PVALB diminishes $[Ca^{2+}]$ and alters mitochondrial features in follicular thyroid carcinoma cells through AKT/GSK3 β pathway

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Abstract

We have identified previously a panel of markers (C1orf24, ITM1 and PVALB) that can help to discriminate benign from malignant thyroid lesions. C1orf24 and ITM1 are specifically helpful for detecting a wide range of thyroid carcinomas, and PVALB is particularly valuable for detecting the benign Hürthle cell adenoma. Although these markers may ultimately help patient care, the current understanding of their biological functions remains largely unknown. In this article, we investigated whether PVALB is critical for the acquisition of Hürthle cell features and explored the molecular mechanism underlying the phenotypic changes. Through ectopic expression of *PVALB* in thyroid carcinoma cell lines (FTC-133 and WRO), we demonstrated that PVALB sequesters free cytoplasmic Ca^{2+} , which ultimately lowers calcium levels and precludes endoplasmic reticulum (ER) Ca^{2+} refilling. These results were accompanied by induced expression of PERK, an ER stress marker. Additionally, forced expression of PVALB reduces Ca^{2+} inflow in the mitochondria, which can in turn cause changes in mitochondria morphology, increase mitochondria number and alter subcellular localization. These findings share striking similarity to those observed in Hürthle cell tumors. Moreover, PVALB inhibits cell growth and induces cell death, most likely through the AKT/GSK-3 β . Finally, PVALB expression coincides with Ca^{2+} deposits in HCA tissues. Our data support the hypothesis that the loss of *PVALB* plays a role in the pathogenesis of thyroid tumors.

Key Words

- ▶ PVALB
- ▶ Hürthle cell adenoma
- ▶ thyroid cancer
- ▶ AKT and GSK β

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Introduction

Thyroid nodules are exceptionally common in the general population. Although only 5% of the adult population develops clinically palpable thyroid nodules during their

lifetime, with the widespread use of the high-resolution ultrasound in clinical practice, an increasing number of impalpable thyroid nodules are being detected and the

reported prevalence is estimated to be as high as 67% (Ezzat et al. 1994, Ross 2002). Once a nodule is discovered, a careful evaluation is needed to surgically treat patients with malignant nodules, while surgery is avoided in patients with benign thyroid nodule.

Fine-needle aspiration (FNA) cytology is the first step in the evaluation of a thyroid nodule. While FNA cytology allows a conclusive diagnosis for papillary thyroid carcinomas (PTCs), cytology alone cannot reliably discriminate follicular thyroid carcinomas (FTCs) or Hürthle cell carcinomas (HCCs) from follicular thyroid adenomas (FTAs) or Hürthle cell adenomas (HCAs). In addition, FNA is also indeterminate for the follicular variant of PTC (FVPTC) (Baloch et al. 2008b). As FNA cytology cannot reliably rule out cancer in nearly 30% of the cases, such nodules are reported as indeterminate (Baloch et al. 2008a). Indeterminate nodules often undergo surgical excision for a definitive diagnosis. For better classification of a disease state and improved clinical decision-making, preoperative molecular markers have been considered.

We have previously identified four carcinoma markers (C1orf24, ITM1, ARG2 and DDIT3) that can accurately distinguish between a wide variety of benign and malignant thyroid lesions (Cerutti et al. 2004). This panel of markers had a sensitivity of 1.00 for distinguishing the benign (FTA and HCA) from the malignant (FTC, HCC and FVPTC) tumors. Nonetheless, HCAs were identified as the reason for keeping specificity at about 0.85 (Cerutti et al. 2006). We later identified PVALB (parvalbumin) as specifically expressed in HCA and, therefore, a new promising marker that could help to discriminate the benign HCA from thyroid carcinomas (Cerutti et al. 2011). In fact, when used in conjunction with two carcinoma markers (C1orf24 and ITM1), it reduced the false-positive rates, yielding high sensitivity and specificity for detecting malignancy (Cerutti et al. 2011).

Despite the clinical significance, the role of these markers in both physiological and pathological conditions is still poorly understood. In this article, we focus particularly on the role that *PVALB* plays in thyroid cancer.

PVALB is a classical member of the EF-hand protein superfamily that has been described as a Ca^{2+} buffer protein (Wang et al. 2009, Schwaller 2010). *PVALB* expression is restricted to a few cell types in the brain, skeletal and heart muscles; parathyroid glands and kidneys (Olinger et al. 2012). *PVALB* gene was assigned to the long arm of chromosome 22 (22q13) (Ritzler & Berchtold 1992).

To provide a better understanding of *PVALB*'s role in the pathogenesis of the thyroid cancer, we have employed ectopic gene transfer. Here, we show that *PVALB* acts as a Ca^{2+} chelator, prevents Ca^{2+} refilling of the endoplasmic reticulum (ER) stores and triggers ER stress with activation of the unfolded protein response (UPR) in thyroid carcinoma cell lines. We also show that forced expression of *PVALB* reduces Ca^{2+} inflow in the mitochondria, which in turn increases the number of mitochondria and promotes changes in mitochondrial morphology and subcellular localization. Moreover, *PVALB* inhibits cell growth and induces cell death, most likely through the AKT/GSK-3 β pathway. We further demonstrate that our *in vitro* results exhibit striking similarities with the results obtained from an analysis performed in Hürthle tumor sections.

Materials and methods

Thyroid samples

HCA ($n=7$) and HCC ($n=7$) samples from patients who underwent thyroid surgery at Hospital São Paulo (Universidade Federal de São Paulo) were obtained with informed consent, according to Independent Ethical Committees of the affiliated institutions.

Cell lines

WRO (human FTC) and human embryonic kidney 293 (HEK-293) cell lines were maintained in Dulbecco's modified essential medium (DMEM), FTC-133 and FTC-238 (human FTC) were maintained in DMEM and Ham's F12 (1:1 mixture) and Nthy-ori 3-1 (human thyroid follicular epithelial derived from normal thyroid tissue) were maintained in RPMI 1640, supplemented with 10% fetal bovine serum (Life Technologies), 100 U/mL of penicillin and 100 $\mu\text{g/mL}$ of streptomycin (Thermo Scientific). Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere. Cells were used in passages 2–5 after transfection. FTC-133 (cat# 94060901), FTC-238 (cat# 94060902) and Nthy-ori 3-1 (cat#90011609) cell lines were purchased from the European Collection of Authenticated Cell Cultures (ECACC). WRO (UCLA RO-82W-1) was kindly donated by Professor Alfredo Fusco (Facoltà di Medicina e Chirurgia, University Federico II, Naples, Italy). Cell line authentication was verified by STR profiling. Cells were also tested for mycoplasma contamination.

Generation of HA-tagged phCMV2-PVALB expression vector

The PVALB full-length cDNA (NM_002854.2) was cloned into phCMV2 expression vector containing a HA tag upstream of the multiple cloning sites. The cDNA was synthesized from mRNA isolated from an HCA sample using Super-Script III Reverse Transcriptase kit (Invitrogen) as described previously (Cerutti et al. 2004, 2011). An aliquot of the cDNA was used in a 50 µL PCR containing 0.2 mM dNTPs mix, 1× PCR buffer, 1 U Platinum Taq DNA Polymerase High Fidelity (Invitrogen) and 10 pmol of each sense and antisense primers. A sense primer, containing a HindIII restriction site (5'CCGAAGCTTTCGATGACAGACTTGCTGAACG3'), and an antisense primer, with a KpnI restriction site (5'CCGGGTACCGGGCAGTCAGTGCTTCTTAGCTT3'), were designed to insert HindIII and KpnI cloning sites (underlined) on, respectively, the 5'- and 3'-termini of the PCR product. The PCR product was purified, digested with the chosen restriction endonucleases and cloned into a double-digested HA-phCMV2 vector. The resulting construct (phCMV2-HA-PVALB) was fully sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Generation of thyroid cells stably expressing PVALB

Follicular thyroid carcinoma cell lines were independently transfected with HA-phCMV2-PVALB construct to generate stable clones that constitutively express the wild-type PVALB protein or HA-phCMV2 (empty vector) to generate clones deprived of PVALB expression (control cells). WRO cells (1.8×10^5) were transfected with 5 µg of each construct using Lipofectamine LTX with Plus Reagent (Life technologies), according to the manufacturer's instructions. FTC-133 cells (5×10^4) were electroporated with 5 µg of each construct using a Gene Pulser Xcell Electroporation System (Bio-Rad Laboratories). The cells were allowed to grow for 48 h and then selected in the presence of G418 antibiotic (800 µg/mL for WRO and 700 µg/mL for FTC-133). Cells were kept under selective medium for 3 weeks. G418-resistant cells were pooled, expanded and maintained in the selective medium. Cell lysates were used for Western blot analysis. Antibody against the HA tag (1:1000, Cell Signaling) was used to confirm the expression of HA-tagged recombinant proteins (HA-PVALB). Antibody against PVALB (1:1000, Sigma-Aldrich) was used to select the cell line with lower basal expression of PVALB. Western blot analysis was

performed as described previously (Latini et al. 2008, Carnevali et al. 2015).

Intracellular Ca²⁺ measurements

WRO cells (1×10^6) were incubated with 5 µM Fluo-4 AM (Life Technologies), an intracellular fluorescent calcium indicator, for 40 min at 37°C in Ca²⁺-free Hank's balanced salt solution (HBSS) with 1 µM Probenecid (Sigma-Aldrich). After a spin down, cells were resuspended in HBSS containing 1 mM CaCl₂ (Merck). To promote intracellular Ca²⁺ mobilization in cells, we used 2 µM Ca²⁺ ATPase inhibitor thapsigargin (THG), a specific ER calcium ATPase inhibitor that blocks calcium refilling of ER. Fluo-4 AM fluorescence ($\lambda_{ex}=505$ nm and $\lambda_{em}=530$ nm) was measured by spectrofluorimeter (Hitachi F-7000; Hitachi). The cytoplasmic calcium concentration ($[Ca^{2+}]_{cyt}$) for each sample was calculated from the fluorescence data (F) using a K_d of 345 nM: $[Ca^{2+}]_{cyt} = K_d \times [(F - F_{min}) / (F_{max} - F)]$. F_{max} is calcium maximum fluorescence obtained after addition of detergent digitonin and F_{min} is the lowest fluorescence obtained after calcium chelation by EGTA in Tris (pH 8) solution. The experiment was carried out in triplicate.

Simultaneous monitoring of cytoplasmic and mitochondrial [Ca²⁺]

WRO cells were simultaneously incubated for 40 min with 5 µM Fluo-4 AM (cytoplasmic) and Rhod-2 AM (mitochondrial) Ca²⁺ fluorescent indicators at 37°C (Molecular Probes). Subsequently, cells were washed twice and maintained in HBSS supplemented with 1 mM CaCl₂. The fluorescence parameters used were $E \times 488$ nm/ $Em 505-530$ nm (Fluo-4 AM) and $E \times 552$ nm/ $Em 580$ nm (Rhod-2 AM). Samples were obtained using Leica TCS SP8 confocal microscopy and LAS AF Lite software (Version 4.0; Leica Microsystems). To promote intracellular Ca²⁺ mobilization from ER stores in the cells, 10 µM THG was added at 30 s after initial reading. The experiment was performed in triplicate.

Transmission electron microscopy

Transmission electron microscopy (TEM) was used to visualize changes in mitochondrial morphology, number and ultrastructure. Cells were fixed with 2.5% glutaraldehyde (Sigma-Aldrich) in 0.1 M sodium cacodylate buffer (pH 7.4, Sigma-Aldrich) and then postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (Sigma-Aldrich). The samples were further dehydrated

through a graded acetone series (50, 60, 70, 80, 90 and 100%) for 10 min in each step and embedded in Spurr's resin (Brenner & Horne 1959). Ultrathin sections (80–90 nm) were cut using an LKB Ultratome and mounted on copper grids. The sections were contrasted with uranyl acetate-lead citrate (Watson 1958, Reynolds 1963). The samples were observed with a Philips EM 208 transmission electron microscope (FEI Ltd, Eindhoven, The Netherlands) under an acceleration of 80 kV.

Mitochondrial morphology and distribution

To analyze changes in mitochondrial morphology, cells were stained with 25 nM (WRO) and 250 nM (FTC-133) Mitotracker Red CMXRos dye (Thermo Scientific) in culture medium at 37°C, for 45 min, according to the manufacturer's instructions, and visualized with a Leica TCS SP8 confocal microscopy $\times 63$ objective (Leica Microsystems), with an excitation/emission wavelength of 579/599 nm, respectively. For analysis of the mitochondrial membrane potential, approximately 4×10^3 cells were also incubated with Mitotracker Red CMXRos dye, as described above. Then, cells were suspended in 200 μ L free-FBS culture medium, and analyzed in Guava PCA flow cytometer (Millipore).

Proliferation assay

To estimate cell proliferation rate, cells were seeded in 35 mm dishes at an initial density of 2×10^4 , in quintuplicate. Cells were harvested each day, incubated with 0.4% trypan blue solution and the number of live cells was counted in a Neubauer chamber at indicated time points.

Apoptosis assay

WRO and FTC-133 cells (4×10^3) were seeded in 24-well plates at an initial density of 4×10^3 per well. Cells were double-stained with Annexin V and 7-AAD. The Annexin V and 7-AAD positive populations were counted using the Guava PCA flow cytometer (Millipore), according to the manufacturer's recommendations. The analysis was performed 120 h (WRO) or 72 h (FTC-133) after seeding. Experiments were performed in quintuplicates.

Senescence assay

Senescence assay was performed as described previously (Latini et al. 2011). Briefly, 2×10^4 WRO and FTC-133

transfected cells were seeded in 35 mm dishes. Cells were incubated with 1 mg/mL X-gal (5-bromo-4-chloro-3-inolyl- β -D-galactopyranoside, Sigma-Aldrich), at 37°C in 5% CO₂ for 18 h. After the incubation time, the cells were examined by a Nikon eclipse 80i microscopy and the stained cells were counted using $\times 100$ objective lens.

Western blot analysis

Protein extraction and quantification were performed in transfected WRO and FTC-133 cells, as described previously (Latini et al. 2008, Carnevali et al. 2015). Briefly, cells were lysed in 50 mM Tris-HCl, 100 mM NaCl, 50 mM NaF, 1 mM NaVO₄ and 0.5% Triton-X solution with protease inhibitors (Complete Cocktail, Roche, Basel, Switzerland). Quantification was performed using BCA Protein Assay Kit (Pierce Biotechnologies). Total protein extracts (50 μ g) were separated on a 10% SDS-PAGE gel electrophoresis using a Bio-Rad Mini-Protein II electrophoresis system (Bio-Rad). Separated proteins were then transferred to 0.45 μ m nitrocellulose membrane (Bio-Rad). Immunoblotting was carried out with the following antibodies: anti-phospho-AKT (Ser473) (1:1000; Cell Signaling), anti-phospho-GSK-3 β (Ser9) (1:1000; Cell Signaling), anti-PERK (1:1000; Cell Signaling), anti-phospho-ERK1/2 (Thr202/Tyr204) (1:1000; Cell Signaling), anti-ERK1/2 (1:1000, Cell Signaling) and anti- α -tubulin (1:10,000, Sigma-Aldrich). The membranes were incubated with anti-mouse (1:10,000, Santa Cruz Biotechnology) or anti-rabbit (1:10,000, Dako) horseradish peroxidase (HRP)-conjugated secondary antibodies. HRP chemiluminescence was detected by adding Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore). The signal was detected by ImageQuant LAS 4000 system (GE Healthcare). The bands were quantified using Image-J Software, as described previously (Carnevali et al. 2015).

Immunohistochemistry analysis

Immunocytochemistry staining was performed in formalin-fixed, paraffin-embedded tissue samples. Tissue sections were deparaffinized with xylene and hydrated through a graded alcohol series. Endogenous peroxidase activity was blocked by placing slides in 15% hydrogen peroxide solution for 10 min. Sections were then incubated with 5% Tween in phosphate-buffered saline for 20 min, to permeabilize the membranes. Antigen retrieval was performed using 0.1 M Tris-HCl

Figure 1

PVALB expression alters calcium homeostasis in thyroid carcinoma cell lines. (A) Representative changes in Fluo-4 AM ratio measured in WRO cells transfected with *PVALB* and empty vector (control). (B) No significant difference in the free Ca²⁺ level was observed in the cytoplasm of *PVALB*-expressing cells after addition of 2 μM thapsigargin (THG), compared with control cells. Representative (C) confocal images and (D) curve of WRO cells treated with 10 μM THG and loaded with Fluo-4 AM (cytoplasm) and Rhod-2 AM (mitochondria) Ca²⁺ indicators, showing low levels of [Ca²⁺]_{cyt} and [Ca²⁺]_{mit} in *PVALB*-expressing cells. (E) Histogram of Ca²⁺-relative fluorescence before and after stimulation with THG (relative to data presented in C and D). Data represent mean ± s.e.m. of three independent experiments. (F) Representative blot of PERK levels in WRO and FTC-133 cells transfected with HA-*PVALB* or empty vector (control). (G) Densitometry of each band was normalized with respect to α-tubulin as the loading control. Data were expressed as mean ± s.d. of three independent experiments. ****P* < 0.001; ***P* < 0.01; **P* < 0.05.

pH 8.0 buffer in steamer for 15 min. After cooling to room temperature, nonspecific binding sites were blocked with 5% BSA in phosphate-buffered saline. Primary antibody (anti-PVALB, 1:1000, Sigma-Aldrich) was incubated overnight and immunostaining was performed using EnVision System (Dako). Slides were counterstained with hematoxylin and analyzed using a light microscope.

Von Kossa staining

Paraffin-embedded tissues of HCA ($n=7$) and HCC ($n=7$) were sectioned at 4 μm thickness, deparaffinized and hydrated, as described above. Slides were placed in a

solution containing 5% AgNO_3 (Sigma-Aldrich) for 1 h under constant light, as described previously (Michalany 1990, Embi & Menes 2013) and then, counterstained with hematoxylin. The slides were examined by light microscopy Carl-Zeiss Axio Scope A1 (Carl Zeiss) and the images were captured using Zeiss Zen Software.

Statistical analysis

All results were submitted to Shapiro-Wilk Normality Test to determine whether the data followed a normal distribution. Student's *t*-test was applied in normally distributed data. Mann-Whitney test was used when the distribution was not normal. For all analysis, $P < 0.05$ was

Figure 2

PVALB-forced expression altered mitochondrial morphology. Representative images from transmission electron microscopy (TEM) analysis of WRO (A) and FTC-133 (B) cells following *PVALB* expression, compared with control. *PVALB* promoted dramatic changes in mitochondrial morphology (black arrowhead, fragmented mitochondria; white arrowhead, interruption of the outer membrane), cell size and subcellular localization. Images increase from 4000 to 20,000 $\times$. Scale bar represents 5, 2 and 1 μm . Arrow: concentric rings; white asterisk: technical artifact. (C and D) Mitochondrial count in accordance with TEM images. Data were expressed as mean $\pm$ s.d. $**P < 0.01$; $*P < 0.05$.

considered significantly different. Statistical software used were GraphPad Prism 5 (Graphpad Software) and SigmaPlot 12.0 (Systat Software Inc, San Jose, CA).

Results

Generation of WRO and FTC-133 cells stably expressing PVALB

To validate our previous findings, *PVALB* expression was tested in a panel of human FTC cell lines (WRO, FTC-133 and FTC-238) and in the cell line derived from normal human thyroid follicular epithelium (Nthy-ori 3-1). As positive control, the human embryonic kidney 293 cells (HEK-293) was transfected with the construct that expresses *PVALB* (HA-phCMV2-PVALB). As expected, *PVALB* expression was not detected in WRO, FTC-133, FTC-238 and Nthy-ori 3-1, while a specific band at 12kDa was detected in the positive control (Supplementary Fig. 1A, see section on supplementary data given at the end of this article). To explore the biological effect of *PVALB* re-expression in FTCs, two follicular thyroid cancer-derived cell lines (WRO and FTC-133) were selected as *in vitro* models. *PVALB* tagged with the HA epitope was then ectopically expressed and anti-HA antibody was tested in cells overexpressing HA-tagged *PVALB* (WRO and FTC-133) vs the empty vector (control). The fusion protein (HA-PVALB) was detected at very high levels in cell lysates obtained from

cells overexpressing *PVALB* but was absent in control cells (Supplementary Fig. 1B).

Ectopic expression of PVALB reduces cytoplasmic free Ca^{2+} levels in thyroid cancer cell line and impairs Ca^{2+} storage in the mitochondria

We next investigated if ectopic expression of *PVALB* could disrupt Ca^{2+} homeostasis in WRO cells. To this end, cells were loaded with Fluo-4 AM, a cytosolic free calcium indicator, and the ER Ca^{2+} -ATPase inhibitor thapsigargin (THG) was added to the cells to elicit a rapid transient rise in cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) from ER stores. In control cells, THG increased Fluo-4 AM fluorescence as it increased $[\text{Ca}^{2+}]_{\text{cyt}}$ ($240 \pm 524 \text{ nM}$) (Fig. 1A and B). Although higher concentrations of free Ca^{2+} were observed in the cytoplasm of *PVALB*-expressing cells compared with control cells, no significant reading was detected before and after exposure to THG (Fig. 1A and B). This interesting data show that *PVALB* acts as a Ca^{2+} chelator, thus compromising the Ca^{2+} homeostasis.

As mitochondria can rapidly sense cytoplasmic Ca^{2+} fluctuations, we investigated whether *PVALB*-induced ER Ca^{2+} depletion could also compromise Ca^{2+} availability for the mitochondria. To measure Ca^{2+} flux from the ER to the mitochondria, WRO transfected cells were loaded with both Fluo-4 AM (cytoplasm) and Rhod-2 AM (mitochondria) Ca^{2+} indicators and evaluated by fluorescence microscopy. The images clearly show that

Figure 3

PVALB-forced expression altered mitochondrial membrane potential and mitochondrial morphology in thyroid carcinoma cell lines. Representative results of the mitochondrial membrane potential in WRO (A) and FTC-133 (C) cells. Data represent mean $\pm$ s.d. of five independent experiments. WRO (B) and FTC-133 (D) cells were stained with MitoTracker red CMXRos dye and analyzed for mitochondrial morphology by fluorescence microscope. Scale bar represents $10 \mu\text{m}$. ** $P < 0.01$

there is an increase in Fluo-4 and Rhod-2 fluorescence after the addition of THG (10 μ M) in control cells compared with *PVALB*-expressing cells (Fig. 1C). The representative curve displays a fluorescence peak approximately 30 s after the addition of the inhibitor (Fig. 1D). The THG-elicited cytosolic Ca^{2+} increase was approximately 36% lower in the *PVALB* cells compared with the control ($P < 0.001$; $n = 3$) (Fig. 1E), thus suggesting that *PVALB*-expressing cells had less ER-stored Ca^{2+} . *PVALB*-expressing cells also displayed a reduction in the Ca^{2+} inflow to the mitochondria compared with control cells (Fig. 1E). The latter result is in agreement with the former observation, as lower Ca^{2+} release from the ER is expected to lead to lower Ca^{2+} inflow to the mitochondria. Our results also suggest that *PVALB* acts as a Ca^{2+} chelator, which reduces the Ca^{2+} -dependent metabolism of the mitochondria in thyroid carcinoma cell lines.

PVALB-induced ER stress

It is well known that the disturbance of calcium homeostasis can lead to the so-called ER stress. We investigated if *PVALB* ectopic expression would trigger ER stress and activate the UPR via PERK (protein kinase RNA-like ER kinase). We observed that *PVALB* ectopic expression significantly increased the expression of PERK in both WRO and FTC-133 cells ($P < 0.05$; Fig. 1F and G).

The data provide consistent evidence that the *PVALB* cellular mechanism in cancer cells involves the induction

of stress response through the disruption of normal uptake of Ca^{2+} by the ER.

PVALB expression leads to an increase in cell size and mitochondria number and promotes changes in mitochondrial morphology and subcellular localization

We first investigated if *PVALB*-mediated changes in cytoplasmic and mitochondrial Ca^{2+} concentration could affect mitochondrial morphology, subcellular localization and mitochondria number. TEM images of thin sections of WRO and FTC-133 transfected cells showed that *PVALB*-expressing cells displayed a sequence of mitochondrial modifications (Fig. 2A and B). In *PVALB*-expressing cells, the mitochondria were broadly distributed through the cytoplasm, whereas in control cells, the well-known association between mitochondria and rough ER was still observed (upper and middle panels). Signs of mitochondrial degeneration, characterized by mitochondria fragmentation, were also observed in *PVALB*-expressing cells (black arrowhead; lower panel). The cristae became disorganized in *PVALB*-expressing cells; the inner membrane structure was composed of one to several layers of concentric rings (black arrows) in *PVALB*-expressing cells (lower panel; Fig. 2A). Intriguingly, the outer membrane was interrupted in FTC-133 cells expressing *PVALB* (white arrowhead; Fig. 2B).

Indeed, TEM images also showed an increase in the number of mitochondria in *PVALB*-expressing cells

Figure 4
PVALB reduced cell proliferation and promoted cell death in follicular thyroid carcinoma cell lines. Growth curves of the WRO (A) and the FTC-133 (B) cell lines. (C) The apoptosis rate was assayed by flow cytometry in WRO (C) and senescent cells were quantified by β -galactosidase activity in FTC-133 (D) Graphics represent the mean $\pm$ s.d. of five independent experiments. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

compared with controls (Fig. 2A and B). TEM images confirmed a dramatic increase in the mitochondrial number in *PVALB*-expressing cells, compared with controls (Fig. 2C and D).

To achieve a comprehensive understanding of the mitochondrial changes found by TEM, cells were stained with the fluorochrome MitoTracker Red CMXRos and then analyzed by flow cytometer and confocal fluorescence microscopy. This organelle-specific fluorochrome is a cell permeable probe that passively diffuses across the plasma membrane of viable cells and accumulates in active mitochondria. Its accumulation is dependent of the membrane potential. Mitochondrial membrane potential was not affected in WRO cells expressing *PVALB*, when compared with the control (empty vector; Fig. 3A). However, fluorescence microscopy images of WRO cells expressing *PVALB* showed that mitochondria undergo morphological remodeling, corroborating the findings of the TEM (Fig. 3B). Also, mitochondria were spread out and covered a larger area of the cytosol, confirming increased number of mitochondria and altered subcellular localization (Fig. 3B). It is also clear that *PVALB* ectopic expression affected mitochondrial shape. Smaller and rounded organelles were observed in *PVALB*-expressing cells, which suggest organelle fragmentation. Images from FTC-133 cells expressing *PVALB* showed a reduction in the fluorescence intensity compared with controls, which is indicative of cells with lower membrane potential (31%; Fig. 3C and D). This result is in accordance with the TEM images, which show a disruption in FTC-133 mitochondrial outer membrane.

Altogether, our data suggest that *PVALB* dramatically affects mitochondrial features, most likely in response to $[Ca^{2+}]$.

PVALB expression leads to apoptosis and senescence

Given that the above-described mitochondrial changes are considered hallmarks of apoptosis, we additionally monitored whether *PVALB* alters cell proliferation and cell death rates (Contreras et al. 2010). Cell growth was determined by cell counting at appropriate intervals, that is every 24 h up to 120 h for WRO cells and up to 72 h for FTC-133. The proliferation rate was significantly lower in WRO cells expressing *PVALB*, compared with control cells ($P < 0.001$). Although less evident, this effect was also observed in FTC-133 cells ($P < 0.01$) (Fig. 4A and B). Based on these results, we next investigated if *PVALB* reduces proliferation by inducing apoptosis. Double staining with annexin V and 7-AAD showed that both early and late

apoptosis rate were increased in WRO cells expressing *PVALB* ($P < 0.001$; Fig. 4C). As similar mechanisms of initiation can trigger different responses of genes, we next assessed whether senescence was the mechanism associated with cell death in FTC-133 cells. Increased senescence-associated beta-galactosidase (SA- β -gal) activity was detected in FTC-133 cells expressing *PVALB*, compared with control cells ($P < 0.01$) (Fig. 4D). These data indicate that prosurvival response in FTC-133 cells, following *PVALB* expression and mitochondrial stress, predisposes cells to senescence.

Whether *PVALB* induces apoptosis or senescence may be dependent of the genetic background of each cell line used. Whatever the molecular mechanism underlying these responses, *PVALB* inhibits cell growth and induces cell death in thyroid cancer cells.

Figure 5

PVALB reduced phosphorylation of AKT/GSK-3 β in thyroid carcinoma cell lines. (A) Representative blot of AKT (Ser473) and GSK-3 β (Ser9) phosphorylation, determined by Western blot. (B) Densitometry analysis of each band was normalized with respect to α -tubulin as the loading control. Data were expressed as mean $\pm$ s.d. ** $P < 0.01$.

Figure 6

PVALB expression coincides with calcium deposits in HCA. Representative results of immunohistochemistry analysis performed in paraffin-embedded samples of Hürthle cell adenoma (HCA; $n=7$) and Hürthle cell carcinoma (HCC; $n=7$) samples. Calcium deposits were detected by Von Kossa staining. Magnification 1000 $\times$. Scale bar represents 5 μ m.

PVALB reduces AKT and GSK-3 β phosphorylation

We next investigated whether ectopic expression of PVALB would modulate some commonly activated signaling pathways that could correlate with our *in vitro* observations. Total protein was isolated from both cell lines and the expression of key proteins of the PI3K/AKT/GSK-3 β pathway was evaluated by Western blot analysis. PVALB ectopic expression reduced the phosphorylation of AKT (Ser473) in WRO (48%; $P<0.01$) and FTC-133 (22%) cells, as well as diminished the phosphorylation of GSK-3 β (Ser9) in WRO (46%; $P<0.01$) and FTC-133 (44%) cells (Fig. 5A and B).

As mutations in genes involved in the MAPK pathway are frequently reported in thyroid tumors, we also investigated whether PVALB had a significant effect in this pathway. Surprisingly, ERK1/2 phosphorylation (Thr202/Tyr204) was not modified after PVALB expression (Supplementary Fig. 2).

Thus, PVALB expression may induce apoptosis and senescence by modulating AKT/GSK-3 β pathway.

PVALB expression correlates with calcium deposits in paraffin-embedded Hürthle cell adenomas

The above-described phenotypes observed in thyroid carcinoma cells following PVALB expression strikingly resemble the mitochondrial abnormalities observed in patients with Hürthle cell tumors (Satoh & Yagawa 1981, Sobrinho-Simões 1985). It is well known that the chelation process converts the ionic Ca^{2+} into Ca^{2+} carbonate (CaCO_3) (Embi & Menes 2013). The von Kossa

staining turns the CaCO_3 black, which allows the visualization of the intracellular calcium. To explore if PVALB works as a Ca^{2+} chelator in thyroid tumor sections, we performed the Von Kossa staining in thyroid tumor paraffin-embedded sections of HCA ($n=7$) and HCC ($n=7$). To verify whether there was a correlation between the presence of Ca^{2+} and PVALB expression, samples were also stained with anti-PVALB. As expected, PVALB was expressed in all HCA (7/7), while its expression was absent in all HCC (7/7) (Fig. 6). Interestingly, granular chelated ionic calcium, expressed as silver granules, was detected in all HCA. No granular black spots were detected in HCC, which are negative for PVALB expression. We here show, for the first time, that disruption of calcium flow, caused by chelation, also occurs in HCA samples and may explain, at least in part, the morphological changes observed in HCA.

Discussion

Our group has identified and validated a panel of molecular markers, which includes *C1orf24*, *ITM1* and *PVALB*, that can accurately distinguish between benign and malignant thyroid lesions (Cerutti et al. 2011). Despite the fact that the identification of new markers may ultimately help patient care, the current understanding of their biological functions remains largely unknown.

To narrow this knowledge gap, in this study, we focused particularly on the role that PVALB plays in thyroid cancer. PVALB was identified as a diagnostic marker that stains specifically for the benign HCA, while

tests negative in a wider variety of thyroid carcinomas (Cerutti et al. 2011).

As *PVALB* expression was not detected in FTCs and its variant HCC, we ectopically expressed *PVALB* in two FTC cell lines (WRO and FTC-133) and investigated whether its forced expression is linked to phenotypic changes that prevent cell transformation.

As *PVALB* functions as a calcium-buffering protein, its ectopic expression is expected to influence the intracellular Ca^{2+} concentration, thus causing a cytoplasmic Ca^{2+} level shift that would ultimately influence a variety of cellular processes (Berridge et al. 2003).

To raise cytoplasmic Ca^{2+} levels, we used thapsigargin, which inhibits Ca^{2+} uptake into ER. Our data showed that ectopic expression of *PVALB* sequesters free cytoplasmic Ca^{2+} , resulting in lower Ca^{2+} levels and precluding ER Ca^{2+} refilling, therefore, compromising the flow of Ca^{2+} into the mitochondria (Fig. 7).

Importantly, ER depends highly on Ca^{2+} concentrations to fold, modify and assemble newly synthesized proteins (Orrenius et al. 2003, Xu et al. 2005, Stutzmann & Mattson 2011). Therefore, changes in intracellular Ca^{2+} concentration can lead to misfolded protein accumulation resulting in ER stress activation. One of the most characterized and highly conserved ER stress responses is the UPR (Hoyer-Hansen & Jaattela 2007, Zhang 2014). In mammalian cells, the UPR seems to be driven by three ER-located membrane proteins (PERK, ATF6 and IRE1). As protein kinase RNA-like ER kinase (PERK) may act as Ca^{2+} sensor in the ER (Liang et al. 2006), we tested whether

depletion of ER Ca^{2+} stores would lead to ER stress and a triggered UPR response. We demonstrated that *PVALB* expression rapidly induced PERK expression, a sensor and transducer of the UPR response.

Given that Ca^{2+} is the central component in the regulation of mitochondrial respond to stress through changes in their subcellular localization, overall number, size, shape, interconnectedness and function (Boland et al. 2013), we hypothesized that *PVALB* expression would contribute, at least in part, to mitochondrial morphological changes commonly observed in Hürthle cells (Satoh & Yagawa 1981, Sobrinho-Simões 1985).

PVALB-forced expression in thyroid carcinoma cells promoted an increase in cell size, in parallel with changes in mitochondrial morphology. Furthermore, a particularly striking similarity to Hürthle cell tumors was the abundance of mitochondria. *PVALB*-forced expression leads to an increase in mitochondrial number. Additionally, TEM of the cells showed that the mitochondria were spread out throughout the entire cytoplasm, as well as smaller and rounded, which suggests organelle fragmentation. Additionally, *PVALB* caused changes in the inner mitochondrial membrane in WRO cells, while promoted disruption of the outer mitochondrial membrane in FTC-133 cells. Moreover, membrane potential-dependent staining of mitochondria confirmed increased fragmentation and altered morphology of *PVALB*-positive cells, as well as disturbance in membrane potential in FTC-133 cell line.

It is well known that Ca^{2+} acts as a second messenger in several signaling pathways involved in cell proliferation

Figure 7

Schematic representation of the proposed events that occurs following *PVALB* expression in thyroid carcinoma cells and normal cells. An abnormal $[\text{Ca}^{2+}]_{\text{cyt}}$ caused by ectopic expression of the Ca^{2+} -binding protein (*PVALB*), compromised Ca^{2+} endoplasmic reticulum (ER) storage and the Ca^{2+} flow into the mitochondria. Decreased $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{Ca}^{2+}]_{\text{mit}}$ promoted multiple mitochondrial morphological changes commonly observed in Hürthle cells. Loss of *PVALB* expression and altered Ca^{2+} signaling may be involved in the progression of thyroid carcinoma, particularly in HCC.

~200 nM

and cell death (Berridge *et al.* 2003). Additionally, it has been suggested that the main player in the finely tuned apoptotic activation process is cytochrome c (Boland *et al.* 2013). As the majority of cytochrome c molecules are tightly bound to mitochondrial inner membrane (Pinton *et al.* 2008), which is altered in WRO cells, we postulated that PVALB would trigger cell death through apoptosis, at least in WRO cells.

To further support our hypothesis that PVALB may prevent cell transformation through cell death, we investigated whether PVALB could promote cell death. PVALB expression diminished cell proliferation rate and induced cell death in both cell lines, these results being more evident in the WRO cell line. Curiously, PVALB promoted cell death through apoptosis in WRO cell line and senescence in FTC-133. These differences are most likely due to genetic background of the cell lines used in this study. In fact, the mutational status of this human thyroid cancer cell lines showed that WRO expresses wild-type PTEN while FTC-133 is PTEN-null (Morani *et al.* 2014). Moreover, PTEN status has been associated with cellular senescence in other cancer types (Lee *et al.* 2011). Another potential explanation is that different Ca^{2+} concentrations within cells may trigger different forms of cell death (Pinton *et al.* 2008).

As AKT is one of the proteins known to promote cell survival through its ability to phosphorylate and inactivate several proapoptotic targets, we tested if PVALB could reduce AKT activity. We here show that forced expression of PVALB significantly reduces phosphorylation of AKT at Ser(473) in WRO cells and slightly reduces its phosphorylation in FTC-133 cells. As AKT can attenuate GSK-3 β enzymatic activity by phosphorylating Ser(9) (Jacobs *et al.* 2012), we next tested whether PVALB reduces GSK-3 β phosphorylation. As expected, PVALB reduced its phosphorylation and therefore, increased its enzymatic activity.

Hürthle cell neoplasms, both benign and malignant, should be composed of at least 75% Hürthle cells (Montone *et al.* 2008), which are large cells with abundant cytoplasm containing accumulation of morphologically abnormal mitochondria (Montone *et al.* 2008, Maximo *et al.* 2012, Ferreira-da-Silva *et al.* 2015). Additionally, it has been suggested that Hürthle cell neoplasms may show dystrophic calcifications (Montone *et al.* 2008). Although it has been considered to represent an unusual reaction, in which biochemically modified colloid produced by the Hürthle cells attracts Ca^{2+} , we here demonstrated that PVALB expression coexisted with Ca^{2+} presence in HCA.

Given that PVALB is expressed in the benign HCA and its expression is lost in the malignant HCC and both tumors have Hürthle cells, we hypothesized that PVALB expression is an early event associated with abnormal mitochondrial biogenesis and cell death. Whether additional changes may contribute to prevent cell death, such as overexpression of antiapoptotic factors, need further investigation.

Although HCC are believed to be a variant of follicular carcinomas, a more aggressive behavior has been reported (Montone *et al.* 2008, Maximo *et al.* 2012). Considering this and that Hürthle cells show low uptake of iodine and, therefore, do not respond well to conventional treatment (Montone *et al.* 2008), we raised the question as to whether PVALB could be a target for HCC therapy.

Together, our results indicated that PVALB plays a role in cellular Ca^{2+} homeostasis, ER stress and cell death, and most likely has a role in mitochondrial dynamics. These data support the hypothesis that the loss of PVALB plays a role in the progression of thyroid tumors.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-16-0181>.

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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Author contribution statement

T B M contributed with the design of the study, performed experiments, analyzed the data and wrote the manuscript. B H N performed Western blot experiments, analyzed the data, performed statistical analysis and drafted the manuscript. A B designed the calcium experiments. R B S provided technical assistance with the transmission electron microscopy (TEM) and Von Kossa experiments. M H B C designed and analyzed the TEM data. R D reviewed the diagnosis of all paraffin-embedded thyroid sections used in this study. M L G contributed to the conception and design of the calcium experiments, analyzed the data and drafted the article. J M C conceived, designed and coordinated the study; analyzed and interpreted the data; acquired funding; and edited and reviewed the final version of the manuscript. All authors reviewed and approved the final version of the manuscript.

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