On reprogramming of tumor cells metabolism: detection of glycogen in the cell lines of hepatocellular origin with various degrees of dedifferentiation

Natalya P. Teryukova · Victoria V. Malkova · Elena I. Sakhenberg ·
Vadim A. Ivanov · Natalia N. Bezborodkina · Sergei A. Snopov

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Abstract The reprogramming of cancer cells includes shifts in glucose and glycogen metabolism. The aim of our work was to check the ability of forming glycogen grains in hepatocellular tumor cell lines of various dedifferentiation levels. We studied the monolayer culture established *in vitro* after explanting cells from rat ascites Zajdela hepatoma strain C (ZH-C) as a “parental” line and its five daughter clonal sublines: the holoclone sublines 3H, 5F, 6H and the meroclone ones 1E, 9C, which possess, respectively, the properties of cancer stem-like cells (CSLCs) and cancer progenitor-like

cells (CPLCs). Besides, we studied four permanent cell lines of a rat hepatoma HTC, two murine hepatomas BWTG3 and MH-22a, and human hepatoblastoma HepG2. We used normal rat hepatocytes as positive control cells that form glycogen. We estimated relative cell dedifferentiation levels of the studied lines via analysis of cell morphology, morphometry and motility character on stained cell preparations and lifetime video files. Glycogen in the cells was detected using a Schiff type Au-SO₂ reagent. All studied hepatocellular tumor lines were not of equal dedifferentiation level as manifested by different nucleus-to-cytoplasm ratio, by epithelium-like or fibroblast-like morphology, by tight or loose intercellular contacts, by cell migration of collective or individual types. Glycogen fluorescence of uneven intensity was observed in all normal rat hepatocytes, but only in some cell groups or in single cells of hepatocellular tumor lines. The large or small fluorescent grains were found not only in relatively less dedifferentiated parental ZH-C line, BWTG3 and HepG2 lines, but also in moderately dedifferentiated 1E and HTC lines and even in severely dedifferentiated 3H, 5F and 6H sublines, as well as in the islets of the rat ascites hepatoma induced *in vivo* by the injection of 3H cells (the tumor-initiating cells). On the other hand, MH-22 and 9C lines, being relatively less and moderately dedifferentiated, showed no glycogen fluorescence. Thus, in 10 tumor cell lines of hepatocellular origin, an ability to reserve glycogen

N. P. Teryukova · E. I. Sakhenberg · V.
A. Ivanov · N. N. Bezborodkina · S. A. Snopov (✉)
Institute of Cytology, Russian Academy of Sciences,
Tikhoretsky ave. 4, Saint Petersburg, Russia 194064
e-mail: npiter@yandex.ru

V. V. Malkova
 Herzen State Pedagogical University of Russia,
 Saint Petersburg, Russia
 e-mail: faculty.of.biology2014@gmail.com

E. I. Sakhenberg
 e-mail: lenasakhenberg@ya.ru

V. A. Ivanov
 e-mail: iva@incras.ru

N. N. Bezborodkina
 e-mail: natalia_bezborodkina@mail.ru

S. A. Snopov
 e-mail: snopov@hotmail.com

manifested no obvious dependency on their dedifferentiation level. Glycogen grains were detected in some cells even of the severely dedifferentiated lines: in single CSLCs of holoclonal ZH sublines grown in vitro and in a majority of tumor-initiating cells derived from ascites hepatoma in vivo. We suggest that dynamic changes in glycogen formation in CSLCs and tumor-initiating cells might be of importance for their dedifferentiation, self-renewal in vitro, survival and metastasis in vivo. The role of glycogen in maintaining viability and metastasis of tumor cells is to be further studied.

Keywords Cancer stem-like cells · Glycogen · Hepatocellular tumor cell lines · Hepatoma Zajdela · Tumor reprogramming · Types of cell migration

Introduction

A reprogrammed metabolism in tumor cells is considered now not as a mere consequence of oncogenic transformation, but as a hallmark of cancer (DeBernardinis and Chandel 2016; Pavlova and Thompson 2016; Ždravlević et al. 2017). A fundamental feature of that metabolic reprogramming is switching to aerobic glycolysis mediated by oncogenic drivers and by the undifferentiated character of tumor cells (Hay 2016). High consumption and active metabolism of glucose in tumor cells end at the stage of lactate formation, regardless of the oxygen presence in the environment (Ward and Thompson 2012; Soga 2013; DeBernardinis and Chandel 2016). Several early studies of the effects of neoplastic process on glucose metabolism and glycogen storage in liver cells reported a reduction of glycogen synthase (GS) and glycogen phosphorylase (GP) activities and a decrease in glycogen content or even a complete loss of it in tumor cells; the latter effect was most pronounced in fast-growing hepatomas, in which it was approaching zero values (Lea et al. 1972; Hammond and Balinsky 1978; Schamhart et al. 1979). On the other hand, study of Favaro et al. (2012) evidenced that glycogen mobilization (in both nonmalignant and cancer cells) is of importance under conditions of oxygen and nutrient deprivation. The role of glycogen formation in cancer progression is so far uninvestigated (Zois and Harris 2016).

The content of polysaccharides (including glycogen) in the cells of a rat ascites ZH was investigated with the periodic acid Schiff (PAS) reaction (Zajdela 1963). Initially, it was a solid hepatoma induced by the addition of 4-dimethylaminoazobenzene to the feeding ratio of two rats. This primary hepatoma gave an ascites with floating islets containing secondary tumor cells. From these islets two ZH cell strains C and D were derived and passed in vivo from an animal to a new animal thus transplanting and reproducing the ascites ZH. By the 32th passage of strain C, the PAS reaction was positive in some islets, while in others it was negative, i.e. those islets behaved as separate clones. Over the passaging, all islets ceased to synthesize polysaccharides: sooner in strain D, and later in strain C, by the 74th passage (Staedel and Beck 1978).

We pay attention to the ascites ZH because of its remarkable 90–100% frequency of metastasis to paratracheal lymph nodes (Kiseleva et al. 1972). Aiming to select a subpopulation of migrating CSLCs from this tumor we transferred the strain C cells from ascites islets into cell culture in vitro and established the monolayer line (ZH-C) and the suspension line (Teryukova et al. 2013). After a long-term cultivation and cloning of ZH-C cells (“parent” line) we obtained holoclonal sublines possessing the properties of CSLCs and meroclonal sublines possessing the properties of CPLCs. Those sublines differed by tumorigenicity, by the types of colony formation, by cell morphology, by intercellular contacts, and by morphometric parameters, in particular, the NC-ratio of the cell nucleus area to the cytoplasm area (Teryukova et al. 2017).

The present study concerns a role of glycogen in the metabolic reprogramming of cells at tumor progression and addresses the question on if the ability to accumulate glycogen may serve as a differentiation/dedifferentiation marker for the tumor cells of hepatocellular origin. We detected and compared a presence of glycogen in 10 cultured cell lines with various levels of cell dedifferentiation: the ZH-C parent line, 3 holoclonal and 2 meroclonal daughter sublines, as well as 4 permanent lines of two murine hepatomas, one rat hepatoma and one human hepatoblastoma. The relative degree of cell dedifferentiation in these lines was estimated by their morphology, by the features of the growing monolayer and cell-to-cell contacts, by their

morphometric parameters, including cell sizes and NC-ratio, and by the types of cell migration.

Methods

Cultivation of cell lines

The parental ZH-C cell line was isolated earlier through a long selection of the attached cells from the floating multicellular islets (Teryukova et al. 2013). Using the method of limiting dilutions we cloned the single cells of parental ZH-C and established its daughter sublines: holoclonal 3H, 5F, 6H and mero-clonal 1E and 9C ones (Teryukova et al. 2017). Permanent cell lines of murine MH-22a and BW7G3 hepatomas, rat HTC hepatoma, human HepG2 hepatoblastoma have been received from the Russian Collection of Vertebrate Cell Cultures (Institute of Cytology RAS, St. Petersburg, Russia, http://www.cytspb.rssi.ru/rkkk/katalog1n_2016_with_figs.pdf).

Cells were cultured in DMEM with L-glutamine containing 4.5 g/L glucose (Biolot, Saint-Petersburg, Russia), 10% calf serum Sus-Biol (Biolot) and 20 µg/mL gentamicin at 37 °C in 5% CO₂ atmosphere.

The cells of the in vitro cultured holoclonal 3H subline were transferred to the peritoneal cavity of male white outbred rats (nursery farm “Rappolovo”, Rappolovo, Leningrad District, Russia) of about 200 g weight by intra-peritoneal injection of 20×10^6 cells for ascites hepatoma generation. After the development of tumor ascites, rats were euthanized by decapitation under ether anesthesia, the cells of ascites fluid were collected in glass tubes, pelleted by centrifugation at 1000 rpm, repeatedly washed in 0.15 M NaCl solution, resuspended in a drop of 0.04 M NaCl solution and then used for a staining of glycogen.

Morphologic and morphometric analysis

Cells were grown on coverslips, fixed and stained with hematoxylin and eosin as described previously (Teryukova et al. 2017). The stained preparations were examined with the LSM 5 Pascal microscope (Carl Zeiss, Oberkochen, Germany) at 40× optical zoom. The area of a whole stained cell and the area of its nucleus were measured on the horizontal plane and

expressed in pixels using image analysis software ImageJ (NIH, Bethesda, MD, USA). For each analysed cell, the ratio of the nucleus area to the cytoplasm area (NC-ratio) was calculated according to the formula: NC-ratio = nucleus area/whole cell area – nucleus area. For each cell line, at least 100 cells were measured.

Perceiving of cell migration type in vitro

The types of tumor cells migration were evaluated during a “wound healing” test. To do this, an “experimental wound” (a cell-free path) was made by a plastic pipette tip in the cell monolayer when it reached 80–90% confluence. The migration properties of the cells were studied with a help of a video microscope AxioObserver.Z1 (Carl Zeiss MicroImaging GmbH, Jena, Germany) as described earlier (Petrov et al. 2017). The images were recorded for 24 h of cell cultivation by means of a time-lapse video shooting through a Plan-Neofluar 10×/0.25 lens with 2 min intervals between frames.

Identification of glycogen

For detection of glycogen, the cultured cells were grown on coverslips until their monolayer reached 80–90% confluence. The subline 3H floating cells isolated from rat ascites were suspended in a drop of 0.04 M NaCl solution and evenly placed to the coverslips (an excess of liquid was removed after 1 min). Then cells were fixed with a cold (– 20 °C) methanol and stained for glycogen by fluorescent variant PAS reaction using a Schiff Au-SO₂ reagent. Within 90 min it binds to the aldehyde groups of both readily accessible and hard-to-reach fractions of glycogen (Bezborodkina et al. 2009). As a positive control, we prepared smear preparations of rat hepatocytes according to the procedure previously described (Kudryavtseva et al. 1983). The fluorescent grains of glycogen within the cells were visualized using LSM 5 Pascal microscope (Carl Zeiss) with an objective of 40×. For every cultured cell line, we analyzed the whole coverslips containing thousands of cells, until our visual search revealed the presence of cells with distinct fluorescent grains or a complete absence of such cells. In order to illustrate the results we show representative images for every cell line studied.

Statistical analysis

For all quantitative parameters, the mean, the standard deviation and the mean error were calculated. The differences between the cell lines were estimated by a Student's *t* test and were considered significant at $p < 0.05$.

Results

Cell morphology and morphometry

In order to estimate cell dedifferentiation degrees of the studied lines we analyzed cell morphology, morphometry and types of cell migration.

The cells of human HepG2 hepatoblastoma as well as murine MH-22a and rat HTC hepatomas are referred in the Catalog of the Russian Collection of Vertebrate Cell Cultures as epithelioid ones. During our cultivation, they all formed distinct epithelium-like structures (Fig. 1g, h, j) although the lines had some difference in cell shape (elongated, rounded, polygonal) and dimensions. The murine BWTG3 hepatoma cell line composed a monolayer consisting of oriented elongated cells of fibroblast-like morphology with a large cytoplasm and cytoplasmic protrusions (Fig. 1i).

As well as in our previous work (Teryukova et al. 2017), we observed that cultured parental ZH-C line (Fig. 1a) and daughter meroclonal 1E and 9C sublines (Fig. 1e, f) grew as a monolayer consisting mostly of cells of epithelium-like morphology with close membrane contacts between neighboring cells and composed the multicellular structures resembling a lobular epithelium.

In contrast, most of the cells in the holoclonal 3H, 5F and 6H sublines had a fibroblast-like morphology, a round or oval shape with a small cytoplasm and long star-like spines of the membrane (Fig. 1b–d). They formed a non-oriented monolayer with rather loose contacts of cell spines, and did not compose the epithelium-like structures. These holoclonal 5F, 3H and 6H sublines as well as HepG2 line could form 3D (multilayer) structures (shown by arrows in Fig. 1), while all other studied cultures grew only as a monolayer of 8–16 nm thickness (estimated on the fixed preparations at confocal laser scanning). Such a

Fig. 1 Features of cell morphology in cultured tumor cell lines of hepatocellular origin: epithelioid-like cells of Zajdela hepatoma parent line (a) and daughter meroclonal sublines 1E (e) and 9C (f), human HepG2 hepatotoblastoma (g), murine MH-22a (h) and rat HTC (j) hepatomas; fibroblast-like cells of Zajdela hepatoma holoclonal 3H (b), 5F (c) and 6H (d) sublines and murine BWTG3 hepatoma (i). Cells were grown, fixed and stained with hematoxylin and eosin on cover slides. Ob. $\times 40$. The arrows point to the 3D (multilayer) cell structures

thickness did not affect the measurement of glycogen fluorescence.

The results of cell morphometry are given in Table 1. The cultured cell lines showed significant differences in average cell area and NC-ratio: out of all cultures, three holoclonal sublines had the smallest cells with the smallest cytoplasm and the highest NC-

Table 1 Morphometric parameters of cultured cells derived from tumors of hepatocellular origin: average cell area, average nucleus area, and average nucleus-to-cytoplasm ratio

Cell lines	Mean $\pm$ SE of cell area (pixels)	Mean $\pm$ SE of nucleus area (pixels)	Mean $\pm$ SE of nucleus-to-cytoplasm ratio (rel. units)
ZH-C daughter holoclonal sublines			
3H	14,495 $\pm$ 647 ^a	8118 $\pm$ 317 ^a	1.65 $\pm$ 0.12 ^d
5F	18,122 $\pm$ 680	11,099 $\pm$ 411	1.96 $\pm$ 0.12 ^d
6H	20,374 $\pm$ 1224	10,932 $\pm$ 587	1.61 $\pm$ 0.10 ^d
ZH-C daughter meroclonal sublines			
1E	52,425 $\pm$ 3419 ^b	16,726 $\pm$ 1403	0.74 $\pm$ 0.05 ^{c,d}
9C	42,087 $\pm$ 2147 ^b	15,835 $\pm$ 611	0.79 $\pm$ 0.05 ^{c,d}
Parental ZH-C line	71,725 $\pm$ 3001 ^b	16,709 $\pm$ 537	0.30 $\pm$ 0.02 ^c
Hepatoblastoma HepG2	26,666 $\pm$ 1808 ^b	10,732 $\pm$ 571	0.67 $\pm$ 0.10 ^{c,d}
Hepatoma MH-22a	28,577 $\pm$ 1157 ^b	9040 $\pm$ 304	0.52 $\pm$ 0.02 ^{c,d}
Hepatoma BWTG3	33,225 $\pm$ 1894 ^b	13,041 $\pm$ 727	0.73 $\pm$ 0.03 ^{c,d}
Hepatoma HTC	51,728 $\pm$ 2883 ^b	21,127 $\pm$ 925	0.85 $\pm$ 0.05 ^{c,d}

^aAverage cell and nucleus areas was sufficiently lower compared to other holoclonal sublines, $p < 0.05$

^bAverage cell area was sufficiently higher compared to each holoclonal subline, $p < 0.05$

^cAverage nucleus-to-cytoplasm ratio was sufficiently lower compared to each holoclonal subline, $p < 0.05$

^dAverage nucleus-to-cytoplasm ratio was sufficiently higher compared to parental ZH-C line, $p < 0.05$

ratio ($p < 0.05$). Among these holoclonal sublines, 3H cells had the lowest average cell area ($p < 0.05$) and nucleus area ($p < 0.05$).

Types of cell migration

Analyzing the results of the “wound healing” test and the time-lapse video records we found that vast majorities of HepG2 hepatoblastoma and MH-22a hepatoma cells did not change their location and did not show a tendency to migrate in all vision areas during the entire period of observation. The cells of BWTG3 hepatoma (Fig. 2g) showed a distinct collective migration within their multicellular sheets formed by the oriented elongated cells.

Most of the epithelium-like cells of parent ZH-C line (Fig. 2a), of daughter meroclonal 1E and 9C sublines (Fig. 2e, f) and of HTC hepatoma (Fig. 2h) also moved towards the free space of “wound” via collective migration of cells within their epithelium-like multicellular sheets. There were only rare single cells that changed their shape from epithelium-like to fibroblast-like and moved as singles by a mesenchymal type.

In contrast, all 3 holoclonal sublines revealed a motility character fundamentally different from other

lines. The majority of fibroblast-like cells of these sublines showed a distinct moving autonomy (Fig. 2b–d). These cells migrated into the free space of “wound” as single cells detaching from the monolayer. The cells of 5F and 6H sublines apparently showed two variants of individual migration, the fibroblast-like and amoeboid-like with protrusions of short pseudopodia (Fig. 2c, d), while the cells of 3H subline (Fig. 2b) predominantly displayed the amoeboid variant. There were no visible shifts of the entire multicellular bulk from the edges of the “wound” to a middle space.

Glycogen storage

To detect glycogen in the cells we treated the cellular preparations with an Au-SO₂ dye that specifically stains glycogen. We have to emphasize that our visual detection was not quantitative, but rather qualitative indicator, since it only revealed either presence or absence of fluorescent glycogen grains in cells. Such a detection did allow us only a modest semi-quantitative estimations of glycogen fluorescence by non-numeric symbols “+” and “–”, just for description and simple classification of heterogeneous images of cultured cells, where only some of them might contain

Fig. 2 The migration character of cultured cells in a “wound healing” test: the amoeboid and/or mesenchymal type of individual cell migration in the holoclone sublines 3H (b), 5F (c) and 6H (d); predominantly collective type of migration (movement of multicellular sheet) in the parental ZH-C line (a), meroclone sublines 1E and 9C (e, f), murine BWTG3 (g) and rat HTC (h) hepatomas. In the case of BWTG3, the cell moving from the group of cells keeps contacting them. Ob. $\times 10$

glycogen granules. Thus, in mature differentiated hepatocytes of normal rat, the ability to deposit glycogen was visualized by an even distribution of small glycogen grains over the cytoplasm in all cells in a vision area (Fig. 3a). We assumed such a level of glycogen content as a high one and denoted it in relative units as (++++) . In a priori dedifferentiated hepatocellular tumor cell lines, we assigned a mark (++) if a majority of cells displayed the glycogen grains, marks (+/– and –/+) if these grains were seen in rare and very rare single cells, respectively, and a

Fig. 3 Detection of fluorescent granules of glycogen in the cytoplasm of normal rat hepatocytes (a) and cells of cultured tumor lines of hepatocellular origin: “parent” line of rat Zajdela hepatoma (b), holoclone sublines 3H (c), 5F (e), 6H (f), meroclone sublines 1E (g) and 9C (h) of rat Zajdela hepatoma, human HepG2 hepatoblastoma (i), murine MH-22a (j) and BWTG3 (k) hepatomas, rat HTC hepatoma (l), as well as multicellular islets isolated from the ascites developed after intraperitoneal injection of the holoclone 3H subline cells into rat peritoneum (d). Ob. $\times 40$. White frames include the rare single cells containing small glucogen granules in 5F (e), 6H (f) and HTC (l) lines

mark (–) if there was no glycogen in cells. A microscopic study of cellular preparations revealed that only 2 from 10 lines displayed no glycogen grains in their cells: meroclonal 9C subline (Fig. 3h) and MH-22a hepatoma (Fig. 3j). These cells grew in a monolayer, hence the result was definitely not due to a possible measurement failure which might be considered if the fluorescent cells were screened by other cells in a multilayer culture. In holoclonal 3H subline and HepG2 hepatoblastoma, the majority of cells contained numerous small glycogen grains (Fig. 3c, i). In all other lines, glycogen was detected only in a part of the cells in several forms: either as rare inclusions, like in the holoclonal 5F and 6H sublines (Fig. 3e, f) and HTC hepatoma (Fig. 3l), or as grains of two types, of the small size and very large size. Both types of grains indicated significant amounts of glycogen in the meroclonal 1E subline (Fig. 3g), in the parent ZH-C line (Fig. 3b) and in BWTG3 hepatoma (Fig. 3k). All glycogen-containing cell lines exhibited a heterogeneity of their cells in a visible load of this polysaccharide.

In a separate experiment we induced an ascites hepatoma in rat by injection of holoclonal 3H subline cells, then collected multicellular islets from the ascites and stained them with an Au-SO₂ dye. The staining revealed a heterogeneity of the islets in the glycogen content: a part of them showed numerous brightly fluorescing inclusions, while others showed a lack of staining or weak staining (Fig. 3d).

Glycogen deposition versus cell dedifferentiation grade

In a diagram (Fig. 4), we compare the levels of glycogen content in all examined tumor cell lines to their migration character and NC-ratio, as parameters reflecting the level of cell dedifferentiation. The comparison indicates rather clearly that there was no distinct correlation between the degree of tumor cell dedifferentiation and their ability to accumulate glycogen.

Discussion

Progression of epithelial-origin tumors down-regulates differentiation genes, induces expression of mesenchymal and pro-migratory genes in their cells

that lose their epithelium-like morphology, dedifferentiate/transdifferentiate phenotypically towards more mesenchymal/fibroblastoid/spindle-shaped cells and gain an increased capacity for invasiveness and motility (Jögi et al. 2012; Bettum et al. 2015; Gandalovičová et al. 2016). Morphologic and morphometric cellular parameters, in particular, NC-ratio, and motility are used for diagnosis and classification of tumors as well as for assessment of cell differentiation/dedifferentiation level (White and Gohari 1981; Jin et al. 1993; Bgatova et al. 2015; Gandalovičová et al. 2016; Layfield et al. 2017).

Besides, during tumorigenesis, cancer cells reprogram their metabolic pathways and nutrient sensing in order to provide bioenergetic and anabolic components for their survival and proliferation. They have modulated expression of metabolism genes, damaged respiration and excessive glycolysis. In contrast to normal, differentiated cells, which under aerobic conditions metabolize glucose mainly via oxidative phosphorylation, many types of cancer cells and tumors largely favor glycolytic pathway, regardless of oxygen availability, and produce lactate for their energy production (DeBerardinis and Chandel 2016). Otto Warburg in 1920s was the first who discovered this effect in tumor slices and ascites cancer cells (Koppenol et al. 2011). A similar glycolytic prevalence has been recently found in primed pluripotent stem cells, but not in naïve and naïve-like pluripotent stem cells (mouse embryonic stem cells and human pluripotent stem cells with altered pluripotent state); whereas some naïve growth conditions may convert the primed cells to naïve-like pluripotent state and elevate glycogen synthetic function in these cells (Zhang et al. 2011; Zhou et al. 2012; Chen et al. 2015a). The formation of glycogen bodies in these cells, however, seems to be independent of cellular differentiation (Chen et al. 2015a). The role of glycogen formation in tumor cells remains unclear.

As has been shown, both non-cancer and cancer cells could accumulate glycogen in order to adapt and survive in an oxygen- and glucose-free microenvironment; a crucial role of glycogen has been proven in ensuring the survival of tumor cells under hypoxic conditions, when mitochondrial respiration in cells get decreased and the production of ATP by anaerobic fermentation of glucose get increased (Pescador et al. 2010; Pelletier et al. 2012; Favaro and Harris 2013). In all types of tumors the viability of tumor cells connects

Fig. 4 Indicators of cell dedifferentiation in 10 tumor cell lines of hepatocellular origin (designated below the columns) that were cultured, fixed and stained for glycogen granules under the same conditions: the average Nucleus-to-Cytoplasm ratio (1) indicated as the column height (mean $\pm$ SE); the type of cell migration (2) shown by the column color (a collective

migration—white, an individual mesenchymal/amoeboid migration—grey, an absence of migration—black); the presence of fluorescent glycogen granules (3) shown above the columns by signs (++), (+), (+/-), (-/+), (-) indicating relative fluorescence levels estimated visually for each of the 10 cell lines as described in “Methods” section

to a high level of GP expression, the key enzyme for the cleavage of glycogen (Lee et al. 2004; Favaro et al. 2012). Inhibition of GP activity and metabolic reprogramming through miR-122 or PDK4 has been proposed for targeted antitumor therapy; such inhibition by RNA interference under hypoxic conditions resulted in a significant accumulation of glycogen in cultured tumor cells, decrease in the rate of culture growth, and induction of the ROS-mediated cell aging process (Ros and Schulze 2012; Song et al. 2015). On the other hand, brain metastases from human breast cancer patients expressed higher levels of glycogen compared to the breast primary tumors (Chen et al. 2015b). The other way round, the MDA-MB231 cells of highly aggressive breast cancer having a low level of glycogen and fast consumption of glycogen were able to survive better than the less invasive MCF-7 cells with a 5–6 fold higher level of glycogen (Pescador et al. 2010).

In the present work, we studied a glycogen storage ability in 10 tumor cell lines of hepatocellular origin of various dedifferentiation levels. To estimate their dedifferentiation levels we analyzed cell morphologic and morphometric parameters (the area size of a whole

cell, of nucleus and of cytoplasm) along with a type of monolayer formed by the cells in culture and with a character of cell migration. The results allowed us to compare cell dedifferentiation degrees of the studied cell lines.

According to average NC-ratio indices and migration types assessed for all hepatoma cell lines, their cell dedifferentiation grades could be ranged as following (Fig. 4). The least dedifferentiated were the cells of the parent ZH-C line (showing the lowest NC-index and predominantly collective migration type) and the most dedifferentiated were the cells of holoclone 5F, 6H and 3H sublines (showing the highest NC-indices and mesenchymal/amoeboid migration types). Whereas the cells of 2 daughter meroclonal ZH-C sublines and of 4 permanent hepatoma lines (showing middle values of NC-indices and mainly collective migration type) all had low-to-moderate levels of dedifferentiation.

Thus, in 3 holoclone sublines of ZH-C, most cells were the most removed from their epithelial nature: they completely lost epithelium-like morphology, acquired the characteristics of fibroblastoid and spindle-shaped cells and showed mesenchymal and

amoeboid variants of individual migration. Apparently, these holoclone sublines cells are most dedifferentiated compared to hepatocytes, and we found in our previous work (Teryukova et al. 2017) that they showed the key traits of CSLC: formation of holoclone-type colonies, self-renewal in culture and tumorigenicity in vivo. One of these holoclone sublines, 3H, consisted of cells of a most dedifferentiated morphology (predominantly fibroblast-like ones), morphometry (the lowest cell area and NC-ratio), and motility (predominantly amoeboid type of migration).

Other cell lines appeared to be dedifferentiated to some lesser extent, inasmuch they retained epithelioid cell morphology, capability to form epithelium-like structures and disability to migrate autonomously as a single cell.

Further, we investigated those 10 hepatoma cell lines for a glycogen storage and looked whether it depended or not on the cell dedifferentiation levels. Before our study, there were very limited data in the literature on the accumulation and metabolism of glycogen in these cell lines.

We found, that in all studied hepatocellular lines a glycogen storage ability was diminished compared to normal hepatocytes. Cells of these lines showed either no glycogen grains at all, or grains only in single cells or in some cell groups.

Our results indicate that both hepatoma MH-22a and hepatoblastoma HepG2 cells were similar in low motility and epithelioid morphology, revealed close NC-ratios (0.52 and 0.67) but were considerably different in glycogen content. Analogously, cells of the migratory phenotype, such as cells of hepatomas HTC (epithelioid ones) and BWTG3 (fibroblastoid ones), had close NC-ratios (0.85 and 0.73) but had also a considerably different glycogen content.

Our finding for ZH-C cell strain (significant glycogen storage) confirmed the earlier observation of Staedel and Beck (1978) for another cell strain, ZH-D, also forming a monolayer in culture. Their study showed a relatively more complex ultrastructure of ZH-D cells (with the grains of glycogen and lipids in the cytoplasm), compared to the islet cells of the ascites ZH, indicating, in the opinion of the authors, a higher differentiation level of ZH-D cells (Staedel and Beck 1978). Since then, there were no reports on such a reversion in glycogen formation in liver tumor cells. Our current results on strain ZH-C cells confirmed that

ZH cells, at forming a monolayer in in vitro culture after their explantation from the ascites, again synthesize and accumulate glycogen, i.e. restore functions which were thought by Staedel and Beck to be characteristic of differentiated hepatocytes only.

However, we revealed that a positive glycogen staining in vitro was peculiar not only to relatively higher differentiated cells (the parental ZH-C line with the lowest average NC-ratio of 0.30), but also to the cells of poor differentiation, CPLCs (the daughter meroclone 1E subline with NC-ratio of 0.74) and even to the most dedifferentiated cells, CSLCs (the daughter holoclone 3H subline with the smallest cells showing a high NC-index of 1.65 and amoeboid migration). Furthermore, we have found out that the glycogen-synthetic function was visibly elevated in the multicellular islets derived from the ascites hepatoma induced in rats by the injection of holoclone 3H subline cells, i.e. it was still preserved in the tumor-initiating cells in vivo (Fig. 3d). To explain this finding we may suggest that the ability to form glycogen in these cells get restored due to a transfer of ZH cells from an in vitro growth (in a nutrient medium with a high oxygen content) to an in vivo reproduction (in a peritoneal ascites at hypoxic conditions) in accord to suggestion of Magnusson et al. (1998). As became clear, cancer cells manifest a high metabolic flexibility, so that growing the same cell line either in culture or in animals alters the fuel these cells use (Davidson et al. 2016). The studies of Van der Jeught et al. (2015) and Chen et al. (2015b) confirmed that modulation of glycogen synthetic function may be due to exogenous factors like various cytokines, epinephrine, inhibitors of fibroblast growth factor, mitogen-activated protein kinase kinase/extracellular-signal regulated kinase (MEK/Erk), inhibitors and activators of GS kinases and other molecules participating in regulation of signaling pathways and shifting the cellular differentiation from progenitor to naïve states. For instance, human pluripotent stem cells grown on the plastic coverslips and treated with naïve growth conditions acquire altered pluripotent states, similar to those naïve-like cells, with increased glycogen synthesis due to GS kinase-3 inhibition however, the formation of glycogen bodies in these cells showed no dependency on cellular differentiation (Chen et al. 2015a). Similar to that observation, we revealed in the present study that formation of glycogen grains

showed no dependence on cellular dedifferentiation levels in 10 hepatocellular tumor cell lines.

The roles of glycogen accumulation in tumor cells do not remain that clear. It is strongly suggested that it plays more complex roles rather than solely acting as an inert intracellular glucose store (Vander Heiden 2011; Obel et al. 2012; Favaro and Harris 2013). The number of cancer and non-cancer cell types preferentially shuttle glucose via “glycogen shunt” (i.e. not directly but through glycogen) even in conditions when extracellular glucose is abundant. The channeling of glucose through glycogen breakdown and through the pentose phosphate pathway generates nucleotides required for sustained proliferation (Lunt and Vander Heiden 2011). The first time it was discovered in a fast-growing ZH culture that following metabolic reprogramming, glucose can be consumed by tumor cells as a precursor of nucleic acids (Bismut et al. 1995). Another possible role of glycogen may be as an important signaling factor within cells. For example, AMP-activated protein kinase, which is an important regulator of cellular energy homeostasis, is directly inhibited by highly branched glycogen granules (McBride et al. 2009).

Furthermore, we suppose that glycogen metabolism may participate in the control of differentiation and dedifferentiation of cancer cells too, analogously to the assumptions of Chen et al. (2015a) that the equilibrium between glycogen accumulation and glycogenolysis represents a metabolic switch to control glycolytic states in human pluripotent stem cells (1); and that inhibition of GS kinase 3 and accumulation of glycogen bodies stimulate the transition of these cells from the primed to the naïve-like pluripotent state with a sustained Oct-4 expression, rather than inducing specific lineage differentiation (2). Our observation of glycogen granules in most dedifferentiated cancer cells of ZH (CSCLs in vitro and tumor-initiating cells in vivo) lead us to anticipate a similar participation of glycogen in their dedifferentiation toward a stem-like state, in their self-renewal in vitro, survival and metastasis in vivo.

In conclusion, our results evidenced that accumulation of glycogen in 10 tumor cell lines of hepatocellular origin was not strictly determined by their dedifferentiation level. We observed glycogen granules in individual cells of the most dedifferentiated clonal cell lines of highly metastatic rat Zajdela hepatoma, while other cell sublines, of less

dedifferentiation, revealed clearly less granules or no granules. These results show a distinct heterogeneity of metabolic state of the cells cloned from single cancer cells of this hepatoma. We discovered for the first time that the cells of holoclone 3H subline of rat ascites ZH, the severely dedifferentiated tumor-initiating CSCLs forming a loose monolayer of fibroblastoid cells without tight contacts, having a high NC-ratio, were able to accumulate numerous glycogen grains under culture conditions. Further studies on the glycogen functions in tumor cells, particularly in the invasive and metastatic CSCLs, may help to bring a light on their metabolic reprogramming that ensures their enormous metabolic flexibility, high motility, invasiveness, drug resistance and survival.

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Compliance with ethical standards

The animal experiments were conducted in conformity with international and Russian laws and policies

Conflict of interest The authors declare that they have no conflict of interest.

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