CAF-Secreted Annexin A1 Induces Prostate Cancer Cells to Gain Stem Cell–like Features

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Abstract

Annexin A1 (AnxA1), a phospholipid-binding protein and regulator of glucocorticoid-induced inflammatory signaling, has implications in cancer. Here, a role for AnxA1 in prostate adenocarcinoma was determined using primary cultures and a tumor cell line (cE1), all derived from the conditional Pten deletion mouse model of prostate cancer. AnxA1 secretion by prostate-derived cancer-associated fibroblasts (CAF) was significantly higher than by normal prostate fibroblasts (NPF). Prostate tumor cells were sorted to enrich for epithelial subpopulations based on nonhematopoietic lineage, high SCA-1, and high or medium levels of CD49f. Compared with controls, AnxA1 enhanced stem cell–like properties in high- and medium-expression subpopulations of sorted cE1 and primary cells, in vitro, through formation of greater number of spheroids with increased complexity, and in vivo, through generation of more, larger, and histologically complex glandular structures, along with increased expression of p63, a basal progenitor marker. The differentiated medium-expression subpopulations from cE1 and primary cells were most susceptible to gain stem cell–like properties as shown by increased spheroid and glandular formation. Further supporting this increased plasticity, AnxA1 was shown to regulate epithelial-to-mesenchymal transition in cE1 cells. These results suggest that CAF-secreted AnxA1 contributes to tumor stem cell dynamics via two separate but complementary pathways: induction of a dedifferentiation process leading to generation of stem-like cells from a subpopulation of cancer epithelial cells and stimulation of proliferation and differentiation of the cancer stem-like cells.

Implications: AnxA1 participates in a paradigm in which malignant prostate epithelial cells that are not cancer stem cells are induced to gain cancer stem cell–like properties. Mol Cancer Res; 12(4); 607–21. ©2014 AACR.

Introduction

Annexins comprise a family of proteins that bind to specific phospholipids in a calcium-dependent manner (1, 2). The calcium-binding and phospholipid-binding sequences of annexins are encoded in the conserved C-terminus (40%–60% homology), whereas an N-terminus of varying length and sequence is unique to each family member and confers the specific biologic activity (3). Annexin A1 (AnxA1), a 37-kDa protein, the first-characterized member of the family, was originally reported for its antiphospholipase activity after glucocorticoid induction (4, 5). Subsequent studies showed that both recombinant AnxA1 and AnxA1-derived N-terminal peptides possess a wide range of anti-inflammatory properties (6–8). In addition to the regulatory region, the N-terminus also contains the sites for phosphorylation (3). Various signal transducing kinases, such as EGF receptor tyrosine kinase, platelet-derived growth factor receptor kinase, hepatocyte growth factor receptor kinase, TRPM7 channel kinase, and protein kinase C (PKC) are known to phosphorylate AnxA1 and contribute to its biologic function (9, 10).

AnxA1 does not possess a recognized signal sequence for targeting to the endoplasmic reticulum (11). It seems to follow a cell-specific novel manner of secretion (12). In neutrophils, gelatinase granules store high levels (>60%) of cytoplasmic AnxA1 for extrusion upon activation (13). Cells that do not store AnxA1 in granules display another distinct secretory pathway. In macrophages and pituitary folliculo-stellate cells, AnxA1 is exported by the ATP-binding cassette A1 (ABC-A1) transporter system or ATP-sensitive K+ channels (14–17). In both leukocytes and pituitary cells, phosphorylation on Ser27 is necessary for protein export and secretion. This action is directed by Ca2+-dependent isoforms of PKC, and subsequent translocation of the serine27–phosphorylated species of AnxA1 to the plasma membrane occurs at specific lipid domains...
that allow for secretion (18–20). Extracellular Ser²⁷-
-AnxA1 undergoes a conformational change in the pres-
ence of ≥1 mmol/L Ca²⁺ causing exposure of the N-
terminal domain from inside the pore created by the four
repeated motifs of the core domain and, thereby, binding
to its receptor (19, 21). Binding of AnxA1 to the receptor
activates downstream signaling via phosphorylation of the
mitogen-activated protein kinase (MAPK) extracellular
signal—regulated kinase (Erk)-1/2 (22–24).

Through its specific N-terminal sequence, AnxA1 directly
interacts with a family of G-protein coupled receptors, the
formyl peptide receptors (FPR), which includes FPR1, FPR2
(also known as ALXR), and FPR3 in humans and Fpr1 and
Fpr-related proteins (Fpr-rs) 1–7 in mouse (25, 26). Endo-
genous and recombinant full-length AnxA1 specifically bind
to FPR2/ALXR and murine Fpr-rs1 (approximately 30%
activity has been selectively demonstrated for Fpr1), whereas
the bioactive N-terminal–derived peptide comprised amino
acids of 2–26, Ac2-26, was shown to activate all members
of the human FPR family and the murine Fpr1 and Fpr-rs1,
and may additionally activate Fpr-rs2 (6, 12, 25, 27).

About tissue distribution, AnxA1 is found in high abun-
dance in lung, bone marrow, intestine, lymphatic tissue, and
reproductive tracts, and interestingly, with highest concen-
tration in the seminal fluid of the prostate (150 µg/mL;
ref. 11). It is also found to have differential expression in
certain cancers (28). In prostate cancers, AnxA1 loss of
expression from the ductal epithelial cells was reported
(29–33). Prostatic stromal cells also display expression of
AnxA1 (11). In ductal carcinoma in situ and invasive
carcinoma breast tumors, stromal AnxA1 expression was
positively correlated with infiltration of both epithelial and
stromal cells (34).

We previously described evidence that cancer-associated
fibroblasts (CAF), derived from the stromal compartment of
prostate tumors, secrete factors that enhance both the
stemness and growth potentials of cancer stem cells (CSC)
from the primary prostate tumor (35). We used the condi-
tional Pten deletion with activated luciferase reporter (cPten
⁻/⁻/L) mouse model of prostatic adenocarcinoma (36, 37).
Here, we identified AnxA1 as one of the pertinent
secreted factors. AnxA1 treatment of a prostate cancer cell
line from the mouse model led to upregulation of epithelial-
to-mesenchymal transcription (EMT) factors as well as
pluripotency transcription factors in vitro. Primary epithelial
tumor cells or cells of the cell line treated with AnxA1
displayed enhanced glandular structure formation and bas-
al/progenitor marker p63 expression in vivo, compared with
controls. Treatment with AnxA1 in vitro and in vivo is
associated with pErk1/2 activation. Together, these findings
indicated that AnxA1 might be involved in de novo generation
of CSCs from cancer cells as well as maintenance of the
CSC population.

Materials and Methods

Animals

The conditional Pten deletion mouse model with lucif-
erase reporter (cPten⁻/⁻/L) used in the current work was
described previously (36). For renal tissue grafting, nonobese
diabetic/severe combined immunodeficient (NOD/SCID)
mice, purchased from National Cancer Institute (NCI;
Frederick, MD), were used. All protocols were approved
by the Animal Care Committee of the University of Southern
California (Los Angeles, CA).

Isolation of murine cells and sorting

Urogenital sinus mesenchyme (UGSM; ref. 38), normal
prostate fibroblasts (NPF), and CAFs (39) were isolated
following published procedures. The generation of the cE1
prostate cancer cell line from the mouse model was described
(40). Preparation of primary cultures of epithelial cells from
single cell suspensions of minced prostate tumors from
cPten⁻/⁻/L mice (36), cell staining, and isolation by fluo-
rescence-activated cell sorting (FACS), with exception of
fluorophores used (see Supplementary Materials and Methods),
were described before (35).

Cell culture and assays for spheroid formation

Growth of epithelial cells in Matrigel has been described
(35). Cells were cocultured with fibroblasts or treated with
conditioned media as indicated in the results. For coculture
experiments, epithelial cells were embedded in Matrigel, and
fibroblasts were grown in chamber inserts at 10:1 fibroblast
to epithelial cells. LNCaP human prostate cancer cells
(American Type Culture Collection, ATCC) and PC3
(ATCC) were plated on Matrigel precoated wells. For
detailed cell culture conditions, see Supplementary Materials
and Methods.

Conditioned media and AnxA1 ligands

CAF and NPF conditioned media (CM) were prepared by
24-hour incubation of serum-free Dulbecco’s modified
Eagle medium per 5 µg/mL insulin with confluent stromal
cultures. Collected medium was centrifuged at 300 × g for 5
minutes to remove contaminants, normalized by protein
quantification using Bradford reagent (Bio-Rad) in a Bench-
mark Plus Microplate Spectrophotometer (Bio-Rad) and
also compared with number of fibroblast cells per plate,
counted at time of collection. After concentration using
Amicon Ultra-15 3K Centrifugal Filter Units (Millipore),
conditioned media was used to treat epithelial cells at 0.04
mg/mL or ratio of 10:1 fibroblast to epithelial cells. Ammon-
ium sulfate conditioned media fractions were prepared
following a published procedure (41). Pelleted proteins were
solubilized in 1× PBS and dialyzed overnight, followed by
centrifugal concentration.

Murine recombinant AnxA1 protein was produced as
an N-terminal 6xHis tag fusion protein. Full-length
mouse AnxA1 cDNA (Invitrogen) was subcloned into
pET/TOPO-D vector in BL21 Star (DE3; Invitrogen)
bacteria. Protein expression was induced by 1 mmol/L
isopropyl-thio-galactoside. Fusion protein was extracted
using 6xHis Fusion Protein Purification Kit and purified
using Pierce High Capacity Endotoxin Removal Spin
Columns, both from Thermo Scientific, followed by
centrifugal concentration. Peptide Ac2-26 (acetyl-
AMVSEFLKQAWFIENEEQYEYQVTYK-OH trifluoracetate salt; M, 3089) was purchased from Bachem. Purity was more than 94% as assessed by high-performance liquid chromatography (data supplied by manufacturer).

Renal grafting
Murine cell line and primary epithelial cells were treated with vehicle control, CAF CM, peptide Ac2-26, or recombinant AnxA1 for 14 days before being passaged for transplantation. During passaging, cells did not receive additional treatment. As published (42), epithelial cells (10^5) were mixed with stromal cells (10^5) in 70 μL neutralized rat tail collagen type I (BD Biosciences) before transplanting under the renal capsule of 8- to 12-week-old male NOD/SCID mice (35). See Supplementary Materials and Methods for full details.

Immunostaining and Western blot analyses
Preparation of spheroids and renal tissues for immunostainings was as previously described (35, 36). Primary antibodies for immunostains, Western blot analyses, and neutralizing antibody (nAb) against N-terminal residues of AnxA1, dilutions, and manufacturers are listed in Supplementary Table S1. Photomicrographs were captured with Spot Advanced software (Spot Imaging Solutions) and quantified with ImageJ software (NIH). Immunoblot analyses were captured and quantified with Image Lab software (Bio-Rad). For Western blot analysis, whole-cell lysates were prepared by addition of radioimmunoprecipitation assay buffer (Sigma) with proteinase inhibitor (Roche) and phosphatase inhibitor cocktails II and III (Sigma) at a 1X final concentration (manufacturer’s protocols).

PCR analyses
Extraction of total cellular RNA, reverse transcription reaction, and quantitative real-time PCR (qRT-PCR) were performed and analyzed as described (35). Primer sets are listed in Supplementary Table S2.

Statistical analysis
All data are presented as mean ± SD. qRT-PCR data are shown as mean ± SD of repeat analyses. Differences between individual groups were analyzed by paired t test or χ^2, as appropriate, using Excel (Microsoft Office) and InStat (GraphPad Software, Inc.) software. P values of <0.05 were considered to be statistically significant.

Results
Effect of CAFs on prostate cancer cells
We previously demonstrated that CAFs could stimulate the proliferation and tumorigenicity of prostate CSCs via a paracrine/juxtacrine interaction in vivo (35). Here, we constituted a test system composed of a prostate tumor cell line and primary cultures of CAF or NPF (39), all from the cPten+/−L mouse model. The tumor cell line, cE1, which was derived from a castration-resistant prostate cancer putative stem cells have been described. The hematopoietic Lineage (Lin) negative (CD31/CD45/TER119)/SCA-1+/CD49f−; LSC) phenotype within prostatic tissue cells was determined to contain cells with properties of self-renewal and differentiation (43, 44). Further enrichment for Lin-/SCA-1+/CD49f+ (LSCmed) cells based on high-expression levels of SCA-1 and CD49f led to increased capability for generation of prostate glandular structures in vivo (35). Subpopulations of cE1 cells displaying high, medium, or no expression of SCA-1 and CD49f (SCchi, SCmed, or SCnone, respectively) were recovered (Fig. 1B). Lineage markers were not necessary for selection by FACS for cE1 cells because contaminating nonepithelial cell types were removed during derivation of the cell line (40).

We described the formation of spheroids after coculture of Lin-/SCA-1+/CD49f−; LSC) phenotype within a Matrigel matrix with either NPFs or CAFs that were grown in inserts (35). Spheroids produced in coculture with CAFs were larger in size and were composed of multiple basal and luminal cell layers as opposed to those spheroids generated with NPFs or without stromal cells (35). After further fractionation of the LS cells into LSCmed and Lin-/SCA-1+/CD49f−; LSC) phenotype within cells derived SCchi and SCmed cell fractions. SCchi and SCmed contained cells that showed spheroid-forming ability, and the growth of the spheroids from both SCchi and SCmed were stimulated by conditioned medium collected from CAF. After CAF CM stimulation, the spheroids formed from SCmed seemed to contain increased number of CK5 and CK8 double-positive cells, possibly indicative of more transit-amplifying cells, relative to those from the SCchi cells (Fig. 1C).

The results of qRT-PCR analyses of SCchi and SCmed indicated some characteristic differences between these two subpopulations. As shown in Fig. 1D, SCchi cells grown under control culture conditions displayed significantly higher basal expression levels of pluripotency transcription factors Ocr4 (P < 0.01), Nanog (P < 0.001), and Sox2 (P < 0.001), and transcription factor Snail (P < 0.01) as compared with SCmed cells. Encouraged by these differences in basal levels, we then proceeded to determine the expression levels of Ocr4, Sox2, Nanog, and EMT transcription factors Snail, Slug, and Twist, and epithelial marker E-cadherin, and...
Figure 1. CAFs induce EMT to cE1 mouse prostate cancer cells in vitro. A, phase-contrast images of representative spheroids formed in cE1, after coculturing with either control media, NPFs, or CAFs. Bar, 100 μm. B, cE1 cells were FACS sorted into subpopulations based on high, medium, and no expression of SCA-1 (S) and CD49f (C; SChi, S Cmed, and SCnone, respectively). C, sorted cells grown in post-FACS 3D culture displayed differential response to CAF CM. cE1 SChi and SCmed spheroids increased in size in the presence of CAF CM. (Continued on the following page.)
mesenchymal markers N-cadherin and Vimentin in SC\textsuperscript{hi}, SC\textsuperscript{med} \textsuperscript{+}, and SC\textsuperscript{med} \textsuperscript{−} cells following exposure to CM from either CAF or NPF cultures. We noted extensive EMT-like phenotypic changes induced in SC\textsuperscript{med} \textsuperscript{+} cells by conditioned media from CMs and much less by that from NPFs (Supplementary Fig. S1A). Correspondingly, EMT\textsuperscript{−} SC\textsuperscript{med} \textsuperscript{−} cells displayed a significant upregulation of Oct4 (P < 0.05), Sox2 (P < 0.001), Snail (P < 0.001), Slug (P < 0.01), Twist (P < 0.01), N-cadherin (P < 0.001), and Vimentin (P < 0.05), significant decrease in E-cadherin (P < 0.01) as compared with SC\textsuperscript{hi} and SC\textsuperscript{med} \textsuperscript{+}, and upregulation of Nanog (P < 0.001) as compared with SC\textsuperscript{hi} (Fig. 1E). These changes were tested over a 14-day period, wherein expression levels of these transcription factors and markers significantly increased in SC\textsuperscript{med} \textsuperscript{−} cells from day 7 to day 14 time points (Fig. 1F).

On the basis of these findings, we inferred that although SC\textsuperscript{hi} did display certain molecular characteristics of stem/progenitor cells, such as spheroid-formation capacity, SC\textsuperscript{med} \textsuperscript{−} cells seemed to be enriched with a tumor cell fraction that was especially susceptible to CAF-induced EMT. More importantly, the factors responsible for induction of this biologic effect seemed to be contained within the conditioned medium prepared from CAF, indicating the presence of paracrine-acting secreted molecules.

**A search for responsible CAF factors identifies AnxA1**

To identify secreted factor(s) from the CAFs that are capable of inducing EMT and possibly contributing toward an EMT-CSC lineage, we decided to use fractionation techniques. Proteins from both NPF and CAF CM were salted out into five fractions following ammonium sulfate precipitation: 20%, 30%, 40%, 50%, and 60%. Increased EMT-like morphology and gene expression level changes consistent with previous experiments were seen when SC\textsuperscript{med} \textsuperscript{−} cells were exposed to CAF CM ammonium sulfate fractions, particularly at the 30% to 40% ammonium sulfate cut (Supplementary Fig. S2). Silver stain visualization of the proteins after fractionation of NPF and CAF CM presented detectable levels of a dark band in both CAF CM and CAF CM fractions, but which appeared weak or absent in the NPF CM and NPF CM fractions. We selected this band as a potential candidate for mass spectrometry, an analysis that pointed to the phospholipid-binding protein, AnxA1 (Fig. 2A and Supplementary Table S3). Western blot analysis using an antibody against AnxA1 confirmed its abundance in the CAF CM fractions as compared with the NPF CM fractions (Fig. 2B).

Phosphorylation of AnxA1 on a specific serine residue (S\textsuperscript{27}) by PKC has been extensively reported to be necessary for extracellular secretion of the protein (18–20). The presence of phosopho-serine within the context of the specific amino acid recognition motif necessary for PKC-mediated serine phosphorylation was assayed by Western blot analysis in CAF and NPF CMs. Multiple CAF cultures were shown to possess variably higher protein expression levels of phospho-Ser\textsuperscript{27}\textsuperscript{−}AnxA1 than NPF cultures, in addition to generally higher total abundance of AnxA1 (Fig. 2C). CAF derived from larger tumors (cells recovered ≥ 20 million) produced 4-fold more phospho-Ser\textsuperscript{27}\textsuperscript{−}AnxA1 than smaller tumors (<10 million cells), after normalization to protein mass (g). Analysis of phospho-Ser\textsuperscript{27}\textsuperscript{−}AnxA1 relative to tumor size gave a linear regression with R\textsuperscript{2} of 0.9914 (y = 4.1298x − 7.9487, n = 6). Known protein phosphorylators of AnxA1 were also assayed for upregulation in CAFs versus NPFs. Transcriptional expression levels of Pten, Trpm7, and selected PKC subtypes, revealed that PKC\textsubscript{\textbeta} is significantly upregulated in CAFs (P < 0.01; Fig. 2D).

**Effect of AnxA1 on cE1 cells in vitro**

nAb specific to the N-terminal portion of AnxA1 was incubated with cE1 cells treated with AnxA1 enriched fractions of CAF CM. Treatment with AnxA1 nAb showed a dose-dependent decrease in the number of spheroids more than 50 μm (P < 0.01 at 5 μg/mL; P < 0.001 at highest dose, 20 μg/mL; Supplementary Fig. S3A). Similarly, exposure of cE1 cells to AnxA1 nAb at the lowest dose, 5 μg/mL, and in the presence of CAF CM led to a significant decrease in spheroid number in both SC\textsuperscript{hi} and SC\textsuperscript{med} \textsuperscript{−} cells grown in 3D cultures (P < 0.01 and P < 0.05, respectively). These results indicated that the increased ability of cE1 cells to form spheroids in the presence of CAF CM could be negated by addition of the AnxA1 nAb (Fig. 3A).

In further support of the ability of AnxA1 nAb to repress spheroid proliferation and EMT-like properties of AnxA1-treated cells, total cE1 cells were allowed to grow in Matrigel for 14 days in the presence of CAF CM, ammonium sulfate-enriched CAF CM fractions, or N-terminal mimetic peptide (Ac2-26) with, or without, addition of AnxA1 nAb. Addition of AnxA1 nAb to cE1 cells grown in the presence of CAF CM, ammonium sulfate-enriched CAF CM fractions, or Ac2-26 led to reduced expression of Snail, Slug, Twist, and...
Oct4, indicating the importance of this protein in promoting EMT and certain features of stemness (Fig. 3B). Consistently, Snail and Oct4 expression was significantly upregulated after treatment with CAF CM, ammonium sulfate-enriched CAF CM fractions, and Ac2-26. Twist upregulation was found to be more prevalent in CAF CM fractions and Ac2-26. Pronounced and significant decrease in expression of Snail, Twist, and Oct4 was consistently observed in cells exposed to AnxA1 nAb in the presence of CAF CM, ammonium sulfate-enriched CAF CM fractions, and Ac2-26, suggesting possible specificity of AnxA1 predominantly upon Snail, Twist, and Oct4-dependent pathways (see P values in Fig. 3B).

Recent analyses have established that downstream signaling from interaction of soluble AnxA1 with its receptor, FPR2/ALXR in human and Fpr-rs1 in mouse, involves activation of phosphorylation of MAPK Erk1/2 (pErk1/2). Both mimetic and full-length proteins are able to bind to the receptor (27). A basal-like breast cancer model was used to evaluate the effect of AnxA1 in the promotion of EMT and...
metastasis. In that study, AnxA1 contributed to EMT, potentially by stimulating an autocrine loop of the TGF-β receptor (45). In our study with cE1 cells dosed with increasing concentration of Ac2-26, we found a 2-fold increase in TGF-β1 at the 50 μmol/L dose, which is physiologic in consideration to the seminal fluid level of 150 μg/mL. This result was further supported by the evidence that increase in TGF-β1 from treatment with Ac2-26 could be reversed by simultaneous addition of AnxA1 nAb (Fig. 3C). Increase in MAPK and TGF-β activity was supported by qRT-PCR results using cE1 cells grown in Matrigel culture with or without Ac2-26 for 14 days (Mapk, P < 0.05; Tgfbr2, P < 0.05; Fig. 3D).

**Effect of AnxA1 on cE1 cells in vivo**

Next, to substantiate the *in vitro* findings of AnxA1 induction of EMT *in vivo*, the SCmed subpopulation of the cE1 cell line was used in renal capsule transplantation technique. Control, CAF CM-, or Ac2-26–treated cE1 SCmed cells were admixed with UGSM and grafts were transplanted under the renal capsule of NOD/SCID male mice (Supplementary Fig. S4A). The incidence of glandular structure formation was 75% when SCmed cells were treated with CAF CM and 100% when treated with Ac2-26; however, no glandular structures were detected in control-treated SCmed grafts. Staining for androgen receptor (AR) and proliferation marker Ki67 confirmed that the
structures seen in CAF CM- and Ac2-26–treated grafts were composed of highly proliferating prostate cancer cells (Fig. 4A and B). In addition, significant increases in graft size ($P < 0.05$; Supplementary Fig. S4B), number of p63$^+$ cells per graft area ($P < 0.05$), and ratio of p63$^+$ to CK8$^+$ cells per graft area ($P < 0.05$) were observed in Ac2-26–treated SC$^{med}$ cE1 cells (Fig. 4C).

The combined results of SC$^{med}$ transplantation when treated with either CAF CM or Ac2-26 suggested that AnxA1 alone might be able to largely recapitulate the stimulation provided by CAFs in glandular structure formation. These results served as a proof-of-principle that AnxA1 could be a potent secreted factor responsible for the gland-forming stimulation provided by CAFs to CSCs.

**Effect of AnxA1 on primary tumor LSC$^{hi/med}$ cells in vitro**

We proceeded to test the effect of AnxA1 on sorted LSC subpopulations from the $Pten^{-/-}$ L model. As previously described, coculture of LSC$^{hi}$ cells with CAF led to increasing biologic complexity, indicated by formation of multiple
Figure 5. Effect of AnxA1 on incidence, types, differentiation and proliferation of glandular structures in LSC<sup>hi</sup> grafts. A, chart depicting the incidence and type of glandular structures (cuboidal or acinar type glandular) scored in LSC<sup>hi</sup> untreated control, Ac2-26–treated, and rAnxA1-treated grafts. B, representative H&E images of the types of structures detected in rAnxA1-treated grafts. Magnification, ×400. Bar, 100 μm. C, tissue sections were analyzed by H&E for basic histology and IHC for expression of basal cell marker p63, luminal cell marker CK8, androgen receptor, and Ki67. Magnification, ×400. Bar, 100 μm. D, calculated percentage of p63<sup>+</sup> cells and ratio of p63<sup>+</sup> to CK8<sup>+</sup> cells detected in the grafts. Potential to undergo dedifferentiation in response to AnxA1 was assessed by increase in p63 expression compared with CK8. Statistical significance is indicated by *, P < 0.05; **, P < 0.01; ***, P < 0.001.
**A**

Glandular structures

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<tr>
<td>LSCmed + Ac2-26</td>
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<tr>
<td>LSCmed + rAnxa1</td>
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Acinar glandular structures

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<tr>
<td>LSCmed + rAnxa1</td>
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**B**

Types of structures in rAnxa1-treated grafts

- Acinar
- Simple cuboidal
- Low cuboidal

**C**

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<th>rAnxA1-treated LSCmed + UGSM</th>
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**D**

% p63+ cells per graft

- Control
- Ac2-26
- rAnxA1

% p63+ : CK8+ ratio per graft

- Control
- Ac2-26
- rAnxA1

**E**

Mean number of glandular structures

- Control
- Ac2-26
- rAnxA1

Mean number of acinar structures

- LSCmed
- LSCmed
- LSCmed
basal and luminal layers, as well as immunofluorescent detectable coexpression of basal and luminal cell markers (35). In the present study, we further observed that spheroids formed after coculture with CAFs could generate acinar-type projections, a phenotype which was also seen before but not specifically described (35). Similarly, LSC\textsubscript{hi} cells grown in the presence of ammonium sulfate-enriched CAF CM fractions, Ac2-26, and rAnxA1 grew complex acinar structures. Expansion of both basal and luminal cell layers after CAF coculture as well as after treatment with sources of AnxA1 was revealed with immunofluorescence (Supplementary Fig. S5).

In the previous study, we reported that LSC\textsubscript{hi} cells cocultured with CAFs formed greater numbers of spheroids than LSC\textsubscript{lo} cells cocultured with UGSM (35). Here, we found that primary cells treated with either mimetic or full-length forms of AnxA1 protein formed more spheroids than the no-treatment control groups. Treatment of LSC\textsubscript{hi} with Ac2-26 led to a 20-fold increase over UGSM control, expressed as a percentage of spheroids per total number of cells seeded (control, 0.37% ± 0.006; 5 \mu mol/L Ac2-26, 14.96% ± 0.35, P < 0.001; 50 \mu mol/L Ac2-26, 23.28% ± 1.7, P < 0.01), and murine recombinant full-length AnxA1 (rAnxA1) led to a 28-fold increase (control, 2.47% ± 1.13; 0.5 \mu mol/L rAnxA1, 9.2% ± 0.85, P < 0.01; 5 \mu mol/L rAnxA1, 13.35% ± 2.15, P < 0.05). Although LSC\textsubscript{med} cells virtually lack the ability to form spheroids in vitro (35), remarkably more than 45-fold significant increase in spheroid number was observed in LSC\textsubscript{med} cells inoculated with rAnxA1 (control, 0.03% ± 0.067; 0.5 \mu mol/L rAnxA1, 3.6% ± 1.7, NS; 5 \mu mol/L rAnxA1, 28.1% ± 9.27, P < 0.05). At least a 2-fold difference in spheroid number was observed in LSC\textsubscript{med} cells treated with Ac2-26 (control, 0.14% ± 0.01; 5 \mu mol/L Ac2-26, 0.46% ± 0.07, P < 0.05; 50 \mu mol/L Ac2-26, 0.32% ± 0.02, P < 0.01; Supplementary Fig. S6A). We observed that LSC\textsubscript{hi/med} treated with Ac2-26 or rAnxA1 could be passaged for at least up to three generations, similar to coculture with UGSM, demonstrating retention of self-renewal ability in the absence of additional treatment with AnxA1 after the first generation (data not shown).

As shown in Supplementary Fig. S6B, after exposure to increasing concentrations of Ac2-26, LSC\textsubscript{hi} spheroids evidenced increasing pErk1/2 and TGF-\(\beta\)1 activation. At 50 \mu mol/L Ac2-26 treatment, more than 3-fold pErk1/2 activation and 2-fold Erk1/2 activation were seen (pErk1/2, 3.2 ± 0.7, P < 0.01), compared with control. pErk1/2 activation could be reversed by addition of the AnxA1 nAb in the presence of Ac2-26 (0.5 ± 0.1, P < 0.01). Other MAPKs, p38, and Jnk/Sapk did not show upregulation of phosphorylation in Ac2-26–treated spheroids relative to control. LSC\textsubscript{hi} cells showed a 4-fold increase in TGF-\(\beta\)1 at 50 \mu mol/L Ac2-26 dose (4.2 ± 1.4, P < 0.001), compared with control, and significant decrease in response to nAb (0.5 ± 0.4, P < 0.01).

**Effect of AnxA1 on LSC\textsubscript{hi/med} cells in vivo**

LSC\textsubscript{hi/med} cells were treated with rAnxA1, Ac2-26, or media control and admixed with UGSM for grafting and transplantation under the renal capsule of NOD/SCID male mice. The histopathology of the grafted cells recovered after 10-week transplantation was determined. Tumor epithelial cells were shown to be capable of forming glandular structures of three distinct morphologic subtypes (Fig. 5 and Fig. 6).

The most frequently observed glandular structure was composed of low cuboidal epithelial cells, characterized by elongated nuclei and low cytoplasmic profile. These structures ranged greatly in size and could comprise sections of the graft up to 300 \(\mu\)m. p63\textsuperscript{+} cells represented 30% to 50% of the cells within these structures.

Less frequently, simple cuboidal epithelium was detectably observed as consistent in size and shape and possessed centrally located nuclei that remained uniformly spherical. The size of these glandular structures was reduced, ranging between 50 to 80 \(\mu\)m. Simple cuboidal epithelial structures had the highest percentage of p63\textsuperscript{+} cells out of all three subtypes, ranging from 70% to 100% p63\textsuperscript{+} staining in serial tissue sections.

The third subtype was observed to be composed of high columnar epithelial cells, which were organized into acinar units and which had basolaterally located spherical nuclei and prominent cytoplasm. They displayed higher recruitment of endothelial cells and blood vessels than other glandular subtypes. Columnar, acinar glandular structures were consistently equal to or more than 300 \(\mu\)m, ranging between 300 to 1,500 \(\mu\)m in size. In these structures, p63\textsuperscript{+} cells comprised 10% to 20% of the total. Interestingly, their detection was 90% positively correlated with detection of simple cuboidal structures. These histologic subtypes were considered noteworthy, although their biologic significance would remain to be further explored.

**AnxA1 stimulates glandular structure formation, differentiation and proliferation in LSC\textsubscript{hi} cells, and dedifferentiation in LSC\textsubscript{med} cells in vivo**

Glandular structures were detected in six of six (100%) of the rAnxA1-treated LSC\textsubscript{hi} grafts, three of five (60%) of the Ac2-26–treated grafts, and two of five (40%) of untreated
controls (Fig. 5A), LSCmed cells were stimulated by AnxA1 to form prostastic glandular structures in four of four (100%) of rAnxA1-treated grafts and four of four (100%) of Ac2-26-treated grafts. Glandular structures were detected in zero of four (0%) of untreated controls.

All three morphologic glandular subtypes were detected in AnxA1-stimulated LSChi and LSCmed cells (Figs. 5A and B and Fig. 6A and B). LSChi and AnxA1-treated grafts were most stimulated to form both cuboidal and acinar type glandular structures, which were found to co-occur in four of six (67%) of the grafts (Fig. 5A and B). In LSCmed grafts, acinar type glandular structures were present in two of four (50%) of acinar type LSC untreated-treated grafts, one of four (25%) of Ac2-26-treated grafts, and zero of four (0%) of control grafts. Simple cuboidal glandular structures were only detected in rAnxA1-treated grafts (Fig. 6A and B).

All glandular structures detected were stained for CRE and PTEN to confirm the presence of CRE/PTEN cells of cPten−/− L origin (representative CRE and PTEN staining depicted in Supplementary Fig. S7). Histologic analyses of all three treatment groups in LSChi and LSCmed grafts revealed AR+/CRE+/PTEN+ prostate epithelial cells. Ac2-26 and rAnxA1-stimulated LSChi cells formed glandular structures with higher abundance of basal marker p63+, luminal marker CK8+, and Ki67+ cells than untreated controls (LSChi Ki67+: control, 6.83±3.62; Ac2-26, 15.64±2.74, P < 0.01; rAnxA1, 14.79±4.84, P < 0.05; LSCmed Ki67+: control, 8.74±11.85; Ac2-26, 7.37±5.99, NS; rAnxA1, 12.27±2.56, NS). In contrast, LSCmed-untreated control grafts were mostly composed of CK8+, Ki67+ cells, indicating less-proliferative, differentiated cells. LSCmed-untreated control grafts had virtually no detectable p63+ staining. Treatment with Ac2-26 and rAnxA1 led to significantly greater expression of p63 (LSChi: Ac2-26, P < 0.01; rAnxA1, P < 0.001; LSCmed: Ac2-26, P < 0.01; rAnxA1, P < 0.01) and ratio of p63+ to CK8+ cells per graft area (LSChi: Ac2-26, P < 0.05; rAnxA1, P < 0.05; LSCmed: Ac2-26, NS; rAnxA1, P < 0.05; Figs. 5C and D and Fig. 6C and D). Among all LSChi/med grafts, highest p63 expression was observed in simple cuboidal glandular structures compared with the other two subtypes (acinar, 17.6%±5.8; low cuboidal, 40.9%±8.5, P < 0.01; simple cuboidal, 83.4%±10, P < 0.001).

In AnxA1-treated grafts where glandular structures were detected, larger areas of the graft were found to be covered by glandular formation compared with untreated controls. LSChi Ac2-26 and rAnxA1-stimulated cells formed larger structures than controls, multiple structures per graft were present, and more CK8+ cells were observed, particularly in acinar glandular structures. AnxA1 treatment stimulated LSCmed cells to form glandular structures compared with controls, and in acinar structures, more CK8+ cells were observed. Observations in acinar type structures accounted for the significant differences in size and number of glandular structures detected in AnxA1-treated groups, especially LSCmed (Fig. 6E and Supplementary Fig. S8).

We next analyzed the status of Erk1/2 and pErk1/2. Glandular structures possessed Erk1/2 and pErk1/2-positive foci, indicating a possible stable upregulation of the pErk1/2 pathway due to stimulation by AnxA1, as similarly seen in vitro. pErk1/2 expression was 2- to 3-fold higher in Ac2-26 and rAnxA1-treated LSChi/med than control LSChi/med grafts, after normalization to Erk expression and proliferation marker Ki67 (normalized fold changes compared with controls: LSChi pErk1/2: Ac2-26, 1.4±0.4, P < 0.05; rAnxA1, 1.6±0.3, P < 0.05; LSCmed pErk1/2: Ac2-26, 3.0±0.3, P < 0.01; rAnxA1, 2.2±0.3, P < 0.01; Supplementary Fig. S9A–S9D).

Effect of AnxA1 on LNCaP and PC3 cells in vitro

We tested the effect of AnxA1 on cells from human-derived prostate cancer. LNCaP and PC3 cell lines were treated with vehicle control, 5 or 50 μmol/L Ac2-26, and were assayed for spheroid formation at 3 and 6 days. Both LNCaP and PC3 cells formed greater numbers of spheroids after treatment with Ac2-26 at both concentrations, at days 3 and 6, compared with controls (Fig. 7A). Moreover, larger spheroids were counted in treated versus untreated wells at 6 days (Fig. 7B).

Discussion

We sought to identify secreted factors from the CAFs that are capable of contributing to the generation of CSCs. We demonstrate that AnxA1 is secreted by the CAF cells of the prostate tumor mouse model. The CSC subpopulation can be induced to increased differentiation potential and proliferation following exposure to extracellular AnxA1. This recapitulates many of the observations made when CSCs were tested before in cocultures with CAFs (35). Thus, it seems that AnxA1 is a potent and prominent factor among the various secretory molecules that are produced by the CAFs in the tumor microenvironment, particularly in relation to the stimulation of CSC activity.

Our data furthermore support that AnxA1 is capable of promoting de novo generation of CSCs from prostate cancer cells in addition to stimulating the CSC population. Presumably, EMT-like transition seen in vitro may be linked to a process of CSC generation. For example, AnxA1-induced EMT in SCmed cells seems to correlate with its capability to enhance formation of prostastic glandular structures when these cells are transplanted in vivo. Similarly, the more differentiated primary subpopulation, LSCmed, when treated with AnxA1 displays a gain of CSC properties not possessed intrinsically. Significant gain of ability to form spheroids in vitro and glandular structures in vivo is seen. Moreover, LSCmed cells stimulated by AnxA1 show plasticity in regards to size, number, and morphologic types of glandular structures formed that is akin to that of the LSChi cells. Because LSCmed cells do not display capability to exhibit these behaviors in the absence of treatment, their response to AnxA1 is even more relevant to the premise that AnxA1 seems to shift the cancer cells back to a more stem cell-like state. LSChi, known to harbor CSCs, also respond to AnxA1.
cancer cell lines, LNCaP and PC-3, was also found to be enhanced by AnxA1.

In this study, LSCmed were stimulated by AnxA1 in vitro. This is in slight contrast with the previous study in which LSCmed cocultured with CAFs were not stimulated for increased spheroid formation (35). However, there were conducted coculture of stromal cells to epithelial cells at a ratio to 1:1. For the in vitro studies here, we used a ratio of 10:1, a composition reported in the literature to be optimal for recapitulating biologic effects (46). LSCmed cells seem to be more sensitive to dose-dependent effects, as spheroid formation was significantly stimulated over controls at the higher concentration of rAnxA1. Similar, but distinctly less pronounced, spheroid formation was noted using the mimetic peptide, again stressing the relative sensitivity of this fraction of tumor cells to concentration and potency of stimulant, compared with LSChi.

AnxA1 treatment significantly increases the incidence of simple cuboidal structures with highest p63 expression and acinar glandular structures, which are greatest in size. Moreover, these structures are more commonly seen in rAnxA1-treated than Ac2–26–treated grafts. Cuboidal and acinar structures are not seen in untreated LSCmed controls and are greatly reduced in LSChi controls. These findings may suggest that exposure to AnxA1 increases the inherent plasticity of tumor epithelial cells to form more complex structures and different morphologies. This additionally supports the supposition that cells exposed to AnxA1 may have undergone an EMT, contributing to their plasticity.

A possible reason for the observed differences in effects seen with the full-length protein versus the mimetic Ac2–26 peptide may be the support of the N-terminal domain of the full-length by the pore from the four α-helical repeats, which create a "doughnut-like" ring around that domain. The pore may, thus, stabilize physical interaction of the N-terminus of the full-length protein with the bound receptor and with an adjacent receptor or protein (47–49). Possibly stabilized interaction of the full-length may selectively lead to greater activation of signaling downstream of Fpr1, whereas the lower stability and promiscuity of mimetic Ac2–26 may lead to activation of Fpr1, Fpr-rs1, and Fpr-rs2 and other downstream pathways, albeit with less potent constitutive activity (6, 12, 21, 25, 27).

Our overall results suggest that AnxA1 from CAFs may contribute to tumor stem cell activity via two separate but complementary pathways: (i) induction of a dedifferentiation process, an intermediate step being akin to EMT, leading to generation of basal stem-like cells, and (ii) enhancement of the proliferation and differentiation of the CSCs. AnxA1 is thus implicated in a paradigm in which cells that are malignant epithelial but not CSCs can be induced to gain CSC-like properties, and cells that already possess the properties of CSCs can be enhanced for their oncologic properties. We find that AnxA1 treatment can significantly increase pErk1/2 activation and TGF-β1/TGFβRII in eC1 and LSC cells. These observations remain to be further developed for a mechanistic scrutiny of AnxA1 effect.

Figure 7. Effect of AnxA1 mimetic peptide on spheroid formation in human prostate cancer cell lines. A, spheroids were counted from LNCaP and PC3 human prostate cancer cells grown in 2% Matrigel/2% FBS/RPMI-1640 with treatment on Matrigel precoated wells of a 24-well plate 3 and 6 days after plating. Cells were treated with vehicle control, 5 or 50 μmol/L peptide Ac2–26. Mimetic peptide Ac2–26 treatment of LNCaP and PC3 cells led to increased numbers of spheroids formed, at both days 3 and 6, in a non–dose-dependent manner. B, the number of spheroids greater than 100 μm for all culture conditions was counted at day 6. Representative experiments are shown, and values represent the mean ± SD of triplicate samples. Statistical significance is indicated by *, P < 0.05; *, P < 0.01.

treatment by exhibiting enhanced CSC-like activity in vitro and in vivo, compared with untreated controls. In this regard, like the mouse prostate cancer cells, spheroid-forming capability of subpopulations present in human prostate


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In addition, as indicated by regression analysis of AnxA1 relative to tumor size, CAF cells may potentially produce higher levels of phospho-Ser27-AnxA1 as disease progresses. Thus, as a possible logical extension of our findings to the study of human prostate, an analysis of stromal AnxA1 expression in normal prostates, stratified by advancing age, and in prostate tumors at different stages of disease progression may be worthwhile. It is noteworthy that AnxA1 is naturally most abundant in the seminal fluid (11) and seminal vesicles are most closely associated with the prostate gland. In this regard, it is possible to speculate that phospho-Ser27-AnxA1 produced in tumors may be detectable in seminal fluid.

Finally, a role for AnxA1 may be viewed in the context of therapy. Cancer cells (or their progenitors) are constantly evolving, thereby developing resistance to therapy. In contrast, fibroblast cells of the tumor microenvironment are less evolving and thus may provide a more stable target for attack. AnxA1 is an important factor coming from the prostate stroma, and is now shown, at least in a model system, to support the prostate CSC niche through maintenance and de novo generation of basal stem-like cancer cells in vivo. Therefore, potential therapies interrupting the CAF-AnxA1-CSC axis may prove to be a rational adjuvant approach for the treatment of prostate cancers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Study supervision: P. Roy-Burman.

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