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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> The purpose of this study has been to determine the efficacy of combined therapy using vitamin D binding protein-macrophage activating factor (DBP-maf) and vitamin D as therapy for prostate cancer using a mouse model of human prostate tumors. In this phase of the study we have tested the ability of vitamin D and DBP-maf to inhibit the formation of endothelial cells into tubes. Both molecules were effective on their own, however, the combination of vitamin D and DBP-maf showed synergistic behavior. Because of its high affinity for actin, the possibility that DBP-maf acted by inhibiting microtubule formation was investigated using an anti-phalloidin antibody. No differences in phalloidin strength or localization were observed. Using immunoassays we were able to identify a 75 kDa protein in tumor cells that is phosphorylated by DBP-maf and a synthetic maf peptide. We are investigating the identity of that protein since the mechanism by which DBP-maf works remains unknown.					
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## Introduction

We have continued to characterize the effect of DBP-maf on both endothelial and prostate tumor cells. Our initial results with DBP-maf demonstrated antiangiogenic activity using a pancreatic tumor model. Previous work by another group had identified antitumor activity and so we have proceeded with the assumption that DBP-maf has two targets in the tumor microenvironment, the tumor and the supporting blood vessels. The in vitro studies have been undertaken to characterize the basis of these activities.

Our first objective was to measure and characterize the direct effects of individual and combination therapy of vitamin D and DBP-maf in endothelial and prostate cancer cells.

## Body

### Preparation of DBP-maf

Due to the unfortunate and untimely death of our collaborator, Narasimha Swamy, whose role in this work was to supply vitamin D binding protein-macrophage activating factor (DBP-maf) for our assays, we were required to generate the protein in our own laboratory. This has slowed down our efforts considerably, since there are no commercial sources of the protein. We have, however, been successful in generating the protein as demonstrated in Figure 1 and by our subsequent data. In addition to the use of the full length protein we have added to our studies a peptide of the protein, which has been shown to have biological activity in other applications. If this peptide shows efficacy it would make the synthesis of the DBP-maf much easier and cheaper than the current process.

We have refined the process of DBP-maf generation as follows:

#### Coupling of (1-3) $\beta$ -D-galactosidase to cyanogen bromide-activated agarose

Note: Type VI-A neuraminidase-agarose (*clostridium perfringens*) was purchased from Sigma.

1. Cyanogen bromide-activated beads (0.5 g) were washed with 1 mM HCl (3X, 10 ml per wash) using suction filtration.
2. The beads were then washed with DDI water and resuspended in 2 ml of coupling buffer (0.1 M  $\text{HNaCO}_3$ , 0.5 M NaCl, pH 8.3).
3. (1-3) $\beta$ -D-galactosidase (1000 units) was added to the beads/coupling buffer and then shaken with an over and under mixer for 2 hours at room temperature.
4. The beads were then washed with coupling buffer and resuspended in blocking buffer (coupling buffer plus 0.2 M glycine). The suspension was shaken overnight at 4 ° C.
5. The beads were then washed with coupling buffer followed by 0.1 M sodium acetate, 0.5 M NaCl, pH 4.0, and the washes were repeated for a total of four times. Finally, the beads were washed with a final wash of coupling buffer.
6. The beads were then spun down and resuspended in 1.0 M NaCl.

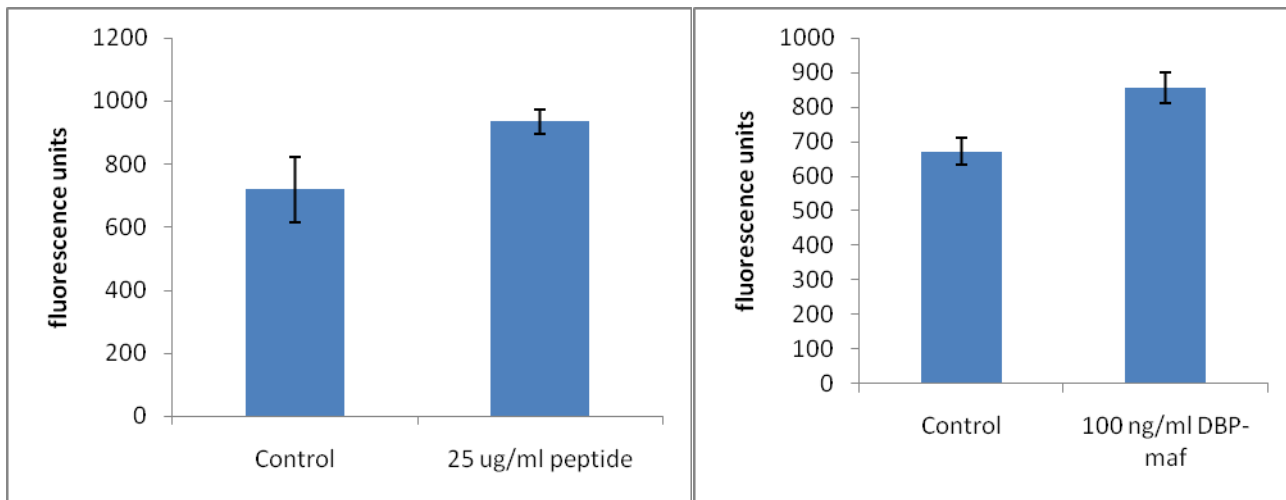
#### Determination of activity (1-3) $\beta$ -D-galactosidase-agarose beads

1. A volume of 50  $\mu\text{l}$  of the bead suspension was added to 0.95 ml of assay buffer/chromogenic substrate (PBS, 3 mM 2-nitrophenyl- $\beta$ -D-galactopyranoside, 10 mM  $\text{MgCl}_2$ , 0.1 mM  $\beta$ -mercaptoethanol), and the suspension was shaken at room temperature for 15 min.
2. The reaction was stopped with 33  $\mu\text{l}$  of 1 M sodium carbonate, and the absorbance was measured at 405 nm.
3. Activity was expressed as units/ml of bead suspension, where 1 unit is defined as the number of  $\mu\text{moles}$  of p-nitrophenol formed per minute. A molar absorptivity of 18380 L/mole cm, and a path length of 0.25 cm corresponding to a 200  $\mu\text{l}$  volume in a 96 well plate were used to calculate the concentration of p-nitrophenol.

#### Synthesis of DBP-maf

1. DBP was added to a final concentration of 0.05 mg/ml in PBS pH 6.0, 10 mM  $\text{MgCl}_2$ , with 0.007 U of (1-3)  $\beta$ -D-galactosidase-agarose and 0.004 U of neuraminidase-agarose, and shaken at room temperature for 4 hours. The total reaction volume was 1 ml.
2. Beads were then spun down and removed. DBP MAF was stored in aliquots at -20 ° C.

As shown in Figure 1, we were able to demonstrate macrophage activity using either the DBP-maf full-length protein or the peptide. Macrophage activity was assessed by oxidation of reduced dihydrofluorescein diacetate by superoxide (excitation: 485 nm emission:530 nm).



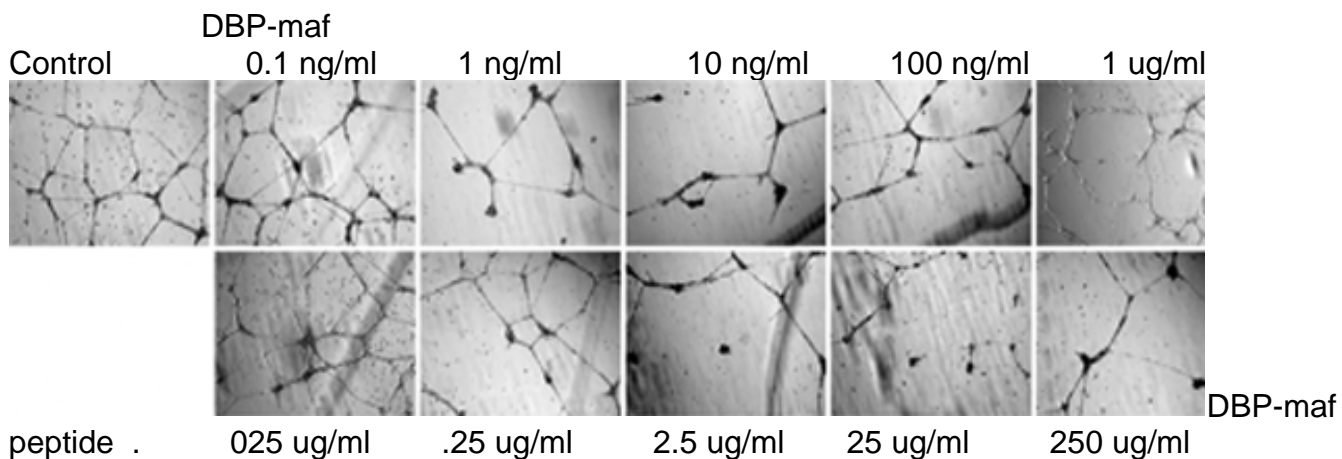
**Figure 1.** DBP-maf stimulates macrophage activity.

**DBP-maf and vitamin D will be tested singly and in combination to determine the effects on endothelial and prostate cancer cells in vitro.**

Endothelial cells use the processes of sprouting, degradation of matrix, migration, proliferation and organization into three dimensional structures to form blood vessels in vivo. Tumor cells, in order to grow and metastasize require matrix degradation and proliferation. Our previous work in this study showed inhibition of LnCaP cell proliferation with both DBP-maf and low concentration (.01 nM) vitamin D, although the effects did not appear to be synergistic or additive. Surprisingly, human endothelial cells showed little response to DBP-maf. Migration assays demonstrated an additive inhibition of DBP-maf and vitamin D. The maf peptide that we have been testing showed less inhibition of proliferation but greater inhibition of migration, also demonstrating an additive effect. We then began to study the effects of these molecules in an endothelial tube formation assay which incorporates a combination of migration and three dimensional organization.

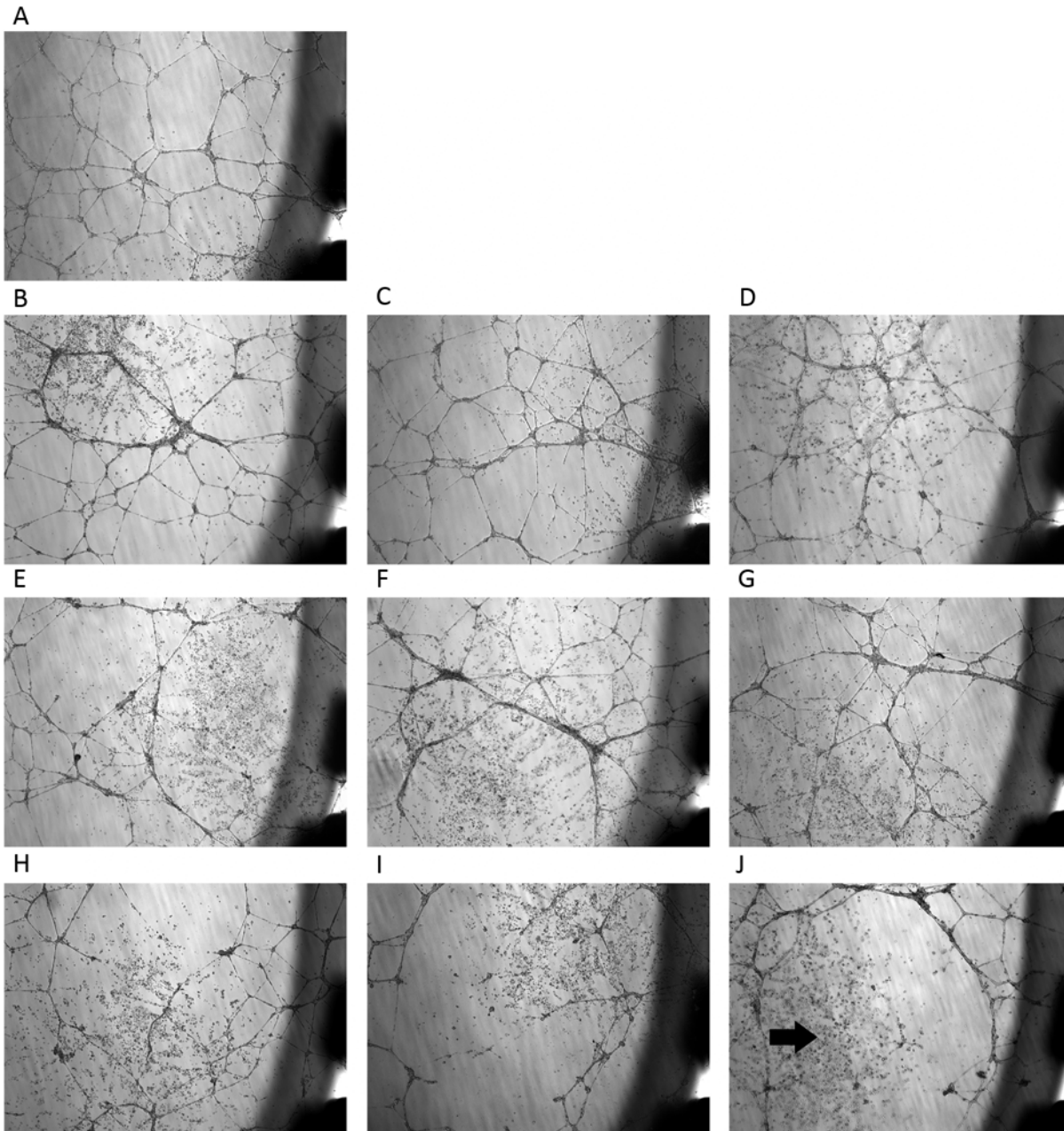
**HUVEC tube formation assay**

A well published and often used measure of antiangiogenic molecules has been their potential to disrupt the spontaneous organization of endothelial cells into tubes when cells are plated on MATRIGEL™, a matrix of the Engelbreth-Holm Swarm mouse tumor. This assay involves both migration of cells and organization into endothelial tubes that have three dimensional structure. As shown in Figure 2, DBP-maf, at low concentration (1-100 ng/ml) effectively disrupted the normal tube formation of HUVEC in this assay and at higher concentration (1 ug/ml) became less effective, suggesting a reversal of the effect. The peptide showed similar disruption but at concentrations much higher than the full length protein (.25-250 ug/ml) and showed no reversal of the effect at higher dose. It is not uncommon for molecules to demonstrate a bell shaped curve that correlates activity with an optimal dose range. Interestingly, the effect on proliferation and migration suggested that higher doses were more effective, whereas the tube formation shows an optimal effect at lower concentrations. This is similar to the original in vivo studies that showed efficacy at very low (ng/kg body weight) concentrations. The maf peptide, although showing inhibitory activity, showed a dose response curve that demonstrated increasing effect with increasing concentration. These results would suggest a more complex role for DBP-maf than is evident in assays measuring a single mechanism.



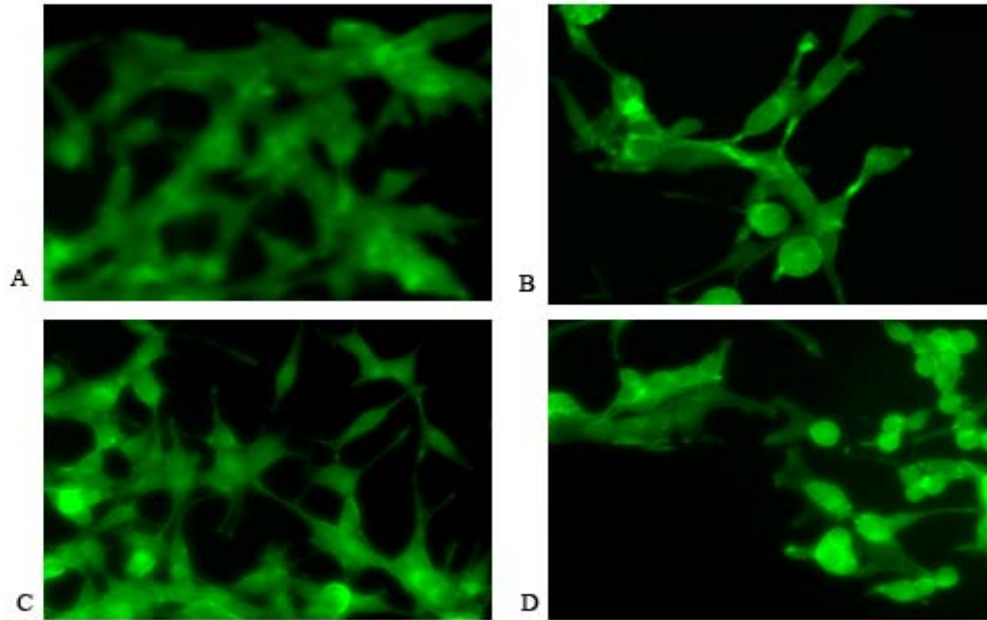
**Figure 2.** DBP-maf inhibits endothelial cell tube formation. HUVEC were plated at a density of 100,000 cells/well in 24 well plates in Medium 199 with 10% FBS +/- DBP-maf or DBP-maf peptide at indicated concentrations. Cells were incubated at 37° C for 12 hours then photographed under a microscope (4x).

When vitamin D was added to the tube formation assay at 10, 100, and 1,000 pM, little effect was seen on tube formation (Figure 3, B,C,D). However, when the combination of either DBP (E,F,G) or DBP-maf (H,I,J) was added with vitamin D there was reduced tube formation. Whereas, DBP-maf alone showed reduced tube formation (Figure 2) with no observed apoptosis or toxicity, in combination with vitamin D there was a significant population of cells that appeared to be dead or dying, although still adhered to the matrix. The fact that this was not observed with vitamin D alone but was also observed with DBP, which by itself had no effect on tube formation, suggests that the potency of vitamin D was amplified by its association with DBP or DBP-maf. Perhaps its association with these binding proteins makes a more efficient moiety for cell surface binding.

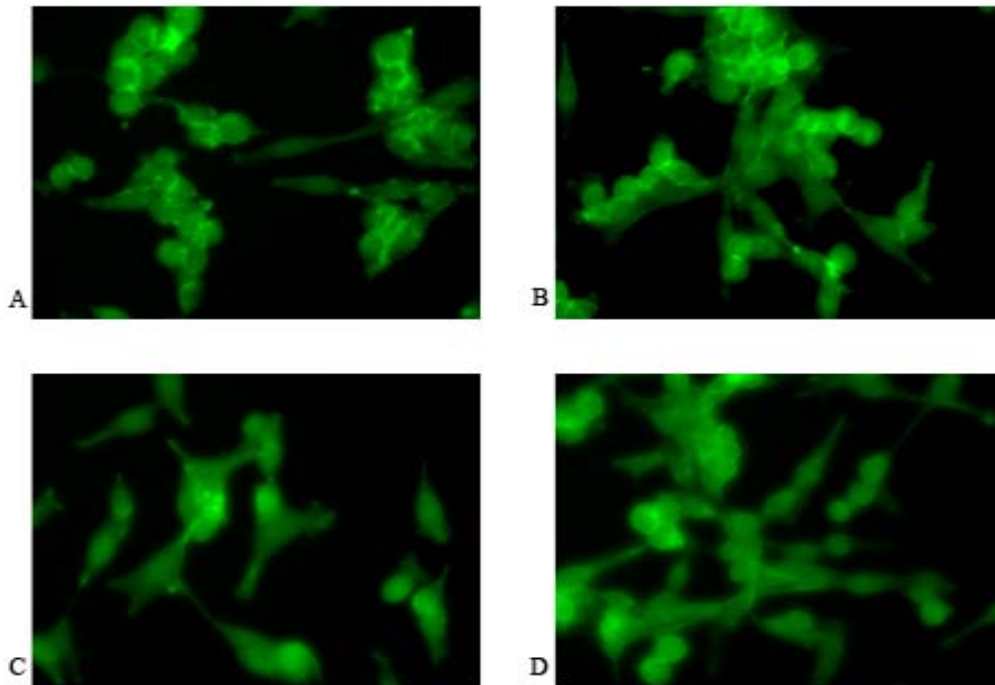


**Figure 3.** DBP and DBP-maf show a synergistic effect on HUVEC tube formation. Vitamin D was added at 10 pM(B,E,H), 100 pM (C,F,I), or 1 nM(D,G,J) to HUVEC culture on MATRIGEL. DBP (E,F,G) or DBP-maf (H,I,J) was added at 100 ng/ml. Arrow in J points to single cells adhered to MATRIGEL.

Our previous results have shown that DBP-maf is able to inhibit the migration of LnCaP cells. We hypothesized that this inhibition involved changes in the actin cytoskeleton since DBP-maf has been shown to have a strong affinity to actin. This possibility was investigated using a phalloidin antibody. LnCaP, LnCaP LN3 cells and HUVECs were plated on tissue culture slides and probed with an anti-phalloidin antibody. The small amount of cytoplasm in the LnCaP (figure 4) and LnCaPLN3

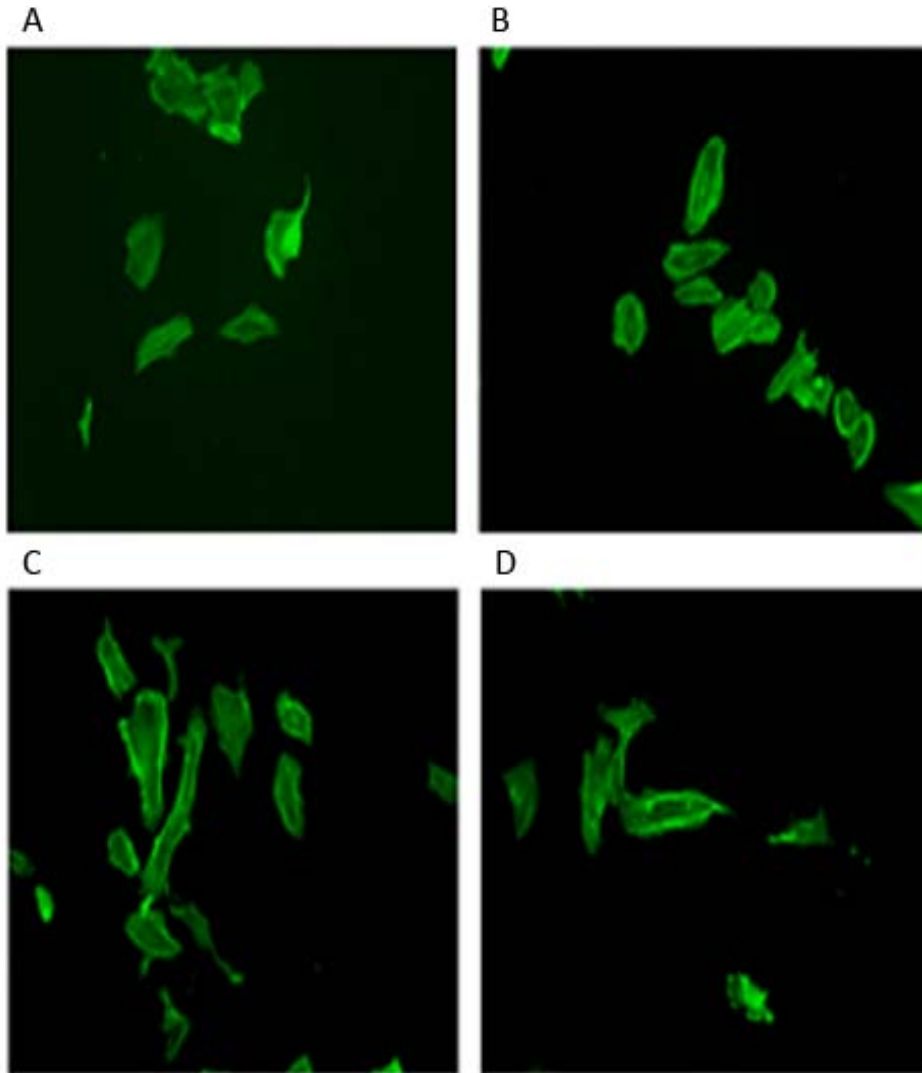


**Figure 4.** DBP-maf effect on LnCaP actin filament formation. LnCaP cells were plated on slides and treated for 4 hours with or without DBP-maf ( A, control; B, 1 ng/ml; C, 10 ng/ml; D, 100 ng/ml). An anti-phalloidin antibody was applied and a secondary antibody that was FITC conjugated.



**Figure 5.** DBP-maf effect on LnCaPLn3 actin filament formation. LnCaPLn3 cells were plated on slides and treated for 4 hours with or without DBP-maf ( A, control; B, 1 ng/ml; C, 10 ng/ml; D, 100 ng/ml). An anti-phalloidin antibody was applied and a secondary antibody that was FITC conjugated.





**Figure 6.** DBP-maf effect on HUVEC actin filament formation. HUVEC cells were plated on slides and treated for 4 hours with or without DBP-maf ( A, control; B, 1 ng/ml; C, 10 ng/ml; D, 100 ng/ml). An anti-phalloidin antibody was applied and a secondary antibody that was FITC conjugated.

### Signaling assays

Although activity has been demonstrated with both DBP-maf and the peptide, it is still not known what mechanism underlies this activity. Since many cell signaling pathways are based on tyrosine phosphorylation we performed assays to measure the effect of DBP-maf on overall cell phosphorylation. LnCaP parental and LnCaP LN3 cells were treated with either DBP-maf or the peptide and, after a 15 minute incubation, harvested cell lysates were electrophoresed and immunoblotted with an anti-phosphotyrosine antibody. As shown in Figure both DBP-maf and peptide blocked the phosphorylation of a band with the approximate molecular weight of 75 KDa. This effect was seen both in the parental LnCaP line (odd lanes) and the metastatic line (even lanes). We are currently attempting to identify this protein.

Lane                    1                    2                    3                    4                    5                    6



**Figure 7.** DBP-maf inhibits tyrosine phosphorylation in LnCaP cells. LnCaP parental and LN3 lines were serum starved overnight then treated with or without DBP-maf (100 ng/ml) or maf peptide (25 ug/ml) for 15 minutes. Cells were lysed in HTG buffer, electrophoresed and immunoblotted using an anti-phosphotyrosine antibody and visualized using an HRP-linked secondary antibody. Lanes were normalized to protein levels. **Lane 1**, LnCaP control; **Lane 2**, LnCaP LN3 control; **Lane 3**, LnCaP with DBP-maf; **Lane 4**, LnCaP LN3 with DBP-maf; **Lane 5**, LnCaP with peptide; **Lane 6**, LnCaP LN3 with peptide.

Determination of optimal concentrations will be made and analysis of the effect of each drug on the binding and signaling of the other. This phase will determine the initial conditions of the in vivo phase.

We have begun our initial in vivo work by implanting orthotopic prostate tumors in mice. Our efforts in this aim have just begun. We will use the doses of vitamin D and DBP-maf that we have determined to be effective and non-toxic in our in vitro studies.

### **Key Research Accomplishments**

One of the first results of interest observed during the characterization of the parental and metastatic prostate cancer lines was the differential response of these cells to similar stimuli. We are collecting more data and preparing a manuscript to describe these differences since they could provide valuable information as to some of the fundamental changes in cell expression that promote metastasis. Since most cancer patients die, not from their primary tumor, but from metastases, this could aid in the design of new therapeutics. We have also demonstrated a synergistic activity between DBP-maf and vitamin D in our in the ability to inhibit endothelial cell tube formation, a crucial step in the growth of new blood vessels.

### **Reportable outcomes**

We are currently collaborating on two prostate cancer funding opportunities based on work supported by this award, an American Cancer Society grant and a Department of Defense grant, both pending. In addition, our initial studies provided weight to our hypothesis of the role of circulating molecules and helped us to be awarded an NIH R01 grant (R01 HL086644-01) to study the regulation of vascular growth by circulating proteoglycans.

### **Conclusions**

Prostate cancer is refractory to traditional chemotherapy. It is, therefore, important to develop new therapeutic strategies. Our work has shown an ability of DBP-maf to inhibit processes crucial to both endothelial and prostate tumor cell activity. Further, we have shown that in combination with vitamin D there is a synergistic inhibition of endothelial tube formation. Although we have not yet discovered the mechanism by which DBP-maf inhibits cellular processes we have found a signaling clue in our phosphorylation experiments that we are pursuing.

### **So What?**

Although vitamin D has been shown to be an effective agent against prostate cancer, the doses needed to be effective are generally toxic due to hypercalcemia. A molecule which, when administered with vitamin D, can amplify its therapeutic effect and have additional antiangiogenic

effects could be an effective means to treat prostate cancer. DBP-maf is a natural protein found at low concentrations in blood. We have shown that a synthesized peptide from the parent protein also shows some inhibitory activity. This is valuable in terms of manufacturing a simple and cost-effective synthetic molecule.