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GENE EXPRESSION PROFILE OF PROSTATE CANCER CELL LINES:
EFFECT OF NERVE GROWTH FACTOR TREATMENT

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Key words : Nerve Growth Factor, DU145 cell line, PC3 cell line, microarray analysis, quantitative RT-PCR.

SUMMARY

A dysregulation of the Nerve Growth Factor (NGF)-mediated control of prostate cell growth is associated with the malignant progression of prostate epithelial cells. Exogenous NGF induced in prostate cancer (PCa) cell lines DU145 and PC3 the expression of p75^{NGFR}, accompanied by a reduction of the cell malignancy. The aim of this study was to analyze the profile of NGF-regulated genes the PCa cell line DU145 by using the cDNA microarray technique.

NGF treatment of DU145 cells decreased the expression of 52 known genes, while the expression of 40 known genes was increased. NGF treatment of the DU145 cell line modified the expression profile of clusters of genes involved in invasion and metastasis, in cell proliferation and apoptosis, inflammation, cell metabolism and transcriptional activity. Interestingly, NGF induced the same pattern of gene modifications in both PCa cell lines. Data presented here may help to identify gene/proteins that dispose to PCa progression and to assess future markers that could allow the development of new clinic diagnostic and therapeutical approaches.

INTRODUCTION

Evidence generated over the past years suggests that growth factor-mediated pathways are modified during prostate cancer (PCa) development and advancement (reviewed in Djakiew, 2000). This dysregulation has been implicated in tumor progression and in the metastatic colonization which, in turn, involve cellular proliferation, adhesion and motility (Montano and Djamgoz, 2004). Therefore, analysis of the role of different growth factors and their receptors in the control of these mechanisms may help to clarify the pathogenesis and the progression of PCa.

Nerve Growth Factor (NGF) plays a physiological role in the control of prostate epithelial cell growth and differentiation (Macgrogan et al., 1992; Paul et al., 1996; Harper, 1999). The observation that NGF production is progressively lost in PCa advancement (Paul et al., 1996; Montano and Djamgoz, 2004) suggests that this neurotrophin plays a role in PCa development. Analysis of NGF receptor expression showed that $p75^{\text{NGFR}}$ expression is decreased in PCa and that the extent of $p75^{\text{NGFR}}$ loss is related to PCa progression (Pflug et al., 1992). By contrast, *trkA* is maintained in all grades of PCa (Pflug et al., 1995), and promotes mitogenesis and invasion (Festuccia et al., 2007). Thus, it could be proposed that, while *trkA* mediates the mitogenic effect of NGF, co-expression of both $p75^{\text{NGFR}}$ and *trkA* represents a crucial condition for the antiproliferative effect of NGF, leading to the suggestion that a dysregulation of NGF-mediated control of prostate growth may be associated with PCa progression (Pflug and Djakiew, 1998). On this line, it has been recently pointed out that the NGF 'circuitry' in PCa is a target for the development of novel therapeutic agents (Papatsoris et al., 2007).

We have previously shown that NGF treatment induces the expression of $p75^{\text{NGFR}}$ and has antiproliferative effects in different tumors including pituitary adenoma (Missale et al., 1993), small cell lung carcinoma (Missale et al., 1998) and thyroid tumor (Paez-Pereda et al., 2000). We have also reported that PCa cell lines are differentiated by NGF into a less malignant phenotype (Sigala et al., 1999; Sigala et al., 2002). In particular, the DU145 and PC3 cell lines responded to NGF with a decrease of telomerase activity and with the expression of functionally-active androgen

and p75^{NGFR} receptors. These molecular changes were accompanied by the reduction of the cell proliferation rate both *in vitro* and *in vivo* and by the reduction of the *in vitro* invasive capacity (Sigala et al., 1999; Sigala et al., 2002). In line with these data, the role of p75^{NGFR} as a tumor suppressor molecule in the PCa cell line PC3 has been recently emphasized (Nalbandian et al., 2005). Indeed, transfection of p75^{NGFR} into PC3 cells has been shown to modulate the expression of genes that are critically involved in cell adhesion, apoptosis, metastasis, signal transduction and transcription (Nalbandian et al., 2005). Regulation of molecular events leading to anchorage-independent growth necessary for invasion involves a temporal and spatial coordination of molecules that are causally involved in the process (Hoon et al., 2006). Thus, identification of the complete pattern of gene alterations and molecular events leading to cancer progression and metastasis is a great challenge in PCa research, that may help the development of new diagnostic and therapeutical approaches. On this basis, the aim of this study was to analyze the profile of NGF-regulated genes the PCa cell line DU145 by using the cDNA microarray technique.

MATERIALS AND METHODS

Cell culture and NGF treatment. PC3 and DU145 cells were purchased from the American Type Culture Collection (ATCC) (LGC Promochem, Sesto San Giovanni, MI, Italy) and maintained in culture as suggested by ATCC. Media and supplements were purchased from Celbio (Milano, Italy). Human recombinant NGF was purchased from Sigma Aldrich Italia (Milano, Italy). On the bases of our previous data (Sigala et al., 1999; Sigala et al., 2002), DU145 cells were treated with 50 ng/ml NGF for 8 days, while PC3 cells were exposed to 100 ng/ml NGF for 14 days. At these concentration and times, the differentiating effects of NGF were maximal, as shown by the extent of inhibition of cell proliferation and telomerase activity. PC12 cells were kindly provided by Prof. Calissano (Neurobiology Institute, CNR, Roma, Italy) and grown in RPMI 1640 medium supplemented with 10% horse serum, 5% fetal calf serum, 4 mM glutamine and 100 U/ml penicillin/streptomycin.

Cell proliferation rate. Cell proliferation rate was evaluated as previously described (Sigala et al., 2002). Briefly, untreated and NGF-treated DU145 and PC3 cells were detached from plates and plated again at the density of 5×10^5 cells/well. Twenty-four hours later, cells were washed with phosphate-buffered saline (PBS), released from the culture wells with trypsin-EDTA and added with Ca^{++} and Mg^{++} -free PBS containing 0.25% trypan blue to stain non-viable cells. Cell suspensions were dropped in a hemocytometer chamber and the viable cells were blind counted under a phase contrast microscope by two different operators. Results are the mean $\pm$ SEM of six different dishes for each cell culture. The number of cells for each dish was the mean $\pm$ SEM of at least five different counts. Data analysis and graphics were obtained using GraphPad Prism 4 software. The one way analysis of variance (ANOVA) followed by the Student's t test was used for statistical evaluation of differences, considering $p < 0.05$ as threshold for significant difference.

Immunocytochemistry. Experiments were carried out as previously described (Sigala et al., 2002). Briefly, untreated and NGF-treated DU145 and PC3 cells and control PC12 cells were plated on poly-L-lysine-treated coverslips. Twenty-four hours later, cells were fixed for 5 min

at -20°C in methanol and washed in phosphate buffered saline (PBS). Endogenous peroxidases were inactivated at room temperature for 30 min with 1% hydrogen peroxide solution. Cells were permeabilized in PBS containing 10% normal donkey serum and 0.2% Tryton X-100 and incubated with a 1:1000 dilution of p75^{NGFR} goat polyclonal antibody (C-20, epitope mapping the C terminus of human p75^{NGFR}; Santa Cruz Biotechnologies, Heidelberg, Germany) (Nickerson Poulin et al., 2006; Hasan et al., 2005; Ohrt et al., 2004). After extensive washes, the donkey anti-goat biotinylated secondary antibody (Dako S.p.A., Milano, Italy) was added. Signal detection was carried out with the ABC kit (Dako S.p.A.). PC12 cells were used as positive internal control, while the negative control was represented by untreated DU145 and PC3 cells (Pflug et al., 1992; Sigala et al., 2002). Moreover, omission of the primary antibody (not shown) and replacement of the primary antibody with goat normal serum were also used to define the non-specific signal.

RNA isolation. Total RNA was isolated from untreated and NGF-treated DU145 and PC3 cells (about 10^7 cells) using the RNeasy kit (Quiagen, Milano, Italy) and digested with the RNase-Free DNase set (Quiagen, Milano, Italy), according to the manufacturer protocol. RNA quality of samples was tested by RNA electrophoresis and RNA LabChip analysis (Agilent Technologies, Palo Alto, USA) to ensure RNA integrity.

Microarray analysis. Five μg of RNA from untreated and NGF-treated DU145 cells were sent to the service provider RZPD (Deutsches Ressourcenzentrum für Genomforschung GmbH, Berlin, Germany) to perform the Affimetrix Expression Profile. Briefly, the quality control of total RNA was run again, using the Eukaryote Total RNA Nano assay (Agilent Technologies) and RNA was quantified with the NanoDrop technology (Agilent Technologies). Synthesis of cDNA for each sample and in vitro transcription was done with the Ambion's Message Amp II kit, while the quality control of labelled cRNA was done with the messenger RNA (mRNA) Smear Nano Assay (Agilent Technologies), followed by the NanoDrop technology for exact quantification. The hybridization solution was prepared according to the Affymetrix GeneChip Expression Analysis Technical Manual. Biotinylated cRNA ($60 \mu\text{g}/\mu\text{l}$ final concentration) was hybridised on the Human

Genome U133 Plus 2.0 Array GeneChip for 16–18 h at 45°C and 60 rpm (Hybridization Oven 640, Affymetrix UK Ltd). Spike controls for *bioB*, *bioC*, *bioD* and *cre* at concentrations of 1.5, 5, 25 and 100 pM were included according to the manufacturer's instructions (Affymetrix UK Ltd, Cat. No. 900454). Array washing and staining were performed with a fluidics station (GeneChip Fluidics Station 400, Affymetrix UK Ltd), which is controlled by the Affymetrix's software GeneChip Operating System (GCOS), using protocols *EukGe_WS2v4* for the test3-array. Scanning of chips was performed with the GeneChip Scanner 3000 (Affymetrix UK Ltd), which is controlled by Affymetrix's software GeneChip Operating System (GCOS), that performed also the primary data analysis. The microarray suite software package (MAS 5.0; Affymetrix UK Ltd) was used to evaluate probe set signals of the array.

Microarray statistical analysis. Starting from the raw data files (.cel files), analysis was performed at the service provider using the Affymetrix Microarray Suite Version 5.0 software. Briefly, signal intensities were scaled to identical trimmed mean intensity of all scaled signals, according to standard procedures (Affymetrix Microarray Suite User's Guide MAS 5.0). For all probe sets of each chip, the median signal was scaled to a target intensity = 100 in the MAS 5.0 software. Default values for the present/absent filter were $\alpha_1 = 0.05$, $\alpha_2 = 0.065$ for 11 probe pairs/probe set. Genes with a P -value < 0.04 were detected as 'present' call, P -value > 0.06 obtained 'absent' calls and P -values ranging from 0.04 to 0.06 were 'marginally present'.

Q-RT-PCR. One μ g of total RNA from untreated and NGF-treated DU145 and PC3 cells was transcribed into cDNA using murine leukemia virus reverse transcriptase (Promega Italia, Milano, Italy) and oligodT₁₅₋₁₈ as a primer (final volume: 50 μ l). Parallel reactions containing no reverse transcriptase were used as negative controls to confirm the removal of all genomic DNA. Human specific primers were designed using the Primer3 software (<http://frodo.wi.mit.edu>) (Rozen and Skaletsky, 2000). The sequences of the primers used are reported in Table 1.

Amplification and detection were performed with the iCYCLER iQ Real Time PCR Detection System (BioRad Italia, Milano, Italia); the fluorescence signal was generated by SYBR Green I.

Samples were run in triplicate in a 25 μ l reaction containing 12.5 μ l 2x SYBR Green Master Mix (BioRad Italia), 12.5 pmol of each forward and reverse primer (Table 1) and 2 μ l of diluted cDNA. Each PCR experiment included serial dilutions of a positive control for construction of the calibration curve, a positive and a negative DNA sample, and water blanks. The PCR program was initiated by 10 min at 95°C before 40 cycles, each for 15 sec at 95°C and 1 min at 60°C. A subsequent dissociation curve analysis checked the specificity of products. Gene expression levels were normalized to β actin expression and data are presented as the fold change in target gene expression in NGF-treated cells normalized to the internal control gene (β actin) and relative to untreated cells. Results were estimated as Ct values, the Δ Ct was calculated as the mean of the Ct for the target gene minus the mean of the Ct for the internal control gene. The $\Delta\Delta$ Ct represented the mean difference between the Δ Ct of untreated cells minus the Δ Ct of NGF-treated cells. The N-fold differential expression in the target gene of NGF-treated cells compared to untreated cells was expressed as $2^{-\Delta\Delta Ct}$. Data analysis and graphics were obtained using GraphPad Prism 4 software and were the results (mean $\pm$ SEM) of three different experiments run in triplicate for each gene in untreated and NGF-treated cell lines.

RESULTS

Effect of NGF on DU145 and PC3 cells

DU145 cells were exposed to 50 ng/ml NGF for 8 days and PC3 cells were exposed to 100 ng/ml for 14 days. These concentrations and times were chosen on the basis of previous data showing that NGF progressively induces cell differentiation with a maximal effect in these conditions. Cell proliferation and p75^{NGFR} expression were measured as an index of differentiation. As shown in Fig. 1A and according to our previous data, exposure of these cell lines to NGF resulted in the reduction of their cell proliferation rate, which was about 55% inhibited in DU145 cells and 45% inhibited in PC3 cells. NGF treatment resulted in the expression of p75^{NGFR} mRNA, as shown by the Q-RT-PCR (Fig. 1B) ($\Delta\Delta C_t$ NGF-treated vs untreated DU145: $4,2 \pm 0,5$; $\Delta\Delta C_t$ NGF-treated vs untreated PC3: $4,9 \pm 0,6$). That the p75^{NGFR} mRNA was translated into protein is demonstrated by the immunocytochemistry experiments reported in Fig. 1C. Untreated DU145 and PC3 cells do not express p75^{NGFR} (Fig. 1C, b, d), while NGF-treated cells showed a positive staining when immunoreacted with the anti-p75^{NGFR} antibody (Fig. 1C, c, e). The non specific signal obtained by incubating cells with the normal goat serum is reported in Fig. 1C, panel a. PC12 cells were used as positive internal control (Fig. 1C, f).

Gene expression analysis by DNA microarray screening in the DU145 cell line

To identify the molecular determinants of NGF-mediated effects on the PCa cell line DU145, we studied the gene expression profile in untreated and NGF-treated cells, using the microarray-mediated gene screening. Genes showing more than a two-fold increase or decrease in their expression were considered significant in the selection criteria. Comparison of set probes contained in the Human Genome U133 Plus 2.0 Array GeneChip, suggested that NGF treatment of DU145 cells decreased the expression of 52 known genes, while the expression of 40 known genes was increased. Sixteen of these genes did not have characterized biological functions (not shown). Table 2 shows that, by clustering analysis based on gene function, exposure to NGF induced in DU145

PCa cells modifications in the expression of genes involved in various aspects of cellular physiology and pathophysiology.

Genes involved in invasion and metastasis. Detailed analysis of gene expression according to their functional annotation evidenced that NGF induced a significant modifications of genes that may facilitate metastasis formation. In particular, LAMP3, that was highly expressed in untreated DU145 cells was inhibited by NGF exposure of about 5-fold. Among NGF-induced genes, we observed that PLEC1, that facilitates cell-matrix adhesion, was about 4-fold increased. Other genes involved in the cell-matrix and cell-cell adhesion such as PXN, LOXL2 and PALPN were induced of about 4-5 fold.

Genes involved in cell proliferation and apoptosis. The inhibition of cell proliferation observed in NGF-treated DU145 cells may find one of its molecular determinant in the reduced levels of genes such as the oncogene 24p3 or LCN2 (about 5-fold decreased compared to untreated cells) and the cyclin-dependent kinase inhibitor Kip2/p57 (about 3-fold decrease). Genes that reduce cell proliferation and may induce apoptosis, such as the Insulin-like Growth Factor (IGF) binding protein IGFBP3, the apoptosis-related cysteine protease CASP2 and a gene involved in the arrest of cell cycle progression, namely GAS2L3, were significantly increased. In particular, the expression of IGFBP3 and CASP2 was increased of about 3,5-fold, while GAS2L3, was increased of about 8-fold.

Genes involved in cell metabolism. Among genes involved in cell metabolism, we would like to highlight on the strong expression of the enzyme that synthesizes prostaglandin E, namely PTGES, in untreated DU145 cells. Interestingly, NGF treatment induced a 4-fold decrease of the levels of this gene. Another gene known to be involved in the PCa progression and in the immune surveillance escape is ARG2 (Bronte et al., 2005), that is highly expressed in untreated DU145 cells and it is reduced by NGF treatment of about 3,5-fold. A number of genes involved in different pathways of cell metabolism that are also reduced of about 3,5-4-fold by NGF treatment are the extracellular glycoprotein SEPP1 and the proteolytic enzyme PGPEP1. GALNT2, that is involved

in the oligosaccharide biosynthesis (2,6-fold induced) and the protease inhibitor SERPINB7 (5-fold induced) are increased by NGF treatment.

Genes involved in signal transduction and of transcription factors. Different genes involved in signal transduction as well as various transcription factors were significantly modified by NGF treatment in DU145 cell line. In particular, the gene encoding the ribosomal protein RPL37, that is a component of the 60S subunit and is expressed in untreated DU145 cells was 3,2 fold reduced by NGF. Transcription factors as HES1, belonging to the basic helix-loop-helix family and NR4A2, a member of the steroid-thyroid hormone-retinoid receptor superfamily, were expressed in untreated cells and reduced by about 4- fold after NGF treatment. On the same line, the signal values of other genes, i.e. the transcription modulator p8, a nuclear protein with mitogenic activity, and TP53INP1, that regulates cell cycle progression and apoptosis, were about 3,5-fold reduced in NGF-treated cells. Furthermore, a number of genes encoding other transcription factors or transcription factor modulators were increased by NGF treatment, i.e. the transcription factor 3 TCF3 (+ 4,2-fold), the triple functional domain TRIO (+ 3,3-fold), the valyl-tRNA synthetase VARS (+ 3,9-fold) and the myc-associated zinc finger protein MAZ (+ 7,9-fold).

Q-RT-PCR validation of DNA array analysis

To validate the gene expression modifications observed with the microarray approach, we chose representative genes for the Q-RT-PCR analysis in control and NGF-treated DU145 cells. In particular, for each cluster, we chose genes that were inhibited as well as genes that were induced by NGF treatment (Table 2, in italic). Preliminary experiments of Q-RT-PCR using serial dilution of cDNA of untreated DU145 were performed, to check specificity and efficiency of our amplification protocol. Fig. 2 reports an example of curves obtained by amplifying the PLEC1 gene (a gene involved in cytoskeletal anchoring), the LCN2 gene (involved in cell differentiation and growth) and the β -actin as housekeeping gene. Q-RT-PCR amplification from serial dilution of cDNA input of each gene showed high linearity, as indicated by the Pearson correlation coefficient.

Fig. 3 summarizes the results obtained with the Q-RT-PCR amplification of selected genes in untreated and NGF-treated DU145 cell line. The gene expression modifications were in direct agreement with the microarray data, although the fold change in the expression level was not superimposable for some genes. In particular, among genes involved in invasion and metastasis, our results demonstrated that the mRNA encoding PLEC1 was increased of 2,5-3-fold by NGF, while the LAMP3 mRNA was decreased of about 3-fold. Among genes involved in cell proliferation and apoptosis, IGFBP3 mRNA was expressed at higher level in NGF-treated cells (about 3-fold), while the mRNA encoding LCN2 was significantly inhibited. Experiments conducted to study mRNA encoding selected genes of the transcription factor family demonstrated that HES1, NR4A2 and p8 were inhibited of about 2-3 fold. The expression of mRNA encoding PTGES and ARG2 mRNAs was inhibited by NGF treatment of about -3-fold and - 5-fold, respectively.

In order to determine whether these genes were selectively modified by NGF treatment only in DU145 cells or whether they may represent general molecular targets for the induced reduction of malignancy in PCa, we investigated their expression by Q-RT-PCR in another NGF-responsive PCa cell line, the PC3 cells. Comparison of gene expression modifications in untreated and NGF-treated PC3 cells showed that NGF induced the same pattern of gene modifications as in DU145 cells (Fig. 4). Interestingly, from a quantitative point of view, we reported different fold of changes of mRNA expression in some genes in PC3 cells compared to DU145 cells after treatment, in line with the different responsiveness to NGF of PCa cell lines (Sigala et al., 1999). Indeed, PLEC1 mRNA was induced at higher level by NGF treatment in PC3 than in DU145 cells, reaching + 7-fold, while mRNA of genes belonging to the transcription factor family were inhibited to a lower level in PC3 cells compared to DU145 cells; in particular: NR4A2, that was 5-fold decrease in DU145 cells, was 2,5-fold reduced in PC3 cells and HES1 was decreased 8-fold in DU145 cells and 2,5-fold in PC3 cells.

DISCUSSION

There is increasing evidence that the NGF system plays a role in the development of prostate cancer. Both NGF and its p75^{NGFR} receptor are, in fact, progressively lost in benign prostate hyperplasia, adenocarcinoma and metastatic cells (Macgrogan et al., 1992; Pflug et al., 1992; Paul et al., 1996; Harper, 1999), while trkA is maintained in all grades of PCa (Pflug et al., 1995), with a suggested mitogenic role (Festuccia et al., 2007). Thus, while trkA mediates the mitogenic effect of NGF, expression of p75^{NGFR} represents a crucial condition for the antiproliferative and differentiating effects of NGF (Sigala et al., 1999; Sigala et al., 2002; Papatsoris et al., 2007), suggesting that p75^{NGFR} could be a tumor suppressor in the prostate (Pflug et al., 1998). On this line, we have reported that NGF treatment of PCa cell lines induces their differentiation into a less malignant phenotype, as shown by the expression of p75^{NGFR} and androgen receptors, decrease of proliferation rate both *in vitro* and *in vivo*, decreased *in vitro* invasiveness, decrease of telomerase activity and expression of the KAI1 metastasis suppressor gene (Sigala et al., 1999; Sigala et al., 2002). Accordingly, it has been shown that p75^{NGFR} transfection in the PC3 cell line results in the modification of the expression of a number of genes involved in metastasis suppression and differentiation (Nalbandian et al., 2005). The target genes identified by Nalbandian et al. (2005) are different from those presented here and this discrepancy can find its rationale in the distinct study design, as the transfection-induced expression of p75^{NGFR} may allow receptor over-expression. Moreover, it cannot be excluded that the different microarray used can influence the results obtained. It should be observed, however, that the genes identified in the above-mentioned paper and in the present work, although different, can be aggregated as functional classes, namely, cell invasion and metastasis, apoptosis, transduction and transcription, suggesting a level of complexity in the intracellular mechanisms underlying PCa progression that could account for the molecular and clinical heterogeneity of this disease.

In this paper, we report that NGF treatment of the DU145 cell line modifies the expression profile of clusters of genes involved in invasion and metastasis, in cell proliferation and apoptosis,

inflammation, cell metabolism and transcriptional activity. Some of the genes modified, i.e. ARG2 and IGFBP3, have been already involved in the advancement of PCa (Bronte et al., 2005; Renehan et al., 2004), while we are first demonstrating a correlation of a number of genes, i.e. LAMP3, PTGES, NR4A2, with the progression and the metastasis-promoting features of PCa, with a significant potential for developing clinical prognostic tests that could help to better classify and treat this tumor. Recently, the up-regulation of LMO2 and ETS-transcription factors, whose expression was reduced by NGF treatment in DU145 cells (Table 2), has been as well involved in PCa progression (Ma et al., 2007; Rostad et al., 2007).

Malignant growth is characterized by several key changes: self-sufficiency of growth signals, insensitivity to anti-growth signals, escape from apoptosis, unregulated proliferation potential, enhanced angiogenesis, metastasis, escape from immune surveillance (Lu et al., 2006). On these bases, among genes modified by NGF treatment in DU145 cells, we validated representative genes playing a role in different cellular functions.

Invasion and metastasis. Cell motility is a critical determinant of PCa progression. Different genes involved in invasion and metastasis were inhibited in DU145 and PC3 cells by NGF treatment. In particular, the mRNA encoding LAMP3/CD63 lysosomal associated membrane protein 3, a cell membrane protein involved in cell adhesion and motility (Maecker et al., 1997), was from 3- to 5-fold less expressed in treated cells. LAMP3/CD63 is located primarily on lysosomal membranes and it is the major carrier for poly-N-acetyllactosamines, which is a critical ligand for E-selectin present on endothelial cells and platelets. Recently, overexpression of LAMP3/CD63 has been associated with an enhanced metastatic profile in cervical cancer (Kanao et al., 2005). In the present paper, we first suggest a link between the LAMP3/CD63 expression and the PCa progression and it could be hypothesized that NGF-induced reduction of *in vitro* invasiveness observed in PCa cell lines could be associated to the inhibition of LAMP3/CD63 expression. The pattern of genes involved in the reduction of invasiveness of NGF-treated PCa cell lines included also PLEC1 (plectin 1), belonging to the plakin family, that is increased in DU145

and PC3 cell lines after NGF exposure. These proteins represent a family of cytolinker proteins that are central components of the connection of adhesive junctions with cytoskeleton, and represent the major mechanism of cell-cell junctions in epithelial cells (Huber, 2003). In line with this, it has been demonstrated that in PCa the composition of basal lamina structure (included PLEC1) is abnormal (Nagle et al., 1995).

Cell proliferation and apoptosis. Among genes involved in DU145 and PC3 cell proliferation and that were inhibited by NGF treatment, we examined LCN2 (lipocalin 2 or oncogene 24p3 or NGAL, Neutrophil Gelatinase-Associated Lipocalin). Lipocalins are small extracellular proteins with a role in cell proliferation and differentiation, that fulfil a number of biological functions, including lipophilic ligand transport, sensory transduction, prostaglandin synthesis and regulation of cellular homeostasis and immunity (Flower, 1994). NGAL overexpression is observed in different human tumors and in ovarian cancer cell lines (Bratt, 2000). The precise role of lipocalins in cancer, however, is still under investigation, as NGAL is overexpressed in tumor cells induced by *neu*, but not by *ras* or in chemically-induced cancers (Stoesz and Gould, 1995). The present data suggest that lipocalin 2 is overexpressed in PCa cell lines and that the differentiating effects elicited by NGF is associated with a reduction of its mRNA expression.

A well known marker of regulation of PCa cell growth is IGFBP3 (Insulin-like Growth Factor Binding Protein 3). Converging results from epidemiological studies and *in vivo* models indicate that there is a strong correlation between the defective regulation of the IGF network and prostate carcinogenesis (Renehan et al., 2004). IGF-1 is an important agent for carcinogenesis and its bioactivity is modulated by IGF-binding proteins (IGFBPs), by regulating the potential ligand function of free IGF-1 for its receptor (Chan et al., 2002). IGFBP3 inhibits prostate tumor growth also by an IGF-independent mechanism (Silha et al., 2006). Local overexpression of IGFBP3 attenuates prostate tumorigenesis (Silha et al., 2006); here, we demonstrated that the mRNA encoding IGFBP3 is 2 to 3-fold increased in DU145 and PC3 cells following treatment with NGF.

Inflammation. Several studies support the concept that there is a link between inflammation, cancers and immunity (Lu et al., 2006). The expression of the enzyme arginase, produced by the gene ARG2, is related to the immune surveillance and it is highly expressed in cancer of different types (Cederbaum et al., 2004) including PCa. The intratumoral arginase induction favors the cancer progression through different pathways: 1) by providing polyamines, it supports tumor growth and development, 2) by negatively affecting the tumor-infiltrating lymphocyte activity, it suppresses antitumor immune response (Bronte et al., 2005). Accordingly, we detected high level of ARG2, NGF treatment that induces cell differentiation significantly reduced ARG2 mRNA expression.

The PTGES (prostaglandin E synthase) gene has been implicated in inflammation and carcinogenesis (Lu et al., 2006). We report here that PTGES was highly expressed in both DU145 and PC3 cell lines and that it was reduced by NGF treatment. Overproduction of PGE may contribute to carcinogenesis and metastasis, by reducing programmed cell death, by stimulating cell migration and angiogenesis and by reducing the immune surveillance (Lu et al., 2006). Moreover, we found that the member of the nuclear receptor superfamily NR4A2, which is a downstream target of PGE, as its expression is increased by PGE in colorectal cancer (Holla et al., 2006), was decreased in NGF-treated PCa cells. These data suggest that the PTGES/NR4A2 system may play a role not only in the colorectal cancer, but also in PCa.

Transcription factors. In addition to NR4A2, NGF treatment reduced the expression of the transcription factor p8 candidate of metastasis, that encodes a highly basic polypeptide of 80 aa, belonging to the HMG-I/Y proteins (Mallo et al., 1997), which are essential for the transcription of several genes. Several functions have been ascribed to p8. Intriguingly, it has been involved in cell cycle regulation, as either a proapoptotic factor (Carracedo et al., 2006) or a factor associated with tumor growth and progression (Mohammad et al., 2004). To account for such different functions, it has been proposed that the small size of the protein, lacking a specific structure, and its

cytoplasmic/nuclear localization allow interactions with different targets; however, it cannot be excluded that cell or tissue-specific factors may determine the role of p8 in the control of cell fate.

Another transcription factor whose expression is inhibited by NGF is HES1, that belongs to the family of helix-loop-helix transcription factors known as hairy/enhancer of split (HES) genes. HES is a downstream target of Notch signalling, which affects the development and function of many organs and it is also involved in cell proliferation and apoptosis (Artavanis-Tsakonas et al., 1999; Weng and Aster, 2004). Alteration of these functions in adults has been associated with various types of cancers, i.e. leukemia, breast cancer, hepatocellular carcinomas, in which Notch may act as an oncogene, although contrasting results have been reported (reviewed in Bolos et al., 2007). Interestingly, HES1 shows elevated expression in different clones of PC3 cell lines and promotes lateral motility (Scorey et al., 2006), indicating that our observation of NGF-mediated inhibition of HES1 gene expression may contribute to the reduction of invasiveness observed in NGF-treated DU145 and PC3 cell lines.

In conclusion, the identification of gene/protein alterations and markers of metastasis-promoting features at the time of diagnosis is a great challenge in PCa research, that may help to predict a negative or positive clinical outcome. Indeed, the PCa clinical behaviour, including disease progression and metastases, androgen sensitivity, response to chemotherapy/radiotherapy and time to relapse cannot still be predicted. Currently, there are no markers that differentiate clinically relevant from clinically benign disease; thus, better indicators of PCa presence and progression are needed to avoid unnecessary treatment, to predict disease course and eventually to develop more effective therapies. The data presented here, using the experimental model of NGF-induced reduction of malignancy of PCa cell lines, may help to identify gene/proteins that dispose to relapse and to assess future markers that could allow the development of new clinic diagnostic and therapeutical approaches, promoting an advantageous and patient-beneficial translational research.

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Table 1. Genes and primer oligonucleotide sequences used for the Q-RT-PCR

Genes	PCR product (bp)	Primer sequence (5' -3')
ARG2	130	TGTCGGGGGACTAACCTATC AGTCTTCGCCTCTTCCTCTG
HES1	111	CAACACGACACCGGATAAAC TTCAGCTGGCTCAGACTTTC
IGFBP3	178	TGCCGTAGAGAAATGGAAGA GAGGCTGCCCATACTTATCC
LAMP3	113	AAAAAACA CTGCAACCACCAG AAGCTAGGGCCGACTGTAAC
LCN2	159	ACCTCCGTCCTGTTTAGGAA AGCATGCTGGTTGTAGTTGG
NR4A2	175	AAGGAACCCAAGAGAGTGGA TCAGGTAGAAAATGCGCTGT
P8	138	CTCTATAGCCTGGCCCATTC CTCTGAATTCTGCAGCTTGG
P75^{NGFR}	105	TCCCTGTCTATTGCTCCATCC CTGTTGGCTCCTTGCTTGTTTC
PLEC1	150	CTTCTGCCACTCACGGATAA CGAGTTGATGATCTCCCAA
PTGES	102	GAACGACATGGAGACCATCTAC AGGAAGACCAGGAAGTGCAT
β ACTIN	149	TCTTCCAGCCTTCCTTCCTG CAATGCCAGGGTACATGGTG

Human specific primers were designed using the Primer3 software (<http://frodo.wi.mit.edu>) (Rosen et al., 2000).

Table 2. Genes modified by NGF treatment (50 ng/ml, 8d) in DU145 cells.

	C signal	T signal	T vs C fold change
Invasion and metastasis			
<u>NM_014398</u> <i>LAMP3, lysosomal-associated membrane protein 3</i>	591,1	114,2	-5,1
<u>NM_005574</u> LIM domain only 2 (rhombotin-like 1) (LMO2)	58,3	19,9	-2,9
<u>NM_001909</u> CTSD, cathepsin D (lysosomal aspartyl protease)	38,3	5,7	-6,7
<u>NM_001195</u> BFSP1, beaded filament structural protein 1	41,8	10,1	-4,1
<u>NM_002348</u> LY9, lymphocyte antigen 9	26,9	3,4	-7,9
<u>NM_000445</u> <i>PLEC1, plectin 1</i>	65,9	268,4	+ 4,0
<u>NM_002859</u> PXN, paxillin	27,3	110,7	+ 4,0
<u>NM_002318</u> <i>LOXL2, lysyl oxidase-like 2</i>	25,9	127,9	+ 4,9
<u>NM_173462</u> PAPLN papilin, proteoglycan-like sulfated glycoprotein	9,2	42,8	+ 4,6
<u>NM_015726</u> WDR42A WD repeat domain 42A	5,5	22,3	+ 4,5
Cell proliferation and apoptosis			
<u>NM_005564</u> <i>LCN2, lipocalin 2 (oncogene 24p3)</i>	648,3	115,1	-5,6
<u>NM_006732</u> FOSB	277,3	82,5	-3,3
<u>NM_058229</u> <i>FBXO32, F-box protein 32</i>	96,3	21,7	-4,4
<u>NM_000076</u> CDKN1C, cyclin-dependent kinase inhibitor 1C (p57, Kip2)	82,5	30,0	-2,9
<u>NM_004165</u> <i>RRAD, Ras-related associated with diabetes</i>	76,8	12,3	- 6,2

NM 000598 <i>IGFBP3, insulin-like growth factor binding protein 3</i>	218,9	767,1	+ 3,5
NM 032982 <i>CASP2, caspase 2</i>	17,9	64,7	+ 3,6
NM 174942 <i>GAS2L3, growth arrest-specific 2 like 3</i>	6,2	50,1	+ 8,1
NM 002612 <i>DLEU2, deleted in lymphocytic leukemia, 2</i>	4,5	10,2	+ 2,2
NM 005967 <i>NAB2, NGFI-A binding protein 2 (EGR1 binding protein 2)</i>	2,4	43,8	+18,2
Different aspects of cellular metabolism			
NM 004878 <i>PTGES, prostaglandin E synthase</i>	1251,5	317	-3,9
NM 001172 <i>ARG2, arginase, type II</i>	408,2	118,3	-3,4
NM 001035530 <i>SGNE1, secretory granule, neuroendocrine protein 1</i>	48,1	8,6	-5,5
NM 015692 <i>C3, complement component 3</i>	47,6	7,4	-6,4
NM 005410 <i>SEPP1, selenoprotein P 1</i>	37,2	9,5	-3,9
NM 017712 <i>PGPEP1 pyroglutamyl-peptidase I</i>	34,7	10,2	-3,4
NM 000663 <i>ABAT, 4-aminobutyrate aminotransferase</i>	31	8,6	-3,6
NM 002557 <i>Oviductin</i>	28,9	10,2	-2,8
NM 018964 <i>SLC37A1 Solute carrier family 37 (glycerol-3-phosphate transporter), member 1</i>	25,2	3,2	-7,8
NM 019080 <i>NDFIP2, Nedd4 family interacting protein 2</i>	23,6	6	-3,9
NM 020133 <i>AGPAT, 1-acylglycerol-3-phosphate O-acyltransferase 4</i>	20,3	2,7	-7,5
NM 001039049 <i>COX8C, cytochrome c oxidase subunit 8C</i>	16,6	1,6	-10,3
NM 002729 <i>MSTP9 macrophage stimulating, pseudogene 9</i>	15,8	2,1	-7,5
NM 004481 <i>GALNT2 UDP-N-acetyl-alpha-D-galactosamine:</i>	73,7	189,8	+ 2,6

polypeptide N-acetylgalactosaminyltransferase 2			
NM_001040147 SERPINB7, serine (or cysteine) proteinase inhibitor	7,9	40,6	+ 5,1
NM_004117 FKBP5, FK506 binding protein	2,8	20,6	+ 13,5
Transcription and translation, signal transduction			
NM_000997 RPL37, ribosomal protein L37	1099,9	339,8	-3,2
NM_012385 <i>p8 protein (candidate of metastasis 1)</i>	608,3	171,4	-3,5
NM_033285 TP53INP1, tumor protein p53 inducible nuclear protein 1	225,1	60,1	-3,7
NM_005524 <i>HES1, hairy and enhancer of split 1</i>	203,6	49,4	-4,1
NM_006186 <i>NR4A2, nuclear receptor subfamily 4, group A, member 2</i>	177,6	37,9	-4,6
NM_017644 DRE1 protein	99,2	27,4	-3,6
NM_172193 KLHDC1, kelch domain containing 1	65,4	19,1	-3,4
NM_198503 KCNT2, potassium channel, subfamily T, member 2	64,8	17,5	-3,7
NM_001001928 PPARA, peroxisome proliferative activated receptor, alpha	62	17,6	-3,5
NM_004433 ETS-related transcription factor (ERT)	61,1	26,4	-2,3
NM_004898 CLOCK, clock homolog	39,1	7,2	-5,4
NM_004915 ABCG1, ATP-binding cassette, sub-family G (WHITE), member 1	32,9	5,4	-6,0
NM_001012957 DISC1, disrupted in schizophrenia 1	28,7	8,4	-3,4
NM_145170 <u>TTC18, tetratricopeptide repeat domain 18</u>	19,1	5,1	-3,7

<u>NM_013304</u> ZDHC1, Zinc finger DHHC-type containing 1	19	4,8	-3,9
<u>NM_018328</u> MBD5, methyl-CpG binding domain protein 5	17,5	3,4	-5,1
<u>NM_006295</u> VARS, valyl-tRNA synthetase	25,7	99,5	+ 3,9
<u>NM_003200</u> TCF3, transcription factor 3	12,8	53,7	+ 4,2
<u>NM_031946</u> CENTG3 centaurin, gamma 3	12	62,3	+ 5,1
<u>NM_015908</u> ARS2 arsenate resistance protein	10,4	57,2	+ 5,5
<u>NM_007118</u> TRIO, triple functional domain	10,3	34,6	+ 3,3
<u>NM_001032283</u> TMPO, thymopoietin	5,4	25,6	+ 4,7
<u>NM_014264</u> PLK4, polo-like kinase 4	4,6	19,2	+ 4,1
<u>NM_002383</u> MAZ, MYC-associated zinc finger protein	3,9	29,1	+ 7,4
<u>NM_006521</u> TFE3, transcription factor binding to IGHM enhancer 3	3,3	17,2	+ 5,2
<u>NM_153034</u> ZFN488, zinc finger protein	3,3	24,2	+ 7,3
<u>NM_006293</u> TYRO3, TYRO3 protein tyrosine kinase	3,2	17,7	+ 5,5
<u>NM_015033</u> FBNP1, formin binding protein 1	3	18,9	+ 6,3
<u>NM_052970</u> HSPA12B heat shock protein 12B	3	29,2	+ 9,7
<u>NM_145185</u> MAP2K7, mitogen-activated protein kinase kinase 7	2,6	26,5	+ 10,1
<u>NM_005967</u> NAB2, NGFI-A binding protein 2	2,4	43,8	+ 18,1
<u>NM_016340</u> RAPGEF6, Rap guanine nucleotide exchange factor (GEF) 6	2	9,7	+ 4,8
Miscellaneous			
<u>NM_001033047</u> NPNT nephronectin	77,6	17,4	-4,4
<u>NM_080546</u> CDW92, member of the choline transporter-like	46,7	13,9	-3,4

protein family			
<u>NM 138796</u> SPATA17 spermatogenesis associated 17	26,6	4,8	-5,5
<u>NM 002260</u> KLRC2, killer cell lectin-like receptor subfamily C, member 2	25,1	3,8	-6,6
<u>NM 017541</u> CRYGS, crystallin gamma S	20	3	-6,6
<u>NM 013433</u> TNPO2, transportin 2	2,8	12,9	+ 4,4
<u>NM 014964</u> EPN, epsin 2	1,8	14	+ 7,7
<u>NM 015120.4</u> ALM1 Alstrom syndrome 1	5,7	17,1	+ 3,0

C: untreated (control) cells; T: NGF-treated cells. In italic, genes studied in the Q- RT-PCR amplification

Fig. 1. Effects of NGF treatment on DU145 and PC3 PCa cell lines.

A. Cell proliferation rate. DU145 cells were exposed to 50 ng/ml NGF for 8 days, PC3 were exposed to NGF for 14 days. Cell count was conducted as described in Materials and Methods. Results are the mean $\pm$ SEM of six different dishes for each cell culture.

* $p < 0.001$ vs untreated cells.

B: Q-RT-PCR detection of p75^{NGFR} mRNA. One μ g of total RNA from untreated and NGF-treated cells was transcribed in cDNA as described in Materials and Methods. Amplification and detection using the SYBR Green I were performed with the iCYCLER iQ Real Time PCR Detection System. Gene expression levels were normalized by β -actin and data were analyzed according to the comparative Ct method, as described in Materials and Methods.

C: Immunocytochemistry. Untreated and NGF-treated DU145 and PC3 cells were plated on polylysine-treated coverslips and processed as described. Cells were fixed for 5 min at -20°C in cold methanol, permeabilized and incubated with a 1:1000 dilution of the goat polyclonal antibody (0,2 μ g/ml) raised against the epitope mapping at the C-terminus of human p75^{NGFR}, as described in Materials and Methods. After extensive washes, the appropriate biotinylated secondary antibody was applied and cells were treated according to the supplier's instructions of the ABC kit. Magnification: 40x. **a:** normal goat serum (blank), **b:** untreated DU145 cells, **c:** NGF-treated DU145 cells; **d:** untreated PC3 cells; **e:** NGF-treated PC3 cells, **f:** PC12 cells (positive control).

Fig. 2. Q-RT-PCR amplification from different amounts of cDNA input from selected genes showed high linearity.

Fig. 3. Comparison of difference in gene expression of selected genes in NGF-treated DU145 vs untreated DU145 cells.

One μg of total RNA from untreated and NGF-treated DU145 (50 ng/ml, 8 days) was transcribed in cDNA as described in Materials and Methods. Amplification and detection using the SYBR Green I were performed with the iCYCLER iQ Real Time PCR Detection System. Gene expression levels were normalized by β -actin and data were analyzed according to the comparative Ct method.

Fig. 4. Comparison of difference in gene expression of selected genes in NGF-treated PC3 vs untreated PC3 cells.

One μg of total RNA from untreated and NGF-treated PC3 cells (100 ng/ml, 14 days) was transcribed in cDNA as described in Materials and Methods. Amplification and detection using the SYBR Green I were performed with the iCYCLER iQ Real Time PCR Detection System. Gene expression levels were normalized by β -actin and data were analyzed according to the comparative Ct method.