Epithelial Mesenchymal Transition Induces Aberrant Glycosylation Through Hexosamine Biosynthetic Pathway Activation

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Running title: EMT activates HBP

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ABSTRACT

Deregulated cellular metabolism is a hallmark of tumors. Cancer cells increase glucose and glutamine flux to provide energy needs and macromolecular synthesis demands. Several studies have been focused in importance of glycolysis and pentose phosphate pathway. However, a neglected but very important branch of glucose metabolism is the hexosamine biosynthesis pathway (HBP). The HBP is a branch of the glucose metabolic pathway that consumes approximately 2–5% of the total glucose, generating UDP-GlcNAc as the end-product. UDP-GlcNAc is the donor substrate used in multiple glycosylation reactions. Thus, HBP links the altered metabolism with aberrant glycosylation providing a mechanism of how cancer cells can sense and respond to microenvironment changes. Here, we investigate the changes of glucose metabolism during epithelial mesenchymal transition (EMT), and the role of O-GlcNAcylation in this process. We show that A549 cells increase glucose uptake during EMT, but instead of increasing the glycolysis and pentose phosphate pathway, the glucose is shunted through the HBP.

The activation of HBP induces an aberrant cell surface glycosylation and O-GlcNAcylation. The cell surface glycans display an increase of sialylation α2-6, poly-LacNAc and fucosylation, all known epitopes found in different tumor models. In addition, modulation of O-GlcNAc levels was demonstrated to be important during EMT process. Taken together, our results indicate that EMT is an applicable model to study metabolic and glycophenotype changes during carcinogenesis, suggesting that cell glycosylation senses metabolic changes and modulates cell plasticity.

Introduction

Altered metabolism represents the first known difference between cancer cells and normal cells (1). The Warburg effect consists of an increase of glucose uptake for producing energy by a high rate of glycolysis followed by lactic acid fermentation even under high oxygen tension (“aerobic glycolysis”). Understanding the metabolism of tumors remains a topic of intense study with important therapeutic
Several advances in cancer metabolism research over past years have enhanced our understanding of how aerobic glycolysis and other metabolic shifts support the anabolic demands of high growth rate (4). Traditionally, the study of glucose metabolism usually focused on the use of glucose for energy needs. However, cancer cells use glucose in anabolic pathways that provide precursors for the synthesis of lipids, proteins, glycans and DNA to satisfy the demands of growth and proliferation. Several studies have been focused in the importance of the pentose phosphate pathway (PPP), to generate NADPH that ensures the cell’s antioxidant defenses and to generate the nucleotides in high demand, or the use of intermediates of the glycolytic pathway to generate molecules such as lipids or amino acids (5). However, a neglected but integral branch of glucose metabolism is the hexosamine biosynthesis pathway (HBP).

Approximately 2-5% of glucose influx is directed to the HBP by the rate limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT), which converts fructose-6P to glucosamine-6-phosphate (GlcN-6P) using glutamine as an amine donor (6). As with glucose, it is widely known that most tumors also increase glutamine uptake (7), and we believe that such an increase indicates the importance of the HBP for cancer cells. To support this hypothesis, Itkonen and colleagues recently showed that several HBP genes were overexpressed in human prostate cancers (8). This pathway generates UDP-GlcNAc, and its derivatives, UDP-GalNAc and CMP-Neu5Ac, donor substrates used in multiple glycosylation reactions such as O-GlcNAcylation, N-linked and O-GalNAc glycans, proteoglycans and glycolipids biosynthesis. Growing evidence demonstrate that alteration of the pool of activated substrates might lead to aberrant glycosylation (9-12). Thus, HBP can link the altered metabolism with aberrant glycosylation providing a mechanism of how cancer cells can respond to a variety of environmental conditions (13). It is already known that aberrant glycosylation is associated with enhanced malignancy and can modulate tumor growth and malignant transformation in different cancer types (14-17). Malignant cells acquire characteristics enabling them to undergo dissociation from tumors, degradation of the extracellular matrix, invasion, adhesion, and metastasis to distant organs. These features prevail during the epithelial to mesenchymal transition.

The epithelial-mesenchymal transition (EMT) process is characterized by a transition from polarized immotile epithelial cells to motile mesenchymal cells, thus leading to increased motility and invasion (18). EMT is also characterized by the reduction of epithelial marker E-cadherin, and by the emergence of mesenchymal markers as N-cadherin and vimentin. The EMT is well documented to play a critical role in tumor invasion and metastasis (19-21). Recently, our group showed that GFAT overexpression induces EMT, indicating the involvement of HBP in tumor progression (9). Cancer cells undergoing EMT present aberrant glycans including glycolipids (22), O-GlcNAc (23) and a specific glycosylation of fibronectin (24). However, studies linking altered metabolism with aberrant glycosylation in the EMT process are missing.

Here, we show an increase of glucose uptake during the EMT with no changes in ATP levels, pyruvate, lactate and glycogen production. Proteomic studies identified the expression of several enzymes involved in the metabolism of glucose and glycosylation were changed in A549 cells after TGF-β stimulus. Unexpectedly, we showed that glucose is shunted through the HBP during EMT. Modulation of O-GlcNAc levels was demonstrated to be important in the EMT process. These observations indicate that cell glycosylation senses metabolic changes and modulates cell plasticity.

**EXPERIMENTAL PROCEDURES**

**Cell culture and treatments**

A549 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (5 mM glucose; Sigma) containing 10% (v/v) fetal bovine serum (Gibco) and penicillin/streptomycin. The media was changed every 24 h to maintain the glucose
concentration. Cells were stimulated with 5 ng/mL TGF-β (R & D System) for 24 h and 48 h.

**Immunoblotting**

Cells were washed with phosphate-buffered saline and homogenized in lysis buffer (150 mM NaCl, 30 mM, Tris-HCl pH 7.6, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and 1 μM PUGNAc with protease inhibitors). Cell lysates were sonicated and centrifuged. Supernatant was collected, protein concentration determined and modified Laemli buffer added. Samples were separated on SDS-polyacrylamide gels, and were subsequently electroblotted to nitrocellulose (Bio-Rad). The membranes were blocked in Tris-buffered saline with 0.1% (v/v) Tween 20 with either 3% (w/v) bovine serum albumin or 3% (w/v) nonfat dry milk. The blocked membranes were then incubated overnight at 4 °C with primary antibodies against N-cadherin (Santa Cruz, USA), E-cadherin (Cell Signaling Technology, USA), GFAT (Cell Signaling Technology, USA), β-actin (Sigma Chemical Co., USA), phosphofructokinase, PFK (Santa Cuz, USA), Glucose-6-phosphate dehydrogenase, G6PD (Cell Signaling Technology, USA), UDP-N-acetylglucosamine—peptide N-acetylglucosaminyltransferase, OGT (AL-28), O-GlcNAcase, OGA (345), O-GlcNAC (CTD 110.6 or RL-2), α-tubulin (Sigma Chemical Co, USA), Glyceraldehyde 3-phosphate dehydrogenase, GAPDH (Cell Signaling Technology, USA). O-GlcNAc competitive assay was conducted by pre-incubation of the antibody with 0.2 M free GlcNAc (Sigma Aldrich) before membrane labelling to confirm CTD 110.6 specificity. The blots were then washed, incubated with the appropriate secondary antibody, developed using ECL (GE Healthcare), and exposed to Image Quant LAS 4000 (GE Healthcare). ImageJ software was used for densitometry analysis of immunoblots and measurements were normalized against β-actin, α-tubulin or GAPDH loading controls.

**Cell Circularity**

The cellular circularity was determined as previously described (25). Briefly, culture dishes of A549 cells treated as above were photographed and images were analyzed using the ImageJ software. The circularity "C" was calculated by the formula P = C / (4πA) 0.5 where P and A are respectively the area and perimeter cells.

**Glucose uptake**

The glucose uptake was assayed using a fluorescent 2-deoxi-glucose analogue (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) as developed by (26). Briefly, A549 cells (1.5x 10^4) were cultivated in 6-well plate and treated with 5 ng/mL TGF-β for 24 h and 48h. We added fresh medium without glucose (DMEM-Sigma D5030) supplemented with 100 μM of 2-NBDG and incubated at 37°C under 5% CO_2 for 1 h. Then, the media was removed, the wells washed twice with PBS, the cells were trypsinized, and centrifuged at 1000 x g for 5 min. The cells were resuspended in 300μl of PBS, and were submitted to Flow Cytometry in a BD FACS Calibur (BD Becton, Dickinson and Company-USA). Each experiment was performed in triplicate. Data was analyzed using the Flow Jo software (Tree Star, USA).

**ATP and lactate quantitative measurements**

The quantification of ATP levels was performed using the CellTiter-Glo assay (Promega) as described by the manufacturer. Briefly, 2 x 10^4 cells were seeded in 96-well plates and treated with TGF-β (5 ng/ml) for 24 or 48 h. Then, the media was removed and replaced with a mixture (1:1) of medium and CellTiter-Glo solution. A well without cells with the mix was used as a blank. The plate was gently shaken for 2 min and the resulting solution was placed in an opaque-walled multiwell plate, incubated for 10 min at room temperature, and measured in a microplate luminometer.

The lactate measurement was based on lactate dehydrogenase-catalyzed oxidation of lactate and formation of NADH as described by (27).

**Glycogen quantification**
To quantify the amount of glycogen present in cell pellets, a method based on the enzymatic hydrolysis of glycogen by amyloglucosidase (EC 3.2.1.3) was employed (28). The cells were homogenized in 200 µL of H buffer (Tris-HCl 50 mM, NaF 5 mM, EDTA 5 mM, EGTA 5 mM, DTT 1 mM, pH 7.2) and an aliquot of the homogenate (100 µL) was incubated for 4 h at 40 °C in the presence of 20 µL of amyloglucosidase (1.4 U). After the incubation, the amount of glucose released was measured with a commercial kit (Doles Reagentes, Goiânia, Brazil) according to the manufacturer’s instructions. Since the measurement included the amount of endogenous glucose, a correction was made to account for the glucose content not related to glycogen, by incubating samples in the absence of amyloglucosidase.

**Pyruvate measurement**

Pyruvate levels were measured using a fluorimetric method as previously described (29). Briefly, the intracellular pyruvate was extracted using 50 µL of ice-cold 0.2 M HClO₄ added to the cell pellet (5 x 10⁶ cells), vortex-mixed and incubated on ice for 5 min. The mixture was neutralized with 1.4 µL of 5 M K₂CO₃ (~pH6.5). Experiments were performed in 96-well black plates using 20 µL of the intracellular pyruvate extracts in 180 µL final volume reactions containing 10 mM potassium phosphate with 1.0 mM EDTA, pH 6.7, 1 mM MgCl₂, 10 µM FAD, 0.2 mM thiamine pyrophosphate, 0.2 U/mL pyruvate oxidase, 50 µM amplex red and 0.2 U/mL HRP. The pyruvate levels were determined by comparison to a standard curve (0, 0.0625, 1.25, 2.5, 5, 10 µmol/well). Blank values were corrected by subtracting the value of the reaction without pyruvate from all sample readings. Fluorescence was measured in a SpectraMax M5e (Molecular Devices) using excitation and emission wavelengths of 535 and 590 nm, respectively.

**Liquid chromatography-tandem MS (LC-MS/MS)**

Protein was digested with Trypsin Gold as described in manufacturer protocol (Promega). LC-MS was performed on peptide samples using a nano-Acquity UPLC nano-capillary high-performance LC system (Waters Corp., Milford, MA, USA) coupled to a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with a TriVersa NanoMate ion source (Advion, Ithaca, NY, USA). Sample concentration and desalting were performed online using a nano Acquity UPLC trapping column (180 µm x 20 mm, packed with 5-µm, 100-Å Symmetry C18 material; Waters) at a flow rate of 10 µl/min for 3 min. Separation was accomplished on a nano Acquity UPLC capillary column (150 µm x 100 mm, packed with 1.7-µm, 130-Å BEH C18 material; Waters). A linear gradient of A and B buffers (buffer A: 1.5% ACN and 0.1% FA; buffer B: 98.5% ACN and 0.1% FA) from 4 to 50% buffer B over 120 min was used at a flow rate of 0.5 µl/min to elute peptides into the mass spectrometer. Columns were washed and re-equilibrated between LC-MS experiments. Electrospray ionization was carried out at 1.65 kV using the NanoMate, with the Q Exactive heated transfer capillary set to 250 °C. Mass spectra were acquired in the positive-ion mode over the range m/z 400–2000 at a resolution of 70,000 (full width at half maximum at m/z 400; ~1 spectrum/s) and AGC target of > 1 x ⁵. Mass accuracy after internal calibration was within 1 ppm. For discovery, MS/MS spectra were acquired at a rate of ~10 MS/MS/s for the 10 most abundant, multiply charged species in each mass spectrum with a resolution of 17,500 and signal intensities > 5 x e⁵ NL at 60 ms. The HCD with MS/MS collision energies was set at 30 V and stepped NCE at 12.0%, nitrogen as the collision gas, and MS/MS spectra acquisition over a range of m/z values dependent on the precursor ion. Dynamic exclusion was set such that MS/MS for each precursor ion species was excluded for 4 s, under fill ratio of 2.0%, intensity threshold 1.7e⁵ and apex trigger 2 to 5s. All spectra were recorded in profile mode for further processing and analysis.
Data analysis

MS and MS/MS data analysis were carried out using Proteome Discoverer (PD) 1.4 software (version 1.4.0.288, Thermo Fisher Scientific) template that used both the Sequest search engine and an in-house Mascot 2.4.0 server (Matrix Science, London, UK). The MS/MS data were searched against the uniprot Human amino acid sequence database for protein/peptide identification. The PD 1.4 search was set up with precursor intensity node for label free quantitation, full tryptic peptides with a maximum of 2 missed cleavage sites with carbamidomethyl cysteine static. Oxidized methionine and deamintated asparagine and glutamine were included as variable modifications for both the Sequ est and Mascot search engines. The precursor mass tolerance was set to 10 ppm for the Q Exactive Orbitrap mass spectrometer method, and the maximum fragment mass error was 0.02 Da. Results were uploaded into Scaffold (Proteome Software version 4.4.8), searched via X!Tandem and label free quantitation. The quantitati on parameters were set for fold change by category with 24h and 48h duplicates time points separately calculated over duplicate controls. Quantification was based on total precursor ions with normalization for each protein and T-test was calculated in Scaffold. Scaffold was set at a minimum of 2 peptides with 95% protein confidence and 90% peptide confidence (0.06% FDR). Quantitative data was exported into excel files and included in supplemental data.

G6PD and GFAT activity assays

G6PD enzyme activity was measured in buffer (0.1 mM NADP^+, 1 mM MgCl_2 e 50 mM Tris, pH 8.1) containing 10 µg of cell lysate. To obtain accurate G6PD activity, we subtracted the activity of phosphogluconate dehydrogenase (PGD) as described previously (30). The NADPH production was monitored (OD 340 nm) in a microplate reader (Spectra Max 190, Molecular Devices) every 20 s for 10 min.

GFAT activity assay was performed with some adaptations as described previously (31). Briefly, A549 cells treated +/- TGF-β were collected in 100 µL of buffer (10 mM Tris-HCl pH 7.4, 0.25 mM sucrose, 20 mM NaF, 5 mM EDTA). Cell lysate was obtained by extraction with syringe and centrifuged at 15,000xg for 30 min at 4 °C. 100 µg of lysate were pre-incubated with 6 mM glutamine, 0.3 mM acetylpyridine adenine dinucleotide (APAD), and 6 U glutamate dehydrogenase for 30 min at 37 °C. After pre-incubation, the reaction was started by adding the substrate fructose-6-phosphate 0.8 mM and the reaction was monitored at 370 nm for 90 min at 37°C in a microplate reader (Spectra Max 190, Molecular Devices) every 20 seconds for 90 min.

Radioassay for PFK activity

PFK activity was measured as described previously (32,33) by adding 80 µg cell lysate to a reaction mixture containing: 50 mM Tris–HCl (pH 7.4), 5 mM MgCl_2, 5 mM (NH_4)_2SO_4, 1 mM fructose 6-P, and 0.1 mM [γ-^32P]ATP (4 µCi/µmol). Quenching was achieved by addition of 1 mL of activated charcoal suspended in 0.1 N HCl (25 g activated charcoal per 100 ml 0.1 N HCl). The suspension was centrifuged at 15,000 xg for 30 min at 4° C for 15 min and 0.4 mL of the supernatant was counted in a liquid scintillation counter. Blanks were obtained in the absence of fructose 6-phosphate. We defined one enzymatic unit (U) as the production of 1 nmol fructose 1,6-bisphosphate per min.

UDP-GlcNAc quantification

Polar metabolites were extracted from 1x10^6 cells with chloroform, methanol and water (2:2:1.8). Polar fractions were dried by centrifugation under vacuum (Speed Vac) and solubilized in water to final concentration of 10 µg/µL. All the samples were spiked with 0.2 mM p-nitrophenol (pNP) as external standard and ATP was taken as internal standard. The samples were subjected to chromatographic separation utilizing a Hypercarb PGC column (3.0 mm x 150 mm, Thermo Scientific) running in Prominence HPLC Shimadzu (34). Briefly, 20 µL of the samples were injected on Hypercarb column and submitted to a gradient of solutions A (0.2% formic acid and 0.75%
ammonium hydroxide in water) and B (95% acetonitrile with 0.1% formic acid and 0.07% ammonium hydroxide) at 0.3 mL/min flow rate as follows: increase from 0.3 to 5% of solution B in 10 min, increase from 15 to 25% of solution B in 60 min, increase from 25% to 35% of solution B in 10 min, increase from 35% to 95% of solution B in 5 min, followed by column reequilibration with 5% of solution B. A calibration curve was constructed by analyzing a series of UDP-GlcNAc dilutions spiked with 0.2 mM pNP according to the aforementioned chromatographic method.

To validate the UDP-GlcNAc chromatographic characterization, we run a control sample collecting the peak corresponding to UDP-GlcNAc and submitted it to tandem mass spectrometry. The collected sample was directly injected at a flow rate of 3 µL/min in Impact 2 mass spectrometer (Bruker) on negative ion mode. The nebulizer gas pressure was set to 0.4 Bar and the dry gas was injected at a 5.0 L/min. The source temperature was set to 180 °C and capillary voltage and end plate offset was adjusted to 4.5 kV and -500 V, respectively.

**Lectins Label and Flow Cytometry**

The changes in cell surface glycoconjugates were analyzed by flow cytometry (35). Cells were treated +/- TGF-β during 48 h were washed 3 times with PBS and fixed in a 3.7% formaldehyde solution in PBS. The cell surface was blocked in a 3% BSA solution in PBS and incubated with the following biotinylated lectins: *Sambucus nigra* agglutinin (SNA), *Erythrina cristagalli* agglutinin (ECA), *Peanut* agglutinin (PNA), *Aleuria aurantia* agglutinin (AAL), *Pisum sativum* agglutinin (PSA), *Maackia a miseris* agglutinin (MAA), Phyto haemagglutinin L (L-PHA), Phyto haemagglutinin E (E-PHA) and *Vicia villosa* lectin (VVL) (EY Laboratories, USA) diluted in PBS-BSA in a concentration of 10 µg/mL. Cells were washed 3 times with PBS and incubated for 1 h with Avidin conjugated to the fluorochrome FITC (1: 2500 - Sigma Chemical Co., USA). Cells were further analyzed by flow cytometry (BD FACScalibur I) and data analyzed using the Flow Jo (Tree Star, USA).

**OGT and OGA silencing**

Both OGT and OGA genes were silenced using short hairpin RNA (shRNA) (Origene Technology, USA). Cells were grown in 6-well plates to 60% confluence and transfected with 3 µg scramble-shRNA, or human OGT-shRNA (5’GGCAACAAACCTGACCACATGATTAGCC3’), or human OGA-shRNA (5’TTGAAGCCAACTACGTTGCTATCCACC3’) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Cultures were harvested after 48 h of transient transfection.

**Colloidal Gold Assay**

Cell motility was determined as the area of phagokinetic tracks on gold particle-coated plates as described (36). Briefly, A549 treated as described above were harvested with trypsin/EDTA, and 4x10^5 cells in 1.0 mL of culture medium were seeded onto gold sol-coated wells (24-well plates). After 18 h cells were observed, and photographed using a light microscope (Zeiss, Germany). Motility track area of 20 cells/well were measured by ImageJ program and expressed as square pixels.

**Statistical analyzes**

All the data reported here are expressed as the mean ± SD from three independent experiments. A significant difference from the respective control for each experimental test condition was assessed by two-way analysis of variance (ANOVA) tested by Bonferroni posttests using GraphPad Prism 5.0 software. Values of *p* <0.05 were considered statistically significant.

**RESULTS**

TGF-β increases glucose uptake but not ATP, pyruvate, lactate and glycogen levels during EMT
As expected, the treatment of A549 cells with TGF-β induced cellular morphology conversion from epithelial cells into fibroblast-like cells, with decreased cellular circularity (Fig. 1A). We also saw a reduction of epithelial marker E-cadherin, accompanied by enhanced expression of mesenchymal markers such as N-cadherin and vimentin (Fig. 1B). To dissect whether EMT might affect cellular metabolism we first examined glucose uptake in A549 cells treated with TGF-β. Interestingly, we observed the increase of the fluorescent analogue of glucose (2-NBDG) after 24 h treatment with TGF-β, returning to control levels at 48 h (Fig. 1C), indicating an increase of glucose uptake during EMT. Next, we investigated if the higher glucose incorporation driven by TGF-β could be affecting the lactate, ATP, pyruvate or glycogen levels. The lactate production (Fig. 1D), ATP levels (Fig. 1E), pyruvate levels (Fig. 1F), and content of glycogen (Fig. 1G) were not changed during EMT, raising the question about the metabolic fate of glucose.

**Proteomic analysis indicates the increase of glucose flux through HBP**

Mass spectrometry analysis of A549 cells induced by TGF-β identified 1742 proteins from 165, 550 spectra with a minimum of 2 peptides at a 0.06% decoy FDR. Ingenuity Pathway Analysis (IPA) of the proteome data also indicated that TGFβ-1 was the primary upstream regulator at both the 24 h (z-score 2.153) and 48 h (z-score 1.929) time points. In addition, IPA predicted the top bio function for both 24 h and 48 h was cellular movement, more specifically cell movement of lung cancer cells (data not shown). We saw an increase in both integrin β-1 (24 h: 1.5 fold; 48 h: 1.4 fold increase) and integrin α-2 (24 h: 7.0 fold; 48 h: 4.5 fold increase) which work along-side cadherins in the cell-cell and cell-extracellular matrix interactions. Furthermore, both cateninα-1 (1.7fold at 24 h and 1.7 fold at 48 h) and cateninβ-1 (1.4 fold at 24 h and 1.4 fold at 48 h) also increased indicating remodeling of epithelial junctions. In addition, we saw an increase in vimentin and actin-alpha proteins, a sign of EMT occurring. Interestingly, we saw a 25 fold increase at 24 h and 21 fold increase at 48 h in CTP synthase, the enzyme responsible for interconverting UTP and CTP for pyrimidine nucleotide biosynthesis which is needed in rapid tumor growth (data not shown).

We focused our data interpretation in three major glucose metabolic pathways and the biosynthetic machinery involved in glycosylation (Fig. 2A and B). Regarding the machinery involved in glycosylation, our data indicated an increase biosynthesis of CMP-Neu5Ac, by increase N-acetylneuraminate cytidylyltransferase (NEUA) levels, and a decrease of UDP-Gal and GDP-Man, by decrease of UDP-Galactose 4-epimerase (GALE) and phosphomannomutase (PMM2) levels. Furthermore, three proteins (OST48, RPN1 and RPN2) of oligosaccharyltransferase (OST) protein complex were upregulated during the EMT suggesting the increase of biosynthesis of N-linked glycans. A decrease of UDP-Glucose glycoprotein glucosyltransferase (UGGG1) was revealed indicating a possible reduction in the ability to correct unfolded glycoproteins in ER, since this enzyme catalyzes the transfer of Glc from UDP-Glc to N-Glycans on incorrectly folded glycoproteins (Fig. 2B). Next, we focused on three metabolic pathways, glycolysis, PPP and HBP. Two hexokinase proteins (HXK-1 and HKDC-1) were upregulated during EMT. However, all others enzymes involved in glycolysis were unchanged or down regulated, indicating that glucose may be channeled to alternate pathways. The PPP enzymes G6PD, 6PGD and TKT were all decreased, indicating reduction of glucose flux through this pathway (Fig. 2B). Interestingly, we saw an increase of GFPT1 (Fig. 2B), the rate-limiting step in the HBP suggesting an alternative pathway to metabolize an increased flux of glucose.

**Hexosamine Biosynthetic Pathway is activated during EMT**

To gain insight into whether TGF-β treatment might influence glucose flux, we evaluated the protein levels and activities of PFK, G6PD and GFAT, the rate-limiting enzymes of the top three glucose metabolic pathways. These enzymes direct and control the carbon flux through the glycolytic pathway, PPP and HBP,

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respectively (Fig. 2A). We observed a decreased by 30% in G6PD protein levels (Fig. 3A and C), and a decrease of G6PD activity after 48 h of TGF-β treatment (Fig. 3F) suggesting a down regulation of PPP during EMT. The PFK protein levels increase by 30% (Fig. 3A and D) while the activity not changed in 24 h and decreased in 48 h (Fig. 3G). As expected, we observed an increase of GFAT protein levels (Fig. 3A and B), and 30% increase in GFAT activity (Fig. 3E) during EMT correlating with the up-regulation of the HBP. To support our hypothesis that glucose is shunted predominantly to HBP during the EMT, we measured the cellular levels of UDP-GlcNAc. Using liquid chromatography with porous graphitic column we were able to distinguish UDP-GalNAc from UDP-GlcNAc (Fig. 4A). UDP-GlcNAc isolated from control cell extract was further characterized by MS. A progressive increase of UDP-GlcNAc levels in TGF-β treated cell extracts compared to control up to 2-fold after 48 h treatment (Fig. 4C and D) was observed. Taken together, our results indicate an up-regulation of the HBP during the EMT process, revealing an uncharacterized regulation of glucose metabolism in A549 invasive cells undergoing EMT.

Glycophenotype changes during EMT

Aberrant glycosylation was one of the first landmarks identified in tumor cells (37). Glycophenotype alterations interfere with cell interactions and regulate numerous pathological events, including cell growth, death, migration, differentiation and tumor metastasis. The increase of the HBP during EMT motivated us to investigate the cell surface glycosylation. Using a panel of lectins that recognize alterations on cell surface glycosylation observed during oncogenesis, we monitored the A549 cell surface terminal sugars during EMT by flow cytometry analysis. Figure 5 shows that TGF-β treatment of A549 cells induced an increase of glycoconjugates terminated with α2-6Neu5Ac as indicated by strong staining with SNA, with simultaneous reduction of PNA binding to terminal β-Gal unities. Moreover, augmented expression of glycoconjugates holding poly-N-acetyl-lactosamine moiety, detected by the ECA lectin, and α-fucose (AAL) were observed. N-glycans containing high mannose structures were also up regulated by TGF-β, demonstrated by increased PSA binding. In conclusion, the observed lectin profile correlated to the remodeling of cell surface glycosylation during TGF-β induced EMT.

EMT promotes hyper-O-GlcNAcylation and OGT over-expression

It is well established that O-GlcNAcylation is highly responsive to the HBP (38). Multiple manuscripts have reported that O-GlcNAcylation and the protein levels of OGT and OGA are aberrant in several types of tumors (39,40). The addition and removal of O-GlcNAc on target proteins is catalyzed by O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), respectively (41). Here, we analyzed the O-GlcNAcylation, OGT and OGA levels during EMT by Western blotting. The O-GlcNAc levels were significantly increased by 2.0-fold after 24 h and 48 h TGF-β induced EMT (Fig. 6A and B). The CTD 110.6 antibody specificity was evaluated by a competition assay with 0.2 M of free GlcNAc (Fig. 6A). We also observed a significant OGT increase (2.2-fold) 24 h and (1.8-fold) 48 h after TGF-β stimulation (Fig. 6C and D). However, no changes were detected for OGA levels during EMT (Fig. 6C and E).

O-GlcNAc modulation induces EMT

To determine if changes in O-GlcNAcylation are the cause or the result of EMT induction we analyzed the EMT parameters, including molecular markers, cellular morphology and cell motility when O-GlcNAc is modulated. Cells were silenced for OGT or OGA (Fig.7A), and then we investigated epithelial and mesenchymal markers (Fig. 7B). The cells silenced for OGT (low O-GlcNAcylation detected) had a decrease of the mesenchymal marker N-cadherin and an increase in E-cadherin. However, cells silenced for OGA
(high O-GlcNAcylation detected), displayed an increase of the mesenchymal marker N-cadherin with a decrease of E-cadherin, indicating that A549 cells are undergoing EMT by modulation of O-GlcNAcylation. We also observed that cells silenced for OGT had an epithelial shape and the same morphology as control cells. More importantly, cells silenced for OGA had a more elongated shape, similar to cells treated with TGF-β, that indicate mesenchymal morphology (Fig. 7C). Cellular circularity was quantified to confirm the similarity between control cells and cells silenced for OGT and between cells treated with TGF-β and silenced for OGA (Fig. 7F). The augmented cell motility is a well-established characteristic of EMT. Therefore, we tested cell motility in cells silenced for OGT or OGA using the colloidal gold assay. When cells were silenced for OGT the motility had the same rate as control cells; however cells silenced for OGA had higher motility, similar to cells treated with TGF-β (Fig. 7D and G). Combined, these results suggest that A549 cells are undergoing EMT by modulation of O-GlcNAcylation.

DISCUSSION

The best characterized metabolic phenotype in cancer cells is the Warburg effect, which consists in a shift of ATP generation from oxidative phosphorylation to glycolysis, even under normal oxygen tension (1). However, it is becoming clear that the metabolic phenotype of tumor cells extends beyond the Warburg effect. Cancer cells need rapid ATP generation to maintain energy status, as well as an increase in the biosynthesis of macromolecules and the NADPH to maintain redox status (42). Tumor cells have a high glucose uptake through increased expression of GLUT transporters (5). Glucose upon entering the cell is phosphorylated by hexokinase forming glucose-6-phosphate (G6P). G6P can be shunted into the PPP by G6PDH or undergo enzymatic isomerization by the action of phospho glucose isomerase, forming fructose-6-phosphate (F6P). The majority of F6P enters the glycolytic pathway through PFK enzyme activity, but 2-5% of glucose that enters the cell is diverted to through the HBP by the rate limiting enzyme GFAT (6). In the past decade, studies of glucose metabolism in tumors have been heavily investigated. However, it is not well understood how cancer cells distribute the glucose through the three major metabolic pathways (glycolysis, PPP and HBP) during crucial events of malignancy, such as EMT.

Here, we demonstrate that glucose is shunted to HBP during the EMT changing the glycophenotype of A549 cells (Fig. 8). We showed an increase of glucose uptake during induced TGF-β EMT. However, no changes in ATP and pyruvate levels or lactate production were detected, suggesting that the increased amount of glucose entering into the cell is not used for energy. Interestingly, our proteomic data showed changes in several proteins involved in the glycosylation process, PPP, and glycolysis during EMT. Regarding the rate limiting enzymes of the top three glucose metabolism pathways, we investigated the protein levels and activity of PFK, G6PDH and GFAT, the three rate-limiting enzymes. Although, PFK activity decreased over time, the protein levels increased. The protein increase in PFK may be in response to the lung cancer cells trying to overcome the decrease in activity. In addition, PFK is known to be down-regulated by O-GlcNAc modification and we may also be seeing a similar effect (43). We showed a decrease in G6PDH protein levels and activity. The PPP plays a critical role in regulating cancer cell growth and helping to maintain the cellular redox status, by supplying ribose-5-phosphate and NADPH (42). Surprisingly, studies relating PPP with EMT currently have not been determined and our results indicate a reduction during EMT. Most studies investigating the PPP in cancer have been related to growth rate and the protection for high levels of ROS (44). Unlike G6PDH, a significant increase of GFAT protein levels and activity were detected during EMT, indicating the potential for increased metabolites to enter the HBP. We showed a significant increase of UDP-GlcNAc supporting our hypothesis of activation of HBP during EMT. Our group previously showed that over-expression of GFAT induces the EMT and high levels of
mesenchymal markers (N-cadherin and vimentin) in A549 cells (9). This data suggests that TGF-β could be activating HBP by increasing GFAT levels and activity, resulting in increased levels of activated monosaccharide donors. The HBP produces UDP-GlcNAc, and its derivatives, UDP-GalNAc and CMP-Neu5Ac, donor substrates used in the production of glycoproteins and glycolipids. Growing evidence demonstrates that alterations in the pool of activated substrates might lead to differential glycosylation.

HBP activation provided by TGF-β could promote aberrant glycosylation favoring malignant cell phenotypes. Indeed, we observed that TGF-β treatment of A549 cells induces an increase of glycoconjugates decorated with α2-6Neu5Ac (SNA labeling) with concomitant decrease of terminal β-Gal units. Increased sialic acid on the surface of tumor cells is well described in the literature of several tumor types (45-47). Sialylation is often associated with the decrease of outer β-Gal units, thus, reducing labeling by PNA. One of the most well characterized functions of this glycosylation is related to inhibition of apoptosis of tumor cells dependent on galectins (a family of proteins defined by their binding specificity for β-galactoside sugars that are secreted by a variety of cells including the immune system), so the sialylation α2-6 on the surface of tumor cells may protect tumor cells from infiltrating immune cells (47). For these reasons, the lectin SNA results suggest that hypersialylation could favor the evasion of the immune system, a known functional consequence of the EMT (48).

We showed an increase of OGT and the O-GlcNAc levels with no changes of OGA levels during EMT by Western-blot. Cho and colleagues showed an increase of O-GlcNAcylation in response to glucose deprivation through glycogen degradation (57). Here, we showed no change in glycogen levels during EMT. Several papers have been published relating O-GlcNAc and cancer (40,58,59). A substantial number of these manuscripts have related the increase of O-GlcNAc to the increase in OGT in several types of tumors (39,40). Many authors have reported that the levels of O-GlcNAc and the protein expression of OGT and OGA are aberrant in different models, including cell lines, murine models, and human tumor samples (17). Further, several cancer types including breast, bladder, prostate and colon display higher levels of OGT or O-GlcNAcylation in grade II or III tumors in comparison to grade I cancers indicating an association with malignancy (17). In addition, OGT silencing inhibits tumor growth in breast, prostate and pancreatic cancer (60-62), indicating that O-GlcNAcylation is important for tumorigenesis and suggesting that OGT represents a novel therapeutic target for these types of cancers (63). However, the role of O-GlcNAc during EMT is still unclear.

Here, we modulate the O-GlcNAc levels of A549 cell by shOGT and shOGA, and investigated the cellular morphology and motility as well as the epithelial and mesenchymal markers. The morphological...
changes involve activation of signaling pathways, cytoskeletal reorganization and surface protein changes. Cells submitted to the shOGT exhibited a similar morphology to control cells. While cells submitted to the shOGA showed a mesenchymal profile, like fibroblasts. In agreement with morphology results, the shOGA cells showed a significant increase of migratory capacity, while shOGT cells displays a similar mobility to control cells. Clearly, there is much to be explored in relation to morphology and the ability of cells to migrate during EMT by studying the effects of O-GlcNAcylation. Some of the cytoskeleton proteins have been described as being modified by O-GlcNAc (64,65). In addition, we showed a decrease of E-caderin with concomitant increase of N-caderin in shOGA cells indicating that the increase of O-GlcNAc levels can trigger the EMT process in A549 cells. However, very few O-GlcNAc molecular mechanisms are known to affect the EMT. Park and colleagues showed in a breast cancer cell line that O-GlcNAcylation at serine 112 of Snail, the repressor of E-cadherin, blocks its phosphorylation by GSK3β and protects Snail from degradation (23). Recently, a study in human ovarian cancer cells showed that O-GlcNAc modulation regulated E-caderin and cell migration (66).

In summary, we show for the first time that A549 cells increased glucose uptake during EMT, increasing the glucose flux through the HBP rather than glycolysis and PPP. The activation of HBP induces an aberrant cell surface glycosylation and O-GlcNAcylation. The cell surface glycans display an increase of sialylation α2-6, poly-LacNAc and fucosylation, which are known epitopes found in different tumor models. In addition, modulation of O-GlcNAc levels was demonstrated to trigger the EMT process. Taken together, our results indicate that EMT is an applicable model to study metabolic and glycophenotype changes during carcinogenesis. This study highlights the potential to target enzymes in the HBP to treat the deleterious effects related to epithelial mesenchymal transition during tumorigenesis.

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CONFLICT OF INTERESTS
The authors have declared that no competing interests exist.

AUTHOR CONTRIBUTIONS
Conceived and designed the experiments: MCL, PCC, JLD, IAO, RQM, SAW, ART, WBD. Performed the experiments: MCL, PCC, JLD, IAO, RQM, MMC, IFP, SAW. Analyzed the data: MCL, PCC, JLD, IAO, RQM, KGC, MEMC, CEC, SAW, ART, WBD. Wrote the paper: MCL, PCC, IAO, RQM, SAW, ART, WBD.

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ABREVIATIONS
2-NBDG, (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose); 6PGD, 6-Phosphogluconate dehydrogenase; ALDO-A, aldolase A; ALDO-C, aldolase C; APAD, acetylpypridine adenine dinucleotide; ASPC1, ATP, adenosine triphosphate; CMP-NeuAc, Cytidine-5'-monophospho-N-acetylneuraminic acid; CTP, citidine-triphosphate; ECA, Erythrina cristagalli agglutinin; EMT, Epithelial Mesenchymal Transition; E-PHA, Phyto haemagglutinin E; Fuc, Fucose; G6PD, Glucose-6-phosphate dehydrogenase deficiency; G6PD, Glucose-6-phosphate dehydrogenase; Gal, Galactose; GALE, UDP-Galactose-4-Epimerase; GALNT2, Polypeptide N-Acetylglactosaminyltransferase 2; GANAB, Neutral alpha-glucoosidase AB; GFAT or GFPT, Glutamine:fructose-6-phosphate amidotransferase; GFPT1, glutamine-fructose-6-phosphate transaminase 1; GFPT2, glutamine-fructose-6-phosphate transaminase 2; GlcNAc, N-acetylglucosamine; Glu, Glucose; GLUB2B, Glucosidase II subunit beta HBP, Hexosamine Biosynthetic Pathway; Hxk-1, hexokinase 1; LDH-B, lactate dehydrogenase b; L-PHA, Phyto haemagglutinin L; MAA, Maackia aconitifolia agglutinin; Man, Manose; MIF, Mean Intensity of Fluorescence; MPRD, Cation-dependent mannose-6-phosphate receptor; NEUA, N-Acetylneuraminic c1yctidyllytransferase; OGA, O-GlcNacase; O-GlcNAc, O-linked N-acetylglucosaminitne; OGT, O-GlcNAc transferase; OST48, Oligosaccharyltransferase 48; PFK, Phosphofructokinase-1; PFK-P, Phosphofructokinase P; PGD, phosphogluconate dehydrogenase; PMM-2, Phosphomannomutase 2; PPP, Pentose Phosphate Pathway; PSA, Pisum sativum; PYK-M, pyruvate kinase; RPN1, Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1; RPN2, Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2; SIAS, N-acetylneuraminate synthase; SNA, Sambucus nigra; TGF-β, Transforming growth factor beta 1; TIGAR, TP53-induced glycolysis and apoptosis regulator; TKT, transketolase; UDP-GalNAc, Uridine 5'-diphospho-N-acetylgalactosamine; UDP-GlcnAc, Uridine diphosphate N-acetylglucosamine; UGGG1, UDP-Glucose Glycoprotein Glucosyltransferase 1; UGPA, UTP-glucose-1-phosphate uridyllytransferase; UTP, uridine-triphosphate; VVL, Vicia villosa lectin.

FIGURE LEGENDS

FIG. 1. TGF-β promotes glucose uptake during EMT. A, Morphology and cellular circularity changes induced by TGF-β (5 ng/ml) treatment over 24 h and 48 h compared with non-treated (control). B, Western blot of cell lysate samples analyzing expression levels of E-cadherin (E-cad), Vimentin (Vim) and GAPDH. C, Glucose uptake of A549 cells incubated with 2-NBDG. MIF = mean intensity of fluorescence. D, Lactate release. E, Relative ATP levels. F, Pyruvate levels. G, Glycogen levels in A549 cells treated with 5 ng/ml of TGF-β over 24 h and 48 h compared to untreated cells (control). The results are representative of 3 independent experiments. Quantitative analyses are shown as mean ± standard deviation. P values were calculated using the Student’s t-test and ANOVA test. *P<0.01.

FIG. 2. Proteomic analyses of time-dependent changes promoted by TGF-β in A549 cells during EMT. Lung A549 cells at Control, 24 h and 48 h TGF-β treatment in duplicate cell
lysates were trypsin digested, analyzed by LC-MS/MS on a Q Exactive hybrid Orbitrap, raw spectra was searched in Proteome Discoverer 1.4 using precursor ion intensity node in both Sequest and Mascot search engines. Results were uploaded into Scaffold software program to view and compare the total precursor ion intensities of each protein in both 24 h TGF-β/Control and 48 h TGF-β/Control. A, Diagram of key enzymes of glycolysis, the hexosamine biosynthetic pathway (HBP) and the pentose phosphate pathway (PPP). B, Quantitative changes of proteins involved in glycosylation and glucose metabolism (log 10). P values were calculated using the Student’s t-test *P<0.05.

FIG 3. TGF-β modulates rate-limiting enzymes of glucose metabolic pathways. A, A549 cells were incubated without (Ct) or with 5ng/ml of TGF-β over 24 h and 48 h. Western blot of cell lysate loads analyzing expression levels of GFAT, G6PD, PFK and β-actin. Signal intensities were normalized, using β-actin as loading control, and relative intensities of GFAT (B), G6PD (C), PFK (D) were presented. GFAT (E), G6PDH (F) and PFK (G) activities in cell lysates were measured as described in Experimental Procedures. Results are shown as mean ± standard deviation. P values were calculated using the Student’s t-test and ANOVA test. *P<0.01.

FIG. 4. TGF-β induces increase of UDP-GlcNAc levels. A, Separation of UDP-GlcNAc (0.1 mM), UDP-GalNAc (0.1 mM) and pNP 1 mM by using liquid chromatography. B, CID mass spectrum of UDP-GlcNAc peak from chromatogram of control cell extract, showing transitions characteristic of this activated nucleotide. C, Chromatograms of polar metabolites cell extracts from control (blue line) and TGF-β treated cells for 24 h (green line) and 48 h (red line), showing regions corresponding to UDP-GlcNAc, UDP-GalNAc and pNP retention times. D, UDP-GlcNAc quantification of control (blue) and TGF-β treated cells for 24 h (green) and 48 h (red). Quantitative analyses are shown as mean ± standard deviation of 3 independent experiments. P values were calculated using one-way ANOVA and the appropriate post-test. * P<0.05.

FIG. 5. Comparison of surface glycan profile in A549 cells before and after induction of EMT by TGF-β. A, Scheme representing binding specificities of the lectins used in this study. A549 cells were incubated without (Ct) or with 5 ng/ml of TGF-β for 48 h and surface glycans were labeled with biotinylated lectins and incubated with FITC-conjugated avidin. B, Histograms indicating the avidin (black line) binding, untreated control (blue lines) cells and cells treated with TGF-β (orange lines). C, Bar graph comparing the fluorescence intensity for lectins in Control and 48 h TGF-β treated cells. The result is representative of three independent experiments. * P <0.01.

FIG. 6. O-GlcNAc levels, OGT and OGA alterations during TGF-β-induced EMT in A549 cells. A, Western blot of OGT, OGA and O-GlcNAc levels; O-GlcNAc competitive assay was conducted by pre-incubation of the antibody with 0.2 M free GlcNAc before membrane labelling to confirm CTD 110.6 specificity. B, C, D, Histograms represent densitometric analyses of western blots of O-GlcNAc levels, OGA and OGT respectively. Signal intensities were normalized with β-actin as loading control. Quantitative analyses are shown as mean ± standard deviation. P values were calculated using Two-way ANOVA and the appropriate post-test.* P<0.05, ** P<0.01, *** P<0.001.

FIG 7. Changes in cellular morphology, motility and molecular markers by O-GlcNAc cycling enzymes, OGT and OGA silencing. A, Protein levels from silencing OGT (lower) and OGA (upper) genes by short hairpin RNAs (shRNAs). B, Silencing OGT and OGA change protein levels of E-cadherin (E-cad) and Vimentin (Vim). C, Cell morphology of A549 cells transfected with scramble shRNA, shOGA or shOGT or the positive control treated with TGF-β 5 ng/ml during 48 h. D, Cell circularity of A549 cells silenced for OGA or OGT or treated with TGF-β 5 ng/ml during 48 h.
h. *E.* Cell motility of A549 cells transfected with scramble, shOGA or shOGT. F. Tracks of 50 random individual cells on gold solution were measured and compared between A549 cell controls and for silenced OGA or OGT. Quantitative analyses are shown as mean ± standard deviation of 3 independent experiments. *P* values were calculated using Two-way ANOVA and the appropriate post-test. *P < 0.05; **P < 0.01; ***P < 0.001.

**FIG 8.** TGF-β drives glucose flow through HBP promoting aberrant glycosylation during EMT. TGF-β increases glucose uptake with no changes in ATP levels, pyruvate, lactate and glycogen production. During EMT we showed a decrease of protein levels and/or catalytic activity of G6PDH and PFK, key enzymes of the PPP and glycolytic pathways. However, we showed an increase of GFAT protein levels and activity combined with increased UDP-GlcNAc levels, indicating that excess Glc is shunted to HBP. The activation of HBP induces an aberrant cell surface glycosylation and O-GlcNAcylation. We showed an increase of OGT and the O-GlcNAc levels with no changes of OGA levels during EMT. (Blue), decreased protein levels; (Red), increased product levels and/or catalytic activity; (Violet) increased protein levels and decreased catalytic activity; (Orange) unchanged products, (Pink) experimentally verified; (Green) probable pathway taken by Glc.
FIGURE 4

Fig. 4
FIGURE 5

A

B

Cell count

α-Fuc

AAL

Galβ1-4GlcNAc

ECA

β-Gal

PNA

α-Man

PSA

α2-6-Neu5Ac

SNA

α2-3-Neu5Ac

MAA

GlcNAcβ1-6Man

L-PHA

Galβ1-4GlcNAc

β1-2Man

GalNAcα-Ser/Thr

E-PHA

VVL

C

MIF x 10^3

Ct

TGF-β

0

50

100

150

200

250

300

350

400

450

500

550

600

650

700

750

800

850

900

950

1000

AAL

ECA

PNA

PSA

SNA

E-PHA

L-PHA

MAA

VVL

* * *
Epithelial mesenchymal transition induces aberrant glycosylation through hexosamine biosynthetic pathway activation
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