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The natural history of prostate cancer involves progression to metastasis and the eventual development of hormone refractory disease. Multiple genetic activities are involved in the molecular cascade that leads to hormone resistance in prostate cancer. One of the gene products we found to be associated with metastasis in a mouse model as well as in human prostate cancer is caveolin-1. Orthotopic tumors with stable mouse prostate cancer clones with antisense caveolin-1 have reduced metastatic activities relative to vector-control clones and parent cell lines. Antisense caveolin clones significantly regressed in response to surgical castration *in vivo*. The antisense caveolin tumors that responded to castration therapy demonstrated increased levels of caveolin-1 and apoptosis relative to either vector-control clones or parental cell lines. Our data indicate that reduction of caveolin-1 levels not only suppresses metastatic activity but also restores androgen sensitivity. We have developed *in vitro* models to further define the pathways to hormone resistance that utilize caveolin-1. Our novel results establish a new paradigm for understanding androgen refractory disease and open the door for new innovations in prostate cancer therapy.

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FOREWORD

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Timothy C. Thompson   10-27-99

PI - Signature   Date

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INTRODUCTION

The natural history of prostate cancer involves progression to metastasis and the eventual development of hormone refractory disease. In normal prostate, removal of androgenic hormones results in regression of the gland involving apoptosis of more than 60% of the luminal epithelial cells. Although often initially sensitive to removal of androgens, prostate cancer cells eventually lose this response and continue to grow and spread in the absence of androgenic steroids. Multiple genetic activities are involved in androgen ablation-induced prostate regression, yet very little is known regarding the rate limiting steps in the molecular cascade that leads to regression or the molecular basis of hormone resistance in prostate cancer. We previously developed a mouse model to identify metastasis-related genes in prostate cancer. This model includes a series of clonal cell lines derived from prostate cancer metastases that developed *in vivo* using the mouse prostate reconstitution model system. One of the gene products we found to be associated with metastasis in this model as well as in human prostate cancer is caveolin. The production of stably selected clones with antisense caveolin resulted in a significant reduction in metastatic activities relative to vector-control clones and parent cell lines. Surprisingly, we discovered that tumors produced by the antisense caveolin clones significantly regressed in response to surgical castration *in vivo*. Eleven days following androgen ablation, tumors derived from three independent antisense clones regressed by approximately 30% relative to the wet weights produced in either vector-control clones or parental clones which did not respond to castration therapy under the same conditions. The antisense caveolin tumors that responded to castration therapy also demonstrated significantly increased levels of apoptosis relative to either vector-control clones or parental cell lines. Therefore, our data indicate that reduction of caveolin levels not only suppresses metastatic activity but also restores androgen sensitivity. We are of the opinion that these novel results establish a new paradigm for understanding androgen refractory disease and open the door for new innovations in prostate cancer therapy. We propose to further elucidate the molecular pathway of castration-induced regression and the mechanism of caveolin-mediated hormone resistance. We will also attempt to develop novel therapeutic approaches for hormone refractory prostate cancer based on this new information.

BODY

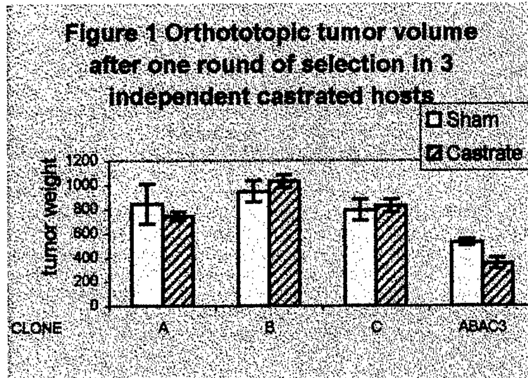
We will provide a detailed account of the research accomplishments for each of the items in the approved statement of work.

STATEMENT OF WORK

(1) **To correlate and determine the reversibility of caveolin expression with castration-induced regression in mouse prostate cancer cell lines (months 1–8).**

- To complete a selection experiment in which ABAC3 is subjected to a series of growth periods in castrate male animals *in vivo* and analyze AR expression, caveolin expression, and androgen sensitivity following the final round of selection (months 1–8). [24 mice for selection, 128 for evaluation]

We injected the antisense caveolin cell line ABAC3 into three separate mice that had been castrated seven days before. Each of these was then allowed to grow for 14 days as an orthotopic tumor at which time it was removed from the animal weighed and portions collected for immunohistochemistry and portions placed in tissue culture. After a brief period of in vitro growth the cells were collected and a single cell suspension reinjected for orthotopic tumor growth in either castrate or intact animals. A portion of the cells was also lysed and Western blotting performed to evaluate caveolin and AR protein expression levels. The tumors were allowed to grow for 14 days and the entire procedure repeated. Thus cells were grown for three cycles in castrated animals and the tumors evaluated at each cycle for androgen sensitivity and AR and caveolin expression. The results of tumor growth at the first round are presented in Figure 1. The antisense caveolin clone ABAC3 quickly reverted to androgen insensitivity in the three experimental groups (see Figure 1). All of the clones also reacquired expression of caveolin (Figure 2). The expression of AR was also regained.



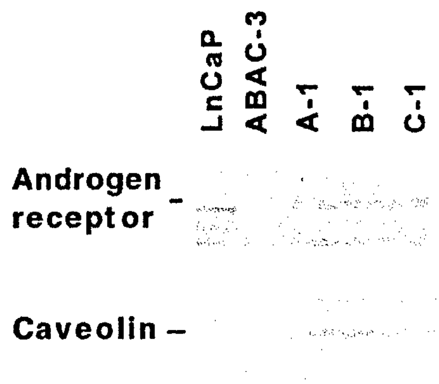


Figure 2. Western blot of cells grown from three independent injections (A-C) for one round of ABAC3 orthotopic tumors grown in castrated animals. Equivalent amounts of protein Western blotted and reacted with antibody against androgen receptor (top) or caveolin-1 (bottom). Control LnCaP and ABAC3 cell extracts showed minimal caveolin protein but the ABAC3 cells re-acquired expression of caveolin-1 after passage in the castrated mice. Expression of androgen receptor was also increased by growth in castrated animals.

- To supertransfect antisense caveolin clones (ABAC3, ABAC5, and BACS4) with sense-caveolin Babepuromycin vector and determine androgen sensitivity of selected stable clones *in vivo* using established hormone manipulation conditions (months 1–8). [288 mice]

We established that the antisense clone ABAC3 rapidly reverted to androgen insensitivity and gained the re-expression of high levels of caveolin after a short time *in vivo*. This result essentially answered the question raised in this specific set of experiments in a highly stringent *in vivo* system. This system is highly preferred to the super transfection approach which can result in considerable clonal selection that can obfuscate interpretation of the results.

- To complete immunostaining of all tissues and quantitate results as well as establish cell lines for future studies (months 1–8).

We have completed the staining and are continuing with the quantitation. Representative immunohistochemical results are presented below (Figure 3).

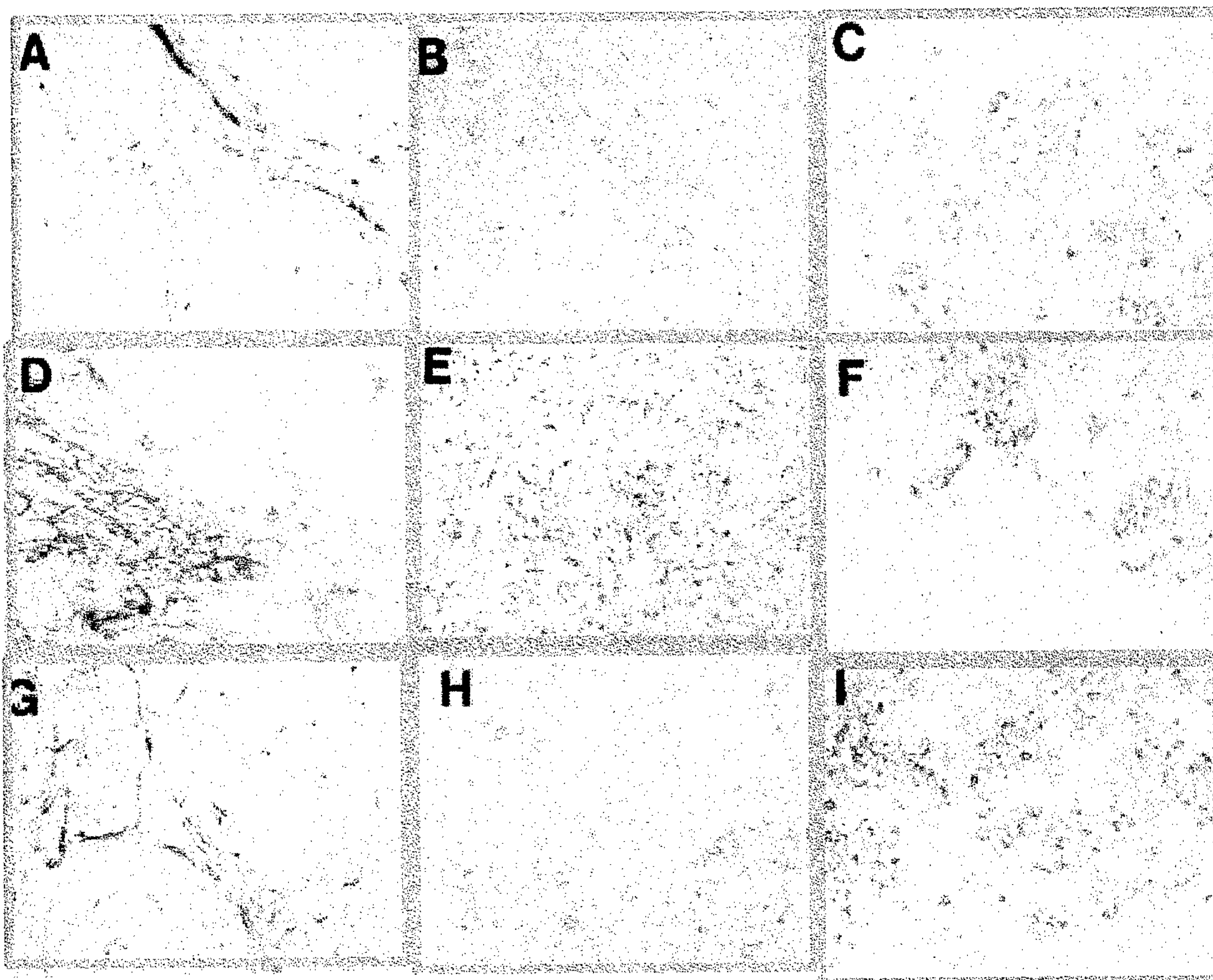
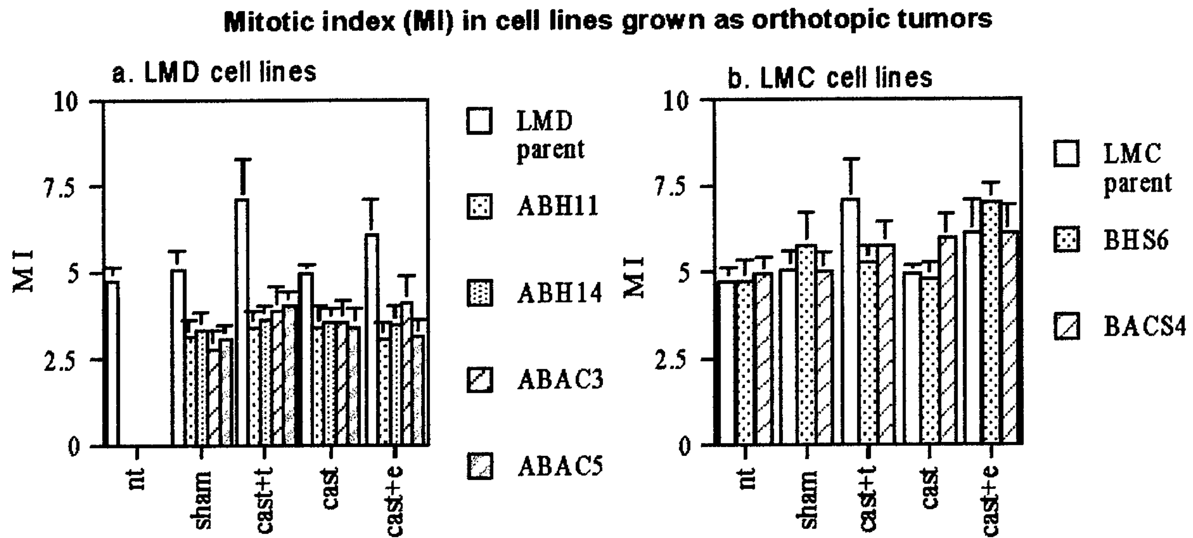


Figure 3. Immunohistochemical detection of caveolin-1 in tissue from normal intact mice (top row) castrated mice (middle row) or castrated mice that also received an implant of silastic tubing with testosterone (bottom row). The tissues stained were from normal prostate (panels A, D and G), the ABAC3 orthotopic tumors (panels B, F and H) and lymph node metastases from the ABAC3 tumors (panels C, G and I). Caveolin-1 in normal prostate tissue is localized in the stroma. . Note the increased staining of caveolin-1 in the castrated as well as testosterone supplemented ABAC3 tumors and lymph node metastases.

(2) To establish the kinetics of castration-induced regression in regard to bioactivity and gene expression (months 3–16).

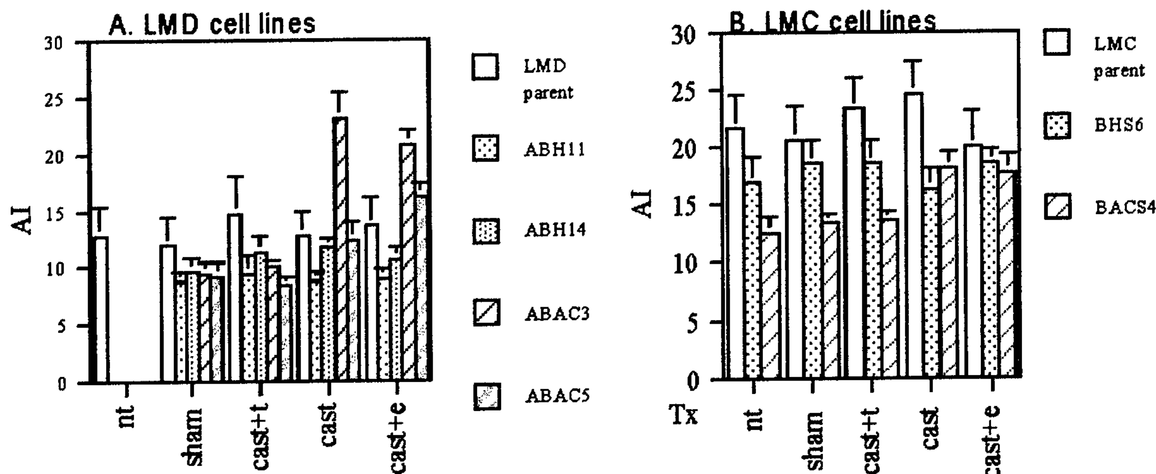
- To determine the mitotic index of all independent animals by counting mitotic figures under a high-power microscope (months 1–4).

The level of mitosis was determined by counting mitotic figures in orthotopic tumors this is presented in the following figure for parental cells 148-1 LMD (a) or 151-2 LMC (b) and the vector controls (ABH11, ABH14, and BHS4) and antisense caveolin clones (ABAC3, ABAC5, and BACS4). The results are indicative of the lack of a profound effect of castration upon the mitotic index irrespective of caveolin-1 status.



- To determine the apoptotic index using the TUNEL methodology by staining representative sections of all tissues described in Figure 1 and counting apoptotic bodies using high-power microscopy (months 3–16).

The level of apoptosis was determined by staining sections with TUNEL and counting apoptotic figures in orthotopic tumors. The antisense cell line with the lowest level of caveolin-1, ABAC3, had the most pronounced increase in apoptosis in castrated animals. This provides a correlate for the reduction in tumor volume in castrates in with this cell line. Increased apoptosis was also observed in ABAC5, the other antisense caveolin-1 cell line derived from 148-1 LMD, in concordance with the reduced tumor volume observed for this cell line. The other antisense cell line, BACS4, derived from 151-2 LMC, also displayed increased apoptosis in castrated hosts.



- To isolate RNA and perform Northern blotting analysis and perform *in situ* hybridization for a panel of selected genes known to be involved in castration-induced regression response (months 6–16).

We are currently using commercial microarrays (Clontech) to initially screen ABAC-3 cells as well as LnCaP cells for changes in gene expression that occurred as a result of testosterone stimulation *in vitro*. This is a preferred method for the questions we are addressing in the proposed work. Commercial microarrays enable us to initially screen many more genes than we otherwise would have using Northern blotting as a first step. Once candidate genes are identified we then proceed with Northern blotting and/or *in situ* hybridization to establish the initial results. Thusfar we have confirmed that cytokeratin-8 and −18 mRNAs are induced by testosterone (data not shown). We are considering testing their potential role as survival factors and their possible regulation by caveolin-1 in prostate cancer.

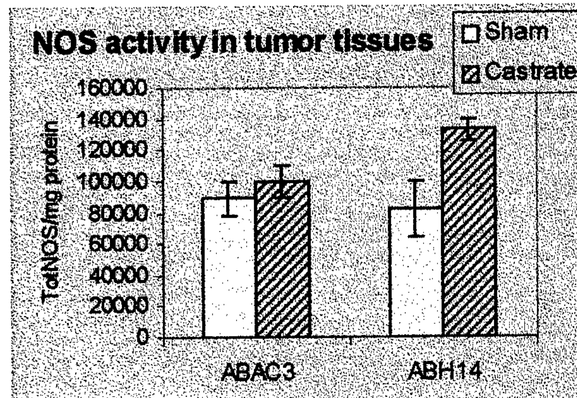
(3) To determine the role of NOS and nitric oxide production in castration-induced regression response (months 6–24).

- To stain representative tissues for three NOS forms, including iNOS (months 6–24).

We have obtained antibody which we have documented to work well for detection of NOS by immunohistochemistry (Nasu et al., Gene Therapy 6:335-43, 1999). Although this work is in progress we have not yet completed the analysis of the tissues from the castration-induced regression.

- To determine nitric oxide production in frozen tissues and/or frozen tissue sections using the arginine to citrulline conversion technique (months 6–16).

We have determined total NOS activity in tumor tissues collected at the time of harvesting (d14) and have detected increased activity in tumors from castrated mice relative to sham operated mice. There was not a difference that related to caveolin levels, as the control cell line ABH14, which did not regress, appeared to have a greater increase in NO production than the antisense caveolin clone ABAC3, which did regress. This may be a reflection of the generation of NO by host cells which have infiltrated the tumor rather than from the tumor per sé. We therefore feel that immunolocalization will provide a more definitive answer to this aim.



- To determine the sensitivity of castration-induced regression mechanism to FK506 *in vivo* using established methodologies (months 6–12). [192 mice]

These experiments are still in the planning stages, before proceeding with these *in vivo* studies we would like to further analyze gene expression patterns in the presence or absence of testosterone *in vitro* to better evaluate the potential for this drug treatment to impact on specific gene activities and castration-induced regression.

(4) To identify gene activities involved in castration-induced regression using DD-PCR (months 6–30).

- To isolate RNase from tissues previously analyzed as well as control tissues, including all lobes of normal prostate under the specific hormone conditions used previously *in vivo* (months 6–30).

We have collected tissues for RNA isolation from all of the tumors generated to date. A selected number have been purified and the amount quantitated.

- To perform DD-PCR using a panel of random primers (10-mers) on mRNA and identify fragments that are increased or decreased in expression (months 8–30).

We have used a series of 10-mer oligonucleotides to perform RT-PCR in the presence of ^{33}P d-ATP and separated the reaction products on polyacrylamide gels then visualized the bands by autoradiography. An example of such a gel with just one primer is shown below.

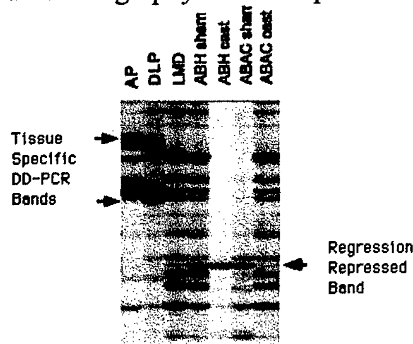


Figure7. DD-PCR analysis with mRNA isolated from orthotopic tumor tissue in sham or castrated mice. Control mRNA from normal 129 mouse anterior prostate (AP) or dorsal lateral prostate (DLP) indicates the ability to detect tissue specific gene expression. The presence of a band which is repressed in the regressing ABAC3 tumor is seen when comparing antisense caveolin clone ABAC3 in the castrated host with the sham host. This same band was present in both sham and castrated mice with the control clone ABH11 or parental LMD tumors.

- To isolate and clone fragments and test clone fragments using a simplified Northern blotting analysis that includes a single sham and castrate vector control clone and antisense caveolin clone (months 9–30).

We have developed in vitro systems for hormone manipulation of ABAC-3 cells (as well as LnCaP cells) in parallel with the basic *in vivo* castration model. Our plan is to compare the two systems using both commercial microarray screens and DD-PCR. To this end we have isolated and cloned numerous DD-PCR fragments from the *in vitro* system, screened several of them by Northern blotting and proceeded with DNA sequencing. We have demonstrated that cytokeratin –8 and –18 are upregulated by testosterone and further shown that caveolin-1 may induce these genes. We have also developed a novel approach to screening DD-PCR fragments using a microarray strategy. DD-PCR fragments are eluted from the original gel and re-amplified these are then gel purified and precise amounts blotted onto nitrocellulose membrane using a commercial slot blotting apparatus. These blots can then be screened with mRNA that has been reverse transcribed to cDNA with radioactive label incorporated.

- To test extensively the cloned fragment by extended Northern blotting, including all eight cell lines as well as four control tissues under all hormonal conditions (months 10–30).

As described above we have tested some cloned fragments isolated from the +/- testosterone *in vitro* model for regulation by testosterone. Indeed we have confirmed that cytokeratins-8 and –18 are upregulated by testosterone at the mRNA level. We are therefore considering these intermediate filaments as "survival factors" or as components of a survival pathway in prostate cancer cells.

- To test selected sequences for cell-specific expression *in vivo* using *in situ* hybridization.

The expression of cytokeratin-8 and –18 has been well characterized in a variety of tissue epithelia. In the prostate these cytokeratins are specific markers for luminal epithelium (the likely precursor of most malignancies). We have proceeded to screen mouse and human primary versus metastatic lesions with specific commercially available antibodies and found both proteins are more highly expressed in metastatic versus primary tumors.

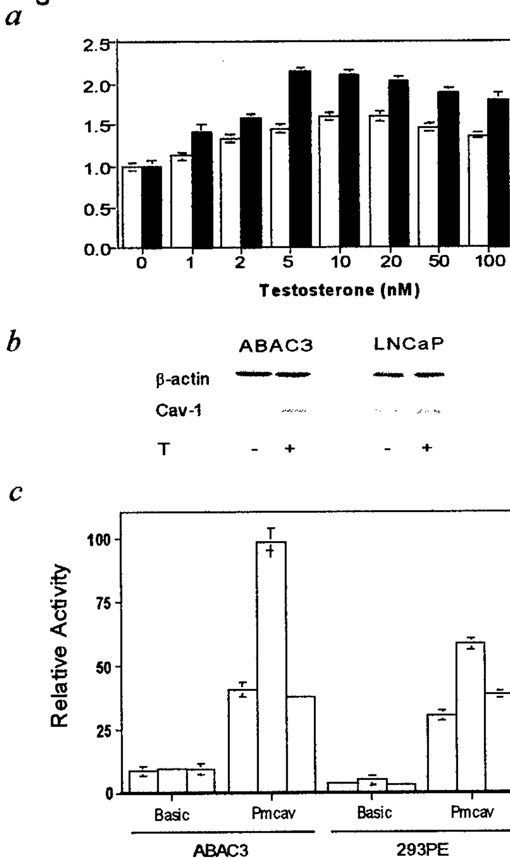
- To clone the full-length cDNA for genes found to be involved in castration-induced response using cDNA libraries that have been previously prepared for 148-1LMD and 148-1PA cell lines (months 8–30).

We are obtaining full-length cDNAs for cytokeratins-8 and –18 in retroviral expression vectors from Dr. Xin Lu, Ludwig Cancer Research Institute, London, U.K. We have not,

as yet, isolated fragments of genes that were both of interest and were not available in full-length clones.

(5) To establish vitro models for androgen sensitivity using selected high and low caveolin producing mouse and human prostate cancer cell lines.

- To determine the response to testosterone in regard to cell growth *in vitro* using all eight mouse prostate cancer cell lines, including parental cells, vector control clones, and antisense caveolin clones and selected human prostate cancer cell lines (ND-1, PC-3 and LNCaP) *in vitro* (months 15–30).



We have completed an intensive evaluation of the androgen responsiveness of mouse and human cell lines with an emphasis on caveolin expression and recently submitted this study for publication (see attached manuscript for results and details). In brief we used ABAC3 and LnCaP as cell lines with low to undetectable levels of caveolin-1 and established the testosterone dose range for enhanced viability in an in vitro assay in which 100-200 cells are seeded in a well of a 96 well plate in serum free medium with or without testosterone. We also evaluated the effects of testosterone on expression of caveolin-1 and found that testosterone promoted increased cell survival in parallel with an increase in the level of caveolin-1 protein. This is depicted in the Figure 8a and b. To determine whether upregulation of caveolin-1 by testosterone occurs at the transcriptional level, a luciferase reporter vector under the transcriptional control of the mouse caveolin-1 promoter, pGL3-Pmcav-1-luc, (Ren *et al*, unpublished data) was used. Forty-eight hours after transfection, ABAC3 or 293PE cells were split, re-seeded at low density ($1x10^5$ cells per 15 cm plate) in SFM or SFMT, and grown at 37°C with 5% CO_2 for 24 hours prior to the luciferase assay. The relative activity of luciferase in ABAC3 cells was increased more than 2-fold by testosterone (Fig. 8*c*). Similarly, the relative activity of luciferase in 293PE cells (androgen receptor (AR) positive) was also increased approximately 2-fold control levels by testosterone (Fig. 8*c*). This enhanced activity could be blocked by the addition of 1μM of Casodex, a direct antagonist of the AR (Fig. 8*c*) indicating that the upregulation of caveolin-1 by testosterone is mediated by AR.

To further correlate caveolin-1 expression with androgen responsiveness we infected ABAC3 cells which express low levels of caveolin-1 with an antisense adenovirus, followed by testosterone stimulation. The results in figure 9*a* show that antisense human caveolin-1 abrogated the up-regulation of caveolin-1 by testosterone. As a consequence of reduced caveolin-1 expression, the survival effects of testosterone were significantly reduced (Fig. 9*b*).

Figure 9 Antisense caveolin adenovirus infected ABAC3

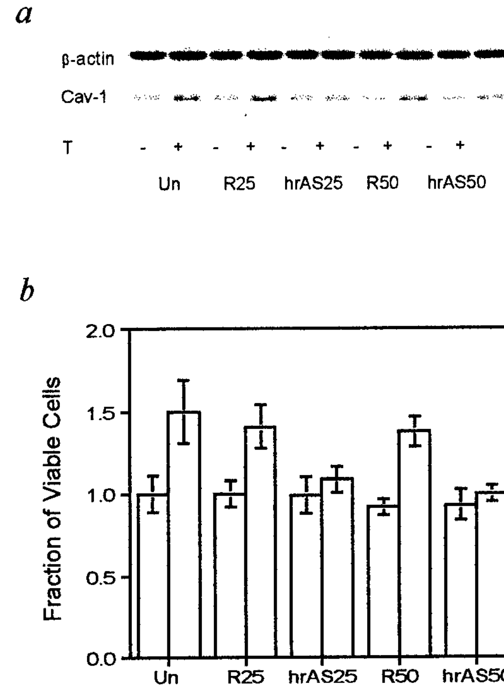
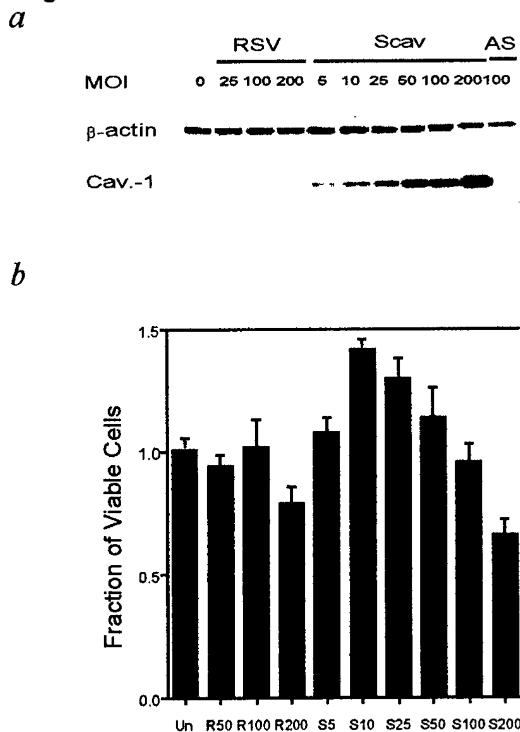


Fig 10 Caveolin-1 adenovirus in LnCaP



To determine whether caveolin-1 could promote survival in human prostate cancer cells in the absence of testosterone, we infected LNCaP cells with an adenoviral vector expressing human sense caveolin-1. Dose-dependent expression of human caveolin-1 was induced by increasing multiplicities of infection (MOI) of the recombinant adenoviral vector. The results demonstrated that the induction of caveolin-1 was detectable at MOI = 5, and gradually increased with increasing MOI (Fig. 10*a*). As expected, caveolin-1 provided significant survival protection to LNCaP cells from growth factor/survival factor depletion-induced conditions. Surprisingly, the maximum protection of caveolin-1 was observed at MOI= 10 (Fig. 10*b*), which represents only a modest level of caveolin-1 (Fig. 10*a*). While survival protection ranged from MOI 5-50, the protection eventually diminished at MOI = 100 and at MOI = 200 reduction of viability was observed (Fig. 10*b*).

- Depending on the results of the studies outlined above, to repeat experiments involving both kinetics of androgen-induced regression regarding bioactivity in gene expression, the measurement of NO acid activities and NO production, and FK506 sensitivity *in vitro* (months 15–30).

The studies that would inform these experiments are still in progress; therefore they have not yet been initiated at this time.

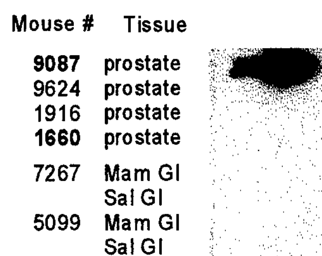
(6) To produce transgenic mice that overexpress caveolin cDNA in prostate and to test these mice for abnormalities and androgen sensitivity (months 1–30).

- To establish 2–3 founder lines of transgenic mice and confirm expression of the caveolin transgene as well as caveolin levels using immunohistochemistry (months 5–12). [36 mice]

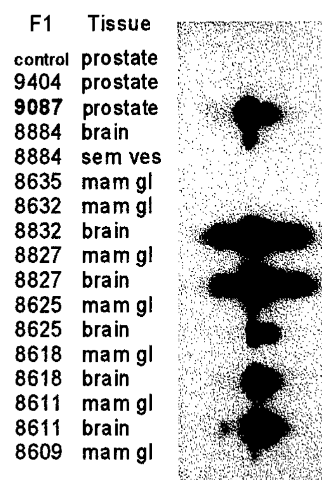
In collaboration with Dr. Franco DeMayo we have injected the caveolin transgene into oocytes and implanted these to attempt to establish founder lines expressing the caveolin-1 gene in a tissue specific fashion. We have screened about 50 offspring for the presence of the transgene. The mice which were positive for the transgene by PCR on tail DNA were raised and bred to develop potential founders. Each was then tested for possible transmission of the transgene and tissue specific expression. Figure 11 demonstrates RT-PCR on tissues from several F1 mice. Just recently we identified two which appear to have good prostatic expression (9087 and 1660). In other F1 offspring the expression appears to more prominent in brain than in mammary gland of females. We will continue to analyze these mice and generate more F1 mice from the founder of 9087 (8042, a male with a probsin-caveolin-1 construct).

Figure 11 RT PCR detection of transgene expression in F1 mice

A. Identification of expression in two F1 mice



B. tissue distribution of expression



- To determine the developmental abnormalities in close consultation with Dr. Luan Truong in transgenic mice (months 7–12). [24 mice]

Since a confirmed founder has not been conclusively identified this has not done yet. We anticipate using the founder 8042 to pursue these studies

- To determine pathological correlates of abnormalities in association with Dr. Luan Truong (months 7–30).

Since a verified founder has not been identified this has not done yet

- To determine the androgen sensitivity *in vivo* of transgenic mouse prostate and genitourinary tissues (months 15–30). [96 mice]

Since a verified founder has not been identified this has not done yet.

(7) **To test therapeutic strategies for antisense caveolin *in vivo* and *in vitro* using a series of adenoviral vector systems as well as other therapeutic tools (months 15–30).**

- To use established adenoviral vector systems, including PCA3 and PAD-12 shuttle vector systems to produce sense and antisense caveolin recombinant adenoviral vectors and test these vectors *in vivo* for potentiation of androgen sensitivity (months 15–30). [192 mice]

We have generated and purified high-titer adenoviral vectors that encode either the human or the mouse caveolin-1 cDNA in both sense and antisense orientation. The adenovirus have either the CMV or RSV promoter driving transcription.

- If available from Statement of Work (Item 5) to use, to use both mouse and human prostate cancer cell line to test adenoviral vector systems for the capacity to potentiate androgen sensitivity (months 20–30).

We have completed an intensive evaluation of the ability to potentiate androgen responsiveness of mouse and human cell lines with adenovirus and recently submitted this study to Nature Medicine (see attached manuscript for results and details).

- To establish additional therapeutic objectives, including antisense oligonucleotides as well as retroviral vector systems, as therapeutic tools (months 15–30).

We have attempted to use antisense S-oligonucleotides in vitro to decrease the expression of caveolin-1 in selected cell lines. We have used several concentrations of oligonucleotide and several different methods to deliver the oligonucleotide into the cells, e.g. various transfection reagents and conditions. Although we have seen transient reductions in caveolin-1 protein by Western blotting we have not yet established conditions to reproducibly decrease caveolin-1 levels to the extent obtained in the antisense clones such as ABAC3.

- To explore the possibility of using small molecules and antibodies as therapeutic tools, depending on the results of mechanistic studies (months 20–30).

We have been making sufficient progress on the mechanistic studies to begin the planning of these experiments. Considerable effort has gone into identifying the role of caveolin-1 in survival during castration-induced regression and we have been gaining some insight into the genes that are active in response to this pathway and in response to testosterone. We anticipate having several candidate genes/pathways in the coming year to begin evaluating small molecules or antibodies that have been commercially developed to block at discrete specific steps in these pathways.

KEY RESEARCH ACCOMPLISHMENTS

- Caveolin-1 expression is associated with hormone resistance
- The establishment of *in vitro* and *in vivo* models for hormone manipulation of prostate cancer cells
- Caveolin-1 is regulated by testosterone at the level of transcription
- Caveolin-1 promotes survival of mouse and human *in vitro* models
- The generation of systems and experimental approaches to identify downstream target genes that are affected by caveolin-1 expression and may play a role in cancer cell survival
- A transgenic mouse with prostate specific caveolin-1 expression has been tentatively identified.

REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations:

Timothy C. Thompson, Terry L. Timme, Likun Li, Alexei Goltsov and Guang Yang: "CAVEOLIN 1: A COMPLEX AND PROVOCATIVE THERAPEUTIC TARGET IN PROSTATE CANCER AND POTENTIALLY OTHER MALIGNANCIES." *Emerging Therapeutic Targets* **3**(2):337-346, 1999.

Li, L., Wang, J, and Thompson, T.C.: "Dose dependent caveolin-1 protection from thapsigargion-induced apoptosis in prostate cancer cell lines. Abstract #2163 90th Annual meeting American Association for Cancer Research. April 1999.

Likun Li, Guang Yang, Chengzhen Ren, Jianxiang Wang, Michael M. Ittmann, Thomas M. Wheeler, Terry L. Timme, Salahaldin Tahir, and Timothy C. Thompson: "TESTOSTERONE PROMOTES SURVIVAL OF PROSTATE CANCER CELLS THROUGH UPREGULATION OF CAVEOLIN-1 AND CAVEOLIN-1 EXPRESSION IS INCREASED IN ANDROGEN RESISTANT HUMAN PROSTATE CANCER" (submitted)

CONCLUSIONS

We have shown that androgen independent prostate cancer cells with high caveolin-1 can become androgen responsive *in vivo* and *in vitro* by suppression of the expression of caveolin-1. We have shown that caveolin-1 is regulated by testosterone both *in vivo* and *in vitro* in prostate cancer and that caveolin-1 is a principle effector of testosterone-mediated prostate cancer cell survival. The mechanism(s) that underlie caveolin-1 mediated cell survival in prostate cancer are likely complex, but could involve molecular transport and/or signal transduction modulation. Based on our preliminary data they could also involve modulation of the cytoskeleton, specifically alteration in cytokeratin expression and function. Further efforts will be mainly focused on understanding the mechanisms that underlie the capacity of caveolin-1 to sustain survival of prostate cancer cells during metastatic progression and in the absence of testosterone. We are attempting to provide a clearer understanding of the molecular changes associated with caveolin-1 and the androgen resistant prostate cancer phenotype through the identification of genes that lie downstream of caveolin-1 and serve as proximal effectors of prostate cancer survival. We have established novel *in vitro* screening systems as well as *in vivo* and transgenic mouse models for these studies. The identification of intermediary molecules in the pathway to hormone resistance may provide a unique target for intervention.

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None cited

APPENDICES

Attached as appendix information are the following publications:

Thompson, TC, Timme, TL, Li, L, Goltsov, A, Yang, G: CAVEOLIN-1: A COMPLEX AND PROVOCATIVE TARGET IN PROSTATE CANCER AND POTENTIALLY OTHER MALIGNANCIES. Emerging Therapeutic Targets 3:337-346, 1999.

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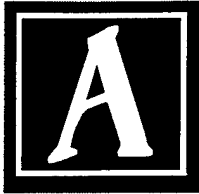

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Caveolin-1: a complex and provocative therapeutic target in prostate cancer and potentially other malignancies

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Review

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Acknowledgements

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1. Introduction

Prostate cancer is the most common cancer diagnosed in adult men in the United States and the second leading cause of cancer deaths [1]. The incidence of prostate cancer increased dramatically during the early 1990s due to the widespread use of prostate specific antigen (PSA) screening, but in recent years has subsequently declined (a likely result of saturation of prostate cancer screening). A slight decrease in prostate cancer mortality has also been observed in recent years with an estimate of 39,200 deaths in 1999 [1]. Curiously, a similar level of decline was also observed for many other malignancies [1]. Current curative therapeutic approaches to prostate cancer, i.e., surgery or radiation, are directed exclusively towards the primary tumour, yet occult metastases are often present at the time of treatment and it is the metastatic spread of the disease to bone and other sites in the body that leads to mortality. The only conventional treatment strategy for metastatic prostate cancer, androgen ablation, is based on experimental observations by Huggins and Hodges over fifty years ago [2]. Although androgen ablation often results in palliative effects, this treatment is not curative. Chemotherapeutic approaches against metastatic cancer have been generally ineffective due to an apparent intrinsic resistance of prostate cancer cells to conventional chemotherapeutic agents [3]. It appears that novel treatment strategies, used alone or as an adjuvant therapy, that are effective against metastatic disease will be necessary to substantially reduce prostate cancer mortality.

1.1 A model system of gene-based therapy for metastatic prostate cancer

We have developed an animal model system for research into the molecular mechanisms of metastasis and development of novel therapeutic approaches for the treatment of prostate cancer metastasis. We refined and extended the *in vivo* metastatic mouse prostate reconstitution (MPR) model system [4] by developing a set of cell lines easily adaptable to molecular methods such as differential display-PCR (DD-PCR) *in vitro* [5,6], but also useful for evaluating metastatic activities *in vivo* [7]. Early passage clonal cell lines were generated from primary and metastatic tumours that developed following initiation of p53 null foetal prostate tissues with the *ras* and *myc*

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oncogenes [8]. Matched pairs of metastasis- or primary tumour-derived cell lines were obtained from cancer tissues in the same animal. These cell lines therefore have an identical genetic background (p53 null foetal prostate tissues initiated with the *ras* and *myc* oncogenes), and in many cases are also clonally related based on Southern blotting evidence that identified unique integration sites of the initiating oncogenic retrovirus (Ziprasmyc 9) [4]. *In vitro* molecular testing of these cell lines has been restricted to early passage cells to avoid the confusing artefacts that could arise from continued passage of cells in tissue culture. For *in vivo* testing of these clonally related cell lines, we adopted an orthotopic model that most accurately reflects their biological activities and metastatic differences [7]. Interestingly, we found that orthotopic tumours produced by metastasis-derived cell lines tended to grow less rapidly but demonstrated greater spontaneous metastatic potential than their matched cell lines derived from the primary tumour [7]. This comparative model system effectively controls for genetic alterations that are unrelated to the metastatic process, and can therefore be used to identify metastasis-related genes for functional analysis. Subsequent evaluation of human prostate cancer tissues has validated the relevance of identified genes to the human disease process.

1.2 Caveolin-1 upregulation in prostate cancer

With the metastatic MPR model system described above we have identified numerous metastasis-related candidate genes. One of the first sequences identified as being upregulated in metastatic mouse prostate cancer using DD-PCR in this system encoded mouse caveolin-1 [5]. Northern blotting confirmed increased caveolin expression in metastasis-derived cell lines relative to primary tumour-derived cell lines. Western blotting with polyclonal and monoclonal caveolin-1 antibodies also confirmed increased caveolin-1 protein in metastasis-derived mouse cell lines and expression in three of four human prostate cancer cell lines. Extensive immunohistochemical analyses of our mouse model for prostate cancer and clinical human prostate specimens obtained at the time of radical prostatectomy revealed minimal caveolin-1 expression in normal prostatic glandular epithelium, with abundant staining in stromal smooth muscle and blood vessel endothelium. Prostate cancer with documented regional spread to lymph nodes had an increased frequency of caveolin-1 positive cells, and lymph node metastatic deposits had markedly increased levels of accumulation of caveolin-1 protein and a granular staining pattern. Additional evaluations of human breast cancer specimens revealed increased caveolin-1 staining in intraductal and infiltrating ductal carcinoma, as well as nodal disease. Caveolin-1 therefore appears to be associated with human prostate cancer progression and is also present in primary and metastatic human breast cancer [5].

2. Caveolins and caveolae

The caveolin-1 gene is a member of a family of three related genes [9]. Caveolins are the major structural proteins of caveolae, specialised plasma membrane invaginations that were first recognised by electron microscopy as non-clathirin coated pits (or 'cave-like' structures) on the cytoplasmic membrane [10]. They have also been characterised as a specific type of membrane microdomain, termed a raft. These are composed of cholesterol and glycosphingolipids, and are involved in organising membrane components for cellular function [11]. Caveolin is a specific protein that is involved in raft domain formation and may function as a scaffolding protein for caveolae [12]; however, caveolin can also be found associated with intracellular membranes such as those of the Golgi complex [10].

2.1 Caveolae structure and cell-specific expression

Caveolae are 50 - 100 nm invaginations of the plasma membrane, usually with a characteristic flask-like appearance in electron micrographs. Caveolae are abundant in endothelial cells, smooth muscle cells and fibroblasts [13]. They are present in most cell types, and are a characteristic feature on the apical surface of many polarised epithelial cells. Because of their unique lipid composition, caveolae are readily isolated as detergent-insoluble glycolipid-enriched domains (DIGs) [14] based on their insolubility in cold Triton X-100. Their high concentration of glycosphingolipids and cholesterol gives DIGs a low density in sucrose gradients and has allowed purification of caveolae-associated proteins. The major protein found in caveolae was termed caveolin, and is a 21 - 22 kDa protein with an intramembrane domain but cytoplasmic amino and carboxy termini [13]. Ectopic expression of the caveolin-1 gene can induce plasma membrane invaginations containing caveolin in cells

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such as lymphocytes, which normally do not have detectable caveolae [15]. This suggests an important role for caveolin-1 in establishing membrane structure and in the formation of caveolae.

The expression of the caveolin gene family has been extensively studied in cell lines [16]. Caveolin-1 and -2 have a widespread expression pattern and are frequently co-localised to the plasma membrane, suggesting the ability to form hetero-oligomeric complexes [16]. All three mammalian caveolin genes contain an invariant domain of eight amino acids in the N-terminal region, and a similar domain is found even in invertebrate caveolin-1 and -2 [17]. Overall, at the amino acid level there is a 58% similarity and 38% identity between caveolin-1 and -2 [9], but specific antibodies are available to distinguish between the two. The genes for caveolin-1 and -2 appear to be localised to the same region of human chromosome 7q31 [18] and the syntenic region of mouse chromosome 6 [19]. Caveolin-3 is a muscle-specific protein, and mutations in the gene have recently been associated with limb-girdle muscular dystrophy [20].

2.2 Regulation of caveolin-1 gene activity in normal cells

In confluent normal human skin fibroblasts, low density lipoprotein (LDL) in plasma was shown to positively regulate the transcription of caveolin-1 mRNA about three-fold within 1.5 h and lead to a similar increase of free cholesterol efflux into the medium. In contrast, oxysterols reduced caveolin-1 mRNA levels and free cholesterol efflux [21]. In a subsequent study, 924 bases of the human caveolin-1 promoter region were sequenced and two functional sterol regulatory elements (SRE) were identified at nucleotide positions 646 and 395 [22]. The site at 395 bp binds SRE binding protein-1 as detected by gel shift analysis. This binding protein is an inhibitor of transcription of the caveolin-1 gene, as opposed to its stimulatory effect on other promoters [22].

2.3 Biological and biochemical activities of caveolin-1

Caveolin-1 was first cloned and characterised because it is a principle substrate for tyrosine phosphorylation by Rous sarcoma virus [23]. There are numerous reports of co-purification or co-localisation of caveolin with proteins that mediate signal transduction, molecular transport and cell-cell communication activities.

2.3.1 Signal transduction activities

Numerous investigators have detected signal transduction-related molecules in association with caveolin *via* co-purification by subcellular fractionation techniques, and visualisation by immunohistochemistry and/or electron microscopy. Shaul and Anderson have recently reviewed these studies [24], and described the four major groups of signalling molecules enriched in caveolae as those involved in G-protein mediated signalling, Ca^{2+}-mediated signalling, tyrosine kinase/mitogen-activated protein kinase (MAPK) signalling pathways, and lipid signalling. Caveolae appear to be a focal point for compartmentalising, organising and modulating signal transduction activities that begin at the cell surface. Given the complexity of each of the different individual signal transduction pathways and the potential for cross-talk and interactions between them, it seems likely that individual cell types will exhibit different responses to stimuli that are potentially mediated by caveolin/caveolae. Cellular context will likely dictate the presence of specific signalling cascades, and caveolins may interact either to stimulate or inhibit signal transduction [9].

2.3.2 Molecular transporter activities

The abundance of caveolae in endothelial cells lead to studies that implicated them in transcytotic pathways for the transport of blood borne macromolecules such as LDL [25]. Folate appears to be taken up by certain cells in a caveolin-mediated process called potocytosis. Receptor-mediated binding of folate is localised in caveolae, which transiently close and release the folate to a cytoplasmic carrier without going through an endocytic pathway [10]. Transport of sterols has also been reported in certain cell types, and caveolin-1 protein has been shown to bind cholesterol [26] and control free cholesterol efflux from cells [21]. These observations may be related to the putative cycle of caveolin movement between the plasma membrane and the *trans*-Golgi network [13].

2.3.3 Cell-cell communication activities

The urokinase-type plasminogen activator receptor (uPAR) is a glycosylphosphatidylinositol (GPI) -linked protein that is an adhesion receptor for vitronectin on the surface of fibroblasts. In migrating cells it is also found at focal adhesions in association with activated integrins. A stable uPAR-integrin-caveolin complex has been detected and may provide a mechanism for localising extracellular activities and generation of an

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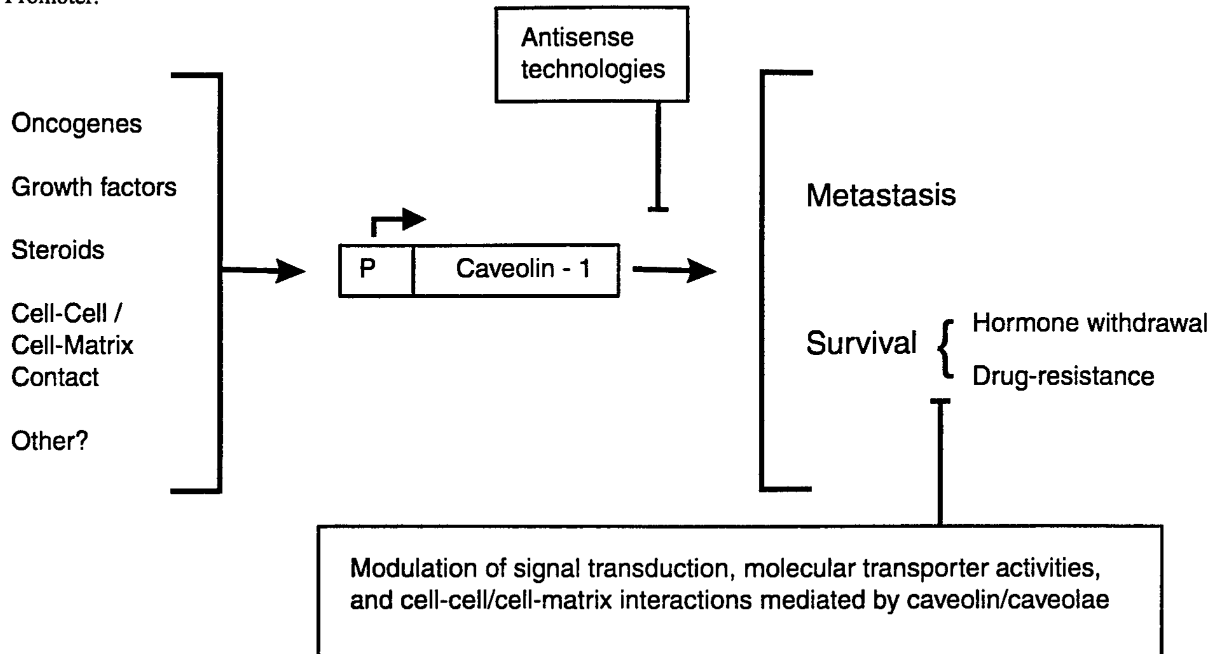
Figure 1: Caveolin-1: a metastasis and hormone/drug-resistance gene.
The central role of the caveolin-1 gene in mediating metastasis and cell survival is depicted. Two possible points of intervention as therapeutic targets are also shown.
P: Promoter.



intracellular signal through integrins [27]. Specific subsets of β 1 integrins have also been implicated in signalling for cell cycle progression and MAPK pathways (linked to the integrins through the adaptor protein Shc and caveolin) [28]. Therefore, the possibility exists for cell-cell and/or cell-matrix initiated, caveolin/caveolae mediated cell signalling that could potentially affect functions related to cell proliferation and cell survival.

3. Consequences of altered caveolin expression in metastatic prostate cancer cells

Through recent studies, a complex picture of caveolin-1 expression and function in malignant cells is emerging. At first consideration, some results appear contradictory. However, a careful analysis of the experimental details of specific studies, including model systems, cell types and physiological context, may resolve apparent inconsistencies. The proposed central role the caveolin-1 gene may play in these processes is discussed below and presented as a schematic summary in **Figure 1**.

3.1 Caveolin expression in transformed cell lines

Caveolin-1 was identified as a major phosphorylation substrate for the v-*src* oncogene [29]. Downregulation of the expression of caveolin-1 has been reported in NIH3T3 cells transformed with activated *abl* or *ras* [30] and *neu* [31] oncogenes. We have also observed decreased caveolin-1 mRNA and protein in *ras+myc* transformed NIH3T3 cells [5]. In contrast, the level of caveolin-2 does not appear to be affected by oncogenic transformation [16]. Further observations in transformed NIH3T3 cells indicated that induced expression of caveolin-1 could lead to abrogation of growth in soft agar, suggesting that caveolin may suppress transformation [32]. In addition, it was recently demonstrated that caveolin-1 transfection resulted in growth suppression of selected human breast cancer cell lines *in vitro* [33]. These observations appear to contradict our report that caveolin-1 is overexpressed in metastatic prostate cancer in both mice and humans and in human breast cancer [5]. However, significant differences in gene regulation are likely to exist between *in vitro* transformed fibroblasts and metastatic epithelial-derived tumours *in vivo*. Furthermore, the selection pressure for growth-related properties may be fundamentally different during tumourigenesis *versus* metastasis, and growth suppression functions may accompany other

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over-riding functions related to the survival of metastatic cells *in vivo*. It is clear that considerably more research is needed to understand the growth-related versus metastasis-related activities of caveolin-1, and that these activities must be placed into a relevant cellular context.

3.2 Caveolin-1 overexpression in metastatic mouse prostate cancer cells is associated with androgen resistance

As discussed above, the results of analysis of caveolin-1 expression in both mouse and human tissues, *in vitro* and *in vivo*, indicated that caveolin-1 was upregulated in metastatic prostate cancer [5]. The functional significance of caveolin-1 overexpression in metastatic prostate cancer was explored using metastatic, androgen-insensitive mouse prostate cancer cell lines. We generated a series of stably transfected antisense caveolin-1 clones with reduced levels of caveolin-1 protein relative to vector controls and parental cells. Surprisingly, antisense caveolin-1 tumours generated in an orthotopic model system regressed following castration, whereas vector control and parental cell tumours did not [34]. Furthermore, when a representative antisense caveolin-1 clone was selected for androgen insensitivity by continued growth in a castrated male host, the cells isolated from the resulting tumour were androgen insensitive *in vivo* and demonstrated high caveolin-1 and androgen receptor expression. We also developed an *in vitro* system to further investigate the response of antisense caveolin-1 clones to hormone withdrawal, and observed that antisense caveolin-1 clones underwent significant apoptosis in the absence of testosterone whereas vector control clones and parental cell lines did not. Infection of a representative androgen sensitive cell line with an adenoviral vector to generate relatively high levels of caveolin-1 expression converted the cells to an androgen insensitive phenotype, confirming the association of caveolin-1 protein with androgen insensitivity [34].

3.3 Caveolin-1 overexpression is associated with drug resistance

Two recent papers, published shortly after our identification of caveolin-1 overexpression in prostate cancer [5] and its association with androgen insensitivity [34], provided strong correlative evidence that caveolin-1 is also associated with the drug-resistant phenotype. The multiple drug-resistant human colon carcinoma cell line, HT-29-MDR, and human breast cancer cell line, MCF-7 AdrR, demonstrated significant caveolin upregulation independent of P-glycoprotein expression [35]. *In vitro*-generated taxol- and epithilone B-resistant human ovarian and lung carcinoma cell lines, as well as the vinblastine-resistant SKVLB1 ovarian cancer cell line, also demonstrated significant caveolin-1 upregulation [36].

Caveolin-1 upregulation therefore appears to be associated with multiple drug-resistant phenotypes. In general, these data are congruent with our previous reports of an association of caveolin-1 upregulation with androgen resistance. Indeed, the two sets of data in regard to androgen and drug resistance suggest a possible overlapping common function for caveolin-1 in cell survival/protection.

Although at this point relevant data are limited, certain concepts can be considered regarding caveolin-1 expression in the androgen and drug-resistant phenotypes. Because caveolin-1-positive cells were found at high frequency in metastatic deposits within lymph nodes of human prostate cancer patients that had not been treated with anti-androgens or other drug therapies, it can be inferred that these cells were selected for metastasic potential based on naturally occurring environmental conditions in the metastatic pathway. This consideration, together with recent experimental data that demonstrate a selection advantage of high caveolin-1 cells for resistance to androgen ablation-induced apoptosis [34], leads to an explanation for co-selection for metastasis and androgen resistance based on common features of the metastatic and the castration-induced environment. The levels of testosterone, a steroid growth/differentiation factor, are abruptly reduced upon castration from the extremely high levels that occur within the normal prostate to relatively low levels [37]. This reduction in testosterone can also lead to significant alterations in the expression of growth factors that are under androgenic control [38] (e.g., epidermal growth factor [39]). Interestingly, under conditions of 'normal' prostatic growth factor and androgen levels when potentially metastatic cells are seeded into either the lymphatics or general vasculature, they encounter similar environmental stresses to those that exist within the prostate gland following castration. Because of the presence of various plasma proteins that can bind steroids [37], when potentially metastatic cells enter vascular compartments they likely encounter an abrupt reduction in testosterone, as well as other growth factors. Other selection pressures which the potentially metastatic cells encounter are

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hypoxia and loss of appropriate cell-cell and cell-matrix interactions. These conditions are also similar in many regards to selective pressures encountered in the prostatic environment upon castration [40]. Thus, cancer cells that seed into the systemic circulation during metastatic progression may be selected for survival in a metastatic environment based on properties that overlap with those necessary for survival in a castrated environment.

4. Caveolin/caveolae pathways as therapeutic targets

Thus far, our laboratory has demonstrated that caveolin-1 is associated with both the metastatic phenotype and the androgen-resistant phenotype in prostate cancer. Subsequent reports from other laboratories suggest that caveolin-1 overexpression may lead to drug resistance in various malignant cells. In the previous discussion, we introduced the concept that selection for the metastatic phenotype may involve co-selection for androgen resistance. Interestingly, the potential biological and biochemical activities of caveolin-1 form the basis for rational therapeutic strategies that could impact on both the androgen-resistant and drug-resistant cancer cell.

4.1 Signal transduction targets

A potential mechanistic link exists between the response of prostatic epithelial cells to androgen ablation and specific molecular signal transduction activities associated with caveolin/caveolae. Within one day following androgen ablation, *in vivo* intracellular Ca^{2+} levels rise within prostatic glandular cells and drive intranuclear Ca^{2+}/Mg^{2+}-dependent endonuclease activities contributing to widespread apoptosis [41,42]. Following on from our previous discussion, one might speculate that Ca^{2+} dysregulation in potentially metastatic cells contributes to the selection of the fully metastatic cell that survives or adapts to these pressures. Interestingly, it has been demonstrated in various non-prostatic cells that caveolae are involved in Ca^{2+} transport, storage and Ca^{2+}-mediated signalling [24]. Although the precise levels at which caveolin/caveolae regulate Ca^{2+}-mediated signalling are largely unknown, it is tempting to speculate that this pathway is involved in androgen/drug resistance. Interestingly, first generation modulators of P-glycoprotein include Ca^{2+} channel blockers and calmodulin inhibitors [43,44]. In addition, many drugs (including taxol) can activate a variety of signal transduction pathways that could be subverted or blocked by caveolin/caveolae-mediated cell signalling. Numerous studies have demonstrated that caveolin/caveolae may regulate multiple signal transduction pathways, including those related to nitric oxide synthase, MAPK and lipid signalling molecules [12,24].

4.2 Molecular transporter targets

The reported association of overexpression of caveolin-1 with drug resistance is consistent with a cell protection/survival function for caveolin/caveolae. Interestingly, there are functional similarities between caveolin-1 and the well-established drug resistance gene, P-glycoprotein [45], that are worthy of discussion. P-glycoprotein is a contributing factor to multi-drug resistance in numerous malignancies [46]. It is a plasma membrane ATPase- and energy-dependent drug efflux pump, and these specific properties are responsible to a large extent for drug resistance in cells that overexpress the gene [47]. These activities can be directly compared to the molecular transport properties of caveolin which has been shown to transport intracellular cholesterol to the cell surface [21]. It is conceivable that this functional aspect of caveolin-1 is somehow related to the extensively studied P-glycoprotein molecular transport activities in regard to drug resistance [46]. Modulators of P-glycoprotein have been developed with the intent of enhancing the efficacy of anticancer drugs without significantly altering the pharmacokinetic interactions of the anticancer agent [48]. In some cases it has been shown that these modulators can inhibit drug efflux, thus establishing the importance of this P-glycoprotein-related activity as a target for drug development. By extrapolation, it may be possible to exploit the molecular transport activities of caveolin/caveolae to further investigate the role of caveolin-1 in drug resistance, and potentially develop caveolin-1-based therapeutics.

4.3 Cell-cell/cell-matrix interaction targets

The cell-cell and cell-matrix interactions mediated by caveolin also represent potential therapeutic targets. However, because of limited information at this time it is difficult to identify either general or specific points of intervention. It has been demonstrated previously that a uPAR-integrin-caveolin complex appears to mediate cell-matrix interactions that affect adhesion and motility functions in fibroblasts [27]. Although these activities may be cell type- and matrix-specific,

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one could speculate that antibodies to specific epitopes and/or small molecules that would block such interactions may have therapeutic potential in suppressing relevant cell-matrix attachments and/or motility functions of malignant cells. However, as with other approaches of this type, it will be challenging to build specificity into this system such that high levels of activity will be achieved without limiting toxicities.

5. Molecular surrogates of caveolin-1-based therapeutic targets

The complexity and lack of specific information regarding the exploitation of caveolin/caveolae as a therapeutic target leads to the consideration of surrogate molecular targets with similar properties to those now associated with caveolin-1. Two specific molecular analogies can serve as a foundation for the conceptual development of the caveolin-1 molecule as a model drug target. These examples are presented and discussed in Sections 5.1 and 5.2.

5.1 P-glycoprotein as a drug target for modulatory molecules

The relatively well-defined cholesterol transport functions of caveolin-1/caveolae can be compared to the well-established and extensively studied drug transporter properties of P-glycoprotein [45]. Overexpression of P-glycoprotein leads to drug resistance in part through stimulation of the efflux of multiple structurally unrelated cytotoxic drugs now in clinical use. The concept of modulating P-glycoprotein and potentially other drug resistance genes of this general class of molecules, has been suggested and exploited in the design and development of multiple modulators of P-glycoprotein including Ca^{2+} channel blockers, immunosuppressive agents (cyclosporin A and FK506), analogues of antihypertensive drugs (reserpine and yohimbine), neuroleptic drugs and anti-oestrogens [43]. More recently, the successful modulation of a variety of cytotoxic agents through direct interaction of P-glycoprotein with a small molecule has been reported [49]. This study demonstrates that P-glycoprotein modulation can result in more potent and efficacious cytotoxic activity compared to the cytotoxic agent alone without unwanted pharmacokinetic interactions [49].

It is possible to consider caveolin-1 as a similar target for modulation by small molecules. As with modulators of P-glycoproteins, it would be possible to devise specific screens for small molecules that modulate calveolin-1 functions. Initial screens, and more specific tests of efficacy for selected molecules, could be based on the molecular transport or anti-apoptotic activities of caveolin-1. Certainly, unique considerations must be made for caveolin-1/caveolae in the design and implementation of such a drug screen and include the multiple pleiotropic pathways through which caveolin/caveolae function. However, many of the concepts used for the identification and characterisation of P-glycoprotein modulators can serve as a conceptual/logistical foundation for the development of modulating agents for caveolin-1.

5.2 Anti-apoptotic *bcl-2* family genes

To provide another molecular surrogate for discussion and to potentially reconcile the survival and growth suppressive functions of caveolin-1, it is interesting to consider the well-established properties of *bcl-2* family genes juxtaposed to those of caveolin/caveolae. Interestingly, BCL-2 and other family members can block entry into the cell cycle and thus inhibit growth, as well as provide well-defined protective functions. This property of BCL-2 may be related to its role during normal differentiation as defined in cells such as myelomonocytic progenitor cells. During differentiation, BCL-2 appears to potentiate cell cycle arrest and irreversible withdrawal into the non-proliferating (G_0) state [50]. Subsequent activities of differentiation inducers then allow for the cell to commit to the differentiation pathway. Functional maturation of these cells appears to ultimately downregulate *bcl-2* levels, and thus lead to apoptosis. However, during the maturation process overexpression of *bcl-2* can clearly induce protection from various apoptotic stimuli [50]. Although analogies to human prostatic cell differentiation are certainly tenuous at this point, the fully differentiated and secretory prostatic cell demonstrates low to undetectable levels of caveolin-1 [5] and, therefore, may be poised to undergo apoptosis following a variety of stimuli, including withdrawal of androgenic steroids. Since BCL-2 has also been shown to protect against cytotoxic drugs, it has been suggested that the properties of BCL-2 that are involved in retarding entry into the cell cycle, as well as its protective activities, could provide double protection against these drugs, as both cell cycle entry and response to apoptotic stimuli are required for effective induction of apoptosis by chemotherapeutic agents [51].

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Bcl-2 gene activities have also been considered as a direct target mainly through the use of antisense technologies. One Phase I clinical trial is in progress for prostate cancer at the Memorial Sloan-Kettering Cancer Center with a *bcl-2* antisense oligonucleotide [52]. Antisense technologies represent a viable approach toward reducing caveolin-1 levels *in vivo*, and thus suppressing caveolin functions with regard to metastatic and survival activities. As with all antisense technologies, non-specific interactions of antisense nucleic acids make it difficult to generate a high level of efficacy, and even more difficult to understand and exploit the mechanism of action. However, as with other proteins identified as drug targets, this approach can and should be pursued.

6. Summary

Recent information indicates that caveolin-1 overexpression is associated with metastasis and the androgen-resistant phenotype in prostate cancer [5,34]. Additional reports have also indicated that caveolin-1 overexpression occurs during the development of resistance to chemotherapeutic drugs in multiple human cancer cell lines [35,36]. Co-selection for metastasis and androgen resistance in prostate cancer cells can be conceptually reconciled through consideration of the relevant characteristics of the metastatic environment which, in general, overlap those induced in prostate by castration. Extrapolation of this overlapping selection criteria concept for metastasis/androgen resistance to drug resistance is possible through consideration of the general properties of caveolin/caveolae with regard to cell type-specific anti-apoptotic functions and antiproliferative activities. We have outlined the fundamental molecular pathways through which caveolin/caveolae may regulate multiple cellular and biochemical activities that appear to be responsible, in part, for malignant progression of prostate cancer and potentially other malignancies.

Caveolin-1 and caveolae represent a novel paradigm in drug targeting as caveolae represent multi-functional 'organelles' that are likely established in some malignant cells *de novo* as a consequence of genetic alterations which stimulate caveolin-1 expression. The presence of increased caveolin/caveolae in the cell may than lead to metastatic progression through enhanced cell survival and potentially other altered properties (**Figure 1**). The possible impact of caveolin-1 overexpression on signal transduction pathways, molecular transport and cell-cell/cell-matrix interactions establishes a general foundation for approaching caveolin-1 as a drug target.

At this stage, certain analogies to approaches that led to the modulation of P-glycoprotein, as well as the targeting of BCL-2 family members through antisense technologies, are possible, and can serve as a starting point for the design of specific approaches used in the development of anticaveolin-1 therapeutic agents. As with other molecular drug targets, issues regarding toxicity and specificity are serious concerns and, in the case of caveolae, warrant extreme consideration. Caveolin-1 is expressed in numerous normal cells and, therefore, strategies to specifically target caveolae in specific cell types with anticaveolae modulators or other anticaveolin-1 drugs are of paramount consideration. Although it is relatively early, the emerging role of caveolin-1 as a metastasis-related, androgen resistance and drug resistance gene certainly and rightfully leads to obvious consideration of blocking specific caveolin functions in an effort to develop more effective therapies for metastatic prostate cancers and potentially other malignancies.

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TESTOSTERONE PROMOTES SURVIVAL OF PROSTATE CANCER CELLS THROUGH UPREGULATION OF CAVEOLIN-1 AND CAVEOLIN-1 EXPRESSION IS INCREASED IN ANDROGEN RESISTANT HUMAN PROSTATE CANCER

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ABSTRACT

Previously we demonstrated that caveolin-1 was associated with prostate cancer metastasis and that suppression of caveolin-1 expression re-established androgen responsiveness in prostate cancer cells. In this study we place caveolin-1 downstream of testosterone in a molecular pathway that leads to survival in mouse and human prostate cancer cells *in vitro*. Testosterone was shown to induce caveolin-1 gene expression at the level of transcriptional activation. In addition, antisense caveolin-1 was able to significantly inhibit the survival effects of testosterone indicating that caveolin-1 is a downstream effector of testosterone-mediated survival activities. Furthermore, adenovirus-mediated caveolin-1 expression alone was also shown to account for a significant component of the survival activities induced by testosterone *in vitro*. Immunohistochemical studies demonstrated a significantly increased frequency of caveolin-1 expression in both human primary prostate cancer specimens and distant metastases following androgen ablation therapy. Overall, our studies establish the testosterone-caveolin-1 pathway and caveolin-1 expression as important determinants of the androgen-resistant phenotype in prostate cancer.

Prostate cancer threatens the lives of tens of thousands of US men, yet unfortunately there are no curative therapies available for androgen resistant disease. Although decades have gone by since prostate cancer was shown to be initially responsive to androgen ablation, there is only minimal understanding at the mechanistic level regarding the ultimate hormone resistant state of prostate cancer that is responsible for the exceedingly high mortality rate. Recently we reported that caveolin-1 levels were elevated in metastatic mouse and human prostate cancer.[1] Further studies demonstrated that suppression of caveolin-1 levels led to re-establishment of androgen sensitivity *in vitro* and *in vivo* and that enforced caveolin-1 expression could convert androgen sensitive prostate cancer cells to androgen insensitive cells.[2] Caveolin-1 is the principal component of caveolae, sub-invaginations of the plasma membrane and trans-Golgi network that have been implicated in sphingolipid-cholesterol transport and specific signal transduction pathways.[3-5] Under some conditions caveolin has been shown to suppress growth of specific cell lines *in vitro*,[6-9] and it has been suggested that caveolin-1 functions as a "tumor suppressor gene".[10] However, specific genetic analysis of caveolin-1 did not support this contention but was consistent with our earlier reports in demonstrating overexpression of caveolin-1 in malignant breast tumors.[11] In addition other reports have shown that caveolin-1 is upregulated in multidrug resistant cancer cells independently of p-glycoprotein.[12,13] Further evidence for a role of caveolin-1 in prostate cancer progression was provided by a recent report demonstrating that caveolin-1 overexpression in primary prostate cancer is an independent predictor of recurrence following radical prostatectomy.[14] Although caveolin-1 can suppress growth of specific cell lines under some conditions, the emerging role for caveolin as a metastasis-related and androgen resistance gene in prostate cancer has consistently been demonstrated.[15,16] In this report, we contribute critical information to the establishment of caveolin-1 as an androgen resistance gene in human prostate cancer. We initially confirmed the biological function of testosterone to promote survival of both mouse and human prostate cancer cells *in vitro* using viability assays in serum free media containing various concentrations of testosterone. As expected, testosterone significantly enhanced cell survival of androgen sensitive prostate cancer cells under conditions of growth/survival factor depletion with maximum protection occurring at 10-20nM of testosterone for 148-

1 ABAC3 (ABAC3) mouse prostate cancer cells and at 5 nM for LNCaP cells, human prostate cancer cell line (Fig 1*a*). To further explore the relationship between testosterone and caveolin-1 expression, we first examined the dose- and time-dependent effects of testosterone on caveolin-1 expression in mouse and human prostate cancer cell lines. ABAC3 cells showed maximal induction of caveolin-1 at 20 nM testosterone after 2 days treatment (Fig. 1*b*). LNCaP cells displayed higher sensitivity to testosterone with maximum induction of caveolin-1 at 5 nM testosterone after 2 days treatment (Fig. 1*b*). These results are in agreement with and extend a previous report demonstrating increased caveolin-1 protein levels following testosterone treatment *in vitro*.[17]

To determine whether upregulation of caveolin-1 by testosterone occurs at the transcriptional level, a luciferase reporter vector under the transcriptional control of the mouse caveolin-1 promoter, pGL3-Pmcav-1-luc, (Ren *et al*, unpublished data) was used. The relative activity of luciferase in ABAC3 cells was increased more than 2-fold by testosterone (Fig. 1*c*). Similarly, the relative activity of luciferase in 293PE cells (also androgen receptor (AR) positive) was also increased approximately 2-fold over control levels by testosterone (Fig. 1*c*). This enhanced activity could be blocked by the addition of 1μM of Casodex, a direct antagonist of the AR (Fig. 1*c*) indicating that the upregulation of caveolin-1 by testosterone is mediated by AR.

After observing induction of caveolin-1 expression by testosterone in parallel with enhanced survival, we asked whether survival activities induced by testosterone were a consequence of the up-regulation of caveolin-1. To address this question, we performed experiments in which ABAC3 cells were infected with adenovirus-mediated antisense caveolin-1, followed by testosterone stimulation. The results in figure 2*a* show that antisense human caveolin-1 abrogated the up-regulation of caveolin-1 by testosterone. As a consequence of reduced caveolin-1 expression, the survival effects of testosterone were significantly reduced (Fig. 2*b*). The results of these experiments together with those described above demonstrate that caveolin-1 is a downstream target gene of testosterone that is largely responsible for the survival activities stimulated by testosterone under these experimental conditions.

To determine whether caveolin-1 could promote survival in human prostate cancer cells in the absence of testosterone, we infected LNCaP cells with an adenoviral vector expressing human sense caveolin-1. Dose-dependent expression of human caveolin-1 was induced by increasing multiplicities of infection (MOI) of the recombinant adenoviral vector. The results demonstrated that the induction of caveolin-1 was detectable at MOI = 5, and gradually increased with increasing MOI (Fig. 3*a*). As expected, caveolin-1 provided significant survival protection to LNCaP cells from growth factor depleted conditions. Surprisingly, the maximum survival protection of caveolin-1 was observed at MOI = 10 (Fig. 3*b*), which represents only a modest level of caveolin-1 (Fig. 3*a*). While survival protection ranged from MOI 5-50, the protection eventually diminished at MOI = 100 and at MOI = 200 reduction of viability was observed (Fig. 3*b*). These data clearly suggest that caveolin-1 can induce survival activities when it is expressed at modest levels, yet when expressed at high levels, caveolin-1 is toxic to the cells resulting in apoptotic cell death (data not shown). The realization of this biphasic nature of caveolin-1 activities may resolve some of the apparently conflicting data regarding the survival versus growth suppressing activities of caveolin-1 expression in specific cell types.

To extend these concepts to clinically relevant *in vivo* conditions, we analyzed a large series of primary prostate cancer tissues and their associated metastases derived from hormone therapy treated versus untreated patients for caveolin-1 immunoreactivity using a well-characterized antibody[1] (see Fig. 4*a*) The results demonstrated that significantly increased frequencies of caveolin-1 positive specimens were observed in primary prostate cancer tissues in stage D patients following hormone therapy (Fig. 4*b*). Frequencies were increased from 38% in the hormonally naive patient group to 74% in the hormone treated patient group. In metastatic tissues caveolin-1 positivity was seen in 62% of the specimens recovered from patients who had not been treated with hormone therapy. This frequency of caveolin-1 expression is in striking agreement with that previously obtained for lymph node metastases.[1,14] As in primary tumor tissues, a significant increase in caveolin-1 positivity to 82% was observed in the hormone treated metastatic specimens (Fig. 4*b*). These results confirm that androgen-

resistant prostate cancer has increased levels of caveolin-1 positivity beyond those relatively high levels that were previously shown to be associated with metastatic disease.

Overall, we have identified a testosterone-regulated survival pathway in prostate cancer cells that is mediated by caveolin-1 expression. We further demonstrated that caveolin-1 expression alone can support a significant component of these survival activities in the absence of testosterone. Importantly, we also demonstrated that this experimental condition mimics that observed following androgen ablation of prostate cancer patients *in vivo* in that primary tumors and metastases from hormone treated prostate cancer patients showed increased frequency of caveolin-1 positivity beyond the relatively high levels previously associated with metastatic progression.[1,14] Since caveolin-1 becomes upregulated during metastatic progression in the presence of circulating testosterone, it appears that prostate cancer cells acquire the capacity for testosterone-mediated caveolin-1 upregulation in a significant number of patients who have not been treated with androgen ablation therapy. However, our new results lead to obvious questions regarding the mechanism(s) of upregulation of caveolin-1 in the absence of testosterone. One possibility for testosterone independent caveolin-1 expression is through ligand independent androgen receptor activation. Caveolin-1 has been previously shown to be involved in the activation of the ras-raf-1-MAP kinase pathway by specific growth factors such as EGF.[18] Interestingly, EGF and other specific growth factors relevant to prostate cancer have been shown to support ligand independent AR activation.[19] Recent reports further indicate the AR-regulated genes can be highly expressed in human prostate cancer xenografts in the absence of testosterone.[20] Therefore, caveolin-1 itself could potentially facilitate growth factor-mediated, ligand-independent AR activation through modulation of growth factor receptor stimulated ras-raf-1-MAP kinase pathway activities. It is also of interest that free cholesterol can upregulate caveolin-1 mRNA levels *in vitro* in fibroblasts.[21] Although at this time it is not clear how caveolin-1 expression is regulated in prostate cancer cells, the results of this study indicate that caveolin-1 mediated survival activities are responsible in part for the selection of caveolin-1 positive prostate cancer cells following androgen ablation *in vivo*. The mechanism(s) that underlies caveolin-1 mediated cell survival in prostate cancer is likely complex, but could involve molecular transport and/or signal

transduction modulation.[4,5] Further efforts should be focused on understanding the regulation of caveolin-1 and the mechanisms of its mediation of survival in prostate cancer cells and potentially other malignant cell types as this report has provided a clearer understanding of its role in the androgen resistant phenotype in prostate cancer.

Methods

Cell lines and cell culture. 148-1ABAC3 (ABAC3) was derived from a metastatic mouse prostate cancer cell line by introduction of an antisense mouse caveolin-1 cDNA as previously described[2] and was grown in DMEM with 10% FCS. The human prostate cancer cell line, LNCaP, was obtained from the American Type Culture Collection, grown in RPMI-1640 with 10% FCS, and used at passage 40-60. 293PE cells were obtained from Dr. Garry Nolan (Stanford University) and grown in DMEM (high glucose) with 10% FCS.

Viability assay. Subconfluent cells were trypsinized, collected by centrifugation and washed once with serum free medium (SFM). A single-cell suspension was then seeded at low cell density (~200 cells per well of a 96-well plate) in SFM, or SFM with testosterone (Sigma, St Louis, MO) (SFMT). Under these conditions, cell proliferation was minimal throughout the assay period. After 3 days, viability of cells was determined by incubation with 0.05 mg/ml of MTT (Sigma, St Louis, MO) at 37 °C for two hours or overnight. The viability was expressed as fraction of viable cells (viable cells/total cells) and normalized to control (control = 1; in caveolin-1 induction experiments, T = 0 nM was used as control; in virus infection experiments, uninfected was used as control). The viability data reported in this study are representatives of at least three independent triplicate experiments. Error bars show standard deviations of a triplicate experiment.

Induction of caveolin-1 protein by testosterone. For the examination of caveolin-1 protein induction by testosterone, cells were seeded at similar density to that in viability assay (1.0 x 10^5 cells/15-cm plate) and grown in SFM with indicated concentrations of testosterone and indicated time.

Western blot analysis. Cells were scraped from culture plates and collected by centrifugation. The cell pellets were washed once with PBS and then lysed with TNES lysis buffer (50 mM Tris (pH7.5), 2 mM EDTA, 100 mM NaCl, 1% NP40, 20 μg/ml aprotinin, 20 μg/ml leupeptin and 1 mM PMSF) on ice for 45 minutes. Proteins were separated on a 15% polyacrylamide-SDS gel and then electrophoretically transferred onto a nitrocellulose membrane. Caveolin-1 (Cav-1) was detected with polyclonal caveolin-1 antiserum (Santa Cruz Biotech, Santa Cruz, CA). A monoclonal β-actin antibody (Sigma, St. Louis, MO) was used to detect β-actin for loading control. All Western blots shown in this study are representatives of at least three independent experiments.

Construction of mouse caveolin-1 promoter. A 5' fragment of the mouse caveolin-1 cDNA was used as a probe to identify mouse caveolin-1 promoter region fragments. A 0.72 kb promoter sequence was subsequently constructed into pGL3 luciferase basic vector to generate a mouse caveolin-1 promoter controlled luciferase reporter vector, pGL3-Pmcav-luc (Ren *et al.*, unpublished data). The orientation and sequence of the constructs were confirmed by sequencing.

Luciferase assay. PGL3-Pmcav-luc or its parental vector, pGL3 luciferase basic vector, was cotransfected with pCMV-β-gal into ABAC3 or 293PE using Lipofectamine (Life Technologies, Grand Island, NY) in SFM according to manufacture's protocol. Four hours after transfection, fresh medium was added and FCS concentration was brought to 10%. Twenty-four hours after transfection, the cells were trypsinized and collected by centrifugation. After washing once with SFM, the single cell suspension was

seeded in SFM or SFMT at low density (1 x 10^5 cell/15-cm plate). Cells were collected after 24 hours of treatment by T, and were lysed in 50 μl of Luclite substrate buffer (Packard, Meriden, CT) at room temperature for 15 minutes and then diluted to desired volume with PBS containing 1 mM Mg^{2+} and 1 mM Ca^{2+}. Luciferase assays were performed using a Luclite kit (Packard, Meriden, CT) and luciferase activities were measured on a TopCount luminescence counter (Packard, Meriden, CT). Light units generated by luciferase activity were normalized to the activity of β-galactosidase as an internal control for transfection efficiency. The data reported in this study are representatives of at least three independent experiments.

Adenovirus-mediated human caveolin-1 expression. Adenoviral RSV-human caveolin-1 constructs (sense and antisense) were generated by recombination of the adenovirus with a pAdL2/RSV plasmid that expressed sense or antisense human caveolin-1 cDNA.

For the dose-dependent induction of caveolin-1, LNCaP cells were grown to subconfluence and detached with 0.05% trypsin and 0.53 mM EDTA. 5.0 x 10^5 cells were seeded onto each well of a 6-well plate. After incubation overnight in regular culture medium at 37°C with 5% CO_2, the medium was withdrawn by aspiration and replaced with 1 ml SFM. Diluted virus with different multiplicity of infection (MOI) was added to the medium and then incubated for 3 hours at 37°C with 5% CO_2. At the end of incubation, medium was removed and replaced with regular culture medium. Cells were split for viability assay and for the preparation of cell lysates 48 hours after infection.

In the experiments testing the antagonistic roles of testosterone and antisense caveolin-1, a prolonged double infection procedure was adopted in order to decay preexisting endogenous caveolin-1 and potentate the function of antisense DNA. 1.0 x 10^5 ABAC3 cells were seeded in each well of 6-well plate and grown in regular medium overnight. After the first infection with an adenovirus expressing antisense caveolin-1 at the indicated MOI on the next day (day 1), cells were grown in regular medium for two days. The second infection were performed on day 3 (MOI calculation was adjusted for increased

cell number), followed by another two day growth in regular medium. On day 5, cells were split, washed with SFM, and then seeded in SFM or SFMT at low cell density as described above for examination of caveolin-1 protein expression and viability. Cell lysates were prepared after 2 days and MTT staining was done after 3 days.

Caveolin-1 immunohistochemistry

In this study, 61 stage D prostate cancer patients were included and 11 of them received androgen ablation therapy (9 orchiectomies; 2 DES treatment). For each patient, one primary prostate cancer and one or more metastatic cancer specimens from different organs were obtained either by radical prostatectomy or autopsy. From the 11 treated patients, a total 33 metastases were derived from lymph node (n=12), lung (n=8), bone (n=1), liver (n=5), adrenal gland (n=1), bladder (n=2), brain (n=1) and soft tissue (n=3). Fifty five metastases were obtained from the 50 untreated patients, which included lymph node (n=48), lung (n=2), bone (n=2), liver (n=1), bladder (n=1), and soft tissue (n=1). All of these tissue blocks were fixed in 10% formalin and embedded in paraffin following a routine procedure. Six micron sections were made from the tissue blocks. The sections were deparaffined and rehydrated. They were then heated in citrate buffer (0.01M, pH 6.5) with in an 800 W microwave oven for 9 minutes for antigen retrieval. Endogenous peroxidase in sections was inactivated in 2% H_2O_2 for 10 min. The sections were then blocked in 3% normal goat serum in 0.2 M phosphate-buffered-saline (PBS) pH7.4 and followed by incubation in primary antibody specific for human caveolin-1 (cat# sc-894, Santa Cruz Biotechnology, Santa Cruz, CA). The caveolin-1 antibody was used at a dilution of 1:400 in PBS with 0.5% normal goat serum. The sections were incubated in the primary antibody for 2 hrs at room temperature. They were then processed following standard avidin biotin complex (ABC) immunostaining procedures with an ABC kit (Vector Lab, CA). Immunoreaction products were visualized in a 3,3'-diaminobenzidine/H_2O_2 solution. To verify the specificity of the immunoreactions, some sections were incubated in primary antibody pre-absorbed with the corresponding caveolin-1 peptide (Santa Cruz) at a concentration of 25 ug/ml. The immunostained sections were evaluated at a power of 200 x, under a Zeiss microscope. For

each specimen, the whole cancer area was scanned. Positive caveolin-1 staining was defined as the presence of any microscopic field in which more than 50% of the cancer cells gave rise to caveolin-1 positive granular immunoreaction products in their cytoplasm.

Statistical analysis

Comparisons in the frequency (%) of caveolin-1 positivity between treated and the non-treated cancer specimens were made by using the chi square test. A p value less than 0.05 was considered statistically significant in all of the analyses. All analyses were performed with statistical software (Statview version 5.0, SAS Institute Inc. Cary, NC).

Figure Legends

Fig. 1 Up-regulation of caveolin-1 by testosterone. ***a,*** Dose-dependent survival protection by testosterone (T). ABAC3 (□) and LNCaP (■) cells were seeded at low cell density (200 cells per well in 96-well plate) in SFM with indicated concentrations of T. Viability of cells was determined on day 3 by MTT staining (see details in **Methods**). ***b,*** Induction of caveolin-1 protein by testosterone (T). Cells were grown in SFM or SFMT (T= 20 nM for ABAC3 and T= 5 nM for LNCaP) for two days. *c,* Transcriptional up-regulation of caveolin-1 by testosterone. PGL3-Pmcav-luc or its parental vector, pGL3 luciferase basic vector, was cotransfected with pCMV-β-gal into ABAC3 or 293PE (see **Methods** for detail). –T (□), +T (■), + T and casodex (dotted bars).

Fig. 2 ***a,*** Antagonistic effects of testosterone and antisense caveolin-1 on the expression of caveolin-1. ABAC3 cells were double infected with an adenovirus expressing antisense caveolin-1, followed by induction of caveolin-1 by T as described in **Methods**. Cell lysates were prepared 48 hours after treatment by T. The levels of caveolin-1 protein were visualized by Western blotting with caveolin-1 antiserum (Santa Cruz Biotech, Santa Cruz, CA). ***b,*** Antagonistic effects of testosterone and antisense caveolin-1 on cell survival. ABAC3 cells were double infected with an adenovirus expressing antisense caveolin-1 as described above and in **Methods**. On day 5, cells were split and seeded onto 96-well plate in SFM (□) or SFMT (■). Viability of cells was determined by MTT staining 3 days after treatment by T.

Fig. 3 ***a,*** Dose-dependent induction of caveolin-1 with adenovirus-mediated human caveolin-1 cDNA. LNCaP cells were infected with an adenovirus expressing human sense caveolin-1 cDNA or with the parental vector (Ad-RSV) at various multiplicity of infection (MOI) as described in**Methods**. Cell lysates were prepared 48 hours after infection. The expression of caveolin-1 protein was detected by Western blotting with caveolin-1 antiserum antiserum (Santa Cruz Biotech, Santa Cruz, CA). ***b,*** Dose-dependent survival protection of caveolin-1 from growth/survival factor

depletion in LNCaP cells. Forty-eight hours after infection with adenoviral vectors, cells were split and seeded onto 96-well plate for viability assay (see details in **Methods**).

Fig. 4 ***a,*** Caveolin-1 immunostaining in a lymph node metastatic deposit from a prostate cancer patient who was treated with androgen ablation therapy. Granular immunoreactivity products were localized to the cytoplasm of cancer cells. ***b***, Incidence of caveolin-1 expression demonstrated by immunohistochemistry as percent of positive patients in 61 primary prostate cancers (50 untreated and 11 treated), or percent of positive specimens from 88 metastatic deposits (55 untreated and 33 treated). * and ** indicate the values significant higher than those of their untreated counterparts ($p=0.0358$ or $p=0.0489$ respectively; chi square test).

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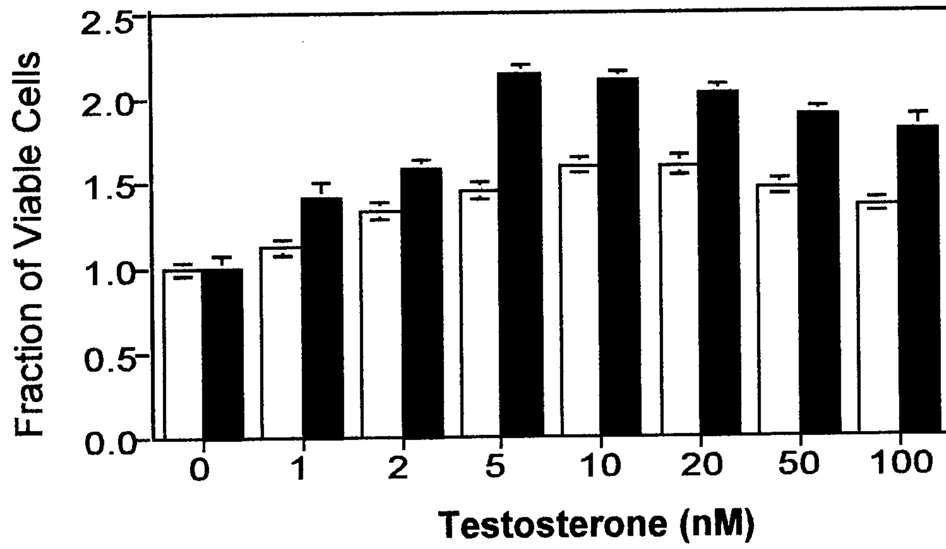

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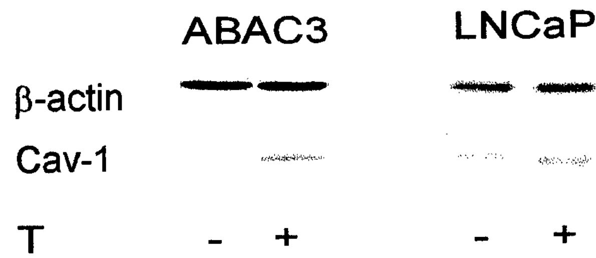

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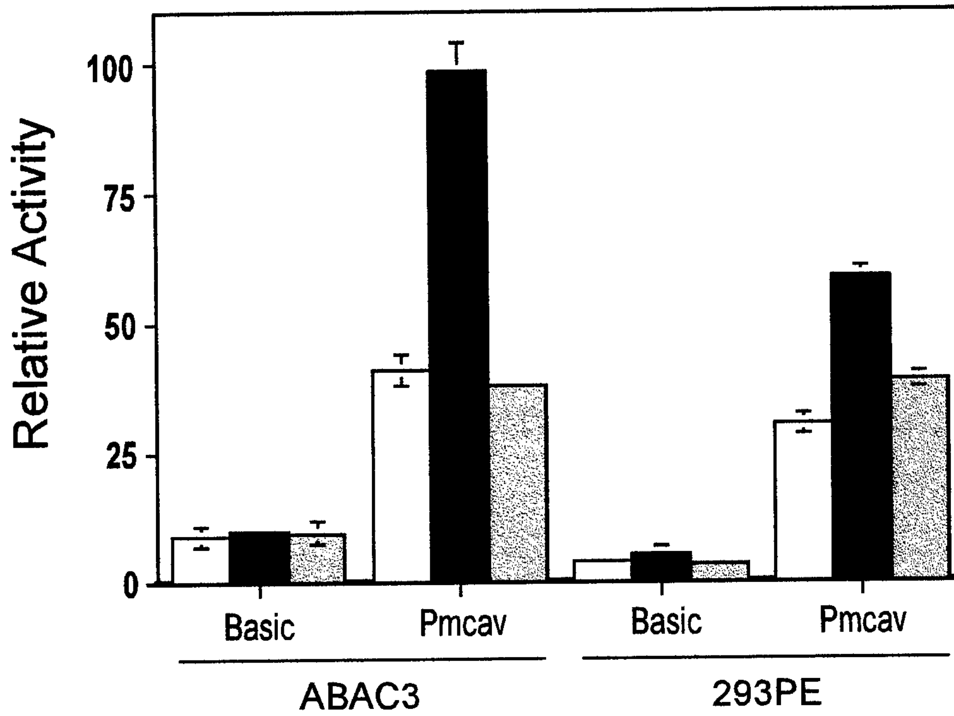

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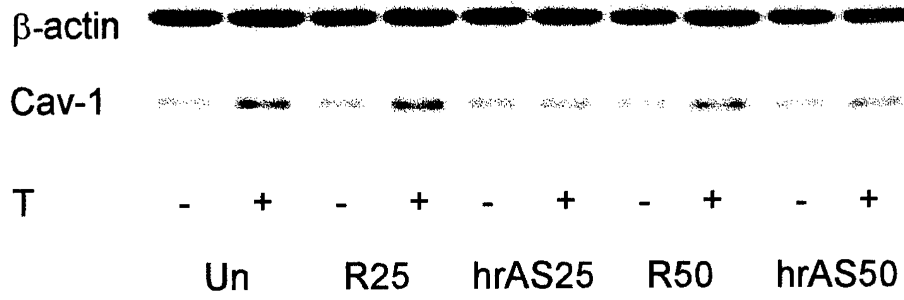

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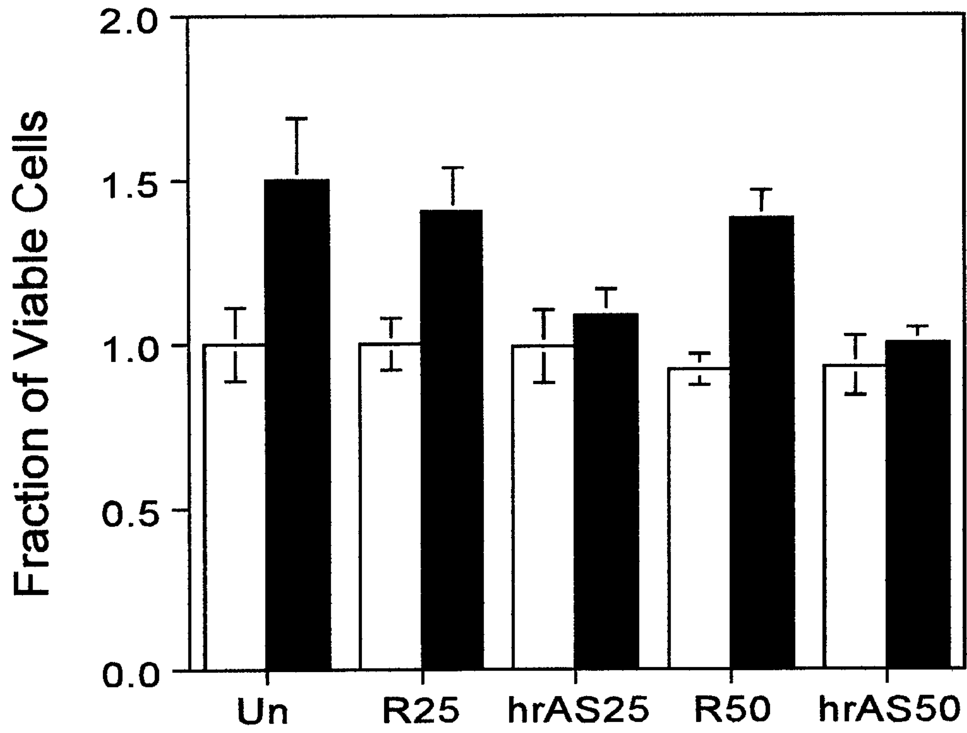

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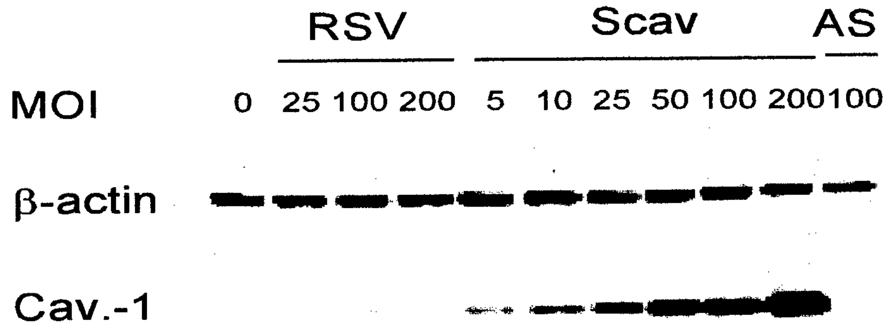

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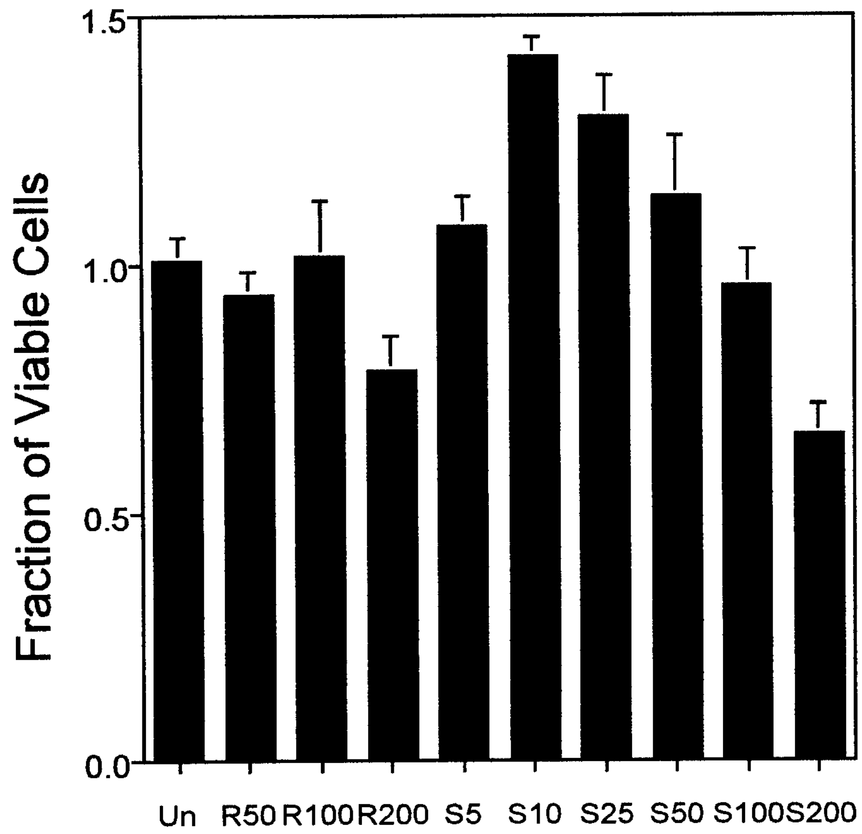

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