DYNAMIC METABOLIC SIGNATURES OF METASTATIC AND NON-METASTATIC BREAST CANCER CELLS

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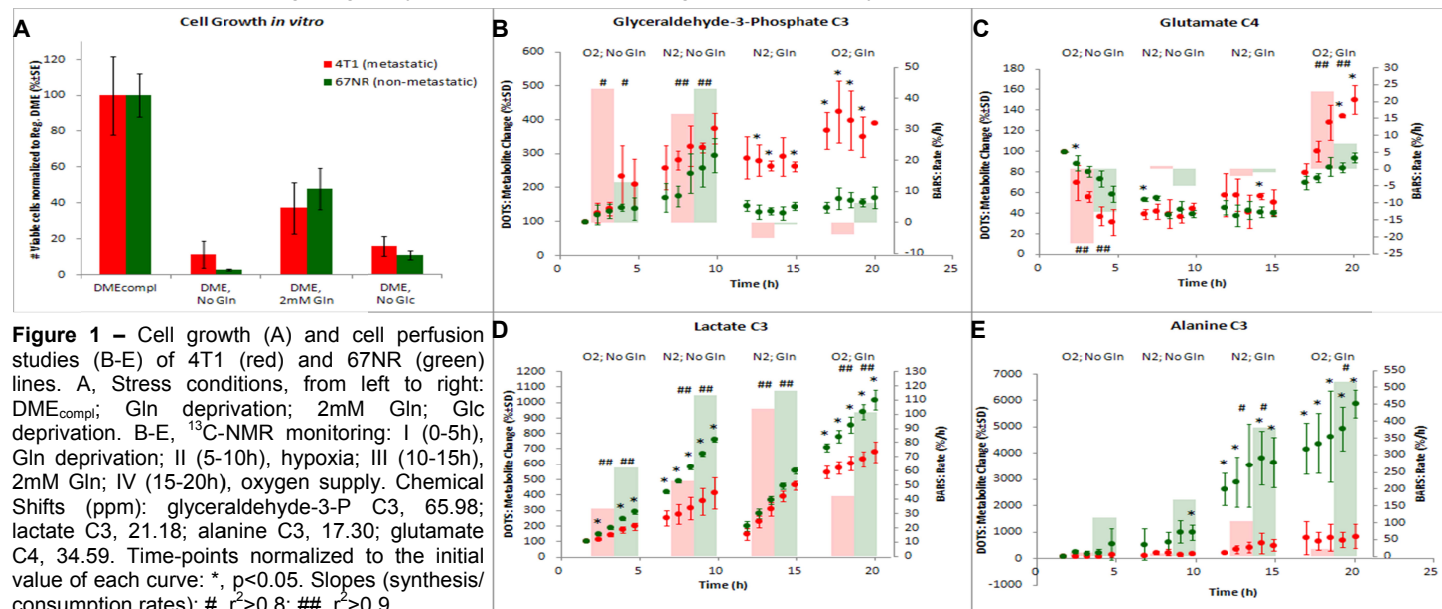
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INTRODUCTION: Distinguishing metastatic from non-metastatic clinical breast cancer, non-invasively, would significantly improve evaluation of patient prognosis. Recent studies with two isogenic murine breast cancer lines, metastatic 4T1 and non-metastatic 67NR, revealed differences at the level of (a) LDH-A expression, in response to changing oxygen tensions *in vitro*, and (b) lactate accumulation, during tumor growth *in vivo* (1). Understanding how each cell line metabolically adapts to dynamic microenvironmental changes, such as oxygen availability vs. hypoxia, and major nutrient supply vs. deprivation (glucose and glutamine (2,3)), could provide unique non-invasive biomarkers of metastatic and non-metastatic breast cancer phenotypes, useful in developing new therapies.

PURPOSE: To study 4T1 and 67NR breast cancer lines in an NMR-compatible cell perfusion system and investigate differences in their dynamic metabolic responses to (a) oxygen availability vs. hypoxia, and (b) glutamine (Gln) supply vs. Gln deprivation.

METHODS: 4T1 and 67NR cells were initially studied *in vitro* to determine the response of cell growth to modified media composition (DME, supplemented with 1% P/S and 10% FBS: DME_{compl}): 1) DME_{compl}, contains 25 mM glucose (Glc) and 6 mM Gln; 2) DME with 2 mM Gln; 3) DME without Gln; 4) DME without Glc. Cells were incubated for 48 h in each medium, harvested, and counted (ViaCount Assay, Guava Technologies). Three independent experiments, with duplicate samples per experiment, were performed for each cell line. Slight modifications were introduced in our cell perfusion system described previously (4) to accommodate its use in a 500 MHz vertical-bore Bruker magnet. For each study, 3.0×10^6 cells were seeded on microcarriers (Plastic Plus, SoloHill) and cultured for 3 days. Cells were then loaded into the perfusion system: a custom-made 10 mm screw-cap tube where culture medium and gas were circulated and the temperature controlled (37 °C) by a heating water jacket. The MR experiments consisted in repeatedly acquiring ^{13}C, ^1H, and ^{31}P spectra (standard Bruker BBO probe) while challenging the perfused cells with different metabolic perturbations. These were carried out in six steps (I – VI, 5 h each): I, DME_{compl} and carbogen gas (95% O_2 / 5% CO_2); II, 100% $^{13}C(1)$-Glc labeled medium, without Gln, and carbogen gas; III, same medium as in II but during hypoxia (95% N_2 / 5% CO_2); IV, still hypoxia but replacing the medium with 100% $^{13}C(1)$-Glc and 2 mM Gln; V, same medium as in IV but shifting the gas back to carbogen; VI, same as in I. Each experiment was repeated 3 times for each cell line. Spectral analysis of peak areas we carried out with AMARES (jMRUI v4.0). Statistical analyses were carried out with the paired t-Test (SPSS 15.0) to compare 4T1 vs. 67NR data (significance level: $p < 0.05$).

RESULTS: As shown in Fig. 1, the *in vitro* cell growth is dependent on the availability of both Glc and Gln, in 4T1 and 67NR lines: cell growth drops by 85-95% in the absence of either Gln or Glc, and remains at 35-45% when Gln alone is decreased by 2/3; no significant differences were observed in growth between the two cell lines in response to each nutrient deprivation. As for dynamic cell perfusion studies, ^{31}P-monitored ATP levels were kept within ±27% of their initial concentration during the experiments (I-VI), in both cell lines, although showing significant differences during stages IV-VI (data not shown). ^1H-visible total choline compounds (3.21 ppm) and mobile lipids (1.29 ppm, mixed contribution with lactate) showed consistent stress-response changes at each stage, similar in both cell lines (data not shown). ^{13}C-labeling studies revealed that 67NR cells are significantly more glycolytic than 4T1 cells in the experimental conditions tested: in 67NR cells, the rate of glucose-derived lactate (Lac) production is 2-fold higher than in 4T1 cells. The glycolytic Lac synthesis during hypoxia is Gln-dependent in 4T1 cells (2-fold higher rate in presence of glutamine vs. deprivation), but not in 67NR cells. Supply of Gln vs. deprivation, generates significantly different dynamic responses between 4T1 and 67NR cells, as far as turnover of glycolytic intermediates, e.g. glyceraldehyde-3-phosphate, alanine synthesis, and TCA cycle activity. In aerobic conditions and presence of Gln, 4T1 cells incorporate more Glc into the TCA cycle (3-fold higher rate of glutamate synthesis) than 67NR cells, whereas the latter show higher glycolytic activity, i.e. 2.4-fold higher rate of Lac synthesis, and also 25-fold faster synthesis of alanine.

Figure 1 – Cell growth (A) and cell perfusion studies (B-E) of 4T1 (red) and 67NR (green) lines. A, Stress conditions, from left to right: DME_{compl}; Gln deprivation; 2mM Gln; Glc deprivation. B-E, ^{13}C-NMR monitoring: I (0-5h), Gln deprivation; II (5-10h), hypoxia; III (10-15h), 2mM Gln; IV (15-20h), oxygen supply. Chemical Shifts (ppm): glyceraldehyde-3-P C3, 65.98; lactate C3, 21.18; alanine C3, 17.30; glutamate C4, 34.59. Time-points normalized to the initial value of each curve: *, $p < 0.05$. Slopes (synthesis/consumption rates): #, $r^2 > 0.8$; ##, $r^2 > 0.9$.

CONCLUSIONS: Although no significant differences in cell growth were observed *in vitro* between 4T1 and 67NR cells, when exposed to defined metabolite stress conditions each cell line adopts different metabolic strategies to cope with said changes, depending on the level of oxygen availability. Mostly, non-metastatic 67NR cells revealed higher glycolytic activity than metastatic 4T1 cells. Unlike in 67NR cells, glycolysis is Gln-dependent in the more aggressive cell line (4T1) during hypoxia, dropping when oxygen becomes available to favor TCA cycle activity. Ongoing $^{13}C(3)$-Gln studies should provide further insight into these metabolic differences.

REFERENCES: (1) Serganova *et al.* Clin. Cancer Res. 2011, 17: 6250-6261; (2) Yuneva et al. Cell Cycle 2008, 7: 2083-2089; (3) DeBerardinis et al. PNAS 2007, 104: 19345-19350; (4) Simões et *al.* Proc ISMRM 2011, p330, a3148.