

## Protein Profiling of Microdissected Prostate Tissue Links Growth Differentiation Factor 15 to Prostate Carcinogenesis

Peter K. Cheung,<sup>1</sup> Bruce Woolcock,<sup>1</sup> Hans Adomat,<sup>1</sup> Margaret Sutcliffe,<sup>1</sup> Terry C. Bainbridge,<sup>2</sup> Edward C. Jones,<sup>4</sup> Douglas Webber,<sup>5</sup> Thomas Kinahan,<sup>4</sup> Marianne Sadar,<sup>1</sup> Martin E. Gleave,<sup>3,4</sup> and Juergen Vielkind<sup>1,6</sup>

Departments of <sup>1</sup>Cancer Endocrinology and <sup>2</sup>Pathology and Laboratory Medicine, British Columbia Cancer Agency, Vancouver; <sup>3</sup>The Prostate Centre, Vancouver General Hospital, Vancouver; <sup>4</sup>Department of Surgery, University of British Columbia, Vancouver; <sup>5</sup>Pathology, Kelowna General Hospital, Kelowna; and <sup>6</sup>Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, British Columbia, Canada

### Abstract

Identification of proteomic alterations associated with early stages in the development of prostate cancer may facilitate understanding of progression of this highly variable disease. Matched normal, high-grade prostatic intraepithelial neoplasia (hPIN) and prostate cancer cells of predominantly Gleason grade 3 were procured by laser capture microdissection from serial sections obtained from snap-frozen samples dissected from 22 radical prostatectomy specimens. From these cells, protein profiles were generated by surface-enhanced laser desorption/ionization-time of flight mass spectrometry. A 24-kDa peak was observed at low or high intensity in profiles of prostate cancer cells in 19 of 27 lesions and at low intensity in 3 of 8 hPIN lesions but was not detectable in matched normal cells. SDS-PAGE analysis of prostate cancer and matched normal epithelium confirmed expression of a prostate cancer-specific 24-kDa protein. Mass spectrometry and protein data-based analysis identified the protein as the dimeric form of mature growth differentiation factor 15 (GDF15). The increased expression of mature GDF15 protein in prostate cancer cells cannot be explained on the basis of up-regulation of GDF15 mRNA because reverse transcription-PCR analysis showed similar amounts of transcript in normal, hPIN, and prostate cancer cells that were obtained by laser capture microdissection in the same set of serial sections from which the protein profiles were obtained. Our findings suggest that early prostate carcinogenesis is associated with expression of mature GDF15 protein.

### Introduction

Cancer of the prostate is currently the most common cancer and the second leading cause of cancer deaths in men in the United States. Major contributors to current mortality and morbidity statistics are the lack of procedures to detect cancer at an early stage and lack of specific treatment strategies. A better understanding of the early developmental stages of prostate cancer at the cellular and molecular levels is therefore of critical importance.

Molecular studies of prostate cancer have been hampered by our limited ability to effectively sample fresh precancerous and cancerous tissue of the prostate gland, and the acquired samples often do not contain the cells of interest. Also, the early stages are generally comprised of a low number of cells and are embedded in a heterogeneous tissue. This has also hindered the study of molecular mechanisms that direct cells into the cancerous pathway, but pure cell populations can now be isolated with the advent of laser capture

microdissection (LCM), and sensitive molecular techniques accommodating the limited number of cells in the early cancer stages have become available.

Altered protein expression is one component that underlies neoplastic changes. To detect these alterations, we have undertaken comparative analyses of proteins in matched normal, high-grade prostatic intraepithelial neoplasia (hPIN), and early cancer epithelial cells procured by LCM. To accommodate the low number of cells, we have used surface enhanced laser desorption/ionization (SELDI), a technology that can detect proteins in the picomole to attomole range. It uses affinity surfaces and time of flight-mass spectrometry to resolve proteins based on their physical or chemical characteristics (1). Our studies revealed a unique 24-kDa protein peak in SELDI protein profiles from cancer cells of Gleason grade 2 to 4 (in 70% of the cases) and hPIN (in 33% of the cases), but it was not detected in profiles from normal epithelial cells. The 24-kDa protein was identified as the dimeric form of mature growth differentiation factor 15 (GDF15), a distant member of the transforming growth factor (TGF)- $\beta$  superfamily with a role in cell growth and differentiation (2, 3).

### Materials and Methods

**Acquisition of Patient Tissue.** Prostate specimens were obtained from 42 patients who were diagnosed with adenocarcinoma and had radical prostatectomies at Vancouver General Hospital and Kelowna General Hospital (Vancouver and Kelowna, British Columbia, Canada). The study was approved by the appropriate local ethics committees, and patient consent was obtained. Excluded from this study were patients undergoing hormone ablation therapy. Within 15 to 30 minutes of surgical removal of the glands, two to four small pieces (average size:  $8 \times 6 \times 2$  mm) of an area likely to contain cancer on the basis of biopsy analysis were obtained by a pathologist, frozen in OCT compound (Tissue Tek, Torrance, CA) over liquid nitrogen vapor, and stored at  $-152^{\circ}\text{C}$ . Each sample underwent histopathological review. Samples with early cancer stages were found in 22 of the 42 radical prostatectomy specimens obtained.

**LCM and Preparation of Cell Lysates.** One frozen section ( $5 \mu\text{m}$ ) from each of the frozen samples was fixed in formalin, stained with H&E, and examined by a pathologist to identify and mark areas that contained morphologically normal, hPIN, and cancer cells of Gleason grades 2 to 4. The majority of samples used for LCM were obtained from peripheral zone. Emphasis was placed that the normal cells were distant to the PIN or cancer cells. The H&E-stained sections served as guidance for LCM and for determining the number of serial sections ( $8 \mu\text{m}$ ) needed for each analysis. For LCM, sections were fixed in 70% ethanol and left unstained, allowing virtually 100% pick up of cells. Using the  $30\text{-}\mu\text{m}$  laser spot size of the PixCell II LCM system (Arcturus Engineering, Inc., Mountain View, CA), two to three cells were procured per laser shot. A total of 2,500 shots consistently yielded 6,000 to 8,000 cells required for each SELDI and reverse transcription-PCR (RT-PCR) analysis, and 8,000 laser shots were used to acquire  $\sim 20,000$  cells for each SDS-PAGE and Western blot analysis. LCM was performed under the direction of a pathologist.

Microdissected cells for SELDI studies were lysed in  $5 \mu\text{L}$  of lysis buffer ( $8 \text{ mol/L}$  urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfo-

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**Requests for reprints:** Juergen Vielkind, Department of Cancer Endocrinology, British Columbia Cancer Agency, 600 West 10<sup>th</sup> Avenue, Vancouver, British Columbia, V5Z 4E6 Canada. Phone: (604) 877-6010, ext. 3038; Fax: (604) 877-6011; E-mail: jvielkin@bccancer.bc.ca.

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nic acid) directly on the LCM cap (in cases when two caps were necessary for procurement of a total of at least 6000 cells, 2.5  $\mu\text{L}$  lysis buffer per cap were used) and incubated for 15 minutes at room temperature in a moist chamber. The larger number of cells, *i.e.*, 20,000 cells, were lysed in 10  $\mu\text{L}$  of lysis buffer again directly on the LCM cap (in cases of two or three caps, 5 or 3  $\mu\text{L}$ /cap were used, respectively) as described above. Cells for RT-PCR studies were lysed in 100  $\mu\text{L}$  of buffer supplied in the Absolute RNA Microprep kit (Stratagene, La Jolla, CA).

**SELDI Analysis.** Each cell lysate was diluted with PBS to a total volume of 50  $\mu\text{L}$  and, using a Bioprocessor (CIPHERGEN Biosystems Inc., Fremont, CA), was placed onto an individual spot of an IMAC3 ProteinChip (CIPHERGEN Biosystems, Inc.) that was pretreated with 100 mmol/L  $\text{CuSO}_4$ . After incubation overnight at room temperature on an orbital shaker, the lysates were removed, each spot washed twice with PBS, rinsed with water, and allowed to dry. A total of 0.5  $\mu\text{L}$  of a saturated solution of sinapinic acid in 50% (v/v) acetonitrile and 5% (v/v) trifluoroacetic acid was added, and SELDI-time of flight mass spectra were generated in a CIPHERGEN Protein Biology System II spectrometer (CIPHERGEN Biosystems, Inc.) using an average of 160 laser shots fired in the positive mode at laser settings of 230. Protein spectra were analyzed using CIPHERGEN's Peaks 3.1.1 software. Over the course of our studies, three different lots of chips needed to be used. SELDI profiles with samples from patients 2 and 18 yielded virtually identical profiles on each lot, demonstrating reproducibility of the SELDI results.

**Mass Spectrometry.** Lysates were resolved under nonreducing conditions by adding 3  $\mu\text{L}$  of 4 $\times$  NuPage SDS sample buffer and under, reducing conditions by adding 3  $\mu\text{L}$  of 4 $\times$  NuPage SDS sample buffer and 1.4  $\mu\text{L}$  of 0.5 mol/L DDT in 4–12% Bis-Tris NuPage gradient gels (Invitrogen Corp., Carlsbad, CA). Gels were silver stained (SilverQuest kit; Invitrogen Corp.), and protein bands of 24 kDa (nonreducing) and 12 kDa (reducing) from the cancer cell lysates, as well as corresponding gel areas from the normal cell lysate lane, were excised. Proteins were identified by in-gel trypsin digestion followed by tandem mass spectrometric analysis carried out by the Genome BC Proteomics Centre (University of Victoria, Victoria, British Columbia, Canada),<sup>7</sup> using an ABI Q-Star with an o-MALDI source and BioAnalyst and ProData software (ABI).

**Western Blot Analysis.** Cell lysates were resolved by SDS-PAGE as described above, transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH), and membranes blocked overnight with 10% nonfat dry milk in TBS [100 mmol/L Tris (pH 7.2) and 150 mmol/L NaCl] at 4°C. For recognition of the dimeric proprotein (62 kDa), membranes were incubated for 1 hour at room temperature with goat anti-PTGF- $\beta$  polyclonal antiserum (Santa Cruz Biotechnology, Santa Cruz, CA), while for the recognition of the 24-kDa mature protein, rabbit anti-NAG-1/PTGF- $\beta$  polyclonal antiserum (Upstate Biotechnology, Lake Placid, NY) was used; both antibodies were diluted 1/1000 with TBS. After washing (TBS, 1% nonfat dry milk, and 0.1% Tween 20), blots were incubated with antigoat or antirabbit antibody conjugated with horseradish peroxidase. Antibody reactivity was detected using an enhanced chemiluminescence system (Santa Cruz Biotechnology). Conditions for GDF15 immunoreactivity were optimized using culture medium of serum-starved LNCaP cells for mature protein and LNCaP whole cell lysate for the pro-GDF15. Because DU145 cells do not express pro- nor mature GDF15, DU145 culture medium and cell lysate were used as negative controls.

**RT-PCR Analysis of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and GDF15 Expression.** Total RNA was extracted using the Absolute RNA Microprep kit (Stratagene), including the DNaseI treatment step. The level of GDF15 transcript was assayed using a one-step RT-PCR method and a housekeeping gene, GAPDH, as an endogenous standard. RT-PCR reactions with RNA from an equivalent of 300 microdissected cells were carried out in a volume of 20  $\mu\text{L}$ , with a final concentration of 1 $\times$  Qiagen OneStep RT-PCR buffer, 2.5 mmol/L  $\text{MgCl}_2$ , 400  $\mu\text{mol/L}$  of each deoxynucleotide triphosphate, 0.6  $\mu\text{mol/L}$  of each GDF15 primer (sense, 5'-GGCCAAC-CAGAGCTGGGAAG-3'; antisense, 5'-GCCCGAGAGATAC-GCAGGTG-3'), and 0.3  $\mu\text{mol/L}$  of each GAPDH primer (sense, 5'-GAGTCAACGGATTTG-GTCGT-3'; antisense, 5'-GACAAGCTTCCCCTCTCAG-3') in a programmable thermal cycler (iCycler, Bio-Rad, Hercules, CA; reverse transcription at 50°C, 30 minutes; PCR activation step at 95°C, 15 minutes; 30 cycles of denaturation at

95°C, 30 seconds; annealing at 60°C, 30 s and extension at 72°C, 1 minute; final extension at 72°C, 10 minutes). These RT-PCR conditions had previously been optimized by performing RT-PCR experiments using various concentrations of GDF15 and GAPDH primers and an increasing number of cycles (two cycle increments from 26 to 34 cycles). After separation of the amplification products on agarose gels and ethidium bromide staining, band intensity was determined using densitometric analysis with the Eagle Eye II Still Video System (data not shown). This established that under selected parameters 30 cycles would be well within the linear range of amplification for both GDF15 and GAPDH PCR products. RT-PCR reactions, including a no-RNA control, were performed at least twice. The GDF15 primers span the transcript region from 229 to 348 bp (GenBank reference sequence NM\_004864). Restriction fragment, as well as sequence analysis, verified amplification of GDF15 sequence.

## Results

**Protein Profiles of Normal, PIN, and Prostate Cancer Cells Reveal Association of a 24-kDa SELDI Protein Peak with Early Prostate Carcinogenesis.** To identify proteins with a possible role in the transformation of normal cells to early cancer cells, SELDI protein profiles were generated from lysates of matched normal, PIN, and prostate cancer cells. Fig. 1 shows representative SELDI profiles ranging from 15 to 35  $\times 10^3$   $m/z$ . Using CIPHERGEN's Biomarker Program, a recurrent major difference was found for a 24-kDa peak; no association of any other peak with any phenotype was apparent. Table 1 summarizes the presence and intensity (normalized to total ion current) of the 24-kDa peak at the various stages of prostate cancer development. It was observed in profiles of 19 of 27 cancer cell foci, the majority of those were of Gleason grade 3 and also in 3 of 8 hPIN lesions that were found in the 22 radical prostatectomy specimens. In contrast, this peak was not detected in any of the matched normal cell protein profiles. In all three 24-kDa-positive PIN lesions, the peak intensity was low and notably came from glands in which the cancer cells also exhibited a low 24-kDa peak intensity (patients 7, 13, and 18). As also seen in Table 1, the 24-kDa intensities range from very high, *e.g.*, patients 2 and 12, to low, *e.g.*, patients 8, 15, and 20. In two cases (patients 2 and 12) where two cancer cell foci of different grades

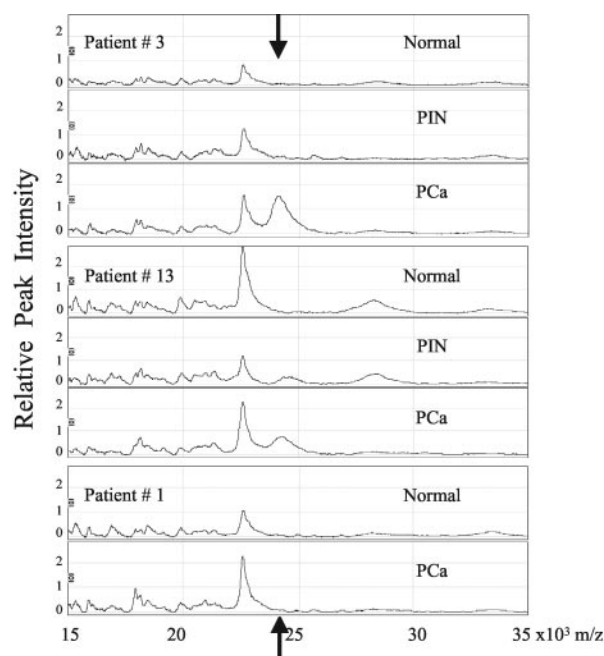


Fig. 1. Representative SELDI-time of flight protein profiles in the mass range of 15 to 35 kDa ( $m/z$ ) obtained from lysates of matched normal, PIN, prostate cancer (PCa) epithelial cells. Relative peak intensities are normalized to total ion current for the entire data set. Arrows indicate the position of the 24-kDa peak observed in the majority of PCa and some PIN cells; this peak was not seen in normal cells.

<sup>7</sup> Internet address: <http://www.proteincenter.com>.

Table 1 Normalized 24-kDa protein peak intensities in lysates of matched normal, PIN, and prostate cancer cells (Gleason grade 2–4) as determined by SELDI analysis

Patient no.	Normal	PIN	Gleason 2	Gleason 3	Gleason 3 + 4	Gleason 4
1	ND			ND		
2	ND	ND		4.539	3.662	
3	ND	ND		1.508		
4	ND					2.553
5	ND			0.574		
6	ND			0.788		
7	ND	0.346		0.375		
8	ND					0.243
9	ND	ND		0.780		
10	ND			ND	ND	ND
11	ND			3.693		ND
12	ND			11.280	7.020	
13	ND	0.138			0.707	
14	ND	ND		2.192		
15	ND	ND		0.127		
16	ND			ND		
17	ND		3.333			
18	ND	0.118		0.383		
19	ND			ND		
20	ND			0.049		
21	ND			ND		
22	ND			3.693		
Fraction of positive cases	0/22	3/8	1/1	13/18	3/4	2/4

Abbreviations: ND, peak not detected; blank cell, phenotype not observed.

were present, high-intensity 24-kDa peaks were observed for both foci of each specimen. However, in another case (patient 11), a high-intensity peak was observed in the Gleason 3 cancer, but no peak was detected in an otherwise ordinary protein profile of the Gleason 4 cancer.

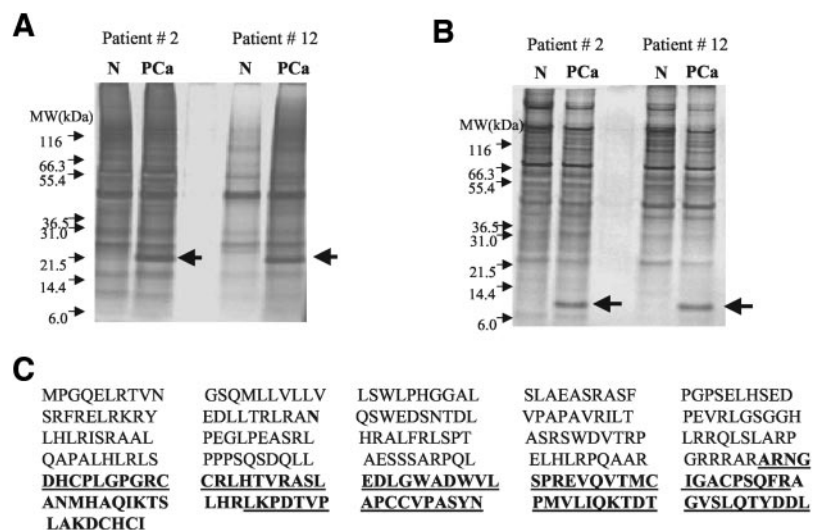
**Mass Spectrometry Identifies the 24-kDa Peak as Mature Form of GDF15.** Mass spectrometry of proteins from complex mixtures resolved by gel electrophoresis typically requires protein in the microgram range (4). Two specimens, which contained a large number of prostate cancer cells and had exhibited high 24-kDa peak intensities, thus considered to be the best candidates for good band visualization, were selected to identify the protein(s) corresponding to the 24-kDa SELDI peak. Prostate cancer and normal epithelial cells (20,000 each) were procured by LCM and resulting lysates resolved by SDS-PAGE. Both cancer samples yielded a strong band in the 24-kDa area (Fig. 2A, nonreducing conditions) that was absent in the normal cell lysates. This finding is consistent with the SELDI data (Table 1). Under reducing conditions (Fig. 2B), the gels showed a

band in the 12-kDa area for the cancer but not for the normal cell samples. This observation suggests that the 24-kDa protein is a dimer of a 12-kDa protein.

The 24- and 12-kDa protein bands from both cancer samples, as well as control gel slices from the same region in the lanes of normal samples, were excised and subjected to in-gel trypsin digestion followed by tandem mass spectrometry analysis. Strong evidence identifying the protein present in the 12/24-kDa gel bands as mature GDF15 was obtained. Two to five peptides corresponding to GDF15 peptides were identified in each of the four digests, with more frequent hits only from keratin. The search engine in the ProID software identified EVQVTMCIGACPSQFR and TDTGVSLQTYDDLLAKD in all four samples with a 90 to 99 best confidence and bin scores of 29 to 42 with one exception of 75 and 25, respectively. ASLEDLGWADWLSR was obtained in three samples, LKPDTVPAPC-CVPASYNPMVLIQK in two samples with best confidence levels 75 to 99 and 50, respectively, and bin scores 20 to 40 and 17 to 19, respectively. ARNGDHCPGPR (confidence 50/bin 19) was present in only one sample along with all previous peptides, representing coverage of 80% of the mature GDF15. The mature 24-kDa dimer arises from proteolytic cleavage of a 62-kDa proprotein dimer (5); however, only peptides from the mature portion were observed. GDF15 is also known by other names PLAB, PDF (2, 3), MIC-1, PTGF, and NAG-1 (5–7). A summary of sequence information is shown in Fig. 2C.

**Expression of Pro-GDF15 and Mature GDF15 in Normal and Prostate Cancer Cells.** The experimental conditions used in our SELDI analyses did not allow good resolution of proteins in the region > 60 kDa. To determine whether the 62-kDa proprotein is present and correlates with the expression of the mature form, Western blot analysis was carried out using commercially available antisera specific for pro-GDF15 and mature GDF15. As shown in Fig. 3A, neither the pro-GDF15 nor the mature GDF15 was found in two lysates of normal cells. The absence of mature GDF15 in the normal cell lysates is consistent with the SELDI protein profiles (Table 1; Fig. 3B) and is apparently not a result of failure of the pro-GDF15 to convert to the mature form. In the cancer cell lysate (patient 2; Fig. 3A), a weak signal for the proprotein was found to be associated with high amounts of the mature GDF15. In another case (patient 13; Fig. 3B), low expression of mature GDF15 was associated with a relatively higher signal of the proprotein. As expected, the signals for the mature GDF15 matched those observed in the SELDI analyses (Fig. 3, A and B).

Fig. 2. In A, nonreducing SDS-PAGE strong bands occur at 24 kDa (arrows) in both prostate cancer (PCa, Gleason grade 3) lysates, bands occur at 12 kDa (arrows) when lysates were subjected to (B) reducing SDS-PAGE; bands were not seen in lysates from normal cells (N). In C, total amino acid sequence of GDF15 is shown; a sequence in *bold* represents mature GDF15 and *underlined* are the sequences identified by mass spectrometry of the 12- and 24-kDa bands.



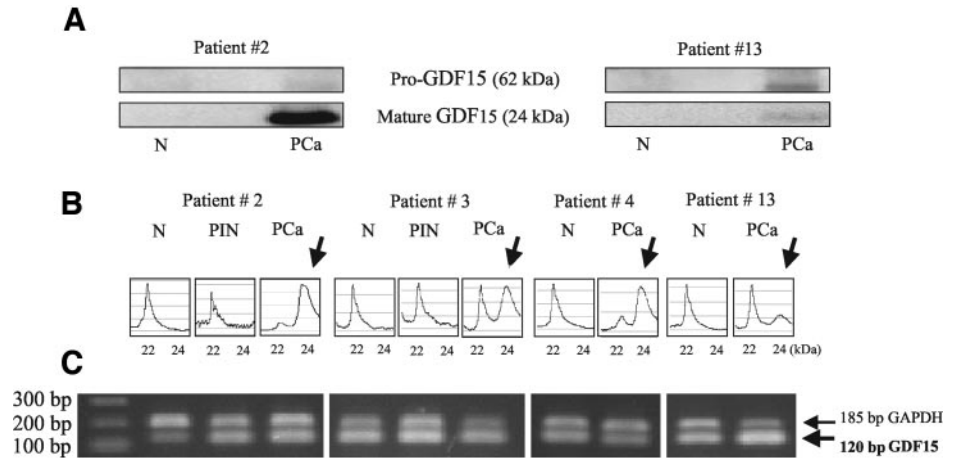


Fig. 3. Expression levels of GDF15 protein as found by Western blotting (A) and SELDI analysis (B) in comparison with GDF15 levels of GDF15 mRNA as determined by RT-PCR (C) in matched normal (N), PIN, and prostate cancer (PCa) cells.

**Expression of Mature GDF15 Does Not Correlate with GDF15 mRNA Levels.** Because we did not detect GDF15 protein in normal epithelium, it might be expected that these cells may not express GDF15 mRNA either. Some researchers (8) found GDF15 mRNA levels higher in prostate cancer than in normal prostatic epithelium, but others (9) found the opposite. To investigate whether the transcriptional levels of GDF15 in matched normal, PIN, and prostate cancer cells correlate with the expression of GDF15 protein, we performed RT-PCR on LCM-procured cells from 11 cases. The transcript level of the endogenous housekeeping gene, GAPDH, served as standard. These experiments were performed using parameters that ensured that both GAPDH and GDF15 were amplified within a linear range and thus a semiquantitative evaluation of GDF15 mRNA levels would be valid. Fig. 3C shows representative results from the RT-PCR experiments in comparison to the SELDI protein data (Fig. 3B). GDF15 transcripts were found in all samples examined and few substantial differences were seen among the levels of the GDF15 transcripts in the normal, PIN, and prostate cancer cells. Thus, the absence of either the pro- or mature GDF15 protein in the normal epithelial cells is not due to the lack of GDF15 message. Furthermore, prostate cancer cells, which were expressing GDF15 mRNA at the highest level, only expressed the mature protein at low levels (see Western blot analysis; Fig. 3A). Thus, there is apparently little correlation between the GDF15 mRNA levels and the levels of the encoded GDF15 protein.

## Discussion

In this study, we focused on the early stages of prostate cancer. Comparing SELDI protein profiles obtained from matched pure cell populations representing normal epithelium, hPIN, and prostate cancer (Gleason grades 2 to 4) lesions, we were able to detect a 24-kDa peak in 3 of 8 PIN lesions and in the majority of, 19 of 27, cancer lesions. Cazares *et al.* (10), also using LCM and SELDI, noted a peak of similar size in a few cancer lesions, of which, the precise histopathological grade was not reported; they did not observe this peak in PIN lesions. Recently, Zheng *et al.* (11) reported a 24-kDa peak in 16 of 17 grossly dissected prostate carcinoma samples. Consistent with our observation, both groups did not observe this peak in normal prostate cells. Because we and these two groups used biochips with the same affinity surface, it is likely that the same protein has been detected.

The data presented here identified this protein as the dimeric form of mature GDF15, a growth factor that belongs to the TGF- $\beta$  superfamily. GDF15 is supposed to play a role in cell cycle regulation (12) and apoptosis (7) and may have an enhancing effect on tumor inva-

siveness (13), metastasis (9), and immunosuppression (5). In this study, expression of GDF15 was found earliest in hPIN and continued to be expressed in almost all cancer cell foci of low Gleason grade where it was expressed at moderate or high levels. Whether these levels of expression relate to tumor aggressiveness is unknown but warrants additional studies. Also, if these differences are related to proprotein convertase activity is at present unknown. The absence of mature GDF15 in normal epithelium, however, appears to be primarily due to a lack of proprotein, although GDF15 mRNA was detected in all cases. We suspect that the same is true for the PIN and prostate cancer lesions that do not show mature GDF15.

Using immunohistochemistry, other groups (14, 15) have also observed GDF15 protein expression, including PIN (14) of prostate carcinogenesis, although no distinction was made between pro- and mature protein. Weak immunoreactivity has been reported (15) in few of the normal cells, but the techniques used in the present study would not have detected a protein signal from a small proportion of weakly positive cells. Ickowski *et al.* (14) suggested that GDF15 expression may correlate inversely with the prostate cancer grade. We were unable to draw such a conclusion because we only had four lesions of Gleason grade 4. However, it is interesting to note that in one prostatectomy sample, GDF15 protein was detected in a Gleason 3 lesion but was missing from a nearby Gleason 4 lesion (patient 11; Table 1), perhaps reflecting the multifocality of prostate cancer.

Our and other GDF15 protein studies are in basic agreement, but there appears to be some differences with regard to mRNA expression studies. GDF15 mRNA has been shown to be commonly overexpressed in prostate cancer by microarray analysis (16) and real-time quantitative RT-PCR (8). However, Nakamura *et al.* (8) found that in 12% of cases, normal tissue expressed GDF15 mRNA at higher levels than matched prostate cancer tissue. Part of the variation observed might be attributable to the use of grossly dissected tissue, which probably contains varying amounts of stromal and other nonepithelial cells that express different amounts of GDF15 mRNA. For example, we determined very low expression of GDF15 mRNA in LCM-procured stromal cells (unpublished observations) consistent with *in situ* hybridization data of Thomas *et al.* (9). Our RT-PCR data using LCM-procured cells, however, also revealed that while some prostate cancer and PIN cells may express GDF15 mRNA at higher levels than matched normal cells, in others, there is little or no difference compared with normal cells. Thus, the mRNA levels may not reflect the level of protein found in the cell. Others (17, 18) have also observed that there is not always a direct correlation between mRNA and protein expression. The reasons for these discrepancies are not known but could be due to differences in, for example, posttranscriptional

modifications or processing of RNA that influence its stability or translation or posttranslational modifications of the protein affecting protein degradation, accumulation, or maturation. However, to reiterate, the absence of mature GDF15 in normal prostate epithelium does not appear to be simply due to a failure of the production of the proprotein to convert.

This study has demonstrated the usefulness of combining LCM and SELDI to screen for and identify proteins associated with cancer development. We focused on the most obvious and consistent difference detected in the SELDI profiles of normal, hPIN, and prostate cancer cells and were able to identify it as a difference in the expression of a growth and differentiation factor, GDF15. The fact that GDF15 expression already occurs in PIN suggests a role in the early steps of the genesis of prostate cancer, although its role is yet to be clarified.

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