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Comparative analysis of the bioenergetics of human adenocarcinoma Caco-2 cell line and postoperative tissue samples from colorectal cancer patients

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Abstract

The aim of this work was to explore the key bioenergetic properties attributed to the mitochondrial respiration in widely used Caco-2 cell line and human colorectal cancer (HCC) postoperational tissue samples. Oxygraphy and Metabolic Control Analysis (MCA) were applied to estimate the function of oxidative phosphorylation in cultured Caco-2 cells and HCC tissue samples. The mitochondria of Caco-2 cells and HCC tissues displayed larger functional activity of respiratory complex (C)II compared to CI, whereas in normal colon tissue an inverse pattern in the ratio of CI to CII activity was observed. MCA showed that the respiration in Caco-2 and HCC tissue cells is regulated by different parts of electron transport chain. In HCC tissue, this control is performed essentially at the level of respiratory chain complexes I-IV, whereas in Caco-2 cells at the level of CIV (cytochrome c oxidase) and ATP synthasome. The revealed differences in the regulation of respiratory chain activity and glycolysis index could represent an adaptive response to distinct growth conditions; this means the importance of proper validation of results obtained from *in vitro* models before their extrapolation to the more complex *in vivo* systems.

Keywords: colorectal cancer, mitochondrial metabolism, OXPHOS, metabolic control analysis.

Abbreviations: ANT, adenine nucleotide translocator; BSA, bovine serum albumin; C, complex; CAT, carboxyatractylide; CSC, cancer stem cells; CM, cardiomyocyte; CS, citrate synthase; Cyt-c, cytochrome-c; FCC, flux control coefficient; HCC, human colorectal cancer; HK, hexokinase; Km, Michaelis-Menten constant; MCA, metabolic control analysis; MOM, mitochondrial outer membrane; uMtCK, ubiquitous mitochondrial creatine kinase; OXPHOS, oxidative phosphorylation; PIC, inorganic phosphate carrier; ROS, reactive oxygen species; SC, supercomplex; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; VDAC, voltage dependent anion channel; Vo and Vm, rates of basal and maximal respiration.

Introduction

Human colorectal cancer (HCC) is one of the main causes of cancer-related mortality worldwide (Jemal et al. 2011). It is a very aggressive neoplasm with high metastatic potential (Vatandoust et al. 2015) and drug resistance (Hu et al. 2016). Despite significant progress in understanding the pathogenesis of HCC, some very important aspects of cancer biology and metabolism remain still poorly understood.

Although the reprogramming of cellular energy metabolism has been widely recognized as an intrinsic hallmark of cancer (Hanahan and Weinberg 2011), there are conflicting data in the literature regarding the metabolic characteristics of cancer cells. Since the first observation of Otto Warburg that cancer cells metabolize glucose to lactate at high rates even in the presence of oxygen (Warburg et al. 1927), the metabolic phenotype of cancer has been considered as mainly

glycolytic. However, various recent studies have shown that many types of tumor cells, instead, depend on oxidative phosphorylation (OXPHOS) as a source of ATP (Moreno-Sanchez et al. 2014; Pasto et al. 2014). Furthermore, it was reported that some tumor cells are characterized by stimulated mitochondrial biogenesis (LeBleu et al. 2014; Luo et al. 2016) and that they can import these organelles or mtDNA from neighboring normal tissue cells (Berridge et al. 2016; Tan et al. 2015). Tumor cells can also shift the metabolism of surrounding stromal cells toward aerobic glycolysis to use metabolic substrates donated by these cells (L-lactate, ketone bodies, and L-glutamine) for cellular energy generation *via* OXPHOS (Martinez-Outschoorn et al. 2014; Pavlides et al. 2009; Whitaker-Menezes et al. 2011). We have previously reported that HCC tissue exhibited obvious signs of stimulated mitochondrial biogenesis (Chekulayev et al. 2015) and it was recently proposed that suppression of mitochondrial function may serve as an efficient strategy for the treatment of this type of cancer (Zhang et al. 2014).

The insufficient elucidation of HCC pathogenesis and its metabolic features is probably due to the limited access to patient samples and the lack of reliable model organisms. Therefore, HCC is usually modeled by means of established cell lines. However, it remains unknown to what extent their metabolic and bioenergetic profile corresponds to that in the primary tumor. *In vitro* studies are also complicated by complex intratumoral heterogeneity (Fessler and Medema 2016) and genetic instability of cancer cells (Vargas-Rondon et al. 2017). *In vitro* research with HCC cell lines often do not take into account the possible impact of (micro) environmental conditions on the tumor gene expression profile and its metabolism. Traditionally, the phenomenon of tumor heterogeneity links with a diverse profile of oncogenic aberrations. However, now there is growing evidence that certain very aggressive tumor cells may arise as a result of adaptation to the unfavorable microenvironment caused by abnormal tumor vascularization triggering corresponding metabolic switches (Eason and Sadanandam 2016; Fluegen et al. 2017). It was also shown that certain stromal cells (fibroblasts) can drive the oncogenic phenotype of colon cancer (Calon et al. 2015).

The main aim of our work was therefore to provide a comprehensive characterization of the human colon cancer derived Caco-2 cell line growing under standard conditions with respect to their phenotype and bioenergetic function of mitochondria – the main source of ATP in HCC tissue (Chekulayev et al. 2015). Oxygraphy together with metabolic control analysis (MCA) were applied to characterize the function of OXPHOS system in cultured Caco-2 cells and HCC tissue samples. By means of MCA, we quantified the control of mitochondrial respiration exerted by different components of the respiratory chain. Although we identified profound alterations in the regulation of the respiratory chain in Caco-2 cells, the basic respiratory properties were very similar between cultured cells and primary tumor. As a whole, our results provide evidence for the upregulation of oxidative mitochondrial metabolism in HCC cells *in vitro* and *ex vivo*. The

side of glycolytic energy production is characterized by glucose index for Caco-2 cells, tumor and control tissues, as well as by hexokinase binding to the VDAC channel.

Materials and methods

Reagents

Eagle's minimum essential medium (MEM) with stable L-glutamine and low glucose was obtained from Corning (REF: 10-010-CVR), 0.05% Trypsin-EDTA, accutase, heat-inactivated fetal bovine serum (FBS), and antibiotics (penicillin, streptomycin and gentamicin) were purchased from Gibco Life Technologies (Grand Island, NY). Primary and secondary antibodies were obtained from Santa Cruz Biotechnology Inc. or Abcam PLC. Unless otherwise stated, all other chemicals were purchased from Sigma-Aldrich Company (St. Louis, USA).

Cell culture

Stock culture of Caco-2 cells was obtained from the American Type Culture Collection; HTB-37™. These human colorectal adenocarcinoma cells were grown in T75 flasks (Greiner bio-one) in MEM containing 20% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml gentamicin at 37 °C in a humidified incubator supplied with 5% CO₂. Cells were passaged every 72 hours by mild trypsinization. At the day of experiments (3-4 days after seeding) Caco-2 cells were harvested by accutase treatment followed by centrifugation at 150 g for 7 min. The cell pellet was resuspended in serum-free medium-B (pH 7.1) consisting of 20 mM Hepes buffer, 3 mM KH₂PO₄, 0.5 mM DTT, 20 mM taurine, 3 mM MgCl₂, 0.5 mM EGTA, 110 mM sucrose, 60 mM K-lactobionate, 2 mg/ml fatty acids free bovine serum albumin (BSA) supplemented with 5 µM leupeptin (a protease inhibitor) and stored on melting ice. The viability of the cells using trypan blue exclusion was never less than 95%.

Clinical material, patients, preparation of tissue samples and their permeabilization

HCC tissue samples (0.1 – 0.5 g) were withdrawn during surgery at the Oncology and Hematologic Clinic of the North Estonia Medical Centre, Tallinn. All patients (n = 64, with ages ranging from 63 to 92 years) had local or locally advanced disease (T2-4 N0-1, M0-1) and only primary neoplasms were examined. The patients in the study had not received prior radiation or chemotherapy. All patients provided written informed consent. Our study was approved by the Medical Research Ethics Committee (National Institute for Health Development, Tallinn) and it was in full accordance with Helsinki Declaration and Convention of the Council of Europe on Human Rights and Biomedicine. Normal colon tissue samples were controlled for the presence of malignant cells; they were normal according to histopathology and cytochemical studies (Chekulayev et al. 2015). Immediately after the surgery, the excised tissues were placed into pre-cooled medium-A. Before oxygraphy, tumor and normal tissue samples were additionally

dissected into small pieces (10-20 mg) and permeabilized in the same medium with 50 µg/ml saponin upon mild stirring for 30 min at 4 °C (Kuznetsov et al. 2008). This permeabilization method allows to study the function of mitochondria *in situ* under nearly physiological conditions and without damaging the organelles (Kuznetsov et al. 2008). The concentrations of saponin used in our studies were specially tested in appropriate preliminary experiments to ensure maximal rates of ADP stimulated respiration and intactness of these organelles.

Assessment of the mitochondrial respiration in permeabilized Caco-2 cells and tumor-derived tissue samples

Rates of O₂ consumption by Caco-2 cells (at a density of ~ 0.5 - 1.0 × 10⁶ cells/ml) and tissue samples were measured as described previously (Gnaiger 2001; Kuznetsov et al. 2008). Saponin concentration of 40 µg/ml for Caco-2 cells was used. All respiration rates were normalized per mg of cell protein or dry weight of tissue.

Determination of apparent Michaelis-Menten constant values for exogenously added ADP and rates of maximal respiration

The apparent Michaelis-Menten constants (K_m) for ADP and maximal rates (V_m) of ADP-activated respiration were calculated by fitting experimental data to a non-linear regression equation.

Metabolic control analysis (MCA) and measurement of flux control coefficients

MCA was applied to characterize the function of OXPHOS system in Caco-2 tumor cells compared to tissue samples from HCC patients. We quantified the control exerted by different components of the respiratory chain and the ATP synthasome complex in these cells by measuring the corresponding flux control coefficient (FCC). This was carried out as described in our previous studies (Kaldma et al. 2014; Koit et al. 2017).

Immunofluorescence analysis for the presence of mitochondrial VDAC and hexokinase-2 in Caco-2 cells

Immunocytochemistry along with confocal microscopy imaging were applied to visualize the expression and colocalization of VDAC and hexokinase-2 (HK2) in Caco-2 cells. For microscopy, these cells were seeded in 12-well plates (Greiner bio-one, at a density of 1.0 × 10⁵ cells/well) on glass chamber slides and were allowed to growth. Two days later, the cells were washed once with preheated PBS and then fixed with 4% paraformaldehyde (PFA) at 25 °C for 10 min. Further, Caco-2 cells were washed with PBS, treated with an antigen retrieval buffer at 95 °C for 3 min, permeabilized with 0.1% Triton X-100 for 15 min at room temperature (RT), blocked with 2% BSA dissolved in PBS for 1 hour at RT, and the following primary antibodies were added: rabbit polyclonal antibodies vs. human VDAC1/2/3 (sc-98708; Santa Cruz Biotechnology, Inc., USA) and mouse monoclonal antibodies against human HK2 (sc-374091);

their dilution factors were 1/100. After overnight incubation (at 4 °C) with the indicated primary antibodies, Caco-2 cells were washed with a 2% BSA solution and co-incubated (for 2 hours at RT) with secondary fluorescent antibodies. After that, coverslips with stained Caco-2 cells were placed over glass microscope slides with ProLong Gold antifade reagent supplemented with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Molecular Probes™) for visualizing the cell nucleus. These cells were then imaged by an Olympus FluoView FV10i-W inverted laser scanning confocal microscope equipped with a 60 x objective.

Immunofluorescent staining of HCC and surrounding normal tissue samples for the presence of VDAC and hexokinase-II

Immunofluorescent analysis along with confocal microscopy was applied to estimate the presence and degree of HK2 co-localization with mitochondrial VDAC in HCC and adjacent healthy tissue samples. Methodologically, this was carried out using paraffin-embedded tissue sections exactly as described earlier (Kaldma et al. 2014).

Assessment of the coupling of HK with OXPHOS in Caco-2 cells

The coupling of HK-catalyzed processes with the OXPHOS system in permeabilized Caco-2 cells, tumor and non-tumorous tissue samples was assayed by oxygraphy through stimulation of mitochondrial respiration by locally-generated ADP (Chekulayev et al. 2015). This effect of glucose on mitochondrial respiration was expressed by glucose index (I_{GLU}) that was calculated according to the equation: $I_{GLU} (\%) = [(V_{GLU} - V_{ATP}) / (V_{ADP} - V_{ATP})] * 100$. Glucose index reflects the degree where glucose-mediated stimulation of mitochondrial respiration is compared to maximal ADP-activated rates of O_2 consumption.

Statistical analysis of data

Data in the text, tables and figures are presented as means $\pm$ standard error (SE) from at least five separate experiments. Significance was calculated by Student's t-test and differences between two data groups were considered statistically significant when $p < 0.05$. Apparent K_m values for ADP were estimated by fitting experimental data to a non-linear regression according to a Michaelis-Menten model equation.

Results

Mitochondrial content and distribution

Firstly, we analysed the content of mitochondria and their profile of intracellular distribution in Caco-2 cells, HCC and non-tumorous tissue samples. The intracellular localization of mitochondria was visualized by immunostaining with specific antibodies against the VDAC channel, while the total mitochondrial content was assessed by measurement of CS activity.

Immunocytochemical studies along with confocal microscopy imaging showed that Caco-2 cells, like HCC tissue cells, contain a large number of mitochondria. In Caco-2 cells these organelles were predominantly located around the nucleus (**Fig. 1A**). Measurement of CS activity showed that Caco-2 cells had the highest content of mitochondria from the investigated material. The value of their CS activity (106 ± 4 mU/mg protein) had similar values with HCC tissue (83 ± 19 mU/mg protein) but exceeded nearly 2 times compared to normal intestinal tissue samples (**Fig. 2**). These observations allow the presumption that cultured undifferentiated Caco-2 cells, similarly to HCC tissue cells, are characterized by stimulated mitochondrial biogenesis.

Respiratory properties of HCC tissues and Caco-2 cells

To test whether cultured Caco-2 cells display similar respiratory characteristics as tissue samples, we measured the rates of oxygen consumption by cells and tissues in the presence of various OXPHOS substrates and inhibitors (**Table 1, Fig. 3**). It can be seen that the tumor samples and Caco-2 cells exhibited higher rates of oxygen consumption compared to control tissues. The addition of 2 mM MgADP notably activated mitochondrial respiration over basal level in all studied samples indicating the presence of functionally active mitochondria (**Table 1**). Addition of rotenone, an inhibitor of CI to Caco-2 cells as well as to HCC and non-tumorous tissue samples resulted in a ~2 times decrease in the rate of maximal ADP-activated respiration, showing that CI of the mitochondrial respiratory chain is functionally-active both in cultured Caco-2 and HCC tissue cells. The following addition of 10 mM succinate shows the presence of active CII. Antimycin suppressed the mitochondrial respiration of all samples, whereas the addition of 1 mM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) with 5 mM ascorbic acid led to an increase in the rates of O₂ consumption confirming the functionally active complexes III and IV. The increased respiratory capacity of tumor tissues and Caco-2 cells compared to normal tissue correlated well with the mitochondrial mass (**Fig. 2**). Altogether, our results showed that the mitochondria preserved their functional properties in both primary tumor and cultured Caco-2 cells despite the occurrence of malignant transformation. The reduced $V_{\text{Glut}}/V_{\text{Suc}}$ ratio in tumor tissue (**Fig. 4**) indicated a relative suppression of the CI-linked respiration. Interestingly, Caco-2 cells displayed even more suppressed $V_{\text{Glut}}/V_{\text{Suc}}$ ratio suggesting CI deficiency to be a common feature of HCC.

Alteration in the control of mitochondrial respiration by outer mitochondrial membrane in cultured Caco-2, HCC and normal colon tissue cells

The VDAC is involved in the transport of ATP, ADP, pyruvate, malate, and other metabolites, and interacts extensively with enzymes from different metabolic pathways (Blachly-Dyson and Forte 2001; Granville and Gottlieb 2003). The ATP-dependent cytosolic enzymes HK, glucokinase, glycerol kinase, as well as the ubiquitous mitochondrial creatine kinase (uMtCK),

have been found to bind to VDAC (Colombini 2004; Pastorino and Hoek 2008). In malignant cells ATP and ADP can diffuse across the VDAC more easily than in normal highly-differentiated cells. Mitochondria of rat CMs have a low affinity for ADP (the apparent $K_m = 297 \pm 35 \mu\text{M}$) whereas for HL-1 cardiac tumor cells this K_m value was significantly lower ($K_m = 25 \pm 4 \mu\text{M}$) (**Table 2**) (Monge et al. 2009). The K_m^{app} (ADP) for HCC tissue cells was measured to $93.6 \pm 7.7 \mu\text{M}$, while normal intestinal tissue cells displayed a low affinity for exogenously added ADP ($K_m^{\text{app}} = 256 \pm 34 \mu\text{M}$) (**Table 2**). These results show that the mechanisms of regulation of the MOM permeability in HCC cells *in vivo* differ from that in healthy colon tissue cells. In the present research we determined to what extent the regulation of mitochondrial respiration in HCC tissues corresponds to cultured Caco-2 cells. For this aim, we measured the apparent K_m value for exogenous ADP for these model cells after the selective plasma membrane permeabilization with saponin. Experiments showed that mitochondria of Caco-2 cells and HCC tissue have an increased affinity for adenine nucleotides as compared to normal intestinal tissue cells; the corresponding K_m^{app} (ADP) values were measured as $39.2 \pm 5.7 \mu\text{M}$ for Caco-2 cells, $93.6 \pm 7.7 \mu\text{M}$ for HCC tissue and $256 \pm 34 \mu\text{M}$ for normal tissue samples. These results show that the permeability of MOM in Caco-2 cells is in the same order of magnitude compared to primary human colorectal tumors.

Coupling of OXPHOS with HK-catalyzed reactions

Immunostaining of paraffin-embedded tissue sections showed that HCC cells contain VDAC-bound HK2 (**Fig. 1**). We have recently demonstrated that the mitochondrial VDAC, a binding partner for HK2, was overexpressed in HCC tissue. An increased binding of hexokinase to mitochondria may be responsible for elevated rates of aerobic glycolysis in HCC cells (Chekulayev et al. 2015). The presence of mitochondrially-bound HK2 was also revealed in cultured Caco-2 cells (**Fig. 1**). In our work, the degree of HK2 colocalization with mitochondrial VDAC was expressed through the Pearson's correlation coefficient (PC). The values of the PC were assayed as 0.56 ± 0.03 for Caco-2 cells, 0.71 ± 0.03 for HCC, and 0.66 ± 0.03 for unaffected tissue cells (**Fig. 1B**).

The high-resolution respirometry was applied to evaluate the coupling between HK and OXPHOS in cultured Caco-2 cells (**Fig. 5A**). Similar experiments were also carried out with HCC and normal tissue samples. We found that the stimulatory effect of glucose on mitochondrial respiration in HCC tissue slightly exceeded the values of adjacent normal tissue samples (**Fig. 5B**) (Chekulayev et al. 2015). This suggests that HCC cells might have stronger inclination to aerobic glycolysis as compared to normal intestinal cells. This result is also supported by Hirayama et al. who revealed low glucose, high lactate and other glycolytic intermediate concentrations in colon malignancies (Hirayama et al. 2009). In our studies, the strength of glucose effect was expressed by means of glucose index (I_{GLU}) that reflects the degree

of glucose effect relative to the maximal rate of ADP-activated respiration (in the presence of 2 mM ADP). Cultured Caco-2 cells displayed the highest value of I_{GLU} (48.2%) in comparison with 19.1% for HCC and 11.7% for normal tissue samples (**Fig. 5B**).

MCA of the function of OXPHOS system in HCC tissue and cultured Caco-2 cells

Our previous work demonstrated that the respiratory chain is regulated differently in breast and HCC tissues (Koitz et al. 2017). To control whether cultured tumor cells display the similar pattern of respiratory chain regulation as primary tumor, we exposed Caco-2 cells to MCA. **Figure 6** illustrates the MCA workflow. This quantification showed that there are relatively large differences between Caco-2 cells, HCC and normal intestinal tissue cells. We found that in HCC tissue the OXPHOS is (Kaambre et al. 2013) controlled essentially at the level of respiratory chain, whereas in normal colon tissue cells this control is carried out predominantly at the level of adenine nucleotide translocator (ANT) and phosphate carrier (**Table 3**). In Caco-2 cells, the CIV (cytochrome c oxidase) seems to play a decisive role in control of mitochondrial respiration, since the value of FCC for this complex (1.57) prevailed significantly over other respiratory chain complexes, as well as the ATP-synthase components. Our MCA studies show that the complexes I and especially IV share larger control over respiration in Caco-2 cells than in HCC tissue (**Table 3**). The sum of FCCs calculated for Caco-2 cells, HCC and normal tissue samples (both for NADH and succinate dependent respiration) were found to exceed significantly the theoretical value, which is estimated to be 1 in the linear systems (Kholodenko and Westerhoff 1993). Caco-2 cells exhibited the highest sum of FCCs ($\Sigma = 3.72$), HCC ($\Sigma = 2.51$) and normal intestinal tissue cells the sum of FCCs = 3.35. Altogether our MCA studies indicate that the control of mitochondrial respiration in Caco-2 cells differs compared to HCC tissue and also in normal intestinal cells.

Discussion

Many years of intensive studies have shown that tumor metabolism is significantly more complicated than just a mitochondrial dysfunction accompanied by elevated glycolysis as was previously thought. Malignant transformation causes profound alterations in numerous metabolic pathways including ATP synthesis, lipogenesis and nucleotide synthesis (Furuta et al. 2010; Vander Heiden 2011). Consequently, the metabolic features of tumors might serve as a promising target for selective anticancer therapy (Martinez-Outschoorn et al. 2017; Weinberg and Chandel 2015). However, the cellular heterogeneity of tumors represents one of the greatest challenges in cancer research. The utilization of cancer cell lines allows the analysis of homogeneous population of cells providing an important tool for studying cancer cell biology. Nevertheless, it should be emphasized that the energy metabolism *in vitro* may differ cardinally from that in primary tumors leading thereby to the deceptive conclusions. To resolve this issue, we performed a comparative bioenergetic analysis of the HCC postoperative material and cultured Caco-2 cells.

The inclination of cancer cells to aerobic glycolysis is often erroneously misinterpreted as an evidence for impaired mitochondrial function. In fact, functional respiratory activity persists in various cancers of the Warburg phenotype (Bonuccelli et al. 2010; Jia et al. 2018; Lim et al. 2011). Moreover, it has been shown that some types of cancer cells require stimulated mitochondrial biogenesis for tumor growth and development (Tan et al. 2015). Our current study demonstrated that cultured Caco-2 cells as well as HCC tissues exhibit characteristics of stimulated mitochondrial biogenesis that has been verified by increased CS activity and higher respiration rates compared to control tissues (**Fig. 2 and Table 1**). In addition, CII activity in HCC tissue and Caco-2 cells predominated over CI activity (**Fig. 4**). This relative deficiency in the respiratory CI activity with improved adenylate control over succinate-dependent respiration was also observed in cancer human gastric corpus mucosa undergoing transition from normal to cancer state and in human gastric cancer cell lines (Puurand et al. 2012). Further studies are needed to elucidate the relation of possible alterations in the fine structure of CI with its activity in HCC cells as compared to untransformed cells. Dysfunction of CI in mitochondria of HCC could be responsible for elevated production of ROS by cancer cells (Inokuma et al. 2009). It is well known that ROS signaling can promote cell proliferation, invasion and survival in many human cancers, including HCC (Inokuma et al. 2009; Sabharwal and Schumacker 2014). Altogether, the revealed properties of the mitochondrial respiration in Caco-2 cells were very similar to those observed in primary human colorectal tumor.

At the same time, both cultured cells and HCC tissues displayed relatively low apparent K_m values for exogenously added ADP resembling glycolytic tissues which also have a higher affinity for ADP (e.g., white gastrocnemius muscle cells) (Kaambre et al. 2012). Very low apparent K_m values for ADP, as compared to non-transformed cells, were also registered for HL-1 cardiac sarcoma cells (Monge et al. 2009), human breast cancer and neuroblastoma cell lines (Kaambre et al. 2012; Klepinin et al. 2014). The high affinity for ADP may be a common feature of malignant tumors and, possibly, some normal cells with a high rate of glycolysis (**Table 2**). Thus, Caco-2 cells seem to be a very attractive and simple model system for understanding the mechanism of this phenomenon which has still remained unclear.

By means of MCA it is possible to elucidate valuable information about the controlling and regulatory mechanisms of cancer cell metabolism. MCA was previously applied to investigate the control of glycolytic flux and mitochondrial respiration in different types of normal and malignant cells growing both *in vitro* and *in vivo* (Cortassa et al. 2011; Kaambre et al. 2013; Marin-Hernandez et al. 2006; Moreno-Sanchez et al. 2010; Rossignol et al. 2000). It was recognized that the process of mitochondrial respiration is controlled differently depending on the histological type of tissue (Rossignol et al. 2000; Varikmaa et al. 2014). In the present study, we showed that the control of mitochondrial respiration in cultured Caco-2 cells does not fully meet that in HCC tissue and differs also from normal colon tissue (**Table 3**). In healthy intestinal tissue

this control is performed essentially at the level of ATP synthasome, whereas in HCC tissue at the level of respiratory chain complexes (**Table 3**). Our observations are in a good agreement with the data from other research groups confirming, that the respiratory chain complexes exert significantly higher flux-control on OXPHOS in cancer cells than in normal cells (Moreno-Sanchez et al. 2014). In Caco-2 cells, the control over respiration was divided between CI, CIV and ATP-synthase (**Table 3**). A high sum of FCCs exceeding one may indicate the presence of respiratory supercomplexes (SC) in Caco-2 cells and colon tissues (Genova and Lenaz 2014; Koit et al. 2017). However, the question about the composition and stoichiometry of protein supercomplexes needs further studies. Recently, this phenomenon was registered for malignant tumors of another histological type – breast cancer (Koit et al. 2017; Rohlenova et al. 2017). Furthermore, these researchers proposed the selective disruption of respiratory SCs as a new strategy to suppress the growth of some human breast cancer subtypes. Nevertheless, we also cannot exclude other possibilities for the high total FCC values, such as the direct channeling of substrates between the protein complexes (Kholodenko and Westerhoff 1993) or reverse electron flow (Lambert et al. 2008; Schonfeld and Wojtczak 2007; Scialo et al. 2016).

Distinct environmental conditions may be responsible for the differences in the regulation of respiratory chain in Caco-2 cells and HCC tissues. While *in vivo* tumor cells must compete for metabolites and growth factors, typical growth mediums provide cells with all nutrients required for an optimal growth. However, the composition of growth medium may influence the gene expression profile, thus modulating cell morphology, proliferation and differentiation (Circu et al. 2017; Danhier et al. 2017; Sambuy et al. 2005). It has been shown that acidification of the growth medium and hypoxia may disrupt respiratory supercomplexes leading to ROS generation (Enriquez and Lenaz 2014; Ramirez-Aguilar et al. 2011). ROS, depending on their concentration, may act as toxic agents or essential signal molecules, promoting tumor progression and metastasis (Genova and Lenaz 2015; Guo et al. 2016). The *in vitro* clonal evolution of cell lines might also explain differences between Caco-2 cells and HCC tumor. The establishment of cell lines leads to the inevitable selection of rapidly proliferating and poorly differentiated cells (van Staveren et al. 2009). It is therefore assumed that the cancer cell lines could originate from a selection of cancer stem cells (CSCs) (van Staveren et al. 2009). Indeed, Caco-2 cells were shown to be especially enriched in CSCs (Ferrandina et al. 2009; Gemei et al. 2013; Haraguchi et al. 2008; Wu and Wu 2009). It was demonstrated that CSCs are responsible for HCC recurrence, distant metastasis and chemoresistance – a main barrier to more effective antitumor therapy (Ong et al. 2010; Pang et al. 2010; Ricci-Vitiani et al. 2008; Zuo-Yi et al. 2016). Studies showed that CSCs have a different metabolic profile. Depending on the cancer type, they may be highly glycolytic or OXPHOS dependent, displaying extremely low (Grazia Cipolleschi et al. 2014) or relatively high (Farnie et al. 2015) levels of mitochondria. In any case, the mitochondria seem to play a central role in the functionality and dissemination of CSCs (Borriello and Della Ragione

2017; Sancho et al. 2016). Interestingly, the high regulatory role of CIV, which was demonstrated in Caco-2 cells (**Table 3**), was also detected in normal embryonic and embryonal carcinoma stem cells (Ounpuu et al. 2017), suggesting the probable involvement of this complex in stemness maintenance. Considering that the survival rates of patients with HCC were negatively correlated with respiratory capacities of corresponding tumor samples (Koit et al. 2017), it can be assumed that the ability of cancer samples to consume oxygen might serve as an important determinant of the HCC aggressiveness. Our experiments on Caco-2 cells support an idea that the colon CSCs exhibit a high mitochondrial content and high rate of OXPHOS. In this regard, we propose that suppression of mitochondrial biogenesis or OXPHOS in colon cancer may be a new strategy for HCC treatment.

Conclusion

Altogether, our data show that cultured Caco-2 cells display similar bioenergetic characteristics as compared to HCC tissue samples. Caco-2 cells, like primary tumors, are characterized by increased respiration rates, decreased O₂ flux through CI and the remodeling of intracellular processes involved in the regulation of MOM permeability to adenine nucleotides. However, we also found that the respiratory chain might be regulated distinctly in Caco-2 cells, tumor and control tissues, difference were also occurring between the ratio of glycolysis and OXPHOS. The variations in their energy homeostasis could represent an adaptive response of tumor cells and tissues to the surrounding microenvironment. Nevertheless, taking into account the correlation between increased O₂ consumption and tumor progression, we suppose that the suppression of mitochondrial biogenesis may be a promising target for colorectal cancer therapy. In this relation, Caco-2 cells can serve as a model for development of new energy metabolism related drug candidates. Further work is needed to clarify the mechanisms of stimulated mitochondrial biogenesis and organization of respiratory chain in HCC.

Conflict of interests

The authors declare no conflict of interest.

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Figure legends

Figure 1. HCC tissues and Caco-2 cells display elevated levels of mitochondria compared to healthy tissue. **(A)** Confocal microscopy images of immunofluorescence staining of paraffin-embedded sections of HCC, surrounding non-tumorous tissue, and Caco-2 cells for the presence of mitochondrial VDAC (green color), hexokinase-2 (HK-II, red color) and their intracellular colocalization; the cell nucleus was visualized with DAPI (blue). Bars are 20 μ M. **(B)** Colocalization of HK with VDAC was determined using the Pearson's coefficient. Data are expressed as mean $\pm$ SEM.

Figure 2. Mitochondrial mass was evaluated by determining the activity of mitochondrial matrix enzyme, citrate synthase (CS). Values are presented as the means $\pm$ SEM. Groups: Control (n = 8) – non-tumorous tissue, HCC (n = 8) – tumor tissue, Caco-2 cells (n = 5). Data were analyzed using unpaired two-tailed Student's t-test; * $p < 0.05$.

Figure 3. Evaluation of the mitochondrial respiratory chain activity in permeabilized Caco-2 cells **(A)** as well as in tumor and surrounding unaffected tissues derived from HCC patients **(B)** (Asc – ascorbic acid; ANM – antimycin-A; Rot – rotenone; Suc – succinate; Cyt-c – cytochrome-c; TMPD - N,N,N',N'-tetramethyl-p-phenylenediamine; Vo – rates of basal respiration); bars are SEM, n = 7. All respiratory substrates and inhibitors were added sequentially as indicated on the X-axis.

Figure 4. Analysis of CI and CII-mediated respiration in permeabilized Caco-2 cells, HCC and surrounding unaffected tissues. $V_{\text{Glu}}/V_{\text{Succ}}$ is the ratio of ADP-stimulated respiration rate in the presence of 5 mM glutamate and 2 mM malate (activity of complex I) to ADP-stimulated respiration rate in the presence of 50 μ M rotenone and 10 mM succinate (activity of complex II). Groups: Control (n = 8) – non-tumorous tissue, HCC (n = 8) – tumor tissue, Caco-2 cells (n = 4). Data were analyzed using unpaired two-tailed Student's t-test; * $p < 0.05$.

Figure 5. (A) Oxygraphic analysis of coupling between HK-catalyzed processes and OXPHOS in permeabilized Caco-2 cells; these experiments were carried out in medium-B with 5 mM glutamate and 2 mM malate as respiratory substrates. Succinate (Suc), glucose (Glu), cytochrome-c (Cyt-c) and corresponding adenine nucleotides (ATP or ADP) were added to the cells sequentially as indicated on the X-axis; bars are SEM, n = 5. The degree of the HK-OXPHOS coupling was quantified by means of glucose index **(B)**. Similar study was carried out on human colorectal cancer (HCC) and surrounding unaffected tissue samples (n = 5); the data concerning postoperative material were taken from our prior work (Chekulayev et al. 2015).

Figure 6. Metabolic Control Analysis; for HCC, control tissue and Caco-2 cells the same protocols were used to address different segments of the respiratory chain. After the respiration was activated with 2 mM ADP, mitochondrial respiratory chain and ATP synthasome complexes were stepwise titrated with specific pseudo-irreversible inhibitors. As it can be seen from the oxygraphy trace, the O₂ flux is progressively diminished by increasing inhibitor concentration. The inhibitor concentrations were plotted against the oxygen flux to estimate the initial steady-state flux value (J_0), inhibitor concentration which gives maximal flux inhibition ($I_{\max}$) and the initial slope of inhibitor/flux curve ($\Delta J/\Delta I$). The flux control coefficients (FCCs) were calculated according to the equation given by (Groen et al. 1982; Moreno-Sanchez et al. 2008).

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Tables

Table 1. Characterization of the respiratory chain function in Caco-2 cells as well as in permeabilized tissue samples derived from CRC patients.

Parameters of OXPHOS	Colorectal cancer patients, n = 7 (Kaldma et al. 2014)		Caco-2 cells, n = 6
	Normal tissue	Tumor	
V ₀	0.82 ± 0.15	1.06 ± 0.14*	2.96 ± 0.19
V _{ADP}	1.39 ± 0.21	2.02 ± 0.21	9.53 ± 0.56
V _{Rot}	0.85 ± 0.14	0.91 ± 0.11	2.30 ± 0.21
V _{Suc}	1.33 ± 0.18	2.22 ± 0.26	11.81 ± 0.86
V _{ANM}	0.69 ± 0.07	1.04 ± 0.09	1.85 ± 0.20
V _{Cox}	3.84 ± 0.58	6.59 ± 0.71	70.41 ± 1.81
V _{Cyt-c}	3.8 ± 0.60	5.92 ± 0.61	65.94 ± 1.43

Notes: here, each data point is the mean ± SEM representing rates of O₂ consumption; values are expressed in nmoles O₂/min per mg of cell protein or dry weight of tissue and are obtained according to the experimental protocol shown in **Fig. 3**. *significant difference between tumor and normal tissue samples, *p* < 0.05.

Table 2. The values of basal respiration rate (V₀), maximal rate of respiration (V_m), and apparent K_m values for ADP for permeabilized Caco-2 cells, human colorectal cancer and adjacent normal tissue samples as well as for some rat muscles of different histological type.

Cells and Tissues ^(a)	V ₀	K _m ^{app} _{ADP} , μM ^(c)	V _m ^(d)	Source
Caco-2 cells	1.2 ± 0.15	39.2 ± 5.7 ^(c)	3.07 ± 0.14	current study
Colorectal cancer	1.99 ± 0.26	93.6 ± 7.7 ^(c)	3.82 ± 0.32*	(Chekulayev et al. 2015)
Healthy colon tissue ^(b)	1.13 ± 0.12	256 ± 34 ^(c)	1.92 ± 0.14	(Chekulayev et al. 2015)
Neuroblastoma cells	1.17±0.13**	20.3 ± 1.4	1.74 ± 0.03	(Klepinin et al. 2014)
Rat heart fibers	6.45 ± 0.19	297 ± 35	28.7 ± 1.1	(Kaambre et al. 2012; Kuznetsov et al. 1996)
Rat soleus	2.19 ± 0.30	354 ± 46	12.2 ± 0.5	(Kaambre et al. 2012; Kuznetsov et al. 1996)
Rat gastrocnemius white	1.23 ± 0.13	14.4 ± 2.6	7.0 ± 0.5; 4.10 ± 0.25	(Kaambre et al. 2012; Kuznetsov et al. 1996)

Notes: ^(a)- all respiratory rates are expressed in nmol O₂/min/mg dry weight of tissue or per mg of cell protein in the case of Caco-2 cells; ^(b)- these samples were taken at a site distant from the tumor locus by 5 cm; ^(c)- apparent K_m values were determined by fitting experimental data to a non-linear regression equation according to a Michaelis–Menten model; ^(d)- V_m values were calculated from a titration curve after step-wise addition of ADP,

up to 2 mM; * significant difference vs. normal colon tissue samples, $p < 0.05$. **- unpublished data. The respiratory control ratio values for Caco-2 cells, HCC and normal tissue samples were determined, respectively, as: 3.56, 2.92 and 2.7.

Table 3. Metabolic control analysis of mitochondrial (ADP-stimulated) respiration in permeabilized Caco-2 cells, human colorectal cancer (HCC) and surrounding healthy tissue samples. Flux control coefficients (FCCs) for different components of the respiratory chain.

MI component	Inhibitor	FCC(s)		
		Normal colon tissue, mucosa (Koit et al. 2017)	HCC (Koit et al. 2017)	Caco-2 cells
Complex I	Rotenone	0.45	0.56	0.81
Complex II	Atpenin A5	0.13	0.12	0.50
Complex III	Antimycin	0.66	0.68	0.17
Complex IV	NaCN	0.5	0.31	1.57
ANT	CAT	0.97	0.28	0.31
ATP synthase	Oligomycin	0.24	0.25	0.71
Pi carrier	Mersalyl	0.53	0.43	0.15
Sum of FCC(s) 1, 3-7^(a)		3.35	2.51	3.72
Sum of FCC(s) 2-7^(b)		3.03	2.07	3.41

Notes: ^a- NADH and ^b- succinate dependent electron transfer pathways, respectively; CAT – carboxyatractyloside; ANT - adenine nucleotide translocator; MI – mitochondrial Interactosome.

Figure 1A

B

Figure 1B

Figure 2

Figure 3A

Figure 3B

Figure 4

A

Figure 5A

Figure 5B

Figure 6

Figure 6

338x190mm (300 x 300 DPI)