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Regucalcin Is Under-Expressed in Human Breast and Prostate Cancers: Effect of Sex Steroid Hormones

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ABSTRACT

Regucalcin plays an important role in maintenance of intracellular Ca^{2+} homeostasis, suppresses cell proliferation, inhibits expression of oncogenes, and increases the expression of tumour suppressor genes. This suggests that regucalcin functions may be altered in cancer tissues. In this study the regucalcin expression in breast and prostate cancer cases was analysed by RT-PCR and immunohistochemistry showing that the mRNA and/or protein are under-expressed in these tumors. The effect of sex steroid hormones on regucalcin expression in breast and prostate cancer cells was determined by real-time PCR. MCF-7 and LNCaP cells were stimulated with 0, 1, and 10 nM of 17β-estradiol (E_2) or 5α-dihydrotestosterone (DHT), respectively, for 0, 6, 12, 24, and 48 h. MCF-7 cells were also stimulated with E_2 conjugated to BSA (E_2-BSA). To explore the mechanisms underlying the sex steroid regulation of regucalcin expression, control treatments with ICI 182,780, flutamide and cyclohexamide were carried out. E_2 effects regulating regucalcin expression were not abrogated in the presence of ICI 182,780, and were similar to those observed with E_2-BSA, which suggests the involvement of a membrane-bound estrogen receptor. In LNCaP cells, DHT down-regulated regucalcin expression, an effect inhibited by the presence of both flutamide and cyclohexamide, suggesting the involvement of androgen receptor and de novo protein synthesis. The loss of regucalcin expression in breast and prostate cancer cases and the regulation of its expression by sex steroid hormones suggest that it may be associated with development and progression of these human tumors. J. Cell. Biochem. 107: 667–676, 2009.

KEY WORDS: REGUCALCIN; SMP30; BREAST; PROSTATE; DHT; 17β-ESTRADIOL; CALCIUM; ALTERNATIVE TRANSCRIPTS

Regucalcin, also known as Senescence Marker Protein-30, was first identified as a calcium (Ca^{2+}) binding protein that does not contain the E-F hand motif as a Ca^{2+} binding domain [Yamaguchi and Yamamoto, 1978]. It plays an important role in the maintenance of intracellular Ca^{2+} homeostasis by enhancing Ca^{2+}-pumping activity in the plasma membrane, endoplasmic reticulum and mitochondria of many cell types [Fujita et al., 1998; Tsurusaki and Yamaguchi, 2000; Yamaguchi, 2005]. Regucalcin regulates the expression of several protein kinases, thyrosine kinases, protein phosphatases, and NO synthase acting also as a regulatory protein in intracellular signalling pathways [Yamaguchi, 2005]. Overexpression of regucalcin suppresses cell proliferation [Nakagawa et al., 2005; Yamaguchi and Daimon, 2005], inhibits expression of oncogenes and increases the expression of tumour suppressor genes [Tsurusaki and Yamaguchi, 2003, 2004]. The disequilibrium in cell death and proliferation is on the basis of carcinogenesis, and the malignant transformation of cells has been associated with alterations in Ca^{2+} signalling [see reviews by Capiod et al., 2007; Monteith et al., 2007]. Therefore, the deregulation of regucalcin function may have a relevant role in cancer initiation and/or progression. Breast and prostate cancers are endocrine cancers which depend on the trophic effects of estrogens and androgens [reviewed by Debes and Tindall, 2002; Platet et al., 2004; Muti et al., 2006], and altered Ca^{2+} homeostasis and signalling have been associated with the development of both pathologies [see reviews by Lee et al., 2006; Prevarskaya et al., 2004]. In a previous report, we demonstrated the expression of regucalcin in rat mammary gland and prostate as well as its regulation by 17β-estradiol (E_2 [Maia et al., 2008]). The present work aims to study the expression of regucalcin in non-neoplastic human breast and prostate as well as in tumors of both tissues. We demonstrate that regucalcin is under-expressed in breast and prostate cancer cases and regulated by E_2 and 5α-dihydrotestosterone (DHT) in breast (MCF-7) and prostate (LNCaP) cancer cells, respectively. These findings support that

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regucalcin may play an important role in the development and progression of breast and prostate tumors.

MATERIALS AND METHODS

CELL LINES AND HUMAN SAMPLES

Human breast (MCF-7, ECACC 86012803) and prostate (LNCaP, ECACC 89110211) cancer cell lines were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK).

Non-neoplastic human mammary gland (cat. No. 636163) and prostate (Cat. No. 636124) poly A^+ RNAs were purchased from Clontech (St-Germain-en-Laye, France).

Breast tissues for RNA extraction (infiltrating ductal carcinoma, n = 42; non-neoplastic breast tissue adjacent to benign lesions, n = 5) were obtained at the Salamanca University Hospital from patients that did not receive preoperative chemotherapy or any other treatment. Immediately after collection, samples were frozen in liquid nitrogen and stored at −80°C until RNA extraction.

Human breast sections for immunohistochemical analysis (n = 85) were retrieved from the archives of Centro Hospitalar Cova da Beira (Covilha, Portugal) and IPATIMUP (Porto, Portugal), and the Human Breast Carcinoma Tissue Microarray (Cat. No. 75-4043, Zymed, San Francisco): non-neoplastic breast (n = 5), fibroadenoma (n = 5), infiltrating ductal carcinoma (n = 75).

Human prostate sections for immunohistochemical analysis (n = 73) were retrieved from the archive of IPATIMUP (Porto, Portugal) and the Human Prostate Cancer and Benign Prostate Hyperplasia Tissue Microarray (Cat. No. 75-5063, Zymed): non-neoplastic prostate (n = 5), benign prostate hyperplasia (n = 17), adenocarcinoma (n = 51).

All human samples were obtained under informed consent of the patients and according to the local ethical committee guidelines.

CELL CULTURE AND TREATMENT

MCF-7 cells were cultured in DMEM medium (Invitrogen, Karlsruhe LMA, Germany), and LNCaP cells were cultured in RPMI 1640 (Invitrogen), in an incubator at 37°C equilibrated with 5% CO_2. Both cultures were supplemented with 10% FBS (Biochrom, Berlin, Germany) and 1% penicillin/streptomycin (Invitrogen). After culturing cells in phenol red-free and serum-free medium for 2 days, MCF-7 and LNCaP cells were exposed to different concentrations (0, 1, and 10 nM) of E_2 (Sigma, Saint Louis) and DHT (Sigma), respectively. For each hormone dosage a time-course experiment was carried out for 0, 6, 12, 24, and 48 h. Control treatments with ICI 182,780 (10 nM, Tocris Cooksob, Bristol, UK), flutamide (1 μM, Sigma) and cyclohexamide (10 μg/ml, Sigma) were carried out with 10 nM of E_2 or DHT for 6 h. Inhibitors were added to cell cultures 1 h before hormone stimulation. MCF-7 cells were also stimulated with E_2 conjugated to BSA (E_2-BSA, 10 nM) for the same period of time. All assays were carried out in hexaplicate.

REVERSE-TRANSCRIPTION PCR

Total RNA was extracted from MCF-7 cells, LNCaP cells and breast tissues using TRI reagent (Sigma) according to the manufacturer's instructions. 2 μg of total RNAs or 100 ng of poly A^+ RNA from human non-neoplastic breast and prostate were denatured for 5 min at 65°C with 500 μM deoxynucleotide triphosphates (Amersham, Uppsala, Sweden) and 250 ng of random primers (Invitrogen). Reverse transcription was carried out at 37°C for 60 min in a 20 μl reaction containing reverse transcriptase buffer (50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$ and 0,1 M DTT), 10 mM DTT, 60 U of RNaseOUT (Invitrogen), and 200 U of M-MLV RT (Invitrogen). Reactions were stopped at 75°C for 15 min. PCR reactions were carried out using 1 μl of synthesized cDNAs in a 25 μl reaction containing 1× Taq DNA polymerase buffer (20 mM Tris–HCl and 50 mM KCl), 500 μM deoxynucleotide triphosphates (Amersham), 3 mM of $MgCl_2$ (Promega, Madison), 300 nM of each primer and 0.125 U of Taq DNA polymerase (Promega). Specific primers for human regucalcin spanning the entire coding region (sense: 5′-CCC TGC GAC CAT GTC TTC CAT TA-3′; antisense: 5′-TCC CGC ATA GGA GTA GGG AG-3′) and for human GAPDH (sense: 5′-CGC CAG CCG AGC CAC ATC-3′; antisense: 5′-CGC CCA ATA CGA CCA AAT CCG-3′) were used to amplify products of 897 and 75 bp, respectively. After an initial denaturation at 95°C for 5 min, the cycling conditions used were as follows: 35 cycles (25 cycles to GAPDH) consisting of denaturation at 95°C for 20 s, annealing at 60°C for 30 s and extension at 72°C for 60 s (10 s to GAPDH). All amplicons were cloned and sequenced for confirmation of RT-PCR specificity.

REAL-TIME PCR

Total RNA extraction and cDNA synthesis were performed as described above. Specific primers to human regucalcin (sense: 5′-GCA AGT ACA GCG AGT GAC C-3′; antisense: 5′-TTC CCA TCA TTG AAG CGA TTG-3′) were used to amplify a fragment of 109 bp. Human beta-2-microglobulin (β_2M; sense: 5′-ATG AGT ATG CCT GCC GTG TG-3′; antisense: 5′-CAA ACC TCC ATG ATG CTG CTT A-C-3′) and GAPDH primers (the same described in reverse-transcription PCR) were used as internal controls to normalize regucalcin expression. Real-time PCR (IQ5 Biorad, Hercules) efficiency was determined for all primer sets using serial dilutions of cDNA samples (1:1, 1:5, and 1:25). Real-time PCR reactions were carried out using 1 μl of synthesized cDNA in a 20 μl reaction containing 10 μl SYBR Green Supermix (Biorad) and 200 nM regucalcin primers or 300 nM β2M and GAPDH primers. After an initial denaturation at 95°C for 5 min, cycling conditions were as follows: 40 cycles consisting of denaturation at 95°C for 10 s, annealing at 60°C for 30 s and extension at 72°C for 10 s. The amplified PCR fragments were checked by melting curves: reactions were heated from 55 to 95°C with 10 s holds at each temperature (0.05°C/s). Samples were run in triplicate in each assay. Fold differences were calculated following the mathematical model proposed by Pfaffl using the formula: $2^{-\Delta\Delta C_t}$ [Pfaffl, 2001].

IMMUNOHISTOCHEMISTRY

Formalin-fixed, paraffin-embedded breast and prostate tissue sections were deparaffinized with xylene and rehydrated using graded ethanol series. Sections were incubated with 0.3% hydrogen peroxide for 5 min to inactivate endogenous peroxidase. Non-specific protein binding was eliminated by incubation with 5% normal goat serum (Santa Cruz Biotechnology, Santa Cruz) for 1 h at room temperature. Sections were then incubated at room

temperature with anti-regucalcin mouse monoclonal antibody (Cellsciences, HM3018) diluted 1:50 in PBS for 1 h at room temperature, washed with PBS for 5 min and incubated with a biotinylated goat anti-mouse IgG antibody (Abcam, ab7067) diluted 1:100 for 1 h. Sections were washed in PBS for 5 min at room temperature, incubated with a Rabbit ExtrAvidin Peroxidase reagent (Sigma) for 30 min and then washed in PBS for 10 min. Color development was carried out using 0.05% 3,3′-diaminobenzidine hydrochloride (Sigma) and 0.0006% hydrogen peroxide in TBS. Sections were counterstained in Mayer's hematoxylin, dehydrated, cleared and mounted. Negative controls were performed by omission of the primary antibody.

IMMUNOHISTOCHEMISTRY STAINING INTERPRETATION

Positivity was indicated by the presence of dark brown staining. Regucalcin immunoreactivity was assessed semiquantitatively using a grade scoring system, based on the intensity of staining and percentage of stained cells [Schmitt et al., 1995]. Staining intensity was divided into moderate (score 1) and strong (score 2). The percentage of stained cells was divided into occasional or up to 1/3 of cells (score 1), up to 2/3 (score 2), and almost all (score 3). Low immunoreactivity indicated that up to 1/3 of the cells were moderately stained (score $1 = 1 \times 1$); moderate immunoreactivity indicated that up to two-third of the cells were strongly stained (scores $2\text{–}4 = 1 \times 2$ or 2×2), or almost all cells were moderately stained (3×1); high immunoreactivity was used when almost all cells were strongly stained (score $6 = 3 \times 2$).

DOUBLE FLUORESCENT IMMUNOCITOCHEMISTRY

For dual labeling with regucalcin and Hoechst 33342 (Invitrogen), which labels the nucleus of cells, MCF-7 and LNCaP cells were prepared as follows. Four to 5 days after plating, cells were fixed on a glass coverslip with 1 drop of 4% PFA in ice-cold medium without FBS for 1 min. Medium was removed, 1 ml of 4% PFA was added and cells were incubated for 15 min. After washing with PBS, cells were permeabilized with 0.1%Triton X-100 for 5 min followed by a blocking step in PBS-T plus 20% FBS for 1 h at room temperature. Cells were incubated at room temperature for 1 h with anti-regucalcin antibody (1:50 dilution), washed with PBS-T and incubated with Hoechst 33342 (2 μM) for 10 min. After washing, cells were incubated with Alexa fluor 488-conjugate goat anti-mouse IgG secondary antibody (Molecular probes; 1 μg/ml), washed and mounted onto microscope slides with Dako fluorescent mounting medium (Dako, Glostrup, Denmark). The preparations were observed on a fluorescence microscope (Carl Zeiss, Germany) and specificity of immunostaining was assessed by omission of primary antibody and Hoechst 33342.

STATISTICAL ANALYSES

The statistical significance of differences in regucalcin mRNA expression among experimental groups was assessed by ANOVA, followed by Dunnett's test. A χ^2 test was applied for comparisons in the immunohistochemistry experiments. SPSS software (version 15) was used in all statistical analysis. Significant differences were considered if $P < 0.05$. All experimental data is shown as mean ± SEM.

RESULTS

REGUCALCIN IS EXPRESSED IN HUMAN BREAST AND PROSTATE AND SEVERAL TRANSCRIPTS ARE DETECTED

Using specific primers and a strategy of RT-PCR we were able to detect regucalcin mRNA in non-neoplastic human breast and prostate, and in human breast (MCF-7) and prostate (LNCaP) cancer cell lines (Fig. 1A). The PCR reaction using primers spanning the regucalcin full-length coding sequence allowed the amplification of regucalcin wild-type cDNA and two additional bands in both non-neoplastic human breast and prostate (Fig. 1A). The 897 bp product corresponded to the regucalcin full-length cDNA (wild-type, WT) while the additional bands of 681 and 549 bp corresponded to mRNAs lacking exon 4 (Δ4) and exons 4 and 5 (Δ4,5), respectively, as inferred from the determination of regucalcin gene structure (Fig. 1B). If translated the Δ4 and Δ4,5 transcripts encode proteins with 227 and 183 amino acids, respectively. Although both MCF-7 and LNCaP cells express the WT regucalcin, MCF-7 cells do not express the Δ4 transcript and in LNCaP cells the expression of the Δ4,5 transcript is not detectable.

The presence of regucalcin protein in human breast and prostate was analyzed by immunohistochemistry. It was possible to confirm its expression in non-neoplastic and tumour samples of both tissues (Figs. 2 and 3). Regucalcin positive immunoreactivity was detected in cytoplasm and nuclei of epithelial cells of breast (Fig. 2A–C) and prostate (Fig. 3A,C,E,G). Both breast and prostate non-neoplastic tissues seem to display stronger staining for regucalcin in comparison with cancer cases. In addition, dual-labeling experiments using anti-regucalcin antibody (Fig. 4A,D) and Hoechst 33342 (Fig. 4B,E), which labels the cell nucleus, showed that regucalcin presents a nuclear localization in MCF-7 and LNCaP cells (Fig. 4C,F, merged image staining light blue).

REGUCALCIN mRNA AND PROTEIN EXPRESSION IS DIMINISHED IN BREAST AND PROSTATE CANCER TISSUES

RT-PCR analysis allowed detection of regucalcin in non-neoplastic breast and prostate, and in human breast (MCF-7) and prostate (LNCaP) cancer cells. Both MCF-7 and LNCaP cells seem to present a lower expression of regucalcin WT mRNA in comparison with non-neoplastic breast and prostate tissues (Fig. 1A). In order to examine if regucalcin mRNA expression is deregulated in breast cancer, real-time PCR was performed using cDNA from non-neoplastic breast and breast cancer specimens. PCR results showed that regucalcin mRNA expression is sixfold lower in breast cancer compared with non-neoplastic breast ($P < 0.05$, Fig. 5).

To test whether regucalcin protein is differentially expressed in breast and prostate cancer tissues, a semiquantitative immunohistochemical analysis was performed. All breast and prostate specimens were histologically classified and the level of regucalcin immunoreactivity (Tables I and II) was established as described in material and methods. The results of regucalcin staining in infiltrating ductal carcinomas of breast (IDC) indicate that 56% of cases present low-, 37% moderate- and 7% high-immunoreactivity. In contrast, 80% of non-neoplastic breast and 100% of benign lesions showed high regucalcin immunoreactivity. A significant difference was found between regucalcin immunoreactivity in cases

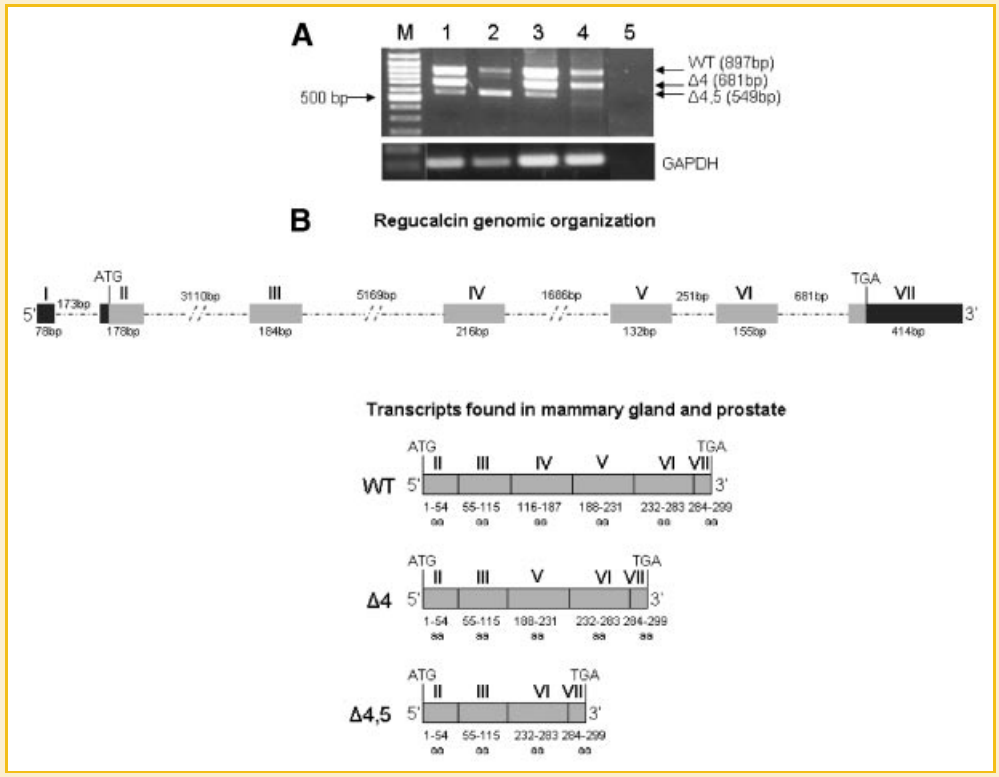

Fig. 1. Regucalcin mRNA is expressed in non-neoplastic human breast and prostate, and in breast (MCF-7) and prostate (LNCap) cancer cell lines: several transcripts are detected. A: RT-PCR analysis using specific primers spanning the entire coding region of regucalcin. Lane M: DNA Molecular Weight Marker; Lane 1: Non-neoplastic breast; Lane 2: MCF-7 cells; Lane 3: Non-neoplastic prostate; Lane 4: LNCaP cells; Lane 5: Negative control with total RNA not reverse transcribed. B: Diagram of the organization of human regucalcin gene and mRNA variants amplified in RT-PCR reactions. The obtained PCR products (WT, Δ4 and Δ4,5) were sequenced and the exon structure of the gene was determined using Blat software [Kent, 2002]. Gray and black boxes indicate coding and noncoding exons, respectively. Dotted lines correspond to introns. Exons are marked with roman numerals. Arabic numerals indicate the number of base pairs per exon or intron, or the number of amino acids (aa) encoded by each exon. WT: wild type regucalcin coding region; Δ4: regucalcin exon 4-deleted variant; Δ4,5; regucalcin exon 4 and exon 5-deleted variant.

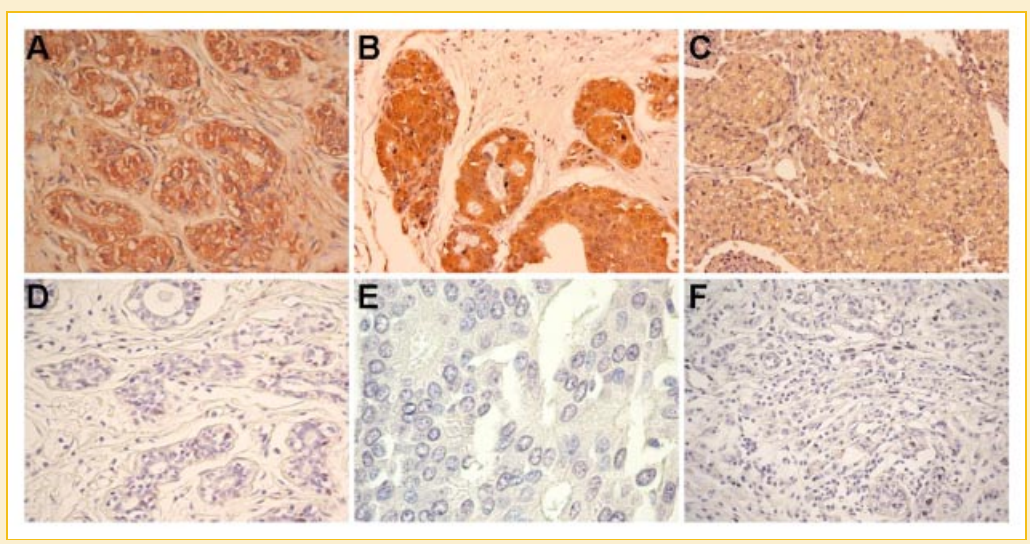

Fig. 2. Regucalcin protein is expressed in non-neoplastic and neoplastic breast tissues. Representative immunohistochemistry sections are shown. A: Non-neoplastic breast. B: Infiltrating ductal carcinoma Grade I. C: Infiltrating ductal carcinoma Grade III. The corresponding negative controls, obtained by omission of the primary antibody, are shown in panels D–F. All tissues are shown in an original magnification of 400×.

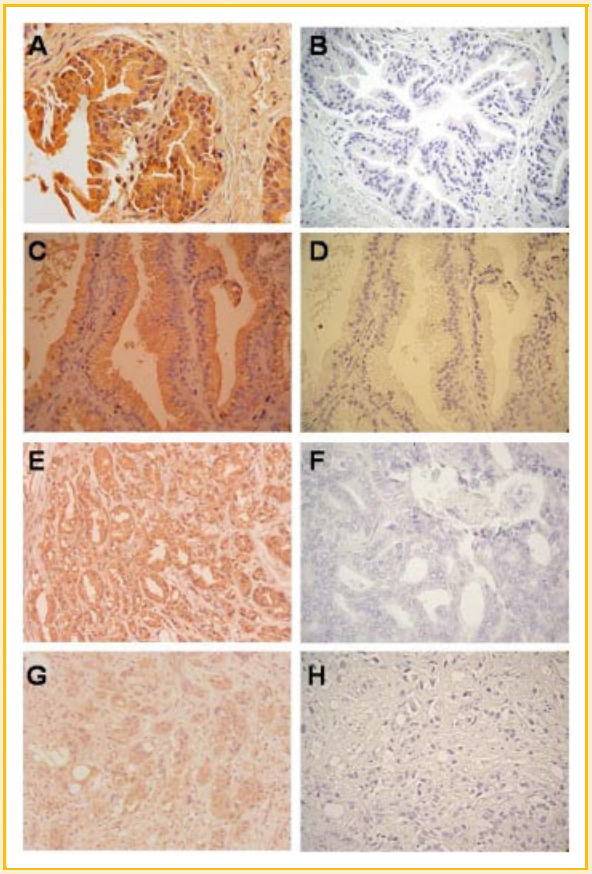

Fig. 3. Regucalcin protein is expressed in non-neoplastic, benign prostate hyperplasia and neoplastic prostate tissues. Representative immunohistochemistry sections are shown. A: Non-neoplastic prostate. C: Benign prostate hyperplasia. E: Well-differentiated adenocarcinoma. G: Poorly differentiated adenocarcinoma. The corresponding negative controls, obtained by omission of the primary antibody, are shown in panels B,D,F,H. All tissues are shown in an original magnification of 400×.

of IDC and non-malignant tissues ($P < 0.001$), which demonstrates loss of regucalcin expression in breast tumors. In addition, a significant negative association between regucalcin immunoreactivity and tumour grading was identified ($P = 0.002$, Table I). Nine out of 13 (69%) grade III tumors had low regucalcin immunoreactivity while 13 out of 26 (50%) grade I tumors expressed moderate or high levels of this protein. In opposition, none of 13 (0%) grade III tumors displayed high regucalcin immunoreactivity.

The majority of prostate adenocarcinomas (53%) showed low-immunoreactivity for regucalcin while 33% and 14% of tumors displayed moderated and high immunoreactivity, respectively. Five out of 17 (29%), 9 out of 17 (53%), and 3 out of 17 (18%) cases of benign prostate hyperplasia showed low, moderate, and high regucalcin immunoreactivity, respectively. None of non-neoplastic prostate samples displayed low regucalcin immunoreactivity. A significant difference exists comparing the regucalcin immunoreactivity in prostate adenocarcinoma with non-neoplastic ($P < 0.001$) or benign prostate hyperplasia tissues ($P = 0.002$, Table II), which demonstrates loss of regucalcin expression in prostate tumors. As shown in Table II a significant negative association ($P < 0.001$) was identified between regucalcin immunoreactivity and cellular differentiation of prostate adenocarcinoma. Seventeen out of 26 (65%) poorly differentiated tumors had low regucalcin immunoreactivity while 5 out of 5 (100%) well differentiated tumors expressed moderate or high levels of this protein.

E_2 AND DHT REGULATE REGUCALCIN mRNA EXPRESSION IN HUMAN BREAST AND PROSTATE CANCER CELL LINES

The effect of E_2 and DHT on the expression of regucalcin in MCF-7 and LNCaP cancer cells, respectively, was analyzed by real-time PCR. For this purpose, a time-course experiment was carried out in both cell lines using 1 and 10 nM of each hormone. In MCF-7 cells similar effects were observed using 1 or 10 nM of E_2 (Fig. 6A). For 6 and 12 h of stimulation, E_2 induced expression of regucalcin mRNA while for longer incubation times a diminished expression was observed in comparison to controls. Since 6 h of stimulation have been described to reflect the E_2 effects mediated by the classical nuclear estrogen receptor [Cascio et al., 2007] we selected 10 nM of E_2 and this exposure period to explore the mechanisms underlying E_2 regulation of regucalcin expression. To test whether this regulation is mediated through ER activation, MCF-7 cells were exposed to E_2, ICI 182,780 and E_2-BSA (Fig. 6B). When administered with 10 nM E_2, ICI 182,780 does not reverse the E_2 induction of regucalcin mRNA expression. Treatment with E_2-BSA stimulated regucalcin expression with an effect similar to what observed with E_2 (Fig. 6B). No significant difference relatively to control was observed when ICI 182,780 was administrated alone.

In LNCaP cells both doses of DHT significantly decreased regucalcin mRNA expression, except for 48h (Fig. 7A). To explore the mechanism underlying the androgenic down-regulation of regucalcin expression, LNCaP cells were exposed to DHT (10 nM) in combination with flutamide or cyclohexamide for 6 h (Fig. 7A). This time of exposure has been demonstrated to be adequate for the observation of DHT effects mediated by the classical nuclear androgen receptor [Jia et al., 2003]. Both flutamide and cyclohexamide prevented the inhibitory effect of DHT on regucalcin mRNA expression, restoring expression levels to those of control. No significant differences were perceived when cells were exposed to flutamide or cyclohexamide alone.

DISCUSSION

In this study we first reported the expression of regucalcin mRNA and protein in non-neoplastic human breast and prostate and also in cancers of both tissues. At the mRNA level we identified the full-length regucalcin transcript and two transcript variants (Δ4 and Δ4,5) in non-neoplastic human breast and prostate, MCF-7 and LNCaP cells. The existence of transcript variants for human regucalcin gene has been previously reported, but the identified transcripts were identical in their coding region differing only in 5′-untranslated regions [Misawa and Yamaguchi, 2000]. Our results, however suggest the occurrence of alternative splicing of the regucalcin pre-mRNA. The Δ4 and Δ4,5 transcripts are likely

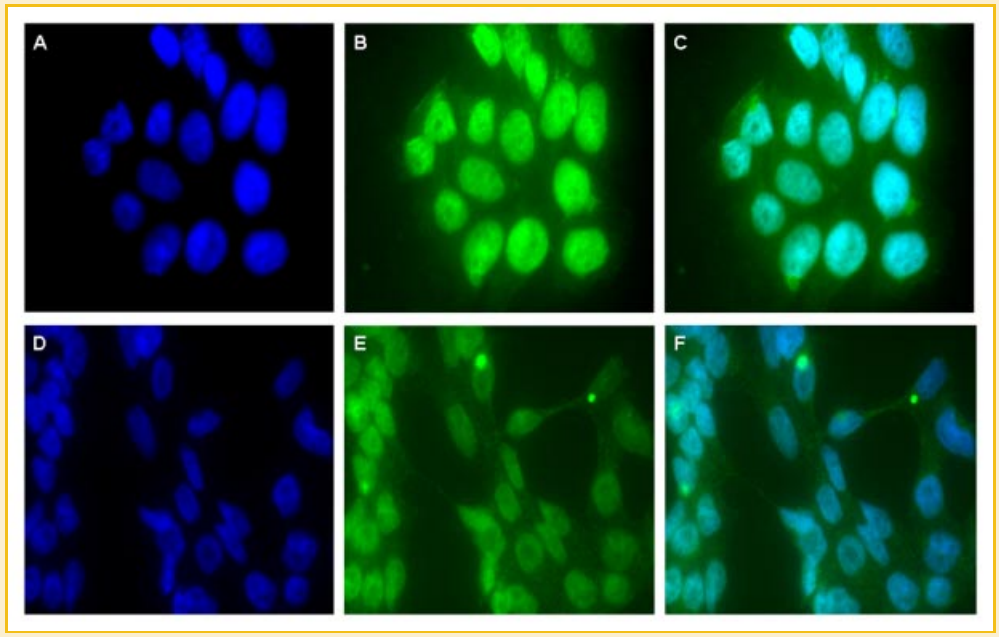

Fig. 4. Regucalcin is localized to the cell nucleus. Dual immunofluorescence localization of regucalcin (green) and nucleus (blue) on MCF-7 (A–C) and LNCaP (D–F) cells. Co-localization of regucalcin and nucleus is shown in the merged figure as light blue areas (C,F). All panels are shown in an original magnification of 630×.

generated by alternative splicing events and if translated may encode proteins with 227 and 183 amino acids, respectively. These proteins could be predicted from the regucalcin gene by a computational methodology for alternative splicing analysis [Kim et al., 2005]. The regucalcin protein is a molecule with hydrophilic character attributed mainly to amino acids between positions 100 and 200 [Shimokawa and Yamaguchi, 1993b]. This hydrophilic region, which has been suggested as the functional domain related to Ca^{2+} binding [Shimokawa and Yamaguchi, 1993b], is deleted in the putative proteins originated from the Δ4 and Δ4,5 transcripts, corresponding to amino acids 116–187 and 116–231, respectively. Ca^{2+} binding was suggested to occur also by involvement of aspartic acid (D) and glutamine (Q) residues [Yamaguchi, 2000] and the putative proteins generated from regucalcin transcript variants may have loss up to 52% of D and Q residues. Thus, it is liable to speculate that these proteins may lack the ability for Ca^{2+} binding. We were able to amplify the Δ4 and Δ4,5 regucalcin transcripts in a variety of human tissues, namely, testis, liver, kidney, lung and heart (data not published). In addition, a differential expression of regucalcin transcript variants in breast and prostate cancer cells was detected. MCF-7 cells did not express the Δ4 transcript, while in LNCaP cells the Δ4,5 transcript is not detectable. Alternative splicing effects range from a complete loss of function or acquisition of a new function to very subtle modulations

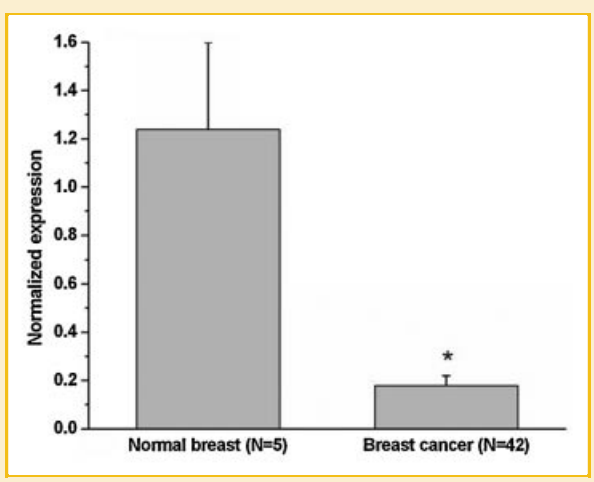

Fig. 5. Regucalcin mRNA is under-expressed in breast cancer. Breast cancer samples: infiltrating ductal carcinoma. Non-neoplastic breast: breast tissue adjacent to benign lesions. Regucalcin mRNA expression was determined by real-time PCR and normalized with GAPDH and β_2M. $^*P < 0.05$.

TABLE I. Relationship of Regucalcin Immunoreactivity With Human Breast Tissues and Histological Grade of Infiltrating Ductal Carcinoma

	Regucalcin immunoreactivity		
Histological diagnostic	Low	Moderate	High
Non-neoplastic tissue	0% (0/10)	10% (1/10)	90% (9/10)
Normal	0% (0/5)	20% (1/5)	80% (4/5)
Fibroadenoma	0% (0/5)	0% (0/5)	100% (5/5)
Infiltrating ductal carcinoma[a]	56% (42/75)	37% (28/75)	7% (5/75)
Grade I	50% (13/26)	38% (10/26)	12% (3/26)
Grade II	56% (20/36)	39% (14/36)	5% (2/36)
Grade III	69% (9/13)	31% (4/13)	0% (0/13)

Parentheses indicated the number of cases over total number of samples in each group.
[a]The regucalcin immunoreactivity is associated with tumour grading ($P = 0.002$, χ^2 test). The regucalcin immunoreactivity in infiltrating ductal carcinoma and non-malignant tissues is significantly different ($P < 0.001$, χ^2 test).

TABLE II. Relationship of Regucalcin Immunoreactivity With Human Prostate Tissues and Cellular Differentiation of Adenocarcinoma

	Regucalcin immunoreactivity		
Histological diagnostic	Low	Moderate	High
Non-neoplastic	0% (0/5)	40% (2/5)	60% (3/5)
Benign hyperplasia	29% (5/17)	53% (9/17)	18% (3/17)
Adenocarcinoma[a]	53% (27/51)	33% (17/51)	14% (7/51)
Poorly differentiated (Gleason scores 8–10)	65% (17/26)	23% (6/26)	12% (3/26)
Moderately differentiated (Gleason scores 5–7)	50% (10/20)	35% (7/20)	15% (3/20)
Well-differentiated (Gleason scores 2–4)	0% (0/5)	60% (3/5)	40% (2/5)

Parentheses indicated the number of cases over total number of samples in each group.
[a]The regucalcin immunoreactivity is associated with the cellular differentiation of tumour ($P < 0.001$, χ^2 test). The regucalcin immunoreactivity in adenocarcinomas and non-neoplastic or benign hyperplasia tissues is significantly different ($P < 0.001$ and $P = 0.002$, χ^2 test).

which are observed in the majority of cases reported [Stamm et al., 2005], and the alteration of splicing patterns of several genes have already been associated with cancer [Srebrow and Kornblihtt, 2006; Venables, 2006]. Altogether our data are indicative of a relevant physiological role for the Δ4 and Δ4,5 regucalcin transcripts in normal and pathologic physiology. Further studies will be necessary to confirm its translation and to decipher the biological function of these proteins. Immunohistochemical analysis confirmed the presence of regucalcin in non-neoplastic and tumour tissues of human breast and prostate with positive staining detected in cytoplasm and nuclei of epithelial cells. In MCF-7 and LNCaP cells, double immunofluorescent labeling confirmed regucalcin localization to the cell nuclei, which is in agreement with the findings in cultured mouse hepatocytes [Ishigami et al., 2003]. However, there is no nuclear localization signal on regucalcin sequence and it was suggested that nuclear translocation is mediated by protein phosphorylation [Feng et al., 2004]. In vitro studies showed that regucalcin is able to bind DNA and nuclear proteins [Tsurusaki and Yamaguchi, 2004] which indicates that it may be involved in the regulation of nuclear functions. Previous reports suggested that regucalcin suppresses cell proliferation by directly inhibiting DNA synthesis [Inagaki and Yamaguchi, 2001; Misawa et al., 2001]. Moreover, regucalcin regulates RNA synthesis and its role as a transcription-related factor has been suggested [Tsurusaki and Yamaguchi, 2002]. Nevertheless, the precise mechanisms by which regucalcin exerts nuclear effects remain to be elucidated.

Real-time PCR and immunohistochemistry analysis demonstrated that regucalcin mRNA and/or protein expression is diminished in breast and prostate cancer cases. Moreover, regucalcin immunoreactivity was associated with either grade of IDC or cellular differentiation of prostate adenocarcinoma, suggesting their role in cancer development and progression. Also, in hepatoma cells a reduced expression of regucalcin was described when compared with non-neoplastic liver [Murata et al., 1997]. More recently, proteomic and oligonucleotide microarray studies have identified regucalcin as a down-regulated gene in mouse

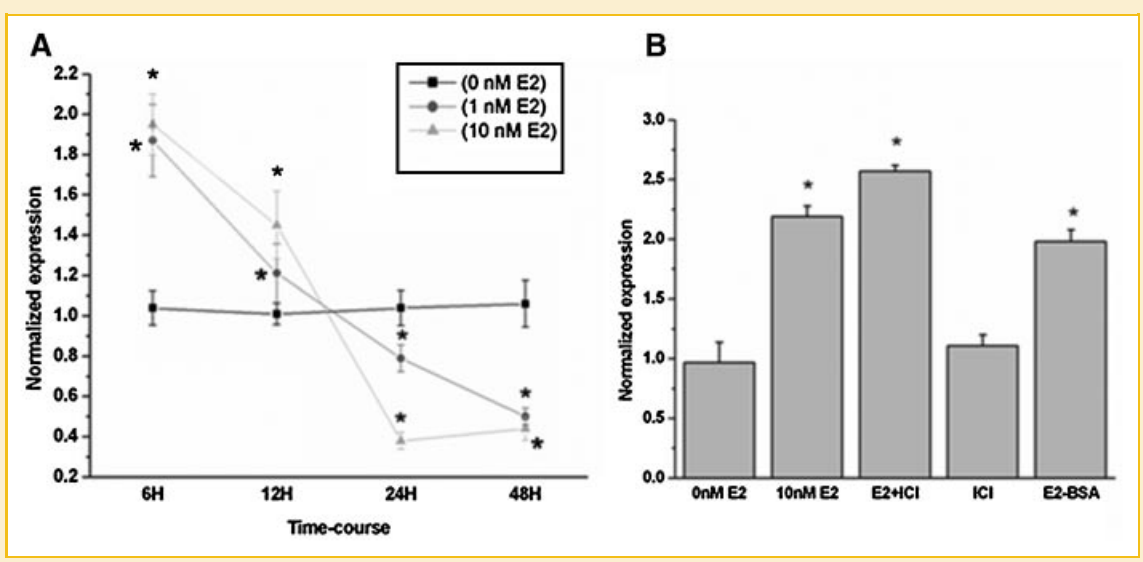

Fig. 6. Effect of E_2 on regucalcin mRNA expression in MCF-7 cells determined by real-time PCR. Regucalcin expression was normalized with GAPDH and β_2M expression. A: Time-course experiment. MCF-7 cells were exposed to 0, 1, and 10 nM of E_2 for 6, 12, 24, and 48 h. $^*P < 0.05$ compared with control values at the same time point. B: Effect of E_2, ICI 182,780 and E_2-BSA conjugated in the regulation of regucalcin expression. MCF-7 cells were exposed to E_2 (10 nM), E_2 (10 nM) plus ICI 182,780 (10 nM), ICI 182,780 alone and E_2-BSA conjugated (10 nM) for 6 h. $^*P < 0.001$ compared with control values.

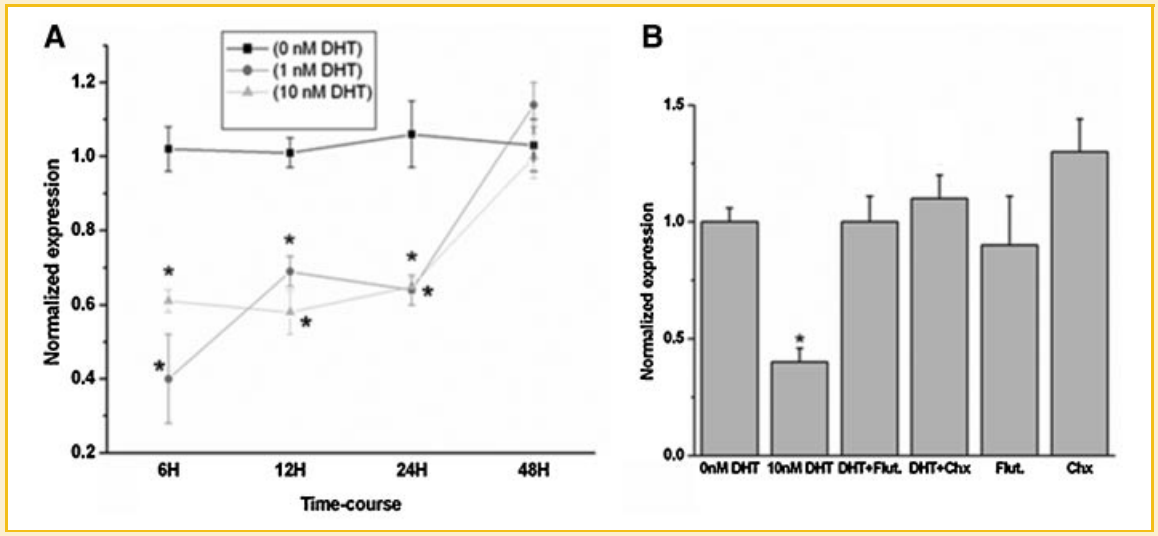

Fig. 7. Effect of DHT on regucalcin mRNA expression in LNCap cells determined by real-time PCR. Regucalcin expression was normalized with GAPDH and β_2M expression. A: Time-course experiment. LNCaP cells were exposed to 0, 1, and 10 nM of for 6, 12, 24, and 48 h. $^*P < 0.05$ compared with control values at the same time point. B: Effect of flutamide and cyclohexamide on DHT regulation of regucalcin expression. LNCaP cells were exposed to DHT (10 nM), DHT (10 nM) plus flutamide (1 μM), DHT (10 nM) plus cyclohexamide (50 μg/ml) and, flutamide (1 μM) and cyclohexamide (50 μg/ml) alone for 6 h. $^*P < 0.01$ compared with control values.

[Elchuri et al., 2007] and human [Kim et al., 2002, 2003] hepatocellular carcinomas, and as an up-regulated gene in human pancreatic cancer cell lines resistant to irradiation treatment [Ogawa et al., 2006]. Studies in hepatoma cell lines showed that regucalcin suppresses cell proliferation [Nakagawa et al., 2005; Yamaguchi and Daimon, 2005], inhibits expression of oncogenes c-myc, H-ras, and c-src, and increases the expression of tumour supressor genes p53 and Rb [Tsurusaki and Yamaguchi, 2003, 2004]. Altogether, these findings suggest that regucalcin may have a protective role against carcinogenesis and, consequently, loss of regucalcin expression may contribute to tumour development. This clearly raises the question of which are the factors involved in the control of regucalcin expression.

The expression of regucalcin mRNA is regulated by several factors, namely Ca^{2+} [Shimokawa and Yamaguchi, 1992, 1993a; Yamaguchi et al., 1994], calcitonin [Yamaguchi et al., 1994], insulin [Murata et al., 1997] and E_2 [Yamaguchi and Oishi, 1995; Kurota and Yamaguchi, 1996; Maia et al., 2008]. E_2 stimulates regucalcin mRNA expression in rat liver [Yamaguchi and Oishi, 1995] while in kidney cortex [Kurota and Yamaguchi, 1996], mammary gland and prostate [Maia et al., 2008] the opposite effect was observed. Breast and prostate carcinogenesis is strongly dependent of estrogenic and androgenic stimulation [Debes and Tindall, 2002; Platet et al., 2004; Muti et al., 2006] and altered Ca^{2+} homeostasis and signalling have been associated with the development of both pathologies [Prevarskaya et al., 2004; Lee et al., 2006], which lead us to investigate the effect of E_2 and DHT on regucalcin expression in MCF-7 and LNCaP cells, respectively. The E_2 stimulation of MCF-7 cells seems to be independent of the dose and dependent of exposure time, as 6 and 12 h of hormonal exposure showed opposite effects to that of 24 and 48 h. The E_2 effects inducing regucalcin expression are not abrogated by the presence of ICI 182,780, and E_2-BSA produces the same effect of E_2, which suggests the involvement of a membrane-bound ER (mER). Several studies have demonstrated that E_2 can induce an increase in intracellular Ca^{2+} in both breast and prostate cells [Audy et al., 1996; Rouayrenc et al., 2000; Huang and Jan, 2001; Rossi et al., 2002; Walsh, 2003] and these effects have been suggested to be associated with rapid non-genomic mechanisms, through a mER [Govind and Thampan, 2003]. Moreover, it has been established that Ca^{2+} administration stimulates regucalcin expression [Shimokawa and Yamaguchi, 1992; Isogai and Yamaguchi, 1995; Yamaguchi and Kurota, 1995]. Nevertheless, E_2-stimulation for periods longer than 12 h induced a down-regulation of regucalcin expression in MCF7-cells, an effect also observed *in vivo* in the mammary gland of ovariectomized rats stimulated with E_2 for 7 days [Maia et al., 2008]. The down-regulatory effect of E_2 in regucalcin mRNA expression may sustain a reduced protein level and may be a relevant mechanism for breast cancer initiation in premenopausal women and postmenopausal women receiving hormonal substitution therapy, both subjected to an E_2 rich environment. In LNCaP cells, both experimental doses of DHT down-regulated regucalcin expression in all exposure times, except for 48h. This down-regulation is inhibited in the presence of both flutamide and cyclohexamide suggesting that the effect is directly mediated through AR, but requires de novo protein synthesis. In rat liver, castration and treatment with testosterone propionate does not modify regucalcin expression [Fujita et al., 1992] and the protein has been described as an androgen-independent factor [Ishigami et al., 2004, 2005]. However, in silico analysis of the regucalcin promoter region (data not shown) allowed the identification of androgen-responsive-elements consensus sequences at −906, −915, −4126, and −5822 bp upstream from transcription initiation site, which further supports the effects observed herein. Primary prostate cancers are dependent of androgenic stimulation and the observed reduction of regucalcin mRNA expression in response to DHT in LNCaP cells suggests that

loss of regucalcin expression may be associated with tumour development.

In conclusion, we demonstrated that regucalcin is under-expressed in breast and prostate cancer cases and regulated by E_2 and DHT in MCF-7 and LNCaP cells. Although further studies are needed to delineate the precise regucalcin functions in human breast and prostate physiology, our data support that loss of regucalcin expression may be associated with development and progression of tumors.

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REFERENCES

Audy MC, Vacher P, Duly B. 1996. 17 beta-estradiol stimulates a rapid Ca2+ influx in LNCaP human prostate cancer cells. Eur J Endocrinol 135:367–373.

Capiod T, Shuba Y, Skryma R, Prevarskaya N. 2007. Calcium signalling and cancer cell growth. Subcell Biochem 45:405–427.

Cascio S, Bartella V, Garofalo C, Russo A, Giordano A, Surmacz E. 2007. Insulin-like growth factor 1 differentially regulates estrogen receptor-dependent transcription at estrogen response element and AP-1 sites in breast cancer cells. J Biol Chem 282:3498–3506.

Debes JD, Tindall DJ. 2002. The role of androgens and the androgen receptor in prostate cancer. Cancer Lett 187:1–7.

Elchuri S, Naeemuddin M, Sharpe O, Robinson WH, Huang TT. 2007. Identification of biomarkers associated with the development of hepatocellular carcinoma in CuZn superoxide dismutase deficient mice. Proteomics 7:2121–2129.

Feng D, Kondo Y, Ishigami A, Kuramoto M, Machida T, Maruyama N. 2004. Senescence marker protein-30 as a novel antiaging molecule. Ann NY Acad Sci 1019:360–364.

Fujita T, Uchida K, Maruyama N. 1992. Purification of senescence marker protein-30 (SMP30) and its androgen-independent decrease with age in the rat liver. Biochim Biophys Acta 1116:122–128.

Fujita T, Inoue H, Kitamura T, Sato N, Shimosawa T, Maruyama N. 1998. Senescence marker protein-30 (SMP30) rescues cell death by enhancing plasma membrane Ca(2+)-pumping activity in Hep G2 cells. Biochem Biophys Res Commun 250:374–380.

Govind AP, Thampan RV. 2003. Membrane associated estrogen receptors and related proteins: Localization at the plasma membrane and the endoplasmic reticulum. Mol Cell Biochem 253:233–240.

Huang JK, Jan CR. 2001. Mechanism of estrogens-induced increases in intracellular Ca(2+) in PC3 human prostate cancer cells. Prostate 47:141–148.

Inagaki S, Yamaguchi M. 2001. Regulatory role of endogenous regucalcin in the enhancement of nuclear deoxyribonuleic acid synthesis with proliferation of cloned rat hepatoma cells (H4-II-E). J Cell Biochem 82:704–711.

Ishigami A, Handa S, Maruyama N, Supakar PC. 2003. Nuclear localization of senescence marker protein-30, SMP30, in cultured mouse hepatocytes and its similarity to RNA polymerase. Biosci Biotechnol Biochem 67:158–160.

Ishigami A, Kondo Y, Nanba R, Ohsawa T, Handa S, Kubo S, Akita M, Maruyama N. 2004. SMP30 deficiency in mice causes an accumulation of neutral lipids and phospholipids in the liver and shortens the life span. Biochem Biophys Res Commun 315:575–580.

Ishigami A, Fujita T, Inoue H, Handa S, Kubo S, Kondo Y, Maruyama N. 2005. Senescence marker protein-30 (SMP30) induces formation of microvilli and bile canaliculi in Hep G2 cells. Cell Tissue Res 320:243–249.

Isogai M, Yamaguchi M. 1995. Calcium administration increases calcium-binding protein regucalcin concentration in the liver of rats. Mol Cell Biochem 143:53–58.

Jia L, Kim J, Shen H, Clark PE, Tilley WD, Coetzee GA. 2003. Androgen receptor activity at the prostate specific antigen locus: Steroidal and non-steroidal mechanisms. Mol Cancer Res 1:385–392.

Kent WJ. 2002. BLAT-the BLAST-like alignment tool. Genome Res 12:656–664.

Kim J, Kim SH, Lee SU, Ha GH, Kang DG, Ha NY, Ahn JS, Cho HY, Kang SJ, Lee YJ, Hong SC, Ha WS, Bae JM, Lee CW, Kim JW. 2002. Proteome analysis of human liver tumor tissue by two-dimensional gel electrophoresis and matrix assisted laser desorption/ionization-mass spectrometry for identification of disease-related proteins. Electrophoresis 23:4142–4156.

Kim W, Oe Lim S, Kim JS, Ryu YH, Byeon JY, Kim HJ, Kim YI, Heo JS, Park YM, Jung G. 2003. Comparison of proteome between hepatitis B virus- and hepatitis C virus-associated hepatocellular carcinoma. Clin Cancer Res 9: 5493–5500.

Kim N, Shin S, Lee S. 2005. ECgene: Genome-based EST clustering and gene modeling for alternative splicing. Genome Res 15:566–576.

Kurota H, Yamaguchi M. 1996. Steroid hormonal regulation of calcium-binding protein regucalcin mRNA expression in the kidney cortex of rats. Mol Cell Biochem 155:105–111.

Lee WJ, Monteith GR, Roberts-Thomson SJ. 2006. Calcium transport and signaling in the mammary gland: Targets for breast cancer. Biochim Biophys Acta 1765:235–255.

Maia CJ, Santos CR, Schmitt F, Socorro S. 2008. Regucalcin is expressed in rat mammary gland and prostate and down-regulated by 17beta-estradiol. Mol Cell Biochem 311:81–86.

Misawa H, Yamaguchi M. 2000. Transcript heterogeneity of the human gene for Ca2+-binding protein regucalcin. Int J Mol Med 5:283–287.

Misawa H, Inagaki S, Yamaguchi M. 2001. Suppression of cell proliferation and deoxyribonucleic acid synthesis in the cloned rat hepatoma H4-II-E cells overexpressing regucalcin. J Cell Biochem 84:143–149.

Monteith GR, McAndrew D, Faddy HM, Roberts-Thomson SJ. 2007. Calcium and cancer: Targeting Ca2+ transport. Nat Rev Cancer 7:519–530.

Murata T, Shinya N, Yamaguchi M. 1997. Expression of calcium-binding protein regucalcin mRNA in the cloned human hepatoma cells (HepG2): Stimulation by insulin. Mol Cell Biochem 175:163–168.

Muti P, Rogan E, Cavalieri E. 2006. Androgens and estrogens in the etiology and prevention of breast cancer. Nutr Cancer 56:247–252.

Nakagawa T, Sawada N, Yamaguchi M. 2005. Overexpression of regucalcin suppresses cell proliferation of cloned normal rat kidney proximal tubular epithelial NRK52E cells. Int J Mol Med 16:637–643.

Ogawa K, Utsunomiya T, Mimori K, Tanaka F, Haraguchi N, Inoue H, Murayama S, Mori M. 2006. Differential gene expression profiles of radioresistant pancreatic cancer cell lines established by fractionated irradiation. Int J Oncol 28:705–713.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45.

Platet N, Cathiard AM, Gleizes M, Garcia M. 2004. Estrogens and their receptors in breast cancer progression: A dual role in cancer proliferation and invasion. Crit Rev Oncol Hematol 51:55–67.

Prevarskaya N, Skryma R, Shuba Y. 2004. Ca2+ homeostasis in apoptotic resistance of prostate cancer cells. Biochem Biophys Res Commun 322:1326–1335.

Rossi AM, Picotto G, de Boland AR, Boland RL. 2002. Evidence on the operation of ATP-induced capacitative calcium entry in breast cancer cells and its blockade by 17beta-estradiol. J Cell Biochem 87:324–333.

Rouayrenc JF, Vignon F, Bringer J, Pujol P. 2000. Non-genomic steroid effects: Estrogen action revisited. Ann Endocrinol (Paris) 61:517–523.

Schmitt FC, Bento MJ, Amendoeira I. 1995. Estimation of estrogen receptor content in fine-needle aspirates from breast cancer using the monoclonal antibody 1D5 and microwave oven processing: Correlation with paraffin embedded and frozen sections determinations. Diagn Cytopathol 13:347–351.

Shimokawa N, Yamaguchi M. 1992. Calcium administration stimulates the expression of calcium-binding protein regucalcin mRNA in rat liver. FEBS Lett 305:151–154.

Shimokawa N, Yamaguchi M. 1993a. Expression of hepatic calcium-binding protein regucalcin mRNA is mediated through Ca2+/calmodulin in rat liver. FEBS Lett 316:79–84.

Shimokawa N, Yamaguchi M. 1993b. Molecular cloning and sequencing of the cDNA coding for a calcium-binding protein regucalcin from rat liver. FEBS Lett 327:251–255.

Srebrow A, Kornblihtt AR. 2006. The connection between splicing and cancer. J Cell Sci 119:2635–2641.

Stamm S, Ben-Ari S, Rafalska I, Tang Y, Zhang Z, Toiber D, Thanaraj TA, Soreq H. 2005. Function of alternative splicing. Gene 344:1–20.

Tsurusaki Y, Yamaguchi M. 2000. Role of endogenous regucalcin in the regulation of Ca(2+)-ATPase activity in rat liver nuclei. J Cell Biochem 78:541–549.

Tsurusaki Y, Yamaguchi M. 2002. Role of endogenous regucalcin in nuclear regulation of regenerating rat liver: Suppression of the enhanced ribonucleic acid synthesis activity. J Cell Biochem 87:450–457.

Tsurusaki Y, Yamaguchi M. 2003. Overexpression of regucalcin modulates tumor-related gene expression in cloned rat hepatoma H4-II-E cells. J Cell Biochem 90:619–626.

Tsurusaki Y, Yamaguchi M. 2004. Role of regucalcin in liver nuclear function: Binding of regucalcin to nuclear protein or DNA and modulation of tumor-related gene expression. Int J Mol Med 14:277–281.

Venables JP. 2006. Unbalanced alternative splicing and its significance in cancer. Bioessays 28:378–386.

Walsh PC. 2003. Calcium intake and prostate cancer risk in a long-term aging study: The Baltimore Logitudinal Study of Aging. J Urol 170: 312.

Yamaguchi M. 2000. Role of regucalcin in calcium signaling. Life Sci 66:1769–1780.

Yamaguchi M. 2005. Role of regucalcin in maintaining cell homeostasis and function (review). Int J Mol Med 15:371–389.

Yamaguchi M, Daimon Y. 2005. Overexpression of regucalcin suppresses cell proliferation in cloned rat hepatoma H4-II-E cells: Involvement of intracellular signaling factors and cell cycle-related genes. J Cell Biochem 95:1169–1177.

Yamaguchi M, Kurota H. 1995. Expression of calcium-binding protein regucalcin mRNA in the kidney cortex of rats: The stimulation by calcium administration. Mol Cell Biochem 146:71–77.

Yamaguchi M, Oishi K. 1995. 17 beta-Estradiol stimulates the expression of hepatic calcium-binding protein regucalcin mRNA in rats. Mol Cell Biochem 143:137–141.

Yamaguchi M, Yamamoto T. 1978. Purification of calcium binding substance from soluble fraction of normal rat liver. Chem Pharm Bull (Tokyo) 26:1915–1918.

Yamaguchi M, Kanayama Y, Shimokawa N. 1994. Expression of calcium-binding protein regucalcin mRNA in rat liver is stimulated by calcitonin: The hormonal effect is mediated through calcium. Mol Cell Biochem 136: 43–48.