METASTATIC BREAST CANCER CELLS HAVE HIGHER MITOCHONDRIAL FUNCTION THAN ISOGENIC NON-METASTATIC CELLS

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INTRODUCTION: Recent work with two isogenic breast cancer cell lines, 4T1 (highly metastatic) and 67NR (non-metastatic), showed that 4T1 cells have higher glycolytic activity and oxygen consumption rate (OCR) than 67NR cells (1), and that their glycolytic lactate synthesis (from 1-^{13}C-glucose) is glutamine-dependent, decreasing after intermittent hypoxia to fuel TCA cycle activity (2). Those studies also showed no significant differences in relative growth between 4T1 and 67NR cells in response to glucose (Glc) and/or glutamine (Gln) deprivation (2). Here we used a variety of techniques to further investigate mitochondrial metabolism in these two cell lines, which may help explaining the different phenotypes of the respective tumors *in vivo*.

PURPOSE:.To detect differences in mitochondrial metabolism of 4T1 and 67NR cells, using ^{13}C MR spectroscopy and labeled substrates (Glc and Gln), OCR and specific inhibitors of the mitochondrial respiratory complexes, and the expression of succinate dehydrogenase (SDH-A) by Western Blot.

METHODS: 4T1 and 67NR cells were grown on microcarriers (Plastic Plus, SoloHill) and studied in our MR-compatible cell perfusion system (~1.1 x10^8 cells), as described previously (2). The MR experiments were carried out on a Bruker 500 MHz spectrometer while perfusing cells with different labelled substrates, either 99% 1-^{13}C-Glc or 99% 3-^{13}C-Gln, under aerobic (~21% O_2) and hypoxic conditions (~1% O_2). The metabolic fate of ^{13}C-labeled nutrients was followed under the various environmental conditions by ^{13}C-MRS, while energy metabolism was observed by ^{31}P-MRS. Each experiment was repeated 3 times for each cell line. Spectral analyses of peak areas were carried out with AMARES (jMRUI v4.0); dynamic ^{13}C isotopomer data were used for modeling metabolic fluxes, with a "bonded cumomer" approach (3) consisting of a 3-compartment network adapted for perfusion experiments (4). Cellular mitochondrial function was also assessed in both 4T1 and 67NR cells using an XF96 Analyzer (Sea Horse Bioscience, Billerica, MA) (5) and the specific inhibitors: oligomycin, FCCP, antimycin and rotenone. Expression levels of SDH subunit A (SDH-A, Cell Signaling) were assessed in the two cell lines by Western Blot by comparing to beta-actin (Abcam), essentially as in (1). Statistical analysis were carried out with the paired t-Test (SPSS 15.0) to compare 4T1 vs. 67NR data (significance level: $p<0.05$).

RESULTS:

Dynamic flux analysis of positional C4, C3, C2, and singlet C4s glutamate isotopomers, from cell perfusion studies with 1-^{13}C-Glc (Fig. 1-A), showed 2.5-fold higher TCA cycle activity in 4T1 cells compared to 67NR cells. Relative to TCA cycle activity, 67NR cells revealed 3-fold higher anaplerotic flux (total exchange at the levels of succinyl-CoA, fumarate, and oxaloacetate), 8-fold higher pyruvate carboxylase flux, and 3-fold higher positional enrichment in acetyl-CoA, than 4T1 cells (predicted). 4T1 cells showed 3.6-fold higher synthesis rate of glucose-derived fatty acids than 67NR cells, and did not show accumulation of succinate (Succ) under aerobic conditions. 67NR cells accumulated Succ C2C3, which was also observed during perfusion with 3-^{13}C-Gln (Fig. 1-A), when cells were exposed to oxygen, whereas Succ C2C3 only became visible in 4T1 cells during hypoxia and presence of glucose in the perfusate, i.e. by reductive synthesis (6). 3-^{13}C-Gln-derived aspartate was also detected in 4T1 but essentially not in 67NR cells. Seahorse microplate measurements of OCR (Fig. 1-B) showed that 4T1 cells have up to 3-fold higher mitochondrial function in terms of basal respiration and ATP synthesis, than 67NR cells. All these observations indicate higher mitochondrial metabolism in 4T1 cells and suggest an impairment of TCA cycle in 67NR cells leading to accumulation of Succ. Expression of SDH-A (Fig. 1-C) was slightly higher in 4T1 cells compared to 67NR. Further experiments are underway, including activity studies of SDH, to explain the metabolic differences observed.

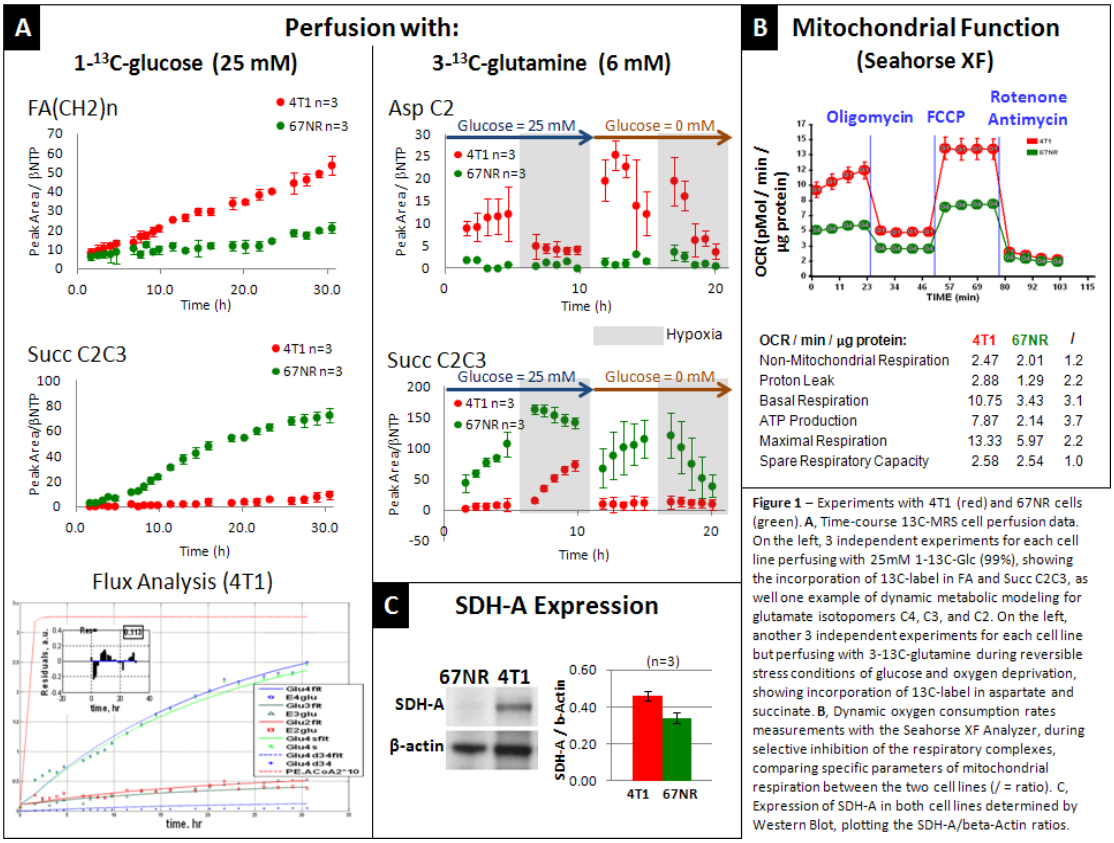

Figure 1 – Experiments with 4T1 (red) and 67NR cells (green). **A**, Time-course 13C-MRS cell perfusion data. On the left, 3 independent experiments for each cell line perfusing with 25mM 1-13C-Glc (99%), showing the incorporation of 13C-label in FA and Succ C2C3, as well one example of dynamic metabolic modeling for glutamate isotopomers C4, C3, and C2. On the left, another 3 independent experiments for each cell line but perfusing with 3-13C-glutamine during reversible stress conditions of glucose and oxygen deprivation, showing incorporation of 13C-label in aspartate and succinate. **B**, Dynamic oxygen consumption rates measurements with the Seahorse XF Analyzer, during selective inhibition of the respiratory complexes, comparing specific parameters of mitochondrial respiration between the two cell lines (/ = ratio). **C**, Expression of SDH-A in both cell lines determined by Western Blot, plotting the SDH-A/beta-Actin ratios.

CONCLUSION: Our results support the association between increased mitochondrial metabolism and metastatic potential, observed recently in breast cancer patients (7).

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