PACE4 Undergoes an Oncogenic Alternative Splicing Switch in Cancer

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Abstract

Inhibition of PACE4, a proprotein convertase that is over-expressed in prostate cancer, has been shown to block cancer progression in an androgen-independent manner. However, the basis for its overexpression and its growth-inhibitory effects are mitigated and uncertain. Here, we report that PACE4 pre-mRNA undergoes DNA methylation–sensitive alternative splicing of its terminal exon 3’ untranslated region, generating an oncogenic, C-terminally modified isoform (PACE4-altCT). We found this isoform to be strongly expressed in prostate cancer cells, where it displayed an enhanced autoactivating process and a distinct intracellular routing that prevented its extracellular secretion. Together, these events led to a dramatic increase in processing of the progrowth differentiation factor pro-GDF15 as the first PACE4 substrate to be identified in prostate cancer. We detected robust expression of PACE4-altCT in other cancer types, suggesting that an oncogenic switch for this proenzyme may offer a therapeutic target not only in advanced prostate cancer but perhaps also more broadly in human cancer. Cancer Res; 77(24); 6863–79. ©2017 AACR.

Introduction

Among malignancies, prostate cancer remains the most common type of cancer in men, with 233,000 new cases each year in the United States, representing 27% of all new cancer cases, as well as the second most common cause of cancer-related mortality (1). When diagnosed in its early progression stages, clinical interventions are able to circumvent disease progression and yield high survival rates over 5–15 years. However, if tumor initiated metastatic dissemination, as occurring at the time of diagnosis or following recurrence, survival rates drop considerably, leading to patient death within 5 years in approximately 75% of cases. The treatment for metastatic prostate cancer involves androgen suppression therapy but remains palliative. Once resistance to castration occurs, the only option remaining is chemotherapy. Novel targeted therapeutic avenues arising from yet unexplored biological pathways may provide a solution, either alone or as cotargets.

Among potential targets that have yet to be fully defined are the proprotein convertases (PC), which are responsible for the post-translational processing of proprotein substrates within the secretory pathway. The PCs are composed of nine members, namely, PC1/3, PC2, furin, PC4, PC5/6, PACE4, PC7, SKI-1, and PCSK9. The first seven are calcium-dependent serine proteases that cleave at paired basic residues at the consensus cleavage site R-X-(K/R)-R (2). The PCs have been loosely associated with malignancies because of their ability to enhance the activity of cancer-associated protein substrates, which are overexpressed by tumor cells, for example, members of the ADAM family of proteases, TGFβ, MMPs, and insulin-like growth factor-1 receptor (IGF1R) family members (3). As PC display increased expression in tumor cells and are required for enhanced processing to sustain tumorigenesis, they have been proposed as attractive antineoplastic targets (4). At this time, the evidence remains indirect as to how oncogenic control by the PCs is executed.

Among the PCs, recent data has made the strongest case yet for PACE4 and its specific association with malignant transformation (5). In prostate cancer, among other PCs, PACE4 is specifically overexpressed and carries nonredundant growth-sustaining functions for cancer cells (6, 7). PACE4 inhibition using either silencing tools or the high affinity PACE4 inhibitor [dL]LLLRVK-aminodibenzylamide (Amba) herein called C23, both prevented prostate cancer tumor progression (8). Despite these important advances, neither the downstream substrates nor the mechanisms associated with sustained PACE4 overexpression in prostate cancer cells have been elucidated. Thus, important questions remain regarding how PACE4 levels can be envisioned as a viable target or have a direct relationship with prostate cancer disease outcomes.

In this study, we uncovered important posttranscriptional changes that have profound effects on PACE4 mRNA and protein as well as its cell trafficking and substrate processing. Our analysis reveals a yet unreported PACE4 splice variant that results in the expression of a C-terminally modified protein isoform (PACE4-altCT), increased autoactivation and retention within cells.
Compared with its parent isoform, PACE4-altCT strongly regulates sustained cell proliferation, further defining this isoform as the molecular target of the PACE4 inhibitor–mediated response of cancer cells and conciliating the thus far unexplainable requirement of PACE4 inhibitor to reach intracellular compartments. This new isoform is strongly upregulated at both the mRNA and protein levels in prostate cancer tissues, which is not the case of any other known PACE4 isoforms. A scan in various malignancy types reveals that PACE4-altCT is expressed in other cancer types, notably in pancreatic, lung, and thyroid cancers. In prostate cancer cells, this splicing activity is regulated through a distinctive intragenic DNA methylaction pattern modulating the binding CTCFC–binding factor CTCF and the inclusion of a novel distal terminal exon. A stable isotope labeling with amino acids in cell culture (SILAC) proteomic approach permitted the identification of progrowth differentiation factor-15 (pro-GDF-15) as a PACE4-specific substrate in prostate cancer. Using primary tissues and xenografted mice treated with the C23 PACE4-inhibitor, blocking the processing of this growth factor suggested that pro-GDF-15 could serve as a target engagement biomarker. The results of this work define the mechanism behind our assertion that PACE4-altCT is a pharmacologically targetable oncogenic driver of prostate cancer–sustained cell growth.

Materials and Methods

Ethics and study approval

Patients agreed to participate and freely signed a consent form, and the research protocol was approved by the Institutional Review Committee for the Use of Human Resected Material at the Centre Hospitalier Universitaire de Sherbrooke (approval #10-017). The experimental protocols were approved by the Université de Sherbrooke Ethics Committee for Animal Care and Use in accordance with guidelines established by the Canadian Council on Animal Care.

Cell culture

Cell lines (DU145, LNCaP, and HT1080) were obtained from the ATCC and HEK293-FT (Life Technologies Inc.) were cultured according to their recommendations. Normal human fibroblasts (C19, derived from radical prostatectomies performed at the Centre Hospitalier Universitaire de Sherbrooke (Québec, Canada). The tissues were frozen at –20°C with optimal cutting temperature (OCT) compound (Tissue-Tek; Miles Scientific), and 5-μm slices were cut and immediately fixed in formalin to perform hematoxylin and eosin staining for pathologic examination. Tumor zones were delimited together with the adjacent noncancerous tissues by a clinical pathologist, and dissection was performed accordingly. Dissected tissues were washed with nano-pure RNase-free water (Wisent) to remove all apparent traces of the OCT compound. The tissues were then finely ground in liquid nitrogen, and RNA extraction was performed using Qiagen RNeasy spin columns (Qiagen) following the manufacturer’s instructions. RNA integrity was assessed by analysis using an Agilent Bioanalyzer with RNA Nano Chips (Agilent Technologies). Normal RNA-standardized preparations were obtained from Clontech Laboratories (Total RNA Master Panel II). Normal and tumoral cDNA samples were obtained from the Origene Cancer Survey cDNA Array covering the entire genome (Agilent Technologies). Normal RNA-standardized preparations were obtained from Clontech Laboratories (Total RNA Master Panel II). Normal and tumoral cDNA samples were obtained from the Origene Cancer Survey cDNA Array covering the entire genome (Agilent Technologies). Normal RNA-standardized preparations were obtained from Clontech Laboratories (Total RNA Master Panel II). Normal and tumoral cDNA samples were obtained from the Origene Cancer Survey cDNA Array covering the entire genome (Agilent Technologies). Normal RNA-standardized preparations were obtained from Clontech Laboratories (Total RNA Master Panel II). Normal and tumoral cDNA samples were obtained from the Origene Cancer Survey cDNA Array covering the entire genome (Agilent Technologies).

DNA constructs, plasmids, transfections, and splicing mapping

Detailed Western blot analysis, PCR, reverse transcription and qPCR conditions, vector information, and procedures for reporter assays can be found in the Supplementary Material. Primers sequence and product lengths are found in Supplementary Tables S1 and S2.

Proliferation assays

For the proliferation assays, the cells were plated in 96-well plates, and the metabolic activity was measured after 72 hours as described in ref. 9. For the colony formation assay, the cells were plated at low densities (50 cells for HT1080, 100–200 cells for DU145 and 500–1000 cells for LNCaP) and allowed to form colonies for 10–14 days in complete medium before being stained with crystal violet counted after with a visible/infrared scanner (Odyssey Image, LI-COR Biosciences). See Supplementary Material for detailed procedures.

Activity assays

For activity assays, medium collected from stable S2 cell cultures expressing the PACE4 isoforms was collected and concentrated to a smaller volume and plated in 96-well plates, and the metabolic activity was measured after 72 hours as described in ref. 10. Identical preparations from wild-type S2 cells were used as blanks.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 7.0 (GraphPad Software), and all data are represented as the mean ± SEM. The one-tailed Student t test was used for the data analysis, and when paired samples were compared, a matched pairs t test was used. For correlation analysis, the Spearman correlation test was used. Values less than 0.05 were considered statistically significant.

Fresh tissue dissection and RNA isolation

Prostate tissues used for RNA extraction were freshly (typically within 30 minutes) dissected from prostate specimens obtained from radical prostatectomies performed at the Centre Hospitalier Universitaire de Sherbrooke (Québec, Canada). The tissues were frozen at –20°C with optimal cutting temperature (OCT) compound (Tissue-Tek; Miles Scientific), and 5-μm slices were cut and immediately fixed in formalin to perform hematoxylin and eosin staining for pathologic examination. Tumor zones were delimited together with the adjacent noncancerous tissues by a clinical pathologist, and dissection was performed accordingly. Dissected tissues were washed with nano-pure RNase-free water (Wisent) to remove all apparent traces of the OCT compound. The tissues were then finely ground in liquid nitrogen, and RNA extraction was performed using Qiagen RNeasy spin columns (Qiagen) following the manufacturer’s instructions. RNA integrity was assessed by analysis using an Agilent Bioanalyzer with RNA Nano Chips (Agilent Technologies). Normal RNA-standardized preparations were obtained from Clontech Laboratories (Total RNA Master Panel II). Normal and tumoral cDNA samples were obtained from the Origene Cancer Survey cDNA Array covering the entire genome (Agilent Technologies). Normal RNA-standardized preparations were obtained from Clontech Laboratories (Total RNA Master Panel II). Normal and tumoral cDNA samples were obtained from the Origene Cancer Survey cDNA Array covering the entire genome (Agilent Technologies).

DNA methylation analysis

DNA was purified from prostate specimens or from cell pellets using the DNeasy Blood & Tissue Kit (Qiagen, #69504). Pyrosequencing assays were performed as described previously (11). The PCR and sequencing primers shown in Supplementary Table S3 were designed using PyroMark Assay Design software v.2.0.1.15. Overall, nine potentially methylated cytosines (in the context of CpG dinucleotides) were analyzed at the PCSK6 gene locus. The detailed procedure is provided in the Supplementary Material.

IHC

Tissues sections (4-μm thick) were stained using Peroxidase Detection Kit (Pierce), counterstained with Harris hematoxylin (Sigma-Aldrich), and scanned with a Nanozoomer (Hamamatsu) using Nanozoomer Digital Pathology software. Antibodies details...
can be found in Supplementary Table S4. For detailed procedure, see Supplementary Material. For custom rabbit polyclonal antibodies, antibodies were raised and purified from serum on a peptide-coated chromatographic column (Pacific Immunology).

**Chromatin immunoprecipitation**

DNA (350 μg) from sonicated and micrococcal nuclease-digested nuclei isolated from formaldehyde-crosslinked cells was immunoprecipitated using Protein A MagnResin beads. Chromatin immunoprecipitation (ChIP)-isolated DNA was further used for qPCR analyses using input as a standard. See Supplementary Material for detailed procedure.

**Confocal microscopy**

Forty-eight hours after transfection, cells were fixed, permeabilized, and stained with the primary antibodies (see Supplementary Table S4) at 4°C. Fluorescent secondary antibodies (Alexa Fluor 488 and 594 antibodies, Thermo Fisher) were further applied for 1-hour incubation at room temperature, followed by DAPI staining and final mounting with SlowFade (Invitrogen). The cells were examined with a Plan Apo 60x oil immersion objective NA 1.42 on an FV1000 inverted spectral scanning confocal microscope (Olympus). See Supplementary Material for detailed procedure.

**LC/MS-MS analysis**

Acquisition was performed with a Sciex TripleTOF 5600 (Sciex) equipped with an electrospray interface with a 25-μm ID capillary and coupled to an Eksigent μHPLC (Eksigent). Analyst TF 1.6 software was used to control the instrument and data processing and acquisition. See Supplementary Material for detailed procedure and Supplementary Tables S6 and S7 for the data from the IP-MS and the SILAC analysis in secretome respectively.

**Peptide synthesis and cleavage analysis**

ML peptide and its derivatives (Peg8-ML and C23) were synthesized as described previously (12). Synthesis and cleavage analysis of the GDF-15 spanning peptide are described in the literature (17), but could be retrieved in some expressed sequence databases.

To our knowledge, this alternative splicing event has not been documented; however, no correlations with clinical parameters, further allowing the upregulation of gene expression through evasion of posttranscriptional regulatory mechanisms such as repression by miRNAs (16). Using 3′ rapid amplification of cDNA ends (3′ RACE) in LNCaP cell cDNA, the PACE4 mRNA 3′ end revealed the presence of the consensual 1335-bp-long 3′UTR as well as two shorter 3′UTRs (164 and 118 bPs; Fig. 1D) generated by alternative splicing. This splicing event, confirmed by 3′RACE product sequencing and three-primer PCR (TP-PCR; Supplementary Fig. S1C), incorporates a distal terminal exon located 6.3 kb downstream of the 25th exon consensually used (Fig. 1E), substituting the 25th exon with this alternative exon (25-alt) and altering the coding region of the C-terminal end of the protein. Thus, two distinct PACE4 proteins can be generated from this splicing event, namely a PACE4 full-length (PACE4-FL) isoform and a PACE4 isoform with an alternative C-terminal end (PACE4-altCT) that has a novel 30 amino acid sequence with 2 Cys residues altering the coding region of the C-terminal end of the protein. To our knowledge, this alternative splicing event has not been documented among the reported PACE4 splice variants in the literature (17), but could be retrieved in some expressed sequence tag (EST) databases.

**Xenograft assay**

Trypsin-harvested LNCaP cells were mixed with ice-cold Matrigel (BD Biosciences) and subcutaneously injected into the shoulders of Nu/Nu male mice (Charles River Laboratories) as described in ref. 8. Prostate-specific antigen (PSA) and GDF-15 levels were determined using Quantikine ELISA Kit (R&D Systems). Tumor lysates were obtained as described in ref. 7.

**Results**

**PACE4 mRNA levels correlate with tumor aggressiveness in prostate cancer tissue specimens**

The overexpression of PACE4 in prostate cancer has been documented; however, no correlations with clinical parameters, such as Gleason grading, have been reported (6, 13, 14). New molecular markers with relevance to prostate cancer progression should show a positive correlation with the established histologic prognostic indicator, that is, Gleason grading, to be considered of prognostic usefulness. Using matched cancerous and adjacent noncancerous tissues (ANCT), PACE4 mRNA levels were analyzed by real-time quantitative PCR (qRT-PCR). In this cohort (Gleason scores 6–9), 34 of 38 samples (>89%) showed significantly increased PACE4 mRNA levels (Fig. 1A). This overexpression pattern was tightly correlated with the tumor Gleason scores (Spearman r: 0.46; P 0.004; Fig. 1B). Our results are supported by similar analyses that were performed using the data from two distinct datasets in the cBioPortal for the Cancer Genomics database (Supplementary Fig. S1A and S1B). IHC analyses of specimens from different tumor grades with an antibody targeting the catalytic domain of PACE4 (i.e., detecting all PACE4 isoforms) corroborated our results as once again overexpression was visible with increasing levels in higher grade foci (Fig. 1C).

Prostate cancer exploits a PACE4 mRNA terminal exon splicing event that is specific to tumor cells

Human PACE4 mRNA is encoded by the 186-kb PCSK6 gene located at the 15q26 3 locus, which is not a locus that has been reported to be susceptible to frequent changes in copy number in prostate cancer specimens (Oncomine databases). While PACE4 overexpression could be due to increased transcription, another mechanism that has gained enormous importance in cancer biology is alternative splicing as a means to promote the expression of genes that sustain proliferation (15). The link between alternative splicing and proliferation is often observed through a shortening of 3′UTR regions of oncogenes and proto-oncogenes, further allowing the upregulation of gene expression through evasion of posttranscriptional regulatory mechanisms such as repression by miRNAs (16). Using 3′ rapid amplification of cDNA ends (3′ RACE) in LNCaP cell cDNA, the PACE4 mRNA 3′ end revealed the presence of the consensual 1335-bp-long 3′UTR as well as two shorter 3′UTRs (164 and 118 bPs; Fig. 1D) generated by alternative splicing. This splicing event, confirmed by 3′RACE product sequencing and three-primer PCR (TP-PCR; Supplementary Fig. S1C), incorporates a distal terminal exon located 6.3 kb downstream of the 25th exon consensually used (Fig. 1E), substituting the 25th exon with this alternative exon (25-alt) and altering the coding region of the C-terminal end of the protein. Thus, two distinct PACE4 proteins can be generated from this splicing event, namely a PACE4 full-length (PACE4-FL) isoform and a PACE4 isoform with an alternative C-terminal end (PACE4-altCT) that has a novel 30 amino acid sequence with 2 Cys residues altering the coding region of the C-terminal end of the protein. To our knowledge, this alternative splicing event has not been documented among the reported PACE4 splice variants in the literature (17), but could be retrieved in some expressed sequence tag (EST) databases.

Upon PACE4 knockdown in cell lines (LNCaP and DU145) using a shRNA targeting the 5′ region (exon #2) of PACE4 mRNA, both splice variants were downregulated (Supplementary Fig. S1D). When assessed in paired prostate ANCT and tumor tissues, PACE4-altCT mRNA was primarily detectable in the tumor specimens with very low or undetectable levels in the ANCT-matched specimens (Fig. 1F). qRT-PCR analyses showed that PACE4-altCT mRNA displayed 8-fold greater amounts between tumor and normal zones compared with PACE4-FL (Fig. 1G), with >95% patients showing increased PACE4-altCT mRNA levels. When stratified by their tumor Gleason scores (Fig. 1H), PACE4-altCT mRNA levels displayed a highly reminiscent pattern to the initial observations (Fig. 1A). Once translated, PACE4-altCT and PACE4-FL have distinct C-termini (Fig. 1E), which were exploited to generate isoform-specific antibodies to validate these observations. IHC on prostate cancer specimens using both antibodies (Fig. 1I).
showed that, as observed for the mRNA, both isoforms were readily overexpressed in tumor cells, and those levels increased along with the grading of the tumor foci. The cellular distribution pattern for PACE4-altCT appeared predominantly vesicular, which was not the case for PACE4-FL, which particularly accumulated in the peritumoral stroma. However, staining for PACE4-altCT showed a strong positive signal within the tumor epithelium and was absent from the normal epithelium. The difference in PACE4-altCT staining intensities between normal prostate glands and tumor cells was much higher than that observed for PACE4-FL (Fig. 1; Supplementary Figs. S2A and S2B). Using a set of prostate cancer specimens, IHC staining for PACE4 (catalytic domain antibody), PACE4-FL, and PACE4-altCT were quantified in normal and cancerous foci using a semiquantitative scoring system (Fig. 1I). Normal epithelium was positive in most cases (11/12 and 10/12 for PACE4 and PACE4-FL, respectively), whereas it was completely negative for PACE4-altCT.

**Figure 1.**
Alternative splicing of PACE4 terminal exon is strongly enhanced in prostate cancer specimens and correlates with tumor aggressiveness. A, qRT-PCR analyses of PACE4 expression levels in fresh prostate tissues specimens showing a significant correlation between levels and tumor Gleason scores (n = 7, 14, 11, and 6 for Gleason scores 6, 7, 8, and 9, respectively). Data, means ± SEM, B, Similar analyses with data retrieved using cBioPortal for Cancer Genomics using the Broad/Coriell Nature Genetics 2012 and MSKCC Cancer Cell 2010 datasets (see data in Supplementary Fig. S1A and S1B). r values are Spearman correlation coefficients. C, Hematoxylin and eosin (H&E) and PACE4 IHC (using catalytic domain targeting antibody) in prostate cancer representative specimens showing the concomitant increase of PACE4 expression at the protein level. Scale bars, 200 μm. D, 3’ RACE PCR showing 3’UTR lengths (expected: 1,564 bps, 3’UTR: 1,335 bps) and the two alternative 3’UTR lengths (341 bps; 3’UTR: 338 bps and 397 bps; 3’UTR: 318 bps) in LNCaP cDNA. E, Representation of the 3’ end of PEG56 transcript encompassing the exons, splicing motifs, 3’UTR lengths, primer design used for the 3’PACE and TP-PCR, and the distinctive protein C-termin generated (PACE4-FL in blue and PACE4-altCT in red; see Supplementary Fig. S1C for TP-PCR design and validation). F, TP-PCR performed on 10 paired ANCT and prostate cancer tissues. G, qRT-PCR on paired tissues. Data, means ± SEM, n = 6, 15, 9, 9, and 6 for Gleason 6, 7, 8, and 9 respectively. H, Subanalysis according to tumor Gleason scores. I, IHC analysis of consecutive slides from FFPE prostate specimens using anti-PACE4 antibodies targeting the catalytic domain. J, A semiquantitative analysis of epithelial foci (low grade, Gleason 3; high grade, Gleason 4–5) from a set of prostate cancer specimens stained with the catalytic domain antibody (used in C).
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for PACE4-altCT (11/12). In cancer foci, whereas PACE4-FL remained constant when comparing with normal epithelium, PACE4-altCT staining was greatly increased, which is consistent with the performed mRNA analysis (Fig. 1H). As PACE4 has been reported to have other splice variants (17), ANCT and prostate cancer tissue pairs were subjected to comparative profiling of PACE4 splicing events by end-point RT-PCR to map all exon–exon junctions (Supplementary Fig. S3A and S3B). Among the reactions, only three potential splice sites were depicted as active (Supplementary Fig. S3C–S3G) named: (i) exon 4 skipping; (Supplementary Fig. S3D) and (ii) exon 18 skipping, known as PACE4-I and PACE4-IL (Supplementary Fig. S3E), having or not the 18th exon, respectively, and (iii) exon 25 substitution, as observed in Fig. 3F. Upon validation by RT-PCR, exon 4 skipping could barely be detected (Supplementary Fig. S3G), and skipping of exon 18, which encodes a short protein segment of approximately 1.5 kDa between the RGD motif and the Cys-rich domain of PACE4, was active but with similar levels between ANCT and tumor specimens (Supplementary Fig. S3H and S3I). Only the substitution of exon 25 was confirmed as a splicing event specific to prostate cancer upon comparison with normal tissues (Supplementary Fig. S3G).

PACE4-altCT is generated by a stabilized mRNA variant that leads to intracellular retention, enhanced stability, and increased autoca-tivation

Consistent with the nature of the 3′UTRs and their roles in regulating mRNA stability, the consensus (PACE4-FL) and alternative (PACE4-altCT) 3′UTR sequences were analyzed for mRNA site prediction using RegRNA and miRDB (see Supplementary Table S5; ref. 18). In the 3′UTR accompanying the alternative terminal exon, 90% less miRNA sites were aligned compared with the consensus 3′UTR (Fig. 2A). The PACE4-targeting miRNA regulatory sites predicted by TargetScan for miR-21, which is overexpressed in prostate cancer (19), and miR-124, a tumor suppressor that is downregulated in prostate cancer (20), were all absent from the transcript after 3′UTR replacement (14) (Fig. 2A). To verify that the 3′UTR substitution could increase mRNA stability by escaping miRNA-dependent degradation, actinomy-cin D (ActD) followed by qRT-PCR showed that transcripts with the alternative 3′UTR were more stable than those carrying the consensus 3′UTR. Luciferase reporter assays using luciferase constructs harboring the shorter and consensus 3′UTRs showed a 2-fold amplification of protein production when the short 3′UTRs were compared with the consensus form (Fig. 2B).

Upon expression as constructs in cell lines, the first major distinction observed between the two isoforms was the lack of secretion of PACE4-altCT accompanied by intracellular retention in comparison with PACE4-FL (Fig. 2C; Supplementary Fig. S4A and S4B). This observation is consistent with the distinct vesicular cell distribution observed for each isoform in IHC in prostate cancer tissues (Fig. 1I; Supplementary Fig. S2A and S2B). PACE4 has been previously reported to be secreted in the medium, although it could be retained in the extracellular matrix through binding to heparan sulfate proteoglycans by its Cys-rich domain while also being displacable with heparin (21). Upon addition of heparin to the medium of transfected cells, PACE4-FL, but not PACE4-altCT, was efficiently displaced from the extracellular matrix to the medium (Supplementary Fig. S4C–S4E), further confirming the inability of the intracellular isoform to exit the cells.

It has been previously reported that the C-terminal end of PACE4 encoded by the 25th exon of PACE4-FL negatively regulates its autocatalytic activation and secretion (22). In this context, we tested the autoactivation rates by addition of cycloheximide to determine the rate of predomain removal for both isoforms. Time-course experiments showed that PACE4-altCT had a higher rate of autoactivation than PACE4-FL (Fig. 2D; Supplementary Fig. S4F–S4H). Moreover, in all tested cell lines, PACE4-altCT stability was considerably higher (more than 2-fold) than that of PACE4-FL (Fig. 2D and E; Supplementary Fig. S4F–S4H). Among previously reported PACE4 isoforms, only isoform A (PACE4-FL) is known to be active or processed from pro-PACE4 to PACE4 (23). Enzymatic activity was thus tested using recombinant (r) PACE4-FL and rPACE4-altCT (S2; Supplementary Fig. S4I), which showed that both isoforms had a similar activity against a fluorogenic PC substrate (pyroEKRARK-amido-methylcoumarin) and were equally inhibited by a PACE4-specific inhibitor, that is, the multi-leucine (ML) peptide (Fig. 2F). Taken together, these data demonstrate that the intracellularly retained PACE4-altCT is generated by an mRNA splice variant that is less susceptible to degradation and that the resulting protein isoform is more rapidly activated and is more stable than its parent isoform (PACE4-FL), while maintaining equal activity levels to the parent isoform. For cancer cells, this may result in higher tumor growth.

As the two PACE4 isoforms are differentially localized, it follows that their intracellular trafficking patterns will be divergent, and it is highly likely that interacting proteins are responsible for this rerouting. To test this hypothesis (Fig. 2I), we carried out coinmunoprecipitation (co-IP) studies using transiently transfected HEK293-FT cell lysates that were subjected to Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH-MS)-based analysis to investigate differences in intracellular interacting proteins of both isoforms (Fig. 2G and H). The proteins identified in each pull-down were compared for their IP enrichment between the two isoforms. We focused on proteins that are typically associated with cell compartments (Fig. 2I). Both isoforms pulled-down endoplasmic reticulum (ER) proteins with similar enrichment. The two isoforms clearly had different patterns in terms of endosomal compartment–associated proteins, such as Arf6, Rab13, and Vps16, with the PACE4-altCT pull-down displaying higher enrichment levels (Fig. 2I and J). However, the PACE4-FL pull-downs demonstrated a stronger association with exocyst complex–associated proteins, such as Exo2 and Vps13. Co-IPs performed in transfected LNCAp cells analyzed by Western blot confirmed differences between the isoforms (Supplementary Fig. S4I).

Consequently, we performed confocal microscopy using known markers of cellular trafficking in colocalization studies with either PACE4-FL or PACE4-altCT. Using RCAS1 as a Golgi marker (24), PACE4-FL was clearly better colocalized within the Golgi than did PACE4-altCT (Fig. 2K). Using Rab GTfase markers, colocalization analysis showed that compared with PACE4-FL, PACE4-altCT accumulated in Rab5-positive endosomal compartments and in Rab9-associated compartments (Fig. 2L–N), suggesting differential routing through an endosomal pathway. The enrichment of secretory pathway–associated proteins from IP-MS studies has been summarized on a cell-secretory pathway map together with the immunofluorescence results (Fig. 2F). These data support the notion that PACE4-FL and PACE4-altCT use different trafficking pathways, accounting for their secretion or...
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Figure 2. PACE4 alternative splicing results in 3'UTR shortening and in the generation of an isoform differentially retained by cells with enhanced stability and increased autoactivation. A, Actinomycin-D chase performed on LNCaP cells. Data, means ± SEM (n = 3). The table below the graph indicates the number of mRNA putative sites retrieved in both 3'UTR using miRDB (3B) and RegRNA2.0 (http://regrna2.mbc.nctu.edu.tw/) tools (see list in Supplementary Table S5). B, Luciferase reporter assay with the different PACE4 3'UTRs; the length refers to the stop codon used; see Fig. 1E (n = 2 at least in each conditions performed in duplicate). C, V5-tagged PACE4-FL or PACE4-altCT secretion kinetic in LNCaP (*" and "C" indicate cell lysate and medium, respectively). See Supplementary Fig. S4A and S4B for other cell lines and quantifications. D, Representative blot of a cycloheximide (CHX) chase in V5-tagged PACE4-FL or PACE4-altCT–transfected LNCaP cell lysates. E, Quantitative analysis of protein content in lysates during the cycloheximide chase in HEK293, DU145, and LNCaP (see Supplementary Fig. S4F; fi = fluorescent confocal images and quantitative colocalization analyzes with V5 and RCAS1, Rab5 (\textsuperscript{1})). Data, means ± SEM of at least 7 individual cells analyzed. IP, immunoprecipitation.

PACE4 splicing is regulated by intraexonic DNA methylation. We then focused our attention on the mechanisms regulating PACE4 terminal exon 25 substitution. According to ChIP sequencing (ChIP-Seq) data in the UCSC genome browser (Fig. 3A),...
CCCTC-binding factor (CTCF) is found to have three reported binding sites in the vicinity of the segment encoding the 25th alternative exon. CTCF has recently been reported to regulate upstream exon inclusion through the binding of nonmethylated CpG dinucleotides in intronic regions (25). The alternative 25th exon genomic segment, which includes the two CTCF binding sites, is located within a DNase hypersensitivity cluster and includes 7 CpG dinucleotides, which suggested a similar...
regulatory mechanism. RNA-seq data assessing the transcription levels in cell lines showed that transcription was sustained from the consensus 25th exon to the alternative 25th exon (Fig. 3A). It is important to note that the alternative 25th exon is only conserved in primates and is completely absent in the other available tested vertebrates, as it is the case for most splice variants (26).

Using bisulfite-pyrosequencing of DNA from both ANCT and cancerous specimens of prostate cancer, the methylation status of the different CpG dinucleotides found either within the CTCF-binding sites in the alternative terminal exon or in the site located in the 24th intron was characterized (Fig. 3B). Significant tumor-specific CpG hypomethylation was observed in both intragenic and upstream CTFC-binding sites (Fig. 3C) but not in other surrounding CpGs. This local hypomethylation suggests a tight regulatory mechanism that is most likely mediated by locus-specific recruitment of DNA-modifying or binding factors that protect DNA from methylation by DNA methyltransferases. Such tumor-specific regulation of CTFC-binding sites by methylation is known (27). It has even been shown that CTFC itself can regulate DNA methylation patterns and that cancerous or immortalized cells, when compared with normal cells, have a distinct CTFC-binding landscape in the genome (28), highlighting the complexity of methylation-related regulation, especially when addressed in a locus-specific manner.

Upon CTFC downregulation in cells, the splicing index (i.e., ratio of PACE4-altCT/PACE4-FL mRNA) was reduced by 70% (Fig. 3D–F), supporting the regulation of exon 25 substitution by CTFC. When these cells were treated with 5-aza-2’-deoxycytidine (5-aza-dc) to force genome hypomethylation (Supplementary Fig. S5A–S5C), splicing increased in a dose-dependent manner, further reinforcing the relationship between the observed alternative splicing and DNA methylation (Fig. 3G and H). After either treating the cells with 5-aza-dc treatment or following CTFC overexpression (Fig. 3I and J), PACE4 terminal exon splicing increased significantly (Fig. 3K). Combining both 5-aza-dc and CTFC expression resulted in even higher splicing levels. ChIP of endogenous CTFC confirmed the binding to DNA within the intronic regions in the 5’ as well as in the 3’ part of the alternative terminal exon and within the alternative exon itself (Fig. 3L), with a clear enrichment in the intragenic and the upstream region upon 5-aza-dc treatment (Fig. 3L; Supplementary Fig. S5D). These findings show the DNA methylation-dependent CTFC binding and its relationship with exon inclusion.

The expression of PACE4-altCT in normal tissues and various cancer types suggests a common tumor mechanism

The question arises as to whether PACE4 alternative splicing is only found in prostate cancer cells, or could also be found in other tissues or cancer types. Thus, we used RNA from normal human tissues and other cancer types to map both PACE4 isoforms. In normal tissues, PACE4-altCT mRNA was only strongly detected in the liver, the testis, and the brain/spinal cord (Fig. 4A; Supplementary Fig. S6A–S6F). Interestingly, the liver, testis, and brain are known for their higher rates of alternative splicing compared with other human tissues. Moreover, very high splicing indices were observed in fetal tissues, for example, liver and brain. Splicing indices were determined on an array of cDNA preparations from various tumor types and normal tissues (Fig. 4B). Some cancer types other than prostate cancer also displayed enhanced splicing activity in the tumor specimens, notably in lung, thyroid, adrenal, and pancreatic cancers. Other PCs were also evaluated in these normal (Supplementary Fig. S6H–S6L) and tumor specimen cDNAs (Fig. 4C). Among all PCs, PACE4, furin, and PC1/3 were the only ones that displayed frequent overexpression levels across numerous types of cancer (Fig. 4C; Supplementary Fig. S6H–S6L).

HIC analyses using normal and tumor specimens originating from different organs supported the results of the mRNA mapping in normal tissues, again with high levels of PACE4-altCT in colon and testis and the lowest levels observed in lungs, adrenal glands, and ovary (Fig. 4D; Supplementary Fig. S6M). Consistent with the observation at the mRNA level (Fig. 4B), the placed placentas as well as lymphoid tissues (thymus, lymph node, lymphomas, and tonsil) were all negative for PACE4-altCT by IHC, whereas organs such as the stomach, pancreas, liver, and kidney were positive to some extent (Supplementary Fig. S6N). Matched tumor specimens also corroborated the expression shift observed at mRNA levels in numerous cancer types, including, among others, lung, esophagus, testicular, and thyroid cancers, thus supporting the concept that PACE4 undergoes an oncogenic alternative splicing event in prostate cancer as well as in some other cancer types where it may act as an oncogenic driver.

PACE4-altCT is the main isoform responsible for prostate cancer cell–sustained growth capabilities

From a functional point of view, we then asked the question as to the phenotype acquired with each isoform. Thus, isoform-specific features were explored by both overexpression and gene silencing studies in cell-based assays. First, PACE4-FL and PACE4-altCT were stably expressed as untagged proteins in cell lines by stable lentiviral transduction. Despite similar mRNA expression levels, PACE4-altCT protein levels were much higher than PACE4-FL protein levels in whole-cell lysates (Fig. 5A and B; Supplementary Fig. S7A). Moreover, very little increase in secreted PACE4 was detected in the PACE4-altCT–overexpressing cells. When tested in cell proliferation assays, PACE4-FL and PACE4-altCT–overexpressing cells displayed enhanced growth and clonogenic capabilities, as depicted by proliferation (Supplementary Fig. S7B and S7C) and colony formation assays (Fig. 5C). However, PACE4-altCT overexpression yielded stronger effects, especially in LNCaP and HT1080 cells (Fig. 5C; Supplementary Fig. S7B and S7C). Levels of cognate PC mRNAs in these stable cell lines were evaluated, as well as varying patterns of PC7 and furin expression, suggesting a cross-talk between the pathways regulating these PCs (Supplementary Fig. S7D–S7F). These findings indicate that the overexpression strategy has some limitations.

Therefore, siRNAs specifically targeting each splice variant were designed to assess the importance of endogenous PACE4-altCT compared with its parent isoform PACE4-FL. Following transfection, each siRNA efficiently silenced its splice variant (i.e., 70%–95% knockdown without affecting the other coexpressed PCs; Fig. 5D; Supplementary Fig. S7G). Despite the lack of Western blot-compatible antibodies to specifically detect PACE4-FL separately from PACE4-altCT, the use of an anti-PACE4 antibody that recognizes the catalytic domain (and thus both isoforms) revealed a pattern that was coherent with observations for the tagged protein (Fig. 2C). The siRNA targeting PACE4-altCT resulted in a stronger reduction in intracellular levels of endogenous PACE4 than the siRNA targeting PACE4-FL (i.e., 45% vs. 13% reduction, respectively; Fig. 5E; Supplementary Fig. S7H). Conversely, in the conditioned medium, siRNA targeting PACE4-altCT had minimal effects on secreted PACE4, whereas siRNA targeting PACE4-FL.
resulted in a very large decrease (i.e., >80%). These data obtained with siRNAs and endogenous PACE4 correlate well with our previous observations concerning the differential secretion of the two isoforms, validating this approach to test the biological significance of PACE4-FL versus PACE4-altCT. Silencing of PACE4-altCT yielded a much stronger reduction in terms of cancer cell growth, in which PACE4-altCT plays a much more important role, allowing the conclusion that (i) the PACE4 knockdown–associated phenotype in prostate cancer cells (7) results from the downregulation of PACE4-altCT (Supplementary Fig. S7B) and (ii) efficacy of PACE4 inhibitors and cell permeability requirements are in fact due to PACE4-altCT–specific functions.

Identification of GDF-15 as a specific PACE4 substrate and pharmacologic target engagement marker
While our work points to the importance of sustained PACE4 activity to maintain prostate cancer cell growth and proliferation (7, 8), the substrate(s) of PACE4 that is responsible for these actions remains to be determined. Secreted factors have previously been suggested to be the main effectors of the PACE4-related cancer cell growth phenotype (7). Therefore, we turned to a SILAC-based proteomic approach to analyze the secretome content in both DU145 and LNCaP prostate cancer cells. The secretomes were pooled 1:1 (shNon-Target:shPACE4) and fractionated, and then each fraction was analyzed by tandem LC/MS-MS. From the protein identified, secreted proteins were retrieved using ProteINsIDE and used to draw a heatmap based on light/heavy (L/H; shPACE4/nontarget) ratio proportions for each cell line (Supplementary Fig. S8A). Proteins having PC-based or PC-like processing events, determined by both Uniprot PTM/Processing
PACE4-high-affinity PC inhibitor decanoyl-RVKR-chloromethylketone (CMK) or the
(iii) cell lines treated with either the nonselective and irreversible
PACE4 inhibitor had no effect, further supporting its
selectivity range (K_i: low nanomolar). In contrast, overexpression of PACE4-FL and PACE4-altCT increased the processing of
IGF1R and ITGA6 pro-forms, highlighting the cautionary inter-
pretation that is needed in overexpression studies. Regarding E-
cadherin, PC7 knockdown showed the best results in blocking the
processing of its pro-form (Fig. 6A; Supplementary Fig. S8B).

Among the candidate proteins with an L/H ratio <1 in either
DU145 or LNCaP, which displayed a PC-like cleavage site(s) (highlighted in bold in Supplementary Fig. S8A), many were
tested by Western blotting using antibodies allowing the discrimi-
nation of human pro- and mature protein forms, when available.

These included low-density lipoprotein receptor–related protein
1 (LRP1), hepatocyte growth factor receptor (HGFR, also known
as Met), clusterin (CLU), desmoglein-2 (DSG2), ADAM10,
ADAM17, and GDF-15 (Supplementary Fig. S8B–S8E). Some
other tested substrates could not be detected adequately by

Figure 5.
PACE4-altCT is responsible for PACE4-associated sustained growth
capabilities in prostate cancer cells. A, PACE4 protein levels (without tags;
using a catalytic domain targeting antibody) in stably overexpressing
LNCaP cells. See Supplementary Fig. S7A for DU145 cells’ data. B, PACE4
mRNA expression levels in qRT-PCR analyses in stable pLenti6-transfected
LNCaP cell lines (means ± SEM, n = 3). C, Colony formation assays on stably
expressing LNCaP and HT1080 cell lines (see Supplementary Fig. S7B for
colony size analysis). The image shows representative stained LNCaP wells
(n = 4 at least). D, PACE4 isoforms and cognate PCs expression levels in
siRNA-transfected LNCaP cells by qRT-PCR (n = 3). See Supplementary
Fig. S7G for knockdown in DU145. E, PACE4 protein levels (endogenous
levels using a catalytic domain targeting antibody) in siRNA-
transfected LNCaP cell lysates and their respective serum-free
conditioned media. See Supplementary Fig. S7H for knockdown in DU145. F, Colony
formation assays on transfected LNCaP at a density of 200 cells/well
for 12 days (n = 2 in duplicate). Representative fields are shown above
the relative quantitation relative to siNon-Target–transfected cells. See
Supplementary Fig. S7I for DU145 results. G, XTT proliferation assays
performed on LNCaP-transfected cells 72 hours after transfection (n = 2 in
triplet). See Supplementary Fig. S7J for DU145 results. Data, means ± SEM.

For validation, we chose to perform Western blotting arrays of
(i) cell lines silenced with shPACE4, shfurin, and shPC7 (7), (ii)
cell lines stably overexpressing PACE4-FL and PACE4-altCT, and
(iii) cell lines treated with either the nonselective and irreversible
PC inhibitor decaanoyl-RVKR-chloromethylketone (CMK) or the
PACE4 high-affinity C23 inhibitor. To test our Western blot arrays,
we selected known PC substrates, namely, IGF1R and integrin
alpha-6 (ITGA6), two well-accepted furin substrates (Fig. 6A;
Supplementary Fig. S8B; ref. 30) and E-cadherin, which has been
shown to be a furin and PC7 substrate (31). For both IGF1R and
ITGA6, only furin knockdown and CMK treatments prevented the
processing of their pro-forms, whereas PACE4 knockdown and the
C23 PACE4 inhibitor had no effect, further supporting its
selectivity towards PACE4 even in cells with concentrations above

data or using the ProP 1.0 server (29), were highlighted as
potential substrates.

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Western blotting. Among these potential PACE4 substrates, only GDF-15 (which is only expressed in the LNCaP line) appeared to be a unique PACE4 substrate (Fig. 6B), whereas the other substrates (LRP1, HGFR, DSG2, and CLU; Supplementary Fig. S8B–S8E) were by far mostly processed by furin and, to a much lesser extent, by PC7.

GDF-15 (also known as prostate differentiation factor; PDF, or macrophage inhibitory cytokine 1; MIF-1) is synthesized as a 35-kDa preproprotein that requires PC-based cleavage at the RRAR [196] site to generate an approximately 17-kDa C-terminal mature form that associates as a disulfide-linked dimer, which is further secreted into the medium (32). Virtually no mature GDF-15 was observed in the medium of shPACE4 knockdown or CMK- or C23-treated cells, whereas processed GDF-15 levels were still evident after furin and PC7 knockdown as assessed by both Western blotting and ELISA (Fig. 6B). In both PACE4-FL and PACE4-altCT–overexpressing cell lines, the amount of secreted GDF-15 was approximately 5 times higher (see ELISA, Fig. 6C) than in the pLenti6 control cells. Under all conditions, the secreted GDF-15 levels inversely correlated with the accumulation of pro-GDF-15 in the cell lysates, confirming that cleavage is a prerequisite for its secretion. These results demonstrate that GDF-15 is
cleaved by PACE4 with very limited redundancy with the other endogenously coexpressed PCs, and are in line with a previous report showing that GDF-15 remained uncleaved in PACE4-deficient PC3 cells (10, 33). Nevertheless, we also tested the in vitro cleavage of a GDF-15–spanning peptide incorporating the PC site (QAARGRRARRARNCG) with recombinant furin and PACE4 enzyme preparations (10). The GDF-15 peptide was cleaved correctly by both PCs (Fig. 6D), further demonstrating the importance of the cellular context and/or full-length substrates to fully comprehend PC functions.

A marked difference in terms of GDF-15 modulation was observed upon PACE4-FL or PACE4-altCT silencing. Following PACE4-FL knockdown, GDF-15 intracellular pro-GDF-15 levels increased by 2.9-fold. However, upon PACE4-altCT depletion, a robust 10.5-fold increase in intracellular pro-GDF-15 levels was observed (Fig. 6E), which was also accompanied by a strong increase in GDF-15 mRNA levels that was not visible under the siPACE4-FL condition (Fig. 6F) but occurred in the shPACE4 line (in which both splice variants were knocked down, see Supplementary Fig. S1D). When treated with the cell-permeable PACE4 peptide inhibitor (ML) and its PEGylated cell-impermeable version (PEG8-ML; refs. 10, 34), cleavage of GDF-15 was more susceptible to the ML-peptide, indicating that an important proportion of cleavage was performed inside the cells (Fig. 6G). Altogether, these data suggest that pro-GDF-15 mostly requires intracellular PACE4 cleavage (predominantly by PACE4-altCT; Fig. 6E), although we cannot exclude the possibility that it can also be retained at the cell surface (35) for potential cleavage by surface PACE4-FL. This phenomenon would be consistent with the data showing that overexpression of PACE4-altCT (mostly retained intracellularly) and PACE4-FL (both secreted and intracellular) resulted in similar increases in pro-GDF-15 processing (Fig. 6A and B). GDF-15 is an important growth factor for prostate tumor progression and resistance to cytotoxic agents and radiotherapy in prostate cancer and other cancers (36–41). Upon GDF-15 silencing (Fig. 6H), both LNCaP growth and colony formation capabilities were reduced to similar extent to those obtained upon PACE4-altCT silencing (Fig. 6I and J), further encompassing the importance of GDF-15 processing/secretion for its activity.

The knowledge that PACE4-altCT expression is strongly elevated in prostate cancer, as well as that it functions as the prominent PC for the processing of pro-GDF-15, provides us with a complete picture from epigenetic and alternative splicing events to substrate activity and biological impact. The highest tissue expression of GDF-15 occurs in the adult prostate gland, and GDF-15 is known to be overexpressed in prostate cancer (42), according to the Human Protein Atlas. However, there is an inverse correlation between pro-GDF-15 deposition in prostate cancer tumors and disease relapse, suggesting that the processing of pro-GDF-15 is an important factor in patient outcomes (35). In pairs of noncancerous and tumoral prostate tissues, the processing of pro-GDF-15 increased along with the tumor grading (Fig. 7A). It has also been reported that serum GDF-15 levels are elevated in patients with prostate cancer (43), which may serve as a companion biomarker with the PSA (44). With the knowledge of GDF-15 as a PACE4-specific substrate, we sought to investigate if it may serve as a target engagement biomarker in vivo to validate PACE4 inhibition in preclinical models. LNCAP-xenografted mice were intraperitoneally treated with 2 or 4 mg/kg of C23 every 24 hours. Both doses strongly inhibited tumor progression (Fig. 7B), which could also be observed on the basis of plasma PSA levels in the mice (Fig. 7C). In the plasma collected at the end of the 28 treatment days, the levels of GDF-15 measured by ELISA displayed a dose-dependent reduction but also correlated with the endpoint of tumor size (Fig. 7D and E). IHC analyses further confirmed the increases in terms of intracellular GDF-15 levels, which appeared as intracellular puncta that were predominately visible in the xenograft obtained from treated compared with control animals (Fig. 7F). Excised xenografts were either lysed and subjected to Western blot analysis or paraffin-embedded for IHC. In tumor lysates, GDF-15 processing displayed a significant reduction along with the PACE4 inhibitor doses (Fig. 7G). Moreover, proliferation, cell quiescence, and apoptosis markers (Ki67, p27, and cleaved PARPAsp214) showed a doserelapse pattern (Fig. 7H–J). Through the impact of C23 on GDF-15, these data validate for the first time that C23 pharmacodynamics is mediated by PACE4 inhibition, more precisely the PACE4-altCT isoform.

Discussion

The PCs are regarded as promising targets for the development of cancer therapeutics because of their position upstream of numerous oncogenic pathways (3, 45). Through the endoproteolytic processing of proproteins, including mediators involved in most of the key hallmarks of cancer, activation by PCs becomes a rate-limiting step following the overexpression of protein pathway components. Thus, when PC substrates are overexpressed by tumor cells, concomitant increases in terms of PC activity may occur to achieve a maximal biological outcome. PC overexpression has been documented in a number of cancer types (3), but the observed variations are not consistent across all cancer types (Fig. 4C; Supplementary Fig. S6G), which may be due to differences in tumor types or, alternatively, due to insufficient data because many studies commonly assume PC activity to be assigned to a single member, namely furin. With such diverse and sometimes fragmentary information, it remains difficult to ascertain the best PCs to target in cancers or cancer subtypes. On the basis of our previous work, we identified PACE4 as critical in prostate cancer, providing strong evidence of its overexpression as an indispensable component for sustained tumor growth. Despite this strong proof-of-concept, the molecular mechanisms were not well defined until now.

Discovery of the novel PACE4-altCT isoform, along with its strong expression in prostate cancer specimens, sheds light on an important mechanism of sustained proliferation that exploits PACE4 activity to promote tumor growth. An alternative splicing epigenetic switch generates the PACE4-altCT isoform (Fig. 3), which has drastically different characteristics in terms of cellular trafficking, autocalytic activation rates, and stability compared with the PACE4-FL isoform (Fig. 2). These processes result in a sophisticated molecular control mechanism that sustains global PACE4 activity through the evasion of several mRNA-regulatory elements (Fig. 8).

It should be noted that a previous study dating back to the late 1990s reported presumed splice variants (46, 47). Many of these encoding truncated isoforms included artefactual/frameshift errors (48, 49). Moreover, these splice variants were isolated using cDNA libraries (17). It is unclear whether these isoforms are produced in tissues or in disease states; however, another study showed that all of these isoforms were tested inactive in vitro.
because of important domain truncations leading to inactivezymogens retained in the ER (23). Our tumor-oriented splicing analysis did not detect any of these variants nor could we validate their tumor-specific expression (Supplementary Fig. S1). The only splice variant that could be validated was the previously not reported PACE4-altCT isoform. This new isoform is readily and actively converted to its active form despite being nonsecreted, which is a completely novel feature. We believe that PACE4-altCT may be a key element in cancer cell proliferation, as our observations were not limited to prostate cancer cells and tissues. Indeed
other cancer types also displayed increased PACE4-altCT/PACE4-FL mRNA expression ratios and positive PACE4-altCT expression by IHC (Fig. 4B–D), suggesting an important, although not ubiquitous, mechanism of action.

The discovery of PACE4-altCT permits us to refine our working model using pharmacologic PACE4 inhibitors. In previous studies, we showed that when our ML-peptide inhibitor was modified by the addition of an N-terminal polyethylene glycol moiety, it lost its antiproliferative activity toward prostate cancer cells, which was correlated with a strong reduction of cell-penetrating properties (10). However, no apparent biological explanation could conciliate this phenomenon. This study shows that the PACE4-altCT isoform is retained inside the cell, while PACE4-FL is secreted at the cell surface (Fig. 2C; Supplementary Fig. S4C–S4E). As PACE4-altCT exhibits most of the cancer cell–associated growth stimulating capabilities (Fig. 5), it is now clear that the antiproliferative effects of the ML-peptide are in fact due to inhibition of the PACE4-altCT isoform, even if the ML-peptide has equal inhibitory activity toward both isoforms (Figs. 2F and 6C).

Unraveling pro-GDF-15 as a PACE4 substrate defines PACE4 activity in the prostate cancer landscape, illustrating how it may sustain tumor progression. As a secreted factor, inhibition of its processing and further secretion likely restrict its action over its surface receptor (presumably TGFβ receptor II; ref. 50), it is though not surprising that PACE4-altCT silencing resulted in similar phenotype as GDF-15 silencing (Fig. 6I and J). GDF-15 is an important factor with immunosuppressive characteristics that supports prostate cancer cell growth (41, 51), but it is also known to protect cancer cells against radiation-induced cell death (36), to mediate tumor-mediated anorexia and weight loss (50) as well as to sustain tumor neovascularization (37). These observations however do not exclude that PACE4 probably have other substrates contributing to the observed effects. Serum GDF-15, which represents the mature form of this protein, has already been suggested as a potential prostate cancer biomarker, highlighting the relevance of the mechanism leading to its cleavage-dependent secretion (43, 44). Our data now extend to the conclusion that measuring pro-GDF-15 processing can serve as a means to monitor PACE4 activity in vivo, where target engagement would be evaluated in pharmacologic interventions using PACE4 inhibitors in cancer therapeutic strategies (Fig. 7). Having such markers is of great importance regarding the preclinical and clinical evaluation and readout of novel therapeutics (52). In our model, GDF-15 correlates directly with the tumor response, implying that its measurement is a direct reflection of PACE4 inhibition (Fig. 7F and G), which is not the case for the other markers of cell cycle/apoptosis (Fig. 7H–J) or for the tumor volume monitoring; the latter was not even able to show the dose-dependent activity of C23 (Fig. 7B).

The study of PC cell biology has often utilized transient or stable overexpression systems (i.e., of PCs and/or their substrates) due to the lack of selective inhibitors. Our study used both approaches and revealed that cautionary interpretations of the data must take into account misbalanced stoichiometries, altered levels of other endogenous PCs (Supplementary Fig. S7D–S7F), and altered cellular trafficking. As an example, we clearly observed the cleavage of ITGA6 by overexpressing PACE4 (both FL and altCT), while PACE4 silencing had no effect (Fig. 6A). In this same vein, a comparison of in vitro versus in cellulo analysis of substrate cleavage patterns also revealed potential misinterpretations of PC functions due to the lack of appropriate substrate–enzyme cellular compartmentalization (Fig. 6D). Previous studies have also made substantial efforts to define PC motifs to distinguish between unique and redundant PC substrates. However, these studies relied solely on in vitro analysis of small peptide cleavages (53), potentially explaining the conclusions of a lack of specific PC functions. As a final point, changes observed with overexpression studies, while useful, do not always reveal underlying mechanisms, as observed in the study by Mahloogi and colleagues, which showed malignant transformation of cells following transfection with PACE4 (PACE4-FL; ref. 5). While we do not dispute this result, we are now convinced that it is the result of an artificial experimental design that would not occur in cancer cells, with the underlying mechanism of malignancy being the genesis of the PACE4-altCT isoform.

While our focus was on the expression of PACE4-altCT in cancer cells and tissues, we also examined the expression of this splice variant in normal tissues. We observed that PACE4-altCT was expressed at levels comparable with those in tumor cells in normal testis, liver, and fetal tissues (Fig. 4). This finding suggests that the epigenetic PCSK6 splicing switch that yields either PACE4-FL or PACE4-altCT regulates underlying physiologic functions that are hijacked by cancer cells to sustain their growth by acting as a proto-oncogene. This hypothesis is also supported by the genomic environment of the 25th alternative exon, which is highly accessible because it is located in a DNase I sensitivity cluster.

Figure 8. Summary and working model. In cancer cells, CpG methylation levels in the intragenic region of the alternative terminal exon are reduced, which favors the binding of the DNA-binding factor CTCF and promote the inclusion of this terminal exon instead of the consensual one. The resulting mRNA then exhibits a distinct and shorter 3′UTR region lacking several of the miRNA sites present on the consensual transcripts. This increases mRNA stability and yields increased protein translation of the resultant isoform PACE4-altCT, which has a higher autocalytic cleavage rate compared with PACE4-FL and is rerouted in the alternative terminal exon instead of the consensual one. The resulting mRNA then exhibits a decrease in intraexonic region of the alternative terminal exon are reduced, which favors the inclusion of the alternative terminal exon instead of the consensual one. This study shows that the PACE4-altCT isoform, which was correlated with a strong reduction of cell-penetrating properties (10). However, no apparent biological explanation could conciliate this phenomenon. This study shows that the PACE4-altCT isoform is retained inside the cell, while PACE4-FL is secreted at the cell surface (Fig. 2C; Supplementary Fig. S4C–S4E). As PACE4-altCT exhibits most of the cancer cell–associated growth stimulating capabilities (Fig. 5), it is now clear that the antiproliferative effects of the ML-peptide are in fact due to inhibition of the PACE4-altCT isoform, even if the ML-peptide has equal inhibitory activity toward both isoforms (Figs. 2F and 6C).

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(Fig. 3A) and bound by DNA-binding elements such as CTCF according to the DNA methylation status. The finding that these epigenetic modifications are distinct between prostate cancer and closed benign zones of the tested patients illustrates the complexly of the genetic regulation, as well as the cancer-associated switching that occurs to solicit PACE4 alternative splicing (Fig. 8). It is noteworthy that PACE4-altCT is not conserved in nonprimate species, in which a similar genomic environment was lacking, thus not allowing terminal exon substitution. As this significant element of human PACE4 biology is absent in mice, murine models of prostate cancer, as well as other cancers or potentially other PACE4-related diseases, will require careful interpretation.

Cancer genomic studies using high-throughput approaches have generated ever-growing databases recording complex somatic genomic alterations. In most cases, it is rare to find common acquired mutations that are widespread across all patients with one type of cancer (54), or to find acquired mutations that are widespread across different types of cancer (55). While the PACE4-altCT isoform is not an acquired mutation, it is a significant change that promotes cancer cell proliferation and was detectable (at least at the mRNA level) in most patient samples that were available to us (>95%). Regarding potential applications, PACE4-altCT (mRNA or protein) could be used as a biomarker to identify PACE4-dependent cancers, which appears to be a nonnegligible constituent of many different cancer types (Fig. 4B). Coupled with a specific pharmacologic inhibitor of PACE4, as we have previously described (6), it may be possible to envisage a potent therapeutic strategy for PACE4-altCT-dependent cancers. Despite the observation that this initial discovery oriented us toward its use as a tissue biomarker (iHC analysis), many useful serum biomarker proteins are not secreted, but are still detectable in the circulation [e.g., HSP70 (56), S100B (57), CA125 (58)]. These proteins are actually secreted by tumor cells upon either tissue structure disorganization, tumor cell necrosis, or even apoptosis, which often occurs as hypoxic conditions arise in solid tumors (59). Thus, we believe that both total PACE4 and, most importantly, PACE4-altCT in patient serum could serve as biomarkers for prostate cancer, especially considering the tumor specificity of the alternative isoform and the correlation between its expression and tumor Gleason score (Fig. 1A, B, and H).

Our growing understanding of the mechanisms that manage the switch from paracrine to autocrine mediation of sustained growth signaling in late androgen-independent stages may provide novel strategies to circumvent androgen resistance and/or serve as biomarkers. PACE4 pharmacologic targeting could provide an alternative to presently used androgen therapies that eventually fail due to resistance mechanisms, after which the only remaining option is chemotherapy. Alternatively, targeting PACE4 could also be considered as an adjuvant to chemotherapy. Our new understanding of the mechanisms involved in PACE4-associated functions in cancer cells provides strong support for these notions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: F. Couture, R. Sabbagh, L. Bouchard, R. Day

Development of methodology: F. Couture, A. Kwiatkowska, S.-P. Guay, R. Day

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Couture, R. Sabbagh, A. Kwiatkowska, R. Desjardins, L. Bouchard, R. Day

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Couture, S.-P. Guay, L. Bouchard, R. Day

Writing, review, and/or revision of the manuscript: F. Couture, R. Sabbagh, A. Kwiatkowska, S.-P. Guay, L. Bouchard, R. Day

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References


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