

Abstract

Cells undergo metabolic reprogramming during oncogenic transformation and cancer progression. Folate one-carbon cycle plays an important role in providing the necessary substrates for proliferating cancer cells. Aberrant folate metabolism has been implicated in the development of several cancer types, though underlying mechanisms remain unclear. Our lab has previously demonstrated that a folate-restricted diet increased the growth and invasion of orthotopically transplanted murine non-metastatic mesenchymal claudin-low breast cancer cells (M-Wnt) *in vivo*. In contrast, it decreased the growth and lung metastasis of transplanted metM-Wnt^{lung} cells, a metastatic subclone of the M-Wnt tumor. The current study explored whether metabolic reprogramming of the two cell lines affects their differential response to folate withdrawal. The non-metastatic and metastatic cells differed in several metabolic pathways, including energetics and autophagy. To examine the effect of long-term folate depletion on M-Wnt and metM-Wnt^{lung} cell metabolism *in vitro*, the two cell lines were grown in standard or folate-depleted media for 14 days. Both M-Wnt and metM-Wnt^{lung} cells exhibited increased autophagy and oxidative stress following long-term folate depletion. However, following long-term folate depletion, only metM-Wnt^{lung} cells showed decreased viability and increased apoptosis. These results suggest that these cells underwent metabolic reprogramming in response to nutrient stress. While the non-metastatic M-Wnt cells were able to withstand nutrient starvation through adaptation involving glutathione metabolism and autophagy, the metastatic cells were less adaptable, and thus folate restriction prevented their growth. This study highlighted that metabolic reprogramming during tumor progression to metastasis may change cells' adaptability under nutrient stress, and folate restriction as a nutrient stress can assess tumors' metabolic phenotypes.

Introduction

Aberrant cellular metabolism can support tumorigenesis and cancer cell progression (Vander Heiden and DeBerardinis, 2017). Cancer cells modify cellular metabolism and modulate a range of signaling pathways to support anabolic growth and bioenergetics for cell survival under limited nutrient availability, and maintain redox homeostasis during oncogenic transformation and cancer progression (DeBerardinis and Chandel, 2016). A growing body of evidence has shown metabolic reprogramming dictates metastatic potential in several cancer types, including breast, cervical and colorectal cancers (Dupuy et al., 2015; Ishikawa et al., 2008; Lin et al., 2012; O'Flanagan et al., 2017; Wu et al., 2015). Increase in reactive oxygen species (ROS) generation caused by mitochondrial DNA mutations can increase metastatic potential in tumor cells (Ishikawa et al., 2008).

Folate, also known as vitamin B9, is a water soluble vitamin naturally found in high concentrations in green leafy vegetables, beans, lentils and some fruits (Mitchell et al., 2009). Folic acid (FA), a synthetic form of folate, is used in many vitamin supplements and is also used in many countries, including the US, to fortify flour, cereals, baked goods and grain products to reduce the risk of birth defects (Crider et al., 2011). Folate coenzymes play a vital role in one-carbon metabolism, supplying one-carbon units for cellular biosynthetic pathways, including nucleotide and glutathione synthesis, and methylation reactions (Locasale, 2013; Tibbetts and Appling, 2010). Altered folate metabolism has been associated with a wide range of diseases such as anemias, neural tube defects, cardiovascular diseases, neuropsychiatric disorders, and cancers (Christensen and MacKenzie, 2006; Sudiwala et al., 2016). Folate deficiency appears to

have differential effects on various cancer types and different stages of cancer development (Duthie, 2011; Miller et al., 2008). However, the detailed mechanisms underlying dietary folate intake and cancer development remain unclear.

Autophagy, an evolutionarily conserved catabolic process in which intracellular membrane structures sequester proteins, macronutrients and organelles for lysosomal degradation, is essential in maintaining cellular homeostasis (Kaur and Debnath, 2015). Caloric restriction and nutrient deprivation stimulate autophagy, which promotes cell survival under metabolic stress (Kaur and Debnath, 2015). The role of autophagy in cancer exhibits context dependency. Autophagy can prevent oncogenesis by preserving cellular and systemic homeostasis, and degrading oncoproteins such as mutant TP53 (White et al., 2010). Yet, autophagy has been shown to modulate tumor initiation and promote cancer cell growth during periods of metabolic stress, including nutrient stress and chemotherapy (White et al., 2010). Proficient autophagic responses can be essential to support cancer cells in tumor progression, invasion and metastasis (Galluzzi et al., 2015).

Triple-negative breast cancer comprises approximately 15% of breast cancers and has a more aggressive nature than other subtypes, with high risks of recurrence and metastasis (Dent et al., 2007; Perou, 2010). Our previous *in vivo* study showed that a folate-restricted diet (0 ppm) exerted differential effects on non-metastatic and metastatic *Wnt1* driven murine claudin-low breast cancer, a subtype of triple-negative breast cancer (Perou, 2010). Specifically, folate restriction increased tumor size and invasion of orthotopically transplanted non-metastatic mesenchymal (M)-Wnt tumor cells, but decreased growth and lung metastasis of transplanted metM-Wnt^{lung} cells, a metastatic derivative of M-Wnt cells. Folate depletion also affected glutathione redox signaling by increasing glutathione, ophthalmate and S-lactoylglutathione in

M-Wnt tumors (O'Flanagan et al., unpublished data). However, when these two cell lines were cultured in folic acid free media for 48 hr *in vitro*, both displayed decreased proliferation, invasion and migration rates. M-Wnt cells also showed increased expression of gamma-glutamyltransferase 5 (GGT5, 7.39-fold), a gene associated with redox defense, following short-term folate depletion (Ashkavand et al., 2016).

Here we examined the effect of long-term folate depletion on M-Wnt and metM-Wnt^{lung} cells *in vitro*. M-Wnt cells retained growth and local invasion upon folic acid withdrawal for 14 days, at least partially through upregulating autophagic flux. In contrast, metM-Wnt^{lung} cells were unable to adapt to the nutrient stress. Folate depletion elevated endogenous ROS levels in M-Wnt, which may underlie a more invasive phenotype *in vivo*. Metabolic reprogramming, including reprogramming of the glutathione metabolism, may contribute to sustaining cell growth and maintaining redox homeostasis in M-Wnt cells. Our findings indicate that metabolic reprogramming during progression to metastasis may underlie differential response to nutrient stress in murine triple-negative breast cancer.

Results

Metabolic alterations in metM-Wnt^{lung} cells

Reactive oxygen species (ROS) in M-Wnt and metM-Wnt^{lung} cells were stained using a redox sensitive fluorescent probe H2DCF-DA. metM-Wnt^{lung} cells exhibited a higher ROS level than M-Wnt cells (Figure 1a, $p < 0.05$). The expression of nuclear factor erythroid 2-related factor 2 (Nrf2) was higher in metM-Wnt^{lung} cells (Figure 1b). Nrf2 is a cytoprotective transcription factor

and stabilized in response to oxidative stress, to maintain cellular redox balance (Gorrini et al., 2013). An increase in Nrf2 expression suggests higher oxidative stress in metM-Wnt^{lung} cells.

Using a differential expression dataset previously generated (O'Flanagan et al., 2017), we determined expression of several enzymes in glutathione metabolism. Glutathione S-transferases (GST) catalyze glutathione's reactions with xenobiotic compounds, resulting in the formation of S-glutathione conjugates (Hayes et al., 2005). metM-Wnt^{lung} cells displayed an increase in mRNA expression of *Gst kappa 1 (Gstk1)* and *microsomal Gst2 (Mgst2)*, compared to M-Wnt cells (Figure 1c, 1d, $q < 0.001$).

Folate depletion exerts differential effects on cell growth and invasion

To determine the short-term and long-term effects of folic acid (FA) depletion on non-metastatic M-Wnt cells and their metastatic derivative, metM-Wnt^{lung}, both cell lines were cultured in folic acid-free RPMI1640 for 48 hours or 14 days. Long-term folic acid depletion decreased colony formation and expression of the cell proliferation marker PCNA in metM-Wnt^{lung} cells, while M-Wnt cells retained some clonogenic growth (Figure 2a, 2b). metM-Wnt^{lung} cells exhibited S phase arrest under long-term folic acid depletion, possibly due to inadequate substrates for nucleotide synthesis and protein synthesis. In contrast, M-Wnt cells were able to proceed from S phase to G2/M phase (Figure 2c). Both long-term and short-term folic acid withdrawal significantly reduced invasion of metM-Wnt^{lung} cells ($p < 0.05$), but exerted no significant effect on the invasion of M-Wnt cells (Figure 2d).

Oxidative stress and autophagy underlies differential response to folate depletion

Consistent with the results of Ashkavand et al. (2017), folic acid depletion for 48 hours caused significant reduction in basal oxidative phosphorylation and mitochondrial respiration capacity in both M-Wnt and metM-Wnt^{lung} cells, as shown by decreased oxygen consumption rate (OCR) (Figure 3a, $p < 0.0001$). However, under long-term folate withdrawal from culture media, both basal and maximal mitochondrial respirations were partially restored in M-Wnt cells, while they were further impaired in metM-Wnt^{lung} cells (Figure 3a, $p < 0.01$). Notably, OCR was higher following 14-day folic acid withdrawal than after 48-hour folate depletion following treatment of antimycin A and rotenone in both M-Wnt ($p < 0.0001$) and metM-Wnt^{lung} ($p < 0.001$) cells. These results indicate an increase in non-mitochondrial oxygen consumption in response to long-term folate depletion in these cells (Figure 3a).

Having observed the elevation of non-mitochondrial OCR following folate deficiency, we explored whether ROS levels change in response to the stress. In M-Wnt cells, 48-hour folic acid depletion resulted in an increase in endogenous ROS level, which was moderately restored upon 14-day folic acid withdrawal. In contrast, metM-Wnt^{lung} cells did not display a significant change in ROS under either 48-hour or 14-day treatment (Figure 3c). Nrf2 expression did not change in metM-Wnt^{lung} cells following 48-hour folate withdrawal but the expression was lost following 14-day folate depletion (Figure 3c). The loss of redox defense protein expression suggests that metM-Wnt^{lung} cells were unable to keep redox balance under the long-term nutrient stress. In contrast, M-Wnt cells did not display changes in Nrf2 expression upon folic acid withdrawal (Figure 3c). mRNA level of *Gstt1*, a member of *Gst* family, was increased by 10-fold in M-Wnt cells upon 14-day folate depletion, while it did not show significant change in metM-Wnt^{lung} cells (Figure 3d). These results suggest reprogrammed glutathione metabolism may play a role in M-Wnt cells' ability to maintain cellular redox balance in response to folate depletion.

Cellular autophagic flux, as indicated by LC3B cleavage, was significantly higher in both M-Wnt and metM-Wnt^{lung} cell lines following 14-day folic acid depletion (Figure 3c), suggesting that cells induced autophagy to withstand nutrient stress caused by folate depletion. However, increased autophagy in metM-Wnt^{lung} cells coincided with cell death, as measured by cleavage of caspase 3 (CC3) (Figure 3c) and reduced cell growth (Figure 2a).

Folate deficiency decreases growth of MDA-MB-231

MDA-MB-231, a human metastatic breast cancer cell line, displayed similar behaviors under folate depletion as the murine metastatic cell line, metM-Wnt^{lung}. A long-term folic acid withdrawal from culture media significantly impaired cell colony formation, proliferation signal, and cell invasion (Figure 4a, b, c). MDA-MB-231 cells exhibited higher autophagy in response to folate starvation, which coincided with cell death (Figure 4b). MDA-MB-231 cell line, similar to metM-Wnt^{lung}, is unable to adapt to folate stress.

Discussion

The metabolic phenotypes of tumor cells are heterogeneous, depending on cancer driver mutations, tissue of origin, metastatic potential, and nutrient availability (DeBerardinis and Chandel, 2016). The intrinsic metabolic variability and flexibility of tumor cells enables them to generate energy, uptake resources for biosynthesis and maintain homeostasis under varying physiologic conditions (DeBerardinis and Chandel, 2016). The two murine claudin-low breast cancer cell lines utilized in this study, non-metastatic M-Wnt and metastatic metM-Wnt^{lung}, although driven by the same oncogene, a number of metabolic pathways are altered during progression to metastasis, including oxidative phosphorylation, glycolysis and xenobiotic

metabolism (O'Flanagan et al., 2017). The current study demonstrated constitutively higher ROS level and higher glutathione-dependent redox defense machinery in metM-Wnt^{lung} compared with M-Wnt cells, which may result from differential levels of mitochondrial respiration, peroxisome metabolism (O'Flanagan et al., 2017).

Folate one-carbon metabolism is essential in anabolic networks comprised of *de novo* purine biosynthesis, *de novo* thymidylate synthesis and activation of methyl groups for DNA methylation (Stover, 2009). Folate deficiency can cause nutrient stress by impairing nucleotide and protein syntheses in proliferating cells (Cossins and Chen, 1997). In metastatic triple-negative breast cancer cell lines, metM-Wnt^{lung} (mouse) and MDA-MB-231 (human), folic acid withdrawal from culture media impaired cell clonogenic growth and proliferation. MetM-Wnt^{lung} cells exhibited arrest in S-phase, suggesting deficiency in supply of nucleotide building blocks and substrates for protein translation. Consistent with the previous animal studies in our lab, a folate-restricted diet significantly ablated tumor growth and metastasis of metM-Wnt^{lung} and MDA-MB-231 tumors in mice (O'Flanagan et al., unpublished data). Surprisingly, M-Wnt cells retained clonogenic growth and were able to proceed from S phase to G2/M phase *in vitro*, which suggests that M-Wnt cells reprogram other metabolic pathways to overcome folate deficiency.

One carbon metabolism is associated with cellular redox balance (Locasale, 2013). The folate cycle interacts with the methionine cycle to provide building blocks for glutathione synthesis (Locasale, 2013). Glutathione is a major contributor to redox defense through neutralizing reactive oxygen species (ROS) and maintains NADPH/NADP⁺ ratio, which is essential in powering reductive biosynthesis (Locasale, 2013). Moderate levels of ROS can be tumorigenic by causing DNA damage, and increasing cellular survival, proliferation and

migration (Storz, 2005). Non-metastatic M-Wnt cells displayed a higher ROS level in response to short-term folate depletion, which was slightly reduced following long-term folate withdrawal. Elevated ROS may have helped retain the invasion potential in M-Wnt cells *in vitro*, and contributed to a more aggressive phenotype *in vivo*. M-Wnt tumor metabolomics revealed an alteration in glutathione metabolism upon a folate-restricted diet (O'Flanagan et al., unpublished data). In the *in vitro* system, M-Wnt cells, but not metM-Wnt^{lung}, displayed significant increase in expression of *GSTT1*, a key enzyme in glutathione metabolism. Changes in glutathione metabolism may contribute to maintaining cellular redox homeostasis upon folate depletion and providing reductive force for biosynthesis in M-Wnt cells.

All three cell lines, M-Wnt, metM-Wnt^{lung} and MDA-MB-231, induced autophagic flux following long-term folate depletion. Autophagy-deficient M-Wnt cells (*ATG5^{wt/mt}*) showed a significant decrease in clonogenic growth following 14-day folic acid withdrawal (O'Flanagan, unpublished data). Depleting *ATG5* via CRISPR did not restore survival in metM-Wnt^{lung} cells under long-term folate starvation. These results suggest that M-Wnt cells are able to upregulate autophagic flux as a survival strategy in response to folate starvation. Yet, in metM-Wnt^{lung} cells, other cellular stress or damage following folate starvation resulted in cell death.

In conclusion, non-metastatic and metastatic cells have different capacities for reprogramming metabolic pathways in response to folate deficiency. Metabolic reprogramming, including autophagy and glutathione metabolism, may allow non-metastatic M-Wnt cells to adapt to nutrient stress to meet their metabolic needs, such as biosynthetic pathways and the redox homeostatic system. Autophagy has been shown to be essential to the survival of M-Wnt cells under folate depletion, indicating that it may be a key pathway through which M-Wnt cells survive nutrient stress. Higher ROS induced by folate depletion may enhance growth and

invasion of M-Wnt tumors. In contrast, metastatic metM-Wnt^{lung} and MDA-MB-231 cell lines have a higher baseline oxidative stress level, and these cells are unable to undergo the adaptation through metabolic reprogramming. These results indicate that folate depletion has differential effects on tumor cells of different metastatic potentials. Cells may have incorporated metabolic modifications during their progression to metastasis that make them more vulnerable to certain types of metabolic stress.

Methods

Cell culture

M-Wnt and metM-Wnt^{lung} cells were maintained in RPMI-1640 with 10% FBS, 25 mM glucose, 2.2 μM folic acid, 2 mM L-Glutamine and 100 U/ml penicillin/streptomycin. MDA-MB-231 cells were maintained in DMEM, supplemented with 10% FBS, containing 25 mM glucose, 9 μM folic acid, 2 mM L-Glutamine and 100 U/ml penicillin/streptomycin. For folic acid withdrawal, M-Wnt and metM-Wnt^{lung} cells were incubated with RPMI-1640 without folic acid (27016021) supplemented as above but with 10% dialyzed FBS. MDA-MB-231 cells were incubated with DMEM without folic acid (D2429) supplemented with 10% dialyzed FBS and, 2 mM L-Glutamine and 100 U/ml penicillin/streptomycin 44 mM sodium bicarbonate. For chronic FA withdrawal, cells were incubated for 14 days in FA-restricted media and surviving cells were used for further experiments.

Flow cytometry

For endogenous reactive oxygen species levels, H2DCF-DA (Invitrogen™ C6827, Hampton, NH) probe was added at 5 μM to the media and incubated at 37°C for 1 hour. Cells were incubated with 15 mM EDTA and harvested by centrifugation. Following centrifugation,

cells were resuspended in PBS/Fixation buffer (eBioscience™ 00-8333, Waltham, MA). Fluorescence was measured using an LSRII analyzer and FACSDIVA software (BD Biosciences, San Jose, CA). Appropriate gates were applied using unstained controls and geometric mean fluorescence intensity was determined for positive cells by a FL-1 channel at 520 nm using FlowJo software (Ashland, OR).

For cell cycle analysis, cells were harvested and cells were resuspended in ice-cold phosphate-buffered saline. Prior to analysis on an LSRII cell analyzer, cells were lysed with 0.1% NP-40 and DNA stained with 50 µg/ml propidium iodide (PI) (Sigma P4170, St. Louis, MO). DNA contents were detected by Alexa 594 channel.

Western blotting

Cellular protein extracts were prepared by lysing cells in RIPA buffer (Sigma R0278, St. Louis, MO), supplemented with Pierce Protease Inhibitor Mini Tablets (Thermo Scientific 88665, Waltham, MA), 1% Phosphatase Inhibitor Cocktail 2 (Sigma P5726, St. Louis, MO) and 1% Phosphatase Inhibitor Cocktail 3 (Sigma P0044, St. Louis, MO) for 20 min on ice. The lysates were centrifuged at 14,000 RPM for 15 minutes at 4°C. Protein concentrations were determined using Bio-Rad Bradford Protein Assay using Bio-Rad Protein Assay Dye Reagent Concentrate (5000006) at the wavelength of 595 nm. Samples were denatured by boiling for 5 minutes in 2X Laemmli Sample Buffer (Bio-Rad, 1610737) with 5% β-mercaptoethanol. Equal amounts (50 µg) of proteins were resolved by Mini-PROTEAN® TGX Stain-Free™ Precast Gels and analyzed by western immunoblotting. Proteins were transferred from gels to PVDF membranes, and the membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% Bovine Serum Albumin for 1 hour at room temperature. All primary antibody incubations were performed overnight at 4°C, and secondary antibody incubations performed were for 1 hour at

room temperature. Primary antibodies PCNA (#2586, 1:1000), Nrf2 (#12721, 1:1000), LC3B (#3868, 1:1000), cleaved-caspase 3 (#9664, 1:1000) were purchased from Cell Signaling Technology. β -actin (1:1000) was purchased from Santa Cruz Biotechnology. Secondary antibodies anti-rabbit IgG (NA934V, 1:4000) and anti-mouse IgG (NA931V, 1:1000) were purchased from GE Healthcare UK. Antibody expressions were detected using ECL™ Western Blotting Detection Reagents (RPN2106) purchased from GE Healthcare, UK.

Microarray

Cells were incubated in triplicate with or without folic acid for 72 hours. RNA was purified from cells from three extractions using RNeasy mini Kit (Qiagen, Hilden, Germany). cRNA libraries were generated using a Low Input Quick Amp labeling kit (Agilent Technologies, Santa Clara, CA) with NIH3T3 cells as reference RNA. Hybridizations were performed by the Lineberger Comprehensive Cancer Center Genomics Core, University of North Carolina using two-color 180K Agilent microarrays (BARCODE25503) and scanned using an Agilent Technologies Scanner G2505C with Feature Extraction software (Santa Clara, CA). Arrays were then mean-adjusted and median-centered. Transcripts with a fold change of three or higher with a q value of less than 0.001 were considered significant.

Colony formation

Cells (1,000) were seeded into each well of a 6-well plate in complete media. After 24 hours, media was changed to either that containing or lacking folic acid and colonies were allowed to form over 21 days. Colonies were photographed and counted using Image J software.

Invasion assay

Corning Biocoat Matrigel Invasion Chamber (354483) was used to determine cell invasiveness. The inserts and the membranes in the well were hydrated with serum-free media

for 2 hrs at 37°C prior to the assay. M-Wnt, metM-Wnt^{lung} and MDA-MB-231 cells suspension (25,000 cells per well) were prepared in respective culture media without serum and added to the upper portion of the chambers. The chambers were placed into wells containing 500 µl media containing 10% FBS and incubated at 37°C for 18 hrs. Cells were photographed and counted using Image J software.

Extracellular flux assay

Cell bioenergetics were carried out in XF96 Seahorse Metabolic Flux Analyzer (Agilent Seahorse Technologies, Santa Clara, CA). Cells (1,000 cells/well) were seeded in RPMI-1640 media with or without folic acid for 48 hours before the assay. Cells were incubated in assay media (serum-free, bicarbonate-free RPMI-1640 media with 10 mM glucose, 2 mM glutamine and 1 mM pyruvate, pH=7.4) in a non-CO₂ incubator for one hour prior to the assay. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured. Oligomycin (1.0 µM), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (1.0 µM), rotenone/antimycin A (0.5 µM) were added sequentially as indicated in Figure 3. Measurements were normalized by total protein amount using a bicinchoninic acid (BCA) protein assay (Thermo Fisher, Waltham MA).

Quantitative RT-PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) and RNA quality and concentrations were determined by NanoDrop Microvolume Spectrophotometers. cDNA was synthesized with equal amounts of RNA using TaqMan™ Reverse Transcription Reagents (ThermoFisher Scientific, Waltham MA). TaqMan™ Fast Universal PCR Master Mix was used to quantify relative amounts of RNAs with the manufacturer's recommended cycling conditions.

Statistical analysis

Means and standard deviations are shown in the figures. Statistical analysis was carried out using GraphPad Prism. Differences between experimental groups were analyzed using Student's t test or one-way ANOVA followed by Dunnett's multiple comparisons test, as indicated under the figures.

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