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We have identified a novel protein, SSeCKS (pronounced *essex*), which is a major substrate of Protein Kinase C (PKC) and tyrosine kinases that plays a key scaffolding role at the juncture of cytoskeletal architecture and proliferative signaling pathways. In untransformed prostate epithelial cells, SSeCKS transcription and protein phosphorylation are cell-cycle regulated, and in many oncogenically-transformed cells, SSeCKS is hyperphosphorylated on select serine and tyrosine residues. We have analyzed whether SSeCKS plays a role in prostate cancer biology. Our current data indicate that SSeCKS is downregulated at the RNA and protein levels in typically studied human prostate cancer cell lines and in undifferentiated (i.e.- advanced) prostate cancers in humans. We have also produced and characterized two monoclonal antibodies specific for PKC phosphorylated regions on SSeCKS. We are attempting to adapt these reagents as diagnostic tools for prostate cancer progression. Lastly, we have produced rat MatLyLu prostate cancer cells with tetracycline-regulated SSeCKS re-expression. Our data indicate that SSeCKS inhibits anchorage-independent growth *in vitro*, and in an athymic mouse model, SSeCKS severely inhibits the appearance of lung metastases. Our data strongly suggest that SSeCKS functions as a suppressor of metastasis.

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ANNUAL PROGRESS REPORT
USARMC Prostate Cancer Initiative
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ANNUAL PROGRESS REPORT
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INTRODUCTION

Normal epithelial cell growth is regulated by intricate interactions between cytoskeletal proteins -which regulate cell shape, cell-cell interactions and adherence to extracellular matrices- and so-called signaling proteins -which induce cell proliferation in response to extracellular growth factors. Prostate cancer cells are marked by the loss of proteins, such as cadherins and catenins, which function within cytoskeletal and signaling pathways. We have identified a novel protein, SSeCKS (pronounced *essex*), which is a major substrate of Protein Kinase C (PKC) and tyrosine kinases that plays a key scaffolding role at the juncture of cytoskeletal architecture and proliferative signaling pathways. SSeCKS exhibits characteristics of a "tumor suppressor" in that its expression is down-regulated in a panel of well-characterized human prostate cancer cell lines relative to controls. Its re-expression in *ras*-induced prostate cells suppresses oncogenic growth (e.g.- colony formation in soft agar) by re-establishing controls on cytoskeletal and signaling pathways and on cadherin-mediated cell-cell interactions. In untransformed cells, SSeCKS transcription and protein phosphorylation are cell-cycle regulated, and in many oncogenically-transformed cells, SSeCKS is hyperphosphorylated on select serine and tyrosine residues. To explore the potential role of SSeCKS in prostate cancer, we propose to i) analyze various grades of primary human prostate cancers for aberrant SSeCKS expression and/or mutations in the SSeCKS allele, ii) target SSeCKS as a diagnostic marker by developing antibodies to phosphorylated forms of SSeCKS expressed constitutively in cancer cells, and iii) develop a therapeutic animal model for human prostate cancer based on the re-expression of SSeCKS. The experiments proposed in this grant are envisioned to elucidate prostate cancer biology through the careful characterization of a strong tumor suppressor candidate, SSeCKS, and through the development of potential diagnostics and therapeutics usable in human prostate cancer.

BODY

In our original Statement of Work, we described three major tasks: 1) to analyze human tumors and tumor cell lines for the loss of SSeCKS expression or for mutations in the SSeCKS gene, 2) develop diagnostic antibodies for SSeCKS based on cancer-specific phosphopeptides and 3) develop *in vivo* models for SSeCKS-induced tumor suppression of metastatic prostate cancer cells. The following will be a description of our lab's progress in regards to these tasks.

As part of Task 1, we have collected DNA, RNA and protein samples from many human prostate cancer cell lines and primary tumors with the collaboration of Dr. Joel Nelson (now Chairman of Urology, University of Pittsburgh School of Medicine) and members of the Mount Sinai Prostate Cancer Study Group (MSPCSG) These include cell lines, LNCaP (grown with or without androgens), PC-3, PPC-1, DU-143 and TSU, and 6-10 samples each from benign prostatic hyperplasia, localized prostatic carcinoma and bony metastases. The MSPCSG has now hired a special surgical pathologist to procure, characterize and store prostate cancer tissues, which will be available to this study. We have also procured a panel of human prostate cancer cells from Joy Ware consisting of P69, (an HPV-immortilized normal prostate epithelial line), M12 (derived from P69 but only producing localized tumors) and M2182 (derived from M12 and producing local and metastatic tumors).

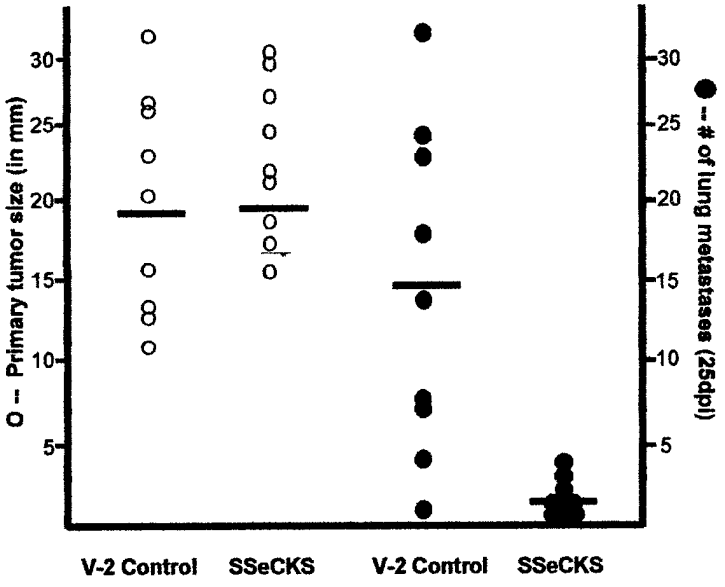
We found a severe loss of SSeCKS RNA in human prostate cancer cell lines compared with RNA from normal human prostate. We first confirmed that the level of SSeCKS RNA and protein increases in confluent untransformed epithelial cells (as we showed before with fibroblasts). Moreover, there was a progressive decrease in steady-state SSeCKS RNA level corresponding with the gain of *in vivo* tumorigenicity (M12 cells) or metastatic potential (M2182). Downregulation at the RNA level was reflected in parallel at the protein level indicating that the major point of regulation is probably the level of transcriptional control. Although are preliminary study size was small, we showed similar results for the loss of SSeCKS protein in undifferentiated, intraprostatic carcinomas compared well-differentiated carcinomas, BPH lesions or normal prostate epithelial cells.

Lastly, we identified a novel proteolytic 80kDa product which is present in the prostate cancer cells only and who relative abundance increases when the cancer cells are grown at high density. Mixing lysates from untransformed and cancer cells leads to the loss of this isoform, but this loss can be blocked if the lysate from untransformed cells is first boiled. Thus, it is likely that the untransformed cells produce a heat-labile protease that normally degrades the 80kDa isoform. We speculate that this is an additional mechanism for some cancer cells to downregulate SSeCKS functions.

Another part of Task 1 was to sequence human SSeCKS genomic clones and produce promoter and intron/exon maps, which we have accomplished fully. Additionally, we have produced reporter constructs (luciferase and CAT) using nested promoter fragments, and currently, we are analyzing how their expression is influenced by mitogens, neuropeptides and *src*-induced transformation. As part of the mapping analysis, we have procured PAC and BAC clones that encode all exon and promoter regions of human SSeCKS. These clones map to human chromosome 6q24-25.2, a well-known deletion hotspot in advanced prostate, breast and ovarian cancer. In conjunction with our sequencing and the data provided by the Sanger Sequencing Centre (Cambridge, UK), we have defined all of the human SSeCKS exon/intron boundaries. We have also produced several PCR primer sets which will allow us to detect gene losses.

In regards to Task 2, we have produced monoclonal antibodies that recognize two phosphopeptides, P-Ser^{300} and P-Ser^{515}, which represent two of the four major PKC phosphorylation sites on SSeCKS. These hybridomas, 65C10 and 5A3, respectively, recognize only the phosphorylated peptides and not the apopeptides or other serine-phosphorylated peptide sequences. Currently, these antibodies work well in ELISA reactions, but we are endeavoring to optimize their reactivity in western blotting, immunofluorescence and immunocytochemistry analyses. Our preliminary data indicate that denaturing fixatives such as those containing 60% acetone can successfully uncover the epitopes recognized by 65C10 and 5A3.

In regards to Task 3, we have produced LNCaP cells with tetracycline-regulated SSeCKS expression to complement our previously analyzed rat MatLyLu prostate cancer cells with tet-controlled SSeCKS expression. We have also inserted both the rat and mouse SSeCKS cDNAs into adenovirus vectors and are producing infectious virus stocks for intratumoral injections. We have begun to analyze the ability of tetracycline-inducible SSeCKS to suppress tumor and metastasis formation by MatLyLu cells in nude mice. Specifically, although SSeCKS decreases the initial growth of s.c. tumors at the primary injection site, these tumors eventually attain the same growth kinetics as control tumors. However, following SSeCKS re-expression, there was a significant decrease in the number of lung metastases (figure below). We showed that the decrease in metastatic potential is unlikely due to SSeCKS-induced changes in motility because the SSeCKS re-expressors were as motile as control cells in monolayer wounding assays. Thus, SSeCKS may function as a suppressor of the metastatic phenotype.



CONCLUSIONS

Our data show a strong correlation at the RNA and protein level for loss of SSeCKS in prostate cancer cell lines. We are endeavoring to analyze a larger number of primary prostate cancer samples in the current year to strengthen the correlation between loss of SSeCKS and advanced prostate cancer. Most importantly, though, our *in vivo* nude mouse model has borne strong data indicating that SSeCKS plays a role in limiting the metastatic spread of prostate cancer

Suppression of Tumorigenic Potential in MatLyLu Prostate Cancer Cells Following the Forced Re-expression of the *Src*-Suppressed C Kinase Substrate, SSeCKS

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ABSTRACT

The molecular mechanisms leading to prostate cancer remain poorly understood, especially how the chronic, malignant form within the prostate converts to a metastatic form. SSeCKS, initially isolated as a negative mitogenic regulator, encodes tumor suppressor activity and is likely the rodent orthologue of human Gravin, an autoantigen in myasthenia gravis that encodes kinase scaffolding activity. Based on our previous mapping of Gravin to 6q24-25.2, a hotspot for deletion in advanced prostate, breast and ovarian cancer, we investigated the possible role of SSeCKS/Gravin in prostate cancer. SSeCKS/Gravin protein was detected in untransformed rat and human prostate epithelial cell lines EP12 and PZ-HPV-7, respectively, as well as in the prostatic epithelium, especially in basal epithelial cells. In contrast, SSeCKS/Gravin protein and RNA levels were severely reduced in panels of human (PC-3, PPC-1, LNCaP, DU145, TSU) and rat Dunning (AT3.1, MatLyLu) prostate cancer cell lines compared to controls. Loss of SSeCKS/Gravin expression correlated with the ability to induce extraprostatic tumor formation and lung metastases in both nude mouse and syngeneic rat models. The forced re-expression of SSeCKS in MatLyLu cells using a tetracycline-regulated vector system caused cell flattening, the formation of filopodia- and lamellipodia-like projections, and a decrease in anchorage-independent growth. In nude mice, SSeCKS re-expression slightly decreased the tumor growth rate at the primary site but severely decreased the appearance of lung metastases. The notion that SSeCKS functions as a tumor suppressor *in vivo* is supported by the findings that I) MatLyLu tumors that progress at the primary injection site lose tetracycline-regulated SSeCKS re-expression and ii) SSeCKS/Gravin expression is lost in poorly-differentiated human prostate

carcinomas *in situ* compared with benign hyperplastic lesions (BPH) and well-differentiated prostatic carcinomas. Our data strongly suggest that the loss of SSeCKS/Gravin promotes prostate cancer tumorigenicity and metastasis.

INTRODUCTION

Prostatic adenocarcinoma (PA) is the most common non-cutaneous malignancy in men in the U.S., associated with roughly 38,000 deaths in the U.S. in 1994 (1) and 42,000 deaths in 1996 (2). Especially hard hit are African American men whose incidence is highest in the world (2). A critical factor in prostate cancer-related mortality is the metastatic potential of the tumor cells and whether the disease will disseminate to secondary sites such as the femur or pelvis. An even larger number of U.S. men show localized, non-disseminated prostatic cancer with much lower risk of mortality. The high frequency of benign prostatic hyperplasia (BPH) in men is associated with the continuous growth of the prostate with age. However, the link between BPH, non-disseminated PA and malignant PA (MPA) remains unclear.

Much data regarding the molecular biology of prostate cancer development and progression comes from a pedigree of Dunning R-3327 rat prostate cancer lines with varying degrees of tumorigenicity, metastatic potential and androgen-dependency for growth (3;4). For example, the G cancer line is poorly tumorigenic in syngeneic, Copenhagen rats, whereas MatLyLu cancer cells induce intra- and extraprostatic primary-site tumors and lungs metastases; cancer cells derived from the AT-3 lineage are highly tumorigenic and metastatic and also androgen-independent.

The molecular pathogenesis of MPA is a multistep process involving the activation of endogenous oncogenes as well as the loss of tumor suppressor/cancer susceptibility genes. No single oncogene has been associated with MPA, yet at least 40% of cases studied contain activating mutations or increased expression of oncogenes such as *ras*,

myc or *fos* (3;5). Activating *ras* mutations in prostate oncogenesis, though still controversial as a significant etiologic factor, are probably less important in MPA progression than the loss of critical tumor suppressor functions such as p53, DCC and Rb, or cytoskeletal/adhesion molecules such as E-catenin/α-cadherin (6;7). These correspond, respectively, to allelic deletions in portions of chromosomes 5q, 17p, 18q and 13q, found in a small but significant population of MPAs, and deletions in 10q and 6q, found in >60% of MPAs.

Several genes have been identified which may function to suppress prostate cancer development and metastasis (8). These include E-cadherin (which maps to 16q24) which is deleted in over 70% of high grade or metastatic prostate cancers (9), KAI1 (which maps to 11p11.2) whose allele suffers loss of herterozygosity (LOH) in over 30% of metastatic prostate cancer (10; 11), CD44 (which maps close to KAI1 at 11p13) whose loss of expression correlates with metastatic potential in the rat Dunning R-3327 cancer cell series (10), PTEN, a protein tyrosine phosphatase mapping to 10q23 (12) and PCTA-1, a gene recently identified using a surface-epitope masking technique (13). The mechanisms by which these gene products regulate normal prostate cell behavior remain unclear. Moreover, the contribution of these products to early cancer formation versus metastatic disease can be complex as exemplified by the up-regulation of KAI1 in early pancreatic cancer contrasting with its decreased expression in metastases (14). Also, whereas some groups report a frequent allelic loss of the KAI1 gene in advanced prostate cancer in humans (10), others show data of infrequent allelic loss yet transcriptional downregulation in metastases (15).

We identified a potential tumor suppressor, SSeCKS, whose expression is downregulated in *src*- and *ras*-transformed fibroblasts (16). Reexpression of SSeCKS suppresses *src*-induced oncogenic growth characteristics such as growth factor- and anchorage-independence, loss of contact inhibition and metastatic potential by reorganizing actin-based cytoskeletal architecture (17). SSeCKS is likely the rodent orthologue of human Gravin, an autoantigen in myasthenia gravis which has been shown to function as a kinase scaffolding protein. We recently mapped the Gravin gene to 6q24-25.2, a hotspot for deletion in advanced prostate, breast and ovarian cancer in humans (18-20), and showed that SSeCKS/Gravin protein is lost in 12/12 human breast cancer cell lines relative to untransformed breast epithelial cells (21). Here we show that loss of SSeCKS/Gravin RNA and/or protein is typical in human and rat prostate cancer cells capable of tumor formation and metastasis. Moreover, reexpression of SSeCKS in MatLyLu rat prostate cancer cells suppresses cell rounding and colony formation in soft agar and restores contact-inhibited growth. SSeCKS also suppresses *in vivo* tumor formation and metastasis. These data suggest a key role for SSeCKS/Gravin in the growth control of normal prostate epithelial cells.

MATERIALS AND METHODS

Cell culture: MatLyLu (MLL) cells (gift of J. Nelson, Johns Hopkins School of Medicine), EP12 (EPYP-1; a gift of K. Pienta, U. of Michigan Comprehensive Cancer Center), LNCaP/tTA (LNGK9; a gift of T. Powell, Memorial-Sloan Kettering Cancer Center; ref. 22), and HeLa (ATCC #CCL2.1) were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Gaithersburg, MD) supplemented with 10% fetal calf serum (GIBCO). P69 (P69SV40T), M2182 and M12 (gifts of J. Ware, Medical College of Virginia) were grown in RPMI 1640 supplemented with insulin, transferrin and selenium (Collaborative Biochemicals), dexamethasone (Sigma; St. Louis, MO) and epidermal growth factor (Collaborative) as described previously (23; 24).

Production of tetracycline-regulated MLL cell lines: MLL/tTAK cells, expressing a tetracycline (tet)-regulated tTA transactivator (25), were produced by transfecting with $CaPO_4$/DNA precipitates containing 3.5 µg of pTet-tTAK and 0.6 µg of pRSV/*hygro* followed by selection of stable transfectants in 400 µg/ml of hygromycin (Sigma). Individual clones were tested for the ability to induce expression of tet_O/luciferase in the absence of tet following transient transfection with pUHD13-3 (26). Clones #2 and #7 were chosen for secondary transfection with 3.8 µg of pUHD10-3/SSeCKS (27) and 1.6 µg of pBABE/*puro* (28), and stable transfectants were isolated after selection in hygromycin and puromycin (8 µg/ml). All cell were maintained in 5 µg/ml tet during the selection processes, and once clones were identified with low background SSeCKS expression in the presence of tet plus high levels of SSeCKS in the absence of tet, the tet level was dropped to 0.7 µg/ml.

Colony assay in soft agar: 10^4 cells were plated into soft agar in 6 cm plates as

described previously (17) and then grown for 3 weeks at 37°C with weekly media feedings.

Northern and western blotting: Total or poly A-selected RNAs were isolated, electrophoresed, blotted and probed with [^{32}P]-rat SSeCKS cDNA as described (16). RIPA lysates containing 40-100 µg of total protein were prepared and immunoblotted using rabbit polyclonal anti-SSeCKS sera as described (29).

Immunofluorescence and immunohistochemistry analyses: Cells seeded onto 22mm^2 coverslips were fixed at -20°C for 20 min with either ice-cold 60% acetone/3.7% formaldehyde or at room temperature for 10 min with 2% paraformaldehyde/0.1% glutaraldehyde/0.05% Triton X-100 (EM Sciences), and then incubated with immunoaffinity-purified rabbit polyclonal anti-SSeCKS (29) followed by staining with FITC-labeled anti-Rb Ig and TRITC-labeled phalloidin as described previously (27). Slides were viewed on an Olympus IX-70 inverted fluorescent microscope and digitized using a Sony Catseye camera connected to a PowerMac G3 (Apple Computers). Image analysis was performed using Adobe Photoshop 4.01.

Tumor and metastasis formation in nude mice: Six-week-old female nude mice (Taconic Farms, Germantown, NY) were injected *s.c.* with 10^5 MLL/vector or MLL/SSeCKS clones. The viability of the injected cells was greater than 90% as determined by the trypan blue exclusion method. All mice were fed water containing 100 µg/ml tet plus 5% sucrose. until the primary tumors were palpable (2-4mm), at which point, tet was withdrawn from the drinking water. The mice were sacrificed 3 weeks after injection. The primary tumors were measured and weighed, and the lungs were stained for metastases by injecting India ink (30 ml ink plus 4 drops of 1 M ammonium hydroxide, diluted into 200 ml of dH_2O) into the trachea for 10 min at room temperature followed by several PBS washes. Metastases,

which exclude dye, were counted.

RESULTS

Loss of SSeCKS/Gravin expression in human and rat prostate tumor cell lines. In order to determine whether the loss of SSeCKS/Gravin expression correlates with prostate oncogenesis, northern blots were prepared containing total or poly A-selected RNA from Dunning rat prostate cancer cell lines or tumors (grown in syngeneic, Copenhagen rats) or from human prostate cancer cell lines, and then probed under stringent conditions with rat SSeCKS cDNA probe. Fig. 1A shows a progressive loss of SSeCKS RNA signal in MatLyLu (MLL) and AT-3.1 cancer cells compared to the mildly transformed G cell line. Specifically, the SSeCKS transcript levels in MLL and AT-3.1 are 5- and >20-fold lower, respectively, than in G cells. Although MLL cells are highly tumorigenic and metastatic, AT-3.1 cells are slightly more metastatic in nude mice and are also androgen-independent (3). The relative levels of SSeCKS RNA in H-, MatLu-, MLL- or AT-3-induced tumors are >5-fold reduced compared to levels in the G cell line. Fig. 1B shows a similar reduction in SSeCKS/Gravin RNA levels in various human prostate cancer cell lines compared to levels in normal human prostate. Growth of LNCaP cells, which are androgen-responsive, in the absence of androgens for >9 months had no effect on SSeCKS/Gravin transcript levels, possibly due to the 6q24-ter deletion reported for the parental LNCaP cells (30), a region which encodes the Gravin gene (21). These data strongly suggest that loss of SSeCKS/Gravin contributes to prostate oncogenesis.

We then compared the relative levels of SSeCKS/Gravin proteins in various prostate cancer cell lines. The P69 series developed by J. Ware (24) consists of a set

of human prostate epithelial cells with increasing oncogenic characteristics in nude mice: P69 (also known as P69SV40T) are normal human prostate epithelial cells immortalized with SV40 T_{ag} that are not tumorigenic; M2182 are non-metastatic variants of P69 that form tumors at the primary subcutaneous injection site; M12 are variants that form lung metastases following intraperitoneal or intraprostatic injection. Fig. 1C shows a severe downregulation (>10-fold) in the levels of the 305/287kDa Gravin isoforms in both M2182 and M12 cells compared with the parental P69 cells. Thus, SSeCKS downregulation correlates with *in situ* tumor growth and not necessarily with metastatic potential alone. Nonetheless, the level of Gravin is significantly higher in M2182 or M12 than in LNCaP cells, where it is below the level of detection (possibly due to homozygous deletion).

Our previous data indicated that the level of SSeCKS RNA and protein increases dramatically under contact-inhibited growth conditions (16; 29; 31). Fig. 1C shows a similar increase in SSeCKS protein levels in increasingly confluent cultures of immortalized EP12 cells. In contrast, SSeCKS levels in MLL cells, which are decreased 3-4 fold in comparison to EP12 cells, are not affected by culture density. However, as shown in Fig. 1D, the abundance of the 240kDa SSeCKS isoform as well as a novel ~80kDa isoform increases following growth at high density. Our previous data suggest that the 240kDa isoform (250kDa in human cells) is a proteolytic cleavage product lacking N-terminal sequences (32). Thus, the current data indicate that i) SSeCKS/Gravin protein levels are induced in epithelial cells as well as fibroblasts in response to increased culture density, ii) this induction mechanism is inactive in prostate

cancer cells, and iii) that generation of SSeCKS proteolytic fragments increases in MLL cells in response to culture density.

Tetracycline-regulated SSeCKS re-expression suppresses MLL-induced oncogenic growth *in vitro*. We previously demonstrated that the re-expression of SSeCKS using a tet-regulated vector system resulted in an inhibition of *src*-induced oncogenic growth characteristics in fibroblasts (17). Because MLL cells encode at least one activated Ha-*ras* allele (4), we addressed whether SSeCKS could suppress *ras*-associated tumorigenic growth by producing MLL cells with tet-regulated SSeCKS expression. Fig. 2 shows similar levels of SSeCKS protein in the MLL/tTAK cells compared to the parental MLL cells. The MLL/tTAK cells express a tet-regulated form of the tTA transactivator (25) which we found to be much less toxic than the constitutively-expressed tTA in most tet-regulated systems. As shown on the left side of Fig. 2, expression of the 290kDa isoform of SSeCKS could be induced 4-20 fold in several independently derived clones grown in the absence of tet. These clones also express background levels of SSeCKS in the presence of tet. Lastly, the ~80kDa isoform found in MLL cells but absent in the mildly transformed EP12 cells was present in the MLL/tTAK cells and in all the MLL[tet/SSeCKS] clones grown in the presence of tet. In contrast, SSeCKS expression following tet removal correlated with a 2-10 fold decrease in the abundance of this isoform. As the data presented below show a correlation between increased levels of SSeCKS and decreased oncogenicity, the current data strongly suggest that the ~80kDa isoform is a marker for prostate oncogenesis.

We investigated whether the ~80kDa isoform was possibly produced by a factor either present or absent in prostate tumor versus untransformed cells. NP-40 lysates from MLL and EP12 cells were mixed at a 1:1 ratio to determine if factors from one lysate would decrease the presence of the ~80kDa isoform. Table shows that incubation of the MLL lysate with EP12 lysate at 30°C for 5 min caused a decrease in the abundance of the ~80kDa isoform (compared to incubation of the MLL lysate alone). Pre-boiling of the MLL lysate did not effect the EP12-induced decrease in the abundance of the ~80kDa isoform, however boiling of the EP12 lysate inhibited the EP12-induced decrease. This indicates that EP12 encodes a heat-labile factor that is antagonistic to the production or stability of the ~80kDa isoform.

Fig. 3 shows that SSeCKS re-expression induces cell flattening, decreased refractility, increased contact-inhibited growth and increased cell-cell interaction following removal of tet from the MLL[tet/SSeCKS] clones. Interestingly, SSeCKS expression induced a fibroblast-like cell morphology rather than the cuboidal/columnar epithelial morphology typified by EP12 cells (compare Fig. 4Bg and 4Cb to 4Bi). Expression of the tTA transactivator did cause mild cell flattening (compare Fig. 4Bd to 4Be), however, this was much less than the flattening induced by SSeCKS (examples: Fig. 4Bg and 4Cb).

In order to analyze the cellular compartmentalization of SSeCKS in control and overexpressor cells, cells were grown on sterilized coverslips, fixed and then subjected to immunofluorescence analysis using immunoaffinity-purified anti-SSeCKS antibody (29). Fig. 4A shows enrichment of SSeCKS in the perinuclear regions of EP12 cells as well as a cortical cytoskeletal distribution within the cytoplasm. Most significantly,

SSeCKS was enriched in actin-dense membrane ruffles (Fig. 4Aa/a') as well as in focal complexes connected to actin stress fibers (Fig. 4Ab/b'). In the overexpressor clones, SSeCKS induces lamellipodia- and filopodia-like projections (Fig. 4Ba-c) with enrichments of SSeCKS in membrane ruffles (Fig. 4Ba-c; Fig. 4Bg) and at the ends of filopodia (Fig. 4Cb). In cells fixed with formaldehyde/glutaraldehyde/Triton X-100 to help retain cytoskeletal filamentation (Fig. 4Cc), the nature of the SSeCKS-associated intermediate/cortical cytoskeletal infrastructure is appreciated even more. These effects are strikingly similar to those induced in NIH3T3 fibroblasts following SSeCKS expression (17; 27), suggesting a conserved set of functions for SSeCKS in the control of cytoskeletal architecture.

We then addressed whether SSeCKS expression affected parameters of *in vitro* and *in vivo* oncogenic growth in the MLL system. Fig. 5 shows that expression of the tTA in vector control cells (V-2 or V-7) inhibited proliferation 40-50% as we and others previously described (17; 27; 33), probably due to squelching of transcription factors by the VP16 moiety of the tTA. With the exception of clone 2-6, SSeCKS expression induced slightly slower proliferation in the remaining clones compared to control cells expressing tTA alone. SSeCKS also induced increased contact-inhibited growth based represented by lower saturation densities. It is unclear, however, whether the lower saturation densities are a direct effect of SSeCKS or indirect effect resulting from the increased cell flattening. These data indicate that SSeCKS induces a slight but significant decrease in proliferation rate and saturation density.

Fig. 6 shows that SSeCKS expression inhibits anchorage-independent growth 2-3 fold over that induced by the tTA alone. Moreover, the inhibition of colony forming ability

cannot be due to decreased proliferation induced by SSeCKS expression. For example, clone 2-6 shows little loss of proliferative potential after SSeCKS expression (Fig. 5) yet is severely deficient in anchorage-independent growth (Fig. 6). This indicates that SSeCKS can inhibit *ras*-mediated anchorage-independent growth in an epithelial cell system, which agrees with our previous findings in a fibroblast system that SSeCKS inhibits *src*-induced anchorage-independent growth (17).

Suppression of *in vivo* MLL tumor growth and metastasis by SSeCKS. Because MLL cells are capable of rapid tumor formation and metastasis to the lung in nude mice (3), we addressed whether SSeCKS expression could inhibit either the growth of primary subcutaneous tumors or the generation of secondary lung metastases. Athymic *nu/nu* mice were injected in their flanks with 10^5 cells *s.c.* and then maintained on tet in their drinking water until tumors were palpable (2-3mm in diameter), whereupon tet was removed from their water source. Fig. 7A shows that SSeCKS expression mildly inhibited tumor growth at the primary site in comparison to MLL containing vector alone (V-2). The most significant suppression of tumor growth was 5-8 days after initial tumor palpation. However, analysis of the primary tumors generated by the MLL[tet/SSeCKS] cells (Fig. 8) revealed an overall loss of tet-regulated SSeCKS overexpression, suggesting that the majority of the proliferating tumor cells were variants that lacked ectopically-expressed SSeCKS. Indeed, *in vitro* growth of cells from these primary tumors (in hygromycin/puromycin selection medium) showed a lack of inducible SSeCKS following growth without tet (data not shown). In contrast, the mice receiving the MLL[tet/SSeCKS] cells contained far fewer lung metastases three weeks

after primary tumor cell injection compared to vector controls (Fig. 7B). These data indicate a role for SSeCKS in *in vivo* tumor suppression, both at the level of primary tumor and metastasis formation.

Our previous data indicated that overexpression of SSeCKS in NIH3T3 cells severely decreased motility probably due to the severe cell flattening and cytoskeletal reorganization (27). However, re-expression of SSeCKS in *src*-transformed fibroblasts did not inhibit cell motility (17), suggesting that SSeCKS selectively inhibits *src*-induced oncogenic pathways but not those governing cell motility. Thus, we investigated whether the SSeCKS-induced decrease in MLL metastasis could be attributed to a decrease in cell motility. Indeed, Fig. 9 shows the opposite: SSeCKS expressing MLL cells displayed slightly increased motility in a monolayer wounding assay compared to either MLL[tet/SSeCKS] grown in the presence of tet or MLL[tet/vector] cells grown either in the absence or presence of tet. This clearly indicates that SSeCKS promotes *in vitro* motility and suggests that the loss of *in vivo* metastatic potential is due to loss of invasive growth parameters.

Loss of SSeCKS/Gravin expression in well-differentiated human prostate cancer. The chromosomal region encoding the putative human SSeCKS orthologue, Gravin, was mapped to 6q24-25.2 by our lab previously (21). This region is deleted in a significant proportion of advanced human prostate cancers using analyses such as fluorescence *in situ* hybridization (FISH), loss of heterozygosity (LOH) at polymorphic microsatellite loci, and standard cytogenetics (GTG-banding) (8; 20; 34-36). Because our polyclonal rabbit anti-rat SSeCKS serum recognizes all Gravin isoforms from human

and monkey cells (32), we analyzed various human prostate tissues and tumors by immunocytochemistry for SSeCKS/Gravin expression. Fig. 10a shows extensive SSeCKS/Gravin staining in prostatic epithelial cells, especially the basal epithelial cells, although cell surface staining is detected in some columnar epithelial cells. Abundant SSeCKS/Gravin staining is also detected in benign prostatic hyperplastic lesions (Fig. 10b) as well as in well-differentiated carcinomas *in situ* (Fig. 10c). In contrast, most SSeCKS/Gravin staining is lost in undifferentiated carcinomas (Fig. 10d) which typically are associated with metastatic disease. Non-cancerous ducts in the same prostate contain basal epithelia with robust SSeCKS/Gravin staining indicating a selective loss of expression in the advanced carcinoma. These data suggest a correlation between loss of SSeCKS/Gravin and the onset of aggressive prostate tumorigenesis.

DISCUSSION

We present evidence that the loss of expression of the SSeCKS/Gravin gene family correlates with increased tumorigenic behavior of prostate cancer in both rodent and human systems. The downregulation of SSeCKS/Gravin agrees with previous results from several groups showing deletions of the 6q23-27 region in many advanced human prostate cancers *in situ* and in cancer cell lines such LNCaP (20;30;34-36). We also show preliminary evidence of a possible selective loss of SSeCKS/Gravin expression in advanced, undifferentiated human prostate cancer *in situ*. Lastly, evidence is presented in the current study that the re-expression of SSeCKS suppresses parameters of *in vitro* and *in vivo* rat prostate cancer growth. These data strongly suggest that the loss of SSeCKS/Gravin contributes to the progression of prostate cancer and that the SSeCKS/Gravin family encode tumor suppressor activity.

Arguments have been put forward that tumor suppressor genes can be either mutated (type 1) or transcriptionally downregulated (type 2) in human cancers (37). Examples of type 1 tumor suppressors include p53, Rb and Mxil. In contrast, whereas $p16^{INK4a}$ is often deleted or mutated in cancer cell lines, it is rarely mutated in human cancer *in vivo*, yet its expression is often lost by transcriptional silencing mechanisms such as promoter hypermethylation (38; 39). Nonetheless, if a candidate type 2 tumor suppressor gene is deleted or mutated in at least some types of human cancer, this strengthens confidence in its tumor suppressor function. Our results show loss of SSeCKS/Gravin at both the RNA and protein levels, yet the data by others of the deletion of the Gravin gene region in advanced prostate cancer and in LNCaP cells

strengthens our belief that SSeCKS/Gravin are *bona fide* tumor suppressors. The current study using a rodent epithelial cell system adds to rodent fibroblast and human epithelial (MCF-7) cell systems (17;21) in which SSeCKS shows tumor suppressive activity.

Although SSeCKS/Gravin expression is severely decreased in the tumorigenic variants of P69, these cells show no loss of chromosome 6q (24), indicating that the mechanism is most likely transcriptional downregulation. However, unlike human prostate cancer which typically metastasizes to the spine, pelvis, femur and ribs, the metastatic M12 cells were selected for metastasis to the lung. This underlines the dearth of nude mouse/prostate cancer cell models that focus on boney metastases, an exception being human PC-3 prostate cancer cells (40) although metastasis in this model is not limited to the bone. Our results, however, indicate that downregulation of SSeCKS/Gravin precedes the onset of metastatic potential.

The ectopic expression of SSeCKS in MLL cancer cells lines leads to cell flattening, reorganization of actin-based cytoskeletal architecture and the production of filopodia- and lamellipodia-like projections. These findings are strikingly similar to those induced by SSeCKS in *src*-transformed NIH3T3 fibroblasts (17). SSeCKS also decreases MLL cell proliferation, saturation density and contact- and anchorage-independent growth. The association of SSeCKS with cortical, actin-based cytoskeletal structures (27) leads us to believe that the tumor suppressive activity of SSeCKS is a direct results of its ability to reorganize cytoskeletal architecture. Interestingly, tumor cells often have normal steady-state levels of major cytoskeletal proteins such as actin and tubulin yet have increased turnover of their polymerized, filamentous forms

compared to untransformed cells (41). In contrast, cancer cells have decreased levels of minor or intermediate cytoskeletal proteins such as vinculin, α-actinin, tropomyosin or cytokeratins (42; 43). Many groups have shown that re-expression of any one of these latter proteins is sufficient to induce a cytoskeletal reorganization that stabilizes microtubule and microfilament structures. SSeCKS seems to function in a similar manner. We postulate that cancer cells are capable of elaborating cytoskeletal ultrastructure although the turnover is more rapid than in untransformed cells. Thus, re-expression of a single downregulated cytoskeletal protein may be sufficient to decrease turnover rates and increase cytoskeletal stability to that in untransformed cells.

The significance of cytoskeletal reorganization in tumor suppression may be explained by the work of Yamada, Ingber and others (44-47) whose data suggests that the activity of proliferative signaling mediators can be modulated by recruitment to specific activation sites such focal adhesion complex, and that these translocations are regulated by cytoskeletal structures such as actin stress fibers.

We also noted that SSeCKS expression induced increased cell-cell adhesion in most of the MLL[tet/SSeCKS] clones. MLL cells are known to have downregulated E-cadherin (48; 49), possibly due to deletion of both alleles (50). We could not detect E-cadherin or α-catenin levels in the MLL[tet/SSeCKS] clones even after ectopic SSeCKS expression; β-catenin levels, which are detectable in the parental MLL cells, did not change following SSeCKS expression (data not shown). Moreover, a pan-cadherin polyclonal antibody (Sigma) which recognizes a conserved region in the cytoplasmic regions of E-, EP-, N-, P, L- and V-cadherins (51) failed to show any increase in cadherin levels after SSeCKS expression. This suggests that the cell-cell adhesion may

be facilitated by one or several of the so-called atypical cadherins (e.g.- cadherin-11) or by non-cadherin moieties such as selectins or integrins. The latter notion is strengthened by data that MLL cells show increased levels of β_1 and α_5 integrins as well as fibronectin (49) which might antagonize mechanisms of normal cell-cell adherence.

Using our polyclonal anti-SSeCKS antibody, an ~80kDa band was detected in tumorigenic human and rat prostate cancer cells (e.g.- MLL, M2182, and M12) but not their untransformed control lines (P69 and EP12). Moreover, reexpression of SSeCKS in the MLL[tet/SSeCKS] clones caused this species to disappear. The abundance of this species increases in MLL cells plated at high densities (i.e.- more contact-independent growth) suggesting that tumor cell crowding similar to focus formation induces production of this species. We postulate that untransformed prostate epithelia express a heat-labile factor which either degrades the ~80kDa isoform or inhibits a tumor-specific SSeCKS protease that generates the ~80kDa isoform. We cannot exclude, however, that the ~80kDa isoform is generated by alternative splicing or by selective usage of internal ATG translational start sites. It is also unlikely that the putative, tumor-specific protease is a known caspase because SSeCKS lacks any of the 3 caspase recognition motifs ($^{W}/_{L}$EHD, $^{I}/_{V}/_{L}$EEXD, or DEXD; ref. 52).

SSeCKS inhibits MLL-induced tumorigenesis *in vivo* inasmuch as growth of primary site tumors in nude mice requires the loss of ectopic SSeCKS expression. More significantly, SSeCKS inhibits the generation of lung metastases in our model. The decrease in metastatic potential cannot be explained by a concomitant loss in motility since we show that SSeCKS promotes *in vitro* cell motility. This agrees with our previous data that SSeCKS could inhibit *src*-induced invasiveness without inhibiting

motility (17), but contrasts with our other data that SSeCKS overexpression in untransformed NIH3T3 inhibits motility (23). These data, though, are not contradictory because SSeCKS overexpression in untransformed cells induces severe cell flattening whereas SSeCKS expression in the *src*-transformed fibroblasts or in the H-*ras*-expressing MLL cells (this study) reverts cells to a relative "normal" shape and cytoskeleton. Additionally, SSeCKS may be capable of modulating oncogenic pathways such as those controlling invasive growth but not capable of affecting motility pathways.

The ease at which the MLL cells override the tet-regulated SSeCKS expression *in vivo* probably belies the plasticity of these tumor cells. Nonetheless, our results suggest that either the sustained expression of SSeCKS or the repeated dosage of SSeCKS in prostate tumors would decrease primary tumor growth and metastatic potential.

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TABLE 1

Effect of lysate mixing on the generation of the ~80kDa SSeCKS isoform

Reactants[a]		Presence of 80 kDa band	Length of Incubation
MLL lysate	+ EP12 lysate	-	5 min
MLL lysate (boiled)	+ EP12 lysate	-	5 min
MLL lysate	+ EP12 lysate (boiled)	+	>30 min

[a]-- MLL and EP12 lysates were added at a 1:1 ratio.

FIGURE LEGENDS

Figure 1. Loss of SSeCKS/Gravin expression in prostate cancer. (**A**) Northern blot analysis of total RNA (25 µg/lane) from Dunning rat prostate cancer cell lines grown in culture or in syngeneic Copenhagen rats, probed with [^{32}P]-rat SSeCKS cDNA. The 28S rRNA species are shown below as a loading control. (**B**) Northern blot analysis of poly A$^+$ RNA (2 µg/lane) from human prostate cancer cell lines or total normal human prostate, probed with [^{32}P]-rat SSeCKS cDNA. "LNCaP - androgen" signifies two independent LNCaP cells lines grown continuously in the absence of androgens for at least 9 months. (**C**) Western blot of protein from various rodent or human prostate cell lines, probed with rabbit polyclonal anti-SSeCKS (rat) serum. Note the loss of SSeCKS/Gravin protein in the tumorigenic M2182 and metastatic variants of the SV40-immortalized but non-transformed P69 human epithelial line. A similar decrease is apparent when comparing HPV-immortalized rat EP12 prostate epithelial cells (slightly transformed) to MLL cells grown in cultures of either low (L), medium (M) or high (H) confluency. The SSeCKS isoforms are identified by arrows relative to protein molecular weight markers (290/280kDa = doublet arrow; 240kDa = triangle) (**D**) Western blot analysis probed with rabbit polyclonal anti-SSeCKS (rat) serum showing that growth of MLL cells at high culture density does not alter the relative abundance of the 290/280kDa SSeCKS isoforms but increases the abundance of the ~80kDa isoform.

Figure 2. Production of MLL cell lines with tet-regulated SSeCKS expression. As described in Materials and Methods, MLL cells stably expressing a tet-regulated tTA

transactivator (tTAK) were first produced and tested for their ability to induce tet_o/luciferase expression in the absence of tet (left side). These clones were then stably transfected with a tet_o/SSeCKS construct, and clones were isolated that showed low background SSeCKS expression in the presence of tet (compare +tet conditions in the 2-6, 2-7, 7-1, and 7-2 clones to the parental MLL cells) as well as inducible expression of the 290kDa isoform in the absence of tet (right side). The 290 and ~80kDA SSeCKS isoforms are identified by arrows (right) relative to protein markers (left).

Figure 3. Reexpression of SSeCKS suppresses morphological transformation and increases cell-cell adhesion. Phase contrast of MLL[tet/Vector] or MLL[tet/SSeCKS] clones grown in the presence or absence of tetracycline. Note the decreased refractility and increased cell flattening in the MLL[tet/SSeCKS] clones grown in the absence of tet. All magnifications are 100X.

Figure 4. Immunofluorescence analysis of SSeCKS and F-actin staining before and after SSeCKS expression. (**A**) Subconfluent cultures of EP12 cells plated onto 22 mm^2 coverslips were fixed in acetone/formaldehyde and stained for SSeCKS (a,b) or F-actin (a'/b') as described in Materials and Methods. Note the cortical cytoskeletal SSeCKS staining marked by enrichments in the perinuclear region, in membrane ruffles (panels a,b; arrows) and in focal contacts (triangle). (**B**) Ectopic SSeCKS expression induces cell flattening and production of lamellipodia- and filopodia- like projections. Compared

to the parental MLL cells grown in the absence of tet (d) or MLL[tet/Vector] cells grown in the presence of tet (f), MLL[tet/Vector] cells grown in the absence of tet show mild cell flattening (e,h). In contrast, growth of MLL[tet/SSeCKS] clones in the absence of tet (a, b, c, g) causes more severe cell flattening and production of lamellipodia- and filopodia-like projections. Enrichments of SSeCKS are found at the leading edge of lamellipodia (c; arrows), in membrane ruffles (a, b, g; arrows) and in focal adhesion complexes (a, g; triangles). A confluent culture of EP12 (l), showing typical cell-cell adhesion and epithelial morphology, is shown for comparison. Note the decreased level of SSeCKS staining in the parental or control cells (d, e, f, h) compared to the MLL[tet/SSeCKS] clones. © MLL[tet/SSeCKS] clone 7-2 grown without tet (b), showing exaggerated filopodia-like projections with enrichments of SSeCKS at their termini (arrows). Growth of these cells with tet results in the typical MLL transformed morphology (a). MLL[tet/SSeCKS] cells (clone 7-2, in this case) grown without tet and then fixed in paraformaldehyde/glutaraldehyde/Triton (Materials and Methods) reveals the cortical, trabecular morphology of the SSeCKS-associated cytoskeleton. Size bar for all panels = 20 μm.

Figure 5. Ectopic SSeCKS decreases proliferation rate and saturation density of MLL in 10% serum. MLL[tet/Vector] control (V-2, V-7) or MLL[tet/SSeCKS] clones were grown in media containing 10% calf serum plus (solid lines) or minus tet (broken lines) and then monitored for proliferation. Growth of the control clones in the absence of tet results in a roughly 40% reduction in proliferation rate and saturation density (compare , probably due to toxic effects of the tTA transactivator). In comparison, the

MLL[tet/SSeCKS] clones grown in the absence of tet showed a 10-40% reduction in proliferation rate and saturation density over that induced by the tTA alone. All experiments were performed in triplicate, and variations at any time point for a given sample were less than 15%.

Figure 6. Ectopic SSeCKS expression decreases MLL-induced anchorage-independent growth. 10^4 MLL[tet/Vector] or MLL[tet/SSeCKS] cells were grown in soft agar overlays for three weeks as described in Materials and Methods at which point the number of colonies formed was determined. In the absence of tet, the control cells formed 50-60% fewer colonies than if grown in the presence of tet. This most likely reflects the tTA-mediated inhibition of proliferation shown in Fig. 5. In contrast, the MLL[tet/SSeCKS] clones produced 7-10 fold fewer colonies than when grown in the presence of tet, indicating a 3-5 fold overall suppression of colony-forming activity by SSeCKS over controls.

Figure 7. Effect of ectopic SSeCKS expression on tumor growth in nude mice. Athymic *nu/nu* mice (6/experiment) were injected *s.c.* with 10^5 MLL[tet/Vector] or MLL[tet/SSeCKS] cells. All mice were maintained on water sources supplemented with 100 µg/ml tetracycline and 5% sucrose (changed every 2 days). After 5-7 days, primary tumors were first palpable (2-3 mm), at which point half the mice were switched to a water source lacking tet (-tet) whereas as the others continued on the tet water source (+tet). The average sizes of tumors at primary injection sites are shown and the

error bars represent the range of tumor sizes in a given cohort. The X axis represents the days following initial tumor palpation.

Figure 8. *In vivo* tumor growth selects for variants which lose tet-regulated SSeCKS expression. Tumors from mice in the experiment in Fig. 7 were excised roughly 2.5 weeks after injection and analyzed by western blotting for SSeCKS as described in Fig. 1. Note the loss of tet-regulated SSeCKS expression in MLL[tet/SSeCKS] clone 2-6 from mice initially fed tet (until tumors were palpable) and then fed water lacking tet (+/-), compared to 2-6 cells grown in culture. The level of SSeCKS in the MLL[tet/SSeCKS]-derived primary tumor was similar to that of the MLL[tet/Vector]-derived tumor, indicating that the MLL[tet/SSeCKS] contained variants that lost tet-regulated SSeCKS expression.

Figure 9. SSeCKS re-expression promotes cell motility in wounding assays. MLL[tet/SSeCKS] or MLL[tet/vector] cells plated at confluency were subjected to wounding with sterile plastic micropipette tip (0 time) and then cell migration was followed for 6 and 18 h into a marked area on the monolayer wound. Note that the MLL cells grown in the presence of tet show a highly transformed morphology (refractility in phase contrast microscopy) yet low motility. In contrast, SSeCKS re-expression promotes motility over that induced by the tTA alone. Magnification = 100X.

Figure 10. Loss of SSeCKS/Gravin in advanced human prostate cancer *in situ*. Formalin-

fixed sections of human prostate representing normal tissue (a), benign prostatic hyperplasia (b), well differentiated prostate cancer (c) and advanced, undifferentiated prostate cancer *in situ* (d) were processed and stained by immunohistochemistry for SSeCKS/Gravin protein as described in Materials and Methods. Note the loss of the SSeCKS/Gravin signal in the undifferentiated prostate cancer compared to normal prostatic ducts, which show cytoplasmic staining in basal epithelia and cell surface staining in most of the columnar epithelia.

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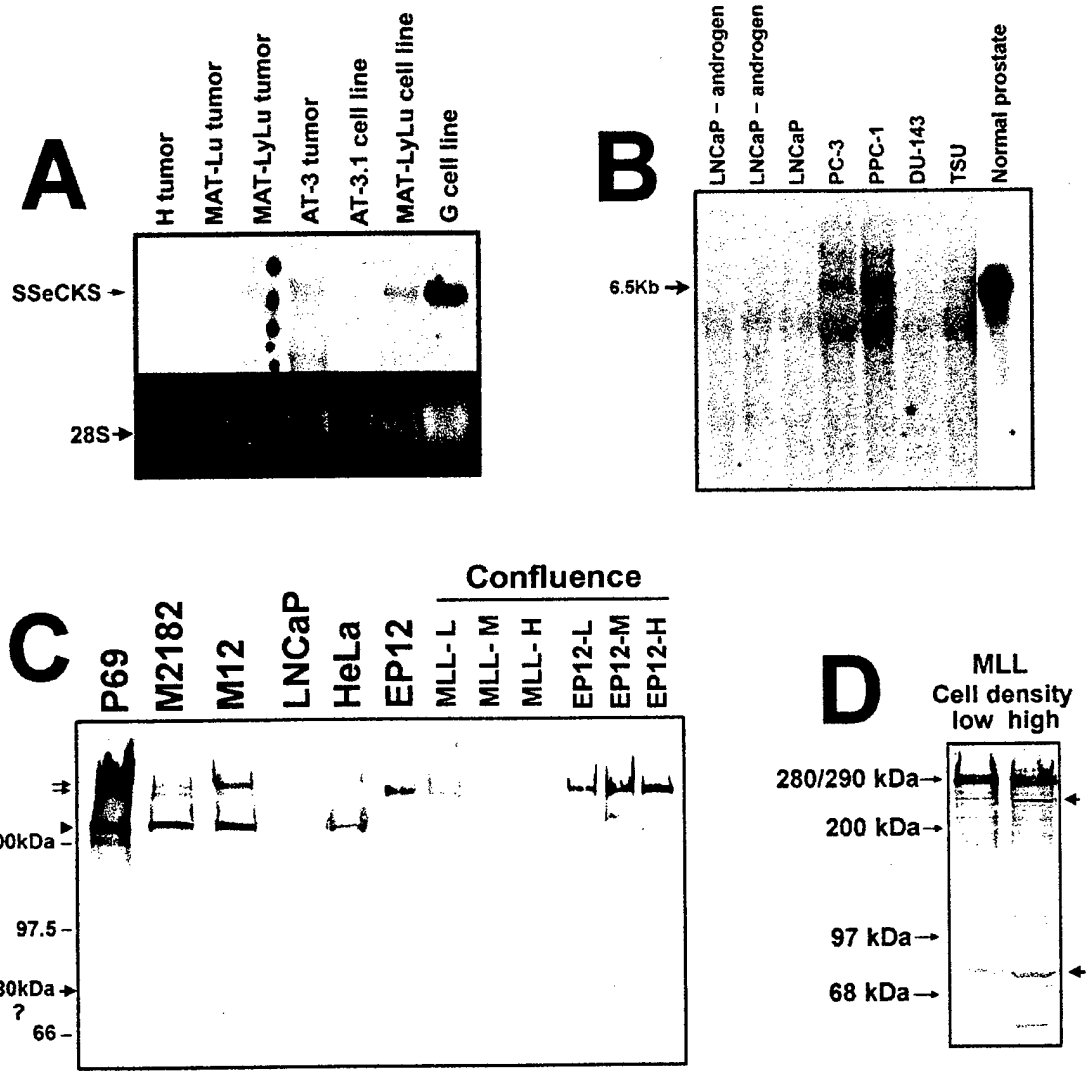
A
H tumor
MAT-Lu tumor
MAT-LyLu tumor
AT-3 tumor
AT-3.1 cell line
MAT-LyLu cell line
G cell line
SSeCKS
28S
B
LNCaP – androgen
LNCaP – androgen
LNCaP
PC-3
PPC-1
DU-143
TSU
Normal prostate
6.5Kb
C
Confluence
P69
M2182
M12
LNCaP
HeLa
EP12
MLL- L
MLL- M
MLL- H
EP12-L
EP12-M
EP12-H
200kDa
97.5
~80kDa
?
66
D
MLL
Cell density
low high
280/290 kDa
200 kDa
97 kDa
68 kDa

Figure 1

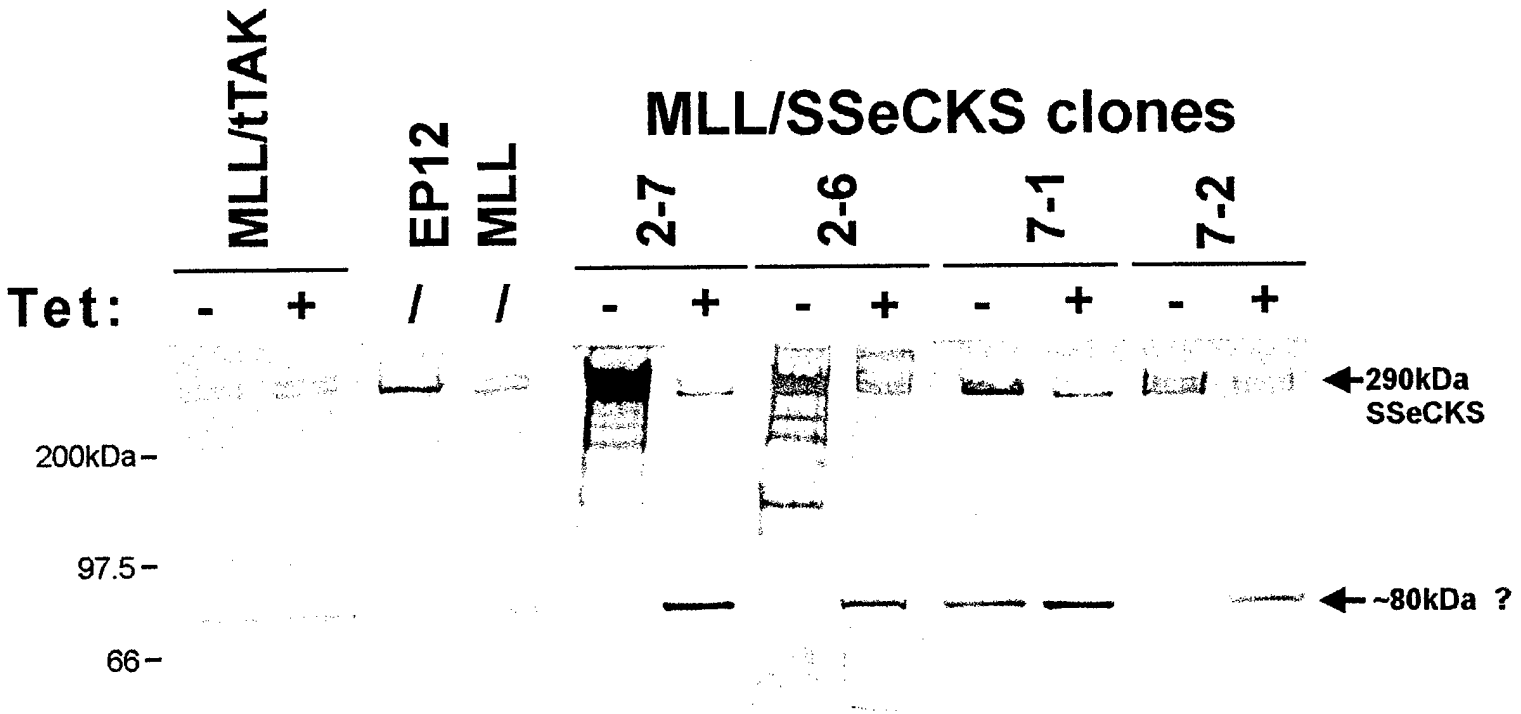
MLL/tTAK
EP12
MLL
MLL/SSeCKS clones
2-7
2-6
7-1
7-2
Tet:
- + / / - + - + - + - +
290kDa
SSeCKS
200kDa
97.5
66
~80kDa ?

Figure 2

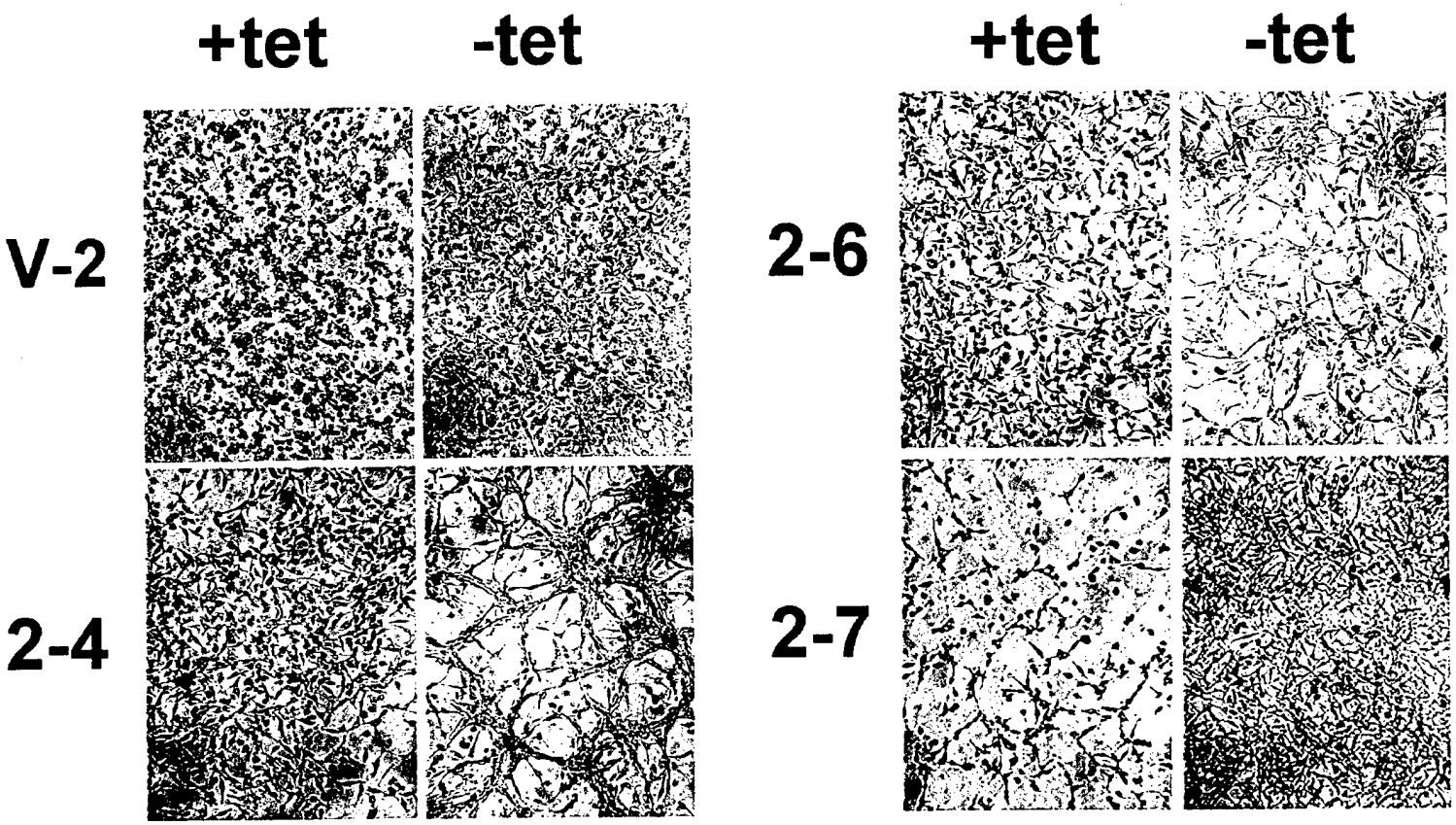

Figure 3

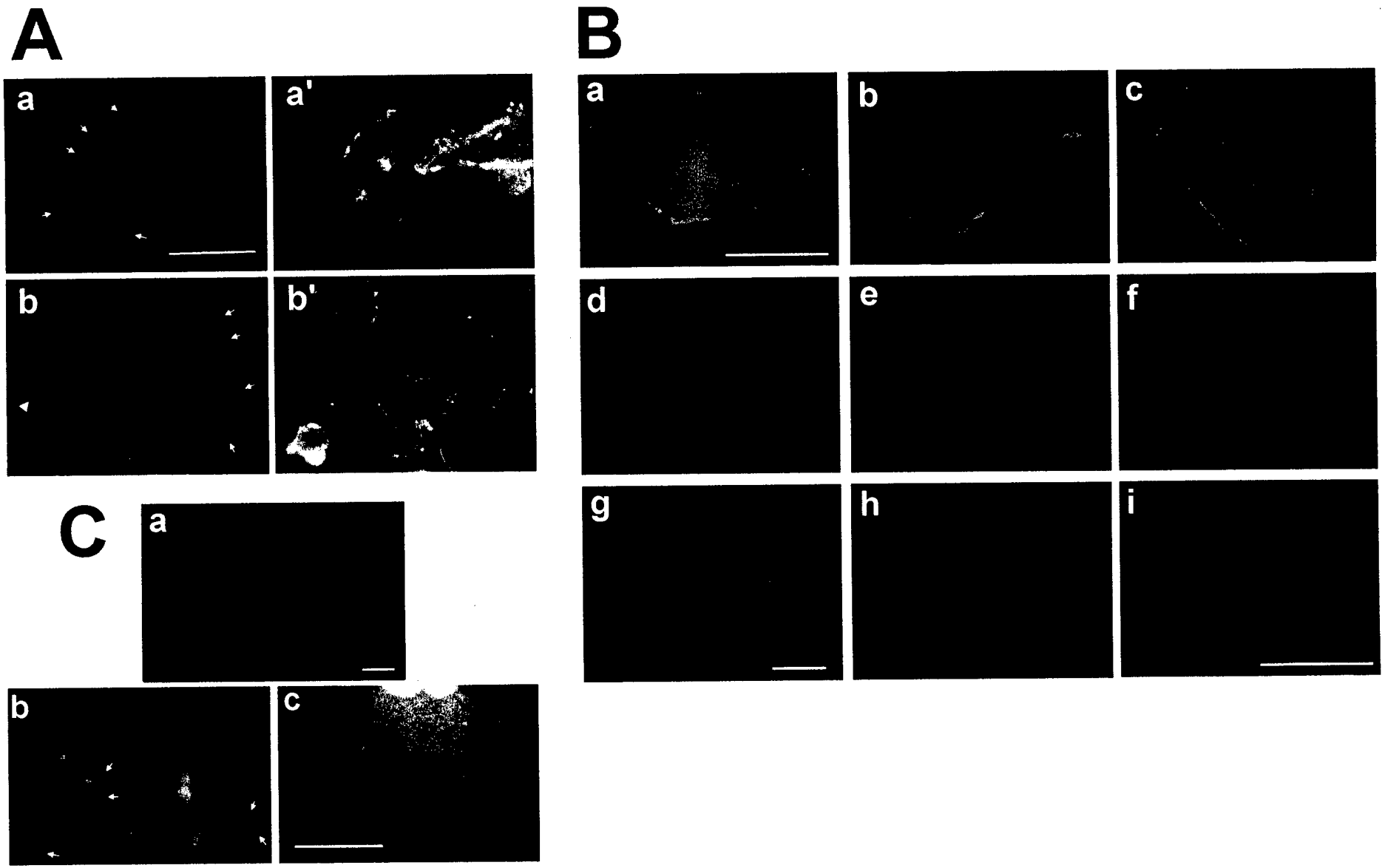

Figure 4

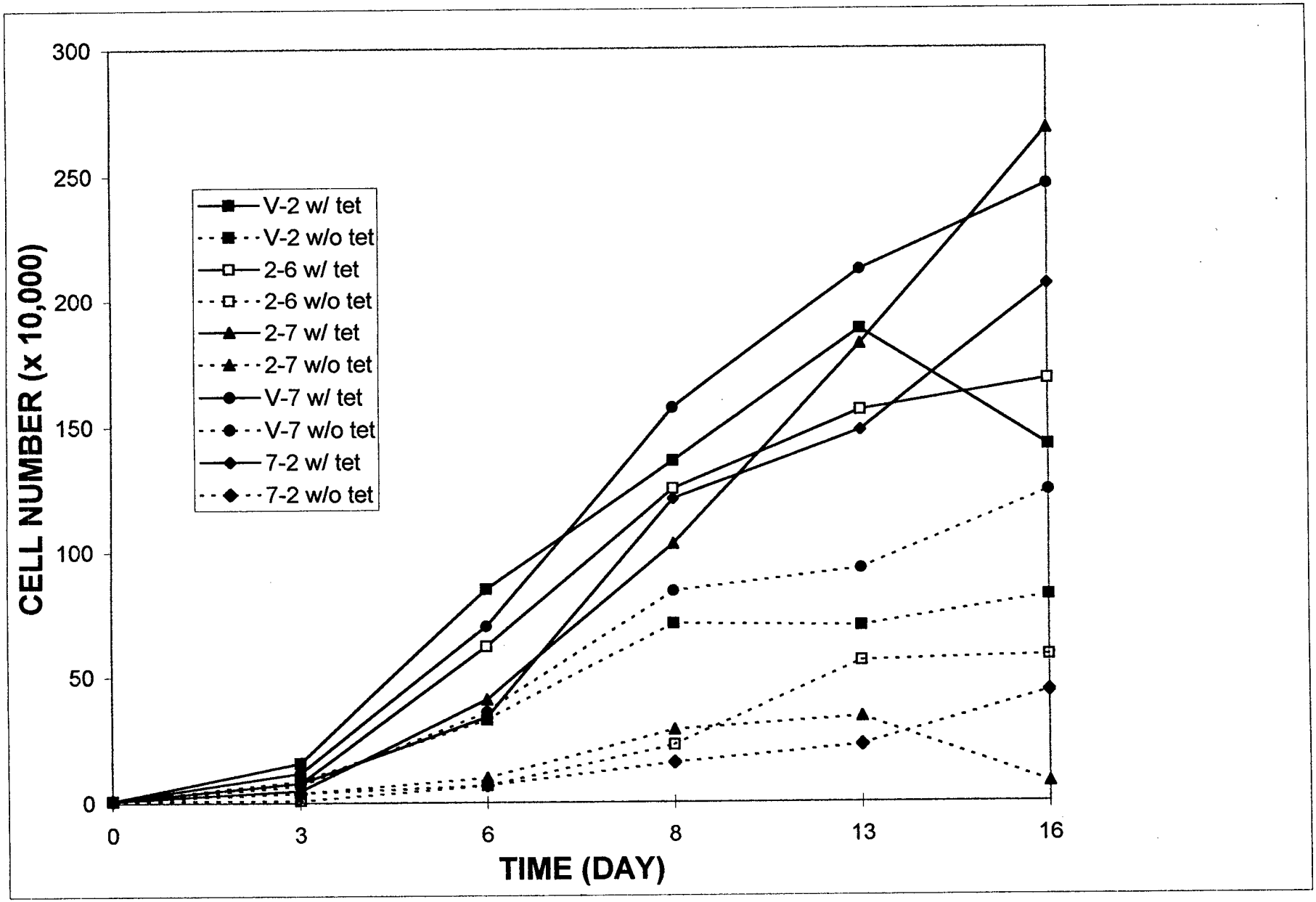
300
250
200
150
100
50
0
CELL NUMBER (x 10,000)
V-2 w/ tet
V-2 w/o tet
2-6 w/ tet
2-6 w/o tet
2-7 w/ tet
2-7 w/o tet
V-7 w/ tet
V-7 w/o tet
7-2 w/ tet
7-2 w/o tet
0
3
6
8
13
16
TIME (DAY)

Figure 5

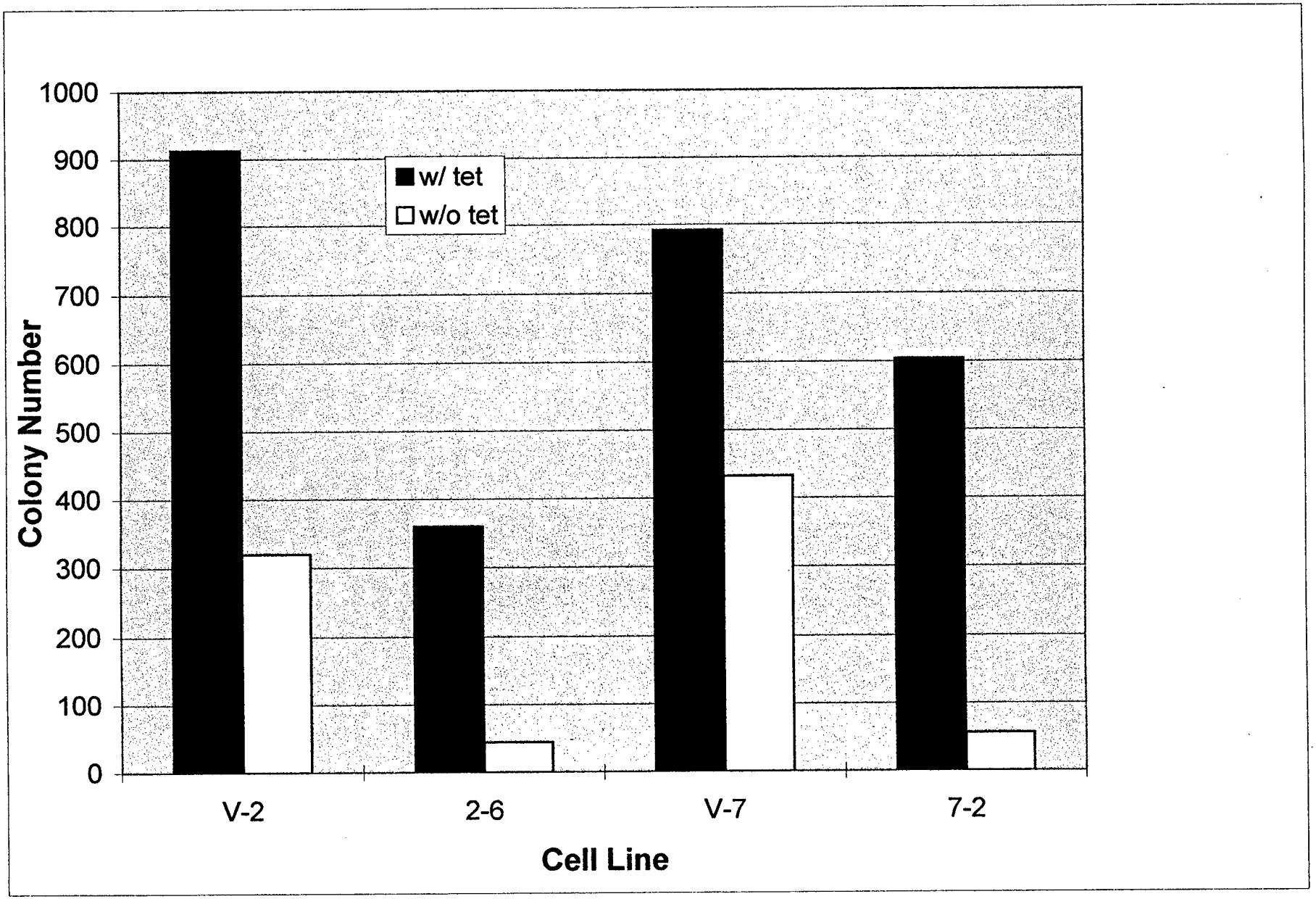
1000
900
800
700
600
500
400
300
200
100
0
Colony Number
w/ tet
w/o tet
V-2
2-6
V-7
7-2
Cell Line

Figure 6

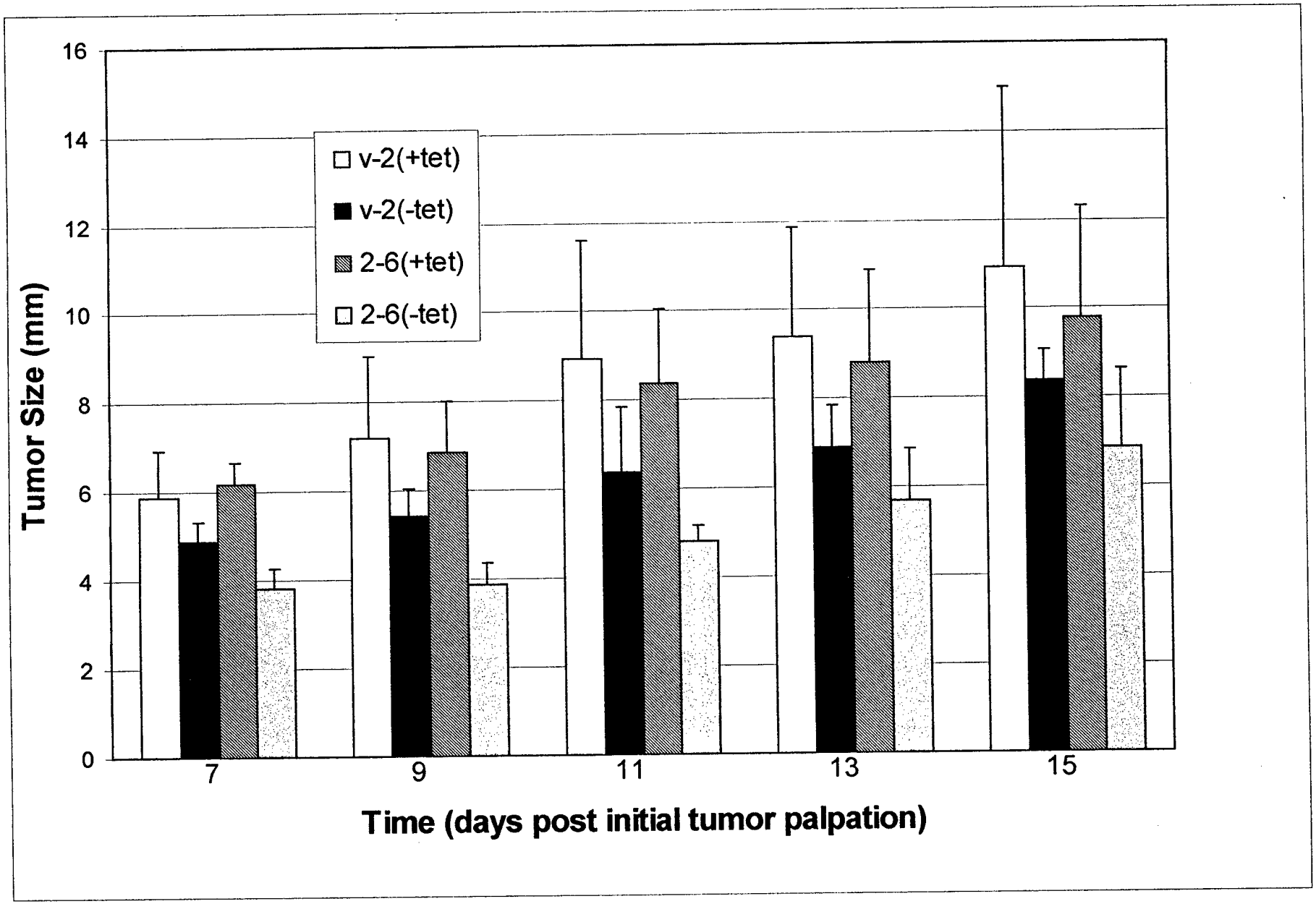
16
14
12
10
8
6
4
2
0
Tumor Size (mm)
v-2(+tet)
v-2(-tet)
2-6(+tet)
2-6(-tet)
7
9
11
13
15
Time (days post initial tumor palpation)

Figure 7A

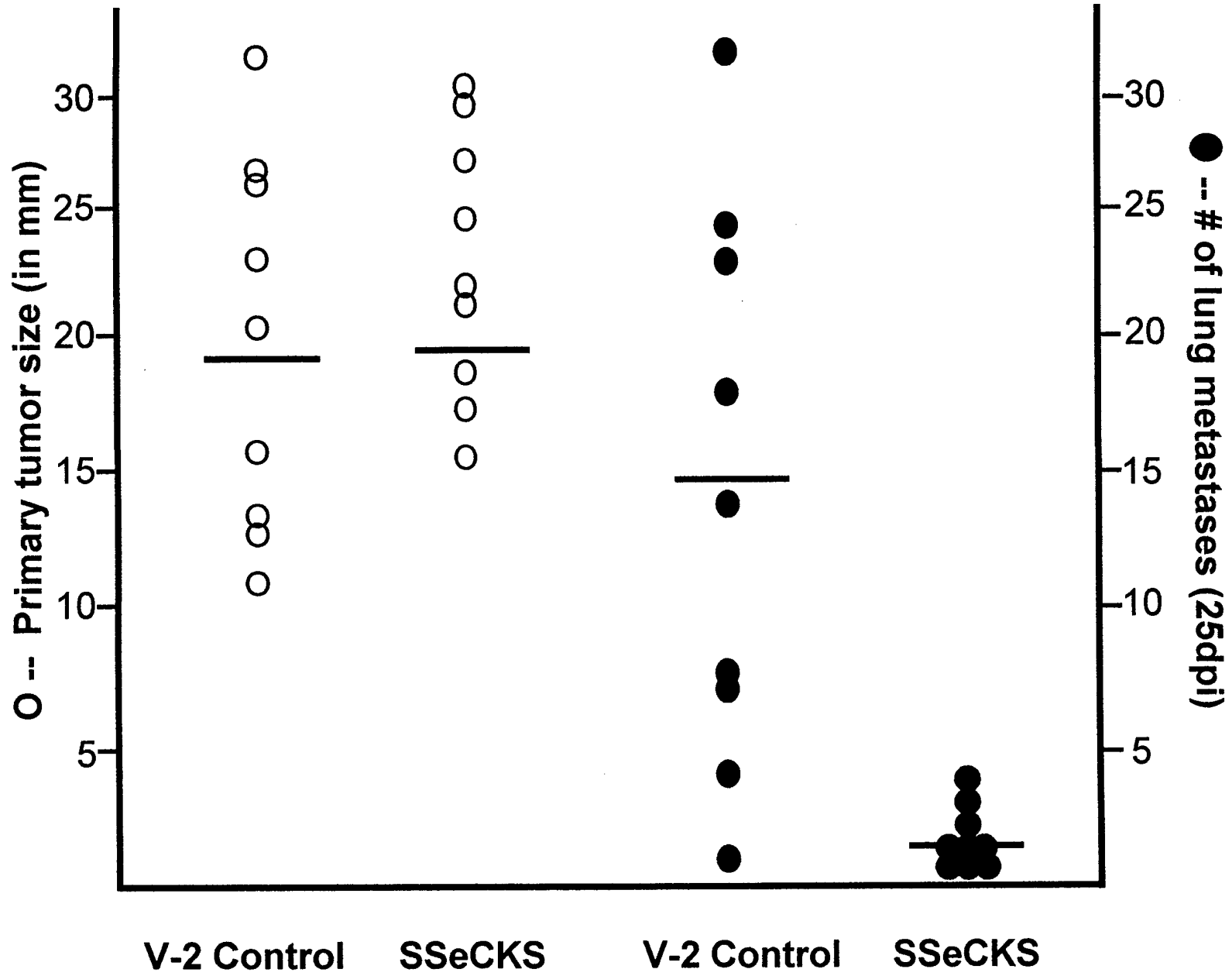
O -- Primary tumor size (in mm)
30
25
20
15
10
5
● -- # of lung metastases (25dpi)
30
25
20
15
10
5
V-2 Control
SSeCKS
V-2 Control
SSeCKS

Figure 7B

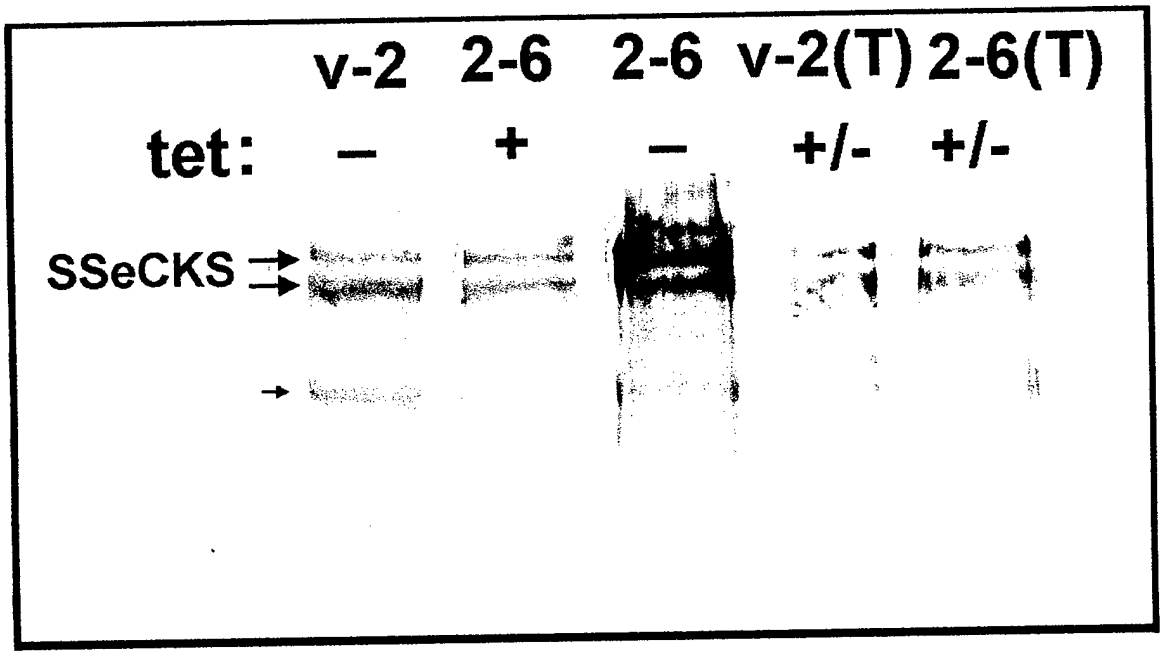

Figure 8

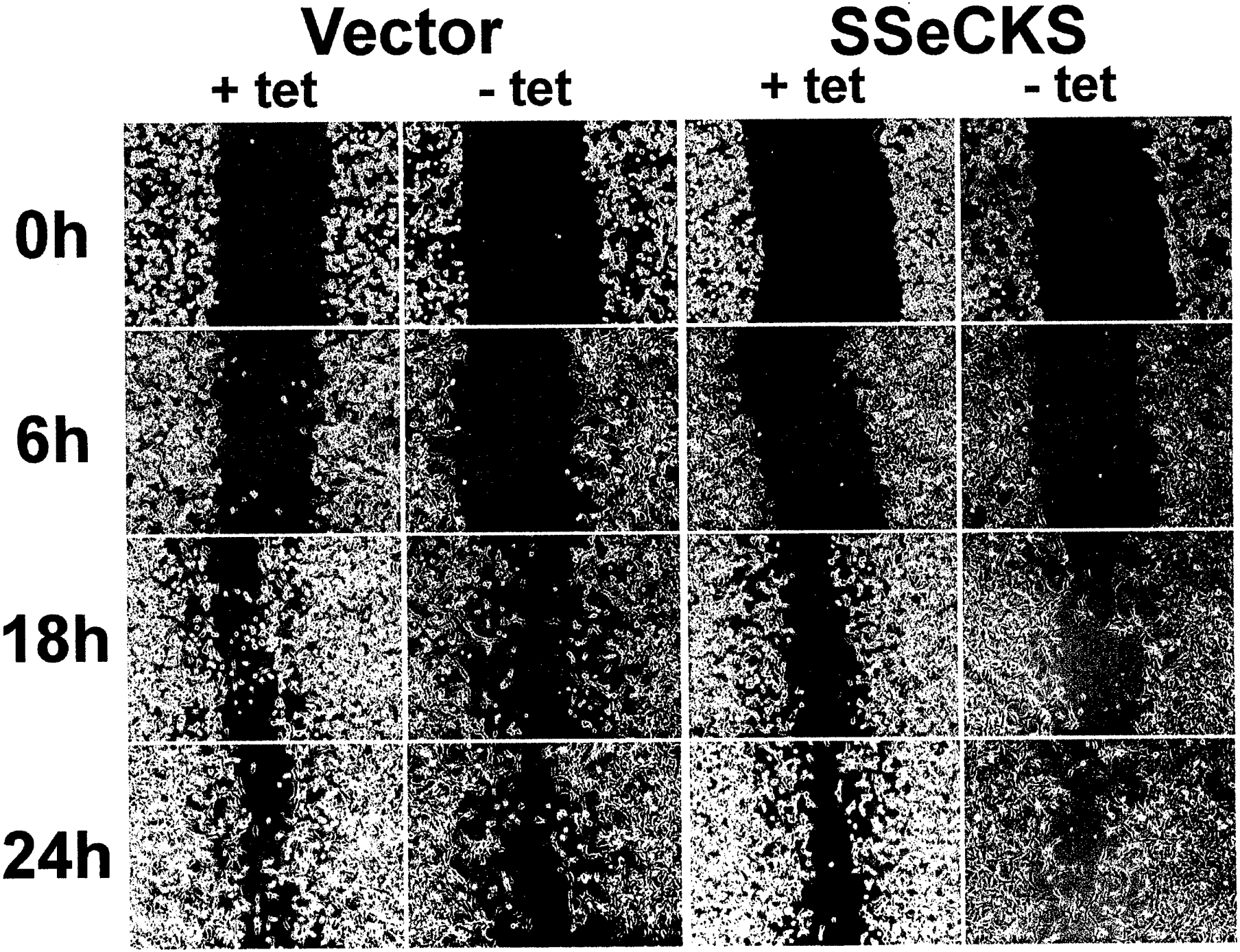

Figure 9

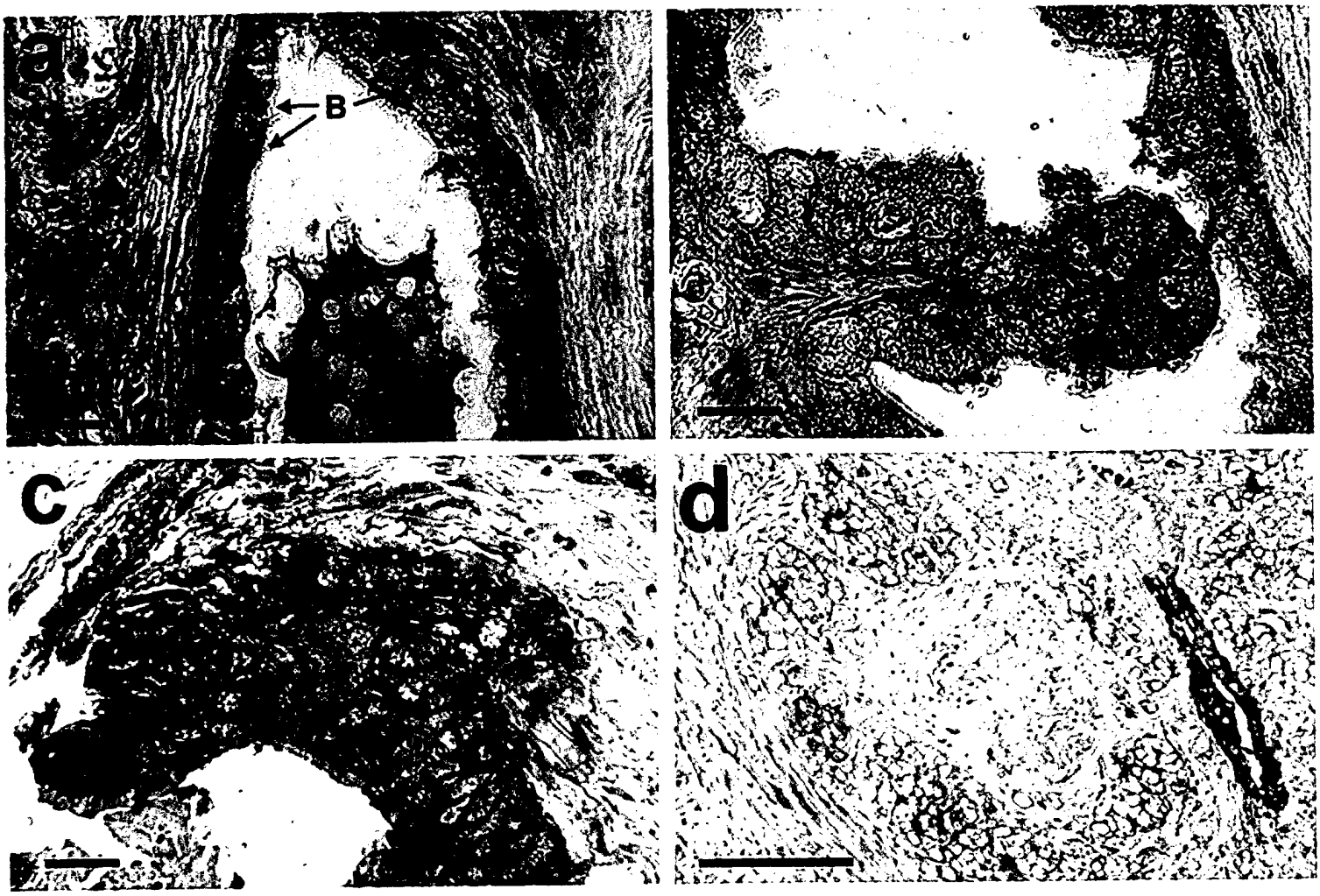

Figure 10

Title:

Suppression of Prostate Cancer Metastasis by the PKC Substrate SSeCKS

Abstract:

The molecular mechanisms leading to metastatic prostate cancer remain poorly understood. SSeCKS, initially isolated as a negative G1→S regulator, encodes tumor suppressor activity and is likely the rodent orthologue of Gravin, a human kinase scaffolding protein. We mapped Gravin to 6q24-25.2, a hotspot for deletion in advanced prostate, breast and ovarian cancer, and thus we investigated the role of SSeCKS/Gravin in prostate cancer. SSeCKS/Gravin protein was detected in untransformed prostate epithelial cell lines EP12 (rat) and PZ-HPV-7 (human) as well as in normal prostate, especially in basal epithelial cells. In contrast, SSeCKS/Gravin protein and RNA levels were severely reduced in panels of human (PC-3, PPC-1, LNCaP, DU145, TSU) and rat Dunning (AT3.1, MatLyLu) prostate cancer cell lines compared to controls. Loss of SSeCKS/Gravin expression correlated with the ability to induce metastases in nude mouse and syngeneic rat models. The re-expression of SSeCKS in MatLyLu cells using tetracycline (tet)-regulated vectors caused cell flattening, the formation of filopodia- and lamellipodia-like projections, and a decrease in anchorage-independent growth. In nude mice, SSeCKS slightly decreased the tumor growth rate at the primary site but severely decreased the appearance of lung metastases. MatLyLu tumors that progressed at the primary injection site lost tet-regulated SSeCKS re-expression. Lastly, the level of SSeCKS/Gravin staining in poorly-differentiated human carcinomas of the prostate was decreased compared to benign hyperplastic lesions (BPH) and well-differentiated prostatic carcinomas. Our data strongly suggest that the loss of SSeCKS/Gravin promotes prostate cancer tumorigenicity and metastasis.

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EDUCATION

Hopkins Grammar School		1972-76
Wesleyan University	Biochemistry B.A.	May 1980
Columbia University	Microbiology M.A.	May 1983
	Microbiology M.Ph.	Oct. 1985
	Microbiology Ph.D.	May 1987

APPOINTMENTS

NIH Graduate Traineeship, Columbia University	1981-1982
NIH Graduate Teaching Fellowship, Columbia University	1982-1983
Graduate Research Fellow, Columbia University	1983-1987
Postdoctoral Associateship, The Rockefeller University	1987-1990
Assistant Professor of Microbiology, Mount Sinai Sch. of Medicine	1990-
Doctoral Faculty, CUNY Biomedical Sciences Program	1991-
Microbiology Course Director, CUNY Physician Assistants Program	1992-
Adjunct Associate Professor, CUNY Dept. of Microbiology	1992-
Faculty, National Medical School Board Review	1993-
Member, Ruttenberg Cancer Center, Mount Sinai Sch. of Medicine	1996-
Assistant Professor, Ruttenberg Cancer Center, MSSM	1999-

SOCIETIES

American Association for the Advancement of Science
New York State Academy of Science
CUNY Academy of Humanities and Science
American Association for Cancer Research
American Society for Microbiology
American Society for Virology
American *In Vitro* Allergy/Immunology Society
American Society for Cell Biology

HONORS/AWARDS

High Honors in Biology- Wesleyan University	1980
American Cancer Society Postdoctoral Fellowship	1987-1990
American Foundation for AIDS Research Scholars Award	1991-92, 1992-93
Section Co-Chairman, AACR Annual Meeting	1995
CapCURE Award	1998

TEACHING EXPERIENCE

Teaching Asst.-Med. Microbiol., Columbia Sch. of Physicians and Surgeons	1981-1982
Tutor in Bacteriology, Virology and Immunology, Columbia P & S	1981-1984
Lecturer in Basic and Transplantation Immunology, Bronx-Lebanon Hospital	1983-1990

Lecturer in Medical Microbiology, Mount Sinai Sch. of Medicine	1990-
Lecturer in Molecular Biology CORE I, Mount Sinai Sch. of Medicine	1994-
Lecturer in Animal Virology, Mount Sinai Sch. of Medicine	1992-
Course Director, Microbiology, CUNY Med. Sch. P.A. Program	1992-
Lecturer in Virology, National Medical School Board Review	1993-
Lecturer, Viral Immunology, American *In Vitro* Allergy/Immunology Society	1994

RESEARCH EXPERIENCE

1975-76: under Dr. G. Osbaldiston, Dept. of Animal Sciences, Yale Univ.: Investigations of Iron Overload Anemias in Inbred Basenji Dogs.

1977-80: under Dr. W. Firshein, Dept. of Biology, Wesleyan University: Studies on DNA-Membrane Complexes in Pneumococcus.

1980-81: under Dr. M. Young, Dept. of Genetics, The Rockefeller Univ.: Studies on Transposable Elements in Drosophila.

1981-87: under Dr. S. Silverstein, Dept. of Microbiology, Columbia Univ.: "Control of Herpes Simplex Virus Gene Regulation" (Doctoral Thesis).

1987-90: under Dr. H. Hanafusa, Dept. of Molecular Oncology, The Rockefeller University: Suppression and Regression of SRC-Induced Tumors.

1991- : Department of Microbiology, Mount Sinai Medical Center: (1) Tumor suppression via the control of cytoskeletal architecture and cell signaling; (2) Involvement of *src*-family and focal adhesion kinases in mitogen- and integrin-induced signaling ; (3) Novel lenti- and retroviruses in Kaposi's Sarcoma and Chronic Fatigue Syndrome.

ACADEMIC/ADMINSTRATIVE EXPERIENCE (Mount Sinai)

Member, Mount Sinai Graduate School Admissions Committee
Member, Mount Sinai Medical Science Training Program Admissions Committee
Chairman, Department of Microbiology Admissions Committee
Chairman, Department of Microbiology Graduate Student Committee
Departmental Representative, Dean's Lecture Series Committee
Member, Departmental Graduate Curriculum Committee
Acting Course Director, 1992-93, Medical Microbiology Course
Departmental Representative, Faculty Council
Member, *Ad Hoc* Curriculum Committee
Faculty Member, Cell & Molecular Sciences Training Program
Member, Steering Committee, Cell & Molecular Sciences Training Program
Member, Mount Sinai AIDS Malignancy Consortium
Faculty Member, Molecular Basis of Disease Training Program
Member, Diagnostics and CORE Facilities Subcommittee
Member, MSSM Prostate Cancer Working Group

EDITORIAL BOARDS/PROFESSIONAL ACTIVITIES

Virology, Assistant Editor
Frontiers in Bioscience, Editor
Viral Immunology, Assistant Editor
Protein Engineering, Contributing Editor
BioTechniques, Contributing Editor

Oncogene, Contributing Editor
Ad hoc reviewer, NIH AIDS Malignancy Study Section
Meeting Organizer, "The Discovery of Oncogenes: Crossroads to the Future (Memorial Symposium for Teruko Hanafusa)", May 7, 1997, The Rockefeller University. Raised $27,000 for one-day event from private and public sources (incl. NIH grant R13-CA75132 for $5,000).

PRIOR FUNDING

1)American Federation for AIDS Research (AmFAR) Scholars Award #001445-11-RG, "Novel Lentiviruses in Kaposi's Sarcoma" ($60,000 annual cost), 1991-1992, P.I.- **I. H. Gelman**.

2)American Foundation for AIDS Research (AmFAR) Scholars Award #001679-13-RGR, "Novel Lentiviruses in Kaposi's Sarcoma" ($60,000 annual cost), 1992-1993, P.I.- **I. H. Gelman**.

3)Total Pharmaceutical Care, #J7-6261, "Expression of Endogenous Virus Sequences in Chronic Fatigue Syndrome", ($30,000 annual cost), 1994-1995, P.I.- **I. H. Gelman**.

4)The CFIDS Association of America, Inc., #J7-6371, "Blinded Pilot Study of Endogenous Retrovirus Marker with Chronic Fatigue Syndrome", ($2,000), 1994-1995, P.I.- **I. H. Gelman**.

5) National Cancer Institute, NIH, R29-CA65787, Minority Graduate Student Supplement, ($204,698 total costs over 4 years), 1996-2000, P.I.- **I. H. Gelman**, Fellow - Jesus Vargas, Jr.

6) CaPCURE Foundation, "Novel Therapeutic and Diagnostic Target in Prostate Cancer", PI: **I.H. Gelman**, ($75,000 total costs), 1998-99.

CURRENT FUNDING

1) National Cancer Institute, NIH, R29-CA65787, "Characterization of a Novel Mitogenic Regulatory Gene", ($575,750 total costs over 5 years), 1995-2000, P.I.- **I. H. Gelman**.

2) Centers for Disease Control and Prevention, "Abnormal Endogenous Virus Expression in Chronic Fatigue Syndrome", PI: **I.H. Gelman**, ($33,750 total costs), 1999-00.

3) Department of Defense, U.S. Army Medical Research and Materiel Command Prostate Cancer Initiative, "A Novel Diagnostic and Therapeutic Marker for Prostate Cancer", #PC970115 ($286,660 total costs over two years), 1999-2000, P.I.- **I.H. Gelman**.

4) NIH, National Institute of Allergy and Infectious Diseases, 1R01AI45343-01A1, "Induction of apoptosis by HIV-1 infected monocytic cells", PI- Kirk Sperber, M.D., Coinvestigator- **I.H. Gelman** (20% effort), ($653,071 direct costs over 5 years), 4/1/00-3/30/05.

PATENTS

1) "Lentivirus-Specific Nucleotide Probes and Methods of Use", Patent #5,478,724, Drs. Stephen S. Morse, **Irwin H. Gelman**, and Hidesaburo Hanafusa; Filing date: 10/29/93, Application date: 12/26/95.

2) "Tumor Suppressor Gene", Patent #5,910,442, **Irwin H. Gelman**, Filed 6/18/96, granted 6/8/99.

PUBLICATIONS

Zerial, A., **Gelman, I.**, Firshein, W., "Glycolipids Stimulate DNA Polymerase Activity in a DNA-Membrane Fraction and in a Partially Purified Polymerase System Extracted from Pneumococcus." *J. Bact.*, 135:78-85 (1978).

Firshein, W., **Gelman, I.**, "Enrichment of DNA Polymerase III in a DNA-Membrane Complex Purified from Pneumococcus: The Possible Existence of Subcomplexes." *Molec. gen. Genet.*, 182:87-96 (1981).

Gelman, I.H., Silverstein, S., "Identification of Immediate Early Genes from Herpes Simplex Virus that

Transactivate the Virus Thymidine Kinase Gene.", *Proc. Nat'l. Acad. Sci.*, 82:5265-5269 (1985).

Gelman, I.H., Silverstein, S., "Coordinate Regulation of Herpes Simplex Virus Gene Expression is Mediated by the Functional Interaction of Two Immediate Early Gene Products." *J. Mol Biol.*, 191:395-409 (1986).

Gelman, I.H., Silverstein, S., "Herpes Simplex Virus Immediate-Early Promoters are Responsive to Virus and Cell Trans-acting Factors." *J. Virol.*, 61:2286-2296 (1987).

Gelman, I.H., Silverstein, S., "Dissection of Immediate-Early Gene Promoters from Herpes Simplex Virus: Sequences that Respond to the Virus Transcriptional Activators." *J. Virol.*, 61:3167-3172 (1987).

Gelman, I.H., Hanafusa, H., "Suppression of Rous Sarcoma Virus-Induced Tumor Formation by Preinfection with Viruses Encoding src Protein with Novel N-Termini." *J. Virol.*, 63:2461-2468 (1989).

Kemp, L.M., **Gelman, I.H.**, Silverstein S.J., Latchman, D.S., "Regulation of HSV Immediate-Early Promoters in Mouse Neuroblastoma Cells", *Neuroscience Letters*, 118:185 (1990).

Gelman, I. H., Zhang, J., Heilman,P.E., Hanafusa, H., Morse, S.S., "Identification and evaluation of new primer sets for the detection of lentivirus proviral DNA", *AIDS Res. Human Retrovir.*, 8:1981-1989 (1992).

Laurence, J., Siegal, F.P., Schattner, E., **Gelman, I.H.**, Morse, S., "Individuals with acquired immune deficiency without evidence for human immunodeficiency virus types-1, -2 by serology, culture, and DNA amplification, *Lancet*, 340:273-274, (1992).

Gelman, I.H., Hanafusa, H., "src-Specific Immune Regression of Rous Sarcoma Virus-Induced Tumors." *Cancer Res.*, 53:915-920 (1993).

Bohensky, R.A., Papavassiliou, A.G., **Gelman, I.H.**, Silverstein, S., "Identification of a promoter mapping within the reiterated sequences that flank the Herpes Simplex Virus Type 1 U_L region", *J. Virol.*, 67:632-642 (1993).

Gelman, I.H., Khan, S. and H. Hanafusa, "Morphological transformation, tumorigenicity, and *src*-specific CTL-mediated tumor immunity induced by murine 3T3 cells expressing *src* oncogenes encoding novel non-myristoylated N-terminal domains", *Oncogene*, 8:2995-3004 (1993).

Frankfort, B. and **Gelman, I.H.**, "Identification of novel cellular genes transcriptionally suppressed by v-src", *BBRC*, 206:916-926, 1995.

Lin, X., Nelson, P., Van Tuyl, A., Johnson, R. and **Gelman, I.H.**, "Isolation and characterization of a novel mitogenic regulatory gene, *322*, which is transcriptionally suppressed in *src*- and *ras*-transformed cells", *Molec.Cell.Biol.* 15(5):2754-2762, 1995.

Lin, X., Tombler, E., Nelson, P., Ross., M., and **Gelman, I.H.**, "A Novel *src*- and *ras*-suppressed protein kinase C substrate associated with cytoskeletal architecture", *J.Biol.Chem.*, 271(45):28,430-28,438, 1996.

Lin, X., and **Gelman, I.H.**, "Re-expression of the major protein kinase C substrate, SSeCKS, suppresses v-*src* induced morphological transformation and oncogenic growth", *Cancer Res.* 57(11):2304-2312, 1997.

Nelson, P. and **Gelman, I.H.** "Cell-cycle regulated expression and serine phosphorylation of the myristylated protein kinase C substrate, SSeCKS: Correlation with cell confluency, G_0 phase and serum response", *Molec.Cell.Biochem*, 175:233-241, 1997.

Gelman, I.H., Lee, K., Tombler, E., Gordon, R. and Lin, X., "Control of Cytoskeletal Architecture by the *src*-Suppressed C Kinase Substrate, SSeCKS" *Cell Motility and Cytoskeleton*, 41(1): 1-17, 1998.

Nelson, P., Moissoglu, K., Vargas, J., Jr., **Gelman, I.H.**, and Klotman, P.E., "The protein kinase C

substrate, SSeCKS, controls actin-based stellate morphology in mesangial cells" *J. Cell Sci.* 112:361-370, 1999.

Gelman, I.H., Tombler, E. and Vargas, J., Jr., "Developmental and Tissue-Specific Expression of SSeCKS, a Major PKC Substrate with Tumor Suppressor Activity, Suggests Roles in Cytoskeletal Architecture, Formation of Migratory Processes and Cell Migration During Embryogenesis ", *The Histochem. J.*, Jan. 2000 (in press).

Krista Rombouts, **I.H. Gelman**, "Trichostatin A suppresses transdifferentiation of rat stellate cells by its effect on the assembly pathway of the actin cytoskeleton" *Hepatology* (in press).

Lin, X. and **Gelman, I.H.** "Regulation of G1→S Progression by the SSeCKS Tumor Suppressor: Control of Cyclin D Expression and Cellular Compartmentalization" (submitted for publication).

Gelman, I.H., Bulua, A. and Wang, A., "Loss of Chromosome 6q24-25.2 in Breast Cancer Cell Lines Correlates with Deletion of the Gravin, the Putative Human Orthologue of the Rodent Tumor Suppressor, SSeCKS" (submitted for publication).

Xia, W. and **Gelman, I.H.**, "Suppression of tumorigenic potential in MatLyLu prostate cancer cells following the forced re-expression of the *Src*-Suppressed C Kinase Substrate, SSeCKS" (submitted for publication).

Irwin H. Gelman, Konstadino Moissoglu, William C. Reeves, Alison Mawle, Rosanne Nisenbaum, Elizabeth R. Unger, "p15 *env* and Immune Function in Chronic Fatigue Syndrome" (submitted for publication).

Frosch, C., Ehrlich, R., Hölzel, F., **Gelman, I.H.**, Oesch, F. and Weiser, R.J., "Molecular mechanisms involved in dexamethasone-inducible contact-dependent inhibition of HH16 fibrosarcoma cells", (submitted for publication).

Wassler, M.J., Foote, C.I., **Gelman, I.H.** and Shur, B.D., "Functional interaction between cell surface β1,4-galactosyltransferase and SSeCKS, a PKC substrate" (submitted for publication).

Xia, W. and **Gelman, I.H.**, "Cell cycle- and FAK-regulated tyrosine phosphorylation of the SSeCKS scaffolding protein modulates its actin-binding properties" (manuscript in preparation).

BOOK CHAPTER

Marius Sudol and **Irwin H. Gelman**, "The v-*src* Oncogene and its Signaling Pathways", in Atlas of Signal Transduction, Bork, P, Margolis, B., Schleuning, W.D., Sudol, M., eds., Walter De Gruyter & Co., publishers, Berlin and New York, in press.

INVITED PAPERS

Gelman, I.H., "Pathogenicity of AIDS-Related Kaposi's Sarcoma", *Oncol.Reports* 2:321-324, 1995.

Gelman, I.H., "Immunological and Virological Parameters of Chronic Fatigue Syndrome", *Clinical Immunology Newsletter*, 15(8):100-104, 1995.

Gelman, I.H. , Qiaoran Xi and C. Chandra Kumar, "Re-expression of the Major PKC Substrate, *SSeCKS*, Correlates with the Tumor Suppressive Effects of SCH51344 on Rat-6/*src* and Rat-6/*ras* Fibroblasts But Not on Rat-6/*raf* Fibroblasts", *N.Y. Acad. Sci.*, Nov. 1999.

INVITED LECTURES

-"Novel Lentiviruses in Kaposi's Sarcoma", *The American Society for Virology*, Ithaca, NY (1992)

-"V-src-encoded tumor regression antigens", American Association for Cancer Research, Orlando, FL (6/22/93).

-"Isolation and characterization of novel cellular genes transcriptionally suppressed in NIH3T3 fibroblasts transformed by the v-src oncogene", Am. Soc. for Virol. 13th Annual Meeting,

Madison, WI, 7/9-13/94.

-"Immunological and Virological Profile of Chronic Fatigue Syndrome", 6th Annual American *In-Vitro* Allergy/Immunology Society, Boston, MA, July 10-13, 1994.

-"Identification and characterization of a novel regulatory/tumor suppressor gene, *322*", American Association for Cancer Research, 86th Annual Meeting, Toronto, Canada, 3/18-22/95.

-"SSeCKS is a novel *src*- and *ras*-suppressed substrate of Protein Kinase-C possibly involved in controlling actin-based cell shape or motility", American Association for Cancer Research 87th Annual Meeting, Washington, DC, April 21-25, 1996. **I.H. Gelman** co-chairs session on "Signal Transduction".

-"SSeCKS, a Novel Mitogenic Regulator", Lecture at Brown University School of Medicine, April 25, 1996.

- "Tumor suppression, growth arrest and regulation of cytoskeletal architecture by the *src*-suppressed Protein Kinase C substrate, SSeCKS", 12th Annual Oncogene Meeting, Frederick, MD, June 18-21, 1996.

-"Suppression of tumor formation via the control of cytoskeletal architecture", Lecture at Wesleyan University, Department of Molecular Biology, Oct. 30, 1996.

-"Suppression of tumor formation via the control of cytoskeletal architecture", Lecture at Harvard University, Dept. of Pathology, Feb. 19, 1997.

-"Suppression of tumor formation via the control of cytoskeletal architecture", Lecture at The National Cancer Institute, Frederick, MD, Feb., 28, 1997.

-"SSeCKS as a target for tumor diagnosis and suppression", seminar at Innovir, Inc., NYC, July 12, 1997.

-"Tumor suppression, Signaling and Cytoskeletal Control by SSeCKS, a Novel PKC and *Src* Substrate with Kinase Scaffolding Activity", seminar at Schering-Plough, Inc., New Jersey, Sept. 11, 1997.

-"Control of cytoskeletal architecture, cell cycle and tumorigenesis by the *src*-suppressed C kinase substrate, SSeCKS", seminar at UC Berkeley, Molecular and Cellular Biology Program, March 30, 1998.

-"Therapeutic Model for Prostate Cancer: Targeting the Novel Tumor Suppressor, SSeCKS", CaP CURE Fifth Annual Scientific Meeting, Lake Tahoe, CA, Sept. 13, 1998.

-"Control of G1→S Phase Transition by the Tumor Suppressor, SSeCKS", Invited lecture, Exelixis Pharmaceuticals, Inc., San Francisco, CA, 12/13/98.

-"The Focal Adhesion Kinase, FAK, Modulates Cytoskeletal Reorganization During v-*src*-Induced Oncogenic Transformation". Cancer Research Campaign, Beatson International Cancer Conference, *Invasion and Metastasis*, Glasgow, Scotland, 6/27-30/1999, oral presentation

- "Tumor Suppression, Control of Mitogenesis and Cytoskeletal Architecture by SSeCKS, a Major PKC Substrate with Scaffolding Activity", University of Mainz, Frankfurt, Germany, July 2, 1999.

"A novel tumor suppression mechanism: Control of cyclin D expression andcompartmentalization by t he Src-Suppressed C Kinase Substrate, SSeCKS", Moffitt Cancer Center, 12/15/99.

"A novel tumor suppression mechanism: Control of cyclin D expression and compartmentalization by the Src-Suppressed C Kinase Substrate, SSeCKS", poster presentation, 39th Ann. ASCB Meeting, Washington, DC, Dec. 11-15, 1999.

Co-Chair, Signal Transduction Poster Discussion Session, 91st Annual Meeting, American Association for Cancer Research, San Francisco, CA, April 1-5, 2000.

Meeting Organizer, "Roles of protein tyrosine phosphorylation in signal transduction- The 25th anniversary of the c-Src discovery", May 10-12, 2000 in Osaka, Japan.

STUDENTS TRAINED

Graduates Students-

Current-

-Wei Xia (Graduate Student, 9/95 -): "Tyrosine phosphorylation of SSeCKS by the focal adhesion kinase, FAK".

-Jesus Vargas, Jr. (Graduate Student, 9/96-): "Developmental and tissue-specific expression of SSeCKS".

-Eugene Tombler (CMBS Graduate Student, 9/96-): "Characterization of the genomic and promoter structure of SSeCKS"

-Kostas Moissoglu (Graduate student, 5/97-): "FAK-dependent pathways involved in v-*src*-induced oncogenic transformation".

Past-

-Jiaojiao Zhang (Graduate student, 6/91-6/93): "*Cis*-acting elements in the collagen type $I_{\alpha}2$ promoter responsive to transcriptional down-regulation by the v-src oncogene". Current affiliation- Postdoctoral fellow, NYU Medical School.

-Xueying Lin (Graduate student, 9/94- 2/99): "Mechanism of tumor suppression, mitogenic control and cytoskeletal organization by the PKC substrate, SSeCKS". Current affiliation: Postdoctoral Fellow, Harvard University, under Dr. Morris White, Rosalyn Diabetes Center.

-Peter Nelson (BioMedical Thesis Student, Mount Sinai Medical School, 5/94-5/97): "Isolation and characterization of *322*-binding genes using the yeast two-hybrid technique". Current affiliation: Clinical Research Fellow, Nephrology, Mount Sinai School of Medicine.

Postdoctoral Students-

Past-

-Jose Serruya, M.D. (Postdoctoral Fellowship, 1/92-3/94): "Search for novel lentiviruses in AIDS-associated Kaposi's sarcoma". Current affiliation: resident, Pediatric Neurology, NYU School of Medicine.

RESEARCH INTERESTS

IRWIN H. GELMAN, Ph.D.

Currently, I have three active research programs in my laboratory: i) identification and characterization of novel mitogenic regulatory genes encoding tumor suppressive functions, ii) control of cytoskeletal architecture and mitogenic signaling by Src-family kinases and the focal adhesion kinase, FAK, in normal and cancer cells, and iii) studies correlating the inappropriate expression of endogenous retrovirus sequences with immune dysfunction disorders in humans. Roughly 80% of the lab's activity involve the first two research interests.

Identification of novel regulatory/tumor suppressor functions: A current theory for the genetic basis of cancer states that cancers arise through a combination of mutations that induce the oncogenic activity of proto-oncogenes and inhibit the control activities of tumor suppressor genes. My assumption was that genes whose expression is downregulated in cells transformed with oncogenes such as *src* or *ras* might be candidate tumor suppressor genes. I also assumed that genes involved in critical steps in mitogenic control would be expressed at low basal levels and would also be serum-responsive. Based on differential screens we developed to satisfy these criteria, my laboratory identified several new candidate control genes, one of which encodes SSeCKS, a new major protein kinase C substrate that also shows tumor suppressive activity. The cell-cycle expression and phosphorylation of SSeCKS, as well as its ability to interact with the actin-based cytoskeletal, suggests that SSeCKS plays a key role in controlling both mitogenic signaling and cytoskeletal architecture. Moreover, SSeCKS seems to work as a "scaffolding protein". In its underphosphorylated form (expressed in early G1 phase or in contact-inhibited cells), it binds key signaling proteins such as PKC, PKA, calmodulin and G1 phase cyclins. Phosphorylation of SSeCKS by mitogen- and integrin-activated kinases inhibits *in vitro* and *in vivo* scaffolding activity. The fact that SSeCKS expression is severely downregulated in *src* and *ras*-transformed fibroblasts and epithelial tumors, and that the human orthologue maps to a deletion hotspot in >60% of cases of advanced prostate, breast, and ovarian cancer, strengthens the notion that SSeCKS is a tumor suppressor. Indeed, we showed that re-expression of SSeCKS using tetracycline-regulated vectors causes a reorganization of cytoskeletal architecture, leading to the suppression of *src*- and *ras*-induced oncogenic growth *in vitro* and the loss of metastatic potential *in vivo* in a rat prostate cancer model.

We are continuing to study how the loss of SSeCKS leads to metastatic behavior in human prostate cancer. We are also characterizing how SSeCKS interacts or competes with p21 and p27 during its binding to cyclin D, and how this affects the formation of kinase active complexes of cyclin D and CDK4 in the cytoplasm. This latter control point in the cell cycle is critical as to whether cells proliferate or differentiate, and indeed, we have found several cell systems in which SSeCKS expression is induced upon differentiation. We are actively researching whether SSeCKS helps establish or maintain the differentiated state of glomerular mesangial cells, hepatic stellate cells or thrombopoietin-induced megakaryocytes.

My lab's interest in cancer genetics, specifically in identifying new mitogenic regulatory genes that are transcriptionally downregulated in transformed cells, continues. We have developed two techniques for selectively screening libraries for relevant mitogenic regulatory genes (i.e.- potential tumor suppressors), and we are actively characterizing several new gene candidates.

Control of cytoskeletal architecture and mitogenic signaling by Src-family kinases and the focal adhesion kinase, FAK, in normal and cancer cells: Oncogenes such as v-*src* induce oncogenic transformation by altering both mitogenic and cytoskeletal signaling pathways. The latter pathways are

less understood, but there is consensus that changes in the actin-based cytoskeleton and in cell-cell interactions correlate with advanced tumor behavior such as metastatic potential and invasiveness. The focal adhesion kinase, FAK, seems to function downstream of v-*src* to regulate cell architecture and cytoskeletal signaling. Thus, we are actively studying how FAK contributes to the control of mitogen- and integrin-induced cytoskeletal and proliferative signaling pathways in untransformed and in *src*-transformed cells. We have made use of mouse embryo fibroblasts from control and FAK knockout mice into which we transduced temperature-sensitive alleles of v-*src*. Our data strongly suggest a novel role for FAK, that of a temporal scaffolding protein that binds and inhibits protein tyrosine phosphatases (PTP) in the absence of mitogen- and integrin-mediated activation signals. In the absence of FAK, at least several of the PTPs are capable of dephosphorylating negative-regulatory phosphotyrosine residues at the C-termini of Src-family kinases, leading to their activation. Inasmuch as many tumors express elevated levels of FAK, my long-term interest is to elucidate the temporal roles of FAK during normal cell cycle progression versus potential roles during oncogenesis or even control of apoptosis.

Expression of endogenous retrovirus sequences in immune dysfunction disorders in humans: The genomes of all mammals contain many copies of so-called endogenous retrovirus sequences that are remnants of germline infections, and thus, passed on vertically. Humans encode many versions of *env* gene sequences that share the potential immunosuppressive "p15E" motifs described in animal retroviruses, however, little is known regarding the natural expression of these sequences or their possible effect on the immune system. Our data indicate that adults do not typically express endogenous retrovirus sequences except for placental expression in the third trimester and in some cancers. My lab addressed whether the inappropriate expression of endogenous 15E-like products might correlate with syndromes displaying immune dysfunctions such as Chronic Fatigue Syndrome (CFS). We studied a well defined group of 20 CFS cases plus 35 controls that were characterized at the Center for Disease Control. Preliminary results with an RT-PCR assay showed significant p15E expression correlating with the CFS population. We currently are developing an ELISA-based test to define baseline and disease thresholds in humans. Although p15E is only a marker for CFS at this time, the similarity between the immune dysfunctions in CFS and the known immunosuppressive effects of p15E in animal models suggests that antagonizing p15E could have therapeutic benefits.