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# Prolactin stimulates prostate cell proliferation by increasing endoplasmic reticulum content due to SERCA 2b over-expression Short title: Prolactin stimulates prostate cell proliferation

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Abbreviations used: PRL, prolactin; ER, endoplasmic reticulum;  $[Ca^{2+}]_{cyt}$ , cytosolic calcium concentration; SERCA 2b, sarcoendoplasmic calcium ATPase 2b; TNT, Tris-NaCl-Tween buffer; HBSS, Hank's Balanced Salt Solution; siRNA, small interference RNA, TG, thapsigargin; PCNA, Proliferating Cell Nuclear Antigen; TRPM8, transient receptor potential melastatin 8

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#### ABSTRACT

Prolactin (PRL) has been shown to be involved in the differentiation and proliferation of numerous tissues, including the prostate gland. Moreover, variations in calcium concentration within the endoplasmic reticulum ( $[Ca^{2+}]_{ER}$ ) may play a role in cell growth. However, few studies have focused on the regulation of calcium homeostasis by prolactin. This study evaluates the regulation of calcium pools as well as the possible role of  $[Ca^{2+}]_{ER}$ variations as a signal for growth modulation by PRL. We show that PRL stimulates the proliferation of normal SV40 immortalized epithelial prostatic (PNT1A) cells with a maximum effect at a dose of 100 ng/ml. We also show that PRL (100 ng/ml) increases the  $[Ca^{2+}]_{ER}$  when measured either by indirect quantification with Fura-2AM after application of 1 µM thapsigargin or by direct quantification with Mag-Fura-2AM within the endoplasmic reticulum. Western blot analysis shows that the sarco-endoplasmic calcium ATPase 2b (SERCA 2b) is over-expressed in PNT1A cells treated with 100 ng/ml PRL for 24 hours. A siRNA SERCA2a/b, used to downregulate endogenous SERCA 2b expression, reduces both PNT1A cell proliferation and  $[Ca^{2+}]_{ER}$ . We thus identify  $[Ca^{2+}]_{ER}$  and SERCA 2b as protagonists in PRL-induced proliferation.

#### **INTRODUCTION**

Due to the constant increase in life expectancy, benign prostate hyperplasia and prostate cancer have become very common diseases. Prostate cancer is now the second cause of cancer-induced death among men. It has been clearly established that the growth, differentiation and programmed cell death of prostatic cells are regulated by androgens [1]. For this reason, the main treatment for prostate tumours consists of cell growth inhibition by suppressing the action or production of endogenous androgens by chemical or surgical castration. This only results in a temporary regression of the disease, since almost all tumours continue to progress.

Therefore, other non-steroid factors or hormones, like prolactin (PRL), are considered to be important for the normal and pathological development of the prostate gland. Indeed, PRL serum levels increase with age, whereas androgen levels decrease, suggesting that the role of PRL in the development of prostate hyperplasia and cancer becomes more important with age. PRL is a polypeptidic hormone, belonging to the cytokine super-family, produced mainly in the pituitary gland, but is also synthesized by peripheral tissues like mammary or prostate glands. Studies by several teams including ours have shown that PRL is one of the non-steroidal factors involved both in prostate cell proliferation [2, 3] and in the development of benign prostate hyperplasia and prostate cancer [4, 5].

Previous research in our laboratory has demonstrated the crucial role played by  $Ca^{2+}$  pools in the prostate [6-8]. Indeed  $Ca^{2+}$  homeostasis modulation is a mechanism common to a number of signal transduction pathways regulating a wide range of cell phenomena. Cytosolic  $Ca^{2+}$  is known to control numerous cell functions, including contraction, gene expression and proliferation [9]. The maintenance of  $Ca^{2+}$  levels within the lumen of the endoplasmic reticulum is another critical factor for cell growth [10]. The endoplasmic reticulum is a

dynamic environment perfectly designed for protein synthesis, post-traductional modifications and folding. Moreover, the maintenance of high intraluminal  $Ca^{2+}$  is essential for these processes [11]. However the role of calcium signalling in the PRL signal transduction has yet to be fully elucidated.

In the present study, we show that PRL stimulates the proliferation of PNT1A prostatic cells via a mechanism involving the increase in the endoplasmic reticulum  $Ca^{2+}$  content due to the over-expression of SERCA 2b proteins. Using siRNA strategy to downregulate the endogenous SERCA 2b expression in PNT1A cells, we show that a reduction in  $Ca^{2+}$  store content leads to a proportional decrease in cell growth. Thus, our study provides the first direct evidence that SERCA expression and endoplasmic reticulum  $Ca^{2+}$  content are major players in the PRL signal transduction controlling prostate cell growth. Considering the recent evidence of PRL involvement in the aberrant proliferation of prostate tumours, our results provide a new insight into the mechanisms of the age-related cancer development.

#### MATERIALS AND METHODS

**Cell culture** - The immortalized human prostate normal cell line PNT1A obtained from the European Collection And Cell Culture (ECACC) was maintained in culture in RPMI 1640 medium (Life technologies, Inc.) supplemented with 10% fetal calf serum (Seromed, Poly-Labo, Strasbourg, France) and 5 mM L-glutamine (Sigma, L'Isle d'Abeau, France) and 1% kanamycine (Sigma, L'Isle d'Abeau, France). Cells were grown at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The medium was replaced every 48 h.

**Proliferation assays** - The assay medium was the same as that used for culture, but supplemented with 1% fetal calf serum. The 96-well plates (Nunc) had a final volume of 200  $\mu$ l or 24-well plates (Nunc) had a final volume of 1 ml and were assayed 48 h after seeding.

PNT1A were cultured for 4 days of treatment with or without reagents, as indicated. The medium was replaced every 48 h. Proliferation was determined with a MTS reagent kit.

**siRNA cell transfection** – PNT1A cells were transfected overnight with 40 nM siRNASERCA 2a/b, using JetSI<sup>TM</sup>ENDO transfection reagent (Polyplus transfection SA, Illkrich, France). Ready-to-use siRNASERCA 2a/b (processing option: A4) with a sense sequence of: 5'- CAAAGUUCCUGCUGAUAUA(dTdT)-3' was synthesized by Dharmacon Inc (Lafayette, USA). Control siRNA experiments were performed either by applying the transfection reagent alone (vehicle) or by transfecting siRNATRPM8 [12].

Ca<sup>2+</sup> measurements using Fura-2 AM - Cells were grown on glass coverslips and before each imaging experiment, the culture medium was replaced by a Hank's Balanced Salt Solution (HBSS) containing (in mM): NaCl, 142; KCl, 5.6; MgCl2, 1; CaCl<sub>2</sub>, 2; Na2PO<sub>4</sub>, 0.34; KH2PO<sub>4</sub>, 0.44; HEPES, 10; glucose, 5.6; and buffered to pH 7.4 with NaOH. Cells were loaded with Fura-2 by exposure to 2 µM fura-2 acetoxymethyl ester (Fura-2 AM) (Calbiochem, Meudon, France) in HBSS for 1 h at 37°C. After incubation, cells were washed three times in the same dye-free solution. When a  $Ca^{2+}$ -free medium was required,  $CaCl_2$  was omitted and replaced by equimolar MgCl<sub>2</sub>. Intracellular Ca<sup>2+</sup> was measured by an imaging system (Quanticell 900, Visitech, UK). The glass coverslip was mounted in a chamber on a Nikon microscope equipped for fluorescence. Fura-2 fluorescence was excited at 340 and 380 nm and emitted fluorescence was measured at 510 nm. The [Ca<sup>2+</sup>]<sub>cvt</sub> was derived from the ratio of the fluorescence intensities for each of the excitation wavelengths ( $F_{340}/F_{380}$ ), and from the Grynkiewicz equation. Thapsigargin (TG), the most potent selective inhibitor of Sarco(endo)plasmic reticulum  $Ca^{2+}$  -ATPase (SERCA) is often used to estimate  $Ca^{2+}$  pool content. In a  $Ca^{2+}$ -free medium, TG induces an increase in  $[Ca^{2+}]_{cvt}$ , due to  $Ca^{2+}$  release from intracellular stores. Endoplasmic reticulum (ER) Ca<sup>2+</sup> content could be estimated in Fura-2

loaded cells by measuring the difference between basal  $[Ca^{2+}]_{cyt}$  and maximum  $[Ca^{2+}]_{cyt}$  at the TG-induced peak.

**Direct quantification of**  $[Ca^{2+}]_{ER}$  - For imaging Ca<sup>2+</sup> within the ER, PNT1A cells were loaded with 2 µM of the AM-ester derivative of Mag-Fura 2 for 45 min at 37°C. After incubation with the dye, the cells were briefly rinsed in a high K+ solution (in mM): 125 KCl, 25 NaCl, 10 HEPES, 0.1 MgCl2 [pH 7.2], and then exposed for 2 min to an "intracellular buffer" at 37°C and 5 mg/ml digitonin. Digitonin-permeabilized cells were continuously perfused with digitonin-free "intracellular buffer" supplemented with 0.2 mM Mg-ATP and free [Ca<sup>2+</sup>] clamped to 170 nM using Ca<sup>2+</sup>/EGTA buffer. The Mag-Fura 2 fluorescence ratio was calibrated using exposure to 10 µM ionomycin and 15 mM Ca<sup>2+</sup> or 10 mM EGTA, assuming a dissociation constant for Ca<sup>2+</sup>-Mag-Fura 2 at room temperature of 53 µM [13]. Ratio imaging measurements of Mag-Fura 2 fluorescence were made using a commercial imaging system (Quanticell 900, Applied Imaging, UK).

Western blot - Following the treatments (PRL or siRNA treatment), PNT1A cells were lyzed with RIPA buffer (Triton X100 1%, Na deoxycholate 1%, NaCl 0.15M, PO4Na2/K pH 7.2 20mM) + 5mM EDTA + anti-proteases cocktail (Sigma P 8340, St Quentin Fallavier, France) for 30 minutes on ice. Insoluble material was discarded by centrifugation at 10,000g for 10 minutes at 4°C and the amount of proteins was assessed by the BCA method (Pierce, Chemical Co., Rockford, IL). Equal amounts of proteins were subjected to 16 or 10% SDS-polyacrylamide gel electrophoresis. Finally, the proteins were transferred onto nitrocellulose membrane using a semi-dry electroblotter (Biorad). After the transfer, the membrane was stained with Ponceau Red and the tracks were cut into thin strips (2mm wide) that were further processed for immuno-detection. The strips were blocked for 30 minutes at room temperature in TNT (15 mM Tris buffer pH 8, 140mM NaCl, 0.05% Tween 20) + 5% skimmed dry milk and then incubated with: mouse antibody anti-Actine (pan Ab-5,

Neomarkers, Fremont, CA), rabbit antibody anti-PCNA (sc-56 S, Santa Cruz), rabbit antibody anti-SERCA 2b (poly clonal rabbit IgG, gift from Prof. Frank Wuytack) or rabbit antibody anti-Calreticulin (Stressgen Biotechnologies, San Diego, CA) diluted in TNT + milk (1/500 for anti-Actine, 1/1000 for anti-PCNA, 1/5000 for anti-SERCA 2b or 1/10000 for anti-Calreticulin) for 1 hour at room temperature. After thorough washes in TNT, the strips were treated with the corresponding horseradish peroxydase-linked secondary antibodies (antimouse AP192P or anti-rabbit AP182P, Chemicon, Temecula, CA) diluted in TNT + milk (1/20,000) for 1 hour at room temperature. After several washes in TNT, the strips were processed for chemiluminescent detection using the Supersignal West Pico chemiluminescent substrate (Pierce, Rockford, IL), according to the manufacturer's instructions. The blots were finally exposed to X-Omat AR films (Eastman Kodak Company, Rochester, NY). The intensity of the signals was evaluated by densitometry and semi-quantified using the ratio between the rate of protein of interest divided by the actin or calnexin rate for each experiment. Each experiment presented was repeated at least twice.

**Indirect immunofluorescence** - PNT1A cells were grown on glass coverslips. After a 10 minute cold (-20°C) acetone permeabilization, the cells were blocked with PBS-gelatine (Phosphate buffer saline, gelatine 1.2%) for 30 minutes at 37°C and then incubated with a mouse antibody anti PNCA diluted in PBS-gelatine (1/100) for 1 hour at 37°C. After thorough washes in PBS-gelatine, the slides were treated with Alexa fluor 488-labeled secondary antibody (Molecular Probes, USA) diluted in PBS-gelatine (1/4000) for 1 hour at room temperature with blue-evans (1/50 000). After two washes in PBS-gelatine and in PBS, the slides were mounted with Mowiol® and watched through a confocal microscope (Zeiss LSM 510). The acquisition parameters were identical throughout the whole experiment to allow comparison of the intensity of the fluorescence level. The intensity of the signals was

directly quantified on the confocal microscope with LSM examiner software (AIM 3.2, Zeiss, Le Pecq, France). Each experiment presented was repeated at least three times.

**Reagents and chemicals** – Human recombinant prolactin (PRL) was kindly provided by Vincent Goffin [14]. Fura-2 AM and Mag Fura-2 AM were obtained from Calbiochem (Meudon, France) and thapsigargin (TG) from Sigma (France).

**Data analysis** - The data were analyzed using Origin 5.0 (Microcal Software Inc., Northampton, MA, USA). Results were expressed as mean  $\pm$  standard error of the mean (sem). Each experiment was repeated several times: n indicates the number of cells used to express the mean and N represents the number of experiments. Statistical analysis was performed using the student's *t*-test (*P*<0.05 considered as significant) and ANOVA tests followed by Tukey-Kramer post-tests.

#### RESULTS

**PNT1A cell growth is stimulated by PRL** - PRL stimulates PNT1A cell growth in a dosedependent manner (Fig. 1 A). 50 - 1000 ng/ml PRL increased cell growth after 4 days' treatment, as estimated by the MTS reagent kit. The maximum effect was observed at a 100 ng/ml dose. At this concentration, PRL induced a proliferation increase of 122.2%  $\pm$  6.5% compared to control conditions. We also checked the effect of PRL on the expression of PCNA (Proliferating Cell Nuclear Antigen), a proliferation marker. Western blot analysis (Fig. 1 B) showed that PCNA was weakly expressed in PNT1A cells under control conditions. PCNA proteins were 2.3 fold over-expressed in PNT1A cells treated for 24 hours with 100 ng/ml PRL (Fig. 1 B) corresponding to the maximum effect observed in the proliferative assay. The PRL-induced increase in the PCNA expression was confirmed by immunocytochemical assays (Fig. 1 C). Indeed, in cells treated with 100 ng/ml PRL for 24 hours, the PCNA nuclear positive cells were more numerous (45%) than in control cells (22%). These data were confirmed using another proliferation marker, the nuclear antigen Ki67 (*data not shown*).

The ER  $Ca^{2+}$  content increases in PNT1A cells pre-treated with PRL - As the maintenance of luminal  $Ca^{2+}$  within the endoplasmic reticulum has been demonstrated to be essential for cell growth in different cell models [7, 10, 15], we investigated whether the Ca<sup>2+</sup> pool content was modulated in PRL-stimulated PNT1A cells. The basal cytosolic  $Ca^{2+}$  level was not affected when PNT1A cell growth was stimulated by PRL (Fig. 2 A). Under control conditions, the  $[Ca^{2+}]_{cvt}$  content of PNT1A cells was 49.7 nM ± 1.2 nM. No significant increase in  $[Ca^{2+}]_{cvt}$  was observed in cells treated with 100 ng/ml PRL (50.2 nM ± 1.2 nM). However, the  $Ca^{2+}$  release from the intracellular stores induced by 1 µM Thapsigargin in cells treated for 24 hours with 100 ng/ml PRL was greater than that in control cells (Fig. 2 B). It is important to point out the remarkable similarity between the extent of cell proliferation (Fig. 1 A) and the amplitude of the TG-induced  $Ca^{2+}$  release from intracellular stores (Fig. 2 B). The maximum amplitude of TG-induced  $Ca^{2+}$  release (669.2 nM ± 44.2 nM), corresponding to 156.1 %  $\pm$  10.3 % of the TG response observed under control conditions (428.8  $nM \pm 18.7 nM$ ), was measured in cells with the highest growth rates (treated with 100 ng/ml PRL). This result suggests that there is a close connection between the  $Ca^{2+}$  pool content and the proliferation of PNT1A cells. Moreover, 50 µM AG490, a potent and specific inhibitor of Jak2 kinase activity (the major PRL receptor associated kinase) rendered PRL incapable of stimulating PNT1A cell growth and the amplitude of the TG-induced Ca<sup>2+</sup> release from intracellular stores in cells treated with AG490 and PRL was the same as under control conditions (data not shown). The higher [Ca<sup>2+</sup>]<sub>ER</sub> in PRL treated cells, estimated by TGinduced Ca<sup>2+</sup> release method, has been confirmed using the direct quantification of luminal  $[Ca^{2+}]_{ER}$  (Fig. 2 C). The luminal  $[Ca^{2+}]_{ER}$  was significantly higher in PRL-treated cells (ratio  $0.95 \pm 0.02$ ) than in control cells (ratio  $0.72 \pm 0.02$ ). The functional activity of the

sarcoendoplasmic calcium ATPase has been confirmed using the direct quantification of luminal  $[Ca^{2+}]_{ER}$  (Fig. 2 D) using the classical protocol to measure the  $Ca^{2+}$  re-uptake into ER after inositol triphosphate (IP3)-induced release [18]. As demonstrated by the d $[Ca^{2+}]/dt_{max}$ , the luminal  $[Ca^{2+}]_{ER}$  is more rapidly refilled in PRL-treated cells (8.5  $\mu$ M.s<sup>-1</sup>) than in control cells (2  $\mu$ M.s<sup>-1</sup>) confirming that the sarcoendoplasmic calcium ATPase are more active in PRL-treated cells than in control cells. Thus, our results clearly demonstrate that PRL induces the increase in the ER Ca<sup>2+</sup> content, suggesting that the cell growth stimulation by PRL could be linked to the high ER Ca<sup>2+</sup> luminal concentration.

SERCA2b protein expression in PNT1A cells is regulated by PRL - We further investigated the effect of PRL on the expression of SERCAs (Sarco(endo)plasmic reticulum  $Ca^{2+}$  -ATPase) proteins known to play an important role in the maintenance of luminal  $[Ca^{2+}]_{ER}$ [16]. Moreover, it has been suggested that SERCA proteins regulate cell growth by controlling the expression of growth and transformation related genes [17]. We therefore investigated the effect of PRL on the expression of three SERCA isoforms: SERCA2a (the cardiac/slow skeletal muscle isoform), SERCA2b (the ubiquitously expressed isoform) and SERCA 3. In our experiments, neither SERCA2a nor SERCA 3 protein expression were detected under control conditions or in PRL-treated cells. However, western blot analysis showed that SERCA2b was constitutively expressed in control cells and over-expressed (3.6 fold) in cells treated with 100 ng/ml PRL for 24 hours (Fig. 3 A). This PRL-induced upregulation of SERCA2b expression in PNT1A cells seemed to be dependent on tyrosine kinase activity. After a brief (3 hour) PRL treatment, western blot analysis showed that Jak2 kinase (the major PRL receptor associated kinase) was more strongly phosphorylated in PRLtreated cells than in control cells (Fig. 3 C). When cells were treated with 100 ng/ml PRL in the presence of 50 µM Jak2 kinase inhibitor AG490, we did not observe an up-regulation of SERCA2b expression (Fig. 3 A). Treating PNT1A cells with AG490 alone had no effect on SERCA 2b expression under control conditions (*data not shown*). We also studied calreticulin expression, the luminal Ca<sup>2+</sup> binding/storage chaperon, to confirm that SERCA2b over-expression was specific to PRL and was not due to the proliferation increase in protein synthesis. We therefore compared the expression of calreticulin in both control and PRL-treated cells, using semi-quantitative western blot analysis. PRL treatment inducing SERCA 2b over-expression did not modify calreticulin expression (Fig. 3 B).

SERCA2b protein expression in PNT1A cells is closely linked to cell proliferation - siRNA technology was used to downregulate the endogenous SERCA 2b expression in PNT1A cells with a siRNA SERCA2a/b. Western blotting with anti-SERCA 2b antibody on the first and 3nd days after transfection demonstrates that the level of SERCA 2b protein was 70% lower than control (Fig. 4 A), indicating the effectiveness of siRNA in downregulating endogenous SERCA 2b expression. Then, we tested the siRNA SERCA2a/b effect on PNT1A proliferation. We observed a 13.6% reduction in the proliferation of the SiRNA SERCA2a/btreated cells compared to control (Fig. 4 B). The result was also confirmed by the PCNA expression method. Western blot analysis shows a down-regulation of PCNA expression in siRNA-treated cells (Fig. 4 C). We next investigated whether the Ca<sup>2+</sup> pool content was modulated in siRNA SERCA 2a/b-treated PNT1A cells. The basal cytosolic Ca<sup>2+</sup> level was affected and increase in [Ca<sup>2+</sup>]<sub>cvt</sub> was observed in cells treated with siRNA SERCA2a/b (111.7 nM  $\pm$  10.1 nM) compared to controls (64.9 nM  $\pm$  2.8 nM) (Fig. 5 A). The Ca<sup>2+</sup> release induced by 1 µM thapsigargin in siRNA SERCA 2a/b-treated cells was about 52 % smaller than that in control cells (Fig. 5 B). Because of the higher basal cytosolic Ca<sup>2+</sup> level, the resulting driving force may reduce the  $Ca^{2+}$  release in siRNA SERCA 2a/b-treated cells. Therefore, these results were confirmed using the direct quantification of luminal  $[Ca^{2+}]_{ER}$ (Fig. 5 C). The luminal  $[Ca^{2+}]_{ER}$  was lower in siRNA SERCA 2a/b -treated cells (ratio  $0.64 \pm 0.02$ ) than in control cells (ratio  $0.87 \pm 0.04$ ). These results show that in PNT1A normal human prostate cells, SERCA2b is a common target of physiological stimuli controlling cell growth by PRL.

#### DISCUSSION

It has been established that prolactin [3, 19] and its receptors [2, 3, 20-22] are expressed in the prostate. These data have provided significant support for the existence of an autocrine/paracrine PRL loop in prostate cells. [23] have shown that prolactin was capable of inducing proliferation and survival of prostate epithelial cells in the long term organ culture of rat prostate tissue explant. In addition, [24] have reported dramatic prostate enlargement in prolactin transgenic mice. In our previous studies we have established that a chronic hyperprolactinemia, caused by sulpiride, induced an enlargement and an inflammation of the lateral rat prostate [5]. PRL stimulates the proliferation of LNCaP prostate cancer cells [2]. In this study, we have shown that PRL stimulates the proliferation of the normal SV40 immortalized prostatic PNT1A cells. Our experiments are consistent with those reported by [25], showing that PRL induces proliferation in LNCaP, DU145 and PC3 cancer cells. These authors have also found that PRL stimulates the proliferation of PC3 cells in a dose-dependent manner. However, the mechanisms by which PRL stimulates prostate cell proliferation are yet to be completely understood.

 $Ca^{2+}$  homeostasis modulation is involved in a number of signal transduction pathways regulating a wide range of cell phenomena. Cytosolic  $Ca^{2+}$  is known to control numerous cell functions, including contraction, gene expression and proliferation [9]. However, it has been documented that  $Ca^{2+}$  pools control numerous cell functions, including protein synthesis and cell proliferation [10, 26] and the maintenance of  $Ca^{2+}$  levels within the lumen of the endoplasmic reticulum is critical for cell growth [10]. Indeed, a range of  $Ca^{2+}$ -binding proteins within the ER lumen are involved in the folding and correct assembly of proteins. The increased effectiveness of these proteins may be the primary reason for growth progression. An alternative or additional possibility is that high  $Ca^{2+}$  concentration within the ER may promote important growth factor-initiated. InsP<sub>3</sub> -mediated Ca<sup>2+</sup> signals that are generated through the release of  $Ca^{2+}$  from the ER and that are essential for cells to progress through cell cycle and to maintain growth. We therefore investigated the potential variation in PNT1A Ca<sup>2+</sup> pool content linked to PRL-increased proliferation. Our results show that the PRL induced proliferation of PNT1A cells is at least partially due to  $[Ca^{2+}]_{FR}$  increase and the SERCA 2b over-expression by the Jak2 pathway. We could not observe any Ca<sup>2+</sup> modifications in the cytoplasm or any modifications in the SERCA 2b activity following quick PRL treatment. Thus, we suggest that PRL promotes, via the Jak2-Stat5 transduction signal, the SERCA 2b gene transcription which increases the  $[Ca^{2+}]_{ER}$  and then cell growth. The intracellular calcium ATPase and the  $Ca^{2+}$  pool content have already been closely associated with cell growth and have been shown to be modulated by hormones or growth factors like platelet-derived growth factor [27]. We have shown that the Epidermal Growth factor modulates prostate cancer LNCaP cell growth and is correlated with intracellular calcium pool content [7]: The rate of cell growth is correlated with an increase in the calcium pool filling state, whereas growth-inhibited cells show a reduced calcium pool load. In addition, we have also demonstrated that the Insulin Growth Factor, which increases prostate cancer LNCaP cell growth, induces an increase in  $[Ca^{2+}]_{ER}$  and is associated with an overexpression of SERCA 2b [6]. To our knowledge, in this study we show for the first time that PRL stimulates cell proliferation via the over-expression of SERCA 2b and the subsequent upregulation of the ER  $Ca^{2+}$  content.

To study the consequence of a reduction in  $[Ca^{2+}]_{ER}$  on the cell growth and to confirm the involvement of SERCA 2b in PRL-induced proliferation, we used the downregulation of SERCA 2b protein by siRNA strategy. Our results show that a reduction in  $Ca^{2+}$  store content leads to a proportional decrease in PNT1A cell growth. The correlation between the decrease in growth rate and the Ca<sup>2+</sup> store content decrease has already been demonstrated in other cell models. However only artificial pharmacological agents have been used in these studies. [15] have shown that depletion of the  $[Ca^{2+}]_{FR}$  stores by the  $Ca^{2+}$  pump inhibitor, thapsigargin or 2.5-di-tert-butyl-hydroquinone (tBHO) promotes growth arrest in DDT1MF-2 smooth muscle cells. In a previous study, we have shown that a long-term treatment with ATP leads to a decrease in the intraluminal endoplasmic reticulum Ca<sup>2+</sup> concentration and induces growth arrest in DU-145 androgen-independent human prostate cancer cells [28]. In our experiments, we also found that cytosolic Ca<sup>2+</sup> in siRNA SERCA 2b-treated cells was higher than in control cells. This could be due to the reduced calcium pool load or to a compensation mechanism for  $Ca^{2+}$  signalling, via the up-regulation of membrane  $Ca^{2+}$  channels and exchangers. [29] have shown that the reduction of SERCA expression by gene silencing is associated with the up-regulation of transient receptor potential (TRP) channel proteins (TRPC4 and TRPC5) and Na<sup>+</sup> / Ca<sup>2+</sup> exchanger in cardiac myocytes. Store deficiency was in this study compensated for by  $Ca^{2+}$  fluxes through the plasma membrane.

To summarize, our study identifies and characterizes, for the first time, the intracellular  $Ca^{2+}$  homeostasis mechanisms involved in the PRL regulation of the PNT1A prostate cell growth. We have shown that PRL increases the intracellular  $Ca^{2+}$  stores in these proliferative cells through the over-expression of SERCA 2b calcium pumps. Using siRNA technology, we have specifically down-regulated SERCA 2b expression and shown that the intracellular  $Ca^{2+}$  stores are proportionally reduced. As a consequence, this reduces the PNT1A cell proliferation. We suggest that  $[Ca^{2+}]_{ER}$  exerts a strict control over cell growth and that it is a crucial element in the PRL signal transduction.

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#### FIGURE LEGENDS

Figure 1: PRL stimulates cell growth. A: Cells were seeded in 96-well plates containing 200  $\mu$ l culture medium supplemented with 1% fetal calf serum. 48 hours after seeding, the cells were treated for 4 days with the indicated dose of PRL. The medium was replaced every 48 h. After 4 days, the number of cells in each well was determined with an MTS reagent kit. B: Western blot analysis of PCNA (proliferation marker) expression. 100 ng/ml PRL for 24 hours induced an over-expression of this protein. C: Regulation of PCNA protein expression was investigated by immunohistochemistry as indicated in the experimental procedures. PCNA proteins were detected in basal conditions (CTL) and 100 ng/ml PRL for 24 hours induced an over-expression of this protein (PRL). PCNA: green. Cells marked with Evans Blue: red. Scale bar: 20  $\mu$ M. \* Significantly different from the control value (P<0.05).

**Figure 2: PRL increases ER Ca<sup>2+</sup> content. A:** Effect of 1  $\mu$ M Thapsigargin (TG) on the  $[Ca^{2+}]_{cyt}$  due to a Ca<sup>2+</sup> release from intracellular stores (ER). The effect of TG was recorded in a Ca<sup>2+</sup> -free HBBS medium. (•) control. PRL ( $\circ$ ) 50 ng/ml. (•) 100 ng/ml. (•)

500 ng/ml. ( $\Box$ ) 1000 ng/ml. **B:** Histograms show the amplitude of the TG-induced Ca<sup>2+</sup> release reflecting the ER content that was estimated by measuring the difference between basal cytosoplasmic calcium and Ca<sup>2+</sup> peak induced by TG. \* Significantly different from the control value (P<0.05). **C:** Measurement of  $[Ca^{2+}]_{ER}$  in response to 100  $\mu$ M InsP<sub>3</sub> (Ca<sup>2+</sup> release) followed by 0.4 mM ATP (Ca<sup>2+</sup> reload) as indicated in the experimental procedures. *Lower trace (grey)*: control. *Upper trace (black)*: PRL 100 ng/ml. **D:** Measurement of d[Ca<sup>2+</sup>]/dt reflecting the Ca<sup>2+</sup> reload into the ER. *Lower trace (grey circle)*: control. *Upper trace (black circle)* : PRL.

**Figure 3: PRL induces SERCA 2b over-expression by Jak2 pathway. A:** Western blot analysis of SERCA 2b expression. 100 ng/ml PRL induced over-expression of this protein. 50 μM AG490 (a specific JAK2 inhibitor) prevents this PRL-induced over-expression. (**CTL**): control. (**PRL**): 100 ng/ml PRL. (**AG490 + PRL**): 50 μM AG490 before 100 ng/ml PRL. **B:** Western blot analysis of calreticulin expression. 100 ng/ml PRL did not induce an over-expression of this protein. 50 μM AG490 before 100 ng/ml PRL had no effect either. (**CTL**): control. (**PRL**): 100 ng/ml PRL. (**AG490 + PRL**): 50 μM AG490 before 100 ng/ml PRL had no effect induce an over-expression of this protein. 50 μM AG490 before 100 ng/ml PRL had no effect either. (**CTL**): control. (**PRL**): 100 ng/ml PRL. (**AG490 + PRL**): 50 μM AG490 before 100 ng/ml PRL.

**Figure 4: siRNA SERCA 2a/b reduces cell growth. A:** Western blot analysis of SERCA 2b expression. siRNA SERCA 2a/b induced a down-regulation of SERCA 2b expression. **(CTL)**: control. **(J1)**: 1 day after transfection. **(J3)** 3 days after transfection. **B:** Cells were seeded in 24-well plates. 48 h after seeding, PNT1A cells were transfected as indicated in experimental procedures and then cultured for 4 days. The medium was replaced every 48 h. After 4 days, the number of cells in each well was determined with the MTS reagent kit. **(CTL)** control. **(SiRNA)**: siRNA SERCA 2a/b. **C:** Western blot analysis of

PCNA expression. siRNA SERCA 2a/b induced a down-regulation of PCNA expression. (CTL): control. (SiRNA J1): 1 day after transfection. (SiRNA J3): 3 days after transfection.

**Figure 5: siRNA SERCA 2a/b reduces ER Ca<sup>2+</sup> content. A:** Effect of TG (1  $\mu$ M) on the [Ca<sup>2+</sup>]<sub>cyt</sub> due to a Ca<sup>2+</sup> release from intracellular stores (ER). The effect of TG was recorded in a Ca<sup>2+</sup> -free HBBS medium. (•) control. ( $\Box$ ) siRNA SERCA 2 a/b. **B:** Histograms show the amplitude of the TG-induced Ca<sup>2+</sup> release reflecting the ER content that was estimated by measuring the difference between basal cytoplasmic calcium and the Ca<sup>2+</sup> peak induced by TG. (**CTL**) control. (**SiRNA**) siRNA SERCA 2a/b. \* Significantly different from the control value (P<0.05). **C:** Histograms show the direct measurement of [Ca<sup>2+</sup>]<sub>ER</sub> in response to 100  $\mu$ M InsP<sub>3</sub> as indicated in the experimental procedures. (**CTL**): control. (**SiRNA**): siRNA SERCA 2a/b. \* Significantly different from the control value (P<0.05).





#### FIGURE 2



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FIGURE 3







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FIGURE 5

