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<th>Sonic hedgehog and androgens signaling in tumor and stromal compartments drives epithelial-mesenchymal transition in prostate cancer (前立腺癌の腫瘍ならびに間質領域にてSonic hedgehogおよびandrogenシグナルは上皮間葉系移行を作動させる)</th>
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Sonic hedgehog and androgens signaling in tumor and stromal compartments drives epithelial-mesenchymal transition in prostate cancer

Keywords: Sonic hedgehog, Prostate epithelial and stroma, Androgens, Epithelial-mesenchymal transition, Prostate cancer
ABSTRACT

Objective  Sonic hedgehog (Shh) signaling, androgens, and epithelial-mesenchymal transition (EMT) are related to prostate cancer (PCa) progression. The aim of this study is to investigate how Shh and androgen (dihydrotestosterone :DHT) signaling act in the prostate epithelial and stromal compartments and whether this signaling pathway drives EMT and promotes PCa progression.

Material and Methods  LNCaP, normal prostate fibroblast (NPF) and cancer-associated prostate fibroblast (CPF) cells were studied with DHT and/or Shh signaling inhibitor, cyclopamine (Cy). Real-time RT-PCR was performed to evaluate the expressions of a potential Shh target gene, osteonectin (ON), and EMT-associated markers (E-cadherin, Vimentin, and N-cadherin). Immunohistochemical (IHC) studies using PCa prostatectomy samples were performed to assess the expression levels of ON, Gli-1, Androgen receptor (AR), Shh, E-cadherin, N-cadherin and Vimentin.

Results  While DHT enhanced cell proliferation in CPF more than LNCaP or NPF, Cy inhibited cell proliferation enhanced by DHT in CPF. Real-time RT-PCR showed whereas both Shh and DHT induced N-cadherin and Vimentin, DHT also induced the expression of ON in LNCaP and Cy blocked these expressions in ON, N-cadherin and Vimentin (p=0.0084, p=0.0002 and p=0.0373, respectively). IHC results showed high expression of stromal ON (ONstr), not epithelial ON (ONepi), was significantly correlated with serum PSA (p=0.031), and high expression of Gli-1 and low expression of ONstr with PSA recurrence (p=0.0114 and p=0.0005, respectively).
Conclusions  Shh and androgen signaling in prostate tumor and stromal compartments drives EMT, and thus may play some role in PCa progression. Shh signaling inhibitor, Cy may be one therapeutic strategy for PCa.
Introduction

Hedgehog (Hh) is a developmental signaling pathway that regulates embryonic cell growth, body pattern formation and organogenesis in certain vertebrate tissues. Hh signaling leads to increased activity of Gli transcriptional factors and increased transcription of Gli target genes (1), and its signaling pathway driven by Hh ligands regulates the genes that are involved in cell proliferation, differentiation and cell motility (2). Although it is involved in the epithelial and stromal compartments during normal prostate development and in regulating growth, maturation and maintenance of the differentiated state in the adult (3), there is increasing evidence that dysregulated Hh signaling plays some role in prostate cancer (PCa) (4). Also, stromal fibroblasts respond to and actively react with stimuli from cancer epithelium (5).

On the other hand, androgen regulates the expression of several key molecules in the Hh signaling pathway including members of the Gli transcription factor family. The potential relationship between androgen and the expression of some critical molecules in the Hh signaling pathway might explain some of the aberrations in Hh signaling reported to occur in PCa. Androgen depletion strongly upregulates expression of Hh ligands and Gli targets, and markedly elevates sonic hedgehog (Shh) expression in human PCa cells (1). This indicates the potential for chronic androgen deprivation therapy to create a Hh signaling environment for primary or metastatic prostate tumors that might improve the response of these tumors to therapy on a long-term basis (4). Therapeutically targeting Hh signaling in human primary and/or bone metastatic PCa could therefore prove to be beneficial (6).

Recently, osteonectin (ON) was identified in interaction between normal human
prostate stromal cells and PCa; moreover, recombinant Shh protein was identified as not only a new Shh-Gli1 target gene but possible Shh target gene in our previous work (7). In addition, epithelial-mesenchymal transition (EMT) is known to promote cancer progression via loss of cell adhesion, repression of E-cadherin expression and increased cell mobility; there are several oncogenic pathways that induce EMT (8).

In this study, we investigated how Shh signaling and representative androgen, dihydrotestosterone (DHT) works in prostate epithelial and stromal compartments and drive EMT in PCa progression, using cell proliferation assays and real time reverse transcription polymerase chain reaction (RT-PCR). We used immunohistochemical (IHC) analyses to explore the significant markers for aggressive clinical PCa, including high serum prostate specific antigen (PSA) and high Gleason scores (GSs), especially focusing on potential epithelial or stromal Shh signaling target genes, ON expression, and biochemical recurrence in PCa patients.
Material and Methods

**Cell culture and conditioned medium (CM) collection**

Established LNCaP PCa cells were cultured by standard methods using the Roswell Park Memorial Institute-1640 medium (RPMI-1640 medium, Sigma Chemical, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (MP Biomedicals Inc, Seven Hills, Australia). Tissues for primary prostate stromal culture were obtained from consenting patients with PCa undergoing radical prostatectomy at the Emory University School of Medicine, Atlanta, GA, USA. This study was approved by the Institutional Review Board (IRB) Committee. The origins of the stromal fibroblasts were: 1) normal prostate fibroblast (NPF) from normal/benign lesions of the human prostate gland and 2) cancer-associated prostate fibroblasts (CPF) from cancerous lesions of prostatectomy specimens as described previously (9). The Hh signaling inhibitor cyclopamine (Cy) (Toronto Chemical Inc., Toronto, ON, Canada) was dissolved and diluted with dimethyl sulfoxide (DMSO) to a 10 mM stock solution. Androgen, DHT (Sigma, St. Louis, MO, USA) was dissolved and diluted with ethanol to a 10 mM stock solution. Recombinant human Shh protein (Neuromics, Edina, MN, USA) was dissolved and diluted by DMSO with 0.1 % weight of PBS at 25 μg/ml of the stock solution. For conditioned medium (CM) collection, cells were cultured in RPMI-1640 medium supplemented with 10% FBS until 50% confluent. The cells were washed once in phosphate-buffered saline (PBS) and twice in T-medium with penicillin. Thereafter, the cells were incubated in 10ml of T-medium with penicillin. After 48 hours of incubation, CM was collected, centrifuged, and aliquat was stored at -20°C until use (9).
**Cell proliferation assay**

Cell proliferation was investigated by Alamar Blue assay (BioSource International Inc. Camarillo, CA, USA) in PCa LNCaP cells and prostate stromal cells (NPF and CPF). The cells were initially seeded in a 100 mm cell culture dish in RPMI-1640 medium supplied with 10% FBS. Near 80% confluence, the cells were seeded in 96-well plates at $5 \times 10^3$ cells/well for 24 hours, then switched to FBS free medium. First, to evaluate the efficacy of both Cy and DHT in a dose-dependent manner in LNCaP cells, 96-well plates were divided into 4 divisions, one division as control and the other three divisions treated with three different doses of Cy or DHT. After 48 hours, Alamar Blue reagent was added at 10 micron per well followed by incubation at 37 °C and 5% CO$_2$ for 1 hour, followed by recording the absorbance of the converted dye at a wavelength of 570 nm. This was also repeated after 96 and 144 hours. For the LNCaP, NPF, and CPF cell plates, each was divided into 4 divisions: Group-1, a vehicle-control group with DMSO added; Group 2, DHT added at a concentration of 10 µM; and Group 3, both Cy added at a concentration of 5µM and DHT added together at the same concentration. In addition, in order to evaluate the effect of stromal cells CM on PCa cell proliferation, CM from NPF and CM from CPF were added to LNCaP and cell proliferation was assayed after 48 hours treatments. CM was prepared according to a previous study (9). All experiments were carried out in triplicate. Data were expressed as mean ± standard deviation (S.D.) and evaluated for statistical significance.

**RT-PCR**

Total RNA was isolated from confluent monolayers of cells using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA), followed the manufacturer’s instructions.
First-strand cDNA was synthesized from total RNA (0.2 μg) using SuperScript II reverse transcriptase (Invitrogen) with random hexanucleotide primers. Quantitative real-time PCR was performed using SYBR Green PCR Master mix and the ABI 7500 Fast Real-Time PCR System (Applied Biosystem., Carlsbad, CA, USA). A hot start at 95 °C for 5 minutes was followed by 40 cycles for denaturation at 95 °C for 15 seconds, annealing of the primers at 60 °C for 30 seconds and elongation at 72 °C for 30 seconds. Data were normalized to β-actin and represented as the average ratio of duplicates, according to the ΔΔ Ct method. The oligonucleotide primer sets are shown in Table I. These primers were designed in our laboratory.

**IHC study**

IHC staining of radical prostatectomy (RP) specimens of 25 patients from the Department of Urology, Akashi Municipal Hospital, was performed as previously described (9). The distribution of GSs was 6 in 3 patients, 7 in 14 patients, 8 in 5 patients and 9 in 3 patients. This research was carried under compliance with the Helsinki Declaration. Briefly, the sections were then incubated with the following antibodies: anti-human androgen receptor (AR) rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-human Gli-1 rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-human ON mouse monoclonal antibody (Abnova, Jhongli, Taiwan), anti-human Shh rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-human E-cadherin mouse monoclonal antibody (DAKO, Carpinteria, CA, USA), anti-human N-cadherin mouse monoclonal antibody (DAKO) and anti-human Vimentin mouse monoclonal antibody (DAKO). The sections were then incubated with biotinylated goat anti-mouse or rabbit immunoglobulin G (Vector Laboratories, Burlingame, CA). After incubation in
avidin–biotin peroxidase complex for 30 minutes, the samples were exposed to
diaminobenzidine tetrahydrochloride solution and counterstained with methyl green
(Vector Laboratories) or hematoxylin.

**IHC analyses**

IHC staining results were scored by two independent observers who were blinded to
the clinicopathological data. If discordant interpretations were obtained, differences
were resolved by joint review and/or consultation with a third observer experienced
in IHC pathology. Both cytoplasmic and nuclear staining for AR and only
cytoplasmic staining for Gli-1 and Shh was considered positive as previously
reported (10, 11). IHC was evaluated by the percentage of positive cells. For Gli-1
and Shh, if more than 10% of the tumor cells were stained, gene expression was
considered to be “positive” (<10 %, −; 10-50 %, 1+; 50-90 %, 2+; >90 %, 3+) (11).
The frequency of ON IHC was scored as 0 when no staining of the tumor cells was
observed, weak staining (score 1) when less than 30 % of the tumor cells were
stained, moderate staining (score 2) when 30-60 % of the tumor cells were stained,
and strong staining (score 3) when more than 60 % of the tumor cells were stained.
The total IHC score was determined as the sum of the frequency and intensity scores
for tumor cells (low expression score: 0-3 and high expression score: 4-6) (12). In
addition, the staining of ON was recorded as positive in epithelial (ONepi) or
positive in stroma (ONstr). The staining localization of E-cadherin, N-cadherin and
Vimentin IHC were classified as nuclear, cytoplasmic and membranous. The staining
intensity was scored as 0 (negative), 1+ (weak), 2+ (medium) or 3+ (strong). The
percentage of stained cells was categorized into; 1= 0-10% stained cells; 2= 11-50%
stained cells; 3= >50% stained cells (13). We showed IHC figures with normal power (x 200) and high power (x 400) images.

The expression levels (histopathological gradings) of ON, Gli-1, AR, Shh, E-cadherin, N-cadherin and Vimentin were investigated including the comparison between normal and cancer area and analyzed in correlation with clinicopathological data such as patients’ initial PSA level, GSs in the prostatectomy specimen, and biochemical recurrence. Biochemical recurrence is defined as PSA rise-up to 0.2 ng/ml or PSA nadir > 0.1 ng/ml after prostatectomy.

**Statistical analysis**

Statistical analysis was conducted with the JSTAT - Java Virtual Machine Statistics Monitoring Tool (Sun Microsystems, Inc., Santa Clara, CA). The results of in vitro experiments were analysed by Student’s t test. The correlations of the protein expression in immunohistochemical analyses (ONepi, ONstr, Gli-1, AR, Shh, E-cadherin, N-cadherin and Vimentin) with clinicopathological data were analysed by the Spearman rank-order correlation. Statistical significance was established at p<0.05.
Results

LNCaP cell proliferation after treatment with cyclopamine

Hh signaling inhibitor, Cy (5, 10, and 20 µM) inhibited LNCaP cell proliferation in 96 hours treatments and revealed its inhibition in a dose-dependent manner especially in 144 hours treatments (Fig. 1).

Comparison of LNCaP, NPF and CPF responses to DHT and cyclopamine

We compared the responses of LNCaP cell lines and NPF or CPF to DHT and DHT + Cy in order to investigate how the prostate tumor and stromal compartments react to DHT and Cy. First, the results showed that DHT tended to enhance cell proliferation in CPF x 1.71 times (significant difference from control; p<0.0001) (Fig. 2C) more than in LNCaP (x 1.14) (significant difference from control; p=0.0001) (Fig. 2A) or NPF (x 1.13) (significant difference from control; p>0.05 (Fig. 2B).

Next, the addition of Cy to DHT showed significantly inhibited effects in LNCaP (x 0.917; p=0.0059) and CPF (x 0.788; p=0.0006), but not NPF (x 0.912; p=0.0887). These data suggested that CPF have potentially more reaction to DHT with the addition of Cy than LNCaP or NPF, indicating that Cy could have more of an effect on cancer associated stromal cells but not necessarily from cancer cells nor normal/benign prostate stromal cells. (Fig. 2).

The comparison of LNCaP cell proliferation in the presence of NPF CM and CPF CM

We compared the LNCaP cell proliferation assay with CM from NPF and CPF. LNCaP cells in the presence of CM from grew faster than those with CM from NPF
(p=0.0017) (Fig 3).

**Shh and cyclopamine effects on EMT in LNCap**

Recombinant Shh protein (1 μg/ml) suppressed the expression of E-cadherin (x 0.29) compared with vehicle control (p=0.0266) and enhanced the expression of N-cadherin (x 5.4) and Vimentin (x 215) in real time RT-PCR, and these changes in N-cadherin and Vimentin were blocked by the Shh signaling inhibitor Cy (p=0.0344 and p=0.0158, respectively) (Fig. 4A).

**ON and EMT expressions by DHT and/or cyclopamine in LNCap**

DHT induced the expression of the potential Shh signaling target gene ON (x 31), N-cadherin (x 5.3) and Vimentin (x 1.5), and suppressed the expression of E-cadherin (x 0.67). Cy blocked these enhanced- or suppressed- expressions. However, the effects of Cy or DHT+Cy were indicated in ON, N-cadherin and Vimentin (in Cy, p=0.0084, p=0.0002 and p=0.0373, respectively) (in DHT+Cy, p=0.0002, p=0.0007 and p=0.0085, respectively) (Fig. 4B and 4C).

**IHC staining results**

Representative findings from IHC staining of PCa specimens for expression of ON, and Gli-1 are shown in Fig. 5. Strong epithelial (Panel A; A-1: normal power image (x 200) and A-2: high power image (x 400); the same shall apply hereafter.), stromal (Panel B (B-1 and B-2)), expressions of ON in PCa area were shown. Panel C showed strong expression of Gli-1 in the PCa area (Panel C-1 and C-2). Panel D showed strong expressions of AR in PCa area (Panel D-1 and D-2). Panel E also showed strong expressions of Shh in PCa area (Panel E-1 and E-2). Panel F showed
down-regulation in the epithelium of E-cadherin in PCa area (Panel F-1 and F-2). Panel G showed strong epithelial expressions of N-cadherin in PCa area (Panel G-1 and G-2). Panel H showed strong stromal expressions of Vimentin in PCa area (Panel H-1 and H-2) (Fig. 5).

**Relationship between IHC analyses and patients’ clinical and pathological data**

Statistical analyses of clinical data and IHC results (Table II) showed that ONstr had a significant correlation with serum PSA ($p=0.031$). Importantly, PCa recurrence had a significant correlation with Gli-1 ($p=0.0114$) and low expression of ONstr ($p=0.0005$), suggesting that the expression of ONstr may correlate to PCa initiation as shown in our previous work (9). The significant correlation was not found between clinicopathological data and EMT marker (E-cadherin, N-cadherin and Vimentin). Also, the significant correlation between serum PSA and IHC stainings was not found in both normal and cancer tissue (Table III). As to cytoplasmic AR stainings, we did not find that cytoplasmic staining was specific to AR in all Gleason grades ($p=0.1489$).
Discussion

The Hh signaling pathway has an axial function in the development and patterning of many endodermally derived tissues, and evidence from several studies suggests that Hh pathway activity in PCa promotes tumor growth, invasion, metastasis, and hormone independence (7). In recent PCa chemotherapy, targeting the Hh pathway has shown promising results, and Smoothened (Smo) and Glis have been considered primary targets for anti-Hh therapeutics (14). A corn lily steroidal alkaloid, Cy, inhibits intracellular Hh signaling by blocking the activity of Smo. Efforts to develop and apply anti-Hh therapeutics have been ongoing for several years (15).

In this study, CPF reacted to androgens (DHT) more than LNCaP or NPF and to Cy in the presence of DHT more than LNCaP or NPF, and also our CM study showed CM from CPF significantly affected LNCapP cell proliferation more than NPF. These results suggest, taken together with our previous study (16), that CPF may be a reactive stroma especially in the presence of androgens (DHT) and may have different proliferative signaling from NPF in this condition. Our findings in CPF may offer a hint for understanding androgen-dependent PCa (LNCaP) cell heterogeneity and the evolution from androgen-dependent to independent PCa. These in vitro results, taken together with our previous studies (5), suggest that Cy and other Hh signaling inhibitors may be a possible therapeutic strategy for PCa not only at an early stage, equivalent to the situation of LNCaP mainly surrounded by NPF in vitro, but in advanced stage cancer, equivalent to where LNCaP is mainly surrounded by CPF in vitro (1, 6, 16) in the presence of androgens (DHT). Interestingly, these findings were partly different from our previous work using androgen-independent PCa cells especially in the response to NPF and CPF including their derived CM (9).
This difference may be a hint for understanding of change of androgen-dependent to 
–independent feature in PCa cells for their growths

The complex roles of ON in different tumors may be attributed to the 
bioavailability of either the whole molecule or its cleavage products (17). Since ON 
was identified as a major bone-derived chemo-attractant for PCa cells in vitro (18), it 
may play a role not only in PCa bone metastasis but also for its growth or 
proliferation. Jacob et al. stated that ON enhanced invasion and migration of PCa 
(19). Consistent with those data, our statistical analysis of clinical data and PCa IHC 
results showed that only ONstr, not ONepi, had significant correlation with the 
serum PSA level of PCa patients (p=0.031). Additionally, our androgens 
(DHT)-induced-ON expression was also abrogated by Cy and it suggests that 
androgens (DHT) might be located upstream of Cy. In addition, our Gli-1 expression 
was significantly related to PCa recurrence and ONstr was significantly related to 
non PCa-recurrence, indicating that Gli-1 expression may have involvement with 
PCa progression while ONstr expression might lead to early status PCa as shown in 
our previous works (9).

EMT is well known to be related to tumor invasiveness and metastasis in human 
carcinomas, including PCa (20-24), by upregulation of mesenchymal genes such as 
Vimentin and N-cadherin and down-regulation of epithelial-associated markers such 
as E-cadherin (21). Maitah et al. showed that transcriptional up-regulation of Shh by 
TGF-β1 induced EMT phenotype and aggressive behavior in non-small-cell lung 
cancer cells (25) and Elkhatib et al. stated that blocking the Hedgehog pathway in 
cyclopamine inhibited EMT (26). We demonstrated that the Shh-induced and 
DHT-induced expressions of EMT markers (E-cadherin, Vimentin, and N-cadherin,)
in LNCaP cells were blocked by Cy, suggesting, along with the results of ON
expression studies, that Cy and its analogues could be a therapeutic strategy for PCa as mentioned above and that EMT markers may locate downstream of Shh and/or androgen (DHT) signaling. However, differently from in vitro data, we could not find the correlation between clinicopathological data and EMT markers in IHC. Lehmann et al. stated that intrinsic, genetically regulated pathways and environmental factors affected physiology and phenotype of tumor cells in EMT transcription factor (27), suggesting our patients group in vivo might have unique environmental factors which were not driven by EMT tested in this study.

We would like to emphasize the limitations of this study. Future studies with a larger number of patient’s samples are needed for definite evaluation of the markers of PCa progression. In addition, although this study focused on the relationship between Shh signaling, androgens (DHT) and EMT in the prostate tumor and stromal compartments in PCa and the relationship of their downstream targets with clinical data (serum PSA, high GSs, and recurrence in PCa patients), there might be other molecules not involved in the regulation of Shh signaling which are more closely associated with PCa patient prognoses. In addition, the mechanistic study was lack of protein level analyses by such as western blots partly because of the problems of antibodies and our study did not obtain convincing results for EMT marker expression in our IHC staining or a signaling link with Shh and/or androgens (DHT). This should also be explored with a greater number of patient samples to further understand the relationship between Hh signaling and EMT markers in PCa progression.

In conclusion, Shh signaling plays a role in PCa through a relationship with androgens and drives EMT in the tumor and stromal compartments. DHT-induced
ON expression or reactive prostate stroma (cancer-associated stroma) may be a possible target in PCa progression especially in androgen independent status and Hh signaling inhibitor Cy might be a potential therapeutic strategy for PCa.
Conflict of interest

We have no conflict of interest.
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isoform of ErbB3 promotes osteonectin expression in bone and enhances the
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Snail-mediated regulation of reactive oxygen species in ARCaP human prostate
localization of p120 catenin during epithelial to mesenchymal transition of colon
Endothelin-1 promotes epithelial-to-mesenchymal transition in human ovarian cancer
growth factor receptor-1 activation mediates epithelial to mesenchymal transition in
hedgehog contributes to TGF-beta1-induced epithelial to mesenchymal transition in

Figure legends

Fig. 1 In vitro cell proliferation assay in LNCaP cell line treated with DMSO (vehicle control) or 3 sets of concentrations of hedgehog (Hh) signaling inhibitor, Cyclopamine (Cy: 5, 10, and 20 µM). Cy inhibited LNCaP cell proliferation in 96 hours treatments and revealed its inhibition in a dose-dependent manner especially in 144 hours treatments. Y axis revealed the relative cell proliferation with setting vehicle control at the initiation of treatments (0 hour) as 1.0 and X axis showed the time course (0, 48, 96, and 144 hours).

Fig. 2 In vitro cell proliferation assay in three cell lines: (Panel A) LNCaP, (Panel B) NPF, normal prostate stromal fibroblasts, (Panel C) CPF, cancer-associated prostate stromal fibroblasts, treated with DMSO (vehicle control) or dihydrotestosterone (DHT (10 µM) or dihydrotestosterone and Cyclopamine (DHT (10 µM) + Cy (5 µM), respectively). Panels A, B, and C demonstrated that DHT enhanced cell proliferation in LNCaP and CPF and the addition of Cy significantly inhibited proliferation in these cells. In them, considering p-value and the ratio of change by DHT and Cy, CPF may respond to DHT and DHT + Cy. Each column represents triplicate averages ± SD. All significant differences were shown as p-value. N.S. = “not significant”. Y axis revealed the relative cell proliferation with setting vehicle control at the initiation of treatments (0 hour) as 1.0.

Fig. 3 The comparison of the growth in LNCaP cells in the presence of NPF- and CPF-derived conditioned medium (CM).
RNA expressions of EMT markers (E-cadherin, N-cadherin and Vimentin,) were investigated in the presence of recombinant sonic hedgehog (Shh) protein with or without Cyclopamine (Cy) in LNCaP cells. Shh induced the expression of N-cadherin and Vimentin and their enhanced expressions were abrogated by Cy. Conversely, Shh suppressed the expression of E-cadherin and its suppression was recovered by Cy (Panel A). RNA expressions of potential Shh signaling target gene, Osteonectin (ON) and EMT markers (E-cadherin, N-cadherin and Vimentin,) were investigated in the presence of dihydrotestosterone (DHT) with or without Cy in LNCaP cells. DHT enhanced the expressions of ON, N-cadherin and Vimentin, and these enhanced expressions were blocked by Cy even though the effect of Cy or Cy +DHT were varied in each marker. Conversely, DHT suppressed E-cadherin expression and Cy or Cy +DHT blocked this suppressed expression. However, the effect of Cy or Cy +DHT was not consistent (Panel B and C).

Typical outcomes of immunohistochemical (IHC) staining of PCa specimens with ON, Gli-1, AR, Shh, E-cadherin, N-cadherin and Vimentin antibodies. Panel A showed strong epithelial expression of ON in the PCa area (An arrow indicates the positive cells in epithelial area.) (Panel A; A-1: normal power image (x200) and A-2: high power image (x400); the same shall apply hereafter.). Panel B revealed strong stromal expression of ON in the PCa area (An arrow indicates the positive cells in stroma.) (Panel B (B-1 and B-2)). Panel C showed strong cytoplasmic expression of Gli-1 in the PCa area (An arrow indicates the positive cells in cytoplasm.) (Panel C-1 and C-2). Panel D showed the epithelial and stromal expression of AR in the PCa area (An arrow indicates the positive cells
in epithelium.) (Panel D-1 and D-2). Panel E showed the epithelial and stromal expression of Shh in the PCa area (Arrows indicate the positive cells in epithelium (single arrow) and stroma (double arrows).) (Panel E-1 and E-2). Panel F showed the low epithelial expression of E-cadherin in the PCa area (Panel F-1 and F-2). Panel G showed the epithelial expression of N-cadherin in the PCa area (An arrow indicates the positive cells in epithelium.) (Panel G-1 and G-2). Panel H showed the stromal expression of Vimentin in the PCa area (An arrow indicates the positive cells in stromal area.) (Panel H-1 and H-2).
Table I  PCR primer

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Table II  Correlation of the protein expression in immunohistochemical analyses with serum PSA or high Gleason scores (≥8) or PSA recurrence

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*bold: statistically significant (p<0.05)
ON epi: epithelial osteonectin
ON str: stromal osteonectin
Table III  Correlation of the protein expression in immunohistochemical analyses of normal prostate area and prostate cancer area with serum PSA

<table>
<thead>
<tr>
<th></th>
<th>serum PSA (normal)</th>
<th>serum PSA (cancer)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p-value</td>
<td>p-value</td>
</tr>
<tr>
<td>Gli-1</td>
<td>0.5326</td>
<td>0.9071</td>
</tr>
<tr>
<td>Shh</td>
<td>0.9003</td>
<td>0.7494</td>
</tr>
<tr>
<td>AR</td>
<td>0.9304</td>
<td>0.7471</td>
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<tr>
<td>ONepi</td>
<td>0.6132</td>
<td>0.1456</td>
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<tr>
<td>ONstr</td>
<td>0.3284</td>
<td>0.1518</td>
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<tr>
<td>E-cadherin</td>
<td>0.0749</td>
<td>0.3583</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>0.4773</td>
<td>0.1804</td>
</tr>
<tr>
<td>Vimentin</td>
<td>0.5177</td>
<td>0.3914</td>
</tr>
</tbody>
</table>

ON epi: epithelial osteonectin
ON str: stromal osteonectin
Fig. 2

A) N.S.  
B) N.S.  
C) N.S.

V: vehicle control  
D: DHT  
D+Cy: DHT+Cyclosporine  
N.S.: not significant
Fig. 3

V: vehicle control
CM: conditioned media
NPF: normal prostate fibroblast
CPF: cancer-associated prostate fibroblast
N.S.: not significant
Fig. 4

A) E-cadherin

V: vehicle control
S: recombinant Shh protein
S+Cy: recombinant Shh protein + Cyclopamine
N.S.: not significant

H-cadherin

Vimentin
Fig. 4
B)

V: vehicle control
D: DHT
Cy: Cyclopaamine
N.S.: not significant
Fig. 4  
C)

N-cadherin

V: vehicle control  
D: DHT  
Cy: Cyclopamine  
N.S.: not significant