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Caveolin-1 secreting LNCaP cells induce tumor growth of caveolin-1 negative LNCaP cells *in vivo*

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Caveolin-1 (Cav-1) was originally identified as a structural protein of caveolae, which is a plasma membrane domain that regulates a variety of signaling pathways involved in cell growth and migration. Here, we show that expression of Cav-1 in the Cav-1-deficient human prostate cancer cell line LNCaP both stimulates cell proliferation and promotes tumor growth in nude mice. Unexpectedly, Cav-1 expressing LNCaP ($LNCaP^{Cav\text{-}1}$) cells injected into one side of a nude mouse promoted tumor growth of Cav-1 negative LNCaP cells injected on the contralateral side of the same animal. The LNCaP tumors were positive for Cav-1, however, this signal was not caused by migrated $LNCaP^{Cav\text{-}1}$ cells, but we show that this Cav-1 was secreted by the $LNCaP^{Cav\text{-}1}$ tumors. We demonstrate that conditioned media from $LNCaP^{Cav\text{-}1}$ cells contained Cav-1 that was associated with a lipoprotein particle ranging in size from 15 to 30 nm and a density similar to high density lipoprotein particle. These results suggest that $LNCaP^{Cav\text{-}1}$ cells secreting Cav-1 particle produce an endocrine factor that stimulates tumor growth.

Key words: prostate cancer; LNCaP; caveolin-1; secretion; lipoprotein particle

Prostate cancer is the most frequently diagnosed cancer and one of the leading causes of cancer-related death in men in the United States. In 2005, an estimated 234,460 new prostate carcinoma cases were reported and an estimated 27,350 men died of this cancer.[1] Prostate cancer first manifests as an androgen-dependent cancer, and androgen deprivation effectively suppresses cancer progression at an early stage. However, as prostate cancer advances to later stages, cancer cells acquire androgen independence, which results in substantially poorer prognoses.[2] Therefore, elucidating mechanistic pathways underlying prostate cancer transition is critical for developing effective therapeutic interventions.

Caveolin-1 (Cav-1) expression has been implicated in a variety of human cancers including breast cancer[3] and lung cell carcinoma.[4] Several lines of evidence suggest that Cav-1 expression is positively correlated with metastasis of human prostate cancer and the development of androgen independence. Expression of Cav-1 has been found to be elevated in prostate cancer specimens from metastatic patients as compared to those from primary tumors.[5] Suppression of Cav-1 expression increases the androgen sensitivity of metastatic prostate cancer cells.[6] Finally, Cav-1 knockout mice appear to be resistant to prostate cancer induction in a transgenic adenocarcinoma of mouse prostate model system.[7]

Cav-1 is a 178-amino acid protein that mediates several pivotal cellular functions. On the cell surface, Cav-1 is a structural component of caveolae, and is required for normal caveola morphology and function.[8,9] Moreover, Cav-1 appears to regulate many signal transduction cascades through its ability to promote the assembly of signaling complexes on the plasma membrane.[10,11] Cav-1 has also been found on endosome-like structures (caveosomes), as well as in mitochondria, endoplasmic reticulum, lipid droplets and cytosol.[12,13] Previously, we showed that Cav-1 is secreted by pancreatic acinar cells after cholecystokinin and secretin stimulation and the protein has also been identified in secretory vesicles of other exocrine cells.[14] It remains unknown whether the different localization of Cav-1 also results in a different structure of the protein; moreover, the physiological significance of multiple cellular localizations of Cav-1 remains unclear. Recently, Tahir *et al.*[15] reported that prostate cancer cells also have the capacity to secrete Cav-1. Moreover, prostate cancer patients appear to have a higher serum level of Cav-1 protein as compared to patients with benign prostatic hyperplasia.[16]

Here, we present evidence that expression of Cav-1 increases LNCaP cell proliferation *in vitro* as well as *in vivo* and that Cav-1 expression promotes prostate tumor growth of Cav-1 negative cells *in vivo*. We show that Cav-1 expressing LNCaP ($LNCaP^{Cav\text{-}1}$) cells secrete Cav-1 in a lipoprotein particle with a density similar to high density lipoprotein (HDL) particle. We conclude that Cav-1 expressed in LNCaP cells is secreted and as a response to this gain of function, the cells become more tumorigenic and can induce tumorigenecity of Cav-1-deficient LNCaP cells.

Material and methods

Materials

Anti-Cav-1 antibody (rabbit polyclonal) was purchased from BD Biosciences (San Jose, CA). Anti-actin (mouse monoclonal) was obtained from Sigma (St. Louis, MO). Anti-myc (rabbit polyclonal) was from Upstate (Lake Placid, NY). Anti-ApoA1 (rabbit polyclonal) was a gift from Dr. Joachim Herz (UT Southwestern Medical Center, Dallas). HDL was prepared as previously described.[17] All other reagents were obtained from Sigma, Invitrogen (Carlsbad, CA) and Calbiochem (La Jolla, CA).

Cell culture and transfection

LNCaP cells were obtained from ATCC (Manassas, VA) and grown in RPMI1640 with 10% fetal bovine serum (FBS) from Hyclone (Logan, UT). Transfection was carried out using Lipofectamine (Invitrogen) according to manufacturer's protocol. The preparation of Cav-1 construct was described previously.[18] For generating Cav-1 stable cell lines ($LNCaP^{Cav\text{-}1}$), LNCaP cells were transfected and then selected in the presence of 600 μg/ml G418. Finally, pooled clones were maintained in a medium containing 200 μg/ml G418.

Preparation of conditioned medium

Equal number (3×10^6) of parental LNCaP and $LNCaP^{Cav\text{-}1}$ cells were seeded into each 10-cm dish and grown in regular media for 2 days. Cells were then washed 3 times with serum-free medium followed by incubation with serum-free medium for 48 hr. Conditioned medium (CM) was collected and contamination of membranous proteins was removed by centrifugation for 1 hr at 220,000g_{av} at 4°C. The supernatant was collected and

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either used directly for analysis (from transient transfectant) or further concentrated 5-fold (from stable transfectant) using Centriprep centrifugal filter devices (Milipore Billerica, MA) with a molecular weight exclusion of 30,000 Da.

Density gradients

For CsCl step gradients, 1 ml of CsCl with the density of 1.35 g/ml was applied to the bottom of a SW41 tube and overlaid with 4.5 ml of 1.21 g/ml CsCl, 3.5 ml of 1.063 g/ml CsCl, and finally topped with 2 ml CM. For Optiprep step gradients, the 60% Optiprep stock solution (AXIS-Shield, Rodeløkka, Sweden) was diluted using HEPES buffer (20 mM HEPES, 100 mM KCl, 2 mM $MgCl_2$, pH7.4 with KOH). Optiprep solution (4 ml of 40%) was loaded into an SW41 tube followed by 3 ml of 20%, 1 ml of 10%, 1 ml of 5% Optiprep solution, and overlaid with 2 ml CM. All gradients were centrifuged for 21 hr in an SW41 rotor at 200,000g_{av} at 4°C. After centrifugation, fractions were collected from the top of the gradient, 500 μl each, and proteins of these fractions were precipitated using trichloroacetic acid (TCA), washed with acetone and dissolved in 20 μl SDS sample buffer containing 2% β-mercaptoethanol. Samples were subjected to SDS-PAGE and processed for immunoblot detection.

Purification of secreted cav-1 using Ni-NTA agarose

LNCaP cells transiently transfected with His_6-tagged Cav-1 were washed 3 times with serum-free medium and then incubated with serum-free medium for 48 hr. After incubation, the CM was harvested and spun for 1 hr at 220,000g_{av} at 4°C. Supernatant (9 ml) was incubated with 1 ml Ni-NTA agarose bead slurry (Qiagen, Valencia, CA), pre-equilibrated with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole pH 8.0). The suspension was rotated on a wheel for 2 hr at 4°C. After incubation, the suspension was loaded into a 10-ml Poly-Prep chromatography column (Bio-Rad, Richmond, CA) and the flow-through was collected. Beads were washed with 6 bed volumes of wash buffer containing 500 mM NaCl, followed by a wash with 3 bed volumes of wash buffer containing 20 mM imidazole. Proteins were sequentially eluted 3 times with 1 ml wash buffer containing 50 mM imidazole, 3 times with 1 ml wash buffer containing 150 mM imidazole and finally 6 times with 1 ml wash buffer containing 250 mM imidazole. For immunoblot analysis, the proteins of all 12 elution fractions, the flow-through, and the combined eluents were subjected to SDS-PAGE.

Negative staining

Purified particles were processed for negative staining by using glow-discharged, formvar-coated, 400-mesh nickel grids as described before.[13] Aqueous uranyl acetate (1%) was used to stain all samples before viewing with the JEOL 1200 CX electron microscope at 80 kV.

Fast protein liquid chromatography

Concentrated CM (500 μl) was loaded onto a Superose 6 HR column (GE Healthcare, Piscataway, NJ) that was pre-equilibrated with degassed HEPES buffer. The sample was then eluted with the same buffer with a flow rate of 400 μl/min. Each 1-ml fraction was collected, and proteins from these fractions were precipitated using TCA and subjected to SDS-PAGE and immunoblot analysis. The column was calibrated with proteins of known molecular weight.

Cell proliferation assay using MTT

Parental LNCaP and LNCaP$^{Cav\text{-}1}$ cells (4,000 cells/well) were plated into 96-well plates. On the indicated day, cell proliferation was measured using MTT assay according to manufacturer's instruction (Roche, Indianapolis, IN). The measurements were made in triplicate.

Nude mice injection

LNCaP or LNCaP$^{Cav\text{-}1}$ cells were trypsinized, washed with serum-free medium and 1×10^6 cells were resuspended in 150 μl matrigel (BD Biosciences, San Jose, CA). Each 4–6-week-old athymic male nude mouse (nu/nu) from Harlan (Indianapolis, IN) received 3 subcutaneous injections per side with 1×10^6 cells. Six weeks after injection, mice were sacrificed and solid tumor formation was examined.

Immunohistochemistry

Tumors were collected from euthanized mice and sliced into small pieces. Tumor tissues were immersion-fixed for 1 hr at 25°C in 4% (w/v) formaldehyde in phosphate-buffered saline (PBS) and then embedded in paraffin. Paraffin sections (5 μm thick) were dewaxed with xylene and rehydrated with PBS. The sections were washed with 50 mM NH_4Cl in PBS for 30 min and blocked by incubation for 1 hr with Tris-buffered saline (TBS) containing 10% (v/v) normal goat serum and 1% (w/v) bovine serum albumin. The sections were then incubated overnight in the presence of either Cav-1 IgG (10 μg/ml) or nonimmune rabbit IgG (10 μg/ml) in blocking buffer. The slides were washed 3 times in TBS, and bound primary antibody was detected by incubation for 2 hr with 10 μg/ml Alexa-Fluor488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) in the blocking buffer. The slides were washed 3 times in TBS, incubated with DAPI to stain DNA, washed again 3 times in TBS and mounted. Images were taken using Leica TCS SP confocal microscope.

Results

Previously Tahir *et al.*[15] reported that high passage LNCaP cells, a human prostate cancer cell line, grew faster than low passage LNCaP cells and that in contrast to low passage cells, high passage cells expressed Cav-1. To test if this increased proliferation was an effect of Cav-1 expression, we expressed Cav-1 both transiently and stably in LNCaP cells, that had no detectable Cav-1 expression (Fig. 3*a*). In contrast to the vector-transfected cells, mixed clones of LNCaP cells stably expressing myc-tagged Cav-1 (LNCaP$^{Cav\text{-}1}$) showed a significant increase in cell growth (Fig. 1*a*).

To test if Cav-1 expression might affect prostate tumor formation *in vivo*, we used a xenograft animal model. Parental LNCaP cells and LNCaP$^{Cav\text{-}1}$ cells were injected into the flanks of nude mice. One group of nude mice received 2 million LNCaP cells/mouse on both flanks, and the other group had an equal number of myc-tagged LNCaP$^{Cav\text{-}1}$ cells. Six weeks after injection, we found only 7% tumor incidence in nude mice injected with parental LNCaP cells (Fig. 1*b*, right panel; $n = 15$), whereas nude mice injected with LNCaP$^{Cav\text{-}1}$ showed 93% tumor incidence (Figs. 1*b*, middle panel, and 1*c*; $n = 15$). Therefore, expression of Cav-1 enhances not only LNCaP cell growth *in vitro* but also tumor formation *in vivo*.

To see if LNCaP$^{Cav\text{-}1}$ cells might affect the growth of Cav-1 null LNCaP cells, we injected equal numbers of parental LNCaP and LNCaP$^{Cav\text{-}1}$ cells on contralateral sides of the same nude mouse. Six weeks after injection, we found 94% tumor incidence on the side injected with LNCaP$^{Cav\text{-}1}$ cells (Fig. 1*b*, left panel; $n = 18$). Unexpectedly, we found significant incidence of tumor formation (66%) of the parental LNCaP cells [$n = 18$; Student's t test, $p < 0.05$, (Figs. 1*b* and 1*c*)]. Moreover, tumor formation of LNCaP cells was only observed in animals with tumor formation of LNCaP$^{Cav\text{-}1}$ cells. These results suggest that LNCaP$^{Cav\text{-}1}$ cells produce factors that influence tumor formation of parental LNCaP cells. Within the double injection experiment (Figs. 1*b* and 1*c*), we also compared the weight of solid tumors derived from parental LNCaP cells and LNCaP$^{Cav\text{-}1}$ cells when injected contralaterally and found that solid tumors derived from LNCaP$^{Cav\text{-}1}$ cells were significantly heavier compared to solid tumors derived from

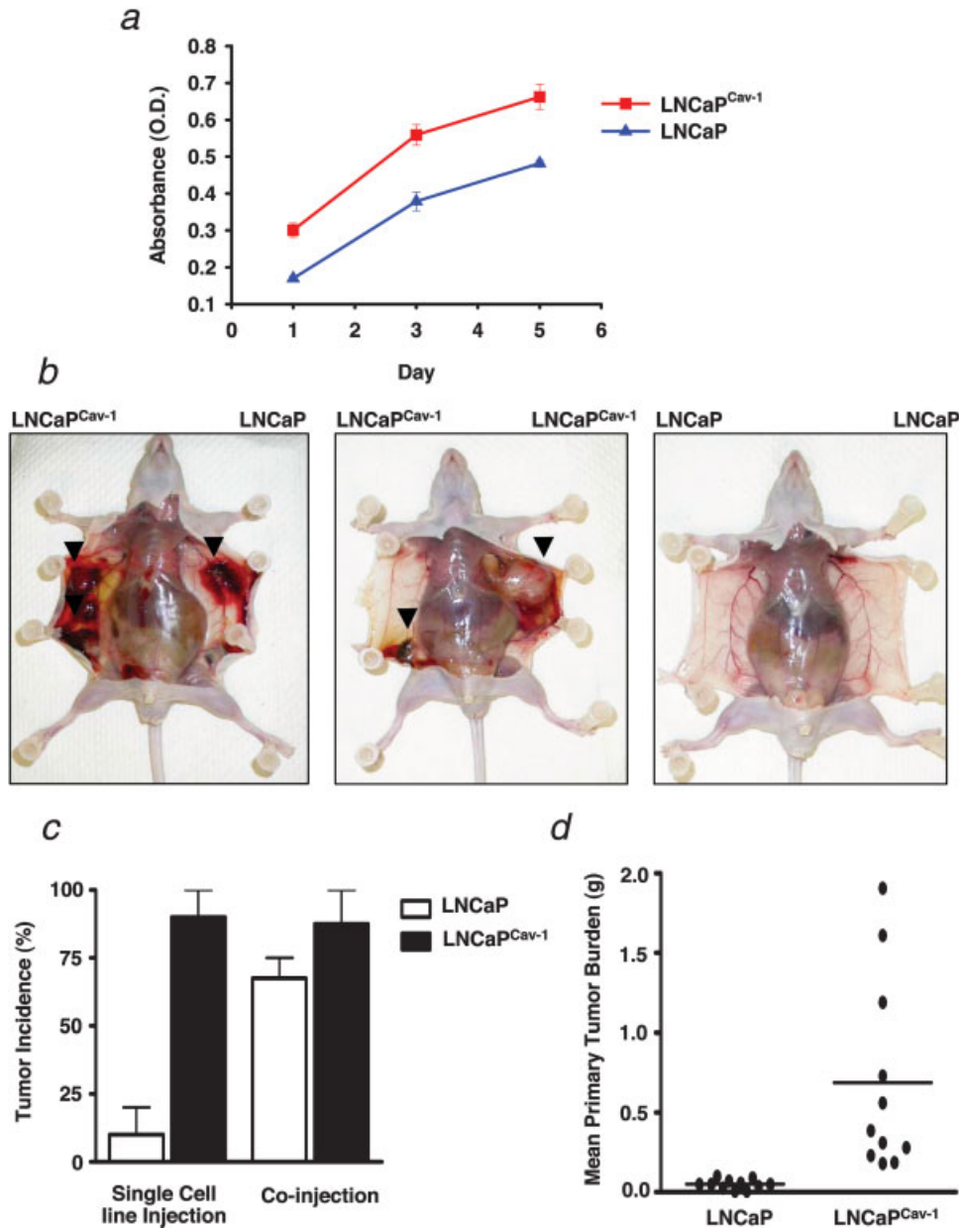

FIGURE 1 – Cav-1 expression stimulates cell growth *in vitro* and promotes tumor formation *in vivo*. (*a*) Four thousand LNCaP cells stably expressing myc-tagged Cav-1 (LNCaP^{Cav-1}) or parental LNCaP cells were plated into each well of a 96-well plate. On the indicated day, cell proliferation was measured using MTT assay according to manufacturer's protocol. Data represent mean of 3 experiment points plus standard deviation. (*b*) Two million parental LNCaP or LNCaP^{Cav-1} cells were injected into nude mice (1 million on each flank distributed into 3 injection spots) as described in section "Material and Methods." Six weeks after injection, mice were examined for solid tumor formation. The representative images showed no solid tumor formed on a mouse injected with parental LNCaP cells (right panel) whereas solid tumors were found on a mouse injected with LNCaP^{Cav-1} cells (center, see arrowheads). Animals that received injections of both cell lines on contralateral sides showed tumor formation on both sides (left, arrowheads). (*c*) Nude mice received injections of either parental LNCaP or LNCaP^{Cav-1} cells indicated as "single cell injection" ($n = 15$ each), and received injections of both cell lines on contralateral sides indicated as "co-injection" ($n = 18$, Student's t test, $p < 0.05$). (*d*) Tumor weight from parental LNCaP or LNCaP^{Cav-1} tumors. Six weeks after co-injection, the weight of each tumor ($n = 11$ each) was determined; LNCaP^{Cav-1} tumors were significantly higher than LNCaP tumors (Student's t test, $p < 0.05$).

parental LNCaP cells [$n = 11$; Student's t test, $p < 0.05$, (Fig. 1*d*)].

Another explanation of these results is that LNCaP^{Cav-1} cells migrate to the injection sites of parental LNCaP cells and form tumors. Therefore, we processed samples from these tumors for immunohistochemistry. Whereas we did not detect any expression of myc-tagged Cav-1 in tumors derived from parental LNCaP cells, we detected abundant expression of myc-tagged Cav-1 in tumors derived from LNCaP^{Cav-1} cells (Fig. 2*a*). We also isolated tumor cells and grew them under tissue culture conditions. Tumor cells derived from the parental LNCaP tumor did not show any detectable myc-tagged Cav-1 expression (Fig. 2*b*) indicating that there were no more rapidly growing LNCaP^{Cav-1} cells in the tumor. In addition, these cells did not express any detectable Cav-1 either, suggesting that the increased growth rate of these cells is not due to the expression of endogenous Cav-1. On the other hand, the cells derived from LNCaP^{Cav-1} tumor were myc-tagged Cav-1 positive (Fig. 2*b*) and grew faster when compared to cells

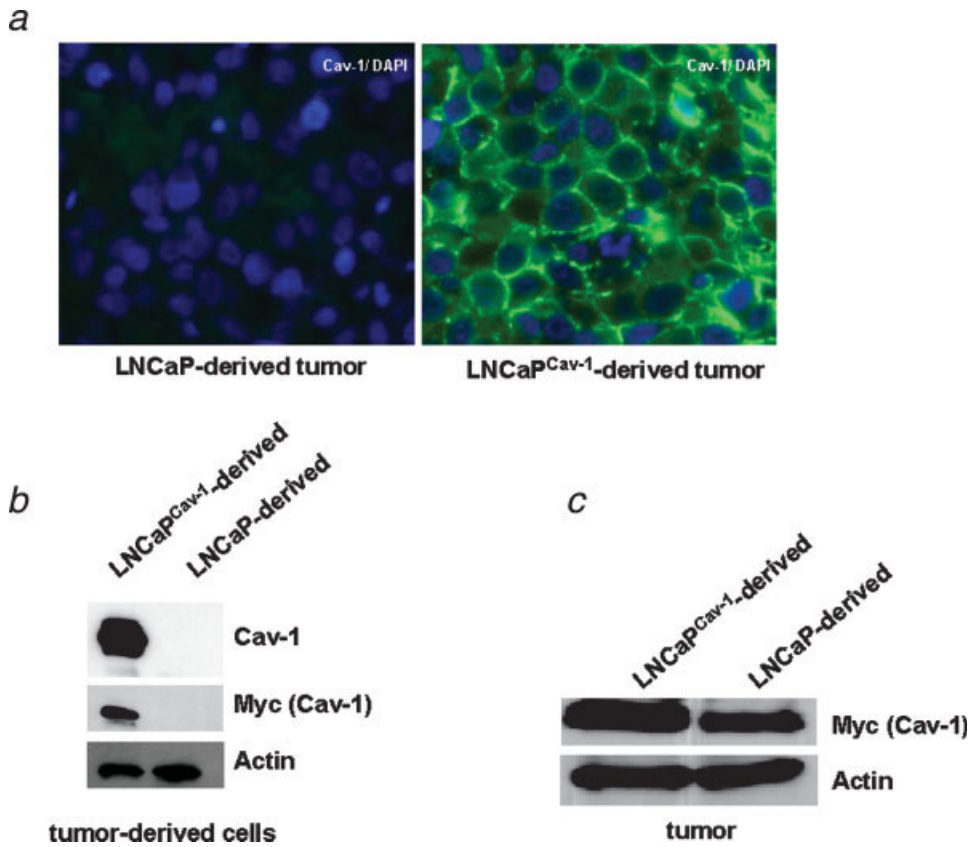

FIGURE 2 – Tumor formation is not caused by migration of LNCaP^{Cav-1} cells. (*a*) Immunohistochemistry analysis of parental LNCaP and LNCaP^{Cav-1} tumors from co-injected mice was performed as described in section "Material and Methods" (blue: DAPI staining; green: Cav-1 staining). (*b*) Excised tumors from both sides of LNCaP/LNCaP^{Cav-1} co-injection animals were cut into small pieces and cultivated in RPMI1640 medium containing 10% FBS under tissue culture conditions (37°C, 5% CO_2, tissue culture plastic). After 8 weeks of culture, equal number of cells (60,000) were lysed in 100 µl SDS sample buffer, separated by SDS-PAGE and analyzed by immunoblot using the indicated antibodies. Actin was shown as a loading control. (*c*) Tumors collected from both sides of co-injected animals as described above were excised and an equal wet weight of tumor (0.1 g) was added in 200 µl SDS sample buffer, sonicated, separated by SDS-PAGE and analyzed by immunoblot using the indicated antibodies. Actin was shown as a loading control.

isolated from LNCaP tumor (data not shown). However, when whole tumors were excised and processed for immunoblotting normalized by wet weight of tumor, we detected the presence of myc-tagged Cav-1 in LNCaP tumor (Fig. 2*c*), suggesting that a secreted form of Cav-1 but not the LNCaP^{Cav-1} cells themselves reached the LNCaP tumors on the contralateral side.

The above results indicate that LNCaP^{Cav-1} cells have a potential endocrine activity that influences tumor growth and the expression of Cav-1 alters the secretory activity of LNCaP cells. Since myc-tagged Cav-1 appears to be produced by LNCaP^{Cav-1} cells and migrates to the opposite side of the animal, we assayed the CM from LNCaP cells transiently and stably expressing Cav-1 for the presence of Cav-1. Immunoblotting detected myc-tagged Cav-1 in the CM from LNCaP cells either transiently or stably expressing Cav-1 (Fig. 3*a*). Since Cav-1 secreted by exocrine cells is a HDL-like particle,[14] we analyzed the density of secreted Cav-1 using CsCl and Optiprep step gradients. Secreted Cav-1 migrated in both gradients in fractions with a slightly higher density as HDL [monitored with anti-ApoA1 antibody, (Fig. 3*b*)]. We then used Superose 6 HR gel filtration fast protein liquid chromatography (FPLC) column to estimate the molecular weight of the Cav-1 containing lipoprotein particles and found them to be larger than 660 kDa (Fig. 3*c*). We conclude that the Cav-1 secreted by LNCaP cells is embedded in a lipoprotein particle.

Since the density gradient or FPLC purification was not sufficient for a morphological analysis of the secreted particle, we further purified secreted Cav-1 from CM of LNCaP cells expressing His_6-tagged Cav-1 using Ni-NTA conjugated agarose beads. The secreted His_6-tagged Cav-1 on Ni-NTA beads was competed away by increasing concentrations of imidazole, thus verifying their specific association (Fig. 4*a*). To test if the purification changed the integrity of soluble Cav-1 particle, we analyzed the eluted fractions in CsCl density gradients as described earlier. The purified Cav-1 resided in the same fractions as the secreted Cav-1 before Ni-NTA beads purification (compare Fig. 4*b* with Fig. 3*b*), showing that secreted Cav-1 particles remain intact after purification. We then performed electron microscope (EM) analysis to visualize the purified Cav-1 particles using negative staining. The EM image showed a homogenous population of particles with a size ranging from 15 to 30 nm (Fig. 4*c*) that is larger than HDL (10 nm). Altogether, our results clearly demonstrate that LNCaP^{Cav-1} cells secreted Cav-1 and the secreted Cav-1 exists in a form of lipoprotein particles.

Discussion

Previously Tahir *et al.*[15] compared low passage LNCaP cells which did not express detectable Cav-1 to Cav-1 expressing high passage LNCaP cells, and found an increased proliferation of the latter. To investigate a possible correlation of Cav-1 expression and proliferation more directly in same cell system, we expressed Cav-1 in LNCaP cells. Using this approach, we show that ectopic expression of the protein in LNCaP cells enhances the growth of these cells *in vitro* as well as their ability to form tumors *in vivo*.

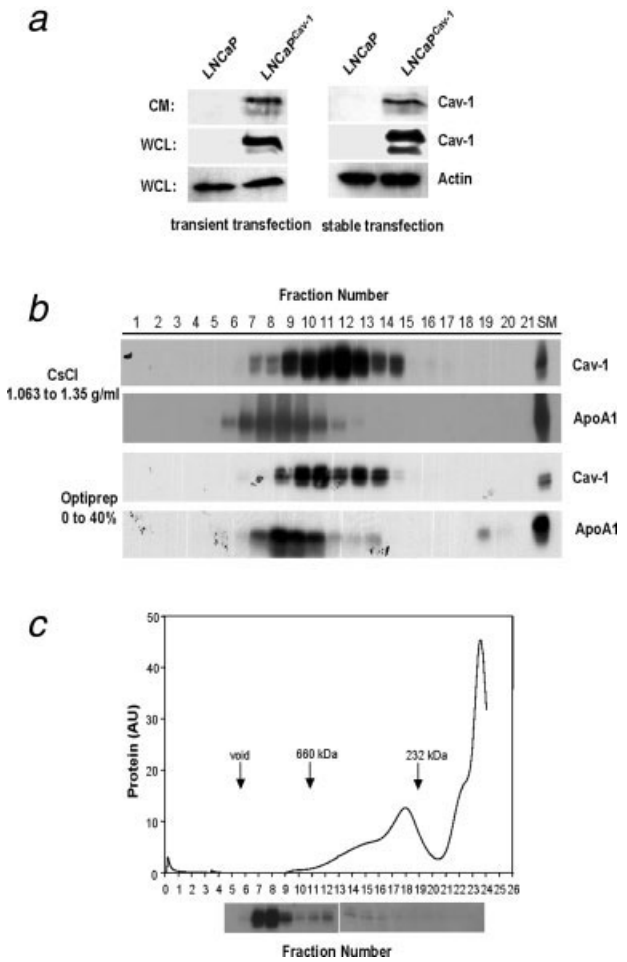

FIGURE 3 – Cav-1 is secreted as a particle. (*a*) Parental LNCaP and LNCaP^{Cav-1} (transient transfected, left panel or stably transfected, right panel) cells were incubated with serum-free medium for 48 hr, starting 1 day after transfection (for transient). The concentrated CM or whole cell lysates was collected, proteins separated by SDS-PAGE and analyzed with immunoblot using the indicated antibodies. (*b*) The CM from LNCaP^{Cav-1} cells (transient transfected) was loaded on CsCl or Optiprep density gradients as indicated and centrifuged for 21 hr in an SW41 rotor at 200,000g_{av} at 4°C. HDL (40 μg) was used in control gradients performed in parallel experiments. Fractions (1, top; 20, bottom) were collected, and proteins precipitated using TCA and subjected to SDS-PAGE followed by immunoblot using anti-Cav-1 and anti-ApoA1 antibodies as indicated (SM, starting material). (*c*) 500 μl concentrated CM was applied to a Superose 6 HR FPLC column. The graph shows the protein elution profile. Fractions were collected, and proteins precipitated using TCA and subjected to SDS-PAGE followed by immunoblot with anti-Cav-1 antibody.

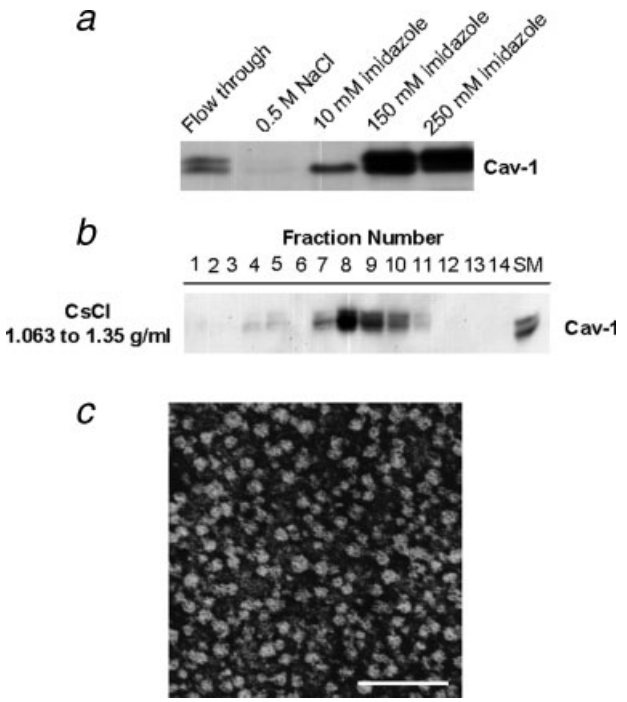

FIGURE 4 – Purification of secreted Cav-1 particles. (*a*) The CM from LNCaP cells transiently expressing His_6-tagged Cav-1 was prepared as described in Figure 3, and then collected and purified using Ni-NTA chromatography as described in section "Material and Methods." The indicated concentration of imidazole was used to elute His-tagged Cav-1 from Ni-NTA beads. The fractions of flow through, 0.5 M NaCl or 10 mM imidazole wash were combined. The proteins of these pooled samples and eluents of 150 and 250 mM imidazole were precipitated using TCA and immunoblotted using anti-Cav-1 antibody. (*b*) The eluents (pooled 150 and 250 mM fractions) from Ni-NTA beads were loaded on CsCl density gradient as described in Figure 3. Fractions were collected, and proteins precipitated and subjected to SDS-PAGE. The presence of Cav-1 was detected by immunoblot using anti-Cav-1 antibody (SM, starting material). (*c*) The eluents (pooled 150 and 250 mM fractions) from Ni-NTA beads were visualized by EM using negative staining (scale bar, 100 nm) as described in section "Materials and Methods."

Importantly, we further demonstrate that LNCaP^{Cav-1} cells stimulate tumor growth of parental LNCaP cells when co-injected into the same animal. We speculate that the expression of Cav-1 in LNCaP cells leads to secretion of a factor which acts at a distance to promote growth of the parental cell line. That stimulation is due to a secreted factor is supported by the observation that we did not detect LNCaP^{Cav-1} cells that might have migrated to the LNCaP injection sites of the same animal to form tumors in the LNCaP cell-derived tumors. However, when we analyzed the whole tumor with its surrounding tissue and body fluids, myc-tagged Cav-1 could be detected. One possible explanation for this observation is that secreted Cav-1 itself migrates to another side of animal and stimulates tumor growth of parental LNCaP cells.

Previously, we described the secretion of Cav-1 from pancreatic acinar cells[14] and Cav-1 secretion has also been reported from high passage LNCaP cells that express Cav-1.[15] Here, we show that Cav-1 is secreted from LNCaP cells when the protein is expressed transiently or stably. Judging from the biochemical properties of the secreted Cav-1, we postulate that Cav-1 is secreted as a lipoprotein particle with a slightly higher density when compared to HDL particles, suggesting a different lipid/protein ratio. Interestingly, the CM from high passage LNCaP cells that express Cav-1 has been shown to stimulate clonal growth and to prevent apoptosis of low passage LNCaP cells that do not express detectable endogenous Cav-1.[15]

As a model of how secreted Cav-1 lipoprotein particle might act as autocrine/endocrine factor, we suggest that secreted Cav-1 lipoprotein particle could be a ligand for a cell surface receptor, similar to lipoprotein ligands from the Wnt and hedgehog protein family.[19,20] In this model Cav-1 particle binds directly to an unknown cell surface receptor on LNCaP cells and subsequently stimulates cell growth. We do not know if Cav-1 itself would facilitate the binding directly or if other proteins or lipids on the same particle bind to the receptors and stimulate proliferation. A further biochemical characterization is necessary to identify the protein- and lipid-composition of the secreted particle to identify these molecules. One possibility would be that the particle is able to reorganize or cluster cell surface receptors into a specific plasma membrane domain (*e.g.* caveolae/lipid rafts). This might promote signaling complex assembly and initiation of downstream

signaling events like increased ERK activity, which can be observed in LNCaP$^{Cav\text{-}1}$ cells (data not shown). At this point, we do not know which receptor is engaged in signaling upon Cav-1 expression that leads to increased ERK activity. Furthermore, we cannot rule out the possibility that the expression of Cav-1 induces the secretion of stimulatory factor(s) not related to the Cav-1 positive particle. Additional experiments, *e.g.* co-injecting the purified particle with LNCaP cells into nude mice or incubation with LNCaP cells *in vitro* are necessary to support one of the suggested hypotheses.

Previous studies showed that Cav-1 reduces androgen sensitivity during prostate cancer progression;[6] therefore, one possible explanation for the rapid proliferation of LNCaP$^{Cav\text{-}1}$ cells is that upon expression of the protein, these cells develop an androgen independency.

Taken together, our findings provide evidence that Cav-1 is secreted as lipoprotein particles from LNCaP cells both transiently and stably expressing Cav-1. Significantly, expression of Cav-1 is sufficient to stimulate LNCaP cell growth *in vitro* and to promote tumor formation in nude mice in an autocrine/endocrine manner. Our results might offer a potential mechanism by which secreted Cav-1 particles act as a potent endocrine factor to promote prostate tumor formation. Thus, our study indicates a novel function and mechanism by which Cav-1 contributes to prostate cancer progression.

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