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				<b>14. ABSTRACT</b> A detailed analysis of ANX7 levels in hundreds of prostate cancer specimens revealed that expression of this candidate tumor suppressor gene is specifically altered in metastatic and hormone refractory prostate cancers. Overexpression of ANXA7 killed prostate cancer cells by apoptosis. So, we hypothesized that the ANX7 gene kills prostate cancer cells by increasing IP3-Receptor expression, thereby potentiating the IP3-dependent apoptotic calcium signaling pathway. We found that the ANX7 induced apoptotic pathway involves calcium and cytochrome c release indicating the probable involvement of mitochondria. ANX7 induces morphological changes including cell shrinkage, nuclear fragmentation and chromatin condensation. Overexpression of ANX7 or the ANX7J mutant in DU145 cells does not alter basal [Ca2+]i levels. However, overexpression of ANX7 reduces the percentage of cells that are capable of responding to the IP3-generating agonist acetylcholine. Furthermore, overexpression of either ANX7 or the ANX7J-mutant may be associated with a reduction in the magnitude of the response to acetylcholine. We identified using cDNA microarrays the apoptosis, metastatic and cell cycle "corrected" genes which show equivalent relative expression in PREC normal prostate cells and transfected metastatic DU145 cancer cells with wt-ANX7 or P53.	
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## INTRODUCTION

Current attempts to develop more effective therapies for prostate cancer have been olorad on the discovery of new tumor suppressor genes, in hopes of using them for gene therapy. We have recently found that the *ANX7* gene, which we discovered and have continued to study in the context of  $\text{Ca}^{2+}$  and GTP mediated exocytosis for many years (Creutz et al, 1978; *ibid*, 1979; Raynal and Pollard, 1994; Caohuy et al, 1996; Srivastava et al, 1996), is defective in 35% of human prostate cancer specimens (Srivastava et al., 2001a) and 40% of human breast cancer specimens. A detailed analysis of *ANX7* levels in hundreds of prostate cancer specimens reveals that expression of this candidate tumor suppressor gene is specifically altered in metastatic and hormone refractory prostate cancers (Srivastava et al., 2001a). These data therefore, strongly suggest that the *ANX7* gene has clinical relevance for prostate cancer in men. This is an important insight because until now the *ANX7* gene has never been thought to play such a role (Raynal and Pollard, 1994). In our preliminary studies with metastatic prostate cancer cells *in vitro*, we find that *ANX7* gene therapy causes these cells to undergo apoptosis, or programmed cell death. Therefore our objective in this proposal is to determine the mechanism and the signaling pathway by which the *ANX7* gene induces death of prostate cancer cells. The rationale behind this study is the hope that such knowledge will enable us to develop therapies based on the use of the *ANX7* gene to treat or prevent prostate cancer. We are best positioned to pursue this work because we not only discovered the *ANX7* gene, but have also recently prepared a tumor-prone *Anx7* knockout mouse. It has fascinated us that the phenotype of this knockout mouse also includes such a high frequency (20-25%) of spontaneous tumors.

Our studies with both human cancer cells and the *Anx7* knockout mouse have indicated the existence of a possible common deficit in calcium regulation (Srivastava et al., 1999). In the case of cancer cells, it is known that the initial signal for the onset of programmed cell death is the release of a pulse of calcium from the internal calcium stores in the endoplasmic reticulum. The calcium is released into the cytoplasm through a protein channel in the endoplasmic reticulum membrane called the IP3-Receptor. A hint regarding the possible connection between the *ANX7* gene and the calcium pulse, comes from our recent work on the *ANX7* (+/-) knockout mouse. Tissues in this mouse express low levels of *Anx7* protein as well as low levels of IP3-Receptors in the endoplasmic reticulum. Intracellular calcium signaling is accordingly defective, in spite of normal levels of extracellular calcium. We are reminded that apoptosis, from whatever cause, is known to be suppressed in the absence of IP3-Receptors. We also found that the effect of the *ANX7* gene on the DU145 prostate cancer cell line enhanced the capacity for undergoing apoptosis in the presence of staurosporine. Additionally, transfection of wild type *ANX7* in adenovirus system enhanced the production of IP3-Receptors, while dominant negative mutant of *ANX7* suppressed IP3-Receptor expression. On the basis of these results **we have hypothesized that the *ANX7* gene kills prostate cancer cells by increasing IP3-Receptor expression, thereby potentiating the IP3-dependent apoptotic calcium signaling pathway.**

## BODY

### **REVISED STATEMENT OF WORK:**

**Task 1.** To define the biochemical requirements and regions in ANX7 that confer apoptosis and tumor suppressor activity in prostate cancer cells.

**Table 1** Test sites of Ca<sup>2+</sup>, GTP and PKC in ANX7 for importance in the induction of apoptosis and cell proliferation. These studies will determine the “hot spot(s)” in the ANX7 coding domain which are necessary for tumor cell proliferation and apoptosis (Months 1-14).

**Task 2.** To elucidate the relationship between ANX7-induced alteration of apoptosis and tumor suppressor activity in prostate cancer cells.

- a. Test prostate cancer cells for biochemical characteristics of apoptosis. These studies will test the hypothesis that changes in ANX7 expression levels affects apoptotic processes in cancer cells. (Months 13-24).
- b. Test tumorigenicity of DU145 metastatic prostate cancer cells expressing wildtype or mutant ANX7. This experiment will define the region required for the biological significance of ANX7 gene expression during prostate tumorigenesis (Months 24-36).

**Task 3.** To investigate the mechanism by which ANX7 permits a pro-apoptotic calcium pulse to be emitted into the prostate cancer cell cytosol.

- a. Test whether ANX7 regulates IP3-Receptor expression in tumor cells (Months 1-13).
- b. Determine subcellular localization of ANX7 induced IP3-Receptors (Months 13-24).
- c. Study the physiological consequences of ANX7 expression on mobilization of intracellular Ca<sup>2+</sup> in DU145 prostate cancer cells expressing wildtype and mutant ANX7. These experiments will examine how overexpression or loss of ANX7 activity control the mechanism by which ANX7 permits a pro-apoptotic calcium pulse to be emitted into the prostate cancer cell cytosol (Months 13-36).

**Task 3.** To identify the signaling pathway by which ANX7 suppresses prostate cancer cell growth.

- a. Perform cDNA microarray studies on cells expressing wildtype and mutant ANX7. These studies will define the downstream targets of ANX7 that constitute the ANX7 signaling pathway by which ANX7 induces apoptosis and suppresses prostate cancer cell growth (Months 1-36).

In the first year of the grant proposal we have focused on the generation of adenovirus vectors containing wild type ANX7 and the dominant negative mutant against calcium and GTP. We investigated the anticarcinogenic effects of the altered ANX7 expression (**SOW for Task #1 and 2**) and studied the expression of IP3-Receptors (**SOW for Task #3**). We also began studies on the signaling pathway using cDNA microarray to gain possible mechanism for ANX7's inhibition of growth (**SOW for Task 4**).

In the second year of the grant proposal we have focused on the ANX7 induced apoptotic pathway involving cytochrome c release, morphological changes including cell shrinkage, nuclear fragmentation and chromatin condensation and subcellular localization using electron microscopy (**SOW for Task #1 and 2**). We investigated the effect of over-expression of ANX7 or the ANX7J mutant in DU145 cells on basal  $[Ca^{2+}]_i$  levels and IP<sub>3</sub>-generating agonist acetylcholine induced calcium response (**SOW for Task #3**). We identified the apoptosis, metastatic and cell cycle related "corrected" genes which show equivalent relative expression in PREC normal prostate cells and transfected metastatic DU145 cancer cells with ANX7J, wt-ANX7 or P53. (**SOW for Aim 4**)

### **Statement of Work. 2.a.**

**To test the hypothesis that changes in ANX7 expression levels affects apoptotic processes in cancer cells, we measured cytochrome c release in DU145 cells transfected with wt-ANX7 and the dominant-negative J mutant against the calcium binding site.**

#### **Mechanism of ANX7 induced calcium and cytochrome c release**

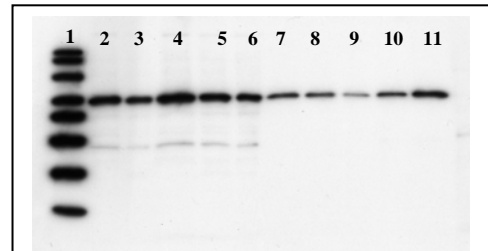
Calcium elevation is a necessary preliminary event in the initiation of the apoptotic cascade (Furuya et al, 1994; Kass and Orrenius, 1999; Scoltock et al, 2000), and compounds that recruit intracellular calcium from the endoplasmic reticulum have therefore been increasingly studied as prototype drugs for induction of apoptosis in cancer cells. An example of such a compound is thapsigargin, which arrests cancer cells in G1/G0 of the cell cycle and induces the apoptotic cascade (Furuya et al, 1994; Lin et al, 1997; Tombal et al, 2000). Thapsigargin raises cytosolic  $Ca^{2+}$  concentration by blocking the SERCA-pump ATPase on the ER, thereby blocking reuptake of calcium into the ER. As a further consequence of elevation of cytosolic free calcium, either by thapsigargin or endogenous mechanisms, plasma membrane-localized store operated calcium (SOC) channels are also activated. The latter process contributes to elevation of intracellular calcium, and thus further pushes the cell into the apoptotic cascade.

Large molecules such as proteins have been shown to have similar effects to those induced by thapsigargin. For example, exogenously added TGF $\beta$  also arrests cancer cells in G1/G0, and induces apoptosis (Furuya et al, 1996). Transfection of the ANX7 gene into the cancer cells also has a very similar sequence of actions (Srivastava and Pollard, 2004). This fact, plus the high prevalence of tumors in the Anx7(+/-) knockout mouse and disorders of calcium metabolism in Anx7(+/-) mouse tissues appear to implicate a specifically thapsigargin-like mechanism for how the ANX7 gene activates human tumor cell apoptosis. For example, in cancer cells, thapsigargin raises cytosolic calcium concentration by preventing reentry of the  $Ca^{2+}$  into the ER and activates SOC channels. Similar studies of  $Ca^{2+}$  metabolism in beta cells from Anx7(+/-) knockout mice, showed that thapsigargin failed to raise cytosolic  $Ca^{2+}$ , and failed to activate SOC channels. The ligand IP<sub>3</sub> also failed to release intracellular  $Ca^{2+}$  from the ER. So, the reason for the lack of efficacy of thapsigargin in the ANX7(+/-) knockout mouse is a documented ten-fold

deficiency in IP3 Receptors. (Srivastava et al, 1999). The importance of IP3 receptors in cancer cells is that IP3 Receptor activation by IP3 is the physiological stimulus needed to release calcium from the ER, thus triggering the mitochondrial permeability transition, which leads to apoptosis (Szalai, et al, 1999). We have therefore hypothesized that the action of the transfected ANX7 gene on tumor cells, may be to elevate cytosolic Ca<sup>2+</sup>, and to potentiate subsequent pro-apoptotic actions of the released calcium.

**Experiment:** Recent studies demonstrates that calcium released from the endoplasmic reticulum synchronizes the mass exodus of cytochrome *c* from the mitochondria, a phenomenon that coordinates apoptosis. Therefore, we examined the effects of altered exogenous ANX7 expression on metastatic prostate cancer cell line DU145. We found that the effect of the ANX7 gene on the DU145 cancer cell line enhanced the release of cytochrome *c* by 3 fold into the cytosol in comparison to vector alone control. P53 used as a positive control increased the release of cytochrome *c* by only 2 fold into the cytosol (**Figure 1**).

**Interpretation:** The ANX7 induced apoptotic pathway involves cytochrome *c* release indicating the probable involvement of mitochondria



**Figure 1:** Western Blot of Cytochrome C release from DU145 prostate cancer cell line transfected with Ad vectors. **Lanes:** 1- MagicMark Standard, 2-6 – cytosol fractions of parental control, Ad control, AdAnx7, AdJ, AdP53 relatively, 7-11 – mitochondrial fractions of parental control, Ad control, AdAnx7, AdJ, AdP53 relatively.

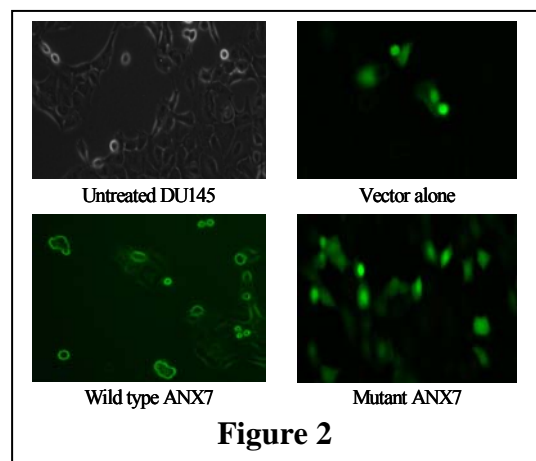
### Statement of Work. 3.b.

#### **To determine subcellular localization of ANX7 induced IP3-Receptors**

#### ANX7 over expression induces morphological changes and DNA fragmentation

It is possible that the morphological features may be altered in ANX7 transfected DU145 cells compared to vector control

**Experiment:** The cells transfected with ANX7 were morphologically different with GFP fluorescence on the membranes and look sick. On the other hand, the GFP fluorescence is distributed throughout upon vector alone or mutant ANX7J transfection (**Figure 2**). The cell shrinkage and nuclear fragmentation can be visualized by Light microscopy with toluidine stain in ANX7 transfected cells (**Figure 3**).



**Figure 2**

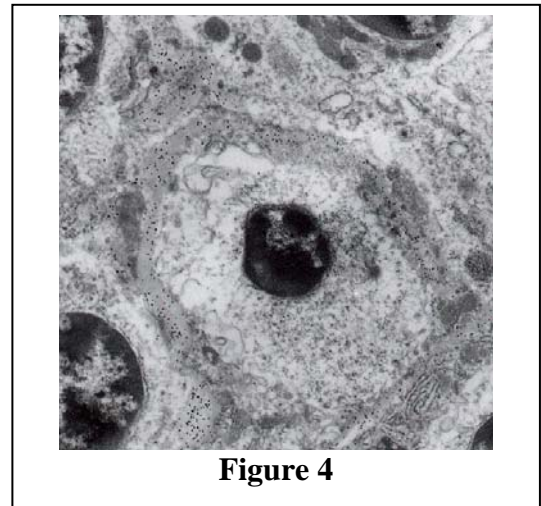
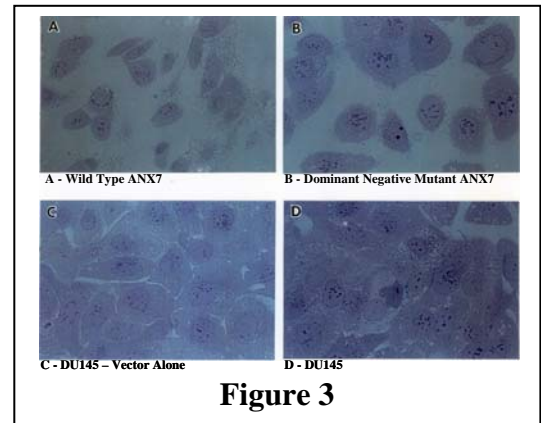
**Interpretation:** ANX7 induces morphological changes including cell shrinkage and nuclear fragmentation

EM-immunogold method to detect and quantitate ANX 7 and IP<sub>3</sub>-Receptors.

It is possible that the an<sub>x</sub>7 or IP<sub>3</sub>-Receptor localization and distribution to plasma membrane and nucleus may be altered in ANX7 transfected DU145 cells compared to vector control.

**Experiment:** we used EM-immunogold method to detect and quantitate ANX 7 and IP<sub>3</sub>-Receptors. As shown in **Figure 4**, ANX7 (large gold particles-15nm gold) and IP3 receptor (small gold particles-10nm gold) are distributed together in tumor cells not only in the plasma membrane, but also in association with secretory granule membranes. Furthermore, EM analysis revealed that ANX7 transfected cells have nuclear condensation and positive staining by TUNEL suggesting that the ANX7 gene has a role to play in inducing apoptosis (**Figure 4**).

**Interpretation:** These studies reveal that while over expression of ANX7 induces apoptosis as evidenced by nuclear condensation, there is no alteration in the localization and distribution of an<sub>x</sub>7 and IP<sub>3</sub>-Receptor expression.

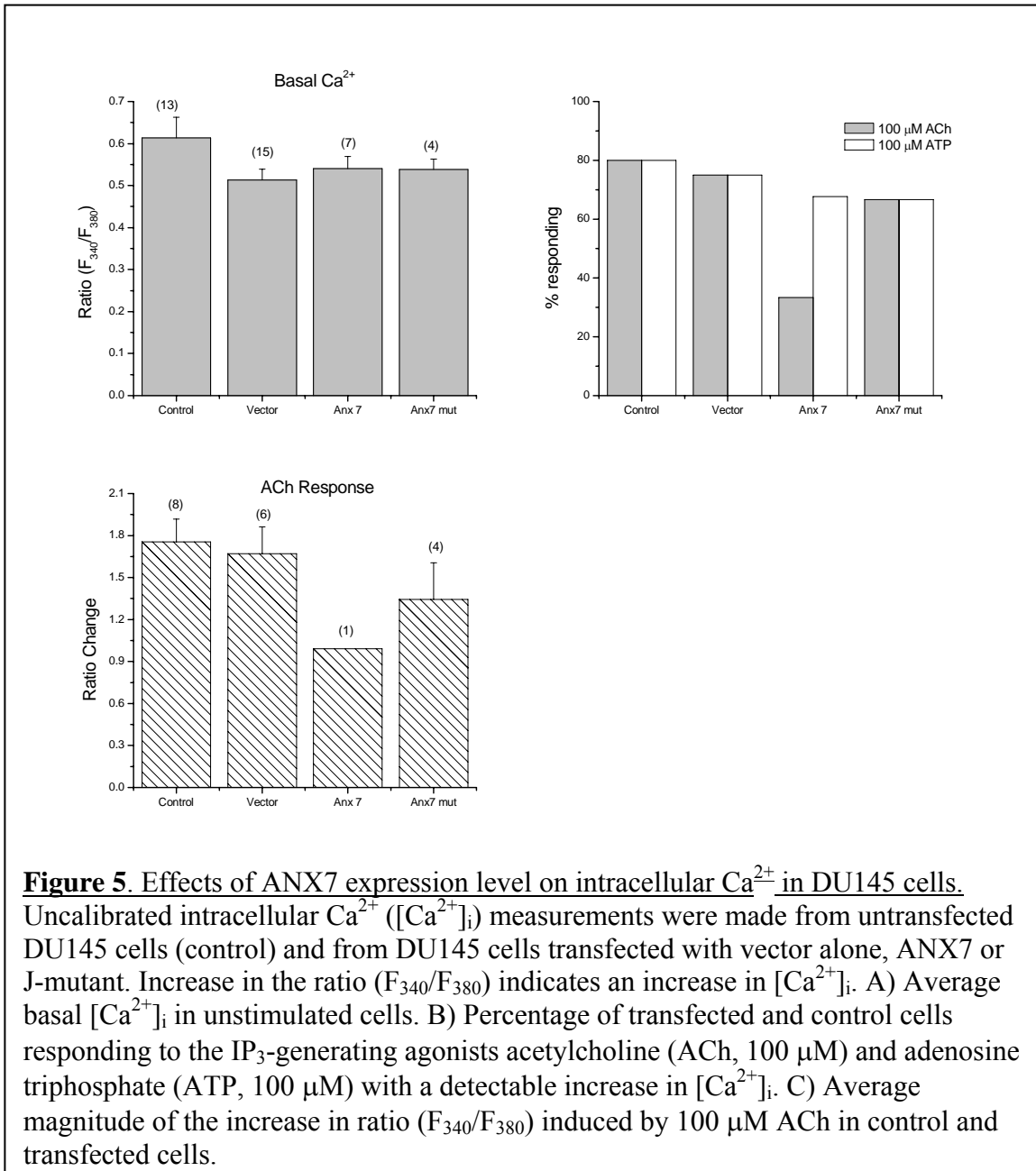


### Statement of Work. 3.c.

#### **To Study the physiological consequences of ANX7 expression on mobilization of intracellular $\text{Ca}^{2+}$ in prostate cancer cells expressing wildtype and mutant ANX7**

To test the hypothesis that changes in ANX7 expression levels alter  $\text{Ca}^{2+}$  homeostasis in cancer cells, we measured intracellular  $\text{Ca}^{2+}$  in DU145 cells transfected with wt-ANX7 and the dominant-negative J mutant.

**Experiment.** Intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) was measured microfluorometrically using DU145 cells loaded with the fluorescent  $\text{Ca}^{2+}$  indicator Fura-2. Cells on glass coverslips were loaded with the membrane-



**Figure 5.** Effects of ANX7 expression level on intracellular  $\text{Ca}^{2+}$  in DU145 cells. Uncalibrated intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) measurements were made from untransfected DU145 cells (control) and from DU145 cells transfected with vector alone, ANX7 or J-mutant. Increase in the ratio ( $F_{340}/F_{380}$ ) indicates an increase in  $[\text{Ca}^{2+}]_i$ . A) Average basal  $[\text{Ca}^{2+}]_i$  in unstimulated cells. B) Percentage of transfected and control cells responding to the  $\text{IP}_3$ -generating agonists acetylcholine (ACh, 100  $\mu\text{M}$ ) and adenosine triphosphate (ATP, 100  $\mu\text{M}$ ) with a detectable increase in  $[\text{Ca}^{2+}]_i$ . C) Average magnitude of the increase in ratio ( $F_{340}/F_{380}$ ) induced by 100  $\mu\text{M}$  ACh in control and transfected cells.

permeable form of the indicator (Fura-2-AM) during a 60-minute incubation in Hank's Balanced Salt Solution containing 2.  $\mu$ M of the dye and 0.04% pluronic acid. The coverslips were then used to form the bottom of a sample chamber that was placed on the stage of an inverted microscope and continuously perfused with Krebs solution at 37  $^{\circ}$  C. A fiber-optic based UV excitation system (DG-4, Sutter Instruments, Novato, CA) was used to excite the dye in the cells with light at 340 and 380 nm, and images of the emitted fluorescence were acquired with a digital camera under computer control. The acquisition software (Metafluor, Universal Imaging, Downingtown, PA) calculated a pixel-by-pixel ratio of the magnitude of the emitted fluorescence at the two wavelengths ( $F_{340}/F_{380}$ ), which provided an uncalibrated measurement of  $[Ca^{2+}]_i$ . Experiments were performed 12-24 hours following transfection of the DU145 cells.

**Figure 5A** shows the average basal  $Ca^{2+}$  measured from control and transfected cells (higher  $F_{340}/F_{380}$  ratio indicates higher  $[Ca^{2+}]_i$ ). The decrease in basal  $[Ca^{2+}]_i$  in the cells expressing the vector compared to controls reflects a non-specific effect of GFP on the emitted fluorescence. Expression of ANX7 or the J-mutant was not associated with changes in basal  $[Ca^{2+}]_i$  when compared to the cells expressing vector alone.

To determine the effects of ANX7 expression level on the dynamics of  $Ca^{2+}$ -sequestering intracellular stores, we measured changes in  $[Ca^{2+}]_i$  caused by the  $IP_3$ -generating agonists acetylcholine (Ach) and adenosine triphosphate (ATP). We found that the majority of control cells (80%) responded to 100  $\mu$ M Ach and 100  $\mu$ M ATP with a rapid rise in  $[Ca^{2+}]_i$  (not shown). As shown in **Figure 5B**, the percentage of responding cells was not markedly affected by expression of the vector. Interestingly, only 1 of 3 cells overexpressing ANX7 responded to Ach, while 4 of 6 cells transfected with the J mutant responded to both Ach and ATP. **Figure 5C** shows the average change in  $[Ca^{2+}]_i$  induced by Ach in the various populations of cells. Expression of the vector had no effect on the response of the cells compared to controls. However, we did note that the response to Ach was markedly smaller in the single ANX7-transfected cell that responded to the agonist. The Ach-induced change in  $[Ca^{2+}]_i$  was also slightly smaller in the 4 of 5 J-transfected cells that responded. No such differences were observed in the magnitude of the response of the various cell groups to ATP (not shown).

**Interpretation:** From these studies we are able to conclude that expression of ANX7 or the J mutant in DU145 cells does not alter basal  $[Ca^{2+}]_i$  levels. Furthermore, although these data are preliminary, they do support the hypothesis that disruption of ANX7 levels alters  $Ca^{2+}$  signaling in DU145 cells. In particular, the data suggest that overexpression of ANX7 reduces the percentage of cells that are capable of responding to the  $IP_3$ -generating agonist acetylcholine. Furthermore, overexpression of either ANX7 or the J-mutant may be associated with a reduction in the magnitude of the response to Ach. Future studies will be carried out to increase the number of observations for statistical purposes as well as to investigate the mechanisms underlying ANX7-induced changes in  $Ca^{2+}$  responses in DU145 cells.

#### **Statement of Work. 4.a.**

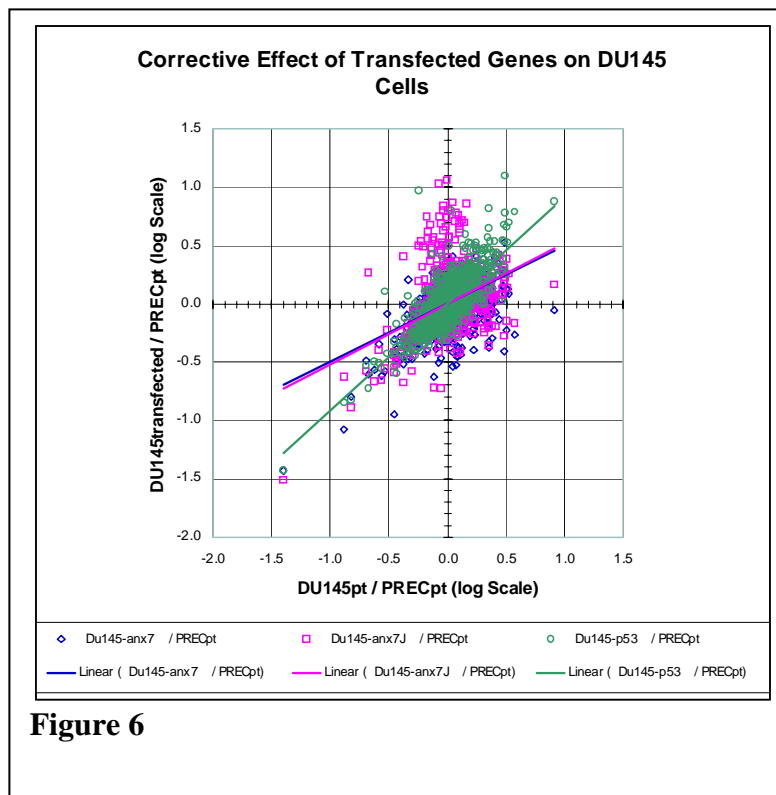
**To identify the downstream targets of ANX7 that constitute the ANX7 signaling pathway by which ANX7 causes apoptosis and suppresses breast cancer cell growth.**

**Rationale:** To investigate the underlying genomic mechanisms of wt-Anx7 action in breast cancer cells, we examined gene expression in breast cancer cells using cDNA microarrays obtained from clontech. We assessed **the downstream targets and signaling pathway** of ANX7 in apoptosis and suppression of breast cancer cell growth in the last year. In this year we compared gene expression in prostate cancer

cells with gene expression in normal cells and asked the question which of the aberrantly expressed genes have been brought back to its normal expression by the addition of ANX7, ANX7J or p53

**Experiment :** In order to assess these targets of ANX7 or p53 in prostate cancer cells, we isolated mRNA's from normal prostate epithelial cell line PREC, parental prostate cancer cell DU145, prostate cancer cell line transfected with either vector alone, wt-ANX7, dominant negative mutant ANX7J or p53 used as a positive control. We used ATLAS™ cDNA expression cancer array to obtain the expression profiles. Comparison of the transcripts between parental, ANX7J, wt-ANX7 or p53 transfected tumor cells with normal prostate epithelial cell line was carried out by GRASP algorithm.

**Table 1** gives the list of genes in metastatic DU145 cells whose expression levels were most corrected towards the respective expression levels in normal PREC prostate cell line by the effect of ANX7J, wt-ANX7 and P53. The genes are ordered according to our list of priorities for validating their involvement in wt-ANX7 induced effects on prostate cancer progression. These data are shown graphically in **Figure 6**, which shows the expression levels in DU145 cells (as log ratio to expression in PREC normal prostate cell line) after treatment with ANX7J, wt-ANX7, P53 or vector alone. The x-axis shows the average expression level of the respective genes in the metastatic DU145 and PREC cells. The transfected DU145 cells are depicted with majenta diamonds (for ANX7J), blue diamonds (for wt-ANX7) and green diamonds (for P53).The improvement in the expression levels is shown by the movement of the expression in the transfected cells towards the x-axis. The movement of a gene towards the x-axis means that it tends to show equivalent relative expression in PREC normal cells and transfected metastatic DU145 cancer cells with ANX7J, wt-ANX7 or P53.



**Figure 6**

**Interpretation:** The data thus indicate that ANX7 or p53 treatment of metastatic DU145 cells makes them greatly resemble PREC normal cells. There is not much difference between ANX7J transfection in correcting the aberrant gene expression in cancer cells. This means that we need to use other mutants against GTP and PKC or combination of these in order to differentiate the requirements of ANX7 action. On top of the lists are genes related to apoptosis and tumor suppression (S6KII-alpha 3 and bak) and cell cycle (CDC25C), and metastasis and invasiveness (Integrins, TGF-beta2, c-myc and mmp-9).

Methods:

**Table 1:** List of Genes that are most effected by transfection of ANX7, ANX7J or P53 in terms of reverting back to expression levels in normal prostate cells.

Gene Name	Corrective Effect of :		
	ANX7	ANX7J	P53
ribosomal protein S6 kinase II alpha 3 (S6KII-alpha 3); ribosomal S6 kinase 2 (RSK2); insulin-stimulated protein kinase 1 (ISPK1)	3.5	1.6	-0.1
bcl2 homologous antagonist/killer (BAK)	-0.2	0.2	3.3
MHC class II HLA-DR-beta (DR2-DQW1/DR4 DQW3) precursor	0.1	0.1	3.1
B4-2 protein	2.8	1.5	0.5
integrin alpha E precursor (ITGAE); mucosal lymphocyte-1 antigen; hml-1 antigen; CD103 antigen	2.7	0.0	-0.1
integrin alpha 8 (ITGA8)	2.5	0.7	-0.1
DNA fragmentation factor 45 (DFF45)	2.5	0.4	0.9
hyaluronan receptor (RHAMM)	2.5	0.0	-0.2
CDC25C; M-phase inducer phosphatase 3	2.4	-0.1	-0.2
cadherin 6 precursor (CDH6); kidney cadherin (K-cadherin)	2.4	0.4	0.1
cation-independent mannose-6-phosphate receptor precursor (CI man-6-P receptor; CI-MPR); insulin-like growth factor II receptor (IGFR II)	0.9	-0.1	2.4
clone PO2ST9 (brain striatum)	-0.2	-0.3	2.3
sonic hedgehog (SHH)	2.1	0.0	0.1
transforming growth factor beta2 precursor (TGF-beta2; TGFB2); glioblastoma -derived T-cell suppressor factor (G-TSF); bsc-1 cell growth inhibitor; polyergin; cetermin	2.1	0.3	0.2
c-myc oncogene	0.0	0.0	2.0
HLA-DR antigen-associated invariant subunit	0.1	2.0	-0.2
heparin-binding growth factor 2 precursor (HBGF2); prostatropin; basic fibroblast growth factor (BFGF; FGFB; FGF2)	0.9	-0.7	2.0
ras-like protein TC10	2.0	0.5	-0.1
interferon-induced guanylate-binding protein 1; guanine nucleotide-binding protein 1	-0.5	1.9	-0.1
skeletal muscle phosphorylase B kinase gamma catalytic subunit	1.9	-0.1	-0.2
interleukin-7 (IL-7)	0.1	1.9	-0.2
cyclin-dependent kinase regulatory subunit (CKS2)	1.9	0.5	1.1
eukaryotic translation initiation factor 3 beta subunit (EIF-3 beta); EIF3 P116	-0.1	-0.2	1.9
semaphorin; CD100	1.9	0.3	-0.3
ephrin type-B receptor 4 precursor; tyrosine-protein kinase receptor HTK	0.9	1.9	0.3

### Preparation and labeling of RNA

Total RNAs from breast cancer cells and normal cells transfected with either vector alone or ANX7, ANX7J or p53 as a positive control were prepared by the method of Champenski et al. and were subjected to DNase 1 digestion to eliminate genomic DNA contamination. Two rounds of purification of poly A+ RNA from total RNA were performed using the mRNA isolation kit from Invitrogen as recommended by the manufacturer. The quality of the RNA were tested by running a formaldehyde denatured agarose gel and quantitated by measuring the optical density at 260nm. A <sup>32</sup>P labeled cDNA probe was synthesized from 1 µg of poly A+ RNA from control and tumor samples using MMLV reverse transcriptase, dNTP mix and CDS primer mix comprising the oligonucleotide sequences for the 1200 cancer related genes spotted on the atlas cDNA microarray. The reaction was carried out in a thermocycler set at 50°C for 25 min. and terminated by the addition of 0.1M EDTA, pH 8.0 and 1mg/ml glycogen. The <sup>32</sup>P labeled cDNA probe was then purified from unincorporated <sup>32</sup>P labeled nucleotides by using a CHROMA SPIN-200 column (clonetech) as recommended by the manufacturer. The human atlas cDNA expression array containing 1200 cancer related genes on a nylon membrane was prehybridized

using Express Hyb (clonetech) at 68°C for 1 hr and hybridized overnight at 68°C with the denatured and neutralized <sup>32</sup>P labeled cDNA probe. The membrane was washed three times with 2 X SSC, 1% SDS at 68°C for 30 min. each and twice with 0.1% SSC, 0.5% SDS at 68°C for 30 min. each. The atlas array will be exposed overnight and the results will be compared with the known distribution of genes.

Imaging and quantitation of the cDNA microarray: Imaging data from the Storm PhosphorImager were downloaded into a Microsoft Excel spreadsheet. Duplicate data points were ratio'ed to the ubiquitin standard. Data were then analyzed using the Stanford University ScanAlyze software. These data were also evaluated in parallel with the PSCAN program for point identification and with the JMP program for graphical organization.

Statistical Data mining from cDNA arrays: The first strategy we employed is embodied in the GRASP methodology (Gene Ratio Analysis Paradigm, Srivastava et al, 1999). The GRASP algorithm allows us to specify the changes in specific intensities of given genes which are greater or less than one standard deviation (S.D.) from the average changes of all genes in the entire array. This technique vastly increases the statistical power of the analysis.

## KEY RESEARCH ACCOMPLISHMENTS

- The ANX7 induced apoptotic pathway involves cytochrome c release indicating the probable involvement of mitochondria.
- ANX7 induces morphological changes including cell shrinkage and nuclear fragmentation.
- Over expression of ANX7 induces apoptosis as evidenced by chromatin condensation.
- Overexpression of ANX7 or the ANX7J mutant in DU145 cells does not alter basal  $[Ca^{2+}]_i$  levels. However, overexpression of ANX7 reduces the percentage of cells that are capable of responding to the  $IP_3$ -generating agonist acetylcholine. Furthermore, overexpression of either ANX7 or the ANX7J-mutant may be associated with a reduction in the magnitude of the response to acetylcholine.
- We identified the apoptosis, metastatic and cell cycle “corrected” genes which show equivalent relative expression in PREC normal prostate epithelial cells and transfected metastatic DU145 cancer cells with *ANX7J*, wt-*ANX7* or P53.

## REPORTABLE OUTCOME

1. We showed that regulating ANX7 levels involves apoptotic events and calcium response. In addition, we identified the apoptosis, metastatic and cell cycle “corrected” genes which show equivalent relative expression in PREC normal prostate epithelial cells and transfected metastatic DU145 cancer cells with *ANX7J*, wt-*ANX7* or P53 for its therapeutic use.

- The results were presented in USUHS research day, 2004 as part of the plenary session talk and in the poster

Meera Srivastava, Ofer Eidelman, Shanmugam Naga, Katya Mezhevaya, Ximena Leighton, Goping, G. and Harvey B. Pollard. (2004) ANX7 signaling pathway in prostate cancer

- The results were also presented in the keystone symposia in Colorado on the “Roles of TGF- $\beta$  in Disease Pathogenesis: Novel Therapeutic Strategies, 2005.

Meera Srivastava, Mirta Glasman, Katya Mezhevaya, Shanmugam Naga, Chang-Hyun Yun, Harvey B. Pollard and Seong-Jin Kim. (2005) ANX7 Activates TGF- $\beta$  Smad-Dependent Transcription. A molecular link between calcium and tumor suppression in prostate cancer

The results formed the preliminary data for the grant, “ANX7 as a molecular target for prostate cancer” that I submitted to NIH on Feb. 1<sup>st</sup>

## CONCLUSIONS

The ANX7 induced apoptotic pathway involves calcium and cytochrome c release indicating the probable involvement of mitochondria. ANX7 induces morphological changes including cell shrinkage, nuclear fragmentation and chromatin condensation. Overexpression of ANX7 or the ANX7J mutant in DU145 cells does not alter basal  $[Ca^{2+}]_i$  levels. However, overexpression of ANX7 reduces the percentage of cells that are capable of responding to the  $IP_3$ -generating agonist acetylcholine. Furthermore, overexpression of either ANX7 or the ANX7J-mutant may be associated with a reduction in the magnitude of the response to acetylcholine. We identified the apoptosis, metastatic and cell cycle “corrected” genes which show equivalent relative expression in PREC normal prostate cells and transfected metastatic DU145 cancer cells with *ANX7J*, wt-*ANX7* or P53.

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