PTEN regulates plasma membrane expression of glucose transporter 1 and glucose uptake in thyroid cancer cells

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Abstract

Glucose represents an important source of energy for the cells. Proliferating cancer cells consume elevated quantity of glucose, which is converted into lactate regardless of the presence of oxygen. This phenomenon, known as the Warburg effect, has been proven to be useful for imaging metabolically active tumours in cancer patients by ¹⁸F-fluorodeoxyglucose positron emission tomography (FDG–PET). Glucose is internalised in the cells by glucose transporters (GLUTs) belonging to the GLUT family. GLUT1 (SLC2A1) is the most prevalent isoform in more aggressive and less differentiated thyroid cancer histotypes. In a previous work, we found that loss of expression of PTEN was associated with increased expression of GLUT1 on the plasma membrane (PM) and probability of detecting thyroid incidentalomas by FDG–PET. Herein, we investigated the molecular pathways that govern the expression of GLUT1 on the PM and the glucose uptake in WRO (expressing WT *PTEN*) and FTC133 (*PTEN* null) follicular thyroid cancer cells cultured under glucose-depleted conditions. The membrane expression of GLUT1 was enhanced in glucose-depleted cells. Through genetic manipulations of PTEN expression, we could demonstrate that the lack of this oncosuppressor has a dominant effect on the membrane expression of GLUT1 and glucose uptake. We conclude that loss of function of PTEN increases the probability of cancer detection by FDG–PET or other glucose-based imaging diagnosis.

Key Words

- ▶ PTEN
- ▶ glucose
- ▶ Warburg effect
- ▶ FDG–PET imaging, cancer
- ▶ GLUT

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Introduction

Glucose is a fundamental source of energy for mammalian cells. Normally, in the presence of oxygen, glucose is oxidised through the mitochondrial respiration pathway with the highest rate of production of ATP. However, in intensely proliferating cancer cells, glucose is preferentially converted into lactate despite the presence of oxygen and functional mitochondria. This aberrant metabolism

of glucose, known as the Warburg effect or aerobic glycolysis, is much less convenient in terms of energy gain and imposes a large consumption of glucose in proliferating cells (Newsholme *et al.* 1985, Koppenol *et al.* 2011). This fact is being exploited for diagnostic imaging of tumours in patients injected with the probe ¹⁸F-fluorodeoxyglucose (FDG), a radioactive analogue of glucose,

which can be detected by positron emission tomography (PET) (Busk *et al.* 2008). As the uptake of FDG competes with that of circulating glucose, the patient undergoing FDG–PET must avoid the intake of any source of glucose during the 6 h preceding the start of the PET study (Boellaard *et al.* 2010). This technique has the potential to reveal unsuspected tumours, commonly indicated as ‘incidentaloma’ because of their accidental discovery.

Glucose, similar to its radioactive analogue ^{18}F FDG and its fluorescent equivalent 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG), is internalised in the cells by glucose transporters (GLUTs) belonging to the GLUT family (Szablewski 2013). GLUT1 (SLC2A1) is the most prevalent isoform in highly aggressive and less differentiated thyroid cancer histotypes (Hosaka *et al.* 1992, Samih *et al.* 2000, Matsuzu *et al.* 2005).

Only a few studies have addressed the signalling pathways involved in the glucose uptake by GLUT1 in thyroid cancer cells. There are indications that the surface expression of GLUT proteins is under the control of the phosphoinositide 3-kinase (PI3K)/AKT pathway (Samih *et al.* 2000). Recently, AMPK has been shown to play a pivotal role in the membrane expression of GLUT1 and glucose uptake in rat follicular thyroid cells (Andrade *et al.* 2012). In a small cohort of patients, we found that loss of expression of the oncosuppressor PTEN, a protein–lipid phosphatase that switches off the AKT pathway, was associated with an increased probability of detecting thyroid incidentalomas by FDG–PET (Morani *et al.* 2012). In this work, we analysed the signalling pathways that govern the expression of GLUT1 on the plasma membrane (PM; and the uptake of glucose) in two follicular thyroid cancer cell lines: WRO, expressing WT *PTEN*, and FTC133 *PTEN* null cells. Genetic manipulations indicated that the lack of PTEN expression has a dominant effect on the membrane expression of GLUT1 and glucose uptake. This finding substantiates our hypothesis that loss of function of PTEN increases the probability of cancer detection by FDG–PET or other glucose-based imaging diagnosis (Morani *et al.* 2012).

Materials and methods

Thyroid cancer cell lines, cell culture conditions and pharmacological treatments

The WRO and FTC133 follicular thyroid cancer cell lines were kindly provided by Dr Francesco Frasca, University of Catania, Italy.

The WRO cell line was cultured in RPMI-1640 media (with L-glutamine) supplemented with foetal bovine serum (FBS, 10%) and penicillin/streptomycin (1%); the FTC133 cell lines were cultured in DMEM, nutrient mixture F-12 (1:1, by volume), supplemented with FBS (10%), penicillin/streptomycin (1%) and L-glutamine (1%). All culture reagents were purchased from Sigma–Aldrich. For studies on glucose deprivation, the cells were incubated in a glucose-free medium (R1383 and D5030, Sigma–Aldrich). For the experiments, growing cells were plated on sterile plastic dishes and allowed to adhere for at least 24 h before use. The PI3K inhibitor 3-methyladenine (3MA; Sigma–Aldrich) was used at a concentration of 10 mM. At the end of the incubations, media and cells or coverslips were collected and processed for protein analysis as detailed below.

siRNA silencing of *PTEN*

Post-transcriptional silencing was achieved by the siRNA technology. Duplexes of nucleotide siRNA were synthesised by MWG Biotech AG (Ebersberg, Germany). The sequence and use of the siRNA for Sham transfection have been described previously (Trincheri *et al.* 2007). Two siRNA oligos targeting two different exons were used for the silencing of *PTEN*, and the sense strands were 5'-AGACUUGAAGGCGUAUACA-3' and 5'-AGAAUCAUCUGGAUUAUAG-3'. Adherent cells (plated at a density of 30 000/cm² in a Petri dish) were incubated for 6 h with 100 pmol RNA duplexes in the presence of 6 µl Lipofectamine 2000 (Invitrogen Co.) in 500 µl OptiMem (Life Technologies Co.). The cells were then washed and treated 36 h after transfection to allow for a maximal effect on protein down-regulation.

Plasmid transfections

pcDNA3.1Zeo+ plasmid containing the full-length WT *PTEN* cDNA were transfected by Lipofectamine 2000 (Invitrogen Co.) following the manufacturer's protocol. The full-length *PTEN* cDNA was cloned from OAW42 ovarian cancer cells mRNA and fully sequenced to confirm the WT sequence. OAW42 total RNA was extracted according to the TRIzol LS reagent protocol (Life Technologies Co.). Total RNA (3 µg) was retrotranscribed using the RevertAiD H Minus FirstStrand cDNA Synthesis Kit (Fermentas, Burlington, CA, USA). RT-PCR was performed according to the manufacturer's instructions with DyNzyme EXT DNA Polymerase (Finnzymes OY, Espoo, Finland) starting from 2 µl cDNA and using a final

concentration of 10 μ M *PTEN* primers (forward primer, CATTTCATCCTGCAGAAGAAG and reverse primer, CCCAATACAGATTCACTTCCTTTAG). *PTEN* cDNA was subcloned into the plasmid pcDNA 3.1 Zeo (Life Technologies Co.) and subjected to automated sequencing (ABI PRISM 3100, Applied Biosystems). Primers were obtained from MWG-BIOTECH AG (Ebersberg, Germany). An empty pcDNA3.1Zeo+ plasmid was used as control transfection (Sham).

Glucose uptake assay

The fluorescent glucose analogue 2-NBDG (Life Technologies Co.) was used to detect glucose uptake in living cells (O'Neil *et al.* 2005). Cells grown on coverslips were incubated with 50 μ M of 2-NBDG for 1 h before the end of the treatments, washed twice with PBS 1 $\times$ and rapidly imaged under a fluorescence microscope (Leica DMI6000B, Leica Microsystem AG, Wetzlar, Germany).

Immunofluorescence staining

At the end of treatments, cells adherent on sterile glass coverslips were fixed in cold methanol for 20 min and permeabilised with 0.2% Triton X-100 in PBS 1 $\times$ for 10 min. Antigens were revealed with a primary specific antibody followed by IRIS2 (green fluorescence)-conjugated goat anti-rabbit IgG or IRIS3 (red fluorescence)-conjugated goat anti-mouse IgG (Cyanine Technologies SpA, Turin, Italy) secondary antibody, as appropriate, for 1 h at room temperature in a humid chamber. The nucleus was evidenced by staining the chromatin with the fluorescent dye 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI). As negative control, the primary antibody was omitted or substituted with pre-immune antiserum. Antibodies were diluted in PBS containing 0.1% Triton X-100 and 10% FBS. The following primary antibodies were used: rabbit polyclonal anti-GLUT1 (Millipore, Darmstadt, Germany), rabbit polyclonal anti-PTEN (Millipore) and mouse monoclonal anti-Rab5 (BD Transduction Laboratories, San Jose, CA, USA). Stained cells were mounted with Slow-FADE (Light AntiFADE Kit, Molecular Probes Invitrogen) and observed under a fluorescence microscope (Leica DMI6000B, Leica Microsystem AG). Observations were performed by two independent investigators. Representative images of at least three independent experiments are shown. Image processing was carried out using the ImageJ Software (freely available at <http://imagej.nih.gov/ij/>, see below).

Western blotting assay

Immunoblotting was carried out following standard procedures as reported previously (Castino *et al.* 2007). Cell homogenates were prepared by freeze-thawing and

Figure 5

Differential uptake of glucose in WRO and FTC133 cells. (A) The uptake of the glucose fluorescent analogue 2-NBDG (in green, 50 μ M) was monitored for 16 h in both cell lines cultured in a glucose-containing or glucose-free medium. The probe was added to the culture medium 1 h before the end of the incubation. Representative microscopic fields are shown in the upper panel. Quantification of 2-NBDG uptake was done using the ImageJ Software directly on the microscopic images of labelled cells (lower panel). (B) A parallel culture of cells plated on Petri dishes and labelled as above was used for cytofluorometric quantification of 2-NBDG-positive cells. The level of 2-NBDG uptake was higher in FTC133 cells than in WRO cells and greatly increased when the cells were pre-incubated in a glucose-free medium. Representative data of four experiments are shown. Scale bar = 20 μ m. Magnification = 63 $\times$. Data shown in this figure have been reproduced six times independently. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-14-0118>.

ultrasonication in a buffer containing detergents and protease inhibitors. Approximately 30 µg of cell proteins were denatured with Laemmli sample buffer, separated by electrophoresis on a 12.5% SDS-containing polyacrylamide gel and then electroblotted onto a PVDF membrane (Carlo Erba Reagents, Milan, Italy).

Protein of interest was detected with the following primary antibodies: rabbit polyclonal anti-PTEN (EX-BIO, Vestec, Czech Republic); rabbit polyclonal anti-phospho AKT (Ser473) (Cell Signaling Technology, Danvers, MA, USA); rabbit polyclonal anti-AKT (Cell Signaling Technology); rabbit polyclonal anti-GLUT1 (Millipore) and rabbit polyclonal anti-HIF1α (GeneTex, Irvine, CA, USA). As an index of homogenate protein loading in the lanes, β-tubulin was used (Sigma-Aldrich). Immunocomplexes were revealed using a peroxidase-conjugated secondary antibody (Bio-Rad), as appropriate, and subsequent peroxidase-induced chemiluminescence reaction (Perkin-Elmer, Waltham, MA, USA). Western blotting data were reproduced at least three times independently.

Quantification analysis: methods and statistics

Unless otherwise specified, all experiments were replicated three times independently and in duplicate or triplicate. For measurement of glucose uptake, two methods were employed. In adherent 2-NBDG-loaded cells, data quantification of the area and of the intensity of fluorescence images was performed using the ImageJ 1.48v Software (freely available at <http://imagej.nih.gov/ij/>). At least five randomly chosen fields for a total of minimum 50 cells were analysed. Fluorescence intensity (FI) is given in arbitrary units as an average value per cell in the selected representative fields.

Alternatively, the 2-NBDG-loaded cells were subjected to cytofluorometry analysis. Adherent cells were incubated with 50 µM of 2-NBDG for 1 h before the end of the treatments, washed twice with PBS 1×, collected by trypsinisation, centrifuged at 100 *g* at 4 °C for 10 min and re-suspended in OPTIMEM (Life Technologies Co.). Cells were analysed using a Becton Dickinson FACS SCAN cytofluorometer (BD Bioscience, Franklin Lakes, NJ, USA).

Figure 2

Differential subcellular localisation of GLUT1 in WRO and FTC133 cell lines. (A) Western blotting of HIF1α in homogenates from WRO (PTEN WT) and FTC133 (PTEN null) thyroid cancer cell lines incubated for 24 h in a glucose-containing or glucose-free medium. Densitometry of three experiments is included. The basal level of HIF1α expression was higher in FTC133 cells than in WRO cells. (B) Western blotting of GLUT1 in homogenates prepared as for A. Densitometry of three experiments is included. Differences in the expression of GLUT1 in WRO and FTC133 cells were not statistically significant. Glucose starvation apparently did not alter the levels of HIF1α and GLUT1 expression in both cell lines. (C) Immunofluorescence assay of GLUT1 expression in WRO and FTC133 cells cultured for 1 or 16 h in a glucose-containing or glucose-free medium. Representative fields of immunofluorescence staining are shown in the upper panels, and the corresponding

fluorescence quantification of GLUT1 in the cytoplasmic (Cyt) and plasma membrane (PM) compartments is shown in the lower panels. In WRO cells cultured in a glucose-containing medium, GLUT1 was localised to the cytoplasm (~85%) and, under glucose-depleted conditions, it translocated onto the PM (from ~15 to ~60%). In FTC133 cells, GLUT1 permanently localised to the PM (~95%), regardless of the presence or absence of glucose in the culture medium. Nuclei are stained with DAPI. The images shown in C have been reproduced four times independently. Fluorescence quantification is based on the analysis of a minimum of 50 cells for each of the three independent experiments. Scale bar = 20 µm. Magnification = 63×. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-14-0118>.

Data were acquired using the CellQuest Software (BD Bioscience) (10 000 gated events/sample, on FL-1) and analysed using the WinMDI 2.9 Software (<http://facs.scripps.edu/software.htm>). Unstained parallel cultures were used for setting the instrument. The proportion of cells positive for 2-NBDG uptake is given as the percentage of the whole population. One (out of three or four) representative cytofluorogram is shown per condition. Alternatively, data are expressed as average $\pm$ s.d. of $n=4$.

Quantification of protein expression in fluorescence images of adherent cells was performed using the ImageJ Software. Data were collected from at least three independent experiments. Five randomly chosen fields for a total of minimum 50 cells were analysed in each coverslip. Data on subcellular distribution of the protein of interest (GLUT1) are expressed as percentage ($\pm$ s.d.) of FI in the PM and cytoplasmic (Cyt) compartments of the cells in the selected representative fields.

Densitometry quantification of western blotting bands was performed using both the ImageJ (1.48v; <http://imagej.nih.gov/ij/>) and Quantity One 4.5.0 Softwares (Bio-Rad), with consistent results. Data are expressed in arbitrary units as average $\pm$ s.d. of at least three independent experiments. The P values <0.05 were considered statistically significant.

Results

FTC133 cells take up glucose more avidly than WRO cells

To monitor the ability of cells to take up glucose, we employed 2-NBDG, a glucose analogue fluorescently labelled at position 2, that is a substrate for GLUTs (Fig. 1). Quantification of 2-NBDG uptake was performed measuring the fluorescence in the cell monolayer (Fig. 1A, lower panel) as well as by cytofluorometry after

Figure 3

Involvement of the PI3K signalling pathway in the glucose-deprivation response in WRO and FTC133 cells. (A) PTEN status was analysed by western blotting in WRO and FTC133 cell lines after 24 h of incubation in a glucose-containing and glucose-free media. FTC133 cells do not express detectable levels of PTEN. In WRO cells, the expression of PTEN is not subjected to glucose-dependent modulation. (B) AKT status, as a readout of PI3K activity, was analysed by western blotting in WRO and FTC133 cells cultured

for 24 h in the presence or absence of glucose and of the PI3K inhibitor 3MA (10 mM). AKT phosphorylation on Ser473 was basally active in FTC133 cells, and it was activated under glucose-depleted conditions in WRO cells. 3MA effectively decreased AKT phosphorylation. Densitometry of the bands corresponding to PTEN (A) and of pAKT and AKT (B), normalised vs β -tubulin, is included. Data shown in this figure have been reproduced three times independently.

re-suspension of the labelled cells (Fig. 1B). In WRO cells cultured in a glucose-containing standard medium, the uptake of 2-NBDG was negligible, while it increased upon incubation in a glucose-free medium (Fig. 1). The simplest explanation of this phenomenon is that 2-NBDG clearly competes with the glucose present in the culture medium. However, we noted that, in FTC133 cells, the basal uptake of 2-NBDG was much higher than in WRO cells, despite the presence of glucose in the culture medium, and it increased further when incubated in a glucose-free medium (Fig. 1).

Glucose depletion differentially affects the membrane localisation of GLUT1 in WRO and FTC133 thyroid cancer cells

Hypoxia triggers a metabolic switch towards aerobic glycolysis through HIF1 α -mediated expression of GLUT proteins and glycolytic enzymes (Semenza 2012, Szablewski 2013). The metabolic stress induced by glucose depletion shares a common signalling pathway with hypoxia. The level of HIF1 α (HIF1A) expression was

Figure 4

3-Methyladenine abrogates glucose starvation-induced plasma membrane (PM) translocation of GLUT1 in WRO cells. The cells adherent on coverslips were incubated in the presence or absence of 3MA for 16 h in a glucose-containing or glucose-free medium. At the end, the coverslips were processed for immunofluorescence staining of GLUT1 (in green) and Rab5 (in red; a marker of endocytic vesicles). Nuclei were stained with DAPI. Glucose starvation induced the PM relocation of GLUT1 in WRO cells.

This effect was abrogated by 3MA. In FTC133 cells, GLUT1 was permanently localised to the PM, regardless of the presence or absence of glucose. Quantification of the relative distribution in cytoplasm (Cyt) vs PM of GLUT1 is included. Representative images and distribution of fluorescence (in percentage) of three independent experiments are shown. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-14-0118>.

higher in FTC133 cells than in WRO cells (Fig. 2A). Then, we looked at the expression of GLUT1, a GLUT that is abnormally over-expressed in highly aggressive and less differentiated thyroid cancers (Hosaka *et al.* 1992, Samih *et al.* 2000, Matsuzo *et al.* 2005). GLUT1 was expressed at a comparable level in WRO and FTC133 cells, and its level slightly increased upon incubation in a glucose-free medium (Fig. 2B). The above data suggested that membrane localisation rather than protein expression of GLUT1 could be the mechanistic cause for the differential uptake of glucose in the two cell lines. In the large majority (>80%) of WRO cells cultured in a glucose-containing medium, GLUT1 was essentially localised to the cytoplasm, and it promptly translocated onto the PM on switching the culture to a glucose-free medium (Fig. 2C). Under glucose-depleted conditions, GLUT1 persisted on the PM of WRO cells for up to 48 h (not shown). On switching to a glucose-depleted medium, the membrane-associated GLUT1 fluorescence in WRO cells increased by approximately fourfold. By contrast, in FTC133 cells, GLUT1 was found permanently residing on the PM (>95% of GLUT1 fluorescence), regardless of whether the cells were cultured in the presence or the absence of glucose (Fig. 2C).

In the following experiments, we investigated the signalling pathway(s) involved in the glucose uptake and the membrane translocation of GLUT1 in response to glucose deprivation in the two thyroid cancer cell lines.

PI3K drives the membrane translocation of GLUT1 in glucose-deprived WRO cells

In FRTL-5 rat thyroid cells, the PM translocation of GLUT1 was shown to depend on the PI3K/AKT pathway (Samih *et al.* 2000). It has been reported that the activation of this pathway leads to increased HIF1 α levels (Zundel *et al.* 2000). Indeed, it is well documented that the PI3K–AKT pathway is abnormally activated in thyroid cancers (Saji & Ringel 2010). Given that this pathway is controlled by the lipid kinase activity of PTEN, the above data could be explained with the altered expression of this oncosuppressor in the two cell lines. In fact, WRO cells express WT *PTEN*, whereas FTC133 cells are subjected to monoallelic deletion and bear an R130STOP mutant allele for *PTEN* (Weng *et al.* 2001, Saiselet *et al.* 2012). The latter leads to a truncated *PTEN* isoform that is not detectable in western blotting (Fig. 3A). The expression level of *PTEN* in WRO cells did not change after 24 h of incubation in a glucose-free medium (Fig. 3A). To determine as to what extent the AKT pathway was driving the membrane expression of GLUT1 in WRO and FTC133 thyroid cancer cells, we pharmacologically inhibited this pathway with 3MA, an inhibitor of PI3K. The PI3K–AKT pathway, as mirrored by Ser473 phosphorylation of AKT, was basally active (regardless of the presence or absence of glucose in the medium) in FTC133 cells, consistent with the lack of *PTEN*, and it was activated in WRO cells upon cultivation under glucose-free condition (Fig. 3B). In WRO cells, Ser473 phosphorylation of AKT was completely abrogated in the presence of 10 mM 3MA (Fig. 3B). By contrast,

Figure 5

3MA differentially affects the uptake of glucose in WRO and FTC133 cells. (A) 2-NBDG uptake was monitored in both cell lines cultured for 16 h in a glucose-free medium in the absence or presence of 3MA. 3MA completely arrested the uptake of 2-NBDG in WRO cells under glucose-depleted conditions, while it had a milder effect in FTC133 cells. ImageJ quantification of 2-NBDG in the cells of representative fields is shown in the

right panel. Scale bar = 20 μ m. Magnification = 63 $\times$. (B) Cytofluorometry assessment of 2-NBDG uptake in the cells cultured as above. Representative cytofluorograms are shown. This experiment further confirmed the inhibitory effect of 3MA on the uptake of glucose. Data shown in this figure have been reproduced four times independently. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-14-0118>.

residual phospho-Ser473-AKT was still detectable in FTC133 cells even in the presence of 3MA, especially in glucose-depleted conditions (Fig. 3B). To be noted, the cellular level of total AKT was reduced in glucose-deprived cultures, especially in FTC133 cells.

We then looked at the phenotypic consequences of the activation and inhibition of the AKT pathway. 3MA had no effect on the membrane expression of GLUT1 in FTC133 cells, whereas, in WRO cells incubated in a glucose-free medium, it strongly limited the membrane translocation of GLUT1, which accumulated in a para-Golgian area (Fig. 4). This observation was confirmed by image quantification of the expression of GLUT1 in the Cyt and PM compartments (Fig. 4, lower panels).

Inhibition of the PI3K-AKT pathway differentially affects the glucose uptake in WRO and FTC133 cells

Then, we assessed the effects of 3MA on the uptake efficiency of glucose in WRO and FTC133 cells. The cells were plated on coverslips and incubated with the 2-NBDG probe in the absence or presence of 3MA in a glucose-free medium. At the end, the cells were imaged under the microscope and cell-associated fluorescence was quantified using the ImageJ Software. 3MA completely abrogated the uptake of 2-NBDG in WRO cells, while it had a milder effect in FTC133 cells (Fig. 5A). Parallel cultures in Petri dishes were used for cytofluorometric quantification of fluorescence-positive cells. Cytofluorometry data indicated that a large

Figure 6

Silencing of *PTEN* in WRO cells determines the membrane translocation of GLUT1. (A) Knockdown of *PTEN* mRNA efficiently reduced (>80%) the expression of the *PTEN* protein in WRO cells (data obtained with one of the two siRNA oligonucleotides targeting two different exons of *PTEN* are shown). The activation status of the AKT pathway was assessed by western blotting after siRNA silencing of *PTEN* and 24 h of incubation in a glucose-containing or glucose-free medium. Basal phosphorylation of AKT increased upon silencing *PTEN* and further increased under glucose starvation. Densitometry quantification of the AKT activation status ($n=3$) is reported in the lower panels (pAKT/AKT was normalised vs β -tubulin).

(B) Subcellular expression of GLUT1 (in green) and *PTEN* (in red) after siRNA silencing of *PTEN* and 24 h of incubation in a glucose-containing or glucose-free medium. Nuclei are stained with DAPI. Down-regulation of *PTEN* promoted the cytoplasmic (Cyt) to plasma membrane (PM) relocation of GLUT1. ImageJ quantification of this phenomenon is included. Note that >80% of the *PTEN* siRNA-transfected cells were negative for *PTEN* staining. Scale bar = 20 μ m. Magnification = 63 $\times$. Representative images and distribution of fluorescence (in percentage) of three independent experiments are shown. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-14-0118>.

proportion of FTC133 cells could take up glucose despite the presence of 3MA (Fig. 5B).

Genetic manipulations of the PTEN expression and trafficking of GLUT1

The above findings are consistent with the involvement of the PI3K/AKT pathway in the trafficking of GLUT1-positive vesicles in both WRO and FTC133 cancer cells.

To determine whether PTEN is involved in the trafficking of GLUT1 in thyroid cancer cells, we genetically manipulated the expression of PTEN in WRO and FTC133 cells. First, we post-transcriptionally silenced *PTEN* in WRO cells. The transfection with a PTEN-specific siRNA elicited (on average) a >80% down-regulation of the expression of PTEN protein (Fig. 6A). The genetic silencing

of *PTEN* expression was reflected on the activation of the AKT pathway. To be noted, under glucose-free condition, AKT was phosphorylated regardless of the expression level of PTEN (Fig. 6A, densitometry in lower panels). Parallel cultures were set on coverslips for immunofluorescence staining of GLUT1 and PTEN. The latter was included to monitor the cells that had been effectively silenced for PTEN. As a result of *PTEN* silencing, in a large proportion of the cells, GLUT1 was found on the PM (Fig. 6B). As predictable, this effect was more evident in the culture incubated in a glucose-free medium (Fig. 6B). Quantification by ImageJ analysis confirmed the PM translocation of GLUT1 upon *PTEN* knockdown.

Then, we transgenically over-expressed *PTEN* in *PTEN*-deficient FTC133 cells (Fig. 7A). The ectopic

Figure 7

Transgenic expression of *PTEN* in FTC133 cells precludes the membrane translocation of GLUT1. (A) Transgenic expression of WT *PTEN* in FTC133 cells was controlled by western blotting assay. The blot was stripped and re-probed to assay the phosphorylation status of AKT. Data show that ectopic expression of PTEN greatly inhibited the phosphorylation of AKT independent of the presence or absence of glucose in the culture medium. Densitometric analysis ($n=3$) of the AKT activation status is included (pAKT/AKT was normalised vs β -tubulin). (B) Subcellular expression of GLUT1 (in green) and PTEN (in red) in transfected FTC133 cells after 24 h of incubation in a glucose-containing or glucose-free medium. Nuclei are

stained with DAPI. Under glucose-containing culture condition, GLUT1 appears clustered in a perinuclear–para-Golgian area and, in glucose-free culture condition, only a small amount of GLUT1 appears to reach the plasma membrane (PM). In *PTEN*-transfected cultures, >60% of the cells were positive for PTEN immunofluorescence. Ectopic expression of PTEN largely precluded the PM relocation of GLUT1. ImageJ quantification of this phenomenon is included. Representative images and distribution of fluorescence (in percentage) of three independent experiments are shown. Cyt, cytoplasm. Scale bar=20 μ m. Magnification=63 $\times$. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-14-0118>.

expression of PTEN was paralleled by the inactivation ($\sim 75\%$) of the AKT pathway (see densitometry in lower panels), probably reflecting the de-phosphorylation of PIP3 in PIP2 operated by PTEN. To be noted, in *PTEN*-transfected cells, AKT remained largely inactive even when cultured in a glucose-free medium (Fig. 7A). In a parallel experiment, the cells adherent on coverslip were stained for GLUT1 and PTEN. In the transfected cells (positive for PTEN), GLUT1 was mainly clustered in a perinuclear–para-Golgian region, though this effect was somehow mitigated when the cells were cultured in a glucose-free medium (Fig. 7B). Based on ImageJ quantification, ectopic expression of PTEN almost completely abrogated the PM-associated GLUT1 fluorescence translocation of GLUT1 in transfected FTC133 cells cultured in the presence of glucose (Fig. 7B).

To determine the functional consequences of the PTEN-dependent relocation of GLUT1 on glucose uptake, we measured the uptake of 2-NBDG in the transfected cell populations. The proportion of cells positive for 2-NBDG almost doubled in *PTEN*-silenced WRO cells compared with control duplex-transfected cells and almost halved in transfected cells expressing transgenic *PTEN* compared with the control Sham-transfected counterparts (Fig. 8).

Figure 8

PTEN drives the glucose uptake in thyroid cancer cells. WRO and FTC133 cells adherent on Petri dishes were transfected with a *PTEN*-specific siRNA or with a plasmid harbouring the *PTEN* cDNA respectively. Sham-transfection was included as a control. The cells were incubated for the optimal time to attain the highest efficiency of endogenous *PTEN* knockdown or of ectopic *PTEN* expression in WRO and in FTC133 cells respectively. The cells were then loaded with the fluorescent 2-NBDG probe and analysed by cytofluorometry. The histogram shows the quantification of uptake (average $\pm$ s.d.) of four experiments. Note that control duplex-transfected WRO cells show an abnormally high uptake of 2-NBDG, probably due to the stress associated with Lipofectamine transfection. The data clearly indicate that PTEN is a master regulator of glucose uptake in WRO and in FTC133 cells.

Discussion

To sustain their high proliferative rate, cancer cells adapt their metabolism towards the aerobic glycolysis (Tong *et al.* 2009). Thyroid cancer cells also show a high rate of glycolysis (Andrade *et al.* 2012). Immunohistochemical studies have shown that enhanced glucose uptake in cancer cells correlates with over-expression of GLUTs, in most cases GLUT1 (Haber *et al.* 1997, Ciampi *et al.* 2008). Herein, we show that the oncosuppressor PTEN plays a dominant role in the membrane expression of GLUT1 and glucose uptake in thyroid cancer cells. It has been shown that the ectopic hyper-expression of PTEN contrasts the uptake and the large glycolytic consumption of glucose observed in proliferating cancer cells (Garcia-Cao *et al.* 2012). Consistently, we found that the transgenic reintroduction of *PTEN* in FTC133 abrogates the membrane expression of GLUT1. This effect was evident in glucose-fed cells, while, in the glucose-deprived cells, the absence of glucose triggered anyhow the membrane translocation of GLUT1 (Fig. 7B). Thus, the absence of glucose is 'dominant' in driving GLUT1 membrane translocation, regardless of the presence of PTEN as in fact occurs in WRO cells as well (Figs 2C, 4 and 6B). Glucose depletion is known to activate the AMPK pathway, which is also involved in the membrane expression of GLUT1 (Andrade *et al.* 2012).

Conversely, we show that the lack of PTEN expression has a dominant effect on the membrane expression of GLUT1 independent of the level of glucose available. Notably, in PTEN-expressing cancer cells, the scarce availability of glucose favoured the expression of GLUT1 on the PM. The PTEN-dependent relocation of GLUT1 had functional consequences in terms of glucose uptake, as the silencing of *PTEN* in WRO cells greatly increased, and conversely the ectopic expression of PTEN in FTC133 cells greatly reduced, the uptake rate of 2-NBDG. These facts have relevance in terms of imaging diagnosis. Imaging by FDG–PET has the potential to reveal unsuspected tumours, including thyroid tumours, commonly indicated as 'incidentaloma' because of their accidental discovery (Bogsrud *et al.* 2010, Hsiao *et al.* 2011, Pagano *et al.* 2011, Bertagna *et al.* 2012). Novel non-radioactive methods for imaging the glucose internalisation by cancer cells are being developed (Walker-Samuel *et al.* 2013), underscoring the importance of glucose uptake as a diagnostic readout of cancer proliferation.

The uptake of glucose in thyroid cancer cells has been shown to be inversely correlated with the uptake of iodide (the so-called 'flip-flop' phenomenon), a feature

of highly aggressive thyroid tumours that are associated with increased lethality (Filetti *et al.* 1986, Frilling *et al.* 2001, Bläser *et al.* 2006, Grabellus *et al.* 2012). In addition, a link between the expressions of GLUT molecules and the pGp responsible for chemoresistance has been shown (Seo *et al.* 2009). Therefore, understanding the signalling pathways that govern the membrane expression of GLUTs and the glucose uptake in thyroid cancer cells also has prognostic and therapeutic implications.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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