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Mgat5 and Pten interact to regulate cell growth and polarity

Pam Cheung[2,3] and James W. Dennis[1,2,3,4]

[2]Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, R988 Toronto, Ontario, Canada M5G 1X5 and [3]Department of Molecular and Medical Genetics and [4]Department of Laboratory Medicine and Pathology, University of Toronto, Ontario, Canada

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Phosphatase and tensin homolog (Pten) phosphatase opposes intracellular phosphoinositide 3-kinase (PI3K)/Akt signaling and is a potent tumor suppressor, while Golgi β1,6 *N*-acetylglucosaminyltransferase V (Mgat5) is positively associated with cancer progression and metastasis. β1,6GlcNAc-branched *N*-glycans on receptor glycoproteins promote their surface residency and sensitizes cells to growth factor signaling. Here we demonstrate that the Pten heterozygosity in mouse embryonic fibroblasts enhances cell adhesion-dependent PI3K/Akt signaling, cell spreading, and proliferation, while Pten/Mgat5 double mutant cells are normalized. However, planar asymmetry typical of fibroblasts and invasive carcinomas is not fully rescued, suggesting that Mgat5 and Pten function together to regulate the membrane dynamics of PI3K/Akt signaling typical of motile cells. Pten heterozygosity was associated with increased surface β1,6GlcNAc-branched *N*-glycans, suggesting positive feedback from PI3K signaling to *N*-glycan branching. In vivo, Mgat5$^{-/-}$ Pten$^{+/-}$ and Mgat5$^{+/-}$Pten$^{+/-}$mutant mice showed a small but significant increase in longevity compared with Pten$^{+/-}$ mice. Taken together, our results reveal that Mgat5 and Pten interact in an opposing manner to regulate cellular sensitivities to extracelluar growth cues.

Introduction

β1,6 *N*-acetylglucosaminyltransferase V (Mgat5) catalyzes the addition of β1,6GlcNAc in tetra-antennary *N*-glycans, a subset of structures with higher affinity for galectins than less branched *N*-glycans (Hirabayashi et al. 2002; Partridge et al. 2004). Although grossly normal at birth, Mgat5$^{-/-}$ mice display multiple deficiencies with age including hypersensitivity to autoimmune disease, higher oxidative metabolism, resistance to weight-gain on a high-calorie diet, and multiple aspects of early aging including osteoporosis, decreased muscle mass, and depletion of adult stem cells (Cheung P, Pawling J, Partridge EA, Sukhu B, Grynpas M, Dennis JW, unpublished manuscript). The Mgat5 deficiency also delayed mammary tumor formation and inhibits metastasis in polyomavirus middle T (PyMT) transgenic mice (Granovsky et al. 2000). The PyMT oncoprotein is a lipid-linked adaptor protein that recruits both p85 and Shc, and thus enhances Ras, phosphoinositide 3-kinase (PI3K), and extracellular response kinase (Erk) growth signaling (Webster et al. 1998). Activation of PI3K/Akt was lower in early-stage PyMT Mgat5$^{-/-}$ mammary tumors, suggesting that up-regulation of Mgat5 and its products enhance growth signaling (Granovsky et al. 2000). Indeed, tumor cells lines derived from the PyMT Mgat5$^{-/-}$ mice were shown to be less responsive to insulin-like growth factor (IGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and transforming growth factor (TGF)-β (Partridge et al. 2004). Complex *N*-glycans on EGF receptor (EGFR) and TGF-β receptors (TβR) bind galectin-3 and opposes their transit into the endosomes and caveolae. Cytokine sensitivity is rescued in Mgat5$^{-/-}$ tumor cells by either blocking endocytosis or by re-expression of Mgat5 (Partridge et al. 2004). Glycoproteins are cross-linked by galectins with avidities dependent on both branching and the number of complex *N*-glycans, thus generating a dynamic lattice that regulates surface residency of receptors and availability to ligands (Lau et al. 2007).

Oncogenic activation of Erk/PI3K pathways in tumor cells promotes autocrine TGF-β signaling and epithelial-to-mesenchymal transition (EMT) (Thiery 2003; Ozdamar et al. 2005). However, PyMT Mgat5$^{-/-}$ tumor cells maintain cell-cell adhesion junctions and columnar epithelium morphology while Mgat5$^{+/+}$ cells show loss of cell–cell adhesion, with a fibroblastic morphology that supports migration and tumor invasion. PyMT Mgat5$^{-/-}$ tumor cells are also deficient in cell spreading, α5β1 integrin activation, and fibronectin (FN) fibrillogenesis (Lagana et al. 2006). EMT, along with receptor tyrosine kinase (RTK) and TGF-β signaling, can be rescued in PyMT Mgat5$^{-/-}$ tumor cells by re-expression of Mgat5 (Lau et al. 2007). Oncogenic transformation increases Mgat5 gene expression (Kang et al. 1996; Chen et al. 1998), and our results indicate that β1,6GlcNAc-branched *N*-glycans are required for EMT.

Microfilament remodeling and cell migration are dependent on PI3K/p85 binding to phosphotyrosine residues in activated growth factor receptors and adaptor proteins (Cantley 2002). The PI3K product, phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3) recruits Akt and GDP exchange factors for Rac which stimulates F-actin remodeling, filopodia extension, and polarity characteristic of motile cells (Wang et al. 2002; Weiner et al. 2002). Cytosolic phosphatase and tensin

[1]To whom correspondence should be addressed; Tel: +1 416-586-8233; Fax: +1 416-586-8588; e-mail: mailto:dennis@mshri.on.ca

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homolog (Pten), phosphoinositide 3-phosphatase, binds with a short half-life to membranes at the trailing end of motile cells (Vazquez et al. 2006), creating regional concentration gradients of PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 (Funamoto et al. 2002). Pten$^{+/-}$ mice display adult phenotypes of hyperinflammation, early development of lymphomas and carcinomas, and loss of adult stem cells (Stambolic et al. 1998; Yilmaz et al. 2006; Zhang et al. 2006). Moreover, molecular abnormalities in Pten and the PI3K/Akt pathway occur frequently in human malignancies (Inoki et al. 2005; Faivre et al. 2006).

Either PyMT oncoprotein expression or loss of Pten enhances PI3K/Akt signaling. Here we have examined the interaction between Mgat5 and Pten mutations in mouse embryonic fibroblasts (MEFs). These are premalignant cells that have not undergone confounding genetic changes associated with transformation in the PyMT transgenic mouse model (Rodriguez-Viciana et al. 2006). Our results demonstrate that the Mgat5 deficiency suppresses Pten$^{+/-}$ phenotypes of increased Akt activation, cell spreading and proliferation in MEFs. However, planar asymmetry of MEF cells was not completely normalized in double mutant cells. These results demonstrate that β1,6GlcNAc-branched *N*-glycans promote, while Pten opposes, PI3K signaling in primary cells, and together they regulate microfilament remodeling and planar asymmetry characteristic of motile cells. In vivo, the Mgat5 deficiency extended the survival time of Pten$^{+/-}$ mice, consistent with a compensatory molecular interaction and genetic epistasis.

Results

Mgat5$^{-/-}$ MEFs and tumor cells migrate more slowly than wild-type cells in scratch-wound assays, suggesting a deficiency in growth signaling (Demetriou et al. 1995; Guo et al. 2005). In contrast, Pten$^{+/-}$ MEFs display increased basal PI3K/Akt activation and cell spreading (Stambolic et al. 1998). To study the interaction between Mgat5 and Pten, primary MEFs with the six possible Mgat5/Pten genotypes were compared for substratum-dependent activation of Akt and Erk, 30 min after their application to FN-coated plastic. Pten$^{-/-}$ embryos arrest early in development (Stambolic et al. 1998), and therefore primary MEFs were only available for the Pten$^{+/-}$ and Pten$^{+/+}$ genotypes. As expected, Pten$^{+/-}$Mgat5$^{+/+}$ MEFs displayed elevated levels of phosphorylation of Akt at Ser473. However, Pten$^{+/-}$Mgat5$^{-/-}$ normalized Akt-p and partial normalization was observed in Pten$^{+/-}$Mgat5$^{+/-}$ MEFs, in both the presence and absence of serum (Figure 1). Serum increased adhesion-dependent activation of Akt in Mgat5-expressing MEFs, but only marginally in Mgat5$^{-/-}$ cells and MEFs with one copy of Pten (Figure 1A and B). These results confirm that β1,6GlcNAc-branched *N*-glycans enhance cellular sensitivity to cues for PI3K/Akt activation. In contrast, Pten heterozygous cells display precocious Akt-p, which can be suppressed in a dose-dependent manner by loss of Mgat5 activity. Therefore extracellular glycoproteins critical to PI3K/Akt/Pten signaling are dependent on modification with β1,6GlcNAc-branched *N*-glycans. Activation of Erk was low on FN substratum alone for all the cell lines, but enhanced in Mgat5-expressing cells in the presence of serum, as expected from previous studies (Partridge et al. 2004) (Figure 1C and D). Erk-p was also lower in Pten heterozygosity following serum-dependent substratum activation, probably due to down-stream positive feedback between the PI3K and Erk pathways, or differences in the kinetics of activation that are not revealed in this experiment (Figure 1D).

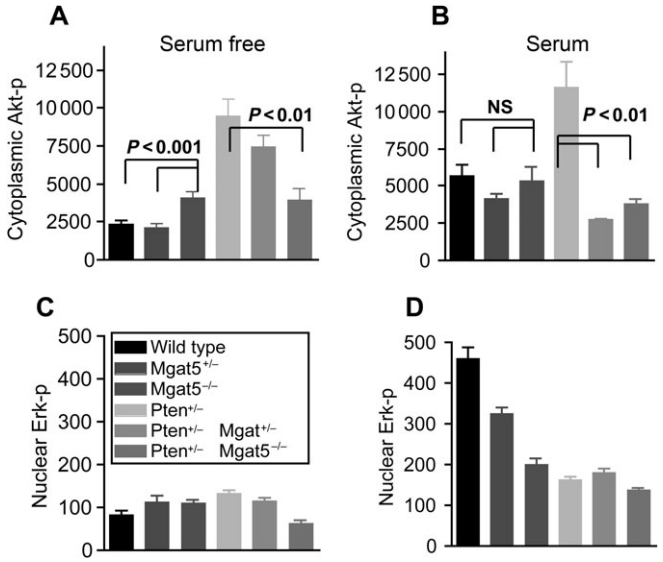

Fig. 1. Adhesion-dependent Akt and Erk activation in Mgat5/Pten mutant MEFs. Cells in (**A, C**) DMEM or (**B, D**) DMEM, 10% serum were added to FN-coated wells, fixed 30 min later, and (A, B) cytoplasmic Akt-p and (B, D) nuclear Erk-p measured by Array scan. *P*-values were calculated using the *t*-test.

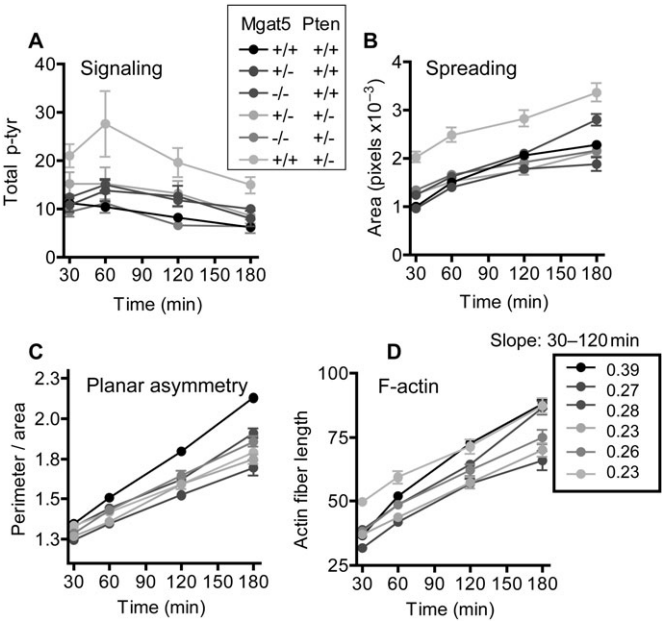

Fig. 2. Dynamics of Mgat5/Pten mutant MEF spreading on FN. Cells were added to FN-coated wells in DMEM, 10% FCS, at the indicated times and stained with antibodies to (**A**) phospho−tyrosine, TRITC−phalloidin to measure (**B**) cell area, (**C**) perimeter to area ratio, and (**D**) F-actin fiber length. Changes in fiber length are measured as the slopes between 30 and 120 min. Cells were imaged by the Array scan microscope, and results are the means ± SE for 500 cells at each time point.

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To examine cellular phenotypes dependent on PI3K signaling, MEFs were applied to FN-coated wells in the presence of serum and several features of cell morphology were quantified. Phospho-tyrosine levels and cell spreading following application to FN-coated plastic were increased in $Pten^{+/-}Mgat5^{+/+}$ compared with wild-type MEFs, but normalized in $Pten^{+/-}Mgat5^{+/-}$ and $Pten^{+/-}Mgat5^{-/-}$ MEFs (Figure 2A and B). The wild-type cells acquired a higher cell perimeter-to-area ratio compared to the other genotypes, a more elongated morphology with fewer but larger membrane protrusions (Figure 2C). Cell morphologies after 3 h on FN confirmed that $Pten^{+/-}$ and $Mgat5^{-/-}$ MEFs were rounded with many small filopodia protrusions and less organized microfilaments (Figure 3A). The $Mgat5^{-/-}Pten^{+/-}$ MEFs displayed larger protrusions, and denser parallel F-actin than either deficiency alone, consistent with a partial normalization of F-actin organization.

Cues that activate PI3K at the leading edges of protrusions are countered by Pten in retracting membranes, which created signaling gradients that promote F-actin remodeling and cell motility (Iijima and Devreotes 2002). If Mgat5 and Pten are opposing activities in the regulation of these signaling gradients, we might expect wild-type cells to display higher rates of F-actin remodeling than either mutation alone. Consistent with this expectation, F-actin fibre length was initially shortest in wild-type cells and increased faster after plating on FN than that of Mgat5 and/or Pten mutants (Figure 2D). Thus, the elongated cell morphology is stimulated under conditions where Pten activity can suppress basal PI3K/Akt activation and β1,6GlcNAc-branched *N*-glycans sensitize to extracellular cues, thus making a regional gradient of PI3K signaling possible in the cell (Figure 3A).

Expression of β1,6GlcNAc-branched *N*-glycans is increased in $Pten^{+/-}Mgat5^{+/+}$ when compared to wild-type MEFs indicating that PI3K signaling exerts positive feedback to Golgi *N*-glycan processing, possibly by increasing Mgat5 expression and/or metabolite flux to the hexosamine pathway (Figure 3B). Probing with concanavalin A (ConA) for the less branched and high-mannose *N*-glycans indicated similar levels of these structures on the cell surface, suggesting the effects of PI3K signaling are specific for GlcNAc-branching (Figure 3C).

Next, we compared acute cytokine signaling in serum-starved MEFs following stimulation with EGF, PDGF, or serum. Wild-type cells displayed the greatest cytokine response, a reflection of the dynamic range between resting and fully activated Erk signaling (Figure 4A). $Pten^{+/-}Mgat5^{+/+}$ MEFs showed the lowest serum dependency for proliferation and the highest rate of DNA synthesis. The wild-type cells ranked next and the combined mutants were poorly stimulated (Figure 4B). Therefore, the Mgat5 deficiency suppresses the hyperproliferative phenotype associated with $Pten^{+/-}$, consistent with an up-stream role for β1,6GlcNAc-branched *N*-glycans in growth promotion by cytokines.

Since the Mgat5 mutation suppresses PI3K activity in $Pten^{+/-}$ cells, tumor formation and mortality in Mgat5/Pten compound mutant mice might be delayed compared with $Pten^{+/-}$ mice. However, median survival for $Mgat5^{-/-}$ male and female mice was reduced at 520 and 522 days, respectively, compared with >650 days for wild-type

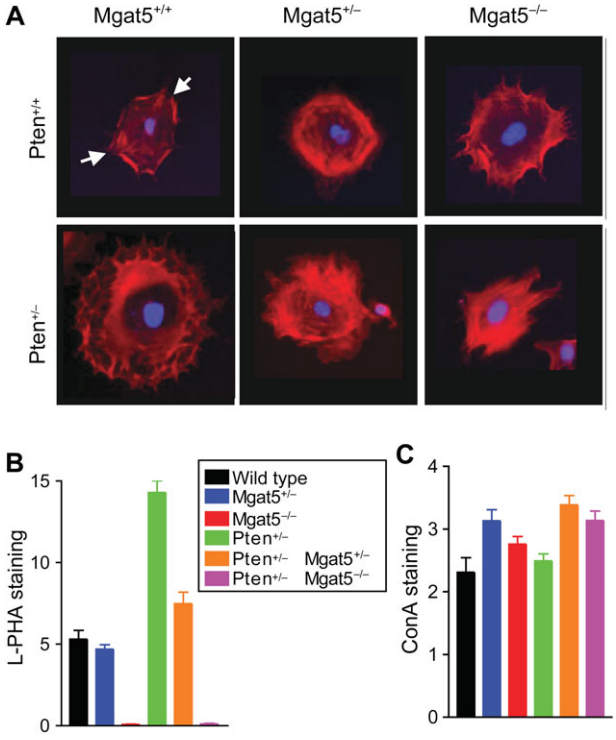

Fig. 3. Morphology and L-PHA reactivity of MEFs. (**A**) Morphology of Mgat5/Pten mutant MEFs on FN. Representative images from the Array scan microscope for six MEF genotypes. Arrows point to membrane protrusions where F-actin fiber ends are oriented. Cells surface (**B**) L-PHA–FITC staining, and (**C**) ConA–FITC staining of surface *N*-glycans in compound Mgat5/Pten mutant MEFs measured by Array scan.

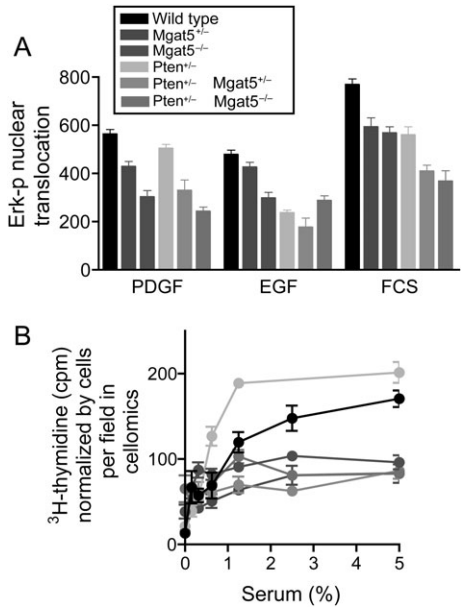

Fig. 4. Cytokine responsiveness and growth rates in Mgat5/Pten mutant MEFs. (**A**) Erk-p nuclear translocation in MEFs following serum starvation for 24 h, then stimulated with 100 ng/mL of the growth factors (5–10 min). (**B**) MEFs proliferation measured by pulse labeling with ^{3}[H]-thymidine for the final 24 h of a 2 day culture period. Results are mean ± SD of triplicate determinations, and representative of three independent experiments.

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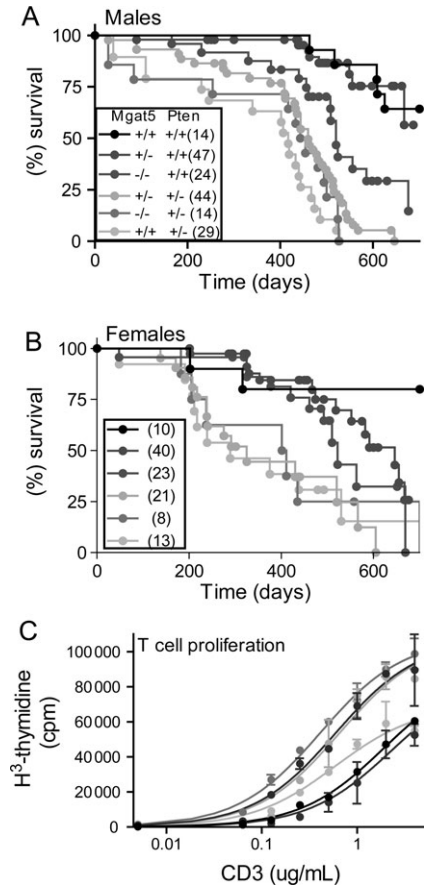

Fig. 5. Phenotypes of Mgat5/Pten mutant mice. Survival of (**A**) male and (**B**) female mice; (*n*) is number of mice; $P < 0.0001$ using Logrank test for survival and Logrank test for trend. Autopsies at death revealed lymphomas and other solid tumors as the likely cause of early mortality. (**C**) Stimulation of splenic T-cell proliferation by anti-CD3 antibodies (key is same as panels above). Results are mean ± SD of triplicate determinations, and representative of three independent experiments.

C57BL6 mice (Cheung P, Pawling J, Partridge EA, Sukhu B, Grynpas M, Dennis JW, unpublished manuscript). In contrast, median survival for $Pten^{+/-}$ male and female mice was 417 and 289 days, respectively, with deaths due to early cancer development, mainly lymphomas. The survival of $Pten^{+/-}$ mice was enhanced by the $Mgat5^{-/-}$ and $Mgat5^{+/-}$ mutations in males and females by 8–40% ($P < 0.0001$), with an apparent delay in the inevitable development of cancer in these mice (Figure 5A and B). Similarly, tumor progression in PyMT transgenic mice was delayed but not blocked on the $Mgat5^{-/-}$ background (Granovsky et al. 2000). Both the Mgat5 and Pten deficiencies enhance T-cell sensitivity to T-cell receptor (TCR) agonists, and result in tissue inflammation in vivo (Di Cristofano et al. 1999; Demetriou et al. 2001). Indeed, the combined deficiencies enhanced T-cell hypersensitivity, and inflammation might limit further rescue of longevity in Mgat5/Pten mutant mice (Figure 5C).

Discussion

The PyMT oncoprotein activates PI3K/Akt promoting transformation and metastasis in mice (Webster et al. 1998), but the metastasis phenotype also depends on Mgat5-dependent modification of surface glycoproteins (Granovsky et al. 2000; Lau et al. 2007). Loss of the tumor suppressor Pten also enhances PI3K/Akt signaling, and here we have examined phenotypes associated with cell motility and proliferation in Mgat5/Pten single- and double-mutant MEFs. Host selection pressures and tumor progression differ depending on the genetic background, and may confound our interpretation of Mgat5 functions in the PyMT model. Therefore, as premalignant cells that have not yet undergone genetic changes associated with transformation, MEFs represent a more homogenous cell model. We report that the Mgat5 deficiency normalized $Pten^{+/-}$ phenotypes including substratum-dependent hyperactivation of Akt and cell spreading, as well as serum-dependent hyperproliferation. The phenotypic interactions were revealed in the presence of extracellular stimuli, serum and substratum adhesion, consistent with the action of Mgat5 as an enhancer of receptor sensitivity to growth cues, upstream of negative regulation by Pten. $Pten^{+/-}$ MEFs display increased β1,6GlcNAc-branching of *N*-glycans suggesting that PI3K signaling is also an upstream regulator of Mgat5 expression and/or complex *N*-glycan biosynthesis. The adaptor protein Grb2 binds activated RTKs, and compound Pten/Grb2 hypomorphic MEFs also show decreased Akt-p compared with $Pten^{+/-}$ MEFs when stimulated with EGF (Cully et al. 2004). However, tumorigenesis was not delayed in $Pten^{+}/^{-}Grb2^{+/-}$ mutant mice, and only delayed in $Mgat5^{-/-}Pten^{+/-}$ compared with $Pten^{+/-}$ mutant mice based on survival time. This suggests that Pten is a critical and dosage-dependent suppressor of growth signaling in tumor cells, and opposes PI3K signaling from a variety of stimuli.

Integrins and cytokine receptors are transmembrane proteins, generally *N*-glycosylated in their extracellular domains, and surface residency is dependent on endocytosis rates and opposing molecular interactions at the cell surface (Partridge et al. 2004). The products of Mgat5, β1,6GlcNAc-branched *N*-glycans are higher affinity galectin ligands, that enhance surface retention of RTKs and TβR, and sensitize cells to cytokines (Lau et al. 2007). Fluorescence recovery after photobleaching experiments revealed that disruption of galectin binding in $Mgat5^{+/+}$ tumor cells by competition with lactose increases EGFR–green fluorescent protein mobility in the plane of the membrane (Lajoie P et al. unpublished material). Interestingly, microfilament remodeling was faster and planar polarity more developed in wild-type MEFs than MEFs with deficiencies in either or both Mgat5 and Pten. This indicates that polar asymmetry of motile cells requires a threshold of surface RTKs- and integrins-dependent signaling via PI3K and opposed by Pten; both are markedly sensitive to dosage, presumably creating the rapid dynamics of PI3K/Akt signaling gradients in the membrane (Weiner et al. 2002; Reynolds et al. 2003) (Figure 6). Thus, wild-type cells are most sensitive to substratum and serum cues for planar polarity compared with Pten, Mgat5, or compound mutant cells. Our results suggest that β1,6GlcNAc-branched *N*-glycans enhance sensitivity to cytokine gradients at ruffling edges of the cell, and when coupled with opposing Pten activity, promote $PI(3,4,5)P_3$ turnover in an asymmetric manner, as necessary for cell polarity (Figure 6). β1,6GlcNAc-branched *N*-glycans are also present on β1 integrin, and binding to galectin-3 and galectin-8 has been shown to enhance β1 recruitment and turnover in focal adhesions (Furtak et al. 2001; Levy et al. 2003;

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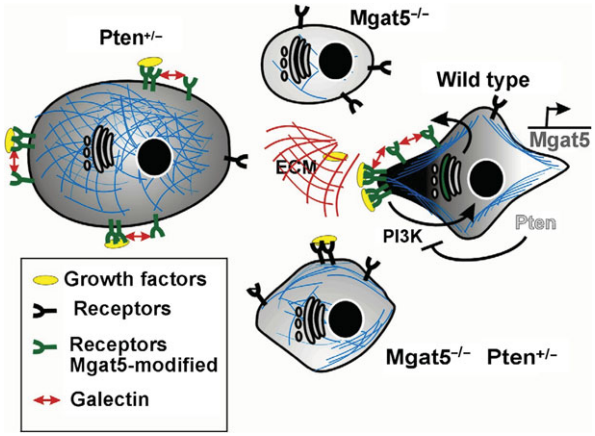

Fig. 6. Model of Mgat5- and Pten-dependent regulation of cell morphology. Cytokine and adhesion receptors with the potential to stimulate PI3K signaling bind to galectins, an interaction that enhances surface residency, and the dynamic of integrin turnover in focal adhesion. Mgat5 expressing cells generate glycoforms (green) with higher affinities for galectin, and availability to ligand. In wild-type MEFs, F-actin (blue cables) is organized in cortical bundles, and the cells display planar asymmetry typical of the motile phenotype. Cytokines and substratum adhesion promote PI3K signaling which is opposed by Pten, and, importantly, cell migration requires dynamic intracellular gradients of membrane PtdIns(3,4,5)P_3 (gray). The arrow depicts positive feedback from PI3K signaling to β1,6GlcNAc-branching, which appears to enhance these dynamics by increasing signaling from surface receptors.

Nishi et al. 2003). RTKs are recruited into focal adhesions where clustering can promote ligand-independent activation of EGFR (Moro et al. 2002). Therefore, galectin binding to β1,6GlcNAc-branched *N*-glycans on both α5β1 integrins and RTKs may sensitize cells in a cooperative manner to substratum and serum growth factors.

Survival of $Pten^{+/-}$ mice is reduced to <40% normal life span due to early tumor development. Inhibition of mammalian target of rapamycin) kinase with rapamycin, a downstream effector of Akt, inhibits lymphomagenesis and extends the longevity of $Pten^{+/-}$ mice (Yilmaz et al. 2006). Although survival of $Mgat5^{-/-}$ mice is also reduced to approximately 80% of wild type, compound Mgat5/Pten mutant mice display a 8–40% increase in longevity relative to $Pten^{+/-}$ mice. Loss of Mgat5 dampens precocious PI3K/Akt signaling in primary $Pten^{+/-}$ MEFs, implying that the delay in mortality may be due to slower proliferation of preneoplastic cells. Indeed, muscle satellite and bone marrow stem cells in $Mgat5^{-/-}$ mice show reduced growth signaling and early loss of self-renewal (Cheung P, Pawling J, Partridge EA, Sukhu B, Grynpas M, Dennis JW, unpublished material). It is not surprising that Mgat5/Pten compound mutant mice are not fully rescued for life span, as both genes affect many molecular targets and different tissues. In naïve T cells, the β1,6GlcNAc-branched *N*-glycans on TCRs slow antigen-induced clustering and thereby the threshold for PI3K/Erk signaling and activation (Demetriou et al. 2001). Unlike MEF phenotypes, the Mgat5 and Pten deficiencies in T cells are additive, with double mutants showing greater hypersensitivity to TCR agonists. Although inflammation may limit longevity in the single-mutant mice, Mgat5/Pten compound mutant mice show significantly enhanced survival relative to $Pten^{+/-}$ mice. Therefore, the compensatory or opposing activities of Mgat5 and Pten are a significant influence on survival time in vivo.

Materials and methods

Mice and cell lines

Mgat5 and Pten mutant strains of mice (Stambolic et al. 1998; Granovsky et al. 2000) on the C57BL/6 background were intercrossed and monitored for survival, analyzed using Prism 4.0 and Kaplan–Meier survival analysis. MEFs were isolated from E14.5 embryos using standard protocols, and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS, serum) (Invitrogen Canada Inc. 2270 Industrial St Burlington, Ontario L7P 1A1). After two passages, the MEFs were cryopreserved in 10% dimethyl sulfoxide and 20% FCS in DMEM, and used in experiments after no more than two further passages or 2 weeks in culture.

Activation of Akt and Erk

For adhesion-dependent signaling, MEFs were trypsinized and suspended in DMEM or DMEM with 10% FCS, and 1000 cells/well added to 96-well plates coated with 1 μg/well of FN (Sigma-Aldrich Canada Ltd. 2149 Winston Park Dr. Oakville, Ontario L6H 6J8). After 30 min at 37 °C, cells were fixed with 3.7% formaldehyde, washed with phosphate-buffered saline (PBS) with 1% serum, and permeabilized using 100% methyl alcohol for 2 min. The cells were washed three times and blocked in PBS plus 10% serum overnight at 4 °C, followed by antibodies to either phospho–Akt Ser473 or phospho–tyrosine (New England Biolabs Ltd. 1815 Ironstone Manor, Unit 6, Pickering, Ontario L1W 3W9) (1/200) overnight at 4 °C. The cells were washed three times with PBS with 1% serum. AlexaFluor 488-labeled anti-mouse Ig secondary antibody (Molecular Probes, Invitrogen Canada Inc. 2270 Industrial St Burlington, Ontario L7P 1A1) was added at 1/1000 with Hoechst 33342 (1/2000) (Molecular Probes Canada Inc. 2270 Industrial St Burlington, Ontario L7P 1A1) for 1 h at 20 °C. After washing three times, AlexaFluor 488 was measured by ArrayScan II fluorescence microscope (Cellomics, Pittsburgh) for 200 cells/well.

For cytokine-dependent signaling, cells plated in 96-well plates at 500 cells/well were serum starved for 24 h, then stimulated with either 100 ng/mL of EGF, PDGF (R&D Systems 614 McKinley Place NE, Minneapolis, MN 55413) or 5% serum. After 7 min, cells were fixed with 3.7% formaldehyde at room temperature, and stained as decribed above using mouse phospho–Erk1/2 (Thr202/Tyr204) (Sigma M-8159) at 1/1000 in PBS with 10% serum for 2 h at 20 °C. Nuclear and cytoplasmic staining intensity were determined by ArrayScan II for 200 cells/well, and cytoplasmic staining subtracted from nuclear staining for each cell.

To measure surface *N*-glycans, cells were fixed as above without permeabilization, and surface β1,6GlcNAc-branched *N*-glycans (Mgat5-specific) were labeled with 10 ng/mL of fluorescein isothiocyanate (FITC)-labeled leukoagglutinin (L-PHA) (E-Y labs 107 N. Amphlett Blvd, San Mateo, CA 94401 USA), or ConA and Hoechst (1/2000) for 1 h at 20 °C, and quantified by ArrayScan II.

Actin microfilaments and cell morphology

Cells were added to 96-well plates at 1000/well coated with the indicated concentrations of FN in serum-free DMEM. Cells were incubated at 37 °C, and at various times thereafter, fixed with 3.7% formaldehyde for 1 h at room temperature,

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and after three washes with PBS, cells were incubated with tetramethylrhodamine isothiocyanate (TRITC)–Phalloidin (1:1000) and Hoechst (1:2000) with 0.2% Triton X-100 for 30 min at room temperature. Cells were scanned with a 10× objective, identified by nuclear stain, and cell area quantified by phalloidin staining using the Cellomics Scan Array cell-spreading (area) and the morphology explore algorithms. Data are the mean ± SEM of 500 cells/well.

T cell proliferation

T cells from spleens of 8–12 week-old mice were at 10^5/well in 96-well plates were stimulated in culturing for 48 h in RPMI, 10% FCS, 10^{-5} M 2-mercaptoethanol in the presence of soluble antibodies, hamster anti-mouse TCRα/β (clone H59.72; Pharmingen BD Bioscience 2280 Argentia Road Mississauga, ON Canada L5N 6h8) and 0.5 μg/mL anti-mouse CD-28 (Pharmingen BD Bioscience 2280 Argentia Road Mississauga, ON Canada L5N 6h8). Two microCi of [^{3}H]-thymidine were added for the last 20 h of incubation, and cells were harvested on fiberglass filters and radioactivity was measured in a β-counter.

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Conflict of interest statement

None declared.

Abbreviations

bFGF, basic fibroblast growth factor; ConA, concanavalin A; DMEM, Dulbecco's modified Eagle medium; EGF, epidermal growth factor; EGFR, EGF receptor; EMT, epithelial-to-mesenchymal transition; Erk, extracellular response kinase; FCS, fetal calf serum; FGF, fibroblast growth factor; FITC, fluorescein isothiocyanate; GlcNAc-T, *N*-acetylglucosaminyltransferase; IGF, insulin-like growth factor; L-PHA, leukoagglutinin; MEF, mouse embryonic fibroblast; Mgat5, β1,6*N*-acetylglucosaminyltransferase V gene; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase; Pten, phosphatase and tensin homolog; PyMT, polyomavirus middle T; ROS, reactive oxygen species; RTK, receptor tyrosine kinase; TβR, transforming growth factor (TGF-β) receptor; TCR, T cell receptor; TRITC, tetramethylrhodamine isothiocyanate; UDP-GlcNAc, UDP-*N*-acetylglucosamine.

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