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Jessy Alonzeau, David Alexandre, Lydie Jeandel, Maïté Courel, Coralie Hautot, et al.. The neuropeptide 26RFa is expressed in human prostate cancer and stimulates the neuroendocrine differentiation and the migration of androgeno-independent prostate cancer cells. *European Journal of Cancer*, 2013, 49 (2), pp.511-519. 10.1016/j.ejca.2012.05.028 . hal-01706422

HAL Id: hal-01706422

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Submitted on 19 Jul 2018

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The neuropeptide 26RFa is expressed in human prostate cancer and stimulates the neuroendocrine differentiation and the migration of androgeno-independent prostate cancer cells

Jessy Alonzeau^a, David Alexandre^a, Lydie Jeandel^a, Maïté Courel^a, Coralie Hautot^a, Fatima-Zohra El Yamani^b, Françoise Gobet^c, Jérôme Leprince^a, Rabia Magoul^b, Afaf Amarti^d, Christian Pfister^e, Laurent Yon^a, Youssef Anouar^a, Nicolas Chartrel^{a,*}

^a *INSERM U982, Laboratory of Neuronal and Neuroendocrine Differentiation and Communication, Biomedical Research and Innovation Institute (IRIB), University of Rouen, 76821 Mont-Saint-Aignan, France*

^b *Laboratory of Neuroendocrinology and Nutritional and Climatic Environment, University Sidi Mohamed Ben Abdellah, Fes, Morocco*

^c *Department of Pathology, University Hospital of Rouen, Institute for Biomedical Research, University of Rouen, Rouen, France*

^d *Department of Pathology, University Hospital Hassan II, Fes, Morocco*

^e *Department of Urology, University Hospital of Rouen, Institute for Biomedical Research, University of Rouen, Rouen, France*

* *Corresponding author: Tel.: +33 235 14 6731; fax: +33 235 14 6946.*

E-mail address: nicolas.chartrel@univ-rouen.fr (N. Chartrel).

1. Introduction

Because androgens stimulate tumoural growth, androgen ablation therapy represents at present the main treatment of advanced prostate cancer (CaP) and is initially effective in slowing down the progression of the disease. But, frequently, CaP recurs as an androgen-insensitive (AI) tumour with an associated life expectancy of only 15–20 months.¹ Prostate tumour cell populations have been reported to be enriched in neuroendocrine cells after a long term anti-androgen therapy.^{2–4} The presence of neuroendocrine cells in CaP points them as potential effectors of paracrine stimulation for the surrounding carcinomatous cells in the absence of androgen stimulation. Indeed, one of the main features of neuroendocrine cells is to secrete neuropeptides, some of which have been reported to influence growth, invasiveness, metastatic processes and angiogenesis in various cancers.^{5–7} Identification of neuropeptides produced in prostate tumours and elucidation of their role in the development of androgen independence may therefore provide clues for improving therapy.

26RFa (TSGPLGNLAEELNGYSRKKGGFSFRF-NH₂) belongs to the RFamide peptide family that includes all the neuropeptides sharing the Arg-Phe-NH₂ motif at their C-terminus. 26RFa is the latest neuropeptide identified in human^{8,9} and the neuropeptide has been identified as the endogenous ligand of the human orphan receptor, GPR103.^{10–12} 26RFa has been shown to be involved mainly in the control of feeding behaviour^{9,10,13,14} and gonadotropic axis function,^{15,16} but also in the regulation of bone formation.¹⁷ mRNA quantification performed on human tissues revealed that 26RFa is appreciably expressed in the prostate¹² and, immunocytochemical data reported on the website ‘Human Protein Atlas’ (www.proteinatlas.org/) indicate that GPR103 is present in glandular cells of the normal prostate as well as in cancer cells of prostate tumours at various stages. Altogether, these observations prompted us to investigate a possible involvement of 26RFa in prostate tumourigenesis.

2. Materials and methods

2.1. Immunohistochemical procedure

Deparaffinised sections (15- μ m thick) from five benign prostate hyperplasia (BPH) and 15 prostate tumours at various stages (Gleason scores: 3 + 3, 4 + 3 and 4 + 5) were used for the immunocytochemical study. All the tissue procurement protocols were approved by the relevant institutional committees (University of Rouen and University of Fez) and were undertaken under informed consent of each patient and all of the participants.

CaP sections were incubated for 1 h at room temperature with rabbit antibodies against 26RFa⁸ diluted

1:600, or GPR103 (#NLS1922; Novus Biologicals, Littleton, CO) diluted 1:100, or EM66¹⁸ diluted 1:600. The sections were incubated with a streptavidin–biotin-peroxydase complex (Dako Corporation, Carpinteria, CA), and the enzymatic activity was revealed with diaminobenzidine. The slices were then counterstained with hematoxylin. Observations were made under a Nikon E 600 light microscope.

The specificity of the immunoreactions was controlled by (1) substitution of the primary antibodies with Tris buffer saline (TBS; pH 7.4) and (2) preincubation of the 26RFa antiserum (diluted 1:600) with synthetic human 26RFa (10⁻⁶ M) (PRIMACEN platform, Mont-Saint-Aignan, France), or preincubation of the EM66 antiserum (diluted 1:600) with recombinant human EM66 (10⁻⁶ M) (INSERM U982, Mont-Saint-Aignan, France). Photomicrographs showing the specificity of the immunoreactions are available in the [Supplemental material \(Fig. 6\)](#).

26RFa- or GPR103-immunoreactive cells present in the sections were quantified. For this, five independent fields of 20 distinct sections of benign prostate hyperplasia (BPH) or CaP were photographed at a 20 \times magnification. The number of immunostained cells present in each image was evaluated by using the cell counter plugin of the image analysis software Image J and expressed as a percentage of the total number of cells present on the photomicrograph.

2.2. Culture of prostate cancer cell lines

Three human-derived CaP cell lines were used, i.e. the androgen-responsive cell line LNCaP, and the androgen-unresponsive cell lines DU145 and PC3. The three cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD). The LNCaP cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin and 1% glutamine. The DU145 and PC3 cells were maintained in Dulbecco modified Eagle’s minimal essential medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were grown at 37 °C in a humidified 95% air/5% CO₂ atmosphere and the culture medium was replaced every 3 days. When the cells reached 70–80% confluence, they were washed with phosphate buffer saline (PBS; pH 7.4), harvested by a brief incubation with 0.25% trypsin-EDTA solution, and seeded as suggested by ATCC.

2.3. RNA extraction, reverse transcription and quantitative polymerase chain reaction (PCR)

Total RNA from LNCaP, DU145 and PC3 cells was extracted with the Tri-reagent (Sigma–Aldrich, Lyon, France), purified by using a Nucleospin kit

(Macherey–Nagel, Hoerdt, France), and quantified with a Nanodrop spectrometer (Nanodrop Technologies, Wilmington, DE). Contaminating genomic DNA was removed by treatment with deoxyribonuclease I, and cDNAs were synthesised from 1 to 5 µg RNA using the ImProm II Reverse Transcriptase (Promega Corp., Madison, WI).

Quantitative PCR was performed by using the 7900 HT Fast Real-time PCR System and Taqman Gene Express-

sion Master Mix 2× assay (Applied Biosystems, Courtaboeuf, France). The TaqMan fluorogenic probes and the Assay-On-Demand (Applied Biosystems) used in this study were as follows: GRP103, Hs01014830_m1; Chromogranin A (CgA), Hs00154441_m1; Secretogranin II (SgII), Hs00185761_m1, Neuron Specific Enolase (NSE), Hs00157360_m1. 26RFa and hypoxanthine ribosyltransferase (HPRT1) primers were designed by using Primer Express software version 3.0: HPRT1 forward primer 5'-

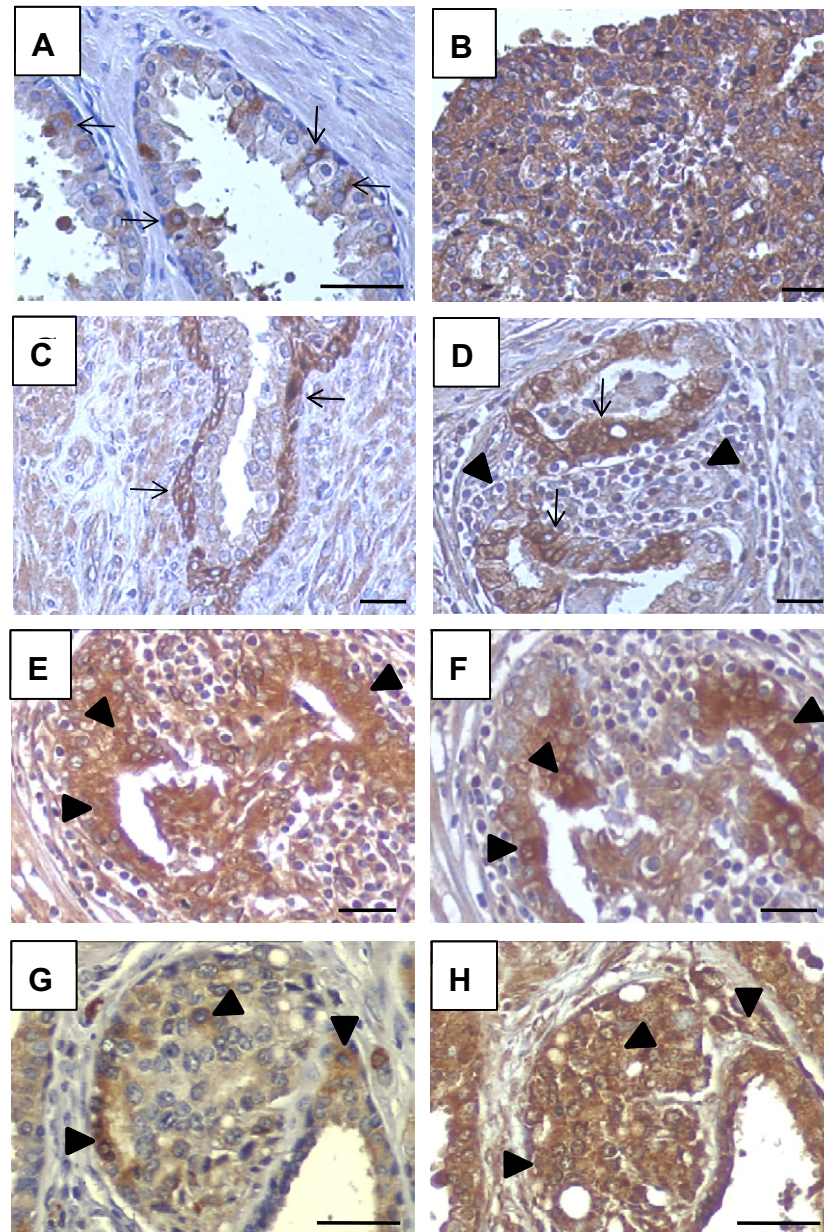


Fig. 1. Representative microscopic fields of prostate tumours at various grades comparing the immunocytochemical distribution of 26RFa, GPR103 and EM66 in consecutive sections. (A) Photomicrograph showing the presence of scattered 26RFa-stained epithelial cells (arrows) in some acini of benign prostate hyperplasia (BPH) sections. (B) Adenocarcinomatous structure in a Gleason's score (4 + 5) prostate cancer (CaP) showing that a large majority of the cancerous cells are intensely labelled with 26RFa antibodies. (C) Photomicrograph of a BPH section showing that GPR103-Li is confined to the basal cells of a few acini (arrows). (D) Photomicrograph of a Gleason's score (4 + 3) CaP showing cancerous cells invading an acinar lumen. Some of these cells display strong GPR103-Li (arrows) whereas others are totally unstained (arrowheads). (E and F) Consecutive sections of a Gleason's score (4 + 3) CaP showing that 26RFa (E) and GPR103 (F) antibodies label the same adenocarcinomatous structure (arrowheads). (G and H) Consecutive sections of a Gleason's score (4 + 3) CaP showing that GPR103 (G) and EM66 (H) antibodies label the same adenocarcinomatous structure (arrowheads). Scale bars: 50 µm.

acacaccacac-3'; HPRT1 reverse primer 5'-acacaccacac-3'; 26RFa forward primer 5'-acacaccacac-3'; 26RFa reverse primer 5'-acacaccacac-3'. The purity of the PCR products was assessed by dissociation curves. The amount of target cDNA was calculated by the comparative threshold (Ct) method and expressed by means of the 2-Ct method according to Applied Biosystems instructions using HPRT1 as an internal control.

2.4. Neuroendocrine differentiation of prostate cancer cell lines

DU145 cells (5×10^4 cells/well) were starved 24 h prior to experiments. Neuroendocrine differentiation of the cells was carried out by adding to the culture medium dibutyl-cyclic adenosine monophosphate (db-cAMP; 1 mM)/3-isobutyl-1-methylxanthine (IBMX; 0.1 mM), interleukin 6 (IL-6; 0.1 $\mu\text{g/ml}$) or 26RFa (10^{-6} M). Medium was changed every day until day 4.

2.5. Wound healing assay

DU145 cells (10^6 cells/well) were starved 24 h before the wound healing assay. Then, the wells were scratched with a thin tip to generate a wound in the cell's monolayer, and they were incubated for 24 h with 26RFa (10^{-6} M) or mitomycin C (1 $\mu\text{g/ml}$). During the experiment, for each well, six independent fields were photographed every 6 h with a Leica video microscope (Nanterre, France) at a $20\times$ magnification. Data were analysed using the Metamorph Acquisition and Analysis software (Molecular Device, Sunnyvale, CA).

2.6. Cell proliferation assay

DU145 cells (10^5 cells/well) were starved for 6 h. Cells were then incubated with 26RFa (10^{-6} M) or mitomycin C (1 $\mu\text{g/ml}$) for 3 days and counted with a Neubauer haemocytometer.

2.7. Evaluation of cell morphological changes

DU145 cells (5×10^4 cells/well) were starved for 24 h prior to experiments. Cells were then incubated in the presence of db-cAMP (1 mM)/IBMX (0.1 mM) or IL-6 (0.1 $\mu\text{g/ml}$) or 26RFa (10^{-6} M) for 4 days. The number of neurite-like extensions and their length were determined under a Leica video microscope, in six distinct viewing fields, at a $20\times$ magnification, by using the Metamorph Acquisition and Analysis software.

2.8. Statistical analysis

All of the experiments were performed in triplicate and repeated at least three times. Results are expressed

as mean \pm SEM. All statistical analyses were performed with GraphPad Prism 4 data analysis software. The Mann-Whitney *U* test was used for comparison of the mean values between two groups. Differences were considered statistically significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

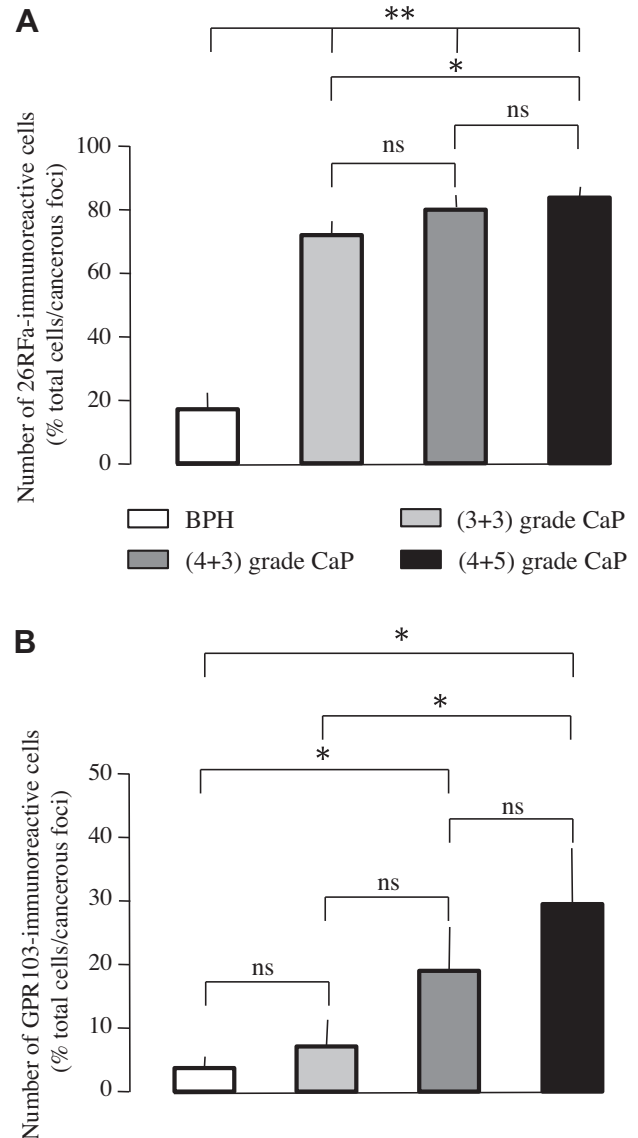


Fig. 2. Quantification of 26RFa- and GPR103-immunoreactive cells in benign prostate hyperplasia and prostate tumour sections at various stages. (A) The number of 26RFa-immunoreactive cells is significantly higher in the prostate cancer (CaP) sections as compared to the benign prostate hyperplasia (BPH) sections whatever the grade of the cancer. In addition, the percentage of cancer cells labelled with the 26RFa antibodies increases with the grade of the CaP. (B) The number of GPR103-positive cells is significantly higher in the medium and high grade CaP sections as compared to the BPH sections. The percentage of GPR103-containing cancer cells tends to increase with the grade of the CaP. Values are mean \pm SEM of five determinations performed on 20 distinct sections of BPH and CaP at various stages. Data were analysed by using the Mann-Whitney *U* test. * $p < 0.05$ and ** $p < 0.01$. ns, Not significant.

3. Results

3.1. Immunohistochemical distribution of 26RFa and GPR103 in BPH and CaP

The distribution and localisation of 26RFa and its receptor, GPR103, have been investigated on sections of BPH and CaP at various stages (Gleason's score: 3 + 3 = low grade, 4 + 3 = medium grade and 4 + 5 = high grade). In BPH, 26RFa-like immunoreactivity (Li) was confined to scattered epithelial cells observed just in a few acini (Fig. 1A). Fig. 1B shows the typical aspect of a high grade CaP with large adenocarcinomatous formations heavily labelled with 26RFa antibodies. In BPH, GPR103-Li was restricted to the basal cells of some acini and was never detected in the epithelial cells (Fig. 1C). In medium grade CaP, a significant proportion of malignant cells invading the acinar lumens were stained with the GPR103 antibodies (Fig. 1D). Treatment of consecutive CaP sections with either 26RFa antibodies or GPR103 antibodies revealed that the neuropeptide and its receptor were present in the same adenocarcinomatous formations (Fig. 1E, F). Treatment of consecutive sections with either 26RFa or GPR103 antibodies, and antibodies directed against a marker of neuroendocrine differentiation, the peptide EM66, revealed that 26RFa, GPR103 and EM66 antibodies labelled the same carcinomatous formations (Fig. 1G, H).

Quantification of immunoreactive cells revealed that the number of 26RFa positive cells was higher than that of cells exhibiting GPR103-Li in BPH or in CaP slices whatever the grade of the tumour (Fig. 2). The number

of 26RFa-Li cells present in the CaP sections was significantly higher ($p < 0.01$) compared to BPH sections (Fig. 2A), and the percentage of 26RFa-positive cancer cells increases with the severity of the CaP (Fig. 2A). The number of cells labelled with the GPR103 antibodies was significantly higher ($p < 0.05$) in the medium and high grade CaP sections as compared to the low grade CaP and BPH sections (Fig. 2B). The percentage of GPR103-stained malignant cells tends to increase with the severity of the CaP (Fig. 2B).

3.2. Expression of 26RFa and GPR103 mRNAs in prostate cancer cell lines

Quantitative real-time PCR (RT-PCR) performed in three distinct prostate cancer cell lines, i.e. the androgeno-dependent (AD) LNCaP, and the AI DU145 and the PC3 cells revealed that the 26RFa gene was expressed in the three cell lines (Fig. 3A) whereas the GPR103 transcript was only detected in the DU145 cells (Fig. 3B).

3.3. Effects of 26RFa on the proliferation/migration of DU145 cells

26RFa (10^{-6} M) induced a highly significant reduction of the wound's width in native DU145 cells ($p < 0.001$) as compared to untreated cells (Fig. 4A). The stimulatory effect of 26RFa was less pronounced when the DU145 cells were initially submitted to a neuroendocrine differentiation (Fig. 4B). Mitomycin C (1 μ g/ml, an inhibitor of DNA synthesis that totally blocks the proliferation of cancer cells) did not affect

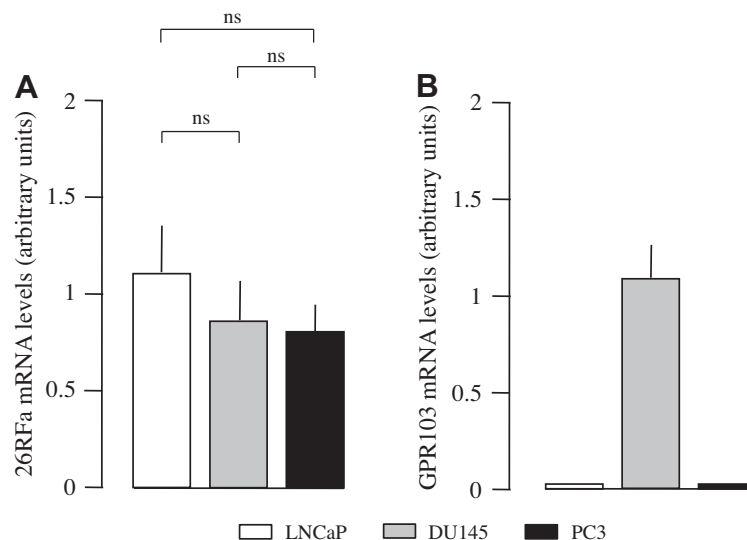


Fig. 3. Expression of 26RFa and GPR103 mRNAs in prostate cancer cell lines. 26RFa and GPR103 mRNA levels were determined by quantitative polymerase chain reaction (PCR) and adjusted to the signal intensity of hypoxanthine ribosyltransferase (HPRT1) in three distinct prostate cancer cell lines, i.e. LNCaP cells which are androgeno-dependent, and DU145 and PC3 cells which are androgeno-independent. (A) The 26RFa gene is expressed in the three cell lines at similar levels. (B) In contrast, the GPR103 gene is only detected in the androgeno-independent DU145 cell line. Values are mean \pm SEM of four independent experiments for each cell line. Data were analysed by using the Mann-Whitney U test. ns, Not significant.

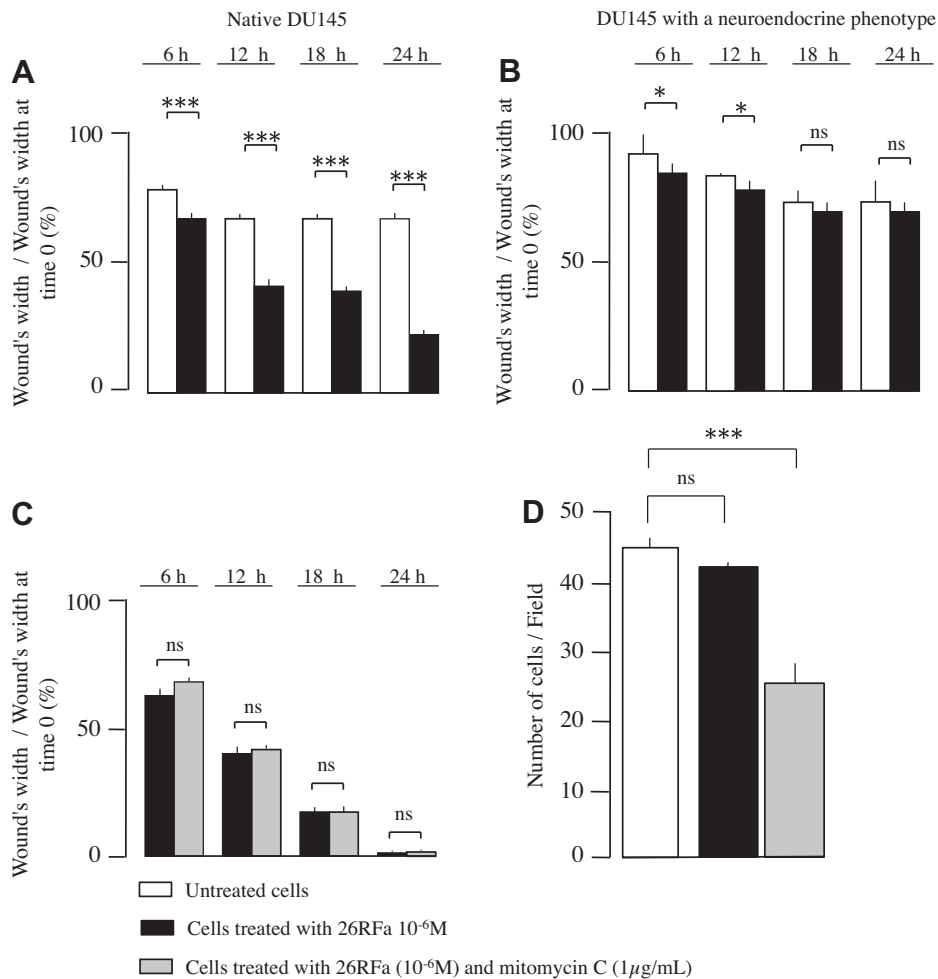


Fig. 4. Effect of 26RFa on the migration and proliferation of the DU145 prostate cancer cells. Ability of 26RFa (10^{-6} M) to induce proliferation/migration of the androgeno-independent DU145 cells, submitted or not to a neuroendocrine differentiation (by addition of dituryl-cyclic adenosine monophosphate (db-cAMP) (1 mM)/3-isobutyl-1-methylxanthine (IBMX) (0.1 mM), was assessed by using a wound healing assay and a cell proliferation assay with a Neubauer haemocytometer. (A and B) 26RFa induces a marked increase in the proliferation/migration rate of native DU145 cells during the 24 h following the treatment (A). In DU145 cells exhibiting a neuroendocrine phenotype, the stimulatory effect of the neuropeptide is less pronounced and is only observed during the first 12 h following the treatment (B). (C) The stimulatory effect of 26RFa on the reduction of the wound's width in the wound healing assay is not altered by addition of the proliferation blocker mitomycin C (1 μ g/ml) in the culture medium for 24 h. (D) After a 3-day incubation, 26RFa does not modify the proliferation rate of DU145 cells whereas mitomycin C, in the same conditions, significantly inhibits cell proliferation. Values are mean \pm SEM of nine determinations performed in three independent experiments. Data were analysed by using the Mann-Whitney *U* test. * $p < 0.05$ and *** $p < 0.001$. ns, Not significant.

the speed of reduction of the wound's width induced by 26RFa (Fig. 4C), suggesting that the neuropeptide promoted the migration of the DU145 cells but not their proliferation. To confirm this hypothesis, DU145 cells were incubated with 26RFa (10^{-6} M) or mitomycin C (1 μ g/ml) for 3 days, and cells were counted. 26RFa did not alter the number of the DU145 cells whereas mitomycin C, in the same conditions, significantly inhibited ($p < 0.001$) cell proliferation (Fig. 4D).

3.4. Effect of 26RFa on the neuroendocrine differentiation of DU145 cells

Ability of 26RFa (10^{-6} M) to induce a neuroendocrine differentiation of the DU145 cells was assessed by quantifying the expression of three markers of neuro-

endocrine differentiation, i.e. CgA, SgII and NSE, and by evaluating the morphological changes of the cells, i.e. the presence of neurite-like extensions and their length. As positive controls, DU145 cells were treated with db-cAMP (1 mM)/IBMX (0.1 mM) or IL-6 (0.1 μ g/ml) which are known to induce a neuroendocrine phenotype in various prostate cancer cell lines.¹⁹ Treatment of the DU145 cells with 26RFa for 4 days significantly stimulated the expression of CgA (Fig. 5A) but had no effect on SgII or NSE transcript levels (Fig. 5B, C). Incubation of the cells with db-cAMP/IBMX induced a significant increase in the expression of the three neuroendocrine markers (Fig. 5A–C) whereas, in the same conditions, IL-6 only stimulated CgA mRNA levels (Fig. 5A). 26RFa as well as db-cAMP/IBMX and IL-6 significantly increased the num-

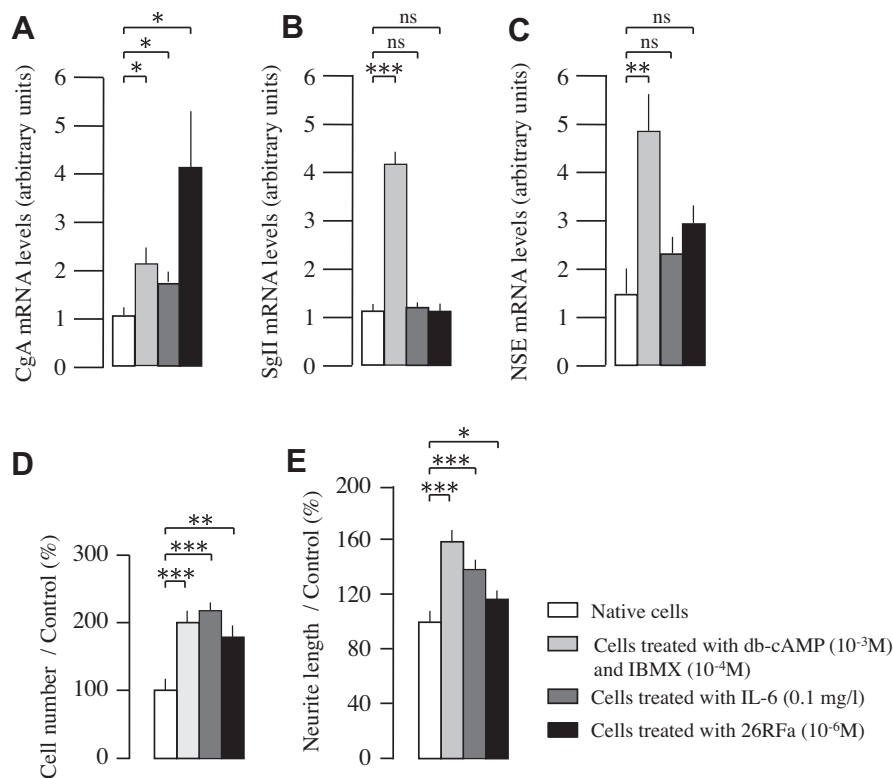


Fig. 5. Effect of 26RFa on the neuroendocrine differentiation of the DU145 prostate cancer cells. Ability of 26RFa (10^{-6} M) to induce a neuroendocrine differentiation of DU145 cells was assessed by quantifying the expression of three markers of neuroendocrine differentiation, i.e. chromogranin A (CgA), secretogranin II (SgII) and neuron specific enolase (NSE), and by an evaluation of the morphological changes of the cells, i.e. the presence of neurite-like extensions and the length of the neurites. As positive controls, DU145 cells were treated with ditiryl-cyclic adenosine monophosphate (db-cAMP) (1 mM)/3-isobutyl-1-methylxanthine (IBMX) (0.1 mM) or interleukin-6 (IL-6; 0.1 μ g/ml). (A) CgA mRNA expression is increased significantly by a 4-day treatment with 26RFa or db-cAMP/IBMX or IL-6. (B) SgII mRNA expression is not modified by addition of 26RFa or IL-6 in the culture medium. In contrast, db-cAMP/IBMX induces a significant increase in SgII mRNA levels after a 4-day treatment. (C) NSE mRNA expression is not altered by addition of 26RFa or IL-6 in the culture medium for 4 days. In contrast, db-cAMP/IBMX induces a significant increase in NSE transcript levels. (D and E) Addition of 26RFa, db-cAMP/IBMX and IL-6 in the culture medium for 4 days induces a significant increase in the number of DU145 cells exhibiting neurite-like extensions (D) and of the length of these neurites (E). The stimulating effects of 26RFa and db-cAMP/IBMX on the number and length of the neurite-like extensions are more pronounced than those of IL-6 (D and E). Values are mean \pm SEM of nine determinations performed in three independent experiments. Data were analysed by using the Mann–Whitney *U* test. * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$. ns, Not significant.

ber of cells exhibiting neurite-like extensions and the length of the neurites (Fig. 5D, E).

4. Discussion

This is the first report investigating the presence and function in CaP of the human neuropeptide, 26RFa.^{9,10} Immunohistochemical experiments indicate that 26RFa and its receptor, GPR103, are present in carcinomatous foci of CaP sections. Consistent with this observation, our *in vitro* data revealed that three distinct CaP cell lines express the neuropeptide and one AI CaP cell line expresses its receptor. In addition, we show that the number of 26RFa- and GPR103-stained cells increases with the grade of the cancer, suggesting that 26RFa is produced at a high rate in cancerous cells. Supporting this view, it is noteworthy that 26RFa is an α -amidated neuropeptide and that the expression of peptidyl-glycine

alpha-amidating monooxygenase, the enzyme responsible for the amidation of neuropeptides is overexpressed in high Gleason's score CaPs.²⁰ We also found that 26RFa and GPR103 are present in the same carcinomatous formations, indicating that 26RFa may act on cancer cells via an autocrine/paracrine mechanism. In addition, 26RFa and GPR103 are exclusively present in cancerous structures labelled with EM66, a fragment of SgII which has been previously identified as a marker of neuroendocrine differentiation,¹⁸ suggesting therefore that the expression of the neuropeptide and its receptor is closely associated with the acquisition of a neuroendocrine phenotype by CaP cells.

Accumulating evidence indicates a correlation between the number of CaP cells with a neuroendocrine phenotype and the transition of CaP to an androgen-refractory status, associated with the severity of the cancer,^{4,7} suggesting that the neuroendocrine differenti-

ation and the neuropeptides produced by neuroendocrine cells play a crucial role in the progression as well as the dissemination of the disease. Indeed, several peptides have been shown to affect CaP cells. For instance, bombesin and endothelin-1 have been shown to promote the migration and invasion of CaP cells.²¹ Neuropeptide Y stimulates the proliferation of the AI prostate cancer cell line PC3 via activation of the Y1 receptor subtype.²² Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide affect proliferation and neuroendocrine differentiation of LNCaP cells.^{23–25} Adrenomedullin stimulates the proliferation and prevents apoptosis of AI prostate cancer cells,^{20,26} and induces a neuroendocrine phenotype in the AD prostate tumour cells LNCaP.²⁷ Finally, oxytocin has been shown to promote the migration of CaP cells.²⁸ Here, we show that 26RFa stimulates the migration of native or transdifferentiated DU145 cells and is capable of inducing a neuroendocrine phenotype in these cells, as assessed by the occurrence of morphological changes (neurite-like extensions) and the overexpression of the neuroendocrine marker CgA. Interestingly, in the same set of experiments, db-cAMP and IL-6, used as positive controls,^{19,29,30} affected differently the expression of the three neuroendocrine markers. IL-6, like 26RFa, stimulates only the expression of CgA whereas db-cAMP/IBMX increases the mRNA levels of CgA, SgII and NSE. This observation suggests that db-cAMP/IBMX, 26RFa and IL-6 induce the neuroendocrine differentiation of the DU145 cells by activating distinct intracellular signalling pathways. Indeed, db-cAMP/IBMX activates the cAMP pathway which has long been recognised to promote neuroendocrine differentiation, whereas IL-6 stimulates Stat3, tyrosine kinase Etk/Bmx and P13K in order to induce neuroendocrine differentiation^{19,29–31} (see Fig. 7 in supplemental material). The discrepancy between the effects of 26RFa and db-cAMP/IBMX on the expression of the three neuroendocrine markers suggest that 26RFa does not promote the transdifferentiation of the DU145 cells via activation of the cAMP pathway. In support of this view, we found that 26RFa was unable to stimulate cAMP production in the DU145 cells whereas forskolin strongly activated this intracellular pathway (data not shown). Whether 26RFa requires the mobilisation of Stat3, like IL-6, to induce the neuroendocrine differentiation of the DU145 cells deserves further investigation (see Fig. 7 in supplemental material).

In conclusion, the present report provides the first evidence that the neuropeptide 26RFa is present in CaP exhibiting a neuroendocrine differentiation. Our data support the view that 26RFa may participate to the development of CaP at the AI state by promoting the neuroendocrine differentiation and migration of cancer cells via autocrine/paracrine mechanisms.

Conflict of interest statement

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

Acknowledgements

This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM U982), the University of Rouen, the LARC-Neuroscience network, the Association pour la Recherche sur les Tumeurs Prostatiques (ARTP), the Plateforme Régionale de Recherche en Imagerie Cellulaire de Haute-Normandie (PRIMACEN) and the Conseil Régional de Haute-Normandie.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ejca.2012.05.028>.

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