Suppression of Cancer Progression by MGAT1 shRNA Knockdown

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Abstract

Oncogenic signaling promotes tumor invasion and metastasis, in part, by increasing the expression of tri- and tetra-branched N-glycans. The branched N-glycans bind to galectins forming a multivalent lattice that enhances cell surface residency of growth factor receptors, and focal adhesion turnover. N-acetylglucosaminyltransferase I (MGAT1), the first branching enzyme in the pathway, is required for the addition of all subsequent branches. Here we have introduced MGAT1 shRNA into human HeLa cervical and PC-3-Yellow prostate tumor cells lines, generating cell lines with reduced transcript, enzyme activity and branched N-glycans at the cell surface. MGAT1 knockdown inhibited HeLa cell migration and invasion, but did not alter cell proliferation rates. Swainsonine, an inhibitor of \(\alpha\)-mannosidase II immediately downstream of MGAT1, also inhibited cell invasion and was not additive with MGAT1 shRNA, consistent with a common mechanism of action. Focal adhesion and microfilament organization in MGAT1 knockdown cells also indicate a less motile phenotype. \textit{In vivo}, MGAT1 knockdown in the PC-3-Yellow orthotopic prostate cancer xenograft model significantly decreased primary tumor growth and the incidence of lung metastases. Our results demonstrate that blocking MGAT1 is a potential target for anti-cancer therapy.

Introduction

Metastatic cancers generally have a poor prognosis, and show limited responses to chemotherapy and newer targeted therapies [1]. The redundancy in growth receptors and signaling contributes to invasion and drug resistance, a problem that might be overcome by considering additional levels of feedback regulation. In this regard, receptors are N-glycosylated in the ER and the N-glycans remodeled in the Golgi on route to the cells surface [2]. Oncogenic transformation increases Ets-driven transcription of N-acetylglucosaminyltransferase V encoded by Mgat5, a medial Golgi enzyme that initiates the \(\beta_1,6\text{GlcNAc}\) antenna in tri- and tetra-branched N-glycans [3–5]. Over-expression of Mgat5 in immortalized epithelial cells has been shown to relax growth controls and promote tumorogenesis when the cells are injected into mice [6]. The oncogenic induced expression of Mgat5 and its \(\beta_1,6\text{GlcNAc}\) tri- and tetra-branched N-glycans products correlate with distant metastasis and reduced survival in human mammary and colon cancers [7,8]. In mice, tumor progression is delayed in the polyoma-virus middle T transgenic (PyMT) and the Pten\textsuperscript{-/-} models of cancer on an Mgsat5 deficient background [9,10]. Moreover, the Golgi \(\alpha\)-mannosidase II inhibitor swainsonine inhibits tumor cell metastasis in mouse models, and was tested with promising results in clinical trials [11,12].

Galectins bind to the N-glycans on receptors and solute transporters with affinities proportional to branching and the number of N-glycans (NXS/T attachment sites) [13,14]. Galectin-3 has been shown to cross-link glycoprotein receptors at the cell surface, forming a dynamic lattice that slows trafficking into coated-pit endosomes and caveolin-1 lipid rafts, thereby promoting surface residency and sensitivity to ligands [14,15]. The lattice is heterogeneous and has been shown to regulate surface retention of EGF, TGF-\(\beta\) and VEGF receptors [14,16] as well as GLUT-2, \(-4\) glucose transporters and TRPV5 Ca\textsuperscript{2+} channel [13,17,18] (Figure 1). Branched N-glycans also enhance turnover of substratum and cell adhesions, supporting the epithelial-mesenchymal transition (EMT) in cancer cells [19,20]. Moreover, Mgat5 transgene expression in mouse skin promotes EMT-like phenotype and wound healing [21]. Mgat5\textsuperscript{+/-} PyMT-mammary tumor cells in culture show reduced surface residency of cytokine receptors, which can be rescued by (i) Mgat5 re-expression, (ii) inhibiting constitutive endocytosis, (iii) depletion of caveolin-1 and (iv) by GlcNAc supplementation to UDP-GlcNAc the common donor for the Mgsat enzymes [13,14].
GlcNAc supplementation in Mga5−/− cells increases the content of bi- and tri-antennary N-glycans, which rescues affinities for galectin-3 without the Mga5 product [13]. This suggests redundancy in N-glycan structures and profiles that can support the malignant phenotype.

Mga1 catalyzes the addition of the first branch, and the product is required for the action of α-mannosidase II and Mga2, and then Mga4 and Mga5 in a catalytically ordered pathway [2] (Figure 1). Low Mga1 activity limits the output of tri- and tetra-branched end products, but high levels can also do the same by depriving UDP-GlcNAc supply to Mga4 and Mga5 enzyme later in the pathway. Indeed, Mga enzymes display decreasing affinity for UDP-GlcNAc, which results in pathway ultrasensitivity, with a characteristic sigmoidal output of Mga5 products in response to this shared metabolite [13]. The hexosamine pathway substrates intersect with basic metabolism which is known to undergo extensive change in cancer cells [22]. UDP-GlcNAc synthesis by the hexosamine pathway depends on glucose, glutamine, and acetyl-CoA supply to the hexosamine pathway, as well as GlcNAc salvage. GlcNAc and GlcNAc-p can be elevated at an intermediate stage in prostate cancer progression [23], and may play a role in promoting tumor progression [24]. Therefore, inhibiting branching early in the pathway may be a viable strategy to avoid metabolic compensation.

To begin exploring the role of MGA1, we generated human tumor cell lines stably expressing MGA1 shRNA, and achieved 70% suppression of branched N-glycans and the invasive phenotype, without altering proliferation and viability in cell culture. MGA1 knockdown decreased growth and metastasis of human prostate cancer cells in a xenograft orthotopic mouse model. Although MGA1 is required for mouse development beyond day E9.5 [25,26], our results suggest that partial systemic depletion of MGA1 in adults may be tolerable and have important anti-cancer effects.

Figure 1. Schematic of N-glycan branching and receptor dynamics at the cell surface. Oligosaccharyltransferase (OST) substitutes NXS/T sites of proteins in the rough endoplasmic reticulum (RER), transferring the pre-assembled glycan from Glc3Man9GlcNAc2-p-dolichol to the Asn. Glycoproteins traffic to the Golgi where the N-glycans are remodeled, and the structural details depend on enzyme expression and UDP-GlcNAc supply. The branching enzymes Mga1, Mga2, Mga4, and Mga5 differ in Km values for UDP-GlcNAc "D" and for glycoprotein acceptor "A". The pathway has evolved for pathway ultrasensitivity to UDP-GlcNAc. The epitopes are completed in the trans-Golgi (small box) where efficient substitution with Gal generates the LacNAc epitope, and can be extended by poly-LacNAc. Further extension with galactose generates epitopes for galectin binding, with effects on receptor dynamics, interactions and trafficking.

doi:10.1371/journal.pone.0043721.g001
Materials and Methods

Cell culture

HeLa human cervical cancer cells (purchased from ATCC) were maintained in Dulbecco’s modified Eagle Medium (DMEM). PC-3-Yellow cells (a highly metastatic clone of PC-3 human prostate cancer cell line stably expressing red fluorescent protein (RFP) and green fluorescent protein (GFP) which was a gift from G. Gimsa, Ordway Research Institute, [27]) were maintained in RPMI 1640. All cells were supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), antibiotics, cultured in a standard humidified incubator at 37°C in a 5% CO2 atmosphere.

L-PHA binding to cell surface glycans

Cells were seeded in 96-well plates at 500 cells per well. After adhering overnight, cells were fixed with 3.7% formaldehyde and washed. Surface tri- and tetra-branched N-glycans were stained with L-PHA (20 μg/mL) conjugated to Alexa Fluor 488 or Alexa Fluor 647 and nuclei were stained with 5 μg/mL of 4',6-diamidino-2-phenylindole, dilactate (DAPI, dilactate) (Molecular Probes, Eugene, OR, USA). The total intensity of L-PHA staining on each cell was quantified using Cellomics ArrayScan II (Cellomics, Pittsburgh, PA) [13].

MGAT1 silencing by lentiviral-delivered RNA interference

Construction of hairpin-pLKO.1 vectors (carrying a puromycin antibiotic resistance gene) containing short hairpin RNA (shRNA) sequences and production of shRNA viruses have been described in detail [28]. The shRNAs targeting the MGAT1 coding sequences are as follows: MGAT1-sh1 (NM_002406), 5’-CCCTGAGATGCTCAAGAAGATGAT-3’; and MGAT1-sh2 (NM_002406), 5’-GCACCTAAGTGTATCAAGGCT-3’. The control shRNA coding sequences are as follows: RFP, 5’-CTACAAGGCGACATCAAGCT-3’ and LacZ, 5’-CCGCTCA- TAGGCGATAACGGTT-3’. Lentiviral infections were done essentially as described previously [28]. Briefly, adherent cells were treated with 0.5 mL of the virus followed by overnight incubation (37°C, 5% CO2) without removing the virus. The next day, viral medium was replaced with fresh medium containing puromycin (1 μg/mL) to select a population of resistant cells.

Reverse-transcriptase real-time PCR

First-strand cDNA was synthesized from 1 μg of DNase-treated total cellular RNA using random primers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. Real-time PCR assays were performed in triplicate with 5 ng of RNA equivalent cDNA, SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA, USA), and 400 nM of gene-specific primers. Reactions were processed and analyzed on an ABI 7900 Sequence Detection System (Applied Biosystems). Forward/reverse PCR primer pairs for human cDNAs were as follows: human GlcNAc-TI: Forward 5’-CGGAGCAGGC-CGAATTC-3’, Reverse 5’-CCCTGAGATGCTCAAGAAGATGAT-3’; human GlcNAc-TII: Forward 5’-CCCTGAGATGCTCAAGAAGATGAT-3’, Reverse 5’-CCCTGAGATGCTCAAGAAGATGAT-3’; human GlcNAc-TIII: Forward 5’-CCCTGAGATGCTCAAGAAGATGAT-3’, Reverse 5’-CCCTGAGATGCTCAAGAAGATGAT-3’. Relative mRNA expression was determined using the ΔΔCT method as described.

GlcNAc-transferase activities

The enzyme activity of MGAT1 was measured using a synthetic receptors as previously described [29]. Briefly, cell lysates were incubated with MGAT1 acceptor Manz[1,3] [Manz[1,6]Glc[3]O-(CH2)2]CH2I (Toronto Research Chemicals, Toronto, ON), in a solution containing 0.5 mM UDP-[6H]GlcNAc(44000 dpm/μmol); 125 mM MES (pH 6.5), 50 mM GlcNAc, 1 mM UDP-GlcNAc, 0.8 mM AMP and 10 mM MgCl2. After incubation, the reaction was stopped by adding ice-cold H2O. Transfer of [6H]GlcNAc to the acceptor was quantified by liquid scintillation counting.

Migration and invasion assays

Invasion and migration assays in HeLa cells were performed as previously described [30]. Briefly HeLa cells (2×105) were harvested and seeded in uncoated invasion chambers for migration assay or in BioCoat Matrigel Invasion Chambers (BD Biosciences, Mississauga, ON) for invasion assays. For both the migration and invasion assays, growth medium containing 10% FBS was used as a chemoattractant in the bottom well. Following 48 hours of incubation, cells that had migrated or invaded the lower surface of the membrane were stained with Diff-Quik Stain (BD Biosciences). The number of migrating or invading cells were imaged and counted using the Aperio ScanScope CS whole slide scanner (Aperio Technologies, Vista, CA) and Image-Pro Plus Software (version 4.5; Media Cybernetics Inc., Silver Springs, MD). In order to test the effect of simultaneous MGAT1 inhibition and swainsonine treatment, HeLa cells were treated with 2 μM swainsonine for 72 hours prior to the migration and invasion assay as described above.

To assess invasion and migration in PC-3-Yellow cells, cells were serum deprived overnight and seeded in the upper chamber of 16-well CIM-Plate (Roche Applied Sciences) for migration assays or Matrigel (BD Biosciences, Mississauga, ON) coated plate for invasion assays. The lower chamber containing 20% FBS as chemo-attractant, and the plates were placed in the Roche xCELLigence Real-Time Cell Analyzer (RTCA) DP platform (Roche Applied Sciences). Real-time measurements were done every 5 minutes for 36 hours. The RTCA Analyzer measures electrical resistance of cells that move to lower surface of the membrane, and compares well with the cell-count method above.

Immunocytochemistry

Cells were seeded on fibronectin-coated cover slips (Sigma-Aldrich, Oakville, ON). After adhering overnight, cells were fixed with 2% paraformaldehyde for 10 minutes, followed by permeabilization using 0.2% Triton-X-100. After blocking by 3% BSA for 1 hour, cells were incubated overnight at 4°C with mouse anti-B1 integrin (1:200, Millipore) or anti-phospho-paxillin (pY31) antibody (1:400, Epitomics). Cover slips were washed with PBS and then incubated with donkey anti-mouse IgG-Cy3 conjugated secondary antibody (1:200, Millipore) for B1 integrin or anti-rabbit IgG-FITC conjugated antibody (1:500, Millipore) for paxillin. Staining for actin was done by phallolidin conjugated to tetramethylrhodamine B isothiocyanate (TRITC, Sigma-Aldrich) for 10 minutes. After washing with PBS, cover slips were mounted on slides using fluorescent mounting medium and imaged using the 40× lens of the Zeiss LSM700 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

In vivo model of prostate cancer metastasis

Distant tumor formation was evaluated in vivo as previously described [31]. Briefly, PC-3-Yellow cells stably expressing RFP with and without MGAT1 knockdown, were injected orthotopically into the prostates of sublethally irradiated (3.5 Gy) SCID mice. Mice injected with tumor cells were maintained for 4 weeks after injection, at which time, the animals were sacrificed via cervical dislocation for complete examination. Red fluorescent tumors were detected via whole body imaging and whole organ imaging using a Leica MZ FLIII fluorescent stereomicroscope.
with a 100 W mercury lamp, a 560/40 excitation filter, and a 610 long-pass emission filter. Images were acquired using an Olympus DP70 digital camera at 0.8x magnification and analyzed using Image Pro Plus 6.0 (Media Cybernetics). A single common threshold was applied to identify and measure fluorescence in each organ as the number of fluorescent spots per lung lobe. Mice were obtained from an in-house breeding program and housed in laminar-flow cage racks under standardized environmental conditions with ad libitum access to food and water. All experiments were approved by the local institutional ethics review board (University Health Network-Ontario Cancer Institute Animal Care and Use committee) and were performed according to the regulations of the Canadian Council on Animal Care. All efforts were made to minimize suffering.

Results and Discussion

MGAT1 shRNA1 reduces N-glycan branching, cell migration and invasion

To assess the cell autonomous effects of MGAT1 depletion on malignant cell growth and invasion, HeLa human cervical cancer cells were infected with lentivirus shRNA vectors targeting MGAT1 or control sequences, and stable cell populations were selected with puromycin. Target knockdown using two independent shRNA sequences was confirmed by qRT-PCR (Figure 2A). shRNA1 and shRNA2 also reduced MGAT1 enzymatic activity and decreased L-PHA cell-surface staining in proportion to the depletion of mRNA and enzyme activity (Figure 2B, C). L-PHA binds to products downstream of MGAT1, the β1,6GlcNAc tri- and tetra- branched N-glycans [32]. MGAT1 knockdown did not alter cell viability or proliferation (Figure 2D and data not shown).

To evaluate the effects of MGAT1 shRNA2 knockdown on HeLa cell migration and invasion, the cells were seeded into chambers on porous filters in serum-free medium, and 10% FBS was placed in the lower chamber as a chemo-attractant. Cell migration into the bottom chamber was measured 48 h later. Similar studies were conducted using Matrigel-coated filters to measure cell invasion though a barrier. Knockdown of MGAT1 decreased cell migration and invasion, in proportion to MGAT1 depletion (Figure 3A, 3B). Blocking the pathway one step downstream of MGAT1, at α-mannosidase II, using swainsonine also inhibited invasion as previously reported [33,34]. Swainsonine treatment alone inhibited migration and invasion, but to a lesser degree than shRNA2. This may be due to the paralog α-mannosidase Ix, which is less sensitive to swainsonine. Importantly, swainsonine had no additional effects in MGAT1 shRNA2 cells, suggesting that optimal suppression of migration and invasion by targeting the branching pathway is observed with ~70% depletion of MGAT1 (Figure 3C, 3D).

Cell migration rates are partly dependent on α5β1 integrin receptor contacts with substratum fibronectin, which stimulate focal adhesions signaling, turnover and propulsion [35]. The branched N-glycans products of MGAT5 are present on α5β1, among other receptors and cell surface proteins, and have been shown to promote α5β1 activation, signaling, and tumor cell migration [19]. Integrin activation results in phosphorylation of paxillin by FAK and Src, leading to F-actin remodeling and cell motility [36]. In HeLa cells with MGAT1 knockdown, the F-actin stress fibers were smaller in diameter, less convergent on cell projections, and tended to circumscribe the cell (Figure 3E). Staining of p-paxillin revealed smaller and more clusters at the edge of cellular projections. In contrast, control HeLa cells had

Figure 2. shRNA MGAT1 suppresses N-glycan branching. A) HeLa cells were infected with lentiviral vectors targeting MGAT1 (shRNA1 or shRNA2) or the control shRNA sequences. Stable cell populations were selected by the addition of puromycin (1 µg/mL). A) MGAT1 mRNA were measured by qRT-PCR, B) MGAT1 enzyme activity, C) L-PHA reactive surface N-glycans by Array scan microscope, D) Proliferation over 4 days Data represent the mean ± 5D relative expression of mRNA relative to control sequence (n = 3 independent experiments performed in triplicate). doi:10.1371/journal.pone.0043721.g002
prominent and long stress fibers that project into pseudopodia ending with larger phospho-paxillin staining focal adhesions, characteristic of a more motile phenotype than the MGAT1 knockdown cells.

MGAT1 knockdown decreases tumor growth and metastasis in vivo

To assess the effects of MGAT1 knockdown in an animal model of cancer metastasis, we used the human PC-3-Yellow prostate cancer cells, a well-established xenograft model. PC-3-Yellow cells were infected with the lentivirus containing MGAT1 shRNA2 or control sequences, and stable cell populations were selected as above. MGAT1 mRNA, enzymatic activity, and cell surface branching were all depleted by ~70% (Figure 4A–C). MGAT1 knockdown decreased both cell migration and cell invasion in an in vitro and in vivo model (Figure 4D,E). MGAT1 knockdown did not alter the growth and viability of PC-3-Yellow cells (Data not shown). Thus, the in vitro phenotypes were remarkably similar to the effects of MGAT1 knockdown in HeLa cells. To assess the effects of MGAT1 knockdown on tumor metastasis, control or MGAT1 knockdown PC-3-Yellow prostate cancer cells were injected orthotopically into the prostate glands of sub-lethally irradiated SCID mice (n = 15 per group). Four weeks after injection, mice were sacrificed and distant tumor formation in the organs was imaged by fluorescent microscopy. Tumors developed in the prostate in all mice injected with MGAT1 knockdown and control RFP-labeled PC-3-Yellow cells. In mice injected with control cells, tumor formation was detected at clinically relevant sites of metastases, including lung, lymph nodes...
and liver. However, tumors volume was reduced and fewer distant lung metastases were observed in mice injected with MGAT1 knockdown cells (Figure 4F,G). Overall, more of the mice in the MGAT1 knockdown group were free of metastases. Thus, taken together, MGAT1 knockdown inhibits distant tumor formation in vivo.

In summary, ~70% MGAT1 knockdown suppress N-glycan branching and the invasive phenotype in HeLa and PC-3-Yellow cells (Figure 4F,G). Overall, more of the mice in the MGAT1 knockdown group were free of metastases. Thus, taken together, MGAT1 knockdown inhibits distant tumor formation in vivo.

Author Contributions
Conceived and designed the experiments: RB JWD ADS DRD HS. Performed the experiments: RB RH MG XW NM FZ. Analyzed the data: RB MAS. Contributed reagents/materials/analysis tools: JM TK CJS JWD ADS CDS. Wrote the paper: RB JWD ADS.
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