream to +125 bp downstream of the start of exon 1. Arrows indicate start positions of amplification. (B) The methylation status of CpG sites in the Lgals3 promoter was determined in GE11, GEβ1, and GEβ3 cells by bisulfite modification followed by methylation-specific PCR and sequencing. Depicted is the representative methylation-status of 21 CpG sites for each cell line, determined from at least 9 individual clones. Closed circles represent methylated CpG sites, whereas open circles represent unmethylated CpG sites. The start of exon 1 is indicated by (0). (C) Top, schematic representation of the Lgals3 promoter, showing the location of 3 HpaII/MspI sites. Bottom; genomic DNA of GE11, GEβ1, and GEβ3 cells was treated with HpaII or MspI, subjected to PCR, and reaction products were analyzed on gel. Shown is a representative experiment (n=3). (D) Expression of Dnmt3b in GE11 and GEβ1 cells was investigated by Western blotting (left). Content of 5-Me-dC, expressed as a percentage of total dC content, in GE11 and GEβ1 cells was determined by HPLC (right). (E) GE11 cells were incubated for 48 hrs with 1 µM decitabine, and the methylation status of the Lgals3 promoter was investigated using digestion with HpaII or MspI, followed by PCR. (F) GE11 cells were incubated for 48 hrs with the indicated concentrations of decitabine, and expression of Gal-3 was investigated by Q-PCR (left) and Western blotting (right). (G) GEβ3 cells were incubated for 48 hrs with the indicated concentrations of decitabine, and expression of Gal-3 was investigated by Q-PCR (left; expressed relative to GE11 without decitabine) and Western blotting (right).
ting. Whereas Dnmt1 and Dnmt3a were not detected, the expression of Dnmt3b was not decreased in GEβ1 cells with respect to GE11 and perhaps even slightly elevated (Figure 3D). As a readout for global Dnmt activity, we then tested the overall content of methylated cytosines in GEβ1 cells by HPLC (36). The ratio of methylated/unmethylated cytosines was not decreased in GEβ1 cells, suggesting that demethylation of the Lgals3 promoter is a specific event, and is not the result of decreased overall Dnmt activity (Figure 3D). To confirm that DNA methylation is the cause for Gal-3 silencing in GE11, we incubated GE11 cells for 48 hrs with the Dnmt inhibitor decitabine, and performed digestion of genomic DNA with HpaII/MspI as described above. The 466 bp fragment was hardly amplified from GE11 cells after treatment with decitabine, indicating that the Lgals3 promoter was demethylated by this treatment (Figure 3E). In addition, decitabine caused mRNA and protein expression of Gal-3 in GE11 cells in a dose-dependent manner, confirming that demethylation of the Lgals3 promoter leads to transcriptional activation of the Lgals3 gene (Figure 3F). Finally, decitabine treatment also increased Gal-3 mRNA and protein in GEβ3 cells (Figure 3G). These findings unambiguously demonstrate that 1) DNA methylation maintains Gal-3 silencing in GE11 and GEβ3 cells, and 2) demethylation is sufficient to activate Gal-3 transcription, even in the absence of β1.

**Gal-3 promotes β1 integrin-mediated cell adhesion**

We next investigated whether Gal-3 contributes to β1-mediated adhesion of GEβ1 cells. The β1-integrin repertoire in GEβ1 cells consists primarily of the FN-binding integrin α5β1, whereas low levels of FN-binding αvβ1, as well as the LN-binding integrins α3β1 and α6β1 are also expressed (7,8). We first tested whether adhesion to FN was affected by knockdown of Gal-3 expression with siRNA. Because β1 also enhanced Gal-1 levels, Gal-1 was depleted in parallel. Both siRNAs caused a strong and specific reduction of their respective targets within 24 hrs after transfection, while a scrambled siRNA-control had no effect (Figure 4A). Interestingly, cell adhesion to FN was reduced to 50% of that of control cells by Gal-3 depletion, but was not affected by the loss of Gal-1 expression (Figures 4B,C). Thus, Gal-3 but not Gal-1 contributes to adhesion of GEβ1 cells to FN. Similar results were obtained using stable cell lines that were infected with shRNAs directed against Gal-3, and subsequently selected with puromycin (data not shown). Because the low amounts of α3β1 and α6β1 on GEβ1 cells allow weak adhesion to laminins, we next tested whether Gal-3 also promotes cell adhesion to LN-511, a ligand for both α3β1 and α6β1. In line with the results obtained on FN, Gal-3 depletion reduced cell adhesion to LN-511, albeit to a lesser extent (Figure 4D). Thus, Gal-3 promotes β1-integrin-mediated cell adhesion to FN and LN-511 in GEβ1 cells.

We then determined whether Gal-3 affects β1-mediated adhesion in epithelial cells that endogenously express Gal-3 and β1-integrins. For this purpose we used a keratinocyte cell line designated MKα3, which we have described previously (39). MKα3 cells are non-transformed keratinocytes isolated from mouse epidermis,
and their predominant β1-integrins are α2β1 and α3β1, whereas α5β1 is not expressed (39). Knockdown of Gal-3 expression in MKα3 cells reduced α2β1-mediated cell adhesion to Col-1 (Figure 4E). In addition, MKα3 adhesion to LN-511, which is primarily mediated by α3β1, was also decreased though less prominently than on Col-1 (Figure 4F). Together, these results show that Gal-3 promotes β1-integrin-mediated cell adhesion to FN and LN-511 in GEβ1 cells, and to Col-1 and LN-511 in MKα3.

**β1-induced Gal-3 expression promotes cell migration, but not FN fibrillogenesis or RhoA-mediated contractility**

Next, we investigated whether Gal-3 is involved in other β1-induced phenomena in GEβ1 cells such as cell migration, FN fibrillogenesis, and RhoA activity. To analyze cell migration, GEβ1 cells

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**Figure 4 Gal-3 promotes β1-integrin-mediated cell adhesion in GEβ1 and MKα3 cells.** (A) GEβ1 cells were transfected with Gal-1, Gal-3 or control siRNAs, and protein expression was analyzed 24 hrs after transfection by Western blotting. (B) Cell adhesion to FN was investigated in GEβ1 cells treated with control siRNAs (squares) or an siRNA directed against Gal-3 (circles). (C) Cell adhesion to FN was investigated in GEβ1 cells treated with control siRNAs (squares) or siRNAs directed against Gal-1 (circles). (D) Cell adhesion to LN-511 was investigated in GEβ1 cells and GEβ1 cells depleted of Gal-3. (E) Gal-3 expression was knocked down in mouse keratinocytes MKα3, and cell adhesion to LN-511 was investigated in an adhesion assay. (F) Adhesion of MKα3 cells and MKα3 cells depleted of Gal-3 to Col-1. Graphs represent the average of 3 independent experiments. Statistically significant differences are indicated by * (p<0.05) or ** (p<0.01).
were transfected with control or Gal-3 siRNAs and grown to confluence, and 24 hrs after transfection the monolayers were scratched with a pipette tip. Migration into the wound was then monitored by time-lapse microscopy, and migration speed was calculated from the movies. The knockdown of Gal-3 expression delayed migration speed by 30%, compared to cells transfected with control siRNAs (Figure 5A). Thus, in addition to cell adhesion, Gal-3 expression in GEβ1 cells also promotes cell migration. We then investigated FN fibrillogenesis in cell monolayers 24 hrs after siRNA transfection by confocal microscopy. Whereas FN fibrils in GE11 cells were not detected, GEβ1 cells transfected with control or Gal-3 siRNAs both demonstrated abundant FN fibrillogenesis (Figure 5B), and quantification demonstrated that the extent of fibril formation was not significantly decreased upon Gal-3 depletion (Figure 5C). Furthermore, Gal-3 knock-
down in GEβ1 cells did not affect RhoA activity, as determined by a RhoA G-LISA (Figure 5D). Consistent with this finding, Gal-3 knockdown did also not disrupt the RhoA-mediated contractile phenotype of GEβ1 cells, characterized by stress fibers and FAs located in the periphery (Figure 5E). Summarizing, these data indicate that Gal-3 expression in GEβ1 cells promotes β1-mediated cell adhesion and migration, but not RhoA-mediated contractility or FN fibrillogenesis.

**DISCUSSION**

In this study, we identify a functional synergistic loop between β1-integrins and Gal-3, consisting of the epigenetic activation of Gal-3 expression during β1-induced EMT, which enhances β1-mediated cell adhesion and migration.

A number of reports demonstrate that galectins can regulate integrin-mediated cell adhesion. However, the exact mechanism underlying this phenomenon is poorly understood and galectins seem to have both promoting and inhibiting effects. For example, whereas overexpression of Gal-3 promotes adhesion and invasion in tumor cells, their adhesion is inhibited by addition of exogenous Gal-3 (27,28). Similarly, soluble Gal-8 has been shown to inhibit cell adhesion, whereas Gal-8-coated surfaces support cell adhesion, cell spreading and integrin signaling in the same cells (15,16). It is conceivable that soluble galectins perform different functions than endogenously expressed galectins that are associated with membrane proteins, or immobilized galectins that are present in the ECM. However, additional complexity arises from observations that galectins can have opposing effects across different cell types (which may have different integrin repertoires), and even on different integrins within the same cell type. An example of the former is that while soluble Gal-8 inhibits the adhesion of CHO, HeLa, and HaCaT cells, it stimulates adhesion of neutrophils (15,17). An example of the latter is that Gal-3 regulates α2β1-mediated adhesion to collagens, but has no effect on FN- or LN-binding integrins in MDCK cells (22).

We find that Gal-3 promotes β1-mediated cell adhesion to FN and LN in GEβ1 cells, and to Col-1 and LN in mouse keratinocytes. Earlier reports have documented that Gal-3 regulates surface expression of α6β1 and α4β7 on cancer cells (26,28,31), but we did not detect differences in β1 expression by flow cytometry upon knockdown of Gal-3 expression (data not shown). Moreover, we found that FN fibrillogenesis was not affected upon Gal-3 depletion, while a previous study has shown that soluble Gal-3 can promote this process in mammary tumor cells (30). The discrepancy between the latter study and ours probably reflects differences in the used cell system, as well as differences in the effects of soluble Gal-3 versus endogenous Gal-3.

It is clear that the effects of galectins on integrin function are complex and depend on the integrin repertoire, the cell type, and the particular galectin involved. Accumulating evidence suggests that the pattern of glycosylation is key to the interactions of galectins with their ligands, and thus to the effects of galectins on cell...
behavior. Galectins bind to N-acetyllactosamine (typically Galβ1,4GlcNAc), which is a recurring sequence in branched N-glycans, and the affinity of galectins increases with the number of branches. Affinity is also regulated by adjacent saccharides, as different galectins show specificity for different oligosaccharides (11,40). Many galectins exist as dimers or oligomers, and Gal-3 can form up to pentamers, which are able to cross-link multiple ligands at the cell surface to form lattices of glycoproteins (41,42). In this way, galectins can cluster integrins but they can also form heterogeneous lattices consisting of integrins, non-integrin proteins that regulate adhesion such as MUC1, and other glycoproteins including cytokine- and growth factor-receptors (43,44). Thus, it is increasingly recognized that the effects of galectins can only be understood completely with knowledge of the molecular composition of the ‘glycoproteome’ at the cell surface, and its pattern and degree of glycosylation (40).

Irrespective of the mechanism whereby Gal-3 enhances β1 function, our data reveal a new aspect of galectin-integrin biology, as we document the transcriptional activation of a galectin by an integrin, which clearly has significance for integrin function. Gal-3 was specifically induced by β1, whereas neither endogenous αvβ3 in GE11 cells, nor overexpression of the β3-subunit in GEβ3 cells was able to activate Gal-3 expression. Moreover, introduction of the β4-subunit in GE11 also failed to induce Gal-3 (data not shown). Neither of these integrins supports the EMT-like phenotypic changes induced by β1 integrins, and the correlation between this phenotype and Gal-3 expression is further strengthened by our observations using chimeric integrins. Surprisingly, the induction of Gal-3 expression was independent of the β1 cytoplasmic tail, RhoA signaling, or common integrin-induced signal transduction pathways. Rather, its expression in GEβ1 cells is regulated in an epigenetic fashion by the demethylation of DNA sequences around the transcription-initiation site of the Lgals3 gene, which are methylated in GE11 and GEβ3. Demethylation of the Lgals3 promoter is a specific event, and is not the result of a loss or reduction of global methylation in GEβ1 cells. Our data emphasize the importance of DNA methylation in the regulation of the expression of various galectins, in line with previous reports (36-38).

In light of our data, 2 previous studies are particularly interesting. First, it has been shown that the cell-surface glycoprotein MUC1 controls Gal-3 expression in an epigenetic manner in cancer cells, be it through a miRNA-dependent mechanism rather than DNA-methylation. In turn, Gal-3 binds MUC1 at the cell surface and bridges it with epidermal growth factor receptor, thereby establishing a functional feedback-loop (44). Second, integrin α6β4 activates the expression of a number of target genes involved in invasion and metastasis, by demethylation of their promoters in MDA-MB-435 human mammary carcinoma cells (45). Thus, these reports provide additional evidence that integrins, as well as other cell-surface receptors, can function in a regulatory feedback-loop that involves the activation of expression of accessory proteins via epigenetic mechanisms rather than through a conventional signal transduction cascade.
Future research should aim at the identification of regulators between the integrin and Lgals3 methylation, and the link between Gal-3 expression and the acquisition of a mesenchymal phenotype. As Gal-3 is considered to be an important player in cancer progression and metastasis, and its expression is frequently regulated by DNA demethylation in tumor cells, it will be interesting to determine whether integrins are responsible for de novo expression of Gal-3 during tumor progression.

MATERIALS AND METHODS

Antibodies and other materials
Antibodies used in this study were directed against actin (Millipore), Gal-1 (R&D systems), Gal-3 (Abcam), integrin β1 (TS2/16) and paxillin (both from BD Transduction Labs), RhoA (Santa Cruz), Dnmt3b (Abcam), and tubulin (Sigma). Texas Red-conjugated phalloidin and DAPI were from Molecular Probes, FITC- or Texas Red-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories, decitabine (5-aza-deoxycytidine), FN, Y-27632, PD98059, UO126, SB203580, LY-294002, PP1, Gö6983, genistein, staurosporine and puromycin were from Sigma, PI-103 was from Merck, and Texas Red- and FITC-conjugated Streptavidin were from Pierce Chemical Co. Collagen (Col)-1 was purchased from Vitrogen and LN-511 from BioLamina. Biotinylated-FN was prepared as described previously (8).

Cells, plasmids, and transient transfections
The pcDNA3-GFP-RhoA-Q63L construct was kindly provided by Dr. Sylvio Gutkind (NIH, Bethesda, Maryland, USA). The β1-3 and β1-3-1 expression plasmids were a kind gift from Dr. Yoshikazu Takada (UC Davis, Sacramento, CA, USA). GE11 cells are epithelial in origin and were obtained by injecting β1-null mouse embryonic stem cells into blastocysts, which were allowed to develop into whole chimeric embryos until day E10.5. Cells were then retrieved from these embryos, immortalized with SV-40, and selected with G418. Polarized cells which had formed small colonies were cloned (7). GE11 cells expressing human β1, human β3, or the chimeric human integrins β1-3 and β1-3-1 were generated by transfecting Phoenix packaging cells with retroviral constructs encoding the indicated integrins, to produce culture supernatants containing virus. GE11 cells were infected with the supernatants, selected with zeocin, and FACS-sorted for expression of the respective integrins at the cell surface (7,8,34). All GE-derived cell lines were cultured in DMEM supplemented with 10% FCS and 100 U/ml penicillin/streptomycin (Gibco BRL). Mouse keratinocytes MKα3 were isolated as described previously (39). Briefly, skin was obtained from newborn mice and epidermis and dermis were separated with trypsin. Keratinocytes were retrieved by gentle shaking and centrifugation, and incubation in keratinocyte serum-free medium (Gibco) supplemented with 50 µg/ml bovine pituitary extract, 5 ng/ml epidermal growth factor, and penicillin/streptomycin. Spontaneously immortalized clones were obtained after several weeks.
These were named MKα3 and cultured routinely on Col-1 (3 μg/ml). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Transient transfections were performed using the Amaxa nucleofector, according to the manufacturers’ instructions.

**Western blotting**
Cells were washed with ice-cold PBS and lysed in protein loading buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5% mercaptoethanol) at 4°C. Proteins were separated on SDS-PAGE gels, transferred to polyvinylidene difluoride membranes (Millipore), and analyzed by Western blotting followed by ECL using the Super signal system (Pierce Chemical Co.).

**Q-PCR analysis**
Q-PCR was performed using the iCycler (Biorad) in a total volume of 25 μl containing 1.5 μl cDNA, 12.5 μl iQ SYBR Green Supermix (Biorad) and 400 nM of both the forward and reverse primer. The primers used were designed and validated according to a previously described protocol (46), and included primers for Gal-1 (5’-TGTTGTGTAACACCAAAGGAA-GAT-3’ forward and 3’-ACCTCTGTGATGCTCCC-5’ reverse), Gal-2 (5’-CAGTATGGGACCGCTTC-3’ forward and 3’-ACAGACAATGGTAGATCATC-5’ reverse), Gal-3 (5’-CAGATTGTTTCT-AGATTTGAGG-3’ forward and 3’-TTGTTGTAGCTGTTGCAG-5’ reverse), Gal-4 (5’-GCTAGAGAGGAG GTAACCCACTGG-5’ forward and 3’-CCATTTGGGATGGCTCTGGAC-5’ reverse), Gal-7 5’-AACAC-CAAAGAACAGGCAA-3’ forward and 3’-TGGGAAGTGGAGATTCGTCAG-5’ reverse), Gal-8 (5’-CTGAGGCTGGCCA-TTGGAA-3’ forward and 3’-AACGTCCGGCATTGGTG-5’ reverse), Gal-9 (5’-CTTTCTACACCCCATCCA-3’ forward and 3’-GATATGGGAACCTCGTAGCATCT-5’ reverse), Gal-10 (5’-CTGGATGTACTTTTGTCATGGG-3’ forward and 3’-AGATGCTCAGTAAATGGTTA-5’ reverse), Gal-12 (5’-CTGAGGCTGAGATGGGG-3’ forward and 3’-GGGAGACAGCTCTTGCA-5’ reverse). As a negative control, the cDNA was replaced by milliQ. Cyclophilin A, β-actin and HPRT-1 were used as reference genes. Each sample was analyzed in quadruplicate. Differences in mRNA expression were calculated by normalizing the Ct value of each sample to the geometric mean of the 3 reference genes ([delta]Ct = Ct,sample - (Ct,cyclo+Ct,actin+Ct,HPRT)/3). Subsequently, the fold difference in expression was calculated as 2^-[delta][delta]Ct, with [delta]Ct = [delta]Ct,Gal11 - [delta]Ct,Galβ1. The Mann-Whitney rank sum test was used to identify statistically significant differences between [delta]Ct-values of GE11 and GEβ1. All statistical analyses were performed in SPSS 12.0.

**Confocal microscopy**
Cells on glass coverslips were fixed in 2% paraformaldehyde, washed with PBS, permeabilized with 0.2% Triton X-100, and washed with PBS. Coverslips were subsequently blocked with 2% BSA in PBS for 1 hr, incubated with primary antibodies for 1 hr at RT, washed with PBS, and incubated with secondary antibodies for 1 hr at RT. After washing with PBS, coverslips were mounted in Mowiol supplemented with
DABCO (Calbiochem), and examined with a confocal Leica TCS NT microscope.

**RNA interference**

For Gal-1 and RhoA knockdown, a SMARTpool® from Dharmacon consisting of 4 siRNAs was used (catalog number M-042642-00-0010 against Gal-1 and M-042634-01-005 against RhoA). For Gal-3 knockdown, a custom siRNA was ordered from Dharmacon with the target sequence GUAACACGAAGCAGGACAA. The standard siCONTROL® siRNA from Dharmacon was used as negative control. Cells were transfected using the transfection reagent DharmaFECT® according to the manufacturers’ instructions. Experiments were performed 24-48 hrs post-transfection and knockdown was verified in each experiment by Western blotting. Alternatively, short hairpins against Gal-3 (target sequences included GCAGTACAACATCGGATGAA, CCCGCTTCAATGAGAACAACA, CCGCATGCTGATCAAACTCAT, CTTTGAGAGTGCGAAAACCACT, CCGGATGAGTGGCGAAAACCACCAT, CCAACGCACACAGGATTGTT), cloned into pLKO.1, were obtained from the TRC shRNA Open Biosystems library and transfected into HEK 293FT cells together with the Virapower™ Packaging mix (Invitrogen), using Lipofectamine 2000 according to the manufacturers’ instructions. Viral supernatant was harvested 48 hrs later, transduced into GEβ1 or MKα3 cells, and positive cells were selected with puromycin.

**Adhesion assays**

96-well plates were coated at 37°C with 1 μg/ml Col-1 (5 min), 1 μg/ml FN (1 hr), or 8 μg/ml LN-511 (overnight). The plates were washed twice with PBS, blocked in 2% BSA for 1 hr at 37°C, and washed twice with PBS. Cells were seeded at a density of 3x10^4 – 1x10^5 per well and allowed to adhere at 37°C for the indicated time-points. Non-adherent cells were washed away with PBS before the addition of substrate buffer (7.5 mM p-nitrophenyl N-acetyl-beta-D-glucosaminide (NPAG) in 0.1 M sodium citrate pH 5, 0.5% Triton-X100). Plates were then incubated overnight at 37°C, after which stop buffer (50 mM glycine, pH 10.4, 5 mM EDTA) was added and the absorbance was measured at 405 nm on a BioRad microplate reader. Alternatively, cells were fixed after washing and processed as described previously (33). Each condition was analyzed in triplicate, and graphs represent the average of 3 independent experiments.

**Scratch assays**

Cells in 24-well plates were grown to confluency and subsequently serum-starved overnight. Proliferation was inhibited with 10 μg/ml mitomycin C (Nycomed Inc.) 2 hrs before scratching the monolayers with a yellow pipette tip. Cells were then washed twice in serum-free medium to remove cell debris, stimulated with fresh medium, and incubated at 37°C. Scratched areas were photographed overnight at 10x magnification. To determine the relative migration, the area that was not covered by cells at the end of the assay (t(0)) was calculated over the open area at the beginning of the assay (t(0)). The resulting ratio in untreated cells was set to 1. Experiments were performed 3 times in triplicate.

**RhoA assay**

Cells were transfected with control or Gal-3 siRNAs, plated in tissue-culture plates, and RhoA activity was assessed...
24 hrs post-transfection using the Rho G-LISA kit (Cytoskeleton), according to the manufacturers’ instructions.

**FN fibrillogenesis assay**

Cells on coverslips were grown to confluence and then incubated overnight in DMEM containing FN-depleted serum, supplemented with 10 μg/ml biotinylated FN. Thereafter, they were processed for immunofluorescence as described above. FN was visualized with Texas Red-conjugated strepavidin and nuclei were stained with TOPRO-3. Several fields were photographed, and total fluorescence for each field was measured using ImageJ software and divided by the number of cells. Graphs represent the averages of 3 independent experiments.

**Analysis of promoter methylation**

For methylation analysis of the Lgals3 promoter, genomic DNA was isolated using the nucleospin tissue DNA isolation kit (Machery Nagel). The purified DNA was bisulfite-converted using the Epitect Bisulfite Kit (Qiagen) according to the manufacturers’ instructions. Next, PCR for regions of the Lgals3 promoter was performed using the following mouse- and bisulfite-specific primers: 5’-TGTTTTAGTT-TATGGGGAAGTT-3’ (forward); 5’-AAAACCCAACCCTTTTAATA-CAC-3’ (reverse), amplifying a 268-bp product which contained 21 CpGs located adjacent to the start of exon 1. PCR products were isolated from gel using a gel extracion kit (Qiagen), cloned into the pCRII-topo TA cloning vector (Invitrogen), and transformed into Top10 E. coli competent cells (Invitrogen). Positive clones were selected by blue-white screening and sequenced using M-13 reverse primer (Invitrogen). At least 9 individual clones per cell line, derived from independent experiments, were analyzed. CpG island prediction and primer design were performed using MethPrimer (47).

For DNA methylation analysis using restriction enzyme digestion, genomic DNA was treated with HpaII or MspI, after which 30 cycles of PCR were performed using the following primers: 5’-TTGGGT-GAGTCTGAGAGTCTG-3’ (forward); 5’-CATCCCAAAGAGACTTAGTATG-3’ (reverse), to amplify a 466 bp product. Reaction products were normalized for the amount of input DNA and analyzed on gel. All experiments were performed in triplicate.

**Analysis of global genomic methylation by HPLC**

To assess global genomic methylation levels, the ratio of 5-methyl-deoxycytosine (5-Me-dC) over total deoxycytosine (dC) was determined using HPLC (48). Cell lysates were depleted of RNA with 1 mg/ml RNase A (Sigma) for 5 min, after which DNA was extracted as described above. Next, 1 µg DNA was digested to single nucleotides using a combination of DNase I (Sigma), Nuclease P1 (Sigma) and alkaline phosphatase (New England Biolabs) treatment, and dC and 5-Me-dC content were quantified with an HPLC-UV system (Shimadzu) equipped with a 125x4mm Nucleosil 100-10 SA column (Macherey-Nagel). The mobile phase consisted of 40 mM acetic acid in 15% acetonitrile (pH 4.8), and the flow rate was 0.6 ml/min. Global 5-Me-dC content was expressed as a percentage of the total dC content. 6 replicates were analyzed per condition.
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ABBREVIATIONS

Collagen-1, Col-1; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; ERK, extracellular signal-regulated kinase; FA, focal adhesion; FN, fibronectin; Gal-1, galectin-1; Gal-3, galectin-3; Gal-8, galectin-8; LN, laminin; MAPK, mitogen-activated protein kinase; PI3-K, phosphatidylinositol-3-OH kinase; PKC, protein kinase-C; ROCK, Rho kinase

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