Role of Lactate in the Regulation of Transcriptional Activity of Breast Cancer-Related Genes and Epithelial-to-Mesenchymal Transition Proteins: A Comparison of MCF7 and MDA-MB-231 Cancer Cell Lines

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Simple Summary: This study shows how endogenous lactate is a major regulator of key genes involved in two main breast cancer cell types: estrogen receptor positive (ER^+) and triple negative breast cancer cells (TPBC). Lactate regulates both gene and protein expression in these cells. Furthermore, lactate is also an important player in the regulation of epithelial-to-mesenchymal transition (EMT), a process involved in metastasis. Targeting lactate production and exchange within and among cancer cells should offer novel therapeutics opportunities.

Abstract:
The Warburg Effect is characterized by accelerated glycolytic metabolism and lactate production and is a hallmark of cancer cells. Recently, we have demonstrated the role of endogenous, glucose-derived lactate as an oncometabolite which regulates gene expression in the estrogen receptor positive (ER+) MCF7 cell line cultivated in glucose media (San-Millan, Julian et al. 2019). Presently, with the addition of a triple negative breast cancer (TNBC) cell line, MDA-MB-231, we further confirm the effect of lactate on gene expression patterns, but extend results to include lactate effects on protein expression. As well, we report effects of lactate on the expression of E-cadherin and vimentin, proteins associated with epithelial-to-mesenchymal transition (EMT). Endogenous lactate regulates the expression of multiple genes involved in carcinogenesis. In MCF7 cells, lactate increased the expression of *EGFR, VEGF HIF-1a, KRAS, MIF, mTOR, PIK3CA, TP53, and CDK4* (The *Ital* is used for genes) as well as decreased the expression of *ATM, BRCA1, BRCA2, E2F1, MET, MYC,* and *RAF* mainly at 48h of exposure. On the other hand, in the MDA-MB-

231 cell line, lactate increased the expression of *PIK3CA, VEGF, EGFR, mTOR, HIF-1α, ATM, E2F1, TP53* and decreased the expression of *BRCA1, BRCA2, CDK4, CDK6, MET, MIF, MYC,* and *RAF* after 48h of exposure. Protein expression of representative genes corroborated mRNA expressions. Finally, lactate decreased E-cadherin protein expression in MCF7 cells and increased vimentin expression in MDA-MB-231 cells.

In this study, we demonstrate that endogenous lactate produced under aerobic conditions (Warburg Effect) is capable of eliciting important regulation of gene and protein expression in both ER+ and TNBC cell lines. The regulation of multiple genes by lactate is widespread and involves those involved in carcinogenesis including DNA repair, cell growth, proliferation, angiogenesis, and metastasis. Furthermore, both cell lines demonstrated changes in the expression of EMT biomarkers representing a shift to a more mesenchymal phenotype with exposure to endogenous lactate.

Keywords: lactate; glycolysis, carcinogenesis, gene expression; protein expression; epithelial-mesenchymal transition

1. Introduction

Described by Otto Warburg a century ago (Warburg and Minami 1923) accelerated glucose consumption and lactate production under fully aerobic conditions is ubiquitous in solid tumors. With discovery of DNA by Watson and Crick in 1953 (Watson and Crick 1953), the landscape of cancer research completely switched from emphasis on metabolism to genetics. At present, while it is recognized that cancer is characterized by mutations of multiple genes involved in cell growth, proliferation, angiogenesis or DNA repair (Vogelstein, Papadopoulos et al. 2013), regrettably despite decades of efforts, the cure of through gene science has not been realized. Hence, others and we, have decided to re-open the gates of cancer metabolism. This reopening including studies of cell metabolism has led to a renaissance in cancer biology.

Observations of accelerated glucose uptake and lactate secretion under fully aerobic conditions led Warburg to posit that cancer could be attributed to an injury to the cellular respiratory system (mitochondria) (Warburg 1925), although that idea could not be quite correct (Hussien, Rihn et

al. 2013). Still, for many decades lactate was considered a “waste” product resulting from lack of oxygen. However, pioneering work and discoveries by George Brooks since the 1980’s have demonstrated that lactate is the preferred fuel for most cells, the main gluconeogenic precursor in the body, and a signaling molecule (a “lactormone,” “exerkine and “myokine”) capable of inducing gene expression (Brooks 1986, Brooks 2018, Brooks, Osmond et al. 2022). In concert, Zhang et al. have reported recently, that the key role of lactate in histone acetylation (“lactylation”) is the acetylation of lysine histone residues, acting as an epigenetic modulator and increasing gene transcription from exposed chromatin in both human and mouse cells (Di Zhang 2019). More recently, Jiang et al. have shown that lactate modulates cellular metabolism through histone lactylation-mediated genes in non-small cell lung cancer (Jiang, Huang et al. 2021). As well, Verma and colleagues have also recently demonstrated that tumor-derived lactate can elicit epigenomic reprograming in cancer-associated fibroblasts from pancreatic ductal adenocarcinoma (Bhagat, Von Ahrens et al. 2019). Moreover, W. Liu and colleagues have recently demonstrated that lactate regulates cell cycle by remodeling the anaphase promoting complex (Liu, Wang et al. 2023).

In 2017, we developed the “lactagenesis hypothesis” (San-Millan and Brooks 2017) by which we posited that accelerated lactate production in tumors was not for bioenergetics (Brooks 2009, Brooks 2018), but rather owing to its powerful signaling properties (Brooks 2018, Brooks 2020), the role of lactate in tumors was to stimulate carcinogenesis. Posteriorly, in 2019 we demonstrated that lactate functions as an “oncometabolite” and regulates the expression of major genes related to carcinogenesis in MCF7 breast cancer cells (San-Millan, Julian et al. 2019). Recognition of the importance of lactate as a hallmark of cancer led several groups to block lactate transport and exchange among and cancer cells and within tumors as a therapeutic approach (Sonveaux, Vegran et al. 2008, Sonveaux, Copetti et al. 2012, Draoui, Schicke et al. 2013, Doherty, Yang et al. 2014, Draoui, Schicke et al. 2014, Mishra and Banerjee 2019, Kuo, Huang et al. 2020).

The first aim of the present study was to investigate the role of endogenous (glucose-derived) lactate as an oncometabolite on important oncogene expression in two representative breast

93 cancer cell lines. Herein, we sought to extend our findings by studying the representative breast
94 cancer cells lines MCF7, an estrogen-receptor positive (ER+) cell line, and MDA-MB-231, a triple-
95 negative cell line (TNBC). To further confirm the importance of lactate in carcinogenesis, we also
96 assessed protein expression for some of the important genes in both breast cancer cells.
97 The second aim of our study was to assess the potential role of lactate in the regulation of the
98 epithelial-to-mesenchymal transition (EMT). EMT is a key process in cancer progression
99 characterized by the loss of epithelial towards mesenchymal properties, thus allowing cells to
100 acquire enhanced capacity for motility, migration, invasiveness, and resistance to apoptosis. This
101 increased migratory capacity from a primary tumor site to a secondary, tumor-free site, involves
102 EMT as an important hallmark of metastasis (Thiery 2002, Mittal 2018). In general, MCF7 cells
103 are characterized by possessing an epithelial phenotype, while MDA-MB-231 a mesenchymal one
104 (Wang, Xu et al. 2019).
105 EMT is a complex multifactorial process with two major proteins representative of the process:
106 E-cadherin and vimentin. E-cadherin is a transmembrane protein which facilitates intercellular
107 (cell-to-cell) adhesions as well as morphogenesis, limiting cell motility (Chao, Shepard et al. 2010).
108 The decrease of E-cadherin expression disrupts cellular adhesion as well as elicits loss of cellular
109 architecture, facilitating motility and metastasis (Wells, Yates et al. 2008, Devlin and Verschuren
110 2018). Furthermore, autocrine signaling reinforces the mesenchymal phenotype and stimulates
111 endothelial growth factor receptor (EGFR), downregulating E-cadherin (Kopfstein and Christofori
112 2006).
113 Vimentin is a structural protein (type III intermediate filament protein) expressed in
114 mesenchymal cells. Vimentin is attached to the nucleus, endoplasmic reticulum, and
115 mitochondria, anchoring organelles in cytosol (Katsumoto, Mitsushima et al. 1990, Cheng, Shen
116 et al. 2016). Vimentin induces cell migration by inhibiting focal-adhesion proteins (Gregor,
117 Osmanagic-Myers et al. 2014). Moreover, it has been shown that vimentin is a key player in
118 metastasis by regulating cell migration and invasion as well as tumor angiogenesis. (Chen, Fang
119 et al. 2021). Furthermore, it has been shown that TNBC cells possess significantly higher amount
120 of vimentin than ER+ cancer cells (Su, Hopfinger et al. 2018).
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122

2. Materials and Methods

2.1 Cancer cell lines

MCF7 and MDA-MB-231, both adenocarcinoma breast cancer cell lines were obtained from the ATCC (Manassas, VA) and stored in liquid nitrogen until use. Cells were grown on tissue culture treated dishes (Genessee Scientific, San Diego, CA) in DMEM 1x media with 1g/L glucose (10-014-CV, Corning, Manassas, VA), 10% FBS, and 1X pen/strep. In experiments requiring high glucose media, cells were grown with DMEM 1X media with either 1 or 4.5 g/L glucose (10-013-CV, Corning). In these experiments, the normal 10% FBS was changed to 10% Nu-Serum (#51000, Corning Life Sciences, Glendale, AZ) to reduce the amount of lactate in the initial medium. Media was also amended with 10 and 20 mM added L-lactate both for 6h and 48h exposures. All cell cultures were grown at 37°C in a laboratory incubator with 5% CO_2 atmosphere (Forma Isotemp 3530, ThermoFisher, Marietta, OH). Trypsin (0.25% trypsin, 25-053-CI, Corning) was utilized during culture expansion.

2.2. QPCR analysis

Cell lines were trypsinized and resuspended in media, followed by low-speed centrifugation to a soft pellet. The cell pellet was then harvested for RNA using the RNeasy Plus Mini Kit (#74134, Qiagen, Hilden, Germany) as per the manufacturer's protocol. The concentration of purified RNA was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). cDNA was prepared from 500ng RNA per reaction using the iScript cDNA Synthesis Kit (#1708891, Bio-Rad, Hercules, CA). QPCR was performed using probe-based primer sets and master mix (Prime Time, Integrated DNA Technologies, Coralville, IA) on a CFX96 real time platform (Bio-Rad). Data was evaluated using Maestro software (Bio-Rad) and compared to a standard dilution curve from cell line cDNA. The appropriate housekeeping genes to use for data normalization were determined employing the Human Reference Gene Panel (Bio-Rad). Data was normalized with TBP and HPRT1 analysis.

153 *2.3. Cell Protein Isolation and Western Blot Protocol*

154 Western blots for cell line protein expression were performed by harvesting cell cultures
155 from growth plates by removal of spent media, washing plates 3X with cold 1X PBS, followed by
156 addition of cell lysis buffer (#9803, Cell Signaling Technologies, Danvers, MA) amended with
157 AEBSF (1 uM, SBR00015, Sigma) rather than PMSF. Cells were scraped and transferred to a
158 microcentrifuge tube and frozen at -80°C until analyzed. Samples were thawed, sonicated for 5
159 seconds at 50% power with a pencil type sonicator (model 450, Branson, Danbury, CT). Lysate
160 samples were centrifuged at maximum speed in a microfuge at 4°C for 15 minutes and the soluble
161 protein supernatant removed to a new microcentrifuge tube. Protein concentrations of samples
162 were determined using the BCA protein assay (#23225, Pierce) as per the manufacturers
163 protocol. Protein specimens were aliquoted in 50 uL amounts and frozen at -80°C until analysis.
164 Western blots were loaded at 2.5 to 50 ug total protein per lane in 4-20% gradient pre-cast
165 polyacrylamide gels (#3450033, Bio-Rad). Gels were also loaded with the Dual pre-stained
166 protein standards (#1610374, Bio-Rad) to evaluate protein separation during electrophoresis and
167 transfer to membranes. Gels were transferred to PVDF membranes (#1620177, Bio-Rad) for 2
168 hours at 250 mAmps followed by blocking in 5% w/v milk in TTBS buffer for 1 hour. Blocked
169 membranes were probed with primary antibodies overnight on a laboratory rocker at 4°C.
170 Membranes were washed 5X with cold TTBS and incubated with anti-Rabbit or anti-mouse
171 secondary antibodies conjugated to HRP (#7074 and #7076, Cell Signaling). After washing,
172 membranes were visualized with ECL reagent (Clarity, #1705060, Bio-Rad) on radiographic film
173 in the dark room. Western blot films were imaged with an Epson 310 scanner (Los Alamitos, CA).
174 Primary antibodies were from Cell Signaling including ATM (#2873), beta Actin (#4970), CDK4
175 (#12790), E cadherin (#14472), EGFR (#4267), HIF1 alpha (#35169), LDHa (#3582), MET (#8198),
176 MIF (#87501), mTOR (#2983), PIK3CA (#4249), RAS (#67648), and vimentin (#5741), from R&D
177 Systems (Minneapolis, MN) including LDHb (MAB9205), and from Abcam (Cambridge, UK)
178 including Citrate Synthase (AB129095).

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182 *2.4. Analysis of lactate and glucose concentrations in spent growth media*

183 Lactate in spent growth media was determined employing the L-lactate Assay Kit I from Eton
184 Biosciences (#120001400A, San Diego, CA). Sample lactate analysis was performed in the 96-will
185 plate format with comparison to a standard curve in the range of 30 – 3000uM. Assay plates were
186 measured for the formazan product at 490 nM using a Synergy 2 plate reader (BioTek, Winooski,
187 VT). Net lactate production was determined by subtracting lactate in the initial media from
188 cancer cell's lactate production at each time point during the experiments.

189

190 *2.5. Seahorse* XFe real-time ATP rate assay

191 For Seahorse experiments, cell lines were maintained in either 1 g/L or 4.5 g/L glucose
192 growth medium for at least 72 hours prior to analysis. Cells were plated at the concentration of
193 about 14,000 cells/well in XFe 96 microplate prior to washing and changing to assay-specific
194 medium sans phenol red and sodium bicarbonate. Brightfield visualization was performed prior
195 to the run utilizing a Cytation Plate Imaging Scanner (BioTek). The real-time measurement of
196 oxygen consumption rate (OCR) and glycolytic extra-cellular acidification rate (ECAR) was
197 performed using the Seahorse XFe 96 Extracellular Flux Analyzer (Agilent Technologies, Santa
198 Clara, CA) with the Seahorse XF real-time ATP rate assay Kit (#103592-100, Agilent Technologies)
199 according to the manufacturer's protocol. At the conclusion of the assay run, cells are counted
200 via fluorescence in the Cytation reader following exposure to Hoechest 33342 cell-permeable
201 DNA stain (2 µM final, #62249, Thermo Scientific). OCR values were normalized to cell number.

202

203 *2.6. Statistical analyses*

204 Independent sample t-tests and analyses of variance (ANOVA) were utilized for group-mean
205 comparisons via GraphPad Prism software (version 9.2.1, GraphPad, San Diego, CA).

206

207 **3. Results**

208 *3.1. Glucose-derived endogenous lactate (Warburg Effect)*

209 Because lactate was not present in treatment media, the lactate present in the spent media was
210 endogenously-derived in both cell lines from glycolysis (glucose-derived lactate). Glycolysis

progressed readily in both cell lines with total and net lactate releases significantly higher after 48h than 6h of incubation ($p<0.001$) (Fig-1A and 1B, respectively). Notably, lactate release from MDA-MB-231 is higher than from MCF7 cells (69%, $p<0.01$ at 6 hours, 72%, $p<0.0001$ at 48 hours) (Fig-1A and Fig-1B). Further, as discussed below, exogenous lactate additions of 10mM and 20mM to incubation media did not affect net lactate production (Fig-1B).

The higher glycolytic phenotype of MDA-MB-231 was confirmed via our Seahorse XFe Real-Time ATP Rate analysis. Overall, the total basal ATP production rates of both low-glucose and high-glucose adapted MDA-MB-231 cells were significantly higher than for MCF7 cells (Fig-2A). In MDA-MB-231 cells, more than half of ATP was derived from glycolysis. Once again, by that measure, MDA-MB-231 cells displayed a more glycolytic phenotype than MCF7 cells (Fig-2B).

Consistent with Seahorse analyses, expression showed increased levels of the "lactagenic" enzyme isoform, LDHA, was higher in MDA-MB-231 cells than in MCF7 cells (Fig-3). Conversely, in terms of oxidative phenotype, MCF7 cells showed increased protein expression of the mitochondrial marker citrate synthase (CS) as compared to MDA-MB-231 cells (Fig-3).

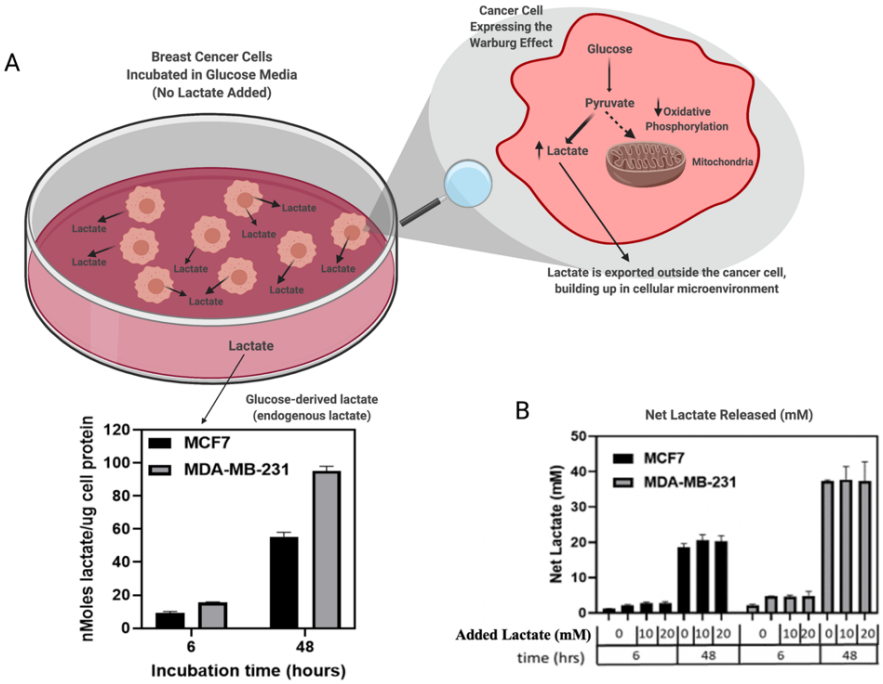


Figure 1. Comparison of lactate production in MCF7 and MDA-MB-231 cell lines. Lactate concentration in the spent media at 6 and 48 hours, normalized to total cell protein concentration (**A**). Comparison of net lactate production in spent media in response to 10 mM and 20 mM added exogenous lactate in 4.5 g/L glucose medium with increasing incubation time (**B**).

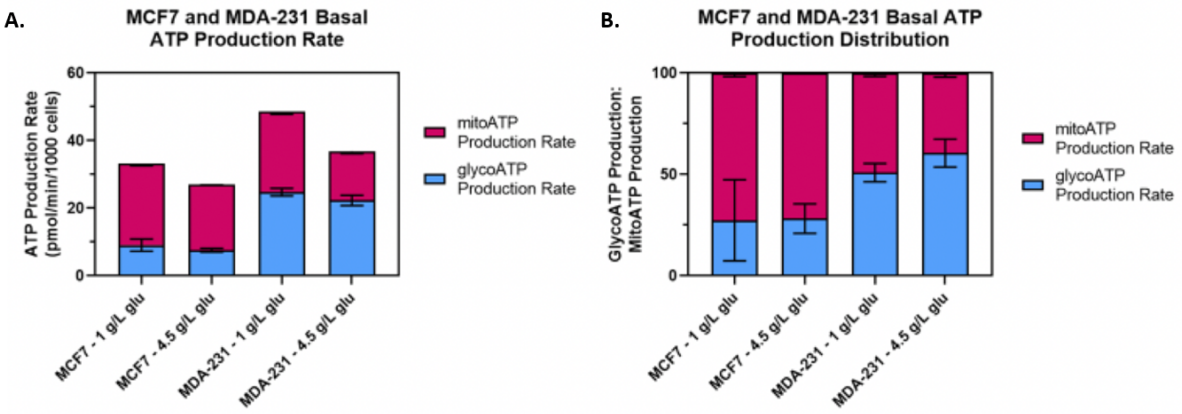


Figure 2. Total basal ATP production rate (**A**) and basal ATP production distribution (**B**) in MCF7 and MDA-MB-231 breast cancer cell lines. Cell lines were maintained in either 1 g/L or 4.5 g/L glucose growth medium for 72 hours prior to analysis.

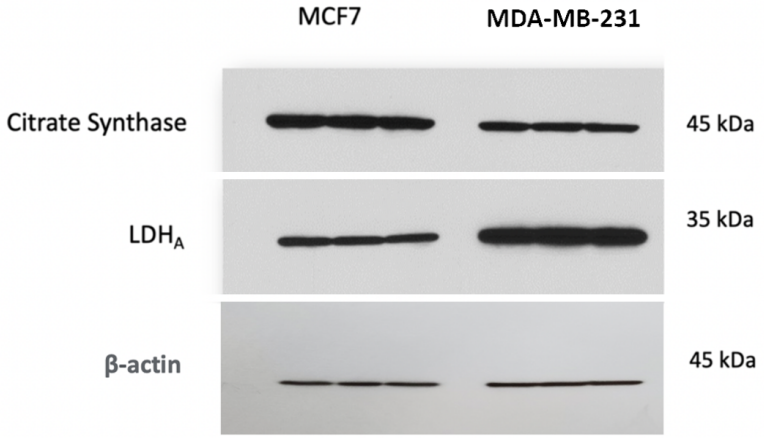


Figure 3. Differences in citrate synthase (CS) and lactate dehydrogenase A (LDHA) protein expression between MCF7 and MDA-MB-231 cells, representing the higher glycolytic nature of MDA-MB-231 compared to the higher OXPHOS capacity of MCF7.

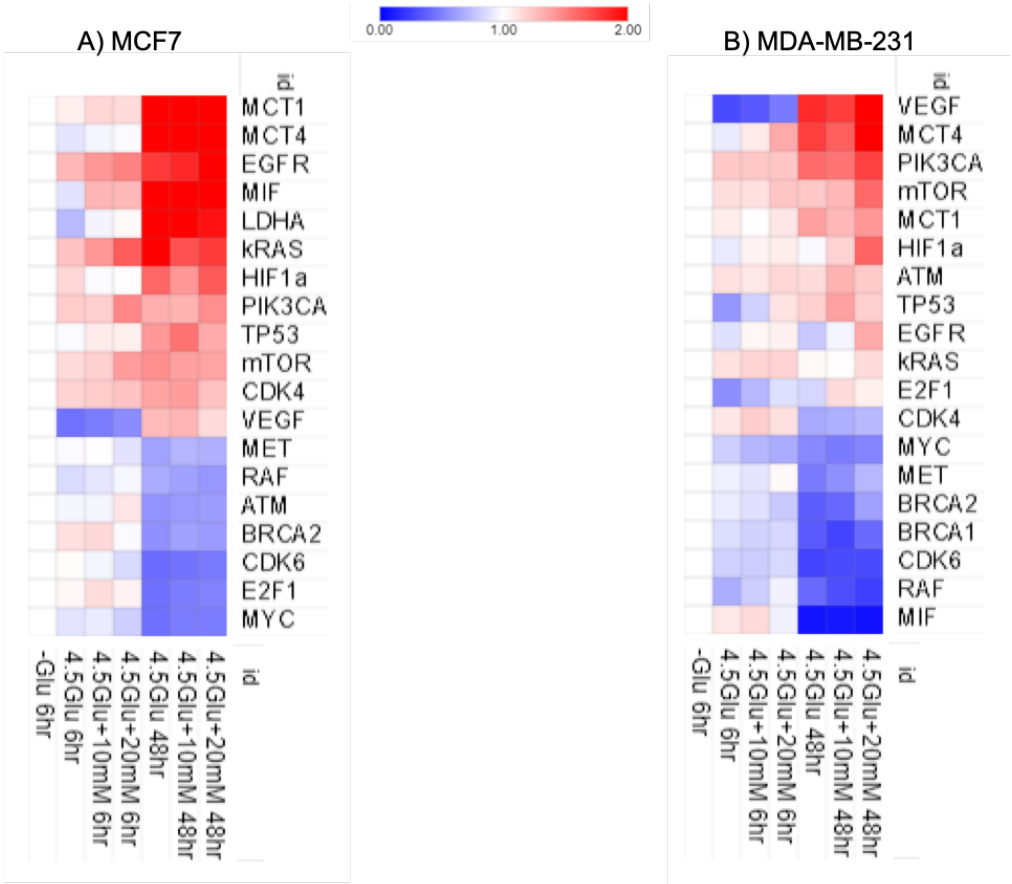
3.2. Gene expression regulation by lactate

Glycolysis in the cancer cell lines we studied readily resulted in lactate excretion and accumulation (Fig-1). Glucose-stimulated lactate regulated the expression of multiple representative cancer-related genes in both MCF7 and MDA-MB-231 cell lines (Fig-4). However, this regulation was heterogeneous and varied between cell lines. MCF7 cell lines showed increased expressions of *EGFR, KRAS, PIK3CA, mTOR, VEGF, HIF-1α, TP53, MIF,* and as well,

253 decreased expressions of *BRCA1, BRCA2, ATM, E2F1, CDK6, MET, MYC, and RAF* when exposed
254 to endogenously produced lactate, mainly at 48h (Fig-1, Fig-4a).

255 In response to glucose-stimulated lactate, MDA-MB-231 cells showed increased expressions of
256 *VEGF, HIF-1α, PIK3CA, mTOR E2F1, EGFR and TP53* (Fig-4b). On the other hand, lactate exposure
257 decreased the expression of *BRCA1, BRCA2, CDK6, MET, MIF, MYC, RA*F, mainly at 48h. However,
258 *CDK4* expression increased at 6h, but decreased at 48h (Fig-4b).

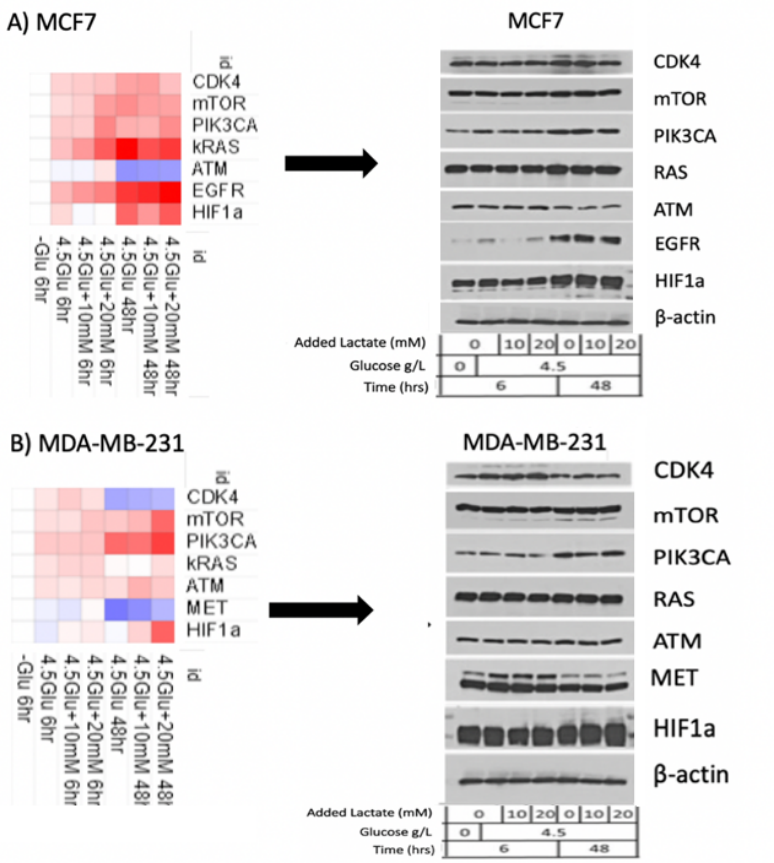
259 The addition of 10mM and 20mM exogenous lactate to glucose-containing media did not
260 increase gene expression compared to endogenously-derived lactate and is further explained in
261 Discussion. Noteworthy, lactate increased the expression monocarboxylate transporters (MCTs)
262 (Fig-4b). Genes encoding for *MCT1*, increased significantly in MCT7 cells, while *MCT4* expression
263 increased in both cell lines in response to increased lactate exposure.
264



265
266 **Figure 4**. Gene expression after 6 and 48h of endogenous (glucose-derived lactate) and exogenous lactate exposure
267 in both MCF7 (**A**) and MDA-MB-231 cell lines (**B**).

268
269 *3.3. Protein expression regulation by lactate*

270 Next, we measured protein expression of several representative genes that were altered in
271 expression by lactate. Western blots confirmed that the protein expression patterns of
272 representative genes mirror the expression patterns of the genes identified as having changed
273 from mRNA analyses (Fig-5). In MCF7 cells, 48h lactate exposure increased protein expression of
274 *EGFR, PIK3CA, RAS, HIF-1α, mTOR* and CDK4 and decreased the expression of *ATM* (Fig-5A),
275 confirming data from mRNA expression analysis. In MDA-MB-231 cells, 48h lactate exposure
276 increased protein expression of *PIK3CA, mTOR, HIF-1α* and *ATM*, did not affect *RAS* expression,
277 and decreased the expression of *MET* and *CDK4* (which increased at 6h) (Fig-5B), reflecting mRNA
278 expressions. Like in the case of gene expression, the addition of 10mM and 20mM exogenous
279 lactate did not further increase protein expression compared to endogenous lactate that is
280 further addressed in Discussion.



281
282 **Figure 5**. Gene and protein expression of representative cancer genes in MCF7 (**A**) and MDA-MB-231 cell lines (**B**)
283 following exposure to endogenous lactate (glucose-derived lactate) plus exogenous lactate at 6 and 48 hours as
284 determined via qPCR analysis and Western blot, respectively.

3.4. Possible role of Lactate in the regulation of epithelial-to-mesenchymal transition (EMT)

Our results can be interpreted to reveal an important role of lactate as a regulator in the EMT process in both cancer cell lines. E-cadherin protein expression was decreased by lactate in MCF-7 cells (p=0.058), (Fig-6). Moreover, while vimentin was significantly increased by lactate exposure in MDA-231 cells (p<0.01), we could not detect vimentin in MCF-7 cells (Fig-6).

Like in the case of gene expression experiments, the addition of 10mM and 20mM exogenous lactate did not affect protein expression compared to endogenous lactate production in either cell line (see Discussion).

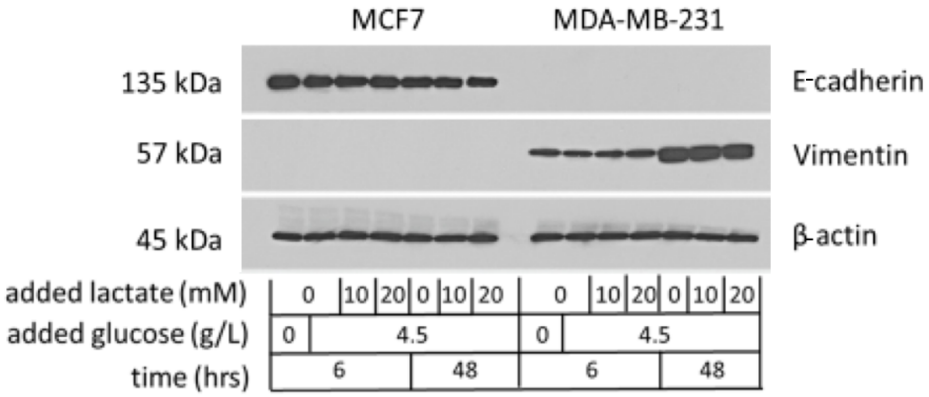


Figure 6. Protein expression of key EMT-related proteins, E-cadherin and vimentin, in MCF7 and MDA-MB-231 breast cancer cell lines in response to 0, 10 mM, and 20 mM exogenous lactate additions at 6 and 48 hours.

4. Discussion

The role of lactate in regulating the expression of multiple genes is not new. Brooks and colleagues already demonstrated that lactate was able to regulate the expression of 630 genes in multinucleated myotubes and striated fibers-L6 cells (Hashimoto, Hussien et al. 2007). Further, Zhang et al. have demonstrated that lactate-derived lactylation of histone-associated lysine residues serves as an epigenetic modification that directly stimulates gene transcription by exposing chromatin and identified 28 lactylation sites on core histones in human and mouse cells (Di Zhang 2019). As well, Jiang et al. have also recently demonstrated that lactate regulates cellular metabolism in non-small lung cancer cells through histone lactylation (Jiang, Huang et al.

310 2021). Moreover, we recently demonstrated the role of lactate as an oncometabolite, regulating
311 the expression of important cancer-related genes in MCF7 cells (San-Millan, Julian et al. 2019). In
312 the present study, we show that lactate is a major regulator of multiple genes and dependent
313 proteins involved in both ER+ and TNBC cells. Furthermore, we show that lactate could play an
314 important role in the epithelial-to-mesenchymal transition and hence, *vice versa* (mesenchymal-
315 to-epithelial transition) by regulating the expression of important proteins, specifically E-
316 cadherin and vimentin, involved in these transformations.

317 As depicted in Figure 1A, lactate is readily produced by both cancer cell lines studied, with the
318 largest lactate accumulation after 48h of exposure to glucose media. Therefore, this lactate is
319 endogenous lactate, or glucose-derived lactate, as a result of the Warburg Effect (Warburg and
320 Minami 1923, Warburg 1925) We also showed this phenomenon in our recent study with MCF7
321 cells (San-Millan, Julian et al. 2019). In the study herein, we observed that MDA-MD-231 cells
322 produce significantly higher quantities of lactate compared to MCF7 cells, with lactate being
323 produced nearly twice as fast as in MCF7 cells per total cellular protein after 48h ($p<0.001$) (Fig-
324 1). This finding is in line with the higher glycolytic nature of MDA-MD-231 cells reported by others
325 (Lunetti, Di Giacomo et al. 2019, Reda, Refaat et al. 2019, Ocana, Martinez-Poveda et al. 2020),
326 which we confirmed via Seahorse cellular respiration analysis (Fig-2). *In vivo*, the glycolytic
327 phenotype is associated with higher cancer aggressiveness and patient mortality (Jang, Han et al.
328 2012, Vlassenko, McConathy et al. 2015, Wang, Jiang et al. 2020, Zhang, Zheng et al. 2020).
329 Similar to what is observed *in vivo*, we found net lactate production at 48h accumulated to 20-
330 40mM in both cell lines (Fig-1B), which is consistent with lactate concentrations found in a variety
331 of tumor microenvironments (Brizel, Schroeder et al. 2001).

332 In our experiments, we added two different lactate concentrations (10mM and 20mM) to our
333 experimental medium in order to assess whether the addition of exogenous lactate would
334 amplify the expression of multiple genes in a dose-dependent manner vs endogenously-derived
335 lactate. Consequently, we concluded that endogenously produced lactate achieved the
336 maximum lactate-stimulated responses to gen and protein expression. The maximum
337 endogenous lactate simulated-response was also true for EMT proteins, vimentin and E-cadherin.

338 Our data shows that endogenous lactate is sufficient to alter genetic and proteomic responses in
339 both cancer cell lines. A main reason for a lack of physiological effect by the additional exogenous
340 lactate in the treatment media could reside in lactate transport kinetics. Lactate is transported
341 into and out of cells by monocarboxylate transporters (MCTs), which are bidirectional,
342 symporters (Brown and Brooks 1994, Halestrap 2012). Lactate is transported through cell
343 membranes following proton and monocarboxylate concentration gradients (Roth and Brooks
344 1990, Roth and Brooks 1990). Hence, metabolic activity and physiological processes such as
345 circulatory transport determine the gradients influencing lactate monocarboxylate (mainly
346 lactate, the physiological Lactate/Pyruvate ranging from 10 to 500, (Bergman, Wolfel et al. 1999,
347 Henderson, Horning et al. 2004) release from lactate net producers (driver cells) and consumers
348 (recipient cells) (Brooks, Osmond et al. 2023). Thus, cellular lactate efflux as a result of lactate
349 production from glucose (i.e., Warburg Effect) could prevent net influx of exogenous lactate
350 which could probably be the case of our experiments (Figs 4 and 5). Related, is that we observed
351 a significant increase in MCT4 expression in both cell lines at 48h (Fig-4), suggesting a feed-
352 forward stimulus by lactate on MCT4 transporter expression in order to export lactate to the
353 extracellular space where it then becomes a main player in acidosis of the tumor
354 microenvironment (TME) and regulator of multiple TME functions (de la Cruz-López, Castro-
355 Muñoz et al. 2019, Pérez-Tomás and Pérez-Guillén 2020).

356 Solid tumors accumulate multiple genetic mutations and in the case of breast cancers, the
357 average number of nonsynonymous mutations is 33, where about 95% of them are single-base
358 substitutions (Vogelstein, Papadopoulos et al. 2013). Within the number of genes mutated,
359 "driver genes" are the key ones conferring cancer cells a selective growth advantage (Haber and
360 Settleman 2007, Vogelstein, Papadopoulos et al. 2013). Within these driver genes, it is thought
361 that breast tumors require mutations in three to six driver genes (Vogelstein, Papadopoulos et
362 al. 2013). In both cells studied herein, lactate regulated the expression of multiple important
363 genes involved in all major processes of carcinogenesis including cell proliferation, growth,
364 angiogenesis, metastasis, and tumor suppression (Fig-4). Most of the genes prevalent in results
365 of our study are considered "driver genes" which are the key genes conferring selective growth
366 advantage to the cancer, as opposed to untransformed cells (Bozic, Antal et al. 2010),.

367 In MCF7 cells, we observed that lactate elicits significant increases in the expression of genes
368 involved in cell signaling, cell growth, and proliferation like *EGFR, PIK3CA, VEGF, mTOR, KRAS,*
369 *MIF*, and *CDK4*. Lactate also elicited increased expression in genes involved in angiogenesis like
370 *HIF1α* and *VEGF*. It is known that lactate stimulates angiogenesis through regulation of the
371 expression of vascular endothelial growth factor (VEGF), a signaling protein that has been known
372 to stimulate the formation and growth of blood vessels (Beckert, Farrahi et al. 2006, Kumar, Viji
373 et al. 2007, Dhup, Dadhich et al. 2012, Polet and Feron 2013). Furthermore, Hypoxia-inducible
374 factor 1 (HIF-1) is a master regulator of angiogenesis including the transcription of VEGF
375 (Forsythe, Jiang et al. 1996, Hirota and Semenza 2006). According to Semenza, HIF-1 is
376 fundamental for activating the transcription of genes encoding glucose transporters and
377 glycolytic enzymes which elicit metabolic reprogramming from OXPHOS to glycolysis and lactate
378 formation (Semenza 2010). In our recent study with MCF7 cells (San-Millan, Julian et al. 2019)
379 and in the study herein, we show that lactate activates the transcription of *HIF-1α* (Figs-4-5).
380 Hence, there appears to be a reciprocal relationship between HIF-1 and lactate and HIF
381 expression.

382 Importantly, we found that lactate elicits overexpression of a key tumor suppressor gene, *TP53*
383 in both cancer cell lines. Although P53 is typically considered a "tumor suppressor" protein that
384 binds to and repairs damaged DNA or signals for apoptosis in irreparable cells, missense
385 mutations in *TP53* elicit mutant p53 proteins with potential gain-of-function properties that
386 stimulate tumor cell proliferation, migration, invasion, survival, chemoresistance, cancer
387 metabolism, and/or tissue architecture disruption (Lee, Park et al. 2019). Consequently,
388 overexpression of P53 may be associated with increased mortality and poor prognosis (Isola,
389 Visakorpi et al. 1992, Friedrichs, Gluba et al. 1993, Wang, Yu et al. 2016). Furthermore, we
390 observed that lactate decreased the expression of two other main tumor suppressor factors,
391 BRCA1 and BRCA2, as well as the expression of ATM, a gene involved in DNA repair (Lavin, Delia
392 et al. 2006).

393 In MDA-MB231 cells, lactate increased the expression of genes involved in cell signaling, cell
394 growth, and proliferation including *VEGF, PI3CKA, HIF-1α, EGFR, mTOR* and *CDK4*, although the

395 latter only at 6h. As in the case with MCF7 cells, we also observed an increase in the expression
396 of tumor suppressor factor, *TP53* which is mutated in ~80% of TNBCs (Li, Zhang et al. 2019). The
397 well-known *TP53* missense mutation of MDA-MB-231 specifically leads to increased cancer cell
398 survival, migration, and invasiveness. Additionally, like in the case of MCF7, lactate decreased
399 gene expression for *BRCA1* and *BRCA2.*

400 To corroborate the findings on gene expression obtained through qPCR analysis, we performed
401 Western blots on a representative number of dependent proteins in both cell lines showing
402 lactate's involvement in protein expression at 6 and 48h of lactate exposure (Fig-5). Overall, it is
403 interesting to observe the differences in gene and protein expression produced by lactate
404 between the two unique breast cancer cell lines. While some key genes and proteins such as
405 *PIK3CA, VEGF, mTOR* and *EGFR* were upregulated by endogenous lactate in , there were
406 differences between cell lines in the expression of other genes such as *ATM, CDK4, and E2F1*.
407 This could be expected given the heterogeneous and different genetic characteristics of both cell
408 lines.

409 We believe it is noteworthy to observe the role of lactate in regulating EGFR in MCF7 cells. EGFR
410 is activated via the binding of its specific ligand at the cell surface, epithelial growth factor, and
411 elicits receptor dimerization and phosphorylation, leading to complex signal transduction
412 necessary for cellular proliferation, differentiation, growth, and migration as well as inhibition of
413 apoptosis within the cell. Therapeutically, EGFR is a frontline target in EGFR-subfamily-positive
414 tumors through a variety of approaches including monoclonal antibodies as well as tyrosine
415 kinase inhibitors (TKI's) (Huang, Armstrong et al. 2004, Lee, Hahn et al. 2014). These direct
416 inhibitors of EGFR bind to and block the ATP-binding pocket of the receptor, resulting in inhibition
417 of downstream signal transduction. EGFR is a "mother" kinase for two main signaling pathways
418 that are often dysregulated in cancer, which are the RAS/RAF/MEK/ERK and PI3K/Akt/mTOR
419 pathways, which are also regulated by lactate in our study herein.

420 In our study, we showed a significant increase in EGFR protein expression in MCF7 cells in
421 response to endogenous lactate production. This is important in ER+ cancer cells as a decrease
422 in drug response over time and eventual resistance to tamoxifen is a major problem in this largely

423 effective cancer therapy. In these type of ER+ cancers, EGFR overexpression can overrule the
424 signaling inhibition of 17β-estradiol (E_2) by tamoxifen, increasing carcinogenic signaling through
425 the PI3K/Akt pathway (Moerkens, Zhang et al. 2014); therefore, anti-EGFR therapy in conjunction
426 with tamoxifen could be preferentially employed in ER+ cancers overexpressing EGFR(Jeong, Bae
427 et al. 2019).

428 Finally, regarding EMT, we observed that lactate exposure elicits a decrease in E-cadherin in
429 MCF7 cells that accompanied by a significant increase in EGFR elicited by lactate. That effect
430 could explain some synergies in the decrease of expression of E-cadherin contributing to the loss
431 of epithelial characteristics and positioning lactate as a possible important regulator in this
432 process. On the other hand, MD-MBA-131 cells possess significantly higher amounts of vimentin
433 than MCF7 cells. Lactate exposure significantly increased the protein expression of vimentin at
434 48h, thus, facilitating a shift to a more mesenchymal, and therefore, metastatic phenotype.
435 Hence, lactate appears to be an important regulator of vimentin and E-cadherin expression in
436 MD-MBA-131 and MCF7 cells, respectively. Therefore, lactate may be an important regulator of
437 EMT in tumor cell lines.

438

439 **5. Conclusions**

440 Endogenous lactate as a result of the Warburg Effect is capable of eliciting important changes of
441 both gene and protein expression in both MCF7 (ER+) and MDA-MB-231 (TNBC) cell lines. The
442 regulation of multiple genes by lactate is widespread and involves main genes involved in the
443 carcinogenesis process including DNA repair, cell growth, proliferation, apoptosis evasion,
444 angiogenesis, and metastasis. Furthermore, a key step in carcinogenesis and metastasis is the
445 epithelial-to-mesenchymal transition (EMT) and herein we show that lactate probably plays an
446 important role in the regulation of EMT in breast cancer.

447 Blocking cellular lactate production as well as the exchange between cancer cells and surrounding
448 stroma cells, affecting the tumor microenvironment, should be a priority in cancer research.

449

450 **Limitations and Challenges**

451 We believe that due to the potent epigenetic-effector properties of lactate on multiple genes
452 and the major metabolic implications of cancer studies there are a variety of elements that need
453 to be better explored and understood. As an example and comparable to many other studies,
454 our previous study employed glycolytic enzyme, GAPDH, as a traditional housekeeping gene (San-
455 Millan, Julian et al. 2019) . In our current study herein, we used other traditional housekeeping
456 genes, TBP and HPRT1, as these genes were determined to be less affected by experimental
457 conditions via a reference gene panel of 15 validated housekeeping genes. Furthermore, the
458 actual methods of glucose media exposure are not physiological as they don't obey the exact
459 metabolic kinetics of glucose uptake and oxidation by normal cells. Furthermore, it is also
460 noteworthy that the accelerated glycolysis and metabolic reprogramming of cancer cells obey
461 different kinetics respect to normal cells. A true physiological glucose delivery to cancer cells
462 mimicking glucose kinetics in cancer should be important to accurately understanding cancer
463 metabolism and bioenergetics as well as lactate production for carcinogenesis.

464

465 **Author Contributions:** Conceptualization, I.S., C.R. and J.M..; methodology, C.R., J.M. and S.L.P.;
466 formal analysis, C.R., J.M, S.L.P, H.Y., G.S. and I.S.; investigation, I.S., C.R., J.M and S.L.P.;
467 resources, C.R., F.H .and I.S..; data curation, C.R., J.M., S.L.P and I.S.; writing—original draft
468 preparation, I.S., C.R. and JM; writing—review and editing, I.S., C.R., J.M. and G.B.; funding
469 acquisition, I.S. All authors have read and agreed to the published version of the manuscript.

470 **Funding:** Funding for this study came from IS-M Cellular Metabolism Laboratory funds, The Tadej
471 Pogačar Cancer Research Foundation and supplementary support from NIH 1 RO1 AG059715-01
472 to GB.

473 **Data Availability Statement:** All data from this study are available in the manuscript text and
474 figures. Further information can be obtained from the corresponding author upon reasonable
475 request.

476 **Acknowledgements**: The authors thank Christina Coughlan, Adriana Solano and Raleigh Jonscher
477 for meaningful discussions of the research results. Use of the Seahorse XFe96 analyzer was

provided by Matthew Jackman in the NORC Molecular and Cellular Analytical Core at the University of Colorado Cancer Center.

Conflicts of Interest: The authors declare no conflict of interest

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