Background Levels of Neomorphic 2-hydroxyglutarate Facilitate Proliferation of Primary Fibroblasts

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Summary

Each cell types or tissues contain certain "physiological" levels of R-2-hydroxyglutarate (2HG), as well as enzymes for its synthesis and degradation. 2HG accumulates in certain tumors, possessing heterozygous point mutations of isocitrate dehydrogenases IDH1 (cytosolic) or IDH2 (mitochondrial) and contributes to strengthening their malignancy by inhibiting 2-oxoglutarate-dependent dioxygenases. By blocking histone de-methylation and 5-methyl-cytosine hydroxylation, 2HG maintains cancer cells de-differentiated and promotes their proliferation. However, physiological 2HG formation and formation by non-mutant IDH1/2 in cancer cells were neglected. Consequently, low levels of 2HG might play certain physiological roles. We aimed to elucidate this issue and found that compared to highest 2HG levels in hepatocellular carcinoma HepG2 cells and moderate levels in neuroblastoma SH-SY5Y cells, rat primary fibroblast contained low basal 2HG levels at early passages. These levels increased at late passage and likewise 2HG/2OG ratios dropped without growth factors and enormously increased at hypoxia, reaching levels compared to cancer HepG2 cells. Responses in SH-SY5Y cells were opposite. Moreover, external 2HG supplementation enhanced fibroblast growth. Hence, we conclude that low 2HG levels facilitate cell proliferation in primary fibroblasts, acting *via* hypoxia-induced factor regulations and epigenetic changes.

Key words

2-hydroxyglutarate • Fibroblast proliferation • SH-SY5Y neuroblastoma cells

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Introduction

It has long been known, that each cell types or tissues contain certain "physiological" levels of R-2-hydroxyglutarate (2HG) (Engqvist *et al.* 2014). However, 2HG has also been recognized as a neomorphic metabolite inhibiting dioxygenases dependent on the regular Krebs cycle intermediate 2-oxoglutarate (2OG), i.e. 2-OG-dependent dioxygenases (Wei *et al.* 2011). Certain cancer cells, notably of grade 2/3 gliomas, secondary glioblastomas and acute myeloid leukemia, exhibit heterozygous point mutations of isocitrate dehydrogenases IDH1 (cytosolic) or IDH2 (mitochondrial) in the active sites and form, presumably in an excessive manner, the accumulating 2HG (Gross *et al.* 2010, Metalo *et al.* 2011, Ward *et al.* 2010). An enormous effort of scientific and medical community led recently to finding of links between the patient's IDH1 or

IDH2 mutation pattern and predicted prognoses for various cancers. However, it has also been recognized that glioblastoma SF188 cells produce 2HG at hypoxia, in spite of lacking the *idh1/2* mutations (Wise *et al.* 2011, Intlekofer *et al.* 2015). Similarly, c-Myc-re-transformed breast cancer cells exhibited also substantial 2HG levels independently of *idh1/2* mutations (Smolková *et al.* 2015, Terunuma *et al.* 2014). These findings call for reconsideration of 2HG "normal" metabolism and possible physiological roles.

Mechanisms of neomorphic effects of R-2HG are given by inhibitions of 2OG-dependent dioxygenases, blocking histone de-methylation; and 5-methyl-cytosine hydroxylation, modulating DNA methylation (Wei *et al.* 2011, Wang *et al.* 2015). The neomorphic activity of mutated IDH1 or IDH2 enzymes probably causes epigenetic changes which in turn lead to a dramatic elevation of 2HG levels. Such levels themselves are sufficient to promote leukemogenesis in the hematopoietic cells through the maintenance of de-differentiation and increased proliferation (Heuser *et al.* 2015, Losman *et al.* 2013, Wang *et al.* 2015). Also, a key component of hypoxia-inducible factor (HIF) regulation pathway, the prolyl hydroxylase domain-2 (PHD2/EglN1) enzyme has been found to be activated by R-2HG (Koivunen *et al.* 2012). Since PHD inhibition initiates HIF-mediated transcriptome reprogramming, promoting also Warburg glycolytic phenotype, consequently, R-2HG should prevent HIF-1α stabilization. In this prediction, R-2HG should act against Warburg glycolytic phenotype and other HIF-mediated transcription changes. However, PHDs exhibit also an important HIF-independent fuel-sensing regulation, which may have an opposite role (Duran *et al.* 2013, Heuser *et al.* 2015).

A few enzymes are known to produce 2HG or its stereoisomer and another class of the enzymes possesses the ability to catabolize 2HG, which can therefore diminish elevated 2HG levels (Engqvist *et al.* 2014). In mammalian cells, R-2HG is produced from 5-aminolevulinate (Chalmers *et al.* 1980). Oxidative degradation of heme precursor 5-aminolevulinate converts it to 2HG through action of glutathione-dependent glyoxylase isoform 1 (GLO1) and 2 (HAGH) (Tafti *et al.* 2003). Also, in mammalian mitochondria hydroxyacid-oxoacid-transhydrogenase (HOT, or ADHFE1, EC 1.1.99.24) forms R-2HG from 2OG, while converts simultaneously 4-hydroxybutyrate to succinic semialdehyde (Struys *et al.* 2004). HOT thus competes with IDH2 for 2OG. Recently, also human phosphoglycerate dehydrogenase (EC 1.1.1.95) has been reported to form R-2HG from 2OG (Fan *et al.* 2015). Under nearly anoxic conditions, lactate dehydrogenase-*A* and mitochondrial malate dehydrogenase have been reported to produce L-2HG, which promotes malignancy similarly as R-2HG (Intlekofer *et al.* 2015).

The specific degradation of R-2HG to 2OG proceeds *via* R-2HG-dehydrogenase (EC 1.1.99.39) localized in the mitochondrial matrix (Achouri *et al.* 2004), while the S-stereoisomer is catalyzed by the enzyme of EC 1.1.99.2. Notably, the loss or heterozygous mutations of R-2HG-dehydrogenase cause 2OG decline and consequent aberrant epigenetic remodeling leading to malignancy (Lin *et al.* 2015). The deficiency of R-2HG-dehydrogenase also causes type I glutaric acidemia (Struys *et al.* 2005) and the enzyme depletion provides IDH1 and IDH2 rather critical roles (Matsunuga *et al.* 2012). Since the R-2HG-dehydrogenase accepts electrons from the electron transfer flavoprotein:ubiquinone oxidoreductase (Struys *et al.* 2005), the oxidoreductase deficiencies lead to glutaric acidemia type II. At present it is not clear, under which conditions or whether at all the non-mutant IDH1 or IDH2 enzymes form R-2HG and which R-2HG levels represent a threshold for each tissue or tumor type above which hyper-accumulation leads to diseases.

In contrast, without any *idh1/2* mutations, breast cancer-associated c-Myc upregulation led to several-fold increased 2HG accumulation and global epigenome changes associated with poor prognosis (Terunuma *et al.* 2014). R-2HG-dehydrogenase depletion resulted in a similar 2HG accumulation (Matsunuga *et al.* 2012, Worth *et al.* 2015) or 2OG decline (Lin *et al.* 2015). In our previous work (Smolková *et al.* 2015), we have clearly demonstrated that 2HG can be formed despite the absence of *idh1/2* mutations in breast carcinoma HTB-126 cells and suggested 2HG as an analytic tumor marker (in serum, urine, or biopsies) predicting malignancy of breast cancer in all patients. So far, 2HG accumulation was considered only in cancer cells bearing heterozygous *idh1/2* mutations, whereas physiological 2HG levels and a possibility of excessive 2HG formation either by non-mutant IDH1/2 or due to mutant R-2HG-dehydrogenase in cancer cells were not studied.

In this work we aimed to study "physiological" effects of 2HG in primary fibroblasts comparing them with model cancer cells, a human neuroblastoma SH-SY5Y cell line. Since cancer cells typically abuse

metabolic and signaling pathways, which otherwise physiologically serve during embryogenesis or tissue repair, we focused on a possible 2HG role in fibroblast proliferation. We found that early passages of cultivated primary fibroblasts contain lower 2HG levels increasing with cultivation, decreasing (likewise the 2HG/2OG ratio) in the absence of growth factors, suggesting that slightly elevated background levels of 2HG facilitate proliferation of primary fibroblasts. Moreover, an enormous increase in fibroblast 2HG occurred at hypoxia, reaching the high levels found in cancer cells, such as in HepG2 cells. Consequently, 2HG most probably act *via* hypoxia-induced factor regulations and epigenetic changes.

Methods

Reagents

All the chemicals and cell culture reagents were obtained from Sigma-Aldrich (St Louis, MO, USA) unless otherwise specified.

Animals

The local animal research committee approved the protocols for all aspects of the animal studies in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the United States National Institutes of Health. Male Wistar rats fed with a standard chow were anesthetized with ketamine/xylazine and skin grafts for establishment of primary culture were taken from the external ear. Ear fragments were washed by ethanol in sterile flow box and cut into small pieces which were cultivated in growth medium. After fibroblasts release, ear matrix was removed and cells were transferred to the sterile flask where proliferated (Huschtscha *et al.* 2012). Freshly isolated cells were used for early culture of fibroblasts (until 20 passages). For old fibroblasts was considered culture after 30 passages.

Cell cultures

Human neuroblastoma SH-SY5Y cells (ECACC94030304) and human hepatocellular carcinoma HepG2 cells (ECACC 85011430) were purchased from ECACC and were cultivated at 37 °C in a humidified incubator with 5 % CO_2 in glucose-free DMEM (Life Technologies, Carlsbad, CA, USA) supplemented with 3 mM glutamine, 10 % (v/v) fetal calf serum (Life Technologies), 10 mM HEPES, 100 IU/ml penicillin, and 100 µg/ml streptomycin, and with 5 mM glucose. Primary fibroblasts were isolated from rat skin and cultured in Dulbecco's Modified Eagle Media (Genetica, Czech Republic) supplemented with 5 $mmol \cdot l^{-1}$ glucose, 3 $mmol \cdot l^{-1}$ glutamine, 10 % fetal calf serum (Biochrom, Berlin, Germany) and 1 $mmol \cdot l^{-1}$ pyruvate in normoxic CO_2 chamber at 37 °C. For specific experiments, glutamine-free media, or fetal calf serum-free media were used, or cells were maintained in a hypoxic chamber (Scitive N, Ruskinn, UK) with 5 % CO_2 and a controlled mixture of air/N_2 to reach stable 5 % O_2. Growth medium with 10 % FCS was used in hypoxia.

Sample preparation and analysis

Cells were harvested after 48 h cultivation in specific conditions. Cell suspension was centrifuged, supernatant was sucked up and pellets were washed by PBS. Samples were freeze after PBS removal and stored at -80 °C till measurement.

Internal standard oxalate was added and cell samples were extracted with water/methanol/chloroform (1:1:2, w/w/w) and centrifuged at 1000 × g for 10 min. The upper polar phase was transferred into glass vial and lyophilized. The analytes were derivatized with pyridine/ *N,O*-Bis(trimethylsilyl)acetamide/chlorotrimethylsilane (9:3:1, v/v/v) at 65 °C for 1 h. Derivatized samples were directly injected into gas chromatography – mass spectrometry (GC-MS, GC 6890N, MD 5973, Agilent technologies, CA, USA).

The 95 % methyl-, 5 % phenyl-polysiloxane column was preconditioned at 100 °C, held 1 minute, then temperature was increased by 10 °C per minute to 180 °C and held for 1 minute. Mobile phase was helium at flow 1 ml per minute. Retention times of selected metabolites were confirmed with commercial standards and detectable specific mass ions were chosen according to their mass spectra (m/z: 190 – oxalate; 273 – citrate; 335 – malate; 349 – 2HG; 347 – 2OG, 245 – fumarate, 219 – lactate). All analytes were measured in one run which lasted 10 min, finally post-run was applied and oven was fired at 300 °C.

For our measurements middle Petri dishes (d=10 cm) were used (1 dish = 1 sample), which means approximately 1.5-2.0.10^6 cells.

Proliferation assay

Cell suspension was divided into proportionate parts. Halves were centrifuged, medium was removed and pellets were washed by PBS. After centrifugation, PBS

was removed and samples were lysed by the lysis buffer. Finally, protein concentration was measured by BCA assay (Bicinchoninic acid kit). Second halves of these parts were cultivated on dishes for two days. Then, cells were harvested and suspension was centrifuged. From this point, algorithm of procedure was same – PBS wash, lysis, protein measurement. Hence, the initial concentration was detected and compared with concentration of cell protein after proliferation during 50 h. Ratio between the final and initial concentration of cell protein was calculated and plotted. 2HG levels for cell treatment were chosen according the experimental procedure – we have checked changes in levels of metabolites by GC/MS. We have picked 2HG range in physiological relevant levels.

Bromodeoxyuridine (BrdU) assay

Alternatively, cell proliferation was measured with bromo-2′-deoxy-uridine. Cells were plated on six-well plates until approximately 80 % confluent, and treated according to the desired experimental schema. Prior to staining, growth medium was exchanged for fresh growing medium containing 10 μM 5-bromo-2′-deoxy-uridine (Roche) and incubated 3 h or 16 h, respectively. After incubation, cells were washed with PBS, fixed with cold ethanol (pH 2), incubated in -20 °C for at least 30 min and washed thoroughly with PBS. Samples were incubated with anti-5-bromo-2′-deoxy-uridine primary antibody (Sigma-Aldrich) for 60 min, washed three times with a washing buffer (PBS supplemented with 0.05 % Triton X-100, 0.05 % Tween20, 0.1 M glycine), incubated with secondary anti-mouse antibody labeled with Alexa Fluor 488 dye for 60 min, and washed three times with PBS. Additionally, fixed samples were stained with 1 mg/ml Hoechst 33342 (Molecular Probes). Samples were analyzed with fluorescence microscope, number of stained nuclei were quantified using ImageJ. Cell proliferation index has been taken as % of 5-bromo-2′-deoxy-uridine-positive nuclei to total nuclei (Hoechst-positive).

Cell cycle analysis

Identification of cell division stages is based on the different DNA amounts in these stages. DNA is stained with propidium iodide. A possible RNA interference is excluded by the RNase treatment. Cells were harvested, washed by phosphate buffered saline (PBS) and divided into equal aliquots ($2 \cdot 10^6$ cells per sample) which were centrifuged, PBS was removed and cells were fixed by 1 ml of 70 % ethanol and left in the refrigerator for 2 h. Then, fixed samples were again washed by PBS, centrifuged at 850 × g and supernatant was removed. Fixed cells were finally treated with 50 $\mu g \cdot ml^{-1}$ RNase *A* for 15 min and then stained by 50 $\mu g \cdot ml^{-1}$ propidium iodide. These suspensions were measured on a 16 color flow cytometer BD LSR II including a FACS Flow Supply of the High Throughput Sampler System (BD Biosciences, San Jose, CA, USA) at excitation at 535 nm and emission at 617 nm. The percentage of each cell cycle phases was calculated using the single cell analysis software FlowJo.

Statistical analysis

Statistical analysis was performed using ANOVA with Tukey test or Student's t-test (for two samples).

Results

Lower 2HG levels exist in rat primary fibroblast when compared to cancer cells

2HG content in rat primary fibroblast attained usually levels below 100 $pmol.10^{-6}$ cells (Fig. 1), whereas levels in cancer cells such as hepatocellular carcinoma HepG2 cells were 500 $pmol.10^{-6}$ cells and intermediated levels were found in neuroblastoma SH-SY5Y cells (Fig. 1). Nevertheless, 2HG increased with the progressing passages of fibroblasts, as exemplified in Figure 1. Hence, we intended to seek, whether this 2HG increase is promoting proliferation or reflects its metabolic consequences.

Increasing basal 2HG levels facilitate cellular proliferation

At first, we tested the effect of serum, naturally promoting cell proliferation. Serum stimulation increased 2HG levels (Fig. 2A) and decreased 2OG levels in late passage fibroblasts, so that their ratio increased up to twice (Fig. 2B). Ratios of 2HG to citrate (Fig. 3A), malate (Fig. 3B) or fumarate (Fig. 3C) dropped, while citrate (Fig. 4A), malate (Fig. 4B) or fumarate (Fig. 4C) accumulation increased with regard to 2-OG accumulation. This means that the corresponding metabolite-consuming reactions were retarded. Yet further enormous 10-fold increase in 2HG levels (Fig. 2A) and two-fold increase in the 2HG/2OG ratio (Fig. 2B) were observed at hypoxia with optimum serum levels (after incubation of these fibroblasts for 24 h at

5 % O_2). In less extent, hypoxia with serum increased 2HG/citrate (Fig. 3A), 2HG/ malate (Fig. 3B) or 2HG/fumarate ratios (Fig. 3C), but did not significantly change other tested ratios (Figs 4A, 4B and 4C).

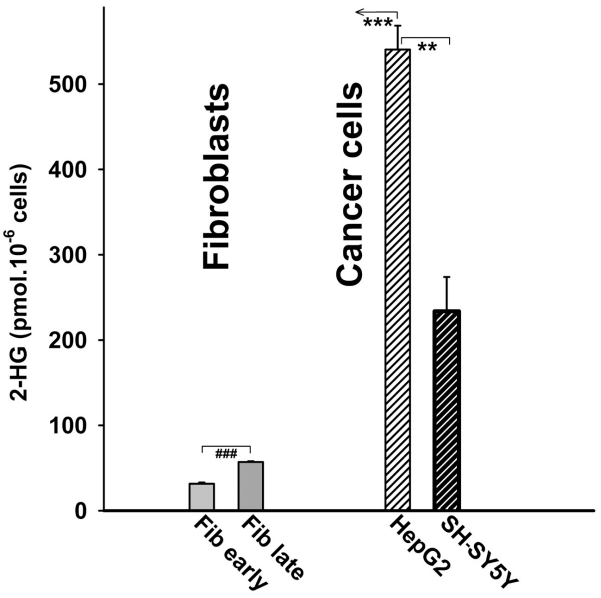

Fig. 1. 2HG levels in rat primary fibroblasts as compared to selected cancer cells. 2HG levels were analyzed for cell types indicated at x-axis. Primary rat fibroblasts were evaluated at early (up to 10) passages vs. later (over 50) passages. ANOVA yielded ***p<0.001, **p<0.01, Student's t-test: ###p<0.001.

Comparing the results for fibroblasts with model cancer cells, such as neuroblastoma SH-SY5Y cells, we found that serum stimulation increased 2HG levels also in SH-SY5Y cells (Fig. 2A). However, considering 2HG/2OG ratios, responses of SH-SY5Y cells to serum withdrawal or hypoxia with serum were just opposite to those in fibroblasts (Fig. 2B). The opposite were also responses for 2HG/citrate (Fig. 3A), 2HG/malate (Fig. 3B) or 2HG/fumarate (Fig. 3C) ratios.

Strengthening of Wartburg phenotype in hypoxic neuroblastoma SH-SY5Y cells, reflected by a relative citrate increase vs. 2OG levels, indicated a relative citrate accumulation in cancer cells but not in fibroblasts (Fig. 4A). Distinct metabolism of SHSY5Y cells and fibroblasts has also been indicated by a relative malate (Fig. 4B) and fumarate (Fig. 4C) accumulation after serum withdrawal from SH-SY5Y cells. In contrast, hypoxia with optimum serum, decreased higher malate accumulation of fibroblasts, established at normoxia with a serum (Fig. 4B). Nevertheless, serum withdrawal always decreased metabolite accumulation in fibroblasts (Figs 4A, 4B and 4C), in contrast to the increased metabolite accumulation in SH-SY5Y cells.

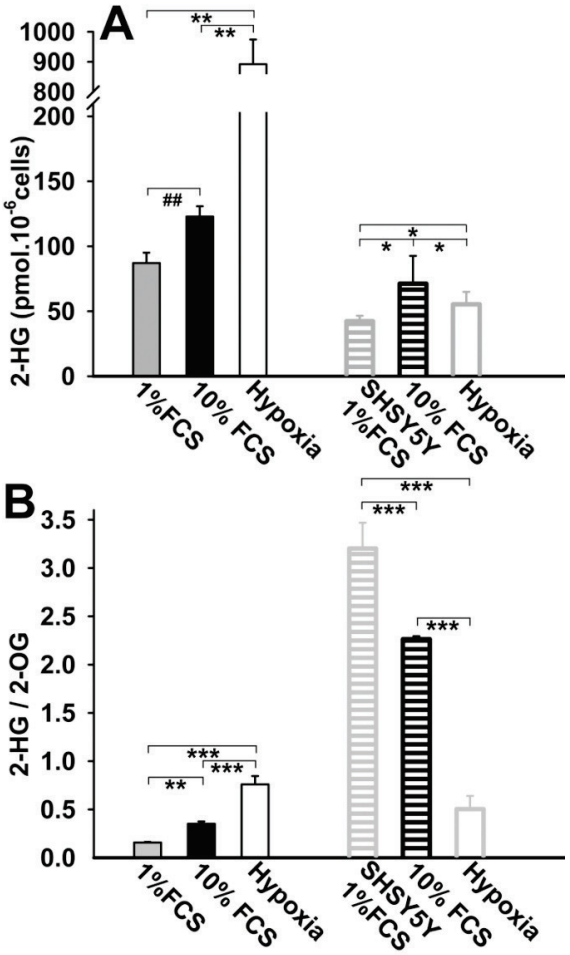

Fig. 2. Physiological changes of 2HG levels and ratios between 2HG to 2OG during serum stimulation or at hypoxia. *Left three bars:* primary fibroblasts and *right three bars:* SHSY5Y neuroblastoma cells. Conditions as indicated (three to six estimations for each). Hypoxia was set at 5 % O_2 in medium with 10 % FCS. **A**) 2HG levels, **B**) 2HG to 2OG ratios. ANOVA yielded ***p<0.001, **p<0.05, *p<0.1, Student's t-test: ##p<0.05.

We have also noticed that the transition of primary fibroblasts from quiescence to proliferation, which is triggered by serum stimulation, is further enhanced by hypoxia (Figs 5A and 5B, Figs 6A and 6C). Since synergy of both these stimuli, i.e. serum stimulation plus hypoxia, increased the 2HG/2OG ratio by about four-fold (Fig. 2B), the ability of 2HG to modulate cellular proliferation was further tested. Surprisingly, addition of exogenous 2HG to the fibroblast cell culture resulted in a dose-dependent acceleration of cell proliferation (Figs 6A and 6C). In turn, proliferation of SH-SY5Y cells decreased at hypoxia (Figs 6B and 6D), but responses to serum starvation and exogenous 2HG were similar (Fig. 5C, Figs 6A, 6B, 6C and 6D). This correlated perfectly with endogenous 2HG levels (Fig. 2A). Note that fibroblasts remained mostly in the G1/G0 phase of the cell cycle (Fig. 5A).

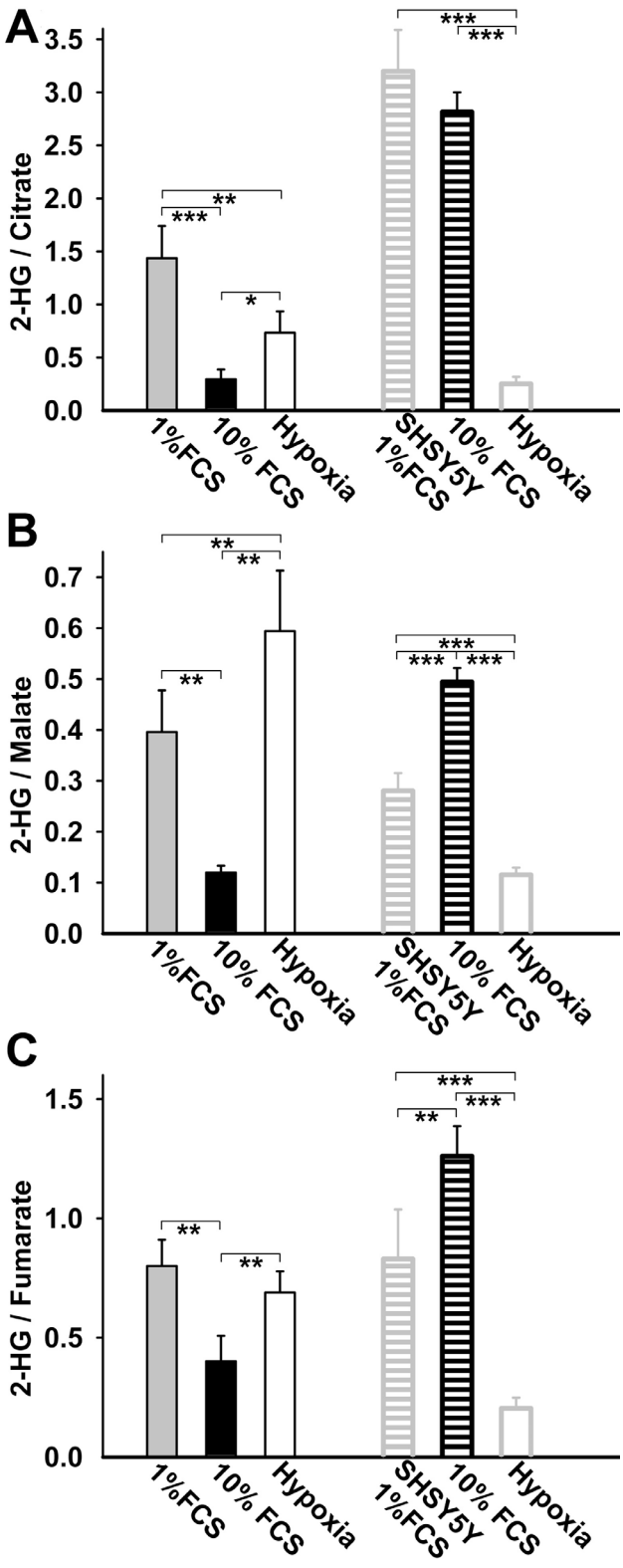

Fig. 3. Physiological changes of ratios between 2HG and citrate or malate or fumarate during serum stimulation or at hypoxia. **A**)-**C**) metabolite ratios as indicated. *Left three bars:* primary fibroblasts and *right three bars:* SH-SY5Y neuroblastoma cells. Conditions as indicated (three to six estimations for each). Hypoxia was set at 5 % O_2 in medium with 10 % FCS. ANOVA yielded ***p<0.001, **p<0.05, *p<0.1.

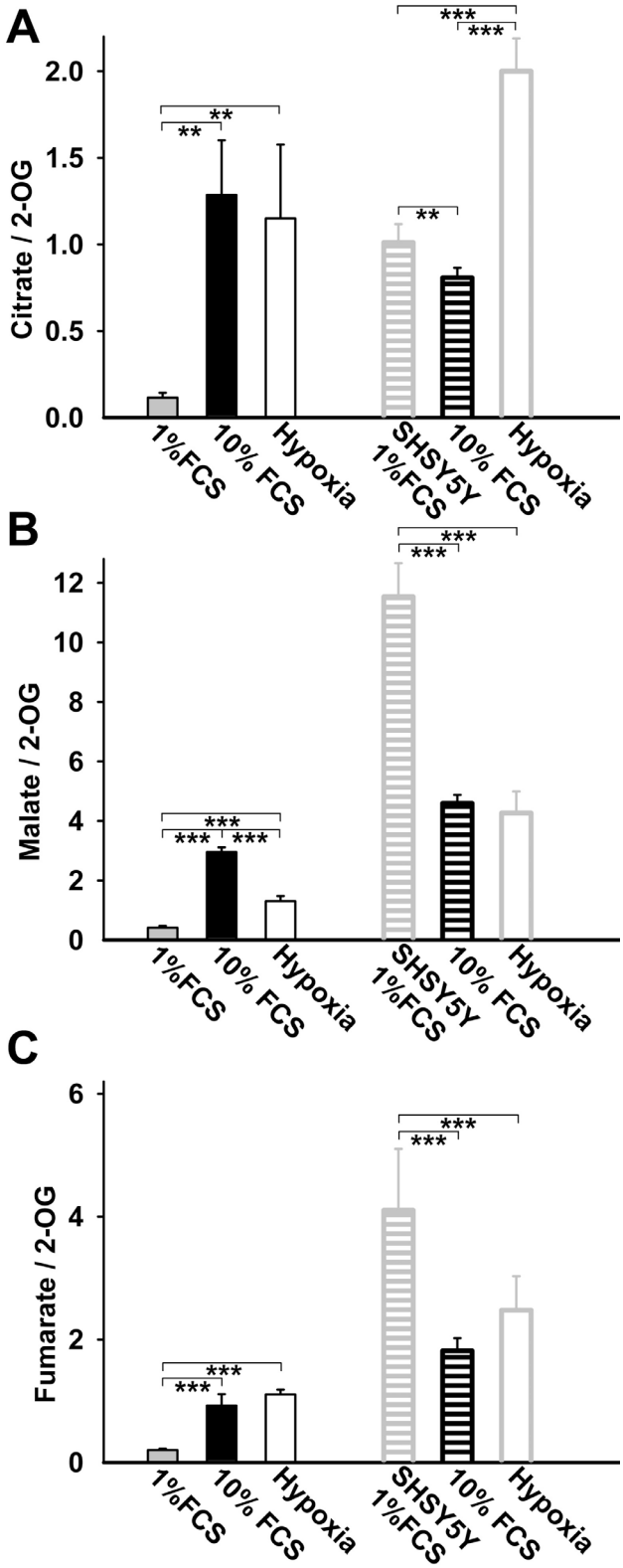

Fig. 4. Physiological changes of ratios between citrate or malate or fumarate to 2OG during serum stimulation or at hypoxia. **A**)-**C**) metabolite ratios as indicated. *Left three bars:* primary fibroblasts and *right three bars:* SH-SY5Y neuroblastoma cells. Conditions as indicated (three to six estimations for each). Hypoxia was set at 5 % O_2 in medium with 10 % FCS. ANOVA yielded ***p<0.001, **p<0.05, *p<0.1.

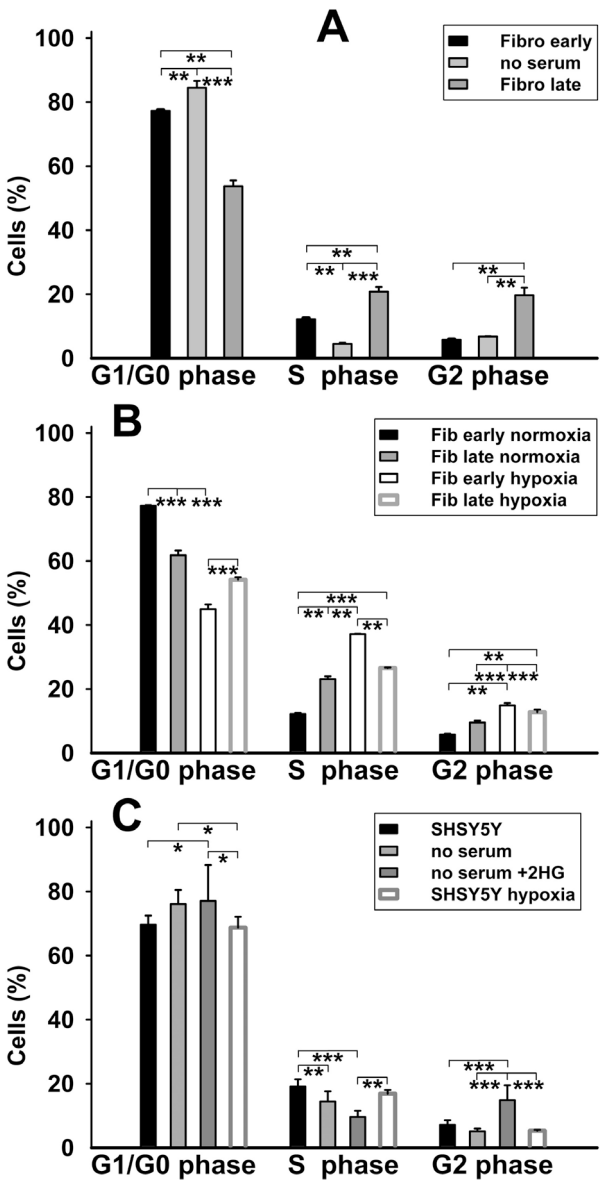

Fig. 5. Cell cycle analyses. **A**), **B**) fibroblasts early and late (early: „Fibro early + no serum", late: „Fibro late"), and **C**) SH-SY5Y cells („SHSY5Y"), without FCS („no serum"), without FCS + 5 mM 2HG („no serum + 2HG"), or in hypoxia („SHSY5Y hypoxia") – % of cells within the culture entered into the ascribed cell cycle phase is depicted for each cell type and condition. ANOVA yielded ***p<0.001, **p<0.05 (n=3).

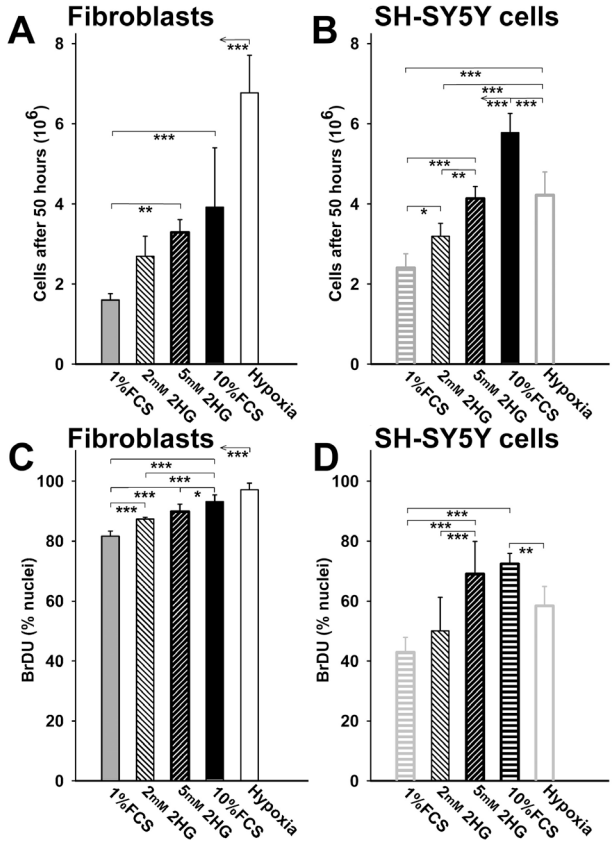

Fig. 6. Proliferation of primary fibroblasts and SH-SY5Y cells as indicated by cell growth or BrdU incorporation. **A**), **B**) cell number after 50 h of treatment, when initially 10^6 cells were seeded, or **C**), **D**) BrdU incorporation during 16 h assayed for primary fibroblasts (**A**, **C**) and SH-SY5Y cells (**B**, **D**) during serum ("10 % FCS") stimulation, serum starvation ("1 % FCS"), stimulation by 2 mM or 5 mM 2HG, and with 10 % FCS serum plus hypoxia set at 5 % O_2 ("Hypoxia"). ANOVA yielded ***p<0.001, **p<0.05, *p<0.1 (n=6-12).

Discussion

Metabolic changes associated with cellular transformation play an important role during oncogenesis (Ježek and Smolková 2012, Hensley *et al.* 2013, Heuser *et al.* 2015, Morin *et al.* 2014, Soga 2013, Smolková *et al.* 2011, Tennant *et al.* 2010, Wahlström *et al.* 2015), which usually includes also hypoxic cell adaptation (Ježek *et al.* 2010). Glutaminolysis and altered redox homeostasis are also frequently hallmarks of tumorigenesis (Ježek and Smolková 2012, Plecitá *et al.* 2015, Smolková *et al.* 2011), as well as reductive carboxylation by isocitrate dehydrogenase-2 (Smolková *et al.* 2015). One of the most intriguing is the overproduction of oncometabolite 2HG, previously associated with neomorphic activity of mutant isocitrate dehydrogenases in the cell cytosol (IDH1) and mitochondria (IDH2) (Gross *et al.* 2010, Losman *et al.* 2013, Metalo *et al.* 2011, Reitman *et al.* 2014, Saha *et al.* 2014, Shim *et al.* 2014, Ward *et al.* 2010, Wise *et al.* 2011). Nevertheless, 2HG formation and resulting metabolic and epigenetic changes also proceed physiologically (Chalmers *et al.* 1980, Engqvist *et al.* 2014, Fan *et al.* 2015, Struys *et al.* 2004, Tafti *et al.* 2003) in normal proliferating cells during embryogenesis, angiogenesis, immune response or tissue repair (Pearce *et al.* 2013). Consequently, we hypothesize that in levels lower than in cancer cells 2HG is a physiological

regulator in normal cells and tissues.

Cancer cells acquire during oncogenesis a specific malignant set up of metabolic and signaling pathways, which otherwise physiologically serve during normal cell regulations, stress and metabolic responses, and specifically those employed in embryogenesis or tissue repair. Our results suggested a possible physiological role of 2HG in facilitating proliferation of primary fibroblasts. In the tissues, fibroblasts are usually dormant until their proliferation is triggered at the sites of tissue growth/repair. These conditions are associated with locally increased cytokine production and hypoxia, which activate transcription factors such as c-Myc and HIF system (HIF-1α accumulation), respectively. Both of them influence numerous targets, triggering cellular proliferation and modulating metabolism (Ježek *et al.* 2010, Smolková *et al.* 2011). Here, we report that these conditions represented by serum stimulation, or hypoxic incubation at optimum serum levels, resulted also in increased 2HG levels and intracellular 2HG/2OG ratios in primary fibroblasts, at their progressing passages.

At first, we found that 2HG in serum-starved fibroblasts was slightly decreased. Under these conditions, OXPHOS is depressed and more lactate is formed (Golpour *et al.* 2014, Herzig *et al.* 2000). Also, 2OG was accumulated in fibroblasts during serum starvation and since 2HG formation did not change significantly, the resulting 2HG/2OG ratio decreased significantly; as well as other metabolite/2OG ratios also diminished. This is reminiscent of situation, when a withdrawal of growth factors in the absence of compensation by glutaminolysis results in metabolic decline affects mTOR signaling and cell division (Durán and Hall 2012). In contrast, serum stimulation induces elevations in lactate dehydrogenase and other glycolytic enzymes of rat fibroblast (Matrisian *et al.* 1985).

Second, the observed profound 2HG increase at hypoxia is parallel to the increased glycolytic (Warburg) phenotype. This might be also related to the increased IDH2 activity at hypoxia (Smolková *et al.* 2015). Note that we have applied only a weak hypoxia with 5 % O_2, for which we have previously described increases in reductive carboxylation in cancer cells (Smolková *et al.* 2015). The increased 2HG can subsequently activate prolyl hydroxylase domain-2 (PHD2/EglN1) enzyme (Koivunen *et al.* 2012), which keeps critical prolines of HIF-1α hydroxylated and thus marked for degradation. During the simultaneously ongoing hypoxia and 2HG elevations after a certain time period, a threshold of 2HG levels is reached when PHD2 will stop HIF-1α stabilization. Hypothetical 2HG-mediated regulations therefore overcome the previously ongoing HIF-mediated transcriptome reprogramming. Among the possible 2HG-mediated regulations, promotion of proliferation seems to be the most prominent. Previous studies reported that 2HG overproduction acts through inhibition of TET2 methylcytosine hydroxylases which catalyze de-methylation of genomic DNA thus maintaining cellular de-differentiation and proliferative potential (Losman *et al.* 2013, Terunuma *et al.* 2014). In this case higher 2HG were established by mutant IDH1/2 or external additions of cell-permeable 2HG analogue. Here, we suggest that 2HG-mediated promotion of fibroblast proliferation acts by the same mechanism. 2HG can act as such or through the 2HG-2OG conversion. It may resemble a described mechanism of mTOR triggering by the membrane-permeant 2OG derivative, resulting in higher proliferation (Durán and Hall 2012).

In contrast, also inhibitory effects of high 2HG levels on PHD2/EglN1 prolyl-hydroxylase have been reported (Wang *et al.* 2014). Hence, one would expect HIF system activation by e.g. HIF-1α stabilization and progressing Warburg glycolytic phenotype. Nevertheless, all reported studies consistently described that 2HG elevation resulted in increased cellular proliferation under serum starvation conditions, the effect ascribed to changes in genome methylation. Our results suggest that even mild elevation of 2HG levels, that might be considered as physiological, or elevation of 2HG/2OG ratio could significantly increase proliferation of serum-starved cells. Moreover, since the increased 2HG levels were associated with serum stimulation, they may hypothetically serve as an independent signaling event. Due to its proliferation-stimulating activity, c-Myc belongs to the most frequently upregulated proteins in cancer cells, including SH-SY5Y neuroblastoma cells (Hammerling *et al.* 1987). We can consider such deregulation as a possible weak point of cancer cells. In contrast to fibroblasts, SH-SY5Y cells decreased proliferation at hypoxia and this was associated with a drop in the intracellular 2HG/2OG ratio. The typical cancer glycolytic cells such as SH-SY5Y had already reset their metabolism during oncogenesis and further exposure to hypoxia transfers them to a rather dormant state (Plecitá *et al.* 2015) while disables their proliferation. Their mitochondria generate a “resistance“ for hypoxia, recognized in the switch between the cytochrome *c* oxidase subunit 4.2 substituting the

normoxic subunit 4.1 (Fukuda *et al.* 2007).

Metabolite/2OG ratios were similar or malate/2OG was slightly lower in hypoxic fibroblasts compared to non-hypoxic ones. Since major metabolites increased only moderately at hypoxia including 2OG, consequently 2HG/2OG ratio was elevated due to the profound 2HG increase. In contrast, typical citrate accumulation (much higher citrate/2OG ratio) was found in hypoxic SH-SY5Y cells.

Since hypoxia is frequent in solid tumor tissues, pharmacological inhibition of enzymes balancing 2HG levels might be suggested as a feasible complementary tumor therapy. This concerns with the enzymes of aminolevulinate degradation, mitochondrial hydroxyacid-oxoacid-transhydrogenase (HOT, or ADHFE1), cytosolic phosphoglycerate dehydrogenase, or finally, cytosolic and mitochondrial IDH1, IDH2, respectively, which all synthesize 2HG; or, alternatively, with the pharmacological induction of R-2HG-dehydrogenase, eliminating 2HG.

Regulation of 2HG levels has not yet been elucidated and remains completely obscure. We can theoretically predict that 2HG levels stem from the balance between the 2HG-producing vs. 2HG-consuming reactions. Besides the IDH1 and IDH2 reactions, to which even the non-mutant IDH1 or IDH2 may contribute (Smolková *et al.* 2015, Terunuma *et al.* 2014), R-2HG is produced from aminolevulinate (Chalmers *et al.* 1980, Tafti *et al.* 2003), or by mitochondrial hydroxyacid-oxoacid-transhydrogenase (HOT) from 2OG (Struys *et al.* 2004), thus competing with IDH2 for 2OG; and by cytosolic phosphoglycerate dehydrogenase from 2OG (Fan *et al.* 2015), competing with IDH1 for 2OG. In turn, R-2HG is specifically degraded to 2OG by the R-2HG-dehydrogenase, localized in the mitochondrial matrix (Achouri *et al.* 2004) as well as S-2HG by S-2HG-dehydrogenase (Worth *et al.* 2015). The fate of cytosolic 2HG produced by mutated IDH1 is not exactly known. The slow reverse phosphoglycerate dehydrogenase reaction and possible non-existence of other active degradation pathways for the cytosolic 2HG most probably leads to its accumulation and oncogenic role. In turn, we may hypothesize that with higher mitochondrial biogenesis in cancer cells the 2HG degradation in the matrix is higher as well.

In conclusion, overall lower basal 2HG levels may participate in the numerous physiological regulations. We have found that 2HG facilitates proliferation of primary fibroblasts.

Conflict of Interest

There is no conflict of interest.

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