Stromal regulation of prostate cancer cell growth by mevalonate pathway enzymes HMGCS1 and HMGCR

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Abstract. It has been suggested that the tumor microenvironment plays an important role in tumor progression, acquisition of androgen independence, and distant metastasis in prostate cancer (PC). However, little is known about the transcriptional basis of cellular interactions in the human PC microenvironment. To clarify the mechanism of PC progression and metastasis, we investigated the interaction of PC, epithelial, and stromal cells using genome-wide gene expression profiling. We hypothesized that PC cells could induce stromal cells to differentiate into so-called cancer-associated fibroblasts (CAFs), which might contribute to cancer invasion and metastasis. Genes upregulated in normal human prostate stromal cells (PrSC) co-cultured with human PC cells (LNCaP) included the mevalonate pathway enzymes 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR). Knockdown of endogenous HMGCS1 or HMGCR in PC cells by shRNA resulted in a significant reduction of PC cell viability. Importantly, exogenous overexpression of HMGCS1 or HMGCR in either PC cells or prostate stromal cells stimulated PC cell growth, suggesting a possible autocrine/paracrine mechanism of action. Immunohistochemical analysis confirmed that HMGCS1 and HMGCR were overexpressed in PC stroma, especially in early stage PC. These results provide clues to the molecular mechanisms underlying PC invasion and metastasis, and suggest that HMGCS1 and HMGCR in PC, as well as in PC stroma, might serve as molecular targets for the treatment of PC.

Introduction

The prognosis of prostate cancer (PC) depends greatly on tumor invasion and distant metastasis. In PC it has been suggested that the tumor microenvironment plays an important role in progression, acquisition of androgen independence, and distant metastasis (1-3). We previously conducted a genome-wide loss of heterozygosity/allelic imbalance (LOH/AI) scan of DNA from the epithelium and stroma of 116 PCs and identified a total of 43 LOH/AI hot/cold spots, of which 17 were associated with both the epithelium and stroma, 18 were unique to the epithelium, and eight were unique to the stroma (4). We also identified 15 LOH/AI markers that were correlated with Gleason scores in that study. However, our understanding of the role of the microenvironment in human PC remains limited.

Epithelial-to-mesenchymal transition (EMT) has gained considerable attention as a conceptual paradigm to explain invasive and metastatic behavior during cancer progression (5). It has been proposed that EMT is induced by cancer cells during metastatic dissemination from a primary organ to secondary sites, but precisely how EMT occurs during PC invasion and metastasis remains uncertain.

To clarify the mechanism of tumor progression and metastasis in PC, and the role of the tumor microenvironment, we investigated the molecular interaction of PC cells, prostatic epithelium, and prostatic stroma through genome-wide gene expression profiling. We hypothesized that PC cells might act on stromal cells to induce their differentiation into cancer-associated fibroblasts (CAFs), thus contributing to tumor invasion and metastasis. Likewise, we hypothesized that CAFs could act on surrounding normal epithelial cells to change their characteristics into PC cells.

Materials and methods

Cell lines. The human PC cell lines LNCaP, PC-3, and 22Rv1 were obtained from the American Type Culture Collection (Rockville, MD, USA). The human normal prostate epithelial cell line PrEC and the human normal prostate stromal cell line PrSC were purchased from Lonza Group Ltd. (Basel, Switzerland). Cells were cultured as monolayers in appropriate medium supplemented with 10% fetal bovine serum (FBS), and maintained at 37˚C in an atmosphere of humidified air with 5% CO₂.

Co-culture experiments. Co-culture experiments were performed as shown in Fig. 1. LNCaP, PrEC, and PrSC cell lines were cultured in 6-well plates or culture plate inserts (0.4 µm
pore size; Corning Inc., Corning, NY, USA). On day 1, PrSC (2x10⁶ cells/well) were plated onto 6-well plates using SCG media (Lonza Group Ltd.) while PrEC (2x10⁶ cells/well) and LNCaP (1x10⁶ cells/well) were plated onto culture plate inserts using PrEGM (Lonza Group Ltd.) or DMEM plus 10% FBS media, respectively. On day two, culture media were replaced with keratinocyte-SFM (Thermo Fisher Scientific, Inc., Waltham, MA, USA) plus 2% FBS and the inserts containing PrEC or LNCaP were then transferred into 6-well plates containing PrSC cells to initiate the experiment. On day five, total RNA was isolated for microarray analysis. For PrEC/PrSC/LNCaP co-culture, the inserts containing PrEC were transferred into 6-well plates containing PrSC previously co-cultured with LNCaP (PrSC/LNCaP), and cultured for three days.

cDNA microarray analysis and data acquisition. Total RNA was extracted using the RNeasy Micro kit (Qiagen GmbH, Hilden, Germany) after co-culture experiments, according to the manufacturer’s instructions. Briefly, total RNA was reverse-transcribed to cDNA with T7-oligo(dt) primer. The cDNA synthesis product was used in an in vitro transcription (IVT) reaction involving T7 RNA polymerase. An unlabeled ribonucleotide mix was used in the first cycle of IVT amplification. Unlabeled cRNA was then reverse-transcribed in the first-strand cDNA synthesis step of the second cycle using random primers. Subsequently, the T7-oligo(dt) promoter primer was used for the second-strand cDNA synthesis to generate a double-stranded cDNA template containing T7 promoter sequences. The resultant double-stranded cDNA was then amplified and labeled using a biotinylated nucleotide analog/ribonucleotide mix in the second IVT reaction. The labeled cRNA products were then fragmented, loaded onto the GeneChip Human Genome U133 Plus 2.0 array (Affymetrix; Thermo Fisher Scientific, Inc.), and hybridized according to the manufacturer’s instructions. GeneChip® array data were compared using the Kudaro custom analysis service (Kudaro Industries Ltd., Osaka, Japan; Kurabo Industries Ltd. is an authorized service provider for Affymetrix Japan K.K., Tokyo, Japan). Differences in gene expression were assessed using the following comparisons: PrSC/LNCaP vs. PrSC/PrEC/LNCaP, PrSC/PrEC vs. LNCaP, and PrEC/PrSC/LNCaP vs. PrEC/PrSC (Fig. 1; PrSC/LNCaP, PrSC co-cultured with LNCaP, PrSC/PrEC, PrSC co-cultured with PrEC, LNCaP/PrSC, LNCaP co-cultured with PrSC, PrEC/PrSC/LNCaP, PrEC co-cultured with PrSC/LNCaP, and PrEC/PrSC co-cultured with PrEC). Raw intensity data from the GeneChip® array were analyzed using the GeneChip® Operating Software (Affymetrix; Thermo Fisher Scientific, Inc.) and hierarchical clustering analyses were conducted using Cluster and TreeView software (http://rana.lbl.gov/EisenSoftware.htm).

Small hairpin RNA-expressing constructs and cell viability assay. We used SureSilencing short hairpin RNA (shRNA) plasmids (Qiagen GmbH) for examining RNA interference effects on the target genes. The target sequences of HMGCS1 and HMGCR were 5’-GAAGGAACTGGTACTTAT-3’ (shHMGCS1) and 5’-CAAGGACATGCAAAGATAT-3’ (shHMGCR), respectively. The scrambled sequence 5’-GGA ATCTCATCGATGCATA-3’, which does not match any human, mouse, or rat gene, was used as a negative control (shNC vector). 22Rv1 cells that highly expressed both HMGCS1 and HMGCR were plated onto 10 cm dishes (2x10⁶ cells/dish) and transfected with 6 µg of each shRNA plasmids (shHMGCS1, shHMGCR, or shNC) using FuGENE6 reagent (Promega Corporation, Madison, WI, USA) according to the supplier’s protocol. Cells were selected in culture medium containing 1.0 mg/ml geneticin for 10 days, fixed with 100% methanol, and stained with 0.1% crystal violet to evaluate colony formation. Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, with absorbance measured at 570 and 630 nm as a reference using a microplate reader (THERMOMax; Molecular Devices, LLC, Sunnyvale, CA, USA). Knockdown effects of these shRNA plasmids on endogenous HMGCS1 or HMGCR expression were calculated 48 h following their transfection, by RT-PCR using the primers described above.

Autocrine/paracrine cell proliferation assay. Full-length human HMGCS1 and HMGCR cDNA (accession nos. NM002130 and NM000859) were amplified and cloned into the pcDNA3.1 (+) vector (Invitrogen; Thermo Fisher Scientific, Inc.). To examine the autocrine effect of HMGCS1 and HMGCR expression on PC cell growth, 22Rv1 cells were seeded into 6-well plates (5x10⁶ cells/well) and transfected with pcDNA3.1 (+) empty vector (mock), pcDNA3.1 (+)-HMGCS1, or pcDNA3.1 (+)-HMGCR expression vectors at a final concentration of 0.6 µg/ml using FuGENE6 reagent (Promega), according to the manufacturer’s instructions. The proliferation of 22Rv1 cells overexpressing HMGCS1 or HMGCR, or mock-transfected cells, was examined using a cell counter (TC10™ Automated Cell Counter; Bio-Rad Laboratories) during days 1-10. To examine the paracrine effect of HMGCS1 and HMGCR on PC cell growth, PrSC cells (1x10⁵ cells/well) transfected with pcDNA3.1 (+) empty vector (mock), pcDNA3.1 (+)-HMGCS1, or pcDNA3.1 (+)-HMGCR expression vector at a final concentration of 0.6 µg/ml and selected with 1.0 mg/ml geneticin, were plated onto culture
plate inserts, while 22Rv1 cells (1x10^5 cells/well) were grown on 6-well plates. The following day, culture media was replaced with keratinocyte-SFM (Thermo Fisher Scientific, Inc.) plus 2% FBS and the inserts containing transfected PrSC cells transferred into the 6-well plates containing the 22Rv1 cells to initiate co-culture. Growth of 22Rv1 cells was calculated during days 1-14 using a cell counter.

Tissue microarray samples and immunohistochemical study. To further investigate HMGCS1 and HMGCR expression in a larger number of tumor specimens, tissue microarray samples containing 80 cases of PC and 16 normal prostate tissue, in duplicate cores per case (PR1921; US Biomax, Inc., Rockville, MD, USA) were obtained. The deparaffinized tissue sections were heated in a microwave for 5 min for antigen retrieval. These sections were incubated with a 1:200 diluted solution of a rabbit anti-HMGCS1 polyclonal antibody (ab87246; Abcam, Cambridge, UK) or a 1:50 diluted solution of a mouse anti-HMGCR monoclonal antibody (C-1, sc-271595; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4˚C and developed with peroxidase labeled-dextran polymer followed by diaminobenzidine (Dako EnVision+ System; Dako, Carpinteria, CA, USA). The sections were counterstained with hematoxylin. For negative controls, primary antibody was omitted. The stromal expression levels of HMGCS1 and HMGCR were determined by calculating average intensities of positive stromal cells, using a BZ-X Analyzer (Keyence Corporation, Osaka, Japan). Three random fields were analyzed with a magnification of 400x. Correlations between HMGCS1/HMGCR expression levels and clinicopathological variables (tissue type, Gleason grade, tumor stage, tumor classification, lymph node metastasis, distant metastasis, and prostate-specific antigen (PSA) expression levels) were evaluated using the Mann-Whitney U and Kruskal-Wallis tests. All statistical analyses were performed using the software JMP® (SAS Institute Inc., Cary, NC, USA). P<0.05 was considered to indicate a statistically significant differences.

Results

Candidate genes identified by cDNA microarray and cluster analysis. Before microarray analysis, we confirmed the overexpression of α-smooth muscle actin (ACTA2) in PrSC/LNCaP, which indicated the transition from normal stromal cells to CAFs (data not shown). We identified 10 genes that were upregulated in PrSC/LNCaP relative to PrSC/PrEC (signal log ratio >2.0), which included insulin induced gene 1 (INSIG1), HMGCS1, solute carrier family 14 member 1 (SLC14A1), and noggin (NOG; Table I). SLC14A1 has been reported to be regulated by androgens and potentially involved in prostate carcinogenesis (6), while NOG is involved in EMT (7). INSIG1 mediates feedback control of cholesterol synthesis by binding sterol regulatory element-binding protein (SREBP) monoclonal antibody (C-1, sc-271595; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4˚C and developed with peroxidase labeled-dextran polymer followed by diaminobenzidine (Dako EnVision+ System; Dako, Carpinteria, CA, USA). The sections were counterstained with hematoxylin. For negative controls, primary antibody was omitted. The stromal expression levels of HMGCS1 and HMGCR were determined by calculating average intensities of positive stromal cells, using a BZ-X Analyzer (Keyence Corporation, Osaka, Japan). Three random fields were analyzed with a magnification of 400x. Correlations between HMGCS1/HMGCR expression levels and clinicopathological variables (tissue type, Gleason grade, tumor stage, tumor classification, lymph node metastasis, distant metastasis, and prostate-specific antigen (PSA) expression levels) were evaluated using the Mann-Whitney U and Kruskal-Wallis tests. All statistical analyses were performed using the software JMP® (SAS Institute Inc., Cary, NC, USA). P<0.05 was considered to indicate a statistically significant differences.

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Unsupervised hierarchical clustering using full gene expression profiles showed that the samples clustered into three distinct groups related to LNCaPs, PrSCs and PrECs, respectively (Fig. 2A). Interestingly, PrECs clustered close to LNCaPs when clustering analysis was restricted to the 767 genes most significantly upregulated or downregulated (i.e.,

### Table I. Upregulated genes in PrSC/LNCaP compared with PrSC/PrEC.

| Gene symbol | Gene title                                      | Function                                   |
|-------------|-------------------------------------------------|--------------------------------------------|
| SCD         | Stearoyl-CoA desaturase (delta-9-desaturase)    | Fatty acid synthesis                       |
| INSIG1      | Insulin induced gene                            | Cholesterol synthesis, steroid metabolism  |
| HMGCS1      | 3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble) | Cholesterol synthesis, lipid metabolism   |
| HTR2B       | 5-hydroxytryptamine (serotonin) receptor 2B     | Serotonin receptor signaling pathway       |
| OXTR        | Oxytocin receptor                                | G-protein coupled receptor                 |
| PTHLH       | Parathyroid hormone-like hormone                | Inhibitor of osteoclastic bone resorption  |
| IGFBP3      | Insulin-like growth factor binding protein 3    | Cell growth regulation                     |
| ALDH1A1     | Aldehyde dehydrogenase 1 family, member A       | Retinol metabolism                         |
| SLC14A1     | Solute carrier family 14 (urea transporter), member 1 (Kidd blood group) | Urea transport |
| NOG         | Noggin                                          | BMP signaling pathway, EMT                 |

BMP, bone morphogenetic protein; EMT, epithelial-to-mesenchymal transition.

### Table II. Upregulated genes in LNCaP/PrSC compared with LNCaP.

| Gene symbol | Gene title                                      | Function                                   |
|-------------|-------------------------------------------------|--------------------------------------------|
| EGR1        | Early growth response 1                         | Transcriptional regulation, BMP signaling pathway |
| SOX9        | SRY (sex determining region Y)-box 9           | Skeletal development, PKB signaling cascade |
| PLA2G2A     | Phospholipase A2, group IIa (platelets, synovial fluid) | Phospholipid metabolism                   |
| STC1        | Stanniocalcin 1                                 | Renal phosphate reabsorption               |
| DIO1        | Deiodinase, iodothyronine, type I               | Hormone synthesis                          |
| FASN        | Fatty acid synthase                             | Fatty acid synthesis                       |
| GINS2       | GINS complex subunit 2 (Psf2 homolog)          | DNA replication                            |
| NFKBIZ      | Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta | Regulation of NF-kappa-B                  |
| UHRF1       | Ubiquitin-like with PHD and ring finger domains 1 | DNA repair                                 |
| AMACR       | α-methylacyl-CoA racemase                       | Lipid metabolism                           |
| CLEC7A      | C-type lectin domain family 7, member A         | Innate immunity                            |

BMP, bone morphogenetic protein; PKB, protein kinase B.

### Table III. Upregulated genes in PrEC/PrSC/LNCaP compared with PrEC/PrSC.

| Gene symbol | Gene title                                      | Function                                   |
|-------------|-------------------------------------------------|--------------------------------------------|
| MAP3K8      | Mitogen-activated protein kinase kinase kinase 8 | Oncogene                                   |
| GABBR1      | γ-aminobutyric acid (GABA) B receptor, 1        | G-protein coupled receptor                 |
| TLR1        | Toll-like receptor 1                            | Innate immunity                            |
| SOD2        | Superoxide dismutase 2, mitochondrial           | Superoxide family member, mutated in cancer |
| HERC5       | Hect domain and RLD 5                          | Interferon-induced E3 protein ligase       |
| CMPK2       | Cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial | dUTP and dCTP synthesis in mitochondria |

Unsupervised hierarchical clustering using full gene expression profiles showed that the samples clustered into three distinct groups related to LNCaPs, PrSCs and PrECs, respectively (Fig. 2A). Interestingly, PrECs clustered close to LNCaPs when clustering analysis was restricted to the 767 genes most significantly upregulated or downregulated (i.e.,
with a log ratio of >2.0 or <-2.0) in PrEC/PrSC/LNCaP, that is PrEC co‑cultured with CAFs (Fig. 2B).

Knockdown of HMGCS1 or HMGCR expression by shRNA suppresses PC cell viability. To investigate the biological role of HMGCS1 and HMGCR in PC cells, we knocked down their endogenous expression in 22Rv1 cells (Fig. 3A) using vector‑based RNA interference technology. Transfection of the shRNA‑expressing vectors shHMGCS1 or shHMGCR, clearly reduced endogenous expression of HMGCS1 and HMGCR, respectively (Fig. 3B), and resulted in significant growth suppression as measured by both colony formation assay and MTT assay (P<0.01; Fig. 3C and D, respectively). By contrast, transfection of the negative control vector (shNC) had little or no effect on HMGCS1 or HMGCR expression and did not affect the viability of 22Rv1 cells (Fig. 3B-D).

HMGCS1 or HMGCR overexpression promotes PC cell proliferation through autocrine/paracrine regulation. To further investigate the potential oncogenic function of HMGCS1 and HMGCR, we examined the autocrine/paracrine effects of these proteins on PC cell growth. The cell proliferation assay revealed that 22Rv1 cells with exogenous overexpression of HMGCR grew more rapidly than 22Rv1 mock‑transfected cells, indicating that HMGCR overexpression promotes PC cell proliferation in an autocrine fashion. HMGCS1 overexpression however, did not significantly stimulate PC cell growth (data not shown). We further examined the influence of HMGCS1 and HMGCR overexpression in PC stroma on PC cell growth using a co‑culture experiment. Overexpression of HMGCS1 or HMGCR in PC stromal cells was found to induce a significantly higher growth rate of 22Rv1 cells than those that were mock transfected (P<0.01; Fig. 4), indicating a paracrine effect.

Immunohistochemical analysis of primary PC specimens. We subsequently performed immunohistochemical staining of 80 primary PC and 16 normal cases on tissue microarrays (a total of 192 cores) with anti‑HMGCS1 or anti‑HMGCR antibodies. Immunohistochemistry confirmed the overexpression of HMGCS1 and HMGCR in PC stroma compared with normal prostatic stroma (P<0.0001; Figs. 5, 6A and B). To investigate the clinicopathological significance of HMGCS1 and HMGCR stromal expression in PC tissues, we next analyzed the relationship between HMGCS1 or HMGCR stromal expression and the clinicopathological variables of PC specimens, which is summarized in Table IV. This analysis revealed a significant association between HMGCS1/HMGCR stromal expression and several clinicopathological factors. Most notably, we observed that HMGCS1 and HMGCR expression levels in PC stroma were inversely associated with tumor stage (P<0.01; Fig. 6C and D), with higher stage PCs associated with

Table IV. Clinicopathological variables of PC cases.

| Variable                                           | Number of cases (cores) | 80 (160) |
|----------------------------------------------------|-------------------------|----------|
| Age, years [median (range)]                        | 69.5 (51‑85)            |          |
| Gleason grade, n, per core                         |                         |          |
| 3                                                  | 16                      |          |
| 4                                                  | 74                      |          |
| 5                                                  | 60                      |          |
| Tumor stage, n, per case                           |                         |          |
| I                                                  | 6                       |          |
| II                                                 | 37                      |          |
| III                                                | 14                      |          |
| IV                                                 | 22                      |          |
| Tumor classification, n, per case                  |                         |          |
| T1                                                 | 4                       |          |
| T2                                                 | 47                      |          |
| T3                                                 | 22                      |          |
| T4                                                 | 6                       |          |
| Lymph node metastasis, n, per case                 |                         |          |
| Positive                                           | 15                      |          |
| Negative                                           | 64                      |          |
| Distant metastasis, n, per case                    |                         |          |
| Positive                                           | 15                      |          |
| Negative                                           | 64                      |          |
| PSA expression level (IHC), n, per core            |                         |          |
| -                                                  | 52                      |          |
| +                                                  | 28                      |          |
| ++                                                 | 45                      |          |
| +++                                                | 32                      |          |

Some data are missing in each clinicopathological variable. PC, prostate cancer; PSA, prostate‑specific antigen; IHC, immunohistochemistry.

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Likewise, HMGCS1 and HMGCR stromal expression was significantly lower in cases with lymph node metastasis \( (P<0.05; \text{Fig. 6E and F}) \). HMGCR stromal expression was also significantly lower in cases with distant metastasis \( (P<0.01; \text{Fig. 6G}) \), however HMGCS1 stromal expression levels had no correlation with distant metastatic status \( (P=0.3113; \text{Fig. 6H}) \).

There was no relationship between HMGCS1 or HMGCR stromal expression and Gleason grade, tumor classification, or PSA expression levels (data not shown).

**Discussion**

Our data suggest that stromal cells may influence PC cell gene expression and contribute to their aggressiveness. In keeping with this concept, AMACR, which is widely used as a biomarker for PC, was included in the genes that were upregulated in PC cells co-cultured with prostate stromal cells. The cluster analyses support our hypothesis that CAFs might induce surrounding normal epithelial cells to change their characteristics towards PC cells. Interestingly, an oncogene, MAP3K8, was included among the genes that were upregulated in prostate epithelial cells when co-cultured with cancer-associated prostate stromal cells (i.e., prostate stromal cells previously co-cultured with PC cells). Among the genes identified in this study, we further investigated the mevalonate pathway genes, HMGCS1 and HMGCR. The mevalonate pathway is included in lipid metabolism and best known as the target of statins. There is a clear association between PCs and lipid metabolism.
or statins, which prompted us to investigate the mevalonate pathway genes, HMGCS1 and HMGCR. Notably, we investigated for the first time the roles of HMGCS1 in human PC cells and tissues. Tables I, II, and III are derived from normal prostate stromal cells, PC cells, and normal prostate epithelial cells, respectively, and these tables indicate that their gene expression could change by co-culture with the different type of cells. Because HMGCS1 and HMGCR were the upregulated genes in stroma, those were not among the genes in Tables II and III, which were derived from PC and epithelium.

HMGCS1 condenses acetyl-CoA with acetoacetyl-CoA to form HMG-CoA, which is the substrate for HMGCR. An association of HMGCS1 with PC has not been previously reported, although links with other cancers have been described (8-14). Lee et al suggested HMGCS1 as one of the candidates involved in tumor stem-like breast cancer cells (8), while Pandyra et al reported that dipyridamole acts as a potentiator of statin anticancer activity in multiple myeloma and acute myelogenous leukemia by attenuating the feedback response that upregulates HMGCS1 and HMGCR after statin treatment (10). In particular, these authors showed that direct targeting of multiple levels of the mevalonate pathway, including blockade the sterol-feedback loop initiated by statin treatment, is an effective and targetable anti-tumor strategy. HMGCS1 has also been linked to drug response or resistance in other studies (14,15). Such observations suggested the possibility that HMGCS1 and HMGCR may similarly represent molecular targets for the treatment of PC.

Figure 5. Immunohistochemical stainings demonstrate overexpression of HMGCS1 and HMGCR in PC stroma. Representative stainings of PC and normal prostate specimens are shown (magnification, x100). PC, prostate cancer; N, normal prostate; HMGCS1, 3-hydroxy-3-methylglutaryl-CoA synthase 1; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase.
HMGCR is the rate-limiting enzyme for cholesterol synthesis and the pharmacological target for statins. The association of HMGCR with PC has been previously described in several studies (16-21). It has been reported that PC cells show high expression of HMGCR and that the inhibition of HMGCR with the use of statins lowers the viability of castration-resistant PC cells (17). Li et al identified two miRNAs, miR-185 and miR-342, that control lipogenesis and cholesterologenesis in PC cells by inhibiting SREBP-1 and SREBP-2 expression and downregulating their targeted genes, which includes HMGCR (18). miR-185 and 342 inhibit tumorigenicity, cell growth, migration, and invasion, and
induce apoptosis through blockade of the SREBP metabolic pathway in PC cells, representing a novel targeting mechanism for PC therapy.

In the present study, overexpression of HMGCS1 or HMGCR in PC stroma promoted PC cell growth (Fig. 4). However, the stromal expression of HMGCS1 or HMGCR was associated with less aggressive PC (Fig. 6C-H). HMGCS1 and HMGCR might be needed for PC growth until PC progresses to aggressive disease, and then downregulated once PC invasion or metastasis has been developed. Several studies that support our findings have been reported on HMGCR (22-26), although there are no studies available on HMGCS1. Previous studies indicated that high HMGCR expression was associated with less aggressive tumor characteristics and HMGCR expression was a good prognostic marker in breast cancer (23-26). Associations of positive HMGCR expression with more favorable tumor characteristics and a prolonged survival were also shown in other type of cancer such as colorectal cancer (22). We analyzed and provided the stromal expression data of HMGCS1 and HMGCR using clinical PC specimens in this study, since PC studies indicate that HMGCS1 and HMGCR stromal overexpression might be key factor in regulating the transition from organ-confined to metastatic disease in PC. We used 22Rv1 cell line for further experiments because only 22Rv1 overexpressed both HMGCS1 and HMGCR and should be relevant to these genes, while we confirmed the knockdown effect of shRNA on HMGCS1 and HMGCR by the mRNA expression results (not protein expression), as seen in the many studies published previously (27-32). The HMGCS1 and HMGCR mRNAs were overexpressed in 22Rv1 cells (Fig. 3A) and their knockdown effect by shRNA was validated on mRNA level (Fig. 3B). The use of a single cell line to verify the hypothesis and the absence of data regarding the knockdown effect on protein expression are two limitations of the present study. Further functional analysis of the role of HMGCS1 and HMGCR in regulating the interaction between PC and PC stroma are required, in order to fully elucidate their potential as molecular targets in the treatment of PC.

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