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ORIGINAL PAPER

Loss of caveolin-1 in prostate cancer stroma correlates with reduced relapse-free survival and is functionally relevant to tumour progression

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Abstract

Levels of caveolin-1 (Cav-1) in tumour epithelial cells increase during prostate cancer progression. Conversely, Cav-1 expression in the stroma can decline in advanced and metastatic prostate cancer. In a large cohort of 724 prostate cancers, we observed significantly decreased levels of stromal Cav-1 in concordance with increased Gleason score ($p = 0.012$). Importantly, reduced expression of Cav-1 in the stroma correlated with reduced relapse-free survival ($p = 0.009$), suggesting a role for stromal Cav-1 in inhibiting advanced disease. Silencing of Cav-1 by shRNA in WPMY-1 prostate fibroblasts resulted in up-regulation of Akt phosphorylation, and significantly altered expression of genes involved in angiogenesis, invasion, and metastasis, including a > 2.5-fold increase in TGF-β1 and γ-synuclein (SNCG) gene expression. Moreover, silencing of Cav-1 induced migration of prostate cancer cells when stromal cells were used as attractants. Pharmacological inhibition of Akt caused down-regulation of TGF-β1 and SNCG, suggesting that loss of Cav-1 in the stroma can influence Akt-mediated signalling in the tumour microenvironment. Cav-1-depleted stromal cells exhibited increased levels of intracellular cholesterol, a precursor for androgen biosynthesis, steroidogenic enzymes, and testosterone. These findings suggest that loss of Cav-1 in the tumour microenvironment contributes to the metastatic behaviour of tumour cells by a mechanism that involves up-regulation of TGF-β1 and SNCG through Akt activation. They also suggest that intracrine production of androgens, a process relevant to castration resistance, may occur in the stroma.

Keywords: caveolin-1; stroma; prostate cancer; prognosis

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Introduction

The tumour microenvironment plays a crucial role in the initiation and progression of prostate and other cancers. It is now clear that cancer stroma promotes increased microvessel density; recruits reactive stromal fibroblasts, lymphocytes, and macrophages; and releases peptide-signalling molecules and proteases [1,2]. Cancer-associated fibroblasts (CAF) produce an altered extracellular matrix (ECM), which can induce epithelial–mesenchymal transition (EMT) or other types of behaviours associated with a more aggressive phenotype in neighbouring epithelial cells [3]. The mechanisms by which the stromal cells adjacent to the cancer epithelium undergo phenotypic alterations and overproduce biologically active proteins, thereby orchestrating stromal–epithelial crosstalk, are still largely unresolved. However, a major role seems to be played by the transforming growth factor β (TGF-β) pathway [4,5]. The myofibroblastic 'reactive' phenotype of prostate cancer stromal cells is not fully characterized, but the predominant cell population expresses increased vimentin and fibroblast activation protein (FAP), and decreased α-smooth muscle actin (α-SMA), desmin, and calponin 1 (CNN1) [3,6]. Reactive prostate cancer stroma synthesizes collagen type I [3] and expresses tenascin C (TNC) and TGF-β,

which are involved in modulation of cell growth and tumourigenesis [5]. However, the use of these proteins as markers of prostate cancer progression is controversial [3,6], and better parameters to define the stroma of aggressive disease are needed.

Reduced levels of Cav-1 in the prostate stroma have been reported [7–9]. A recent global gene expression analysis of prostate cancer stroma identified a significant down-regulation of Cav-1 levels in grade 3 reactive stroma [6]. In line with this, and with the association between reactive stroma and prostate cancer progression [1], we recently reported that loss of stromal Cav-1 correlates with the presence of metastasis and is predictive of increased expression of Cav-1 and active Akt, both of which are overexpressed in advanced disease, in the tumour [9,10].

To elucidate the clinical significance of Cav-1 loss in prostate cancer-associated stroma, we analysed a unique tissue microarray (TMA) comprising specimens from 724 patients with follow-up for biochemical relapse [11]. In this large cohort, we observed an inverse correlation of Cav-1 loss with relapse-free survival. We also used immortalized prostatic myofibroblasts as a model system to investigate the molecular mechanism by which reduced expression of Cav-1 influences tumour progression. We found that when Cav-1 was silenced, the stromal gene expression profile dramatically changed, with overexpression of genes involved in metastasis and angiogenesis. Levels of cholesterol, steroidogenic enzymes, and testosterone were also increased. Furthermore, silencing of Cav-1 in stromal cells induced tumour epithelial cell migration. These results suggest that stromal Cav-1 is an important mediator within the tumour microenvironment that is protective against disease progression.

Materials and methods

Cav-1 expression in human tumours

Patient cohort enrolment and follow-up under the Prostate SPORE data bank at the Baylor College of Medicine, as well as pathological analysis of radical prostatectomies for TMA construction, approved by the Baylor College of Medicine Institutional Review Board (YRB H-11436), have been described in detail [11]. Clinical follow-up data included prostatic specific antigen (PSA) relapse (defined as PSA > 0.4 ng or two consecutive rises), clinical metastasis, and death. TMA sections were immunostained with Cav-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using an LSAB kit (DAKO, Carpinteria, CA, USA). Immunoreactive staining of Cav-1 was analysed using a 0–3+ scoring system for staining intensity and percentage of positive cells (labelling frequency) per field.

Reactive stroma

The tumour-specific stroma was defined as stroma found in the invasive component of the cancer that was not part of the normal pre-existing host stroma, graded as reported previously [3], and analysed independently of the epithelial grade of differentiation of the tumour.

Cell culture

LNCaP, DU145, PC3, WPMY-1, RWPE-1, and RWPE-2 cells were from the American Type Culture Collection (ATCC, Manassas, VA, USA).

RNA interference

For silencing of Cav-1 expression, WPMY-1 cells were transfected with ON-TARGETplus SMARTpool siRNA duplexes J-003467-06 Cav-1, or with control siGENOME Non-Targeting siRNA #3 (Dharmacon Inc, Lafayette, CO, USA) using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA), for 72 h. For generation of cell lines with stable Cav-1 silencing, WPMY-1 cells were infected with Cav-1 shRNA(h) and shRNA(h2), or with control shRNA lentiviral particles (Santa Cruz Biotechnology), in the presence of Polybrene (Santa Cruz Biotechnology) [12], and clones selected with puromycin 0.2 µg/ml (Invitrogen).

PCR array

Template cDNAs from WPMY-1 cells were characterized in technical triplicates using the Human Cancer PathwayFinder™ RT^2 Profiler™ PCR Array and RT^2 Real-Time SyBR Green/ROX PCR Mix (SA Biosciences, Frederick, MD, USA). Real-time PCR was performed on an ABI Prism StepOnePlus Sequence Detector (Applied Biosystems, Foster City, CA, USA). The resulting raw data were then analysed using the web-based PCR Array Data Analysis Template (SA Biosciences).

qRT-PCR

Total RNA from stable WPMY-1/shCAV-1 or WPMY-1/shRNA cells was isolated, purified, and reverse-transcribed as described elsewhere [13]. cDNA was amplified using primers for tenascin-C (TNC); calponin 1 (CNN1); glyceraldehyde-3-phosphate dehydrogenase (GAPDH); angiopoietin 1 (ANGPT1); TGF-β1 (TGFB1); contactin 1 (CNTN1); TEK tyrosine kinase, endothelial (TEK); γ-synuclein (SNCG); thrombospondin 1 (THBS1); and seladin-1. Gene expression of CYP17A1 was evaluated as previously described [14]. The relative abundance of a given transcript was estimated using the $2^{-\Delta\Delta Ct}$ method, following normalization to GAPDH.

Cholesterol and testosterone levels

Cholesterol content in WPMY-1/shCAV-1 or control was determined by gas chromatography–mass spectrometry (GC–MS) [15,16]. In addition, a Filipin-based detection kit (Cayman Chemical Company, Ann Arbor, MI, USA) was used to stain intracellular cholesterol in full media (FM), serum-free

media (SFM) or SFM + liposomes [17,18]. Cell membranes were labelled with FITC-conjugated cholera toxin B (CTxB) subunit (Sigma, St Louis, MO, USA) [13,19]. Testosterone was determined using a modified ID LC-MS/MS method [20].

Migration assay and proliferation assay

Cell migration of epithelial cells was performed using conditioned medium from WPMY-1 cells infected with shCav-1 or control shRNA [9,13]. WPMY-1 cell proliferation was assessed using crystal violet (Sigma).

Co-culture assays and flow cytometry

For proliferation assays, we co-cultured WPMY-1/shCAV-1 or WPMY-1/shRNA with LNCaP/GFP cells (ratio 2 : 1) [21]. Stromal cells were pre-labelled with CellTracker Red CPMTX (Invitrogen) [13]. After 48 h of co-culture, LNCaP cell proliferation was assessed by counting GFP-positive cells in 20 randomly selected fields, in three independent experiments [22]. To test the contribution of WPMY-1-derived androgens to cancer cell proliferation, WPMY-1 cells, treated with shCav-1 and exposed to abiraterone acetate (10 μM) [23], were co-cultured with LNCaP/GFP cells for 24 h. For cell cycle analysis, Hoechst 33342 (5 μg/ml)-stained cells [24] were quantified by flow cytometry using an Aria III Cell Sorter (Becton-Dickson, San José, CA, USA), and the percentage of gated LNCaP/GFP cells in the G1/0, S, and G2/M phases were analysed by FlowJo software (Treestar), in three independent experiments.

Statistical analysis

Correlation of stromal Cav-1 with clinico-pathological parameters and other markers was evaluated using Spearman correlation coefficient testing. For survival analysis, time to recurrence was defined as the interval between the date of surgery and the date of identification of biochemical recurrence. Cox univariate and multivariate proportional hazard models were used to evaluate the predictive value of Cav-1 (original score and dichotomized) for recurrence-free survival and to determine the hazard ratios. Kaplan–Meier survival curves were constructed for patients with no and detectable levels of Cav-1. The hazard ratio (HR) and its 95% confidence interval (95% CI) were recorded for each model. p values less than 0.05 were considered statistically significant. Evaluations were performed using SPSS 16.0 software (SPSS Inc, Chicago, IL, USA).

Results

Loss of stromal Cav-1 correlates with clinico-pathological parameters and is predictive of recurrence-free survival

We interrogated a TMA containing benign and tumour prostate tissues from 724 patients, for which histological and clinical parameters were available. Cav-1 was strongly expressed in smooth muscle cells and endothelial cells, as well as in the stroma surrounding the non-cancerous epithelial ducts (Figure 1A). However, high-levels of Cav-1 in the reactive stroma of prostate cancer were rare, being identifiable in only 3% of the samples. Stromal Cav-1 expression was reduced in 17.3%, low in 35.4%, and completely lost in 44.5% of the tumours (Figure 1B). In association with reduced Cav-1, we observed loss of PTEN, shown to be a crucial alteration for malignant transformation in the stroma of the mouse mammary gland [25,26], and increased levels of NF-κB and Akt in epithelial cells (Figure 1C). Levels of stromal Cav-1 were inversely correlated with Gleason score ($r^2 = 0.93$, $p = 0.0124$), and clinical stage ($r^2 = 0.96$, $p = 0.0099$), confirming that expression of the protein declines substantially in the stroma with tumour progression. Notably, poorly differentiated tumours almost invariably exhibited a loss or reduced expression of Cav-1 in the reactive stroma. Recurrence-free survival was significantly lower in patients with higher expression of Cav-1 [HR (95% CI) = 0.75 (0.61, 0.93), $p = 0.009$], which is equivalent to a 33% increase in the estimated risk of recurrence over the time of follow-up (Figure 1D). A multivariate model, adjusting for clinical stage, pre-operative PSA, extracapsular extension, tumour margins, and Gleason grade, showed that loss of Cav-1 in the stroma is not an independent prognostic marker for disease progression ($p = 0.0706$). Detection of stromal Cav-1 predicted a 54.5% increase in the estimated risk of recurrence.

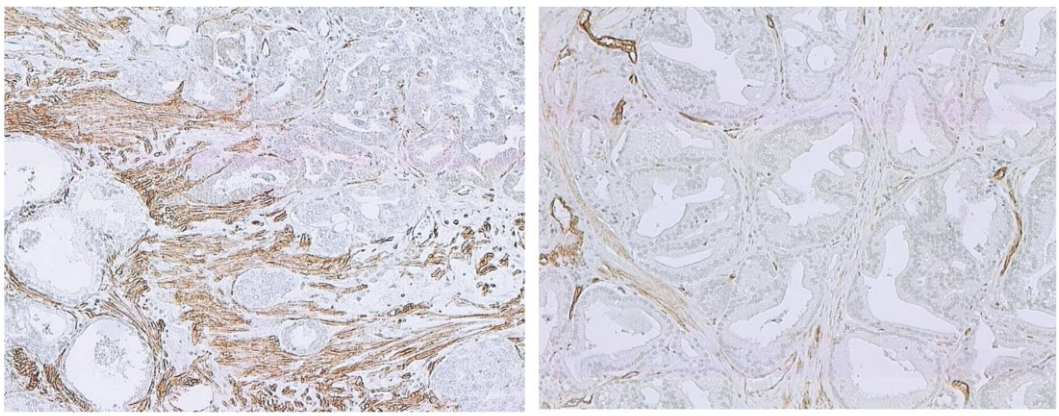
Cav-1 depletion in prostate stromal cells induces a 'reactive stroma-like' phenotype

In order to elucidate the mechanism by which loss of stromal Cav-1 may play a role in disease progression, we used WPMY-1 immortalized benign prostatic myofibroblasts [27]. WPMY-1 cells express Cav-1 at high levels and release it into the medium (Figure 2A), suggesting the interesting possibility that secreted stromal Cav-1 might influence the biological behaviour of epithelial cells. To investigate the functional significance of Cav-1 loss in the prostate stroma, we employed RNA interference. Transient silencing of Cav-1 in WPMY-1 cells induced up-regulation of tenascin C (TNC) and calponin 1 (CNN1), and down-regulation of contactin 1 (CNTN1), markers of reactive stroma [6,7]. In addition, we observed modest but reproducible activation of Akt (Figure 2A).

Gene expression profile of Cav-1-deficient prostate stromal cells reveals activation of oncogenic pathways

Cav-1 was stably silenced in WPMY-1 cells using two independent lentivirus-encoded shRNA constructs [12]. shCAV-1(h) induced more than 90% protein knockdown in WPMY-1 populations (not shown). We then

A



B

	Stromal Cav-1 status			
	High	Medium	Low	Absent
Reactive Stroma	2.9%	17.3%	35.4%	44.5%

C

	PTEN	NF-κB	AKT
Corr. Coeff.	0.112	-0.093	-0.097
Sig. (2-tailed)	0.0500	0.0285	0.0321
N	307	556	490

D

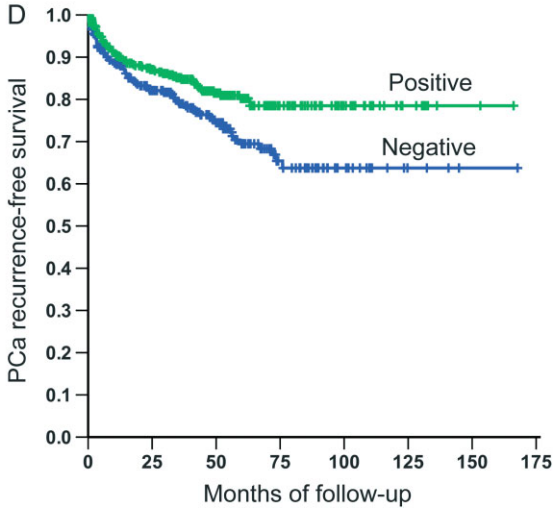
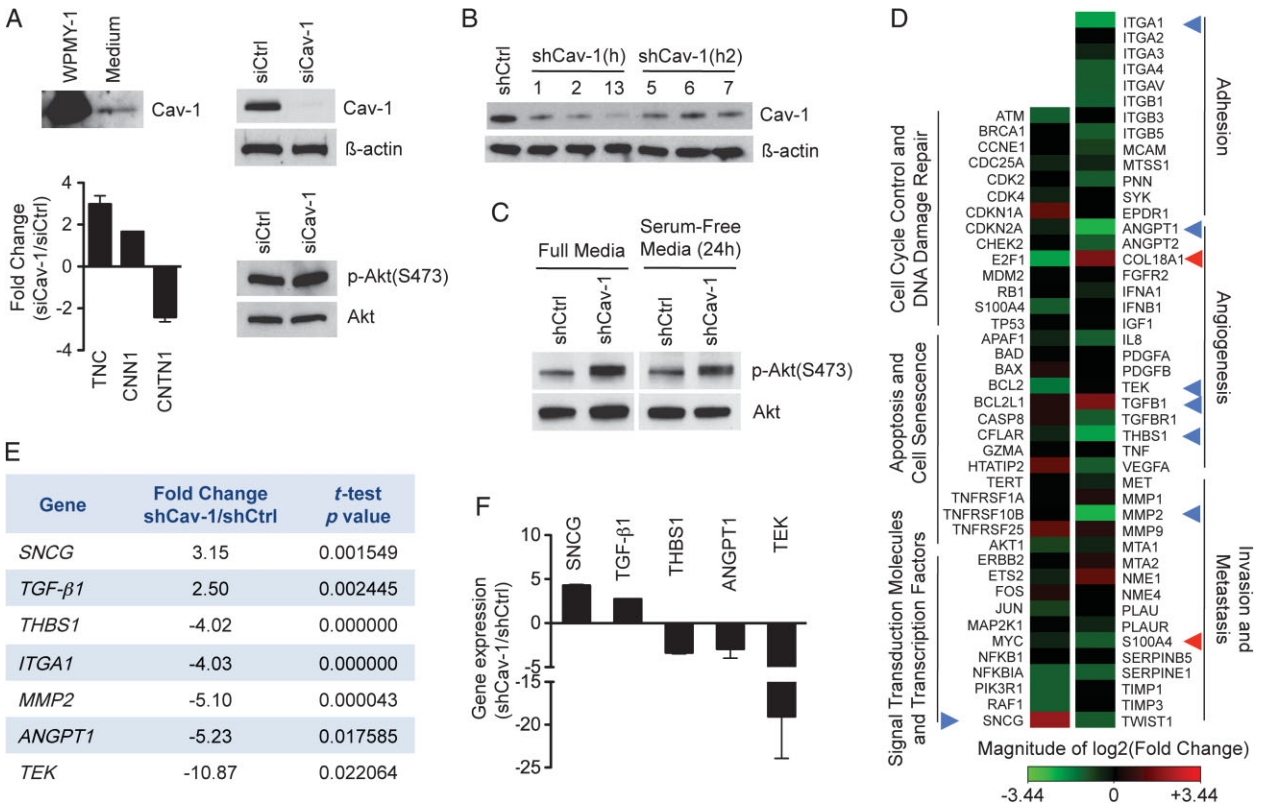


Figure 1. Loss of Cav-1 is predictive of disease-free survival. (A) Cav-1 immunostaining of prostate cancer sections showing high expression of the protein in the stroma of normal prostate glands. Protein levels are dramatically decreased in the tumour stroma. (B) Cav-1 levels are invariably low or absent in reactive stroma. (C) Cav-1 levels are directly correlated with PTEN levels and inversely correlated with Akt and NF-κB levels. (D) Loss of Cav-1 is significantly associated with decreased disease-free survival ($p = 0.0385$).

generated several independent single cell clones, and shCAV-1(h) # 13, which displayed nearly 90% down-regulation of Cav-1, was selected for biochemical and phenotypic analyses (Figure 2B). Akt activation was confirmed in Cav-1 stable knockdown WPMY-1 cells, as indicated by increased levels of p-Akt (S473), both in full medium and in serum-starved conditions (Figure 2C), suggesting that the effect of Cav-1 on Akt activation is not influenced by soluble factors such as those present in the serum.

We then employed the 84-gene Cancer Pathway Finder PCR Array [28,29] to analyse the expression profile of WPMY-1 cells in which Cav-1 had been stably silenced. This array includes genes involved in biological processes relevant to tumourigenesis and cancer progression, such as cell cycle, adhesion, angiogenesis, invasion, and metastasis (Figure 2D). Seven genes exhibited at least a 2.5-fold change in expression in WPMY-1 cells in which Cav-1 was silenced in comparison with cells infected with control shRNA (Figure 2E), with two up-regulated and five down-regulated genes. Four of these genes (TGF-β1, THBS1, ANGPT1, and TEK) are involved in angiogenesis [30–34]. Interestingly, levels of thrombospondin-1 (THBS1), an angiogenesis inhibitor [35], were down-regulated in Cav-1-silenced cells, as were levels of both the TEK receptor tyrosine kinase and its ligand angiopoietin-1 (ANGPT1). Conversely, levels of TGF-β1, known to promote angiogenesis and growth of cancer cells [4,32], and levels of the metastasis-associated gene *synuclein-γ* (SNCG) were up-regulated in Cav-1-knockdown cells. Levels of five of these genes were investigated by qRT-PCR, which confirmed the results of the array (Figure 2F). Notably, we also observed a 2.5-fold increase in COL18A1 levels, and a 2-fold decrease of S100A4 levels in fibroblasts upon Cav-1 knockdown. Both genes have been linked to aggressive cancer and metastasis [36,37].

Because several genes involved in angiogenesis were influenced by Cav-1 knockdown, and to determine whether this observation had biological consequences, we measured the migration of mouse dermal endothelial cells (MDECs), a standard *in vitro* angiogenesis assay [38]. MDEC cell migration was 10% higher when Cav-1-silenced WPMY-1 cells were used as attractants in comparison with shRNA control cells (Figure 3A). This result suggests that loss of stromal Cav-1 might be involved in the establishment of a



Gene	Fold Change shCav-1/shCtrl	*t*-test *p* value
SNCG	3.15	0.001549
TGF-β1	2.50	0.002445
THBS1	-4.02	0.000000
ITGA1	-4.03	0.000000
MMP2	-5.10	0.000043
ANGPT1	-5.23	0.017585
TEK	-10.87	0.022064

Figure 2. Cav-1 knockdown in WPMY-1 cells induces altered expression of genes involved in angiogenesis and oncogenesis. (A) WPMY-1 cell lysates and conditioned medium were blotted with Cav-1 antibody (top left). Cell lysates of WPMY-1 cells transfected with Cav-1 siRNA and control siRNA were blotted with the indicated antibodies (top right). qRT-PCR analysis of WPMY-1 cells transfected with Cav-1 siRNA and control siRNA, using primers for tenascin (TNC), calponin (CNN1), and contactin (CNTN1) (bottom left). Cell lysates of WPMY-1 cells transfected with Cav-1 siRNA and control siRNA were blotted with the indicated antibodies (bottom right). (B) Cell lysates of WPMY-1 cells transfected with control shRNA and different clones from two different Cav-1 shRNAs (h and h2) were blotted with the indicated antibodies. (C) Cell lysates of WPMY-1 cells stably transfected with control shRNA and shCav-1 (h) #13 in full or serum-free media were blotted with the indicated antibodies. (D) Heat map of a human Cancer PathwayFinder PCR Array after Cav-1 silencing in WPMY-1 cells. (E) Expression of seven genes was significantly altered in Cav-1-silenced WPMY-1 cells in comparison with control cells. (F) qRT-PCR analysis of WPMY-1 cells transfected with Cav-1 shRNA and control shRNA, using the indicated primers.

tumour microenvironment characterized by vasculogenesis.

Cav-1 silencing in prostate stromal cells stimulates proliferation and perturbs oncogenic cell signalling

Having observed increased levels of active Akt in Cav-1 knockdown WPMY-1 cells (Figures 2A and 2C), we asked whether activation of this signalling pathway affected WPMY-1 cell proliferation. Consistent with Akt activation, Cav-1 silencing resulted in increased proliferation (Figure 3B and data not shown), down-regulation of p53 and p21 (Figure 3C), and decreased levels of cleaved PARP (Figure 3C), suggesting that loss of Cav-1 confers pro-survival properties to stromal cells. To demonstrate whether up-regulation of TGF-β1 and SNCG in Cav-1-depleted stromal cells was directly mediated by activation of the Akt pathway, we inhibited Akt activation in Cav-1-silenced WPMY-1 cells by a non-toxic dose (10 μM) of triciribine (API-2) (Figure 3D). TGF-β1 and SNCG RNA levels were both significantly down-regulated after 4 h of treatment with API-2, and down-regulation of TGF-β1 was sustained after 8 h, suggesting that up-regulation of these genes induced by Cav-1 silencing is mediated by Akt signalling (Figure 3E).

Cav-1 silencing in WPMY-1 cells stimulates cancer cell migration

Because Cav-1 loss in prostate cancer stroma *in vivo* is associated with reactive stroma and metastatic disease, and coincides with activation of oncogenic signalling, we investigated whether Cav-1 loss in the stroma influences the migratory abilities of cancer cells. LNCaP, DU145, and PC3 cell migration was analysed in response to WPMY-1 cells used as attractants (Figure 4A) [13,39]. LNCaP cells did not migrate, either in the absence or in the presence of WPMY-1 cells, consistent with the poor metastatic potential of this cell type [40]. Conversely, both DU145 and PC3 cells exhibited increased migration in response to the stromal cells. Interestingly, a stronger effect was elicited from WPMY-1 cells on DU145 cells, whose autonomous migration in the absence of stromal cells was extremely low. Therefore, we used DU145 cells to investigate the consequences of Cav-1 down-regulation in WPMY-1 cells used as attractants for tumour cell

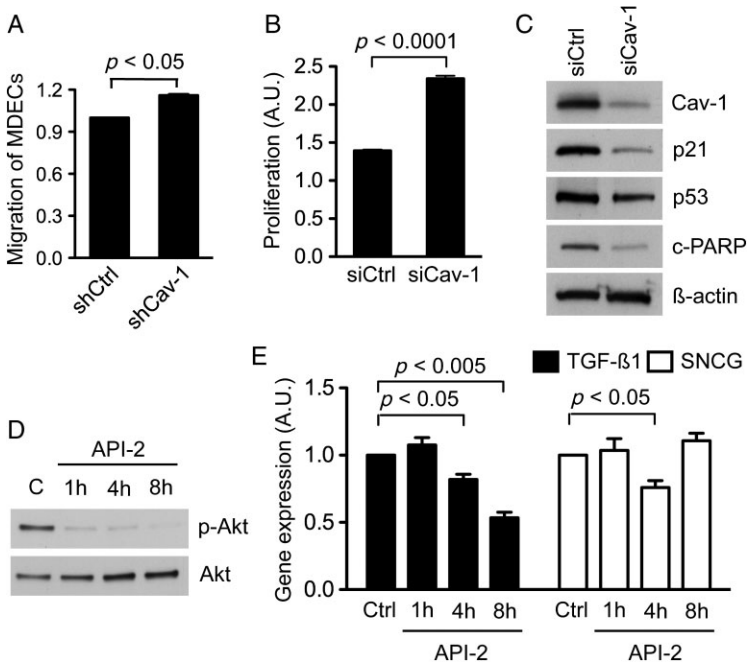


Figure 3. Loss of Cav-1 in WPMY-1 potentiates the migration of endothelial cells. (A) Migration of mouse dermal endothelial cells (MDECs) was significantly increased by Cav-1-depleted WPMY-1 cells. (B) Cell proliferation was assessed by using a crystal violet assay in WPMY-1 cells transfected with control siRNA or Cav-1 siRNA, showing increased proliferation in Cav-1-depleted stromal cells. (C) Cell lysates from WPMY-1 cells transfected with Cav-1 siRNA and control siRNA were blotted with the indicated antibodies. (D) Cell lysates from Cav-1-depleted WPMY-1 cells, treated with 10 μM triciribine (API-2) for the indicated times, were blotted with the indicated antibodies. (E) TGF-β1 and SNCG levels in Cav-1-depleted WPMY-1 cells treated with 10 μM triciribine (API-2) were assayed by qRT-PCR.

migration. Cav-1 depletion in stromal cells induced a 15% increase in DU145 cell migration, which was significantly higher than the DU145 migration elicited by parental stromal cells (Figure 4B), suggesting that Cav-1 loss in the tumour stroma may influence tumour cell migration. We also determined whether Cav-1 loss in the stroma affected the migration of benign epithelial cells, using tumourigenic RWPE-2 cells and their isogenic non-tumourigenic counterpart, RWPE-1 cells [41]. Interestingly, increased migration was observed in the tumourigenic cell line as a consequence of stromal Cav-1 knockdown, while the non-tumourigenic epithelial cells were not affected by Cav-1 silencing in stromal cells (Figure 4C).

Cav-1 knockdown in stromal cells leads to increased intracellular cholesterol, steroidogenic enzymes, and testosterone

Because Cav-1 binds to cholesterol and regulates the intracellular transport of cholesterol to and from the plasma membrane [42,43], and because of increasing evidence of a role for cholesterol in prostate cancer progression [44–46], we asked whether loss of Cav-1 in stromal cells could alter cholesterol levels and/or transport. Silencing of Cav-1 induced an increase of intracellular cholesterol in stromal cells, as shown semi-qualitatively by imaging (Figure 4D) and quantitatively by gas chromatography–mass spectrometry (GC–MS) (Figure 4E). The effect of Cav-1 depletion in increasing cholesterol levels in fibroblasts was abolished when the cells were cultured in serum-free medium (Figure 4D), and treatment with cholesterol-rich liposomes reversed this effect, increasing cholesterol levels in Cav-1-silenced WPMY-1 cells but not control cells. Levels of seladin-1, a key enzyme in cholesterol synthesis, were reduced under the above conditions (Figure 4F), while the expression of proteins involved in sterol metabolism and biosynthesis, including SREBP2 and HSD3β1, was increased. FASN and AMPK, enzymes that regulate fatty acid metabolism, were unaffected (Figure 4G). Levels of StAR and 5-α-reductase type 1 (SRD5A1), key enzymes in the synthesis of steroids, were increased in Cav-1-silenced stromal cells (not shown). Importantly, mRNA levels of CYP17A1, a cytochrome P450 enzyme implicated in *de novo* androgen synthesis in castration-resistant prostate cancer (CRPC) [47], were significantly higher in Cav-1-silenced cells (Figure 4F), suggesting that Cav-1-deprived prostate stromal cells might synthesize androgens endogenously. Consistent with this hypothesis, we observed increased testosterone production and secretion in Cav-1-silenced stromal cells (Figure 4H).

Proliferation of cancer cells induced by Cav-1-depleted stromal cells is mediated by testosterone

Co-culture experiments of LNCaP and WPMY-1 cells demonstrated that proliferation of the prostate cancer epithelial cells was significantly induced by Cav-1 silencing in stromal cells (Figures 5A and

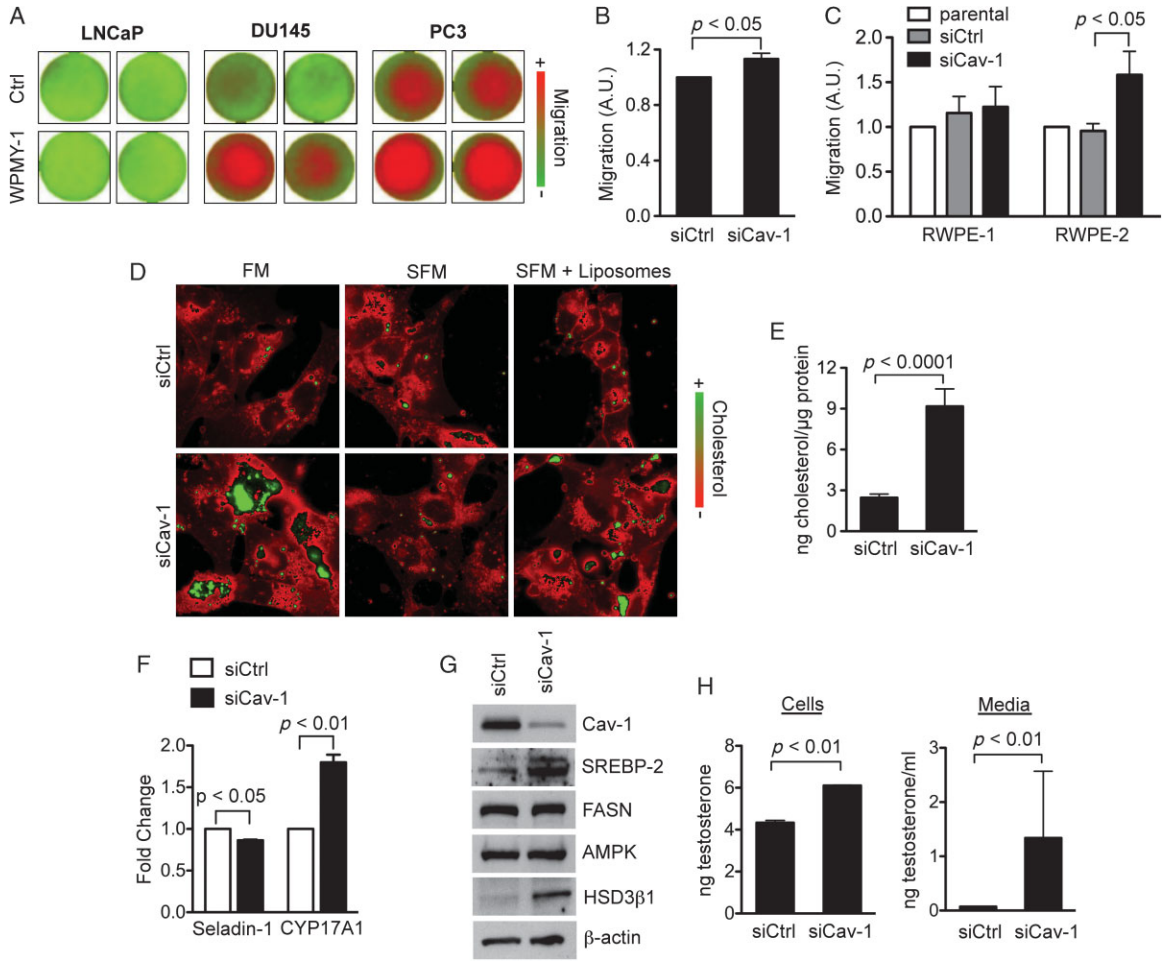


Figure 4. Functional consequences of Cav-1-silencing in WPMY-1 stromal cells. (A) LNCaP, DU145, and PC3 cell migration induced by WPMY-1 cells, used in the bottom chamber as an attractant. The image depicts a heat map of a 24-well plate showing an increase in CellTracker fluorescence (red) in migrating cells. (B) WPMY-1-induced DU145 cell migration, quantitatively analysed, was significantly potentiated by depletion of Cav-1 in the migration-inducer stromal cells. (C) WPMY-1-induced migration of RWPE-2, but not RWPE-1, cells was significantly potentiated by depletion of Cav-1 in the stromal cells. (D) Filipin staining of WPMY-1 cells transfected with Cav-1 siRNA and control siRNA cultured in full media (FM), serum-free media (SFM), and serum-free media supplemented with liposomes (SFM + liposomes). Intracellular cholesterol levels, which increased when Cav-1-depleted cells were cultured in FM or in the presence of cholesterol-rich liposomes, were unchanged in the SFM condition. (E) Gas chromatography–mass spectrometry (GC–MS) of intracellular cholesterol levels in Cav-1 siRNA and control siRNA cells showing a significant increase of intracellular cholesterol in Cav-1-depleted cells. (F) qRT-PCR for seladin-1 and CYP17A1 in WPMY-1 cells transfected with Cav-1 shRNA and control shRNA. (G) Cell lysates from WPMY-1 cells transfected with Cav-1 shRNA and control shRNA were blotted with the indicated antibodies. (H) Isotope dilution–liquid chromatography–tandem mass spectrometry (ID–LC–MS/MS) of intracellular (left panel) and secreted (right panel) testosterone levels in Cav-1 shRNA and control shRNA cells showing a significant increase of testosterone production in Cav-1-depleted cells.

5B), suggesting a potent stimulatory effect of loss of stromal Cav-1 on the proliferation of cancer cells. We then tested whether the excess of androgens detected in Cav-1-depleted stromal cells was responsible for the observed increased proliferation. Pharmacological inhibition in stromal cells of CYP17A1, a critical enzyme in androgen synthesis, by abiraterone acetate significantly suppressed the G2/M transition in tumour cells (Figure 5C), suggesting that loss of stromal Cav-1 promotes the proliferation of cancer cells by stimulating androgen synthesis. In order to determine whether these findings reflect the clinical disease, we examined five RNA expression microarray data sets from the Gene Expression Omnibus database [48] for stromal expression of Cav-1 and CYP17A1 in prostate cancer. Among them, GSE6099 had no CYP17A1 data (no probe) and in GSE11682 and GSE25136 the percentage of stromal content was not indicated. GSE8218 and GSE17951, with more than 50% stromal content, showed a significant negative correlation between Cav-1 and CYP17A1 gene expression ($p = 0.002$, Figure 5D).

Discussion

We recently demonstrated that loss of Cav-1 in the stroma correlates with high Gleason score and presence of metastasis [9], suggesting that down-regulation of Cav-1 in prostatic stroma is clinically relevant. Herein we investigated the clinical and functional significance of stromal Cav-1 in prostate cancer using

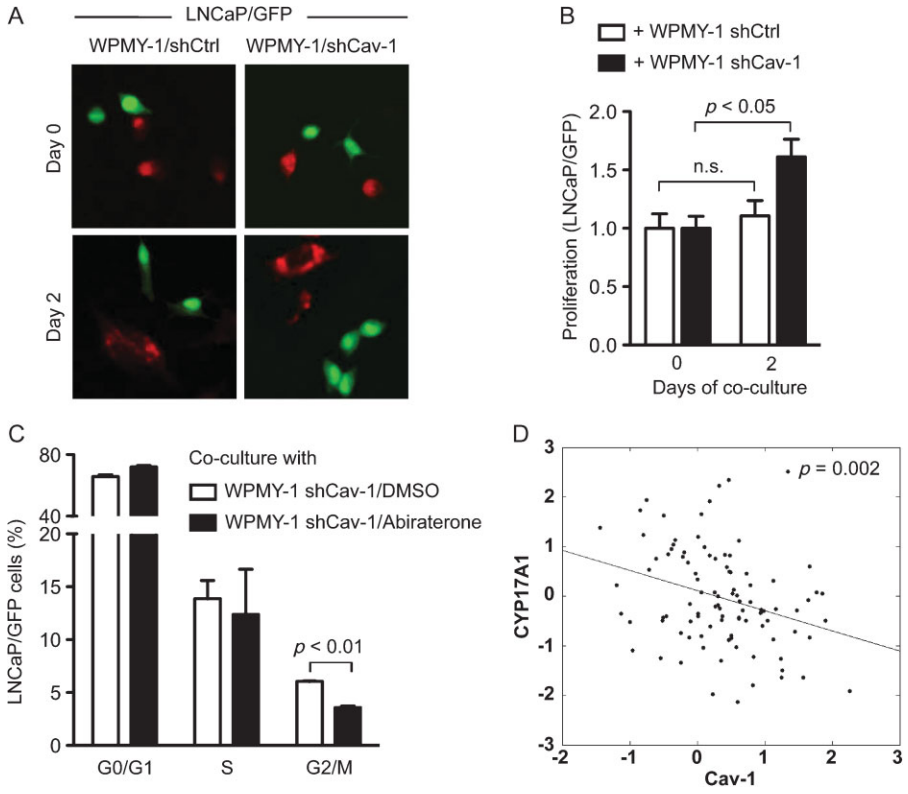


Figure 5. Proliferation of cancer cells induced by Cav-1-depleted stromal cells is mediated by testosterone. (A) Microphotographs of LNCaP/GFP cells co-cultured with WPMY-1 cells treated with Cav-1 shRNA(h) or control shRNA, stained with CellTracker Red CPMTX fluorescence dye, and quantified in B. (C) Flow cytometry analysis of LNCaP/GFP cells, co-cultured with Cav-1-silenced WPMY-1 with and without pharmacological inhibition of CYP17A1. The plot shows the percentage of LNCaP/GFP cells in the G0/G1, S, and G2/M phases of the cell cycle. Mean results of three independent experiments are shown ± standard errors. (D) Scatter plot showing a significant inverse correlation between Cav-1 and CYP17A1 gene expression in patients with more than 50% stromal content ($p = 0.002$).

the largest series of human prostate cancers ever analysed for stromal Cav-1 in combination with an *in vitro* model system. Our results show that the inverse correlation between Cav-1 levels and Gleason score is a reproducible feature of disease progression. Importantly, they indicate that loss of Cav-1 in the prostate tumour microenvironment is indicative of reduced biochemical relapse-free survival. This result is in agreement with the finding that loss of stromal Cav-1 is clinically relevant to breast cancer progression [49]. Intriguingly, our groups demonstrated that Cav-1 expression in the prostate cancer epithelium increases with tumour progression and that Cav-1 is a marker of metastatic disease [9,45]. The inverse correlation between levels of Cav-1 in the stroma and in the tumour suggests that stromal Cav-1 functions to protect the host from the malignant tumour. We also observed decreased levels of PTEN and increased levels of NF-κB and Akt in the tumour epithelium of specimens with low stromal Cav-1 levels. Decreased PTEN expression and activation of the PI3K pathway have been associated with increased risk of recurrence [50], and NF-κB is a major oncogenic pathway, activated in prostate cancer metastasis [51]. This is an important result that supports the finding that reduced levels of stromal Cav-1 are associated with features of advanced disease in the tumour epithelium.

Functional studies presented here suggest that down-regulation of stromal Cav-1 is likely to alter stromal influences on tumour epithelium, tumour angiogenesis, and cholesterol and androgen metabolism. In order to determine the mechanisms by which loss of Cav-1 in the stroma influences prostate cancer progression, we used prostatic myofibroblasts, often employed to study the relationship between cancer cells and the tumour microenvironment [52]. Our results indicate that Cav-1 down-regulation in the stroma, which coincides with increased expression of the reactive stroma marker TNC, induces a number of gene alterations, including up-regulation of TGF-β1 and SNCG, and down-regulation of THBS1, ANGPT1, and TEK, suggesting a pro-angiogenesis effect. The observed increase in endothelial cell migration, showing that endothelial cells are attracted by Cav-1-silenced stromal cells, confirms this result functionally. We also show that Cav-1 silencing stimulates proliferation and provokes oncogenic cell signalling in prostate stromal cells, coincident with increased levels of intracellular cholesterol and activation of steroidogenic enzymes. These stromal alterations result in increased tumour cell migration, demonstrating effects on stromal–epithelial crosstalk.

Our observation of altered levels of TNC, CNN1, and CNTN1 in Cav-1 knockdown cells is consistent

with recent reports showing that Cav-1 down-regulation occurs in reactive stroma [6,7]. While the expression of other markers of reactive stroma was not affected by Cav-1 knockdown, gene expression profiling indicates that Cav-1-silenced stromal cells undergo a number of additional alterations, including overexpression of TGF-β1, an immunosuppressive cytokine that plays a regulatory role in cell differentiation by stimulating angiogenesis and inhibiting the immune response to the tumour [53,54]. Several Cav-1-dependent fibrotic phenotypes have been ascribed to aberrant activation of the TGF-β pathway, which is involved in prostate cancer progression [5,55]. In addition, Cav-1 has been proposed as a negative regulator of TGF-β signalling [56]. However, ours is the first report showing a significant increase of TGF-β levels in myofibroblasts in response to Cav-1 knockdown. Our results support the hypothesis that stromal cells lacking Cav-1 could be functionally similar to cancer-associated fibroblasts (CAFs). This hypothesis needs further investigation and would benefit from tumour reconstitution studies.

Another gene significantly up-regulated in Cav-1-silenced stromal cells was SNCG, a member of the neuronal protein synuclein family, a protein highly expressed in human cancers [57–60]. In breast carcinoma, SNCG is often overexpressed as a consequence of gene demethylation and is linked to increased proliferation, metastasis, and drug resistance [61–63]. SNCG expression can be also induced by IGF-I through activation of MAPK and PI3K, and SNCG knockdown results in decreased IGF-I-induced activation of Akt [64]. Our results point for the first time to increased SNCG in the prostate stroma through Akt activation induced by Cav-1 loss and suggest that the SNCG pro-survival role might involve activation of paracrine signalling in the tumour microenvironment. Given its primary role in neural tissue, SNCG overexpression in the stroma might be involved in cancer-related neurogenesis, a feature recently associated with aggressive prostate cancer [65].

This study shows that loss of Cav-1 in myofibroblasts results in increased levels of intracellular cholesterol, activation of steroidogenic enzymes, and testosterone. The role of Cav-1 as a cholesterol transporter is well known [43]. It has gradually emerged that loss of Cav-1 in breast CAF results in oxidative stress, which induces oxidative metabolism in adjacent cancer cells, also known as the 'reverse Warburg effect' [66]. However, the finding of increased cholesterol levels in prostate-derived myofibroblasts in response to Cav-1 loss is novel. Cholesterol is a precursor for androgenic hormones; thus, our findings suggest that reactive stroma may supply androgens to the epithelial tumour cells under castrate conditions. Cav-1 loss increased stromal levels of cholesterol and steroidogenic enzymes, including CYP17A1, a rate-limiting enzyme in androgen synthesis from cholesterol or other adrenal precursors. Importantly, CYP17A1 has been suggested as a mediator of cell-to-cell communication in the tumour microenvironment and is secreted in human serum exosomes [67], but this is the first study demonstrating that CYP17A1 levels increase as a result of Cav-1 loss. Our results favour the hypothesis that one of the mechanisms by which abiraterone inhibits intracrine production of androgens is by blocking the function of CYP17A1 in the stroma. The sustained loss of Cav-1 could be responsible, at least in part, for the clinical resistance to androgen ablation in patients with CRPC. We believe that this new finding has potentially major clinical implications by suggesting that Cav-1 loss in the prostate stroma plays a role in mechanisms of intracrine androgen metabolism in CRPC [68–70].

In summary, our findings indicate that Cav-1 loss in prostate cancer stroma coincides with more aggressive disease in humans. Functional analysis of prostate stromal cells in which Cav-1 was depleted suggests that stromal Cav-1 may inhibit progression to aggressive disease and that loss of the stromal protein results in multiple effects on the tumour epithelia, including resistance to hormone ablation.

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Author contribution statement

GA conceptualized the histology study, coordinated sample input, scored IHC, and analysed the human data, in collaboration with RL, AF, and TCT. MM executed most of the *in vitro* experiments and was involved with writing the manuscript. GD, FR, and GB performed the quantitative analysis of cholesterol and testosterone. RMA, SY, MPL, and MRF helped with the data analysis, and MRF was involved with writing the manuscript. DDV conceived the study, supervised the experiments, and wrote the manuscript. All authors contributed to the final manuscript.

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