FEBS Letters 580 (2006) 3308–3314

PFKFB3 gene silencing decreases glycolysis, induces cell-cycle delay and inhibits anchorage-independent growth in HeLa cells

M.N. Calvo[a], R. Bartrons[a,*], E. Castaño[c], J.C. Perales[b], A. Navarro-Sabaté[a], A. Manzano[a]

[a] *Unitat de Bioquímica, Departament de Ciències Fisiològiques II, Campus de Ciències de la Salut, IDIBELL-Universitat de Barcelona, Feixa Llarga s/n E-08907 L'Hospitalet, Barcelona, Spain*

[b] *Unitat de Biofísica, Departament de Ciències Fisiològiques II, Campus de Ciències de la Salut, IDIBELL-Universitat de Barcelona, Feixa Llarga s/n E-08907 L'Hospitalet, Barcelona, Spain*

[c] *Serveis Científico-Tècnics, Campus de Ciències de la Salut, IDIBELL-Universitat de Barcelona, Feixa Llarga s/n E-08907 L'Hospitalet, Barcelona, Spain*

Received 30 March 2006; revised 27 April 2006; accepted 28 April 2006

Available online 8 May 2006

Edited by Judit Ovádi

Abstract **The high rate of glycolysis despite the presence of oxygen in tumor cells (Warburg effect) suggests an important role for this process in cell division. The glycolytic rate is dependent on the cellular concentration of fructose 2,6-bisphosphate (Fru-2,6-P_2), which, in turn, is controlled by the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2). The ubiquitous PFK-2 isoenzyme (uPFK-2, alternatively named UBI2K5 or ACG) coded by the *pfkfb3* gene is induced by different stimuli (serum, progesterone, insulin, hypoxia, etc.) and has the highest kinase/phosphatase activity ratio amongst all PFK-2 isoenzymes discovered to date, which is consistent with its role as a powerful activator of glycolysis. uPFK-2 is expressed in brain, placenta, transformed cells and proliferating cells. In the present work, we analyze the impact of small interfering RNA (siRNA)-induced silencing of uPFK-2 on the inhibition of cell proliferation. HeLa cells treated with uPFK-2 siRNA showed a decrease in uPFK-2 RNA levels measured at 24 h. uPFK-2 protein levels were severely depleted at 48–72 h when compared with cells treated with an unrelated siRNA, correlating with decreased glycolytic activity, Fru-2,6-P_2, lactate and ATP concentrations. These metabolic changes led to reduced viability, cell-cycle delay and an increase in the population of apoptotic cells. Moreover, uPFK-2 suppression inhibited anchorage-independent growth. The results obtained highlight the importance of uPFK-2 on the regulation of glycolysis, on cell viability and proliferation and also on anchorage-independent growth. These data underscore the potential for uPFK-2 as an effective tumor therapeutic target.**

Keywords: Glycolysis; Tumor cell proliferation; PFK-2; *pfkfb3*; Cell cycle; Apoptosis; Anchorage-independent growth

1. Introduction

Cancer cells maintain an abnormally high glycolytic rate even in the presence of oxygen, a phenomenon first described by Otto Warburg and subsequently known as the Warburg effect [1], and observed in a wide spectrum of human tumors. Although the underlying mechanisms responsible for this effect are rather complex and have not been definitively elucidated, mitochondrial defects and hypoxia are the most consistent causes [2]. In physiological conditions, the cellular energy metabolism is based, preferentially, on oxidative phosphorylation, which is more efficient than glycolysis. In cancer cells, the factors responsible for the Warburg effect compromise the ability of cells to generate ATP via mitochondrial respiration, which triggers alternative metabolic pathways by, among other processes, preferential overexpression of glycolysis enzymes [3]. The high glycolytic rate allows the cells to balance their energy demands and supply the anabolic precursors for de novo nucleotide synthesis [4]. This strategy is considered to provide a growth advantage for the tumor cell, although it renders cells highly dependent on glycolysis for survival [5]. Another feature of malignant transformation is the ability of cells to grow in an anchorage-independent manner [6].

Phosphofructokinase-1 (PFK-1), a rate-limiting enzyme of glycolysis, is allosterically activated by Fru-2,6-P_2, a metabolite that can override the inhibitory effect of ATP on PFK-1 [7]. Fructose 2,6-bisphosphate (Fru-2,6-P_2) levels are increased in proliferative and transformed cells [4,8–10] as well as in various tumors [11]. Production and degradation of Fru-2,6-P_2 depend on several PFK-2 isoenzymes coded by four genes (*pfkfb*1–4) (reviewed in [12,13]). The expression of these genes is dependent on tissue and on development stage [14]. Importantly, tissue-specific isoenzymes are not totally exclusive and several tissues express more than one isoenzyme. This pattern of expression suggests that each isoenzyme plays a key role under different physiological conditions or in response to different hormonal stimuli.

Pfkfb3 gene codes for ubiquitous (uPFK-2) –the isoenzyme with the highest kinase/bisphosphatase ratio [15]- and inducible (iPFK-2) PFK-2 isoenzymes [16]. The occurrence of six alternative splicing variants of human *pfkfb3* gene, two of which are the ubiquitous PFK-2 (alternatively named UBI2K5 or ACG) and the inducible PFK-2 (UBI2K4 or ACDG), has been described [17]. There is evidence for upregulation of *pfkfb3* expression in response to hypoxia and other stimuli [4,12,13,18]. It has been suggested that tumor cells upregulate glycolytic enzymes by increasing protein synthesis and by switching to the preferential expression of the isoenzymes with a higher tendency to catalyze a reaction in the direction that is more favorable for the tumor [19], which has been demonstrated for PFK-2 [9,18]. Moreover, *pfkfb3* gene is distinguished by the presence of multiple copies of the AUUUA sequence in the 3′ UTR of its mRNA. The AUUUA motif is

*Corresponding author. Fax: +34 93 4024268.
E-mail address: rbartrons@ub.edu (R. Bartrons).

0014-5793/$32.00
doi:10.1016/j.febslet.2006.04.093

typical of proto-oncogenes and proinflammatory cytokines and confers instability and enhanced translational activity [4].

Herein, we report the use of RNA interference to knock-down uPFK-2. Our results corroborate its putative role in sustaining the high glycolytic flux in proliferative cells [20] and provide evidence of its involvement in cell viability preservation, cell-cycle progression and anchorage-independent growth.

2. Materials and methods

2.1. Cell culture

HeLa, HEK-293, MCF7 and T47D cell lines obtained from the American Type Culture Collection (Manassas, VA) were cultured in a 10% CO_2 humidified atmosphere at 37 °C as exponentially growing monolayers in Dulbecco's modified Eagle's medium (DMEM) with glutamax (Invitrogen), supplemented with 10% fetal calf serum (Invitrogen) and penicillin/streptomycin (100 U/ml and 100 μg/ml).

2.2. siRNA design and transfection

Small interfering RNAs (siRNAs) were designed according to criteria outlined elsewhere [21]. Specificity was checked by using BLAST and comparing positions in the alignment of the different human PFK-2 sequences. siRNA against uPFK-2 (u-siRNA) targets a sequence in the ubiquitous PFK-2 mRNA located between nucleotide positions 1501 and 1520 relative to the first nucleotide of the start codon of *pfkfb3* gene [AF109735] (AGCUGCCUGGACAAAACAUG) that is found truncated in the mRNA of iPFK-2 (UBI2K4 or ACDG). Also, an siRNA against a common region of all mRNAs generated from *pfkfb3* (co-siRNA) and, as a negative control, two non-functional siRNAs with no human target (c(−) siRNA) were used. All siRNAs were synthesized by Dharmacon Research, Inc. Transfections were performed with Oligofectamine (Invitrogen) as specified by the manufacturer.

2.3. RNA extraction and real-time PCR

Total RNA was extracted using Ultraspect (Biotecx Laboratories, Inc), according to the manufacturer's protocol. The RNA was reverse transcribed using Ready-To-GoTM You-Prime First-Strand Beads (Amersham Biosciences) and oligo$(dT)_{12-18}$ primer (Amersham-Pharmacia Biotech). *pfkfb3* was specifically amplified by using the probe/primer set for human *pfkfb3* (Hs0019079_m1) (Applied Biosystems). TBP (housekeeping control gene, TATA-box binding protein) (Hs00427620_m1) was used for normalization. PCR data were captured using an ABIPRISM 7700 Sequence Detection System.

2.4. Protein extraction and Western blot analysis

Equal amounts of total protein extracts, prepared from the same number of untransfected cells and cells transfected with siRNA, were analyzed in 10% (w/v) SDS–PAGE. Western blot was performed according to [22] using the rabbit polyclonal sera against uPFK-2 (1:500) [23], commercial antibodies against liver (Santa Cruz) and heart (Abgent) PFK-2, and a polyclonal specific antibody against testis PFK-2 [24] and, for protein loading control, a mouse monoclonal antibody against α-tubulin (1:1000) (Sigma). Peroxidase-conjugated secondary antibodies against mouse and rabbit (Amersham Bioscience) were used at 1:5000. Immunostaining was carried out using the ECL technique (Amersham–Pharmacia Biotech).

2.5. Metabolite determination

Fru-2,6-P_2 was determined following the method described by Van Schaftingen et al. [25]. Lactate and ATP were measured spectrophotometrically in neutralized perchloric extracts by using standard enzymatic methods [26]. Protein concentration was determined by the Bradford-based Bio-Rad assay.

2.6. Viability and proliferation assays

2.6.1. Cell number assay. Cell number was determined by crystal violet staining as previously described [27]. The absorbance read at 550 nm was proportional to the number of cells that remained attached to the plate.

2.6.2. Staining by annexin V and propidium iodide. The appearance of apoptotic or necrotic cells was determined by the annexin V binding assay [28]. The affinity of annexin V for phosphatidylserine residues allows the percentage of cells undergoing apoptosis to be quantified by flow cytometry. Apoptotic and necrotic cells were distinguished on the basis of double-labeling for annexin V-FITC (Bender) and propidium iodide (PI), a membrane-impermeable DNA stain. Floating and freshly trypsinized cells were pooled, washed twice in binding buffer and processed following manufacturer's instructions. Cells fluorescence was analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Mountain View, CA) using the Cell Quest Pro software.

2.6.3. Cell-cycle analysis by flow cytometry. After trypsinization, cells were washed in PBS containing 1% calf serum and fixed by adding cold ethanol to a final concentration of 80%. After washing and resuspending fixed cells in 0.5 ml of PBS containing 1% calf serum, the DNA was stained by adding 200 μg/ml of propidium iodide and 10 mg/ml of DNAse-free RNAse A. Stained cells were analyzed using FACSCalibur system (Becton Dickinson, MountainView, CA). The percentage of cells in each phase of the cell cycle was determined using the Modfit 5.2 computer program.

2.7. Anchorage-independent growth assay

HeLa cells treated with the corresponding siRNA (u-siRNA, co-siRNA and two different non-functional c(−)1 and 2 siRNAs as negative controls) or with Oligofectamine alone (control condition) were seeded 24 h after transfection in triplicate in 12-well plates. For this purpose, 5×10^3 cells/well were plated in 0.3% agarose DMEM medium onto a semisolid 0.6% agarose DMEM layer. Complete medium containing 2.5 nM paclitaxel was added to positive control wells and maintained for 24 h. The remaining wells were supplied with complete medium alone or containing 1 nM siRNA (siRNA redosing) [29]. Medium was changed every two days. After 10 days, colonies of 50 cells or more were counted from at least four randomly selected fields per triplicate well.

2.8. Data analysis

Results are shown as the mean ± S.E.M. of the values obtained from the indicated number of experiments. Differences between groups were analyzed by Student's t test. Significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$ vs. negative control are indicated by *, **, and ***, respectively.

3. Results and discussion

The aim of our work was to investigate the biological consequences of the specific knock-down of uPFK-2 (UBI2K5 or ACG). Firstly, we determined the presence of different isoenzymes of PFK-2 in several cell lines (HEK-293, HeLa, T47D and MCF7) by Western blot (Fig. 1A). HeLa cells expressed all the isoforms analyzed (Fig. 1A and E). Initial validation using u-siRNA and co-siRNA at 100 nM demonstrated high levels of silencing (60–90%) in most of the cell lines assayed (data not shown). We focused on HeLa cells due to their high growth rate and transfection efficiency. We next tested the silencing dose–response and time-course by real-time PCR and Western blot, respectively. Total RNA was extracted from HeLa cells 24 h after transfection and analyzed with specific probe/primers for *pfkfb3* and for TATA-box binding protein housekeeping control gene. There was a significant decrease in mRNA levels from 10 to 100 nM siRNA (Fig. 1B). In light of these data, we chose 100 nM as the optimal working concentration, also in agreement with manufacturer's instructions. Western blots from cells transfected at 100 nM siRNA revealed decreased levels of uPFK-2 48 h after transfection, which were lowest at 72 h (Fig. 1C). In order to monitor the possible non-specific inhibition of translation due to an interferon-mediated response [30], we measured the activation of the small subunit of eucaryotic initiation factor 2-α (eIF2-α).

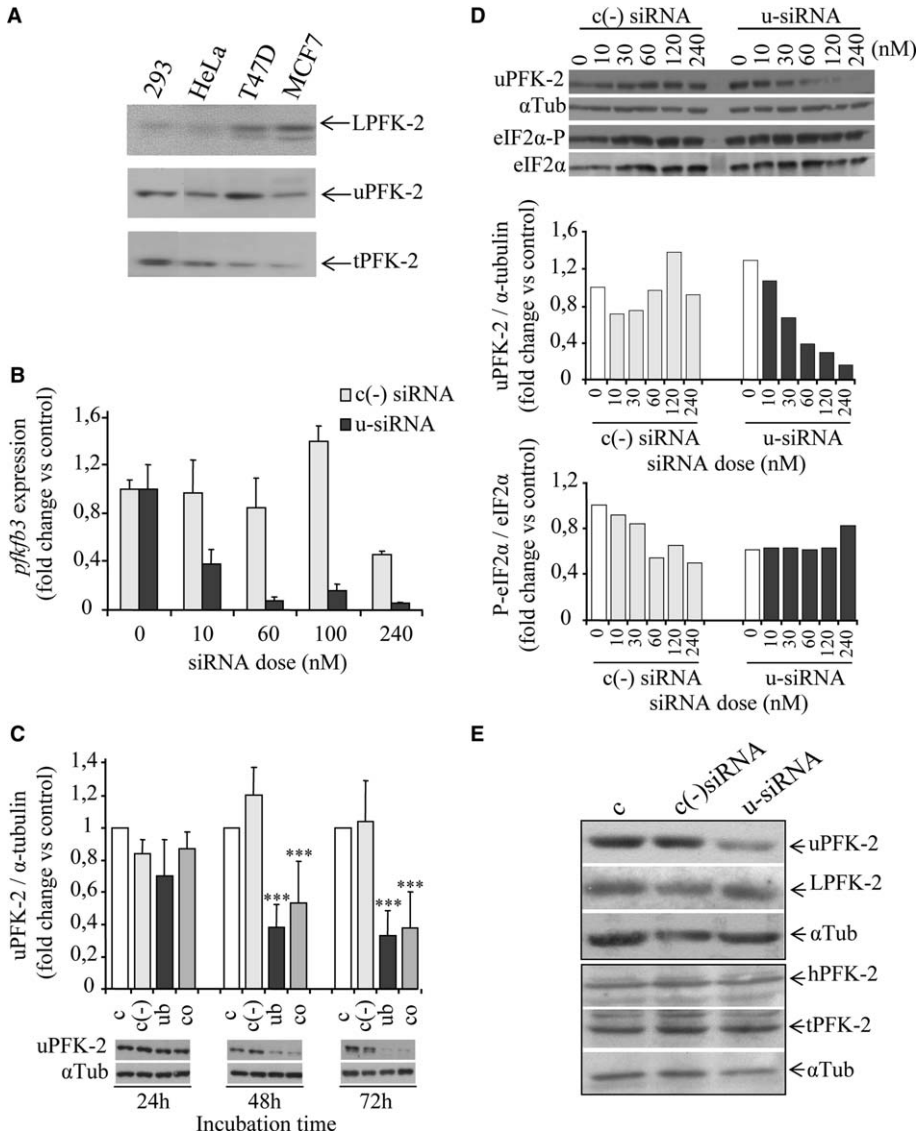

Fig. 1. Western blot and real-time PCR analysis. (A) PFK-2 isoenzymes (liver PFK-2, LPFK-2; ubiquitous PFK-2, uPFK-2 (UBI2K5 or ACG), and testis PFK-2, tPFK-2) expression in different cell lines (HEK-293, HeLa, T47D and MCF7). (B) *pfkfb3* silencing measured by real-time PCR 24 h post-transfection. Results are normalized to the housekeeping control gene TBP and expressed as fold change versus control. (C) uPFK-2 silencing measured by Western blot at various times after transfection (100 nM siRNA). Bars diagram represents average of at least three independent experiments. Differences $P < 0.001$ (***) were considered significant. (D) uPFK-2 silencing dose–response analyzed by Western blot. Non-specific inhibition of translation was checked by analyzing P-eIF2α/eIF2α ratio. Quantification based on densitometric analysis of the Western blot above is represented by the bars diagram. (E) PFK-2 isoenzymes expression (ubiquitous PFK-2, uPFK-2; liver PFK-2, LPFK-2; heart PFK-2, hPFK-2 and testis PFK-2, tPFK-2) in HeLa cells treated with siRNA measured by Western blot. α-tubulin (αTub) is shown as a protein loading control. In all cases, unless differently stated, a representative of at least three experiments is shown. Abbreviations: c, Oligofectamine control; c(−), negative control siRNA; ub, siRNA against uPFK-2; u-siRNA; co, siRNA against all *pfkfb3* mRNAs.

This protein is phosphorylated by double-stranded RNA-activated protein kinase (PKR) in the presence of double-stranded RNA longer than 30 bp. The dose–response (Fig. 1D) showed a decrease in the levels of uPFK-2 from 30 to 120 nM without alterations in the P-eIF2α/eIF2α ratio. Besides, it has been reported that chemically-synthesized siRNAs do not usually activate the antiviral response in mammal cells [31,32]. Finally, we examined whether the silencing of uPFK-2 would upregulate the expression of other PFK-2 isoenzymes and no significant differences were found (Fig. 1E).

Silencing uPFK-2 is expected to decrease Fru-2,6-P_2 levels. The response of Fru-2,6-P_2 concentrations to extracellular and metabolic signals or to the inhibition of its producing enzyme depends on the PFK-2 isoenzymes present in the cell, whose distinctive kinase/bisphosphatase ratio and different regulatory N- and C-terminal domains are adapted to the function of different organs [12,13]. A significant 20–30% decrease in Fru-2,6-P_2 levels was observed 72 h after transfection (Fig. 2). To establish whether the changes in Fru-2,6-P_2 levels had an immediate effect on the cellular glycolytic activity, we measured lactate levels from cell culture medium and cellular ATP content. uPFK-2 knock-down cells showed a decrease of 23% and 20% in lactate and ATP levels, respectively (Fig. 2). Pérez et al. [33] showed that overexpression of a truncated form of PFK-2 carrying only the bisphosphatase domain in Mv1Lu cells induced a decrease in Fru-2,6-P_2 levels that cor-

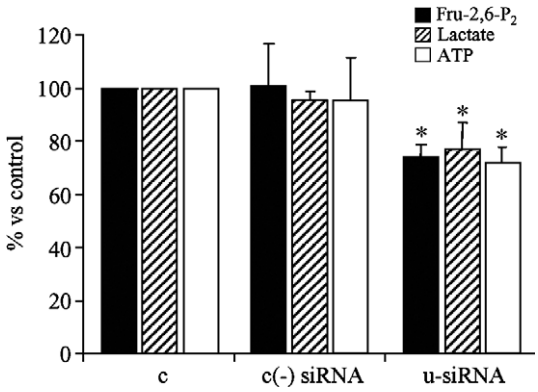

Fig. 2. Effect of uPFK-2 silencing on Fru-2,6-P_2, lactate and ATP levels in HeLa cells. Fru-2,6-P_2, lactate, and ATP were determined enzymatically 72 h after treatment and the results were normalized to protein concentration and expressed as fold change versus control. Data are means ± S.E.M. from at least three experiments. Differences $P < 0.05$ (*) were considered significant. Abbreviations: c, Oligofectamine control; c(−), negative control; u, uPKF-2.

related with lower lactate production and ATP concentration similarly to the results shown in Fig. 2.

An increase in Fru-2,6-P_2 has an important role on the activation of glycolytic flux in proliferating cells [34]. Therefore, we evaluated the effect of u-siRNA on the proliferation rate of transfected HeLa cells harvested 24, 48, or 72 h after plating. Anchorage-dependent growth was assessed by counting the number of cells in the culture dishes, which were measured by the crystal violet assay and plotted versus time. A representative growth curve is shown in Fig. 3A. Cells treated with u-siRNA exhibited a 20% reduction in the proliferation rate compared to control (Fig. 3B). Recent in vitro and in vivo experiments using specific inhibitors of PFK-2 [35] or blocking glycolysis directly with the non-metabolizable glucose-analogue 2-deoxy-glucose (2-DG) [36] have underlined the relationship between glycolysis inhibition and cell damage and survival.

The mechanism responsible for the antiproliferative effect of the u-siRNA was assessed, firstly, by cell staining with annexin V-FITC and PI. A 40–50% decrease in cell viability (annexin V-negative/PI-negative) was observed 48 h after transfection (Fig. 4) and corroborated by staining with PI and FDA (fluorescein di-O-acetate) (data not shown). According to Vander Heiden [37], stress-induced cell death is proportional to the change on the glycolytic rate resulting from growth factors or glucose depletion. Indeed, the mitogenic response of growth factors is concomitant with the modulation they exert on PFK-2 expression and it has thus been suggested that Fru-2,6-P_2 intracellular levels could determine the glycolytic rate of proliferating cells by coupling hormonal signals with the metabolic demand [10]. The same cell staining technique let us explore whether the decrease in the percentage of viable cells was related to an apoptotic process. As illustrated in Fig. 4, more than 22% of HeLa cells transfected with u-siRNA were early apoptotic (annexin V-positive/PI-negative). These results are consistent with similar experiments carried out with the glucose analogue 2-deoxy-glucose (2-DG) [38]. In contrast, cells that overexpressed fructose-2,6-bisphosphatase and thus had a low content of Fru-2,6-P_2 showed a diminished tendency to undergo both spontaneous and induced apoptosis, although their ATP levels were 20% lower than control cells [39]. This

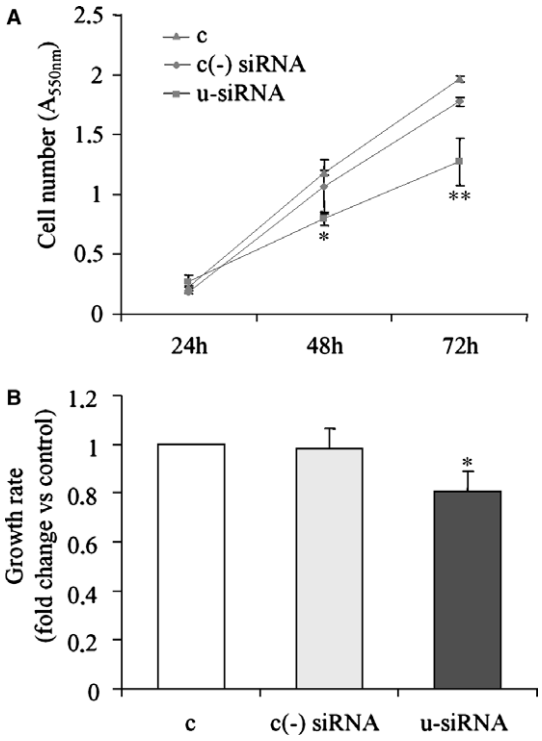

Fig. 3. Effect of uPFK-2 silencing on HeLa cells growth. Cell number was assayed over time by staining of cells with crystal violet and measure of its absorbance at 550 nm. Upper graph (A) represents the growth curves plotted from a representative experiment. Error bars stand for intraexperimental replicates standard deviation. (B) Slope of growth curves was defined as growth rate and expressed as fold change versus control. Average of four experiments is shown. Differences $P < 0.05$ (*) and 0.01 (**) were considered significant. Abbreviations: c, Oligofectamine control; c(–) negative control; u, uPFK-2.

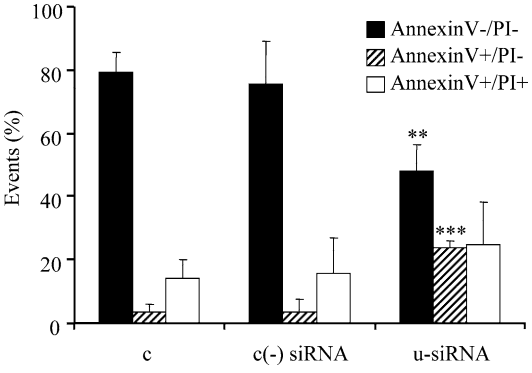

Fig. 4. Effect of uPFK-2 silencing on HeLa cells apoptosis. Apoptosis was assessed 72 h after treatment by staining cells with annexin V-FITC and PI and subsequent FACS analysis of their fluorescence, with cells annexin V-/PI- considered as viable cells, cells annexin V+/PI- considered as early apoptotic cells and cells annexin V+/PI+ considered as late apoptotic or necrotic cells. Histogram plots the average distribution in the three states versus control from four experiments. Differences $P < 0.01$ (**) and 0.001 (***) were considered significant. Abbreviations: c, Oligofectamine control; c(–) negative control; u, uPFK-2.

difference with our results may arise, first, from the fact that the stable transfection performed by Boada et al. may promote the adaptation to a prolonged Fru-2,6-P_2 decrease – unlike the acute effect of uPFK-2 silencing on HeLa cells. Secondly, the cell line they used was Mv1Lu, a non-tumor epithelial cell line.

A feature of many tumor cells is their persistent metabolism of glucose to lactate, which is independent of oxygen availability and is assumed to entail a growth advantage for tumor expansion [4,10]. Nevertheless, the shift from oxidative energy metabolism to glycolysis leads tumor cells to depend on extracellular glucose to maintain their ATP levels. In the case of HeLa cells, a decrease in ATP levels results in an apoptosis increase [40], which is consistent with our data (Fig. 2).

The effect of u-siRNA on cell death could account for the antiproliferative consequences seen so far. However, an effect on the cell cycle could also contribute to that result. Indeed, when quiescent cells are stimulated to proliferate, the expression of several genes is induced at the G_1/S transition. These genes code for proteins involved in cell-cycle progression, for enzymes of DNA synthesis and, as it has previously been shown, for PFK-2 [41]. To check whether silencing of uPFK-2 affected the cell cycle, HeLa cells were synchronized in G_0/G_1 by serum depletion for 48 h post-transfection. Subsequently, cells were challenged to re-enter the cell cycle for 12 h by adding 10% FBS medium. Finally, cells were fixed on ethanol, stained with PI and processed by FACS to establish the distribution of cells in the cell-cycle phases. Treatment with u-siRNA decreased the ability of cells to progress from G_0/G_1 to S, which resulted in an accumulation of cells in G_0/G_1 (Fig. 5).

Finally we examined the effects of uPFK-2 on anchorage-independent growth, one of the hallmarks of transformation. HeLa cells grow and form foci in a semisolid culture. This capacity is not altered when cells are transfected with 100 nM negative control siRNA. However, treatment with 100 nM u-siRNA or co-siRNA (also positive control, 2.5 nM paclitaxel) clearly abolished this ability (Fig. 6A). Quantification as fold change versus control of the mean colony counts of three independent experiments is summarized in Fig. 6B.

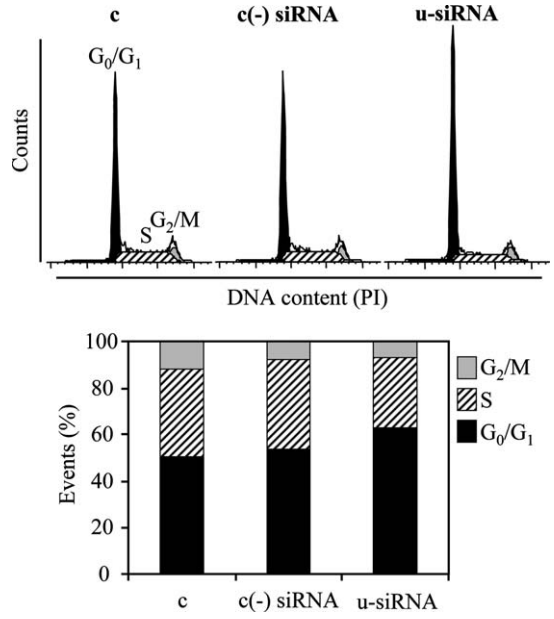

Fig. 5. Effect of uPFK-2 silencing on HeLa cells cycle. Cell cycle was studied 72 h after treatment by staining DNA with PI and subsequent FACS analysis. Cells were synchronized in G_0/G_1 by total FBS depletion during 48 h and induced to re-enter the cell cycle by addition of complete medium for 12 h. Histograms represent the distribution of cells in G_0/G_1, S and G_2/M. A representative experiment of three is shown. Abbreviations: c, Oligofectamine control; c(–) negative control; u, uPFK-2.

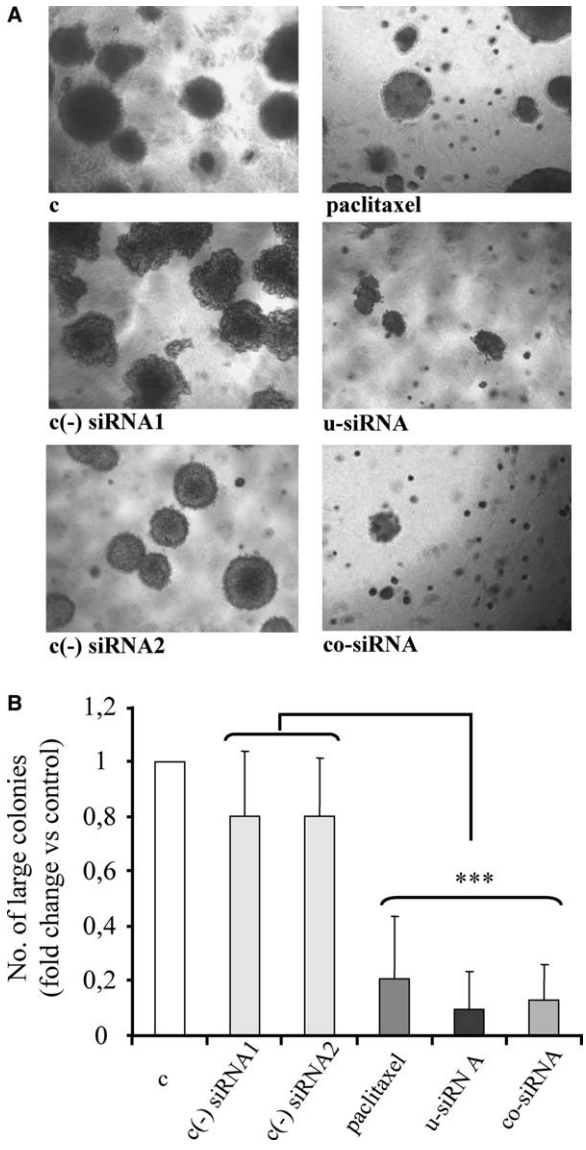

Fig. 6. Effect of uPFK-2 silencing on anchorage-independent growth of HeLa cells. (A) Representative pictures of soft agar colonies formed by HeLa cells grown for 10 days (40×). (B) Bars represent fold change versus control from at least three independent experiments. Differences $P < 0.001$ (***) were considered significant. Abbreviations: c, Oligofectamine control; c(−)siRNA1 and c(−)siRNA2, negative control siRNAs; paclitaxel (2,5 nM for 24 h), positive control; u, uPFK-2, and, co-siRNA, common siRNA against all *pfkfb3* mRNAs.

Despite the relatively modest effects obtained at the metabolic level, the siRNA against uPFK-2 decreased cell proliferation and greatly inhibited anchorage-independent growth, suggesting that the substantial decrease of uPFK-2 (Fig. 1B) could have additional non-glycolytic consequences. In this regard, it has been shown that the heart PFK-2 isoenzyme and the isoenzyme from *Arabidopsis thaliana* bind 14-3-3 proteins [42], which have been implicated in promoting cell survival [43]. At present, there are no studies with other isoenzymes in this field. Numerous studies, reviewed in [44], are providing intriguing evidence of the unexpected nature of glycolytic enzymes but current understanding of the functions of these

enzymes beyond glycolysis is still insufficient and will surely expand in the near future. In this sense, it is interesting that most glycolytic enzymes that display non-glycolytic functions contain evolutionarily conserved consensus Myc-binding sites or E-boxes among their regulatory DNA sequences [45] and these sequences are also present in *pfkfb3* gene [18]. Work is in progress to elucidate other roles this gene may have.

In conclusion, uPFK-2 has a relevant implication in the relationship between glycolysis, cell proliferation and transformation. This isoenzyme is one of the metabolic effectors that facilitates the adaptation and survival of proliferating and tumor cells in their hypoxic microenvironment. Suppression of glycolysis may be an effective alternative to traditional cancer therapies and, accordingly, the inhibition of *pfkfb3* expression could be one of the targets of such an approach.

Acknowledgements: We are grateful to E. Adanero and J. Boada for helpful technical advice and for the skillful technical assistance. We thank Robin Rycroft and J.L. García for English editing, and J. Boada and J.L. Rosa for reading the manuscript and providing helpful comments. We also thank the Scientific/Technical Service at the University of Barcelona for technical support. M.N.C. was recipient of research fellowship from Ministerio de Educación y Ciencia (MECD) (F.P.U.). This work was supported by Ministerio de Ciencia y Tecnología (BMC 2003/01442).

References

[1] Warburg, O. (1956) On the origin of cancer cells. Science 123, 309–314.
[2] Xu, R.H., Pelicano, H., Zhou, Y., Carew, J.S., Feng, L., Bhalla, K.N., Keating, M.J. and Huang, P. (2005) Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia. Cancer Res. 65, 613–621.
[3] Altenberg, B. and Greulich, K. (2004) Genes of glycolysis are ubiquitously overexpressed in 24 cancer classes. Genomics 84, 1014–1020.
[4] Chesney, J., Mitchell, R., Benigni, F., Bacher, M., Spiegel, L., Al-Abed, Y., Han, J.H., Metz, C. and Bucala, R. (1999) An inducible gene product for 6-phosphofructo-2-kinase with an AU-rich instability element: role in tumor cell glycolysis and the Warburg effect. Proc. Natl. Acad. Sci. USA 96, 3047–3052.
[5] Chiaradonna, F., Magnani, C., Sacco, E., Manzoni, R., Alberghina, L. and Vanoni, M. (2005) Acquired glucose sensitivity of k-ras transformed fibroblasts. Biochem. Soc. Trans. 33, 297–299.
[6] Wang, L. (2004) Molecular signaling regulating anchorage-independent growth of cancer cells. Mt. Sinai J. Med. 71, 361–367.
[7] Van Schaftingen, E., Jett, M., Hue, L. and Hers, H.G. (1981) Control of liver 6-phosphofructokinase by fructose 2,6-bisphosphate and other effectors. Proc. Natl. Acad. Sci. USA 78, 3483–3486.
[8] Nissler, K., Petermann, H., Wenz, I. and Brox, D. (1995) Fructose 2,6-bisphosphate metabolism in Ehrlich ascites tumour cells. J. Cancer Res. Clin. Oncol. 121, 739–745.
[9] Hirata, T., Kato, M., Okamura, N., Fukasawa, M. and Sakakibara, R. (1998) Expression of human placental-type 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase in various cells and cell lines. Biochem. Biophys. Res. Commun. 242, 680–684.
[10] Hue, L. and Rousseau, G. (1993) Fructose 2,6-bisphosphate and the control of glycolysis by growth factors, tumor promoters and oncogenes. Adv. Enzyme Regul. 33, 97–110.
[11] Atsumi, T., Chesney, J., Metz, C., Leng, L., Donnelly, S., Makita, Z., Mitchell, R. and Bucala, R. (2002) High expression of inducible 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (iPFK-2; PFKFB3) in human cancers. Cancer Res. 62, 5881–5887.
[12] Rider, M.H., Bertrand, L., Vertommen, D., Michels, P.A., Rousseau, G.G. and Hue, L. (2004) 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: head-to-head with a bifunctional enzyme that controls glycolysis. Biochem. J. 381, 561–579.
[13] Okar, D.A., Manzano, A., Navarro-Sabate, A., Riera, L., Bartrons, R. and Lange, A.J. (2001) PFK-2/FBPase-2: maker and breaker of the essential biofactor fructose-2,6-bisphosphate. Trends Biochem. Sci. 26, 30–35.
[14] Goren, N., Manzano, A., Riera, L., Ambrosio, S., Ventura, F. and Bartrons, R. (2000) 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase expression in rat brain during development. Brain Res. Mol. Brain Res. 75, 138–142.
[15] Sakakibara, R., Uemura, M., Hirata, T., Okamura, N. and Kato, M. (1997) Human placental fructose-6-phosphate,2-kinase/fructose-2,6-bisphosphatase: its isozymic form, expression and characterization. Biosci. Biotechnol. Biochem. 61, 1949–1952.
[16] Navarro-Sabate, A., Manzano, A., Riera, L., Rosa, J.L., Ventura, F. and Bartrons, R. (2001) The human ubiquitous 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene (PFKFB3): promoter characterization and genomic structure. Gene 264, 131–138.
[17] Kessler, R. and Eschrich, K. (2001) Splice isoforms of ubiquitous 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in human brain. Brain Res. Mol. Brain Res. 87, 190–195.
[18] Obach, M., Navarro-Sabate, A., Caro, J., Kong, X., Duran, J., Gómez, M., Perales, J.C., Ventura, F., Rosa, J.L. and Bartrons, R. (2004) 6-Phosphofructo-2-kinase (pfkfb3) gene promoter contains hypoxia-inducible factor-1 binding sites necessary for transactivation in response to hypoxia. J. Biol. Chem. 279, 53562–53570.
[19] Geschwind, J.F., Georgiades, C., Ko, Y.H. and Pedersen, P.L. (2004) Recently elucidated energy catabolism pathways provide opportunities for novel treatments in hepatocellular carcinoma. Expert Rev. Anticancer Ther. 4, 449–457.
[20] Shim, H., Chun, Y., Lewis, B. and Dang, C. (1998) A unique glucose-dependent apoptotic pathway induced by c-Myc. Proc. Natl. Acad. Sci. USA 95, 1511–1516.
[21] Elbashir, S.M., Harborth, J., Weber, K. and Tuschl, T. (2002) Analysis of gene function in somatic mammalian cells using small interfering RNAs. Methods 26, 199–213.
[22] Burnette, W. (1981) "Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate–polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. 112, 195–203.
[23] Riera, L., Manzano, A., Navarro-Sabate, A., Perales, J.C. and Bartrons, R. (2002) Insulin induces PFKFB3 gene expression in HT29 human colon adenocarcinoma cells. Biochim. Biophys. Acta 1589, 89–92.
[24] Gomez, M., Manzano, A., Navarro-Sabate, A., Duran, J., Obach, M., Perales, J.C. and Bartrons, R. (2005) Specific expression of pfkfb4 gene in spermatogonia germ cells and analysis of its 5′-flanking region. FEBS Lett. 579, 357–362.
[25] Van Schaftingen, E., Lederer, B., Bartrons, R. and Hers, H.G. (1982) A kinetic study of pyrophosphate: fructose-6-phosphate phosphotransferase from potato tubers. Application to a micro-assay of fructose 2,6-bisphosphate. Eur. J. Biochem. 129, 191–195.
[26] Gutmann, I. and Wahlefeld, A.W. (1974) L-(+)-Lactate. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis, pp. 1464–1468.
[27] Dalmau, M., Joaquin, M., Nakamura, T., Bartrons, R. and Gil, J. (1996) Nitric oxide inhibits DNA synthesis and induces activation of poly(ADP-ribose) polymerase in cultured rat hepatocytes. Exp. Cell Res. 228, 14–18.
[28] Van Engeland, M., Nieland, L., Ramaekers, F.C., Schutte, B. and Reutelingsperger, C.P. (1998) Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry 31, 1–9.
[29] Hiroi, N., Funahashi, A. and Kitano, H. (2006) Comparative studies of suppression of malignant cancer cell phenotype by antisense oligo DNA and small interfering RNA. Cancer Gene Ther. 13, 7–12.
[30] Wang, Q. and Carmichael, G. (2004) Effects of length and location on the cellular response to double-stranded RNA. Microbiol. Mol. Biol. Rev., 432–452.
[31] Samuel, C. (2004) Knockdown by RNAi-proceed with caution. Nat. Biotechnol. 22, 280–282.

[32] Dahlgren, C., Wahlestedt, C. and Thonberg, H. (2006) No induction of anti-viral responses in human cell lines HeLa and MCF-7 when transfecting with siRNA or siLNA. Biochem. Biophys. Res. Commun. 341, 1211–1217.
[33] Perez, J.X., Roig, T., Manzano, A., Dalmau, M., Boada, J., Ventura, F., Rosa, J.L., Bermudez, J. and Bartrons, R. (2000) Overexpression of fructose 2,6-bisphosphatase decreases glycolysis and delays cell cycle progression. Am. J. Physiol. Cell Physiol. 279, 1359–1365.
[34] Dalmau, M., Bartrons, R. and Gil, J. (1994) Control of fructose 2,6-bisphosphate metabolism by different mitogenic signals in Swiss 3T3 fibroblasts. Exp. Cell Res. 212, 93–96.
[35] Hirata, T., Watanabe, M., Miura, S., Ijichi, K., Fukasawa, M. and Sakakibara, R. (2000) Inhibition of tumor cell growth by a specific 6-phosphofructo-2-kinase inhibitor, *N*-bromoacetylethanolamine phosphate, and its analogues. Biosci. Biotechnol. Biochem. 64, 2042–2047.
[36] Aft, R.L., Zhang, F. and Gius, D. (2002) Evaluation of 2-deoxy-D-glucose as a chemotherapeutic agent: mechanism of cell death. Br. J. Cancer 87, 805–812.
[37] Vander Heiden, M.G., Plas, D., Rathmell, J.C., Fox, C.J., Harris, M.H. and Thompson, C.B. (2001) Growth factors can influence cell growth and survival through effects on glucose metabolism. Mol. Cell. Biol. 21, 5899–5912.
[38] Maschek, G., Savaraj, N., Priebe, W., Braunschweiger, P., Hamilton, K., Tidmarsh, G.F., De Young, L. and Lampidis, T.J. (2004) 2-Deoxy-D-glucose increases the efficacy of adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancers in vivo. Cancer Res. 64, 31–34.
[39] Boada, J., Roig, T., Perez, X., Gamez, A., Bartrons, R., Cascante, M. and Bermudez, J. (2000) Cells overexpressing fructose-2,6-bisphosphatase showed enhanced pentose phosphate pathway flux and resistance to oxidative stress. FEBS Lett. 480, 261–264.
[40] Izyumov, D.S., Avetysian, A., Pletjushkina, O.Y., Sakharov, D.V., Wirtz, K.W., Chernyak, B.V. and Skulachev, V.P. (2004) Wages of fear: transient threefold decrease in intracellular ATP level imposes apoptosis. Biochim. Biophys. Acta 1658, 141–147.
[41] Darville, M.I. and Rousseau, G. (1997) E2F-dependent mitogenic stimulation of the splicing of transcripts from an S phase-regulated gene. Nucleic Acids Res. 25, 2759–2765.
[42] Pozuelo Rubio, M., Peggie, M., Wong, B.H., Morrice, N. and MacKintosh, C. (2003) 14-3-3s regulate fructose-2,6-bisphosphate levels by binding to PKB-phosphorylated cardiac fructose-2,6-bisphosphate kinase/phosphatase. EMBO J. 22, 3514–3523.
[43] Masters, S.C., Subramanian, R.R., Truong, A., Yang, H., Fujii, K., Zhang, H. and Fu, H. (2002) Survival-promoting functions of 14-3-3 proteins. Biochem. Soc. Trans. 30, 360–365.
[44] Kim, J.W. and Dang, C.V. (2005) Multifaceted roles of glycolytic enzymes. Trends Biochem. Sci. 30, 142–150.
[45] Kim, J.W., Zeller, K.I., Wang, Y., Jegga, A.G., Aronow, B.J., O'Donnell, K.A. and Dang, C.V. (2004) Evaluation of myc E-box phylogenetic footprints in glycolytic genes by chromatin immunoprecipitation assays. Mol. Cell. Biol. 24, 5923–5936.