BBA - Molecular Basis of Disease 1866 (2020) 165886

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

Activation of glycogenolysis and glycolysis in breast cancer stem cell models

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ARTICLE INFO

Keywords:
Cancer stem cell
Glycogenolysis
Glycolysis
Breast cancer
Metabolic reprogramming
HIF1a

The most general metabolic reprogramming observed in tumor cells is the glycolytic switch, which induces cells to change from oxidative phosphorylation (OXPHOS) to increased aerobic glycolysis [1]. Otto Warburg [2], who described this phenomenon in some neoplasms in vivo, hypothesized that this increase is due to mitochondrial dysfunction, which helps cancer cells compensate for energy production [3]. In light of many other discoveries made in the area of bioenergetics and cell metabolism for the last decades, Warburg's hypothesis has been significantly challenged [4]. In particular, enhanced glycolysis was not observed in all tumor types and dependence on OXPHOS was demonstrated in glioma [5], cervical cancer [6] and leukaemia [7]. Another word, the mitochondria of some tumors can be fully functional and at the same time operate at low capacity. In this case, the amount of ATP generated from OXPHOS is sufficient to supply the energy for slowly proliferating or quiescent cancer cells such as cancer stem cells (CSC) [8].

It is generally accepted that a small subset of cancer cells with stem-like properties, known as CSC, is responsible for cancer recurrence and metastasis [9]. These cells differ from the corresponding parental cells and exhibit a different response to the same microenvironmental stimuli, which allows them to reduce proliferating rate and survive chemotherapeutic treatment [10]. In this study, we attempted to identify metabolic differences between cancer cells and correspondingly derived tumorspheres, representing CSC model. To this end, we applied a functional OMICs approach to our previously described models of CSC derived from triple negative breast cancer cell lines BT-549 and MDA-MB-231 [11]. First, we performed quantitative proteomic analyses of these cells and identified differentially expressed proteins in CSC as compared with their parental cancer cells. To determine the characteristics of these differentially expressed proteins, we performed pathway enrichment analyses using Reactome software. The analysis of hierarchically clustered heatmaps revealed association of encoded proteins (rows) with enriched pathways (columns), representing several metabolic clusters (Fig. 1A). When Reactome2016 was applied to rank upregulated pathways of CSC versus parental cells, a “metabolic pathway” was identified on top of others for both cell lines (Supplementary Table 1). However, when various different bioinformatic platforms were used to determine top pathways shared between parental and CSC of both cell lines, glycogenolysis (P = 0.00008583, BioPlanet 2019 and P = 0.00009898, Reactome2016), starch and sucrose metabolism (P = 0.0005864, KEGG 2016), and glycogen synthesis and degradation (P = 0.0007243, WikiPathways 2019) were among the highest scoring pathways (Supplementary Table 2).

Glycogenolysis is the breakdown of glycogen, a branched polymer of glucose residues, while glycolysis is the breakdown of carbohydrates. These two processes are coupled as the glucose 6-phosphate derived

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https://doi.org/10.1016/j.bbadis.2020.165886
Received 8 March 2020; Received in revised form 6 June 2020; Accepted 21 June 2020
Available online 24 June 2020

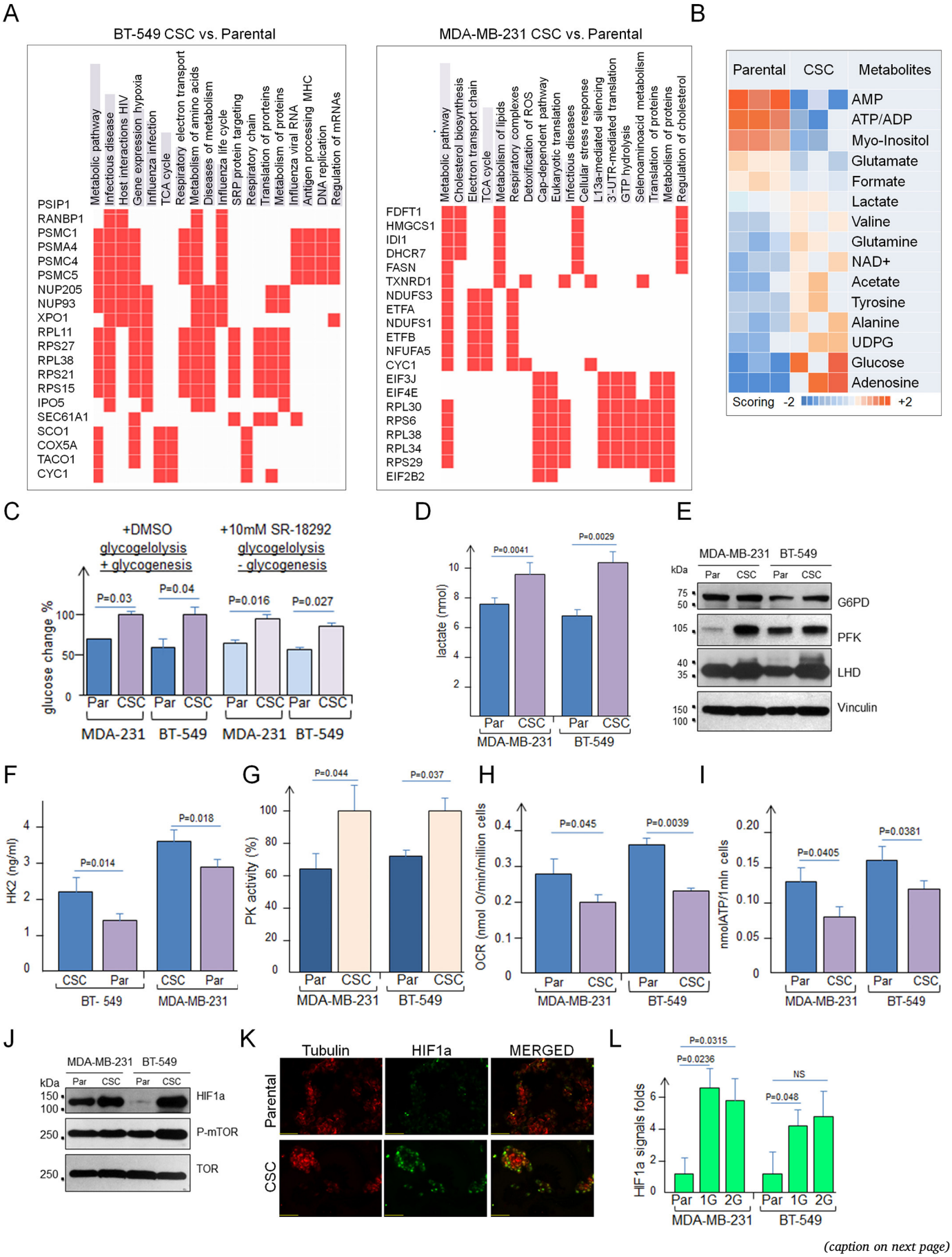

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Fig. 1. Upregulation of glycogenolysis and glycolysis in CSC versus parental breast cancer cells. The human BT-549 and MDA-MB-231 (further called parental) cell lines cultured in Dulbecco's modified Eagle's medium/F12 (Gibco) and correspondingly derived CSC-like cells, cultured in Cancer Stem Cell medium (PromoCell) in non-adherant plates were processed through LC-MS/MS analyses (the data have been deposited to the ProteomeXchange Consortium, PRIDE identifier PXD009442). Clustergrammers obtained with Reactome software indicate association of encoded proteins (rows) with the enriched pathways (columns) in CSC vs. parental cells arranged by the minimal *P*-values (**A**). In parallel, ^{1}H NMR spectra of cellular fractions were recorded. Metabolites with high (red) and low abundance (blue) normalized to the total number of cells are shown (**B**). Glycogenolysis was tested by measuring extracellular glucose levels using glucose assay kit (Abcam) in the absence or presence of SR-18292 inhibitor (10uM, Cayman Chemical) to exclude the influence of glycogenesis (**C**). Secreted lactate in the cell culture supernatants was measured in low serum conditions with a lactate estimation kit (BioVision) (**D**). Western blot analyses of corresponding samples show increased expression levels of glycolytic enzymes (**E**). Concentration of Hexokinase II (HKII) was measured with a spectrophotometer using ELISA Kit (Abcam) in triplicates (**F**). Activity of pyruvate kinase was measured by monitoring fluorescence (Ex/Em 535/587 nm) in microplate assay kit (Abcam) (**G**). Oxygen consumption was determined by the Instech oxymeter. Respiration rates were plotted on the graph after normalization of the polarographic oxygen consumption rates to cell number (**H**). ATP production was measured fluorimetrically according to the enzyme coupling method, following NAD(P)/NAD(*P*)H reduction/oxidation at 340 nm and normalization to the cell number (Promega) (**I**). Representative Western blots show activation of mTOR and HIF1a (***J***). Representative picture of immunofluorescence staining of CSC vs. parental MDA-MB-231 cells revealed increased co-localization HIF1a (red) over abundant tubulin signals (green) and corresponding merged images (orange) (**K**). Red/green signals of 50 cells from G1, G2 CSC generations and parental cells were counted and results were plotted on the graph (**L**). Unless otherwise stated, statistical analyses of data from four replicate trials were carried out by ANOVA and Fisher's protected least significant difference test using the STATVIEW program (Abacus Concepts, Inc.). A probability of $P < 0.05$ was considered to be statistically significant.

from the glycogenolysis is utilized for the first step of the glycolysis [12]. In order to provide in depth understanding between increased glycogenolysis and metabolic pathways, we performed NMR-based metabolomics approach and demonstrated higher levels of AMP, ATP, and other intracellular metabolites in parental cancer cells, in comparison to CSC (Fig. 1B). These results suggest that parental cancer cells could be more dependent on OXPHOS.

We compared the rates of glycogenolysis by measuring extracellular glucose levels as earlier described [13]. To exclude glycogenesis, the indicated levels were adjusted with the levels of glucose accumulated in the presence of gluconeogenesis inhibitor. Altogether, our results revealed enhanced glycogenolysis in CSC of both cell lines in comparison to corresponding parental cells (Fig. 1C). Glycogen synthesis requires an activated form of glucose, uridine diphosphate glucose (UDPG). In turn, glycogenolysis is accompanied by a decline in UDPG, thus corroborating with our NMR data. In parallel, we measured lactate level as described earlier [14]. The observed increase of lactate in CSC might represent upregulation of the glycolytic pathway (Fig. 1D). These results prompted us to test the main metabolic events in CSC in comparison to parental cancer cells.

In order to estimate the impact of glycolysis, expression levels and activities of several glycolysis enzymes have been measured. We demonstrate that glucose-6-phosphate dehydrogenase (G6PDH), phosphofructokinase-1 (PFK) and lactate dehydrogenase (LDH) have increased expression levels in CSC versus parental cancer cells (Fig. 1E). In addition, the activity of key enzymes of anaerobic glycolysis, including hexokinase II (HK2) and pyruvate kinase M2 isoform was measured as previously described [15]. Both enzymes revealed increased activities in CSC as compared to parental cancer cells (Fig. 1F,G). To further investigate the respiratory chain function of cancer mitochondria, oxygen consumption rate (OCR) was measured in accordance with the previously described method [16]. It was found that the OCR was reduced in the CSC, when compared to parental cancer cells (Fig. 1H). The efficiency of OXPHOS was tested by ATP levels. We observed that ATP production in CSC was low compared to ATP generated in parental cancer cells (Fig. 1I). These results suggest that glycolytic pathway is preferable for CSC. From the previous studies we learned that a switch from mitochondrial respiration to glycolysis decreases the levels of ROS in breast cancer cell lines [14]. This could represent an essential mechanism for the maintenance of stemness. The compact morphology of CSC-like cells implies a lack of oxygen which in turn can cause a hypoxic environment inside the mammospheres. Numerous observations suggest that, with limited oxygen availability, Hypoxia-Inducible Factor (HIF)-1 regulatory pathway can play a role in maintaining stemness. To explore such possibility, we studied expression levels of HIF1a and its close co-activator, the mechanistic Target of Rapamycin (mTOR) coordinating eukaryotic cell growth and metabolism with environmental inputs. Indeed, we observed that CSC have higher expression levels of HIF1a and concomitant activation of mTOR pathway (Fig. 1J). Furthermore, we created CSC of the first (G1) and second (G) generations to compare the expression of HIF-1a in tumorspheres and parental cancer cells using the immunofluorescence approach (Fig. 1K). Our results indicate accumulation of HIF1a signals in CSC of both generations suggesting a connection between hypoxia and the cancer stemness (Fig. 1L).

In summary, our data provide compelling evidence that although the metabolic activity of CSC and parental cancer cells involve both OXPHOS and glycolysis, CSC-like cells are more prone to the glycolytic pathway. A simplified model describing how lower levels of oxygen or nutrient deprivation in CSC may activate HIF pathway is provided in the graphical abstract. Under hypoxic conditions, mitochondria cannot fully rely on the OXPHOS and cells obtain energy through glycolysis, which is partially provided by glycogenolysis. These observations corroborate well with some recent data on altered glucose homeostasis with high rates of glycogen metabolism and glycogenolysis in glioma CSC [17]. In addition, our results may provide some ground to explain the difference observed in “liquid cancers” with normoxic microenvironment and in solid tumors, where hypoxia inside tumor lump can provoke glycolytic switch for a subset of CSC. Moreover, these data may have pharmacological implications and suggest the use and/or development of certain drugs targeting the early stages of glycolysis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2020.165886.

CRediT authorship contribution statement

Etna Abad: Investigation, Writing - original draft, Formal analysis, Writing - review & editing, Validation.**Sara Samino:**Investigation, Writing - review & editing, Validation.**Oscar Yanes:**Investigation, Writing - review & editing, Validation.**David Potesil:**Investigation, Writing - review & editing, Validation.**Zbynek Zdrahal:**Investigation, Writing - review & editing, Validation.**Alex Lyakhovich:**Investigation, Methodology, Writing - original draft, Formal analysis, Funding acquisition, Writing - review & editing, Validation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We are grateful to Drs. Trond Aasen and Ana Janic for assisting with cell line authentication. This work was supported by grants from the

Instituto de Salud Carlos III: PI17/02087 (AL). The proteomic part of the work was supported by the project CEITEC 2020 (LQ1601) from MEYS CR. CIISB research infrastructure project LM2018127 funded by MEYS CR is gratefully acknowledged for the financial support of the LC-MS/MS measurements at the CEITEC Proteomic Core Facility. Computational resources for processing of proteomics data were supplied by the project "e-Infrastruktura CZ" (e-INFRA LM2018140) provided within the program Projects of Large Research, Development and Innovations Infrastructures, Czech Republic.

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