Pyruvate kinase isoenzyme M2 is a glycolytic sensor differentially regulating cell proliferation, cell size and apoptotic cell death dependent on glucose supply

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Running title: PKM2 regulates cell population growth parameters.

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Abstract

The glycolytic key regulator pyruvate kinase M2 (M2-PK or PKM2) can switch between a highly active tetrameric and an inactive dimeric form. The transition between the two conformations regulates the glycolytic flux in tumor cells. We developed specific M2-PK-binding peptide aptamers which inhibit M2-PK, but not the 96% homologous M1-PK isoenzyme. In this study we demonstrate that, at normal blood glucose concentrations, peptide aptamer-mediated inhibition of M2-PK induces a significant decrease of the population doubling (PDL rates) and cell proliferation rate as well as an increase in cell size, whereas under glucose restriction an increase in PDL and cell proliferation rates but a decrease in cell size was observed. Moreover, M2-PK inhibition rescues cells from glucose starvation-induced apoptotic cell death by maintaining the metabolic activity. These findings suggest that M2-PK is a metabolic sensor which regulates cell proliferation, cell growth and apoptotic cell death in a glucose supply-dependent manner.

Key words:

Apoptosis, caloric restriction, PKM2, proliferation, growth, metabolic activity.

Abbreviations

ADP, Adenosindiphosphat; ATP, Adenosintriphosphat; Dox, doxycycline; M2-PK or PKM2, pyruvate kinase M2; M1-PK, pyruvate kinase M1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAD(P)H, Nicotinamidadenindinukleotid(phosphate); PDL, population doubling rate; PEP, phosphoenolpyruvate.

Introduction

The microenvironment of a spheroid tumor mass is characterized by heterogeneous nutrient supply with relatively high concentrations of oxygen and nutrients, such as glucose, in the periphery and very low oxygen and nutrient concentrations in the inner regions [reviewed in 1]. Nutrient deprivation reduces cell growth and survival. Accordingly, caloric restriction was found to correlate with cancer resistance in rodents [2] and to induce apoptotic cell death and inhibition of cell proliferation in tumors of mice and humans [3,4]. However, like unicellular eukaryotes normally proliferating cells and tumor cells can adapt their metabolism to variations in nutrient supply. A well known adaptation is the shift from oxidative energy regeneration to glycolysis even in the presence of oxygen [5,6]. Accordingly, quantification of regional glucose uptake in humans by positron emission tomography (PET) using 2-[(18)F]-fluoro-2-deoxy-D-glucose (^{18}F-FDG) demonstrated a significantly higher glucose uptake rate in tumors relative to normal differentiated tissues [7]. In tumor cells, aerobic glycolysis is triggered by growth-signaling, mitogenic, and *p*O2-sensing pathways which induce overexpression of glycolytic enzymes [8-10, reviewed in 11-13]. Activating ras mutations, which are frequently found in human cancers [14], induce upregulation of the glycolytic enzyme machinery [10,11,15-20] and increased level of fructose-2,6-bisphosphate (F2,6BP) the principal reaction product of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2) which is a potent allosteric activator of the rate-limiting glycolytic enzyme 6-phosphofructo-1-kinase (PFK1) and thereby glycolysis [10,21]. Oncogenic signalling through the PI3K/Akt pathway was shown to induce increased glucose uptake by increasing surface expression of glucose transporters and supports a high glycolytic flux by activating hexokinase II and PFK2 [11,12,22]. Moreover, hypoxia-inducible transcription factors (HIFs) are highly induced by hypoxia and activated oncogenes in human cancers. HIFs were shown to induce aerobic glycolysis [8,11,23,24] as well as inhibitors of mitochondrial metabolism [23]. The resulting increased glycolytic capacity allows tumor cells to proliferate, grow, and survive

under hypoxia, as frequently occurs in solid tumors. However, the vast majority of those cells exhibit slower proliferation and undergo apoptosis under glucose restriction [25,26]. Yet a small number of tumor cells can overcome glucose deprivation, suggesting that additional adaptations exist which facilitate cell proliferation and survival under such conditions. In fact, nutrient availability has been shown to be an important determinant for the control of cell proliferation, growth (size), and death [27,28]. In the presence of appropriate amounts of nutrients and growth stimuli, cells display high synthesis rates of cell building blocks. They increase in size and show enhanced proliferation rates, which are often controlled relative to size. In contrast, cells respond to nutrient limitation by a downregulation of biosynthetic processes and enhancement of mass turnover, which together result in decreased cell growth. Thus, the control of cell growth as well as cell proliferation involves a balanced regulation between anabolic and catabolic processes [28]. The activities of metabolic enzymes in tumor cells are accordingly regulated in response to nutrients, metabolites, as well as oncogenes [29,30].

In tumor cells, the pyruvate kinase isoenzyme M2 (M2-PK or PKM2) (EC 2.7.1.40) is one important regulator of the balance between glycolytic energy regeneration and the synthesis of cell building blocks. Pyruvate kinase catalyses the dephosphorylation of phosphoenolpyruvate (PEP) to pyruvate and is responsible for net ATP production in glycolysis. Four pyruvate kinase isoenzymes (M1, M2, L, and R) are known. M2-PK is the embryonic form which is progressively replaced during differentiation by M1-PK in muscle and brain, L-PK in liver and kidney, and R-PK in erythrocytes. When quiescent cells re-enter the cell cycle the tissue-specific isoenzymes are replaced by M2-PK [30]. In most, if not all, tumors M2-PK is overexpressed. Knockdown of M2-PK and replacement of M2-PK by M1-PK were shown to reduce the ability of human tumor cell lines to form tumors in nude mouse xenografts [31]. The M2 and M1-PK isoenzymes are different splicing products of the same gene and differ only in 23 out of 531 amino acids. In contrast to the 96% homologous M1-PK

isoenzyme, which exists only in a highly active tetrameric form, M2-PK can shift between a tetrameric form, which is characterized by a high affinity for its substrate PEP, and a dimeric form with a low PEP affinity [30]. Under physiological conditions the tetrameric form is highly active whereas the dimeric form is almost inactive. Here the tetramer:dimer ratio of M2-PK determines whether glucose carbons are degraded to pyruvate and lactate with production of ATP (tetrameric form) or are channeled into synthetic pathways debranching from glycolysis (dimeric form) [15,16]. Tumor cells are characterized by high levels of the dimeric form of M2-PK and the shift into this conformation as well as an inhibition of the enzyme was shown to be induced by viral oncoproteins (HPV-16 E7 and pp60v-src kinase) [29,30] as well as by cellular phosphotyrosine signalling [32]. The ras oncogene induces the expression, tetramerization and thereby activation of M2-PK [15,16]. In cooperation with oncogenic factors that induce M2-PK inactivation ras signaling is important for the fine tuning of M2-PK activity [16]. This regulatory network generates a glycolytic phenotype which achieves efficient ATP synthesis as well as a high capacity for the biosynthesis of nucleic acids, lipids and amino acids. This is crucial for the proliferation, growth and survival of tumor cells under the heterogeneous oxygen and nutrient supply frequently occurring in solid tumors in vivo [1,6,12,13]. Evidence was also presented for the existence of a crosstalk between oncogenic regulation of M2-PK and cellular regulator metabolites. Thus, oncogenic A-Raf modulates the quaternary structure of M2-PK dependent upon the intracellular levels of serine, which is a strong allostric activator of M2-PK [33]. Another important metabolic regulator of M2-PK is fructose 1,6-P2. High levels of this glycolytic intermediate reflect sufficient amounts of phosphometabolites and induce re-association of the inactive dimeric form of M2-PK to the highly active tetrameric form, thus shifting tumor metabolism from synthesis of cell building blocks to energy regeneration [30].

In a screen for isotype-specific inhibitors of M2-PK we identified 14 peptide aptamers out of 6.5 million different variants which interact specifically and with high affinity with

M2-PK but not with the 96% homologous M1-PK isoenzyme [34]. Peptide aptamers consist of a variable structurally constrained peptide loop integrated into a scaffold protein, thereby conferring an affinity for a given target protein which is several orders-of-magnitude higher compared to the free peptide of the same amino acid sequence. These synthetic molecules can modulate sophisticated functions of a given protein. Binding of the specific peptide aptamers to M2-PK inhibits re-association of the inactive dimeric form of M2-PK to the highly active tetrameric form, thereby reducing the pyruvate kinase mass action ratio ([ATP] * [Pyruvate]):([ADP] * [PEP]) as well as the ATP:ADP ratio [34]. Because of the key regulatory role of the tetramer:dimer ratio of M2-PK on the balance between the catabolic and anabolic utilization of glucose carbons, we investigated the impact of the tetramer:dimer ratio of M2-PK on cell proliferation, cell size, and apoptotic cell death in dependency on the glucose supply. To address this question M2-PK was fixed in the dimeric form using a specific M2-PK binding peptide aptamer.

Materials and methods

Cell culture and retroviral gene expression system

NIH 3T3 cells were obtained from A.T.C.C. (Rockville, MD, USA). The basic cell culture medium was glucose-free DMEM (Sigma, Vienna, Austria) supplemented with 10 % (v/v) CS and 2 mM glutamine. Glucose was added into the medium as indicated and the medium was changed every 2 days. Antibiotics and doxycycline (Dox) were added as described. Polyclonal NIH 3T3 cell lines containing the doxycycline-inducible retroviral expression vectors pSITV-Neo/Pep.Apt.9 and pSITV-Neo/Pep.Apt.26, respectively [34] were generated as follows: cDNAs encoding for aptamer 9 and aptamer 26, respectively, were inserted in the retroviral vector pSITV-Neo (neomycin) under control of a Dox-regulated promoter (tet-CMV). NIH 3T3 cells were sequentially infected with the pSITV-Neo/Pep.Apt. retroviruses and the pLIB-rtTA-M2-ires-TRSID-ires-PURO retrovirus vector which expresses the tet-transactivator rtTA-M2, the tet-repressor TRSID and the puromycin resistance gene. The polyclonal cell lines were selected in puromycin (2 μg/ml) and neomycin (800 μg/ml) containing medium for two weeks. Afterwards these cells were cultivated in puromycin (2 μg/ml) and neomycin (100 μg/ml) containing medium. In the absence of Dox the tet-repressor binds to the tet-CMV-promoter thereby actively repressing aptamer transcription. In presence of 1 μg/ml Dox the tet-transactivator (rtTA) replaces the repressor and the peptide aptamers are expressed.

Western blot analysis

Westernblot analysis was conducted as described [34]. Peptide aptamers were detected using antibodies against the Thioredoxin A scaffold (α-Thioredoxin A clone T0803, Sigma, Vienna, Austria).

Determination of population doublings (PDLs)

Polyclonal cell lines expressing Apt.9 or Apt.26 (NIH 3T3/pSITV-Neo-Apt.9 or NIH 3T3/pSITV-Neo-Apt.26) were cultivated in puromycin (2 μg/ml) and neomycin (100 μg/ml) containing medium. To determine population doublings (PDLs) cells were cultivated for 6 cultivation days and the PDLs were calculated from the initial (I) and final cell number (E) according to the following equation: PDL per 6 days = (log(E)-log(I))/log 2 [34].

BrdU incorporation

Peptide aptamer expression was induced by Dox in logarithmically proliferating NIH 3T3 cells, which need ~19.5 hours for one cell reduplication. The cells were grown on 96 well plates (10.000 cells per well) in medium containing either 5.5 mM or 0.6 mM glucose and labelled in the presence of 10 μM BrdU for 18 hours. BrdU incorporation was analyzed using the Roche 5-Bromo-2´-deoxy-uridine Labelling and Detection Kit III (Roche, Vienna, Austria).

Apoptosis assays

Apoptotic cells were detected A) according to Nicoletti and B) by Annexin V staining and counted by flow cytometry using a Becton Dickinson FACSCalibur. A) Adherent and floating cells were harvested, washed with PBS and suspended in propidium iodide buffer (0.1% Triton X-100, 0.1% Na Citrat, 50 μg/ml propidium iodide in H_2O). After incubation for 30 min at 4°C, flow cytometry was performed with a Becton Dickinson FACSort. Apoptosis was determined by using the CellQuest software package. B) Cells were trypsinized, washed with PBS and suspended in 50 μl 1x Annexin V binding buffer containing 2 μl Annexin V-APC (BD, Vienna, Austria). After 15 min incubation at room temperature in the dark 200 μl of 1x binding buffer were added and cells analyzed by flow cytometry.

MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is used for measuring the metabolic activity of cells as a parameter of viability [35]. This approach is based on the reduction of the tetrazolium salt MTT within active mitochondria of living cells by succinate dehydrogenase [36] as well as by the reduction of cytoplasmic pyridine nucleotides [37] to an insoluble blue formazan derivative. The same cell numbers were seeded and cultured 4 days with or without Dox. 10 µl of MTT reagent was added per well and the plates were incubated for 4 hours at 37°C, 5% CO2. MTT is reduced to formazan by the action of cellular enzymes using predominantly NAD(P)H as coenzyme. Cells were lysed and formazan was solubilised overnight at room temperature in the presence of 100 µl 10%SDS/0.01 N HCl per well. In each well absorbance was measured at 570 nm using 655 nm as reference wavelength.

Determination of cell size

Cell size was determined by forward scattering and flow cytometry from 10.000 exponentially growing cells as described in Valentinis *et al*., [38].

Statistical analysis

Statistical significances were calculated using the unpaired t-test or the Mann-Whitney test (GraphPad PRISM version 3.03).

Results

Effect of the M2-PK-inhibiting aptamer on the population doubling rate

To analyze the effects of peptide aptamer-induced inhibition of M2-PK on the population doubling rates (PDLs) polyclonal cell lines were generated from NIH 3T3 cells [39], which express either the M2-PK-binding peptide aptamer 9 (Apt.9) or, as control, the non-M2-PK binding peptide aptamer 26 (Apt.26) (see Table 1) [34] from doxycycline (Dox)-inducible retroviral expression vectors (pSITV-Neo/Pep.Apt.9 and pSITV-Neo/Pep.Apt.26). The Dox-induced expression of the peptide aptamers was confirmed by western blotting (Fig. 1). As shown in Fig. 2A, the PDLs of the NIH 3T3 cells strongly depend on glucose supply. In cells expressing the non-M2-PK binding Apt.26, PDLs decreased from 7.3 PDLs/6 days when cultivated in the presence of 5.5 mM glucose (normal blood glucose concentration) to 0.4 PDLs/6 days when cells were cultivated in the presence of 0.6 mM glucose (glucose starvation). When the cells were cultured under conditions of glucose starvation (0.6 mM glucose), the PDL rate was two times higher in M2-PK-binding Apt.9 expressing cells (0.96 PDLs/6 days) relative to the non-M2-PK binding Apt.26 expressing cells (0.44 PDLs/6 days (Fig. 2B). In contrast, in the presence of 5.5 mM glucose Apt.9 expression induced a moderate but significant decrease of the PDL rate from 3.9 PDLs/5 days to 3.5 PDLs/5 days [34].

Effect of the M2-PK-inhibiting aptamer on cell proliferation

To study whether enhanced cell proliferation contributes to the Apt.9-induced increase of the PDLs in glucose-restricted cells, we compared the DNA synthesis rate by BrdU incorporation into Apt.9- and Apt.26-expressing NIH 3T3 cells. In glucose-restricted NIH 3T3 cells, Dox-induced expression of the M2-PK-binding Apt.9 led to a significant (approximately 20%) increase in cell proliferation when compared to NIH3T3 cells without

Dox and to NIH 3T3 cells with Dox-induced expression of the non-M2-PK-binding Apt.26 (Fig. 3A). In the absence of Dox, when neither Apt.9 nor Apt.26 were expressed, no differences were found in the proliferation rates between the two polyclonal NIH 3T3 cell lines, indicating a specific effect of the M2-PK-inhibiting Apt.9. In contrast, at normal glucose concentrations (5.5 mM) the expression of Apt.9 significantly decelerated cell proliferation from 100.0 ± 6.6 to 91.8 ± 3.8% ($p<0.05$) (Fig. 3B) [34].

Effect of the M2-PK-inhibiting aptamer on apoptotic cell death

To address the question whether protection against apoptotic cell death contributes to the increased PDLs found in Apt.9-expressing NIH 3T3 cells under glucose restriction, we studied the apoptotic cell death rates in Apt.9 and control Apt.26-expressing polyclonal NIH 3T3 cells. When cultivated for 72 hours in the presence of 5.5 mM glucose neither Apt.9 nor Apt.26 had a noteworthy effect on apoptotic cell death (Fig. 4A and C). However, when cultivated for 72 hours under glucose restriction a strong induction of apoptotic cell death was observed in NIH 3T3 cells expressing control Apt.26. This is reflected by the increased number of cells with Annexin V staining (Fig. 4B) and nuclei with sub-G1 DNA content (Fig. 4D). Similar results were obtained with NIH 3T3 cells which had been transfected with the empty vector (data not shown). Interestingly, in glucose-restricted Apt.9-expressing NIH 3T3 cells a considerably lower apoptotic cell death rate was observed than in glucose-restricted Apt.26-expressing cells (Fig. 4B and D).

Effect of the M2-PK-inhibiting aptamer on metabolic activity

Apoptotic cell death and proliferation is regulated by the complex interplay of cellular signaling processes and metabolism, especially glucose metabolism [11,12]. Moreover, tumor cells have been shown to display a decreased susceptibility to stimuli that induce apoptosis due to their capability to maintain high metabolic activity [12,40,41]. It was interesting to

analyze whether the enhanced proliferation and protection against apoptotic cell death, observed in the M2-PK-inhibiting Apt.9-expressing cells under glucose starvation, correlates with maintenance of the cellular metabolic activity. The MTT assay was used to measure the impact of Apt.9 and Apt.26 expression on this parameter. Intracellular reduced succinate within active mitochondria of living cells as well as cytoplasmic pyridine nucleotides, NAD(P)H, are rate limiting for cellular reduction of MTT [36,37], with the reduction of MTT involving mitochondrial and cytoplasmic enzymes. On cultivation in the presence of 0.6 mM glucose, expression of Apt.9 induced a significant increase in MTT reduction by 37% (Fig. 5A), indicating that the inhibition of M2-PK by Apt.9 increases the metabolic activity of the glucose starved cell population. In contrast, in 5.5 mM glucose Apt.9 induced a decrease in MTT reduction by 17% (Fig. 5B). The expression of control Apt.26 did not change MTT reduction, neither at 0.6 mM nor at 5.5 mM glucose. The data show that the inhibition of M2-PK enhances the maintenance of the metabolic activity in the cell population under glucose starvation. On the other hand, the inhibition of M2-PK leads to decreased metabolic activity at the high glucose concentrations found in circulating blood.

Effect of the M2-PK-inhibiting aptamer on cell size

M2-PK plays a key role in the channeling of glucose carbons either into catabolic or into anabolic pathways. A high level of the low-activity dimeric form of M2-PK coincides with increased amounts of glycolytic phosphometabolites and hence facilitates biosynthetic processes branching off from the glycolytic pathway [15,16]. Therefore, we investigated whether changes in cell size (as parameter for biosynthetic capacity) correlate with the increased PDLs observed in M2-PK-inhibiting Apt.9-expressing glucose-restricted cells. Indeed, under glucose restriction the expression of Apt.9 induced a significant decrease in cell size (Fig. 6A) compared to the control Apt.26 whereas in the presence of 5.5 mM glucose Apt.9 caused a significant increase in cell size (Fig. 6B).

Discussion

Proliferating cells double their cell size before dividing [28]. Both cell growth (increase in size) and cell division consume large amounts of ATP. Moreover, the increase in cell size is due to an increase in the synthetic rates of cell building blocks such as phospholipids, proteins, and nucleic acids. In normal proliferating cells and in tumor cells glycolysis is important for energy regeneration as well as the provision of precursors for synthetic processes. A key regulator of this bi-functional role of glycolysis is M2-PK. To study the importance of M2-PK for cell proliferation, cell growth, and apoptotic cell death an isotype-specific M2-PK-binding peptide aptamer (Apt.9) was employed which interacts only with M2-PK but not with the 96% homologous M1-PK isoenzyme [34]. The binding of Apt. 9 to M2-PK leads to fixation of the enzyme in its inactive dimeric form. High levels of the dimeric form of M2-PK favor the channeling of glucose carbons into synthetic processes. However, glycolytic energy regeneration is restricted when M2-PK is fixed in the inactive dimeric form [30]. Accordingly, when glycolysis is the main energy source, as in NIH 3T3 cells cultivated in a medium with normal blood glucose concentrations (5.5 mM) [39], Apt.9-induced fixation of M2-PK in the dimeric form leads to a reduction of the ATP:ADP ratio and a downregulation of the PDL and cell proliferation rates relative to Apt.26 cells (Fig. 2 and 3; ref. 34). In tumor cells and normal proliferating cells a second main pathway of energy regeneration is glutaminolysis. Accordingly, also the NIH 3T3 cells used in our study are characterized by a high intrinsic glutaminolytic capacity [39]. However, in contrast to glycolysis glutaminolytic energy production depends on oxygen. Low glucose supply was shown to activate glutaminolytic energy regeneration in different tumor cells under normoxic conditions [42,43]. Glutamine and oxygen were not limited in our experiments. Therefore in glucose starved NIH 3T3 cells energy could be provided by glutaminolysis. Hence, under glucose starvation Apt.9 induced fixation of M2-PK in the inactive dimeric form facilitated the complete channeling of the less glucose available into synthetic processes. This can explain the increase in the proliferation

rate of the Apt.9-expressing glucose-starved NIH 3T3 cells (Fig. 2 and 3), because biosynthesis was optimized by inhibition of M2-PK and energy could be provided by glutaminolysis under normoxic conditions.

As in other cell types [44], also in NIH 3T3 cells glucose deprivation strongly induced apoptotic cell death (Fig. 4). However, in Apt.9-expressing glucose-starved NIH 3T3 cells a downregulation of apoptotic cell death was observed which indicates that the fixation of M2-PK in the dimeric inactive form rescues cells from glucose starvation-induced apoptotic cell death. In several studies the decreased susceptibility of tumor cells to apoptotic stimuli was linked to their capability to maintain a high metabolic activity and this has been shown to activate survival pathways and inhibit pro-apoptotic proteins [12,41,45]. Similarly, also in our cell model at low glucose supply the rescue from apoptosis in the M2-PK-inhibiting Apt.9-expressing cell population was accompanied by a significant increase in the metabolic activity, as shown by increased reduction of the formazan MTT (Fig. 5), whereas the metabolic activity was reduced by Apt.9-expression in the cell population growing under high glucose supply. MTT reduction reflects cytoplasmic as well as mitochondrial redox capacities and is often used as parameter for survival of a cell population [35,36]. Due to our results a role of M2-PK within the regulation of the cellular metabolic activity which influences apoptotic cell death as well as proliferation is conceivable.

Besides NADH + H^+ and $FADH_2$ glutaminolysis also generates NADPH + H^+ (malic enzyme reaction) which serves as co-substrate in the fatty acid de novo synthesis [22] as well as for the recycling of glutathione during ROS detoxification [46]. Another source for NADPH + H^+ regeneration is the oxidative pentose P-Pathway (PPP). It was suggested that a large amount of the dimeric form of M2-PK favors the channeling of glucose into the oxidative PPP, thereby enhancing NADPH concentrations [47]. The importance of the activity of glucose-6-phosphate dehydrogenase, the rate limiting enzyme of the PPP, was demonstrated for NADPH + H^+ production and cell proliferation [48] as well as survival [40,

reviewed in 22]. Together these data suggest that a mechanism maintaining the cellular metabolic capacity contributes to the rescue from apoptotic cell death induced by M2-PK-inhibitor Apt.9.

Several studies have shown a high glycolytic flux to be correlated with a high cellular metabolic activity or redox potential [for a review, see for example ref. 41]. This can explain the relative inhibitory effect of the M2-PK-binding peptide aptamer on cell proliferation when glucose is abound. Cell survival was related with oncogenic Akt which induces an increase of mTOR-dependent nutrient uptake 49,50]. However, the pro-survival function of activated Akt depends on its stimulatory effect on glucose metabolism [12] and decreases in medium with low glucose concentrations [41,51]. This suggests that not only the activation of glycolysis by ras, Akt, HIF1 and probably other oncogenic factors, is an advantage for tumor cell survival, especially not within hypoglycemic regions of solid tumors, but also the fine tuning of the glycolytic flux is important. Accordingly, in different studies with humans and mice nutrient deprivation was shown to reduce tumor cell growth and tumor cell survival [2,3,4]. The data presented in the present study, suggest that at low glucose supply, the Apt.9 induced inhibition of M2-PK optimized tumor cell metabolic activity, proliferation, growth and survival by economizing the less glucose available to synthetic processes.

However, in solid tumors in areas with low glucose supply also the oxygen supply is limited [reviewed in 1]. Hypoxia leads to a stabilization of HIF1α which favors glycolytic energy production and suppresses mitochondrial energy regeneration due to an induction of the pyruvate dehydrogenase (PDH) inhibiting protein kinase PDK1. Furthermore, oxygen dependent glutaminolysis is inhibited under hypoxic conditions [reviewed in 23]. In this scenario, when both glucose and oxygen supply are limited as frequently found in a spheroid tumor mass, the Apt. 9 induced inhibition of glycolytic energy production can not be replaced by glutaminolytic energy regeneration. Hence, in glucose and oxygen starved Apt.9 expressing cells an inhibition of proliferation and an increase of apoptotic cell death is likely.

The increase in cell size found in Apt. 9 expressing NIH 3T3 cells when cultivated at high glucose supply can be explained by the expansion of glycolytic intermediates and increase of cell building blocks in cells with a high amount of the dimeric form of M2-PK [22,30]. Activated Akt was shown to maintain cell size by increasing nutrient uptake and stimulation of glycolysis at the level of glucose import, Hexokinase II and PFK-1 under normoglycemic conditions [12,13,41]. Together with these findings, our results suggest that both the inhibition of M2-PK and the activation of the upper part of glycolysis facilitate cell growth when sufficient glucose is available. Under glucose starvation the fixation of M2-PK in the inactive dimeric form leads to an enhancement of cell cycle progression as well as survival, obviously at the expense of cell size. The modulation of cell size by the inhibition of M2-PK suggests that the glycolytic pathway is linked with cell size-controlling systems via M2-PK. Moreover, the impact of the inhibition of M2-PK on cell size varies depending upon glucose supply, suggesting that the glycolytic pathway is also linked with glucose-sensing systems via M2-PK. This is supported by the result that fine tuning of M2-PK activity determines whether glucose carbons are used for glycolytic ATP synthesis or are channeled as glycolytic phosphometabolites into synthetic pathways for the synthesis of cell components [29,30].

Cell growth (size) control is often, if not always, linked to the target of rapamycin complex (TORC in yeasts or mTORC in mammals), a protein kinase complex that regulates growth in response to nutrient availability in all eukaryotic cells examined [27,28]. In turn mTOR has been shown to control cellular homeostasis of glucose and other nutrients. Nutrient deprivation leads to an inhibition of the mTOR kinase and a downregulation of anabolic and activation of catabolic processes, and thus to a reduction of cell growth and protein synthesis [28,41], conserving cellular energy stores and promoting survival [41]. mTOR was recently shown to control also lipid synthesis [52]. This might contribute to cell size control (reduction) under glucose starvation. An inverse correlation between the cellular redox ratio and cell size was demonstrated [53]. Moreover, evidence was provided that redox-sensitive signaling

mechanisms participate in regulating the nutrient-sensitive TOR complex in yeast [54] and mammals [55]. Thus it is conceivable that the intracellular metabolic activity or redox-capacity may link M2-PK activity with cell size control.

Taken together we show in the present study that, at high glucose concentrations, when glycolysis is the main energy-generating pathway, peptide aptamer-mediated fixation of M2-PK in the inactive dimeric form reduces cell proliferation and increases cell size, whereas under glucose starvation, when oxygen dependent glutaminolytic energy regeneration is ensured, the inhibition of M2-PK increases cell proliferation accompanied by decreasing cell size. Moreover, the maintenance of a high metabolic activity and a rescue from glucose deprivation-induced apoptotic cell death is shown in M2-PK-inhibited cells. Modulation of the cellular metabolic activity, proliferation, size as well as apoptosis by M2-PK-sensing pathway(s) could be a mechanism(s) contributing to regulation of the growth of a cell population. The elucidation of the precise mechanism(s) by which the activity of M2-PK is communicated to pathways regulating cell proliferation, cell size, and cell death warrants further studies.

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Table 1: Amino acid sequences (single letter code) of the variable region of the M2-PK-binding peptide aptamer 9 and the non-M2-PK-binding control peptide aptamer 26.

Peptide aptamer	Amino acid sequence
M2-PK binding Apt.9	EGMVLPTVWQPANWMCRLSN
M2-PK non-binding Apt.26 (negative control)	KVPVHRTCVACLLVVNSRCM

Legends to the figures

Fig. 1. Verification of Apt.9 and Apt.26 expression in Dox-regulated NIH 3T3 cells

Apt.9 and Apt.26 expression was induced by addition of 1 µg/mL Dox into the medium. After 24 hours Apt.9 and Apt.26 expression was confirmed by western blotting using a monoclonal antibody against the peptide aptamer scaffold α-thioredoxin A. Actin served as input control.

Fig. 2. Effect of the M2-PK-binding peptide aptamer 9 on the cell population doubling rate of NIH 3T3 cells

(A) Population doublings (PDLs) of Dox-induced Apt.26-expressing cells cultivated in the presence of 5.5 mM and 0.6 mM glucose, respectively. $\bar{x} \pm$ SD; $n = 6$. (B) PDLs of Dox-induced Apt.9- and Apt.26-expressing NIH 3T3 cells cultivated in the presence of 0.6 mM glucose in the cultivation medium. $\bar{x} \pm$ SD; $n = 6$.

Fig. 3. Effect of the M2-PK-binding peptide aptamer 9 on cell proliferation

BrdU incorporation was measured in Dox-induced Apt.9- and Apt.26-expressing polyclonal NIH 3T3 cells cultivated either in the presence of 0.6 mM glucose (A) or in the presence of 5.5 mM glucose (B). For calculation of the percentual cell proliferation rate of Apt.9- and Apt26-expressing cells, BrdU incorporation of NIH 3T3 cells cultivated in the absence of Dox. was set to 100%. $\bar{x} \pm$ SD; $n = 6$ in A) and $n = 5$ in B).

Fig. 4. Effect of the M2-PK-binding peptide aptamer 9 on apoptotic cell death

Polyclonal NIH 3T3 cells containing the Dox-inducible Apt.9 or Apt.26, respectively, were pre-cultivated in medium with 5.5 mM glucose without Dox. The medium was exchanged by a medium containing either 5.5 mM glucose or 0.6 mM glucose, as indicated, as well as Dox (1 µg/mL) in order to induce Apt.9 and Apt.26 expression, respectively. Apoptotic cell death was determined directly after medium exchange (t = 0 hours) as well as after 72 hours of cultivation either by Annexin V staining (A and B) or by PI-staining and counting of sub G1 cells (C and D). ($\bar{x}$ ± SD; n = 4).

Fig. 5. Effect of the M2-PK-inhibiting aptamer on metabolic activity

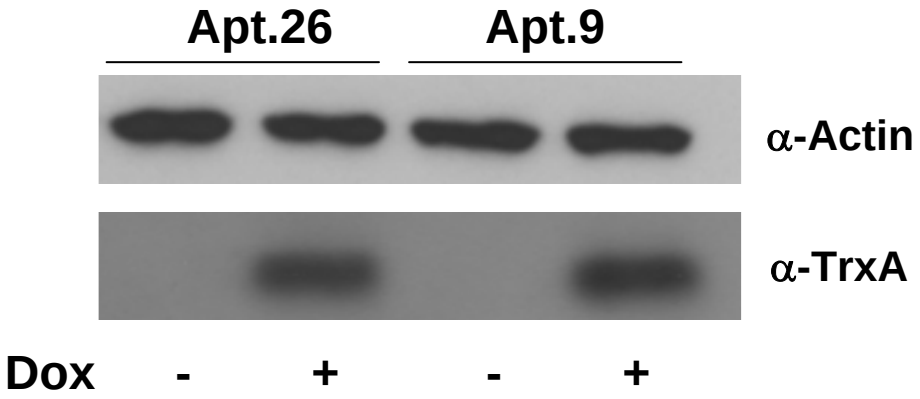
The intracellular metabolic activities were determined in Dox-induced Apt.9- und Apt26-expressing NIH 3T3 cells cultivated in medium containing either 0.6 mM glucose (A) or 5.5 mM glucose (B), as indicated, by measuring the reduction of MTT. The absorbance measured at 570 nm in the Apt.26-expressing cells was set to 100% at each point ($\bar{x} \pm$ SD; $n = 6$).

Fig. 6. Effect of the M2-PK-binding peptide aptamer 9 on cell size

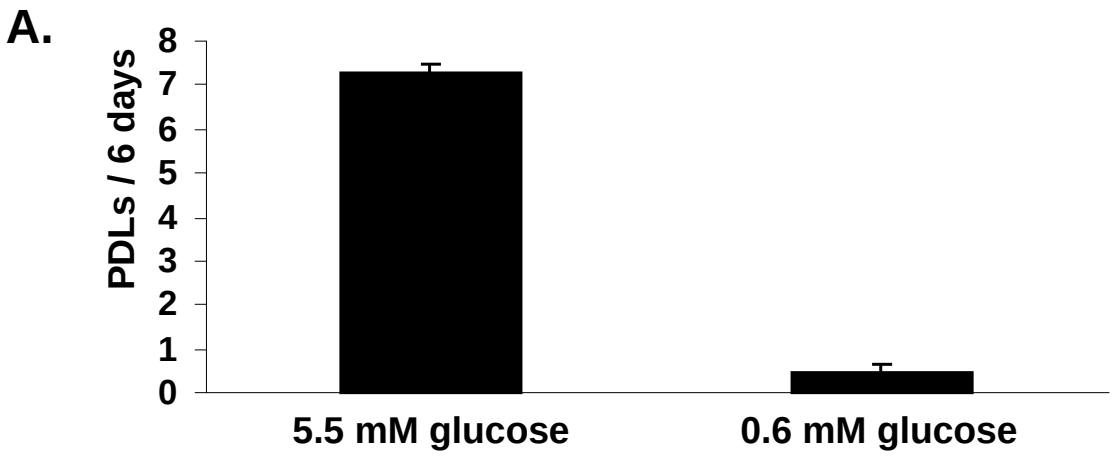
Polyclonal NIH 3T3 cells containing the Dox-inducible Apt.9 or Apt.26, respectively, were pre-cultivated in medium with 5.5 mM glucose without Dox. The medium was replaced by fresh medium containing either 0.6 mM glucose (A) or 5.5 mM glucose (B), as indicated, as well as Dox (1 µg/mL) to induce Apt.9 and Apt.26 expression, respectively. Cell volumes were determined by FACS analysis at $t = 0$ hours as well as after 72 hours of cultivation as indicated. The cell size of Apt.26-expressing cells was set to 100% at each time point ($\bar{x} \pm$ SD; $n = 4$).

Fig. 7. Metabolic scheme

Glycolysis (black color) with debranching synthetic pathways (red color) as well as glutaminolysis (blue color) as source for NAD(P)H and energy.

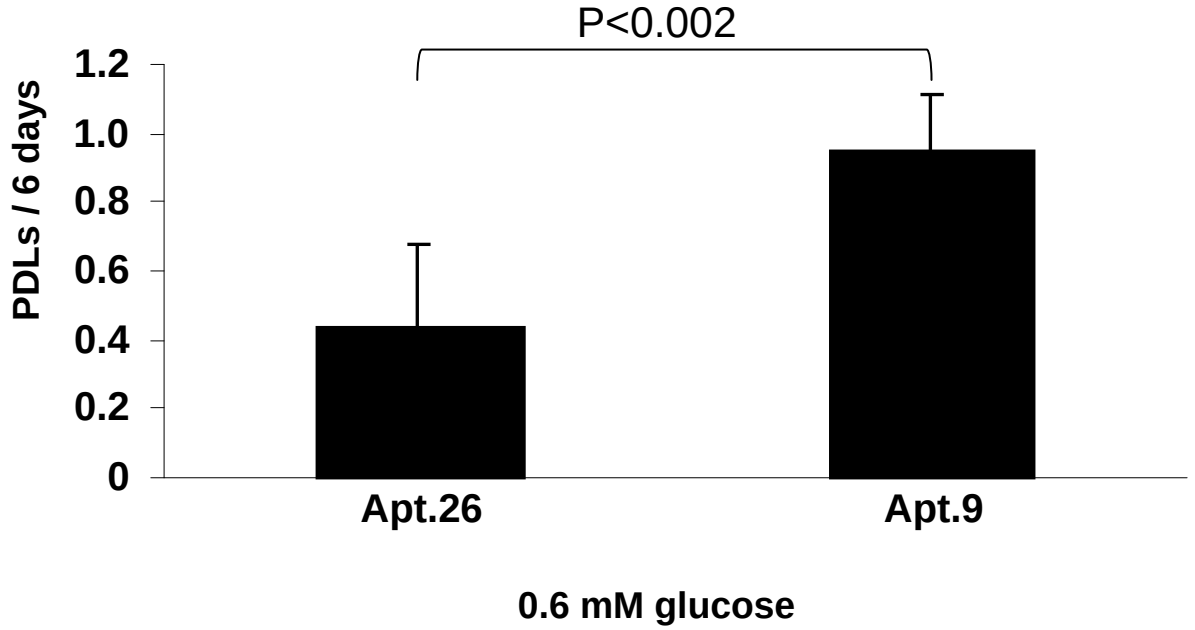


Spoden et al., 2009, Fig. 1



Spoden et al., 2009, Fig. 2

B.



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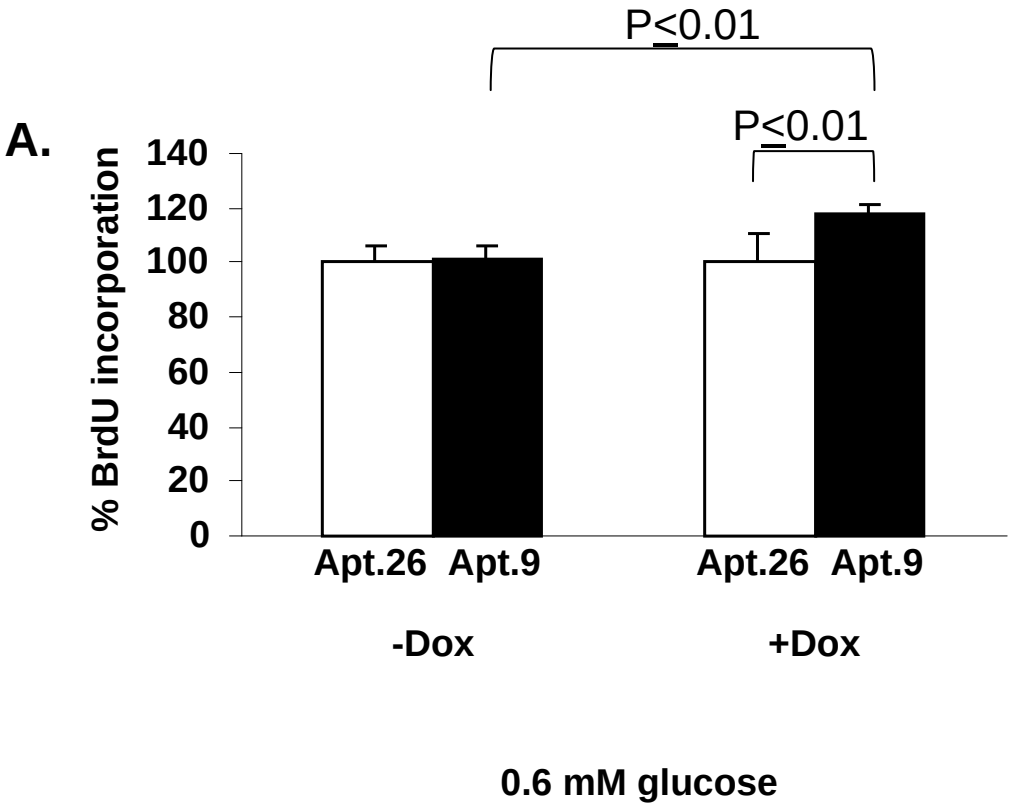

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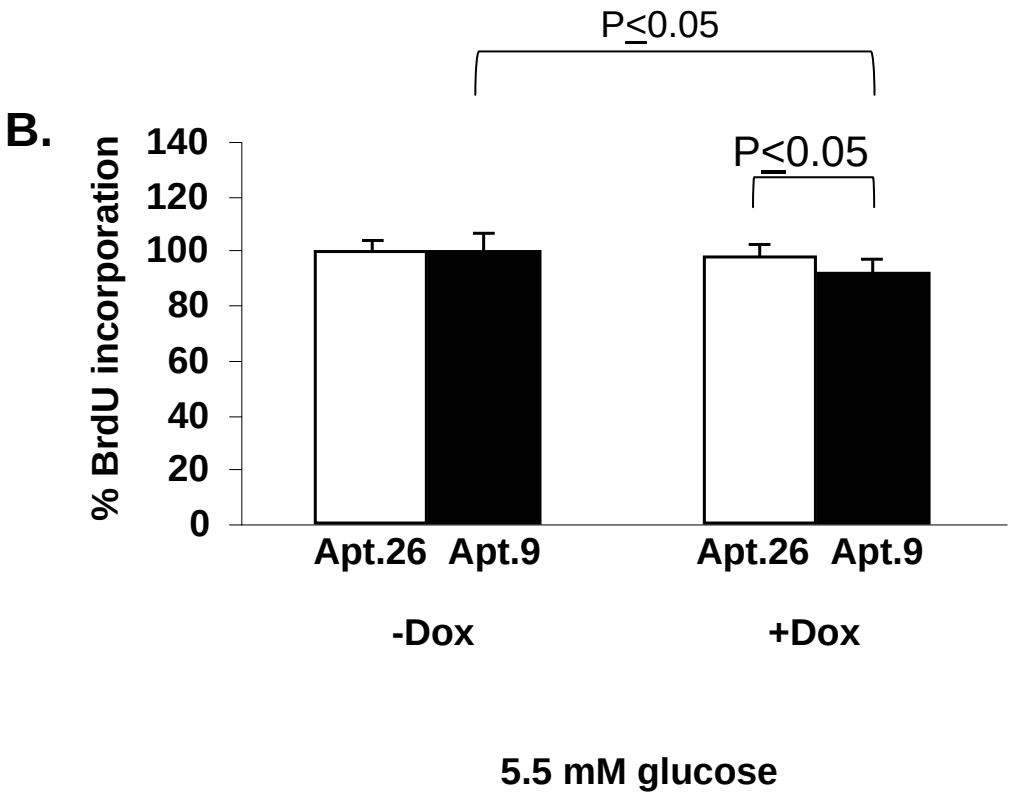

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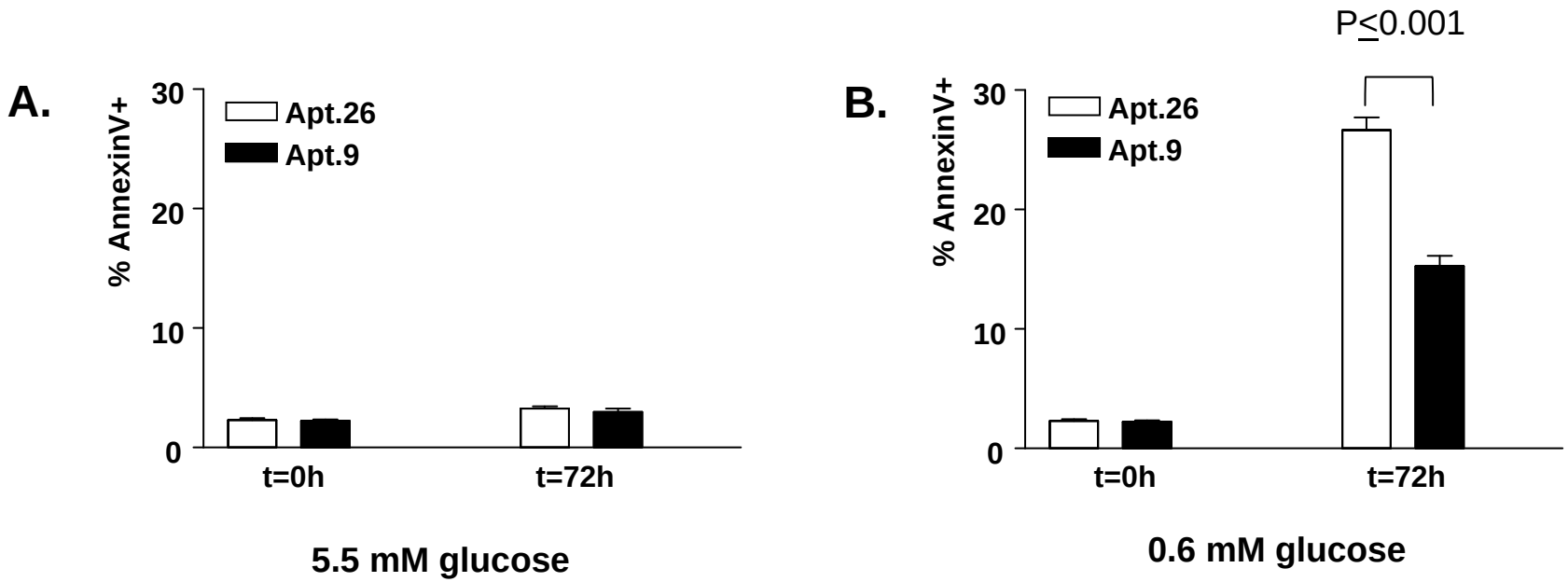

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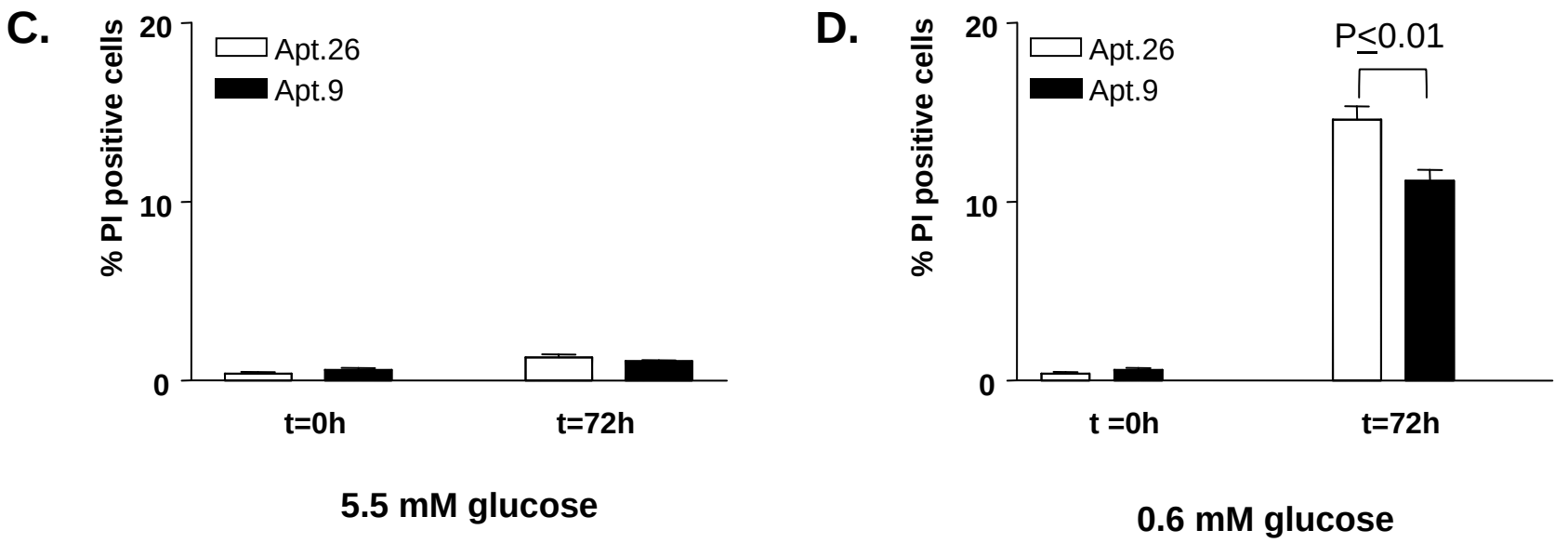
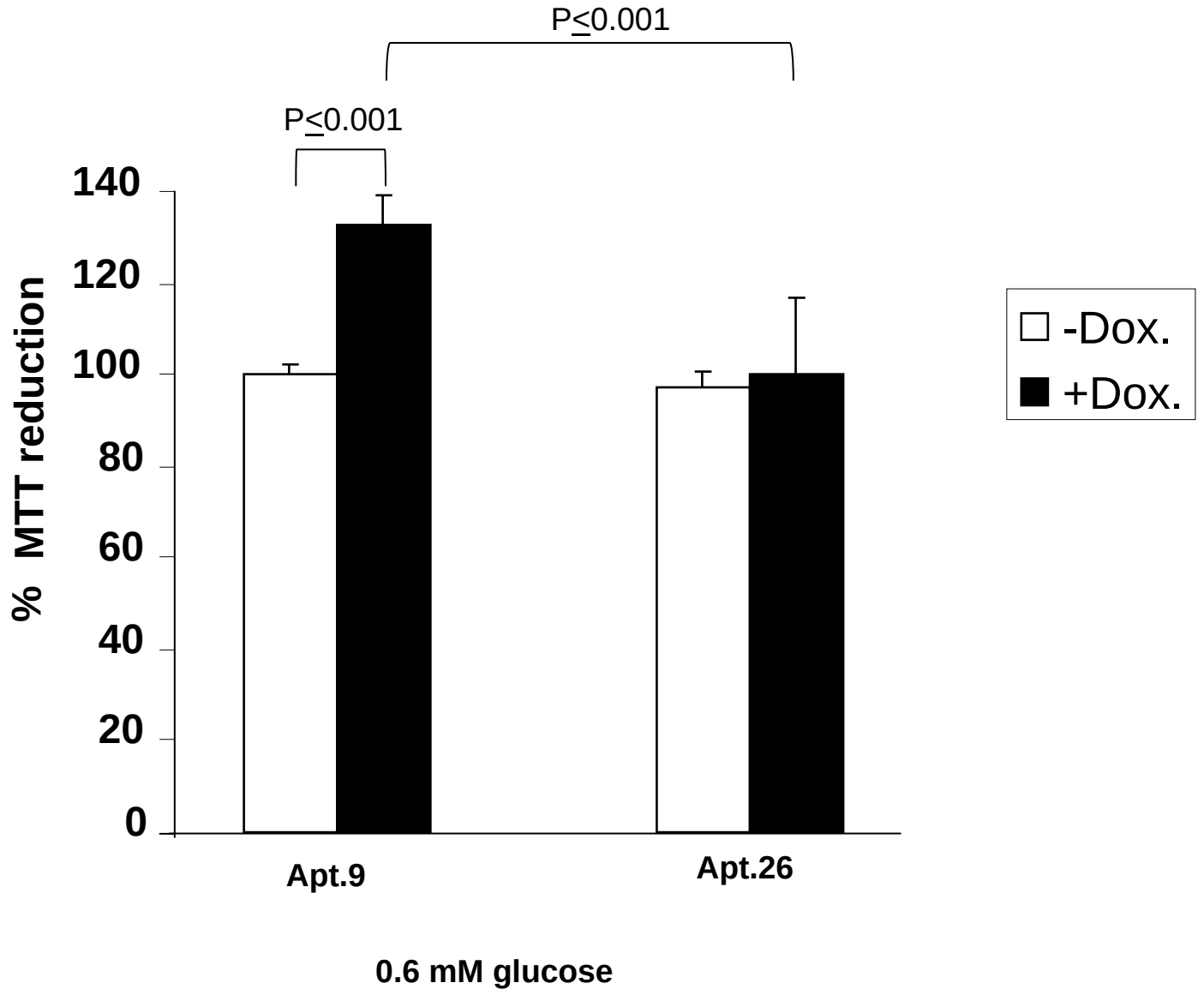


Spoden et al., 2009, Fig. 4

A.



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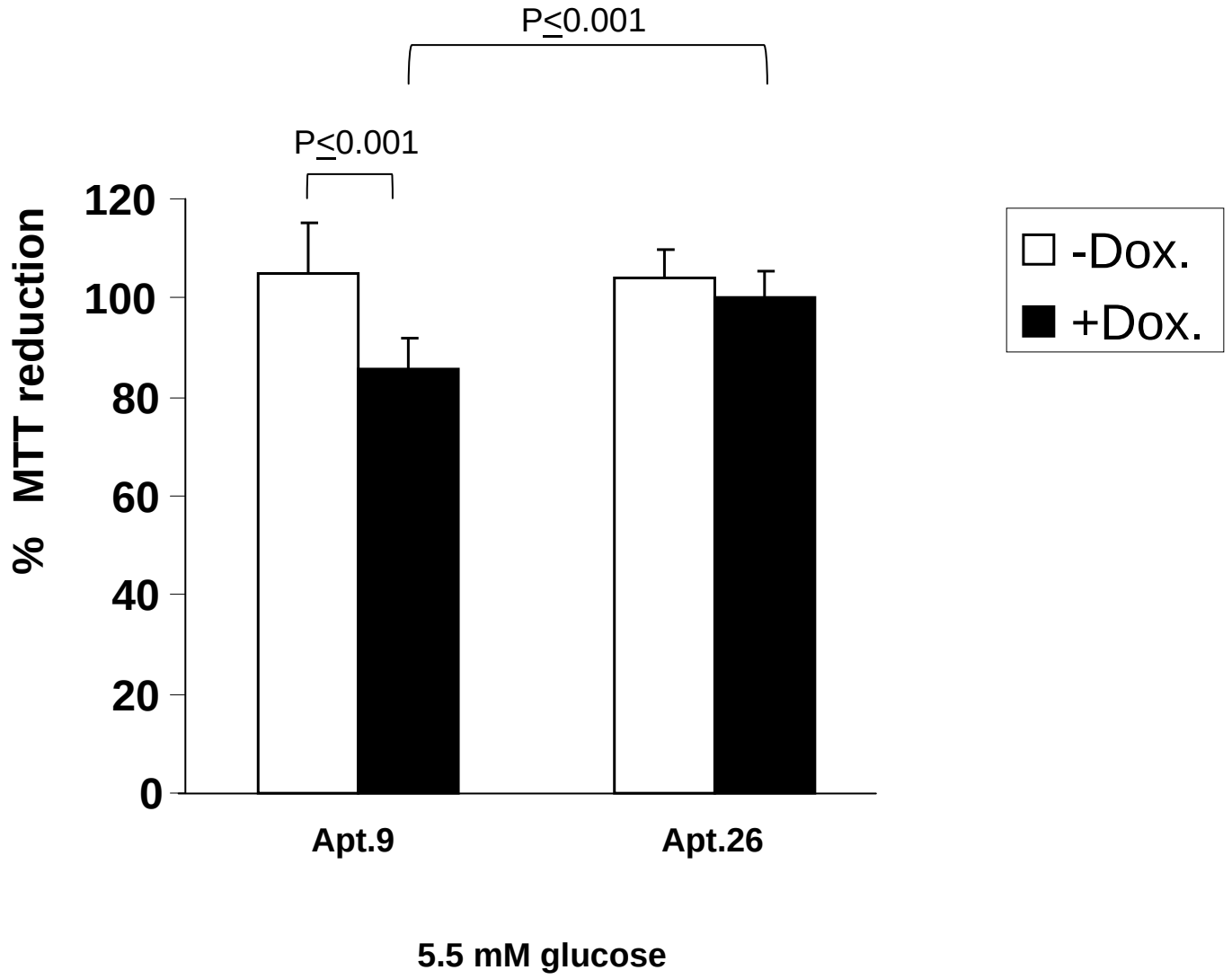
B.



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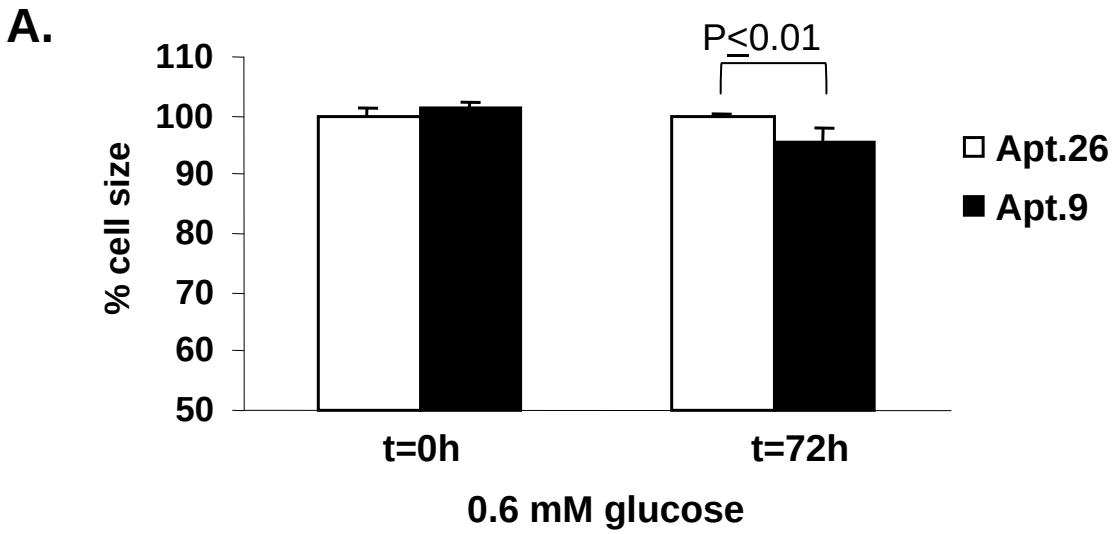

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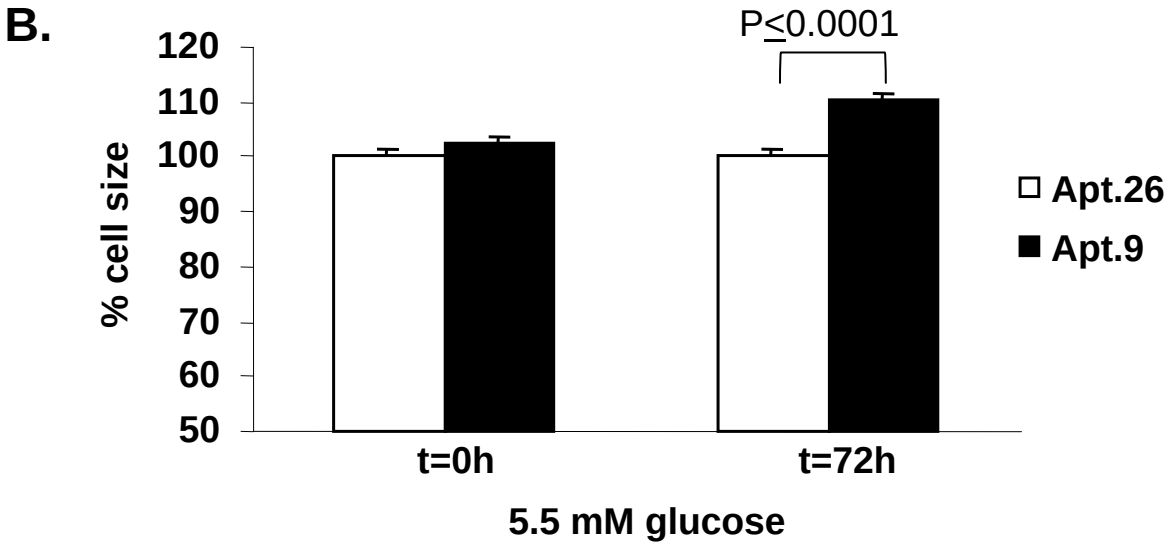

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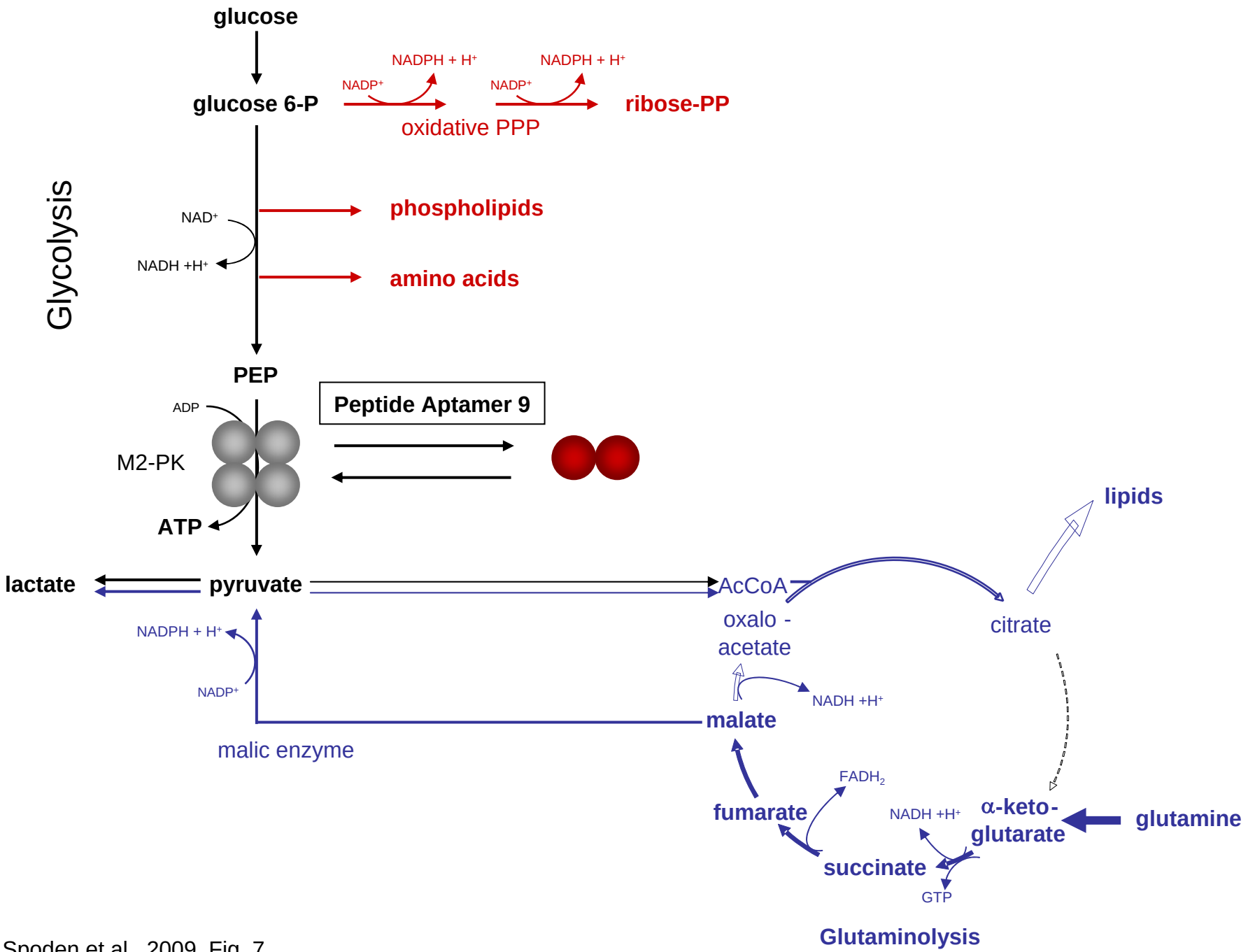

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