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TITLE: The Effect of eIF4E Antisense RNA Expression on Prostate Tumor Angiogenesis and Growth

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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) We set out to investigate the role of the translation initiation factor eIF4E in prostate cancer angiogenesis and to determine if suppression of eIF4E could have clinical benefit. This is highly relevant to both early and late stages of prostate cancer, for tumor angiogenesis is critical to primary tumor growth, as well as metastasis. We found elevated eIF4E levels in a significant number of prostate tumors where it co-localized with both increased vascular endothelial growth factor (VEGF) and vessel density. We set up cell line models and saw that prostate cancer cells overexpressing eIF4E had higher levels of secreted VEGF and grew faster than control cells when injected into mice, while prostate cancer cells expressing an eIF4E anti-sense RNA (and thus with lower eIF4E levels), had depressed VEGF levels and did not grow in the mouse model. Unlike other cell types, however, in prostate cells, eIF4E expression was not associated with any change in the expression pattern of isoforms of FGF-2 or VEGF. In addition, eIF4E expression did not appear to be correlated with p53 status, however, we are continuing to investigate whether levels of p53 protein may be in part regulated at the level of translation.				
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INTRODUCTION

The purpose of this proposal was to investigate the role of the translation initiation factor eIF4E in prostate cancer angiogenesis. Since other groups had reported that high levels eIF4E protein conferred “oncogenic properties” on other tumor types, e.g., breast, bladder, and head and neck, we sought to determine if the same would hold true for prostate cancers. In those tumors, elevated levels of eIF4E increased the production of protein from many mRNA with complex 5' untranslated regions. The complex sequences of the mRNA looped into bulky secondary structures that were energy-inefficient templates for translation, and were thus poorly translated to protein. Many of the mRNA affected by eIF4E levels were known oncogenes, cell cycle regulators, and most importantly to our hypothesis, angiogenic growth factors. If eIF4E were elevated in prostate tumors as well, causing the same mRNA to be translated more efficiently, we hypothesized that eIF4E could be an interesting candidate gene to study. As a positive regulator of important angiogenic growth factors, eIF4E becomes a target for therapeutic intervention whereby eIF4E suppression might have potential for clinical benefit.

This is highly relevant to both early and late stages of prostate cancer, for tumor angiogenesis is critical to primary tumor growth, as well as metastasis. In fact, we found elevated eIF4E levels in a significant number of prostate tumors where it co-localized with both increased vascular endothelial growth factor (VEGF) and vessel density. We set up cell line models to test the effects of eIF4E modulation and saw that prostate cancer cells overexpressing eIF4E had higher levels of secreted VEGF and grew faster than control cells *in vitro* and when injected into mice, while prostate cancer cells expressing an eIF4E anti-sense RNA (and thus with lower eIF4E levels), had depressed VEGF levels and did not grow in the mouse model. Unlike other cell types, however, in prostate cells, eIF4E expression was not associated with any change in the expression pattern of isoforms of the angiogenic growth factors FGF-2 or VEGF. In addition, eIF4E expression did not appear to be correlated with p53 status, as eIF4E had virtually the same function in all cell lines tested. We are, however, continuing to investigate whether levels of p53 protein may be in part regulated at the level of translation by eIF4E.

BODY

Research accomplishments by Aim:

1. Construct retroviral vectors

Purpose: The purpose of this aim was to provide a method of delivery for the antisense eIF4E that would be versatile *in vitro* and *in vivo*.

Accomplishment: A retrovirus model was chosen to ensure long-term expression of the antisense oligonucleotide, as it would incorporate into the host cell DNA. In collaboration with our co-investigator, Michael Mathis, we generated 4 retroviruses that contained either the β -galactosidase gene (β -gal) as a positive control vector, eIF4E in the sense orientation, eIF4E in the antisense orientation, or an “empty” control (negative, promoter only control) vector.

2. Infection of cells and growth assays

Purpose: These viruses were used to infect cultured cells in order to develop stable expressing cells for further studies.

Accomplishments: Stable sublines of PC-3, DU 145, and ALVA 101 were generated. The cells were tested for expression of the eIF4E construct after antibiotic selection for those cells with a viral construct incorporated into the DNA. Pools of cells infected with the “sense virus” had approximately 3-fold higher levels of eIF4E and those infected with the “antisense virus” had approximately the same level of eIF4E suppression (Figure 1). These cells were used in *in vitro* studies outlined in Aims 3 and 4, as well as in the animal studies outlined in Aim 5 below.

Problems: The infectivity of the virus was rather low and did not have any real advantage over traditional (and less complex and time-consuming) transfection methods. We performed the experiments as outlined in the statement of work using the retroviral vectors, but we ultimately went to other methods of delivery in parallel experiments. Generation of eIF4E sense and antisense adenoviruses, also made in collaboration with our co-investigator Michael Mathis, *are still underway*. The adenoviruses have some advantages over the retroviruses, including

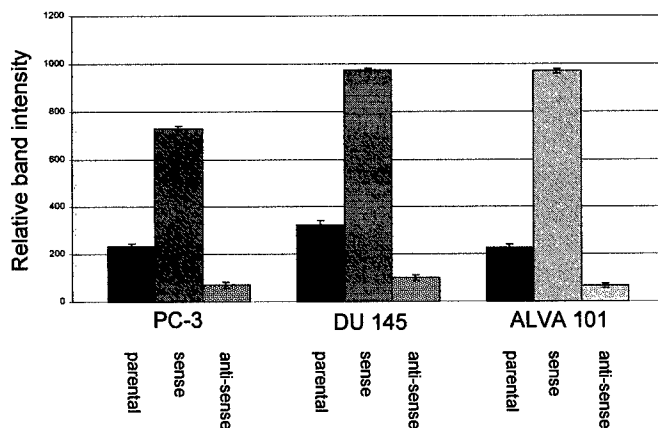


Figure 1. Relative eIF4E protein levels in parental, sense, and antisense expressing sublines of PC-3, DU 145, and ALVA 101 cells. The relative intensity of densitometric bands was measured for each condition. Approximately 20 µg of total protein was electrophoresed, blotted to PVDF membrane, and hybridized with a monoclonal anti-eIF4E antibody (Transduction Laboratories, Lexington, KY).

higher levels of infectivity, infection of non-replicating cells, and usefulness in pre-formed tumors. Their major limitation is that they are only appropriate for transient eIF4E gene expression. Because of the overall success of the antisense approach, however, we plan to continue investigation of eIF4E antisense delivery after termination of this grant.

Alternative Approaches Used and Accomplishments: As an alternative non-viral antisense delivery mechanism we investigated the use of an antisense oligonucleotide made from non-ribose bases. The oligonucleotide backbone is made up of bases with morpholine ring structures. These have distinct advantages over ribose-containing oligonucleotides as they are resistant

to degradation via the normal Nuclease H pathway. We used this morpholino eIF4E antisense oligonucleotide in a study with similar results to those obtained using the retroviral vector presented below in Aim 4.

3. Utilization of FGF-2 alternative translation start sites (the HUVEC model)

Purpose: The purpose of this aim was to test the angiogenic potential of an FGF-2 alternative isoform potentially expressed in response to eIF4E elevation. In previous studies our collaborator, Ricky DeBenedetti, had conducted similar studies but using a different (non-tumor) cell line model in which elevation of eIF4E was shown to shift translation of FGF-2 protein to an alternative form (Kevil *et al.*, 1995). This alternative FGF-2 form was more potent in causing proliferation and differentiation of HUVEC (endothelial) cells into "capillary-like structures" (CLS). The shift to a secondary FGF-2 form was easily identified in western blots as a change in intensity of a lower molecular weight band. In their model, treatment of HUVEC cells with conditioned media from eIF4E overexpressing cells (i.e., with high levels of the alternative FGF-2 form) resulted in significantly more CLS formation. Their model cells (CHO cells) produced more FGF-2 than VEGF protein.

Accomplishments: In general, we found that prostate cancers do not behave as the model CHO cells used in previous experiments. We tested our prostate cancer cell line models for FGF-2 and VEGF protein levels and found that most of them produced more VEGF than FGF-2. For example, PC-3, DU 145, and ALVA cells produced similar levels of VEGF₁₆₅ protein (range: 19-47 pg/mL/10⁶ cells), but varied more widely in the level of FGF-2 they secreted (range: 1.7-1,150 pg/mL/10⁶ cells) with ALVA having the highest level of FGF-2. There did not appear to be any eIF4E-mediated switch to alternative mRNA isoforms of either angiogenic growth factor, as

had been described in CHO cells and we proposed could account for a more potent protein form. It is therefore possible that this phenomenon was cell type-limited as well.

In spite of the fact that our cells did not appear to switch mRNA isoforms of VEGF or FGF-2 in response to eIF4E levels, it is important to note that eIF4E elevation was sufficient to increase

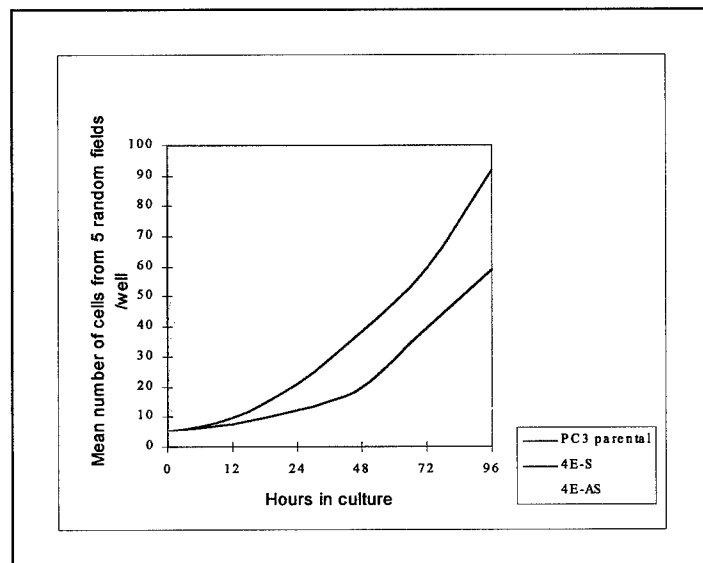


Figure 2. Proliferation of HUVEC cells in response to conditioned media from parental PC-3 cells, or sublines transfected with eIF4E sense or antisense vectors.

total levels of FGF-2 and/or VEGF protein. In turn, modulation of eIF4E was sufficient to influence functional *in vitro* measures of effects on endothelial cells, including proliferation of HUVEC cells (Figure 2) and CLS formation (Figure 3). The CLS formation was at least partially blocked by the addition of VEGF antibodies, but was not significantly blocked by addition of FGF-2 antibodies alone. This may be another reflection of cell type-specific behavior that is different for prostate tumor angiogenesis vs. previous studies using CHO cells.

4. VEGF, FGF-2, and thrombospondin-1 modulation using antisense 4E and/or p53 sense expression

Purpose: The purpose of these experiments was to determine: 1) if antisense-mediated eIF4E suppression

was feasible; 2) if it would be effective in modulating pro- (VEGF and/or FGF-2) or anti-angiogenic (thrombospondin-1) protein levels; and, 3) if p53 expression would have a synergistic effect on these proteins.

Accomplishments: Antisense eIF4E oligonucleotides were generated that spanned the translation start site of the eIF4E mRNA, as described above. Different forms of these oligonucleotides were expressed from an episomal vector, a retrovirus, and will be produced in an adenovirus we are presently generating. In addition, a modified, longer morpholino version of this oligonucleotide was also generated.

All of the versions were able to suppress eIF4E levels by at least 3-fold and all decreased VEGF and/or FGF-2 levels by approximately 2-fold to 3-fold in all cell lines tested.

Thrombospondin-1 levels did not change in response to eIF4E modulation. With regard to p53 expression, the cell lines used were wild-type (ALVA 101), mutant (DU 145), or null (PC-3). The p53 genetic background did not appear to have much effect on the ability of eIF4E to modulate angiogenic growth

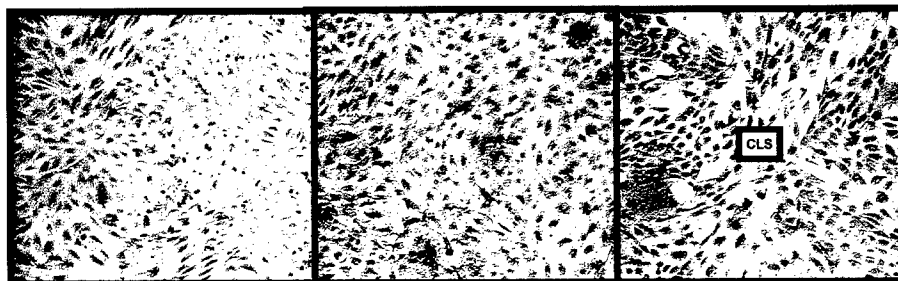


Figure 3. Capillary-like structure (CLS) formation in response to conditioned media from PC-3 cells (left), or sublines expressing the sense (right) or antisense (middle) eIF4E vectors. Only cells incubated for 5 days with conditioned media from the sense-containing cells were stimulated to form CLS, see CLS at arrows.

factor expression. The effect of eIF4E suppression on endothelial CLS formation was described above in Aim 3. The effect on tumor growth and angiogenesis *in vivo* is described in Aim 5 below.

Problems: Our original experimental design was to use the LuCap23 xenograft cell model for wild-type p53 expression. Since we did not have a delivery vector capable of high expression *in vivo*, we did not use this model system. The use of xenografts that grow only in nude mice is expensive and time-consuming even when high-quality reagents are available. We used the ALVA 101 cells instead.

5. 4E anti-sense *in vivo* on vascularization and *in vitro* analysis of Aim 5—
immunohistochemical analysis of the sponge experiment.

Purpose: We used cells that stably carried the retroviral construct to test the effect of eIF4E levels on tumor growth and angiogenesis.

Accomplishments: The studies summarized below in Table 1 were very successful. The tumors carrying the sense version of eIF4E, expressing only about 3 times the parental control level of eIF4E grew more quickly to the maximum size allowed in our animal protocol (2000

TABLE 1. The effects of eIF4E on *in vivo* growth of human PC-3 prostate tumors. Cells transfected with the eIF4E sense vector grew more quickly than parental PC-3 cells and formed large tumors in virtually all animals. Conversely, cells expressing the antisense eIF4E vector did not form tumors in any of the animal after 120 days post injection.

Parental	9/10	2000mm ³ volume	60 day latency
4E sense	9/10	2000mm ³ volume	41 day latency
4E antisense	0/10	--	120 days

mm³). Not only was the tumor growth more rapid, the tumors were more highly vascularized. Cells carrying the antisense version of eIF4E had approximately 3-fold less eIF4E, had approximately the same depression of VEGF, and the tumors did not grow during the experimental period (120 days after injection). This was a sufficient time frame for the parental control animals to have such large tumors that they had to be sacrificed (at 41 or 60 days post injection) per our animal protocol. Essentially the same results were obtained for the DU 145 and ALVA 101 cell lines.

Problems: Our original experimental design was to use polyurethane sponges as a solid support for tumor vessel growth. The number of vessels in the sponges was more difficult to evaluate than expected because of some inherent technical problems that we encountered. The sponges frequently trapped excess colorimetric reagents used in the immunohistochemical staining protocol giving a huge amount of non-specific staining.

Alternative Approaches Used and Accomplishments: We changed to a Matrigel semi-solid support that gave much more reliable and interpretable results. For example, cells were mixed with ice-cold Matrigel, an artificial basement membrane mixture of collagen, fibronectin, and other extracellular matrix components. Matrigel is liquid at 4°C, but gels at body temperature, therefore, in subcutaneous injections, the cells are held in a semi-solid suspension. Vessels can invade the mass and the entire "plug" can be removed nearly intact. In these experiments conducted in the mice for up to 10 days, cells carrying the sense version of eIF4E had more vessels than controls. Conversely, those carrying the antisense version had lower levels of eIF4E, VEGF, and fewer vessels as presented in Table 2. The number of vessels was obtained by counting the total number of vessels in each of 5 random low power fields decorated with the CD34 antibody, specific for endothelial cells.

TABLE 2. In vivo assay of angiogenesis. Mean microvessel counts from 5 random low power (200x total magnification) microscope fields from Matrigel plugs containing 1×10^6 PC-3 cells injected subcutaneously and removed after 5 or 10 days.

	<u>Mean microvessel count</u>	<u>5 days</u>	<u>10 days</u>
PC-3		23	36
PC-3/eIF4E sense		52	112
PC-3/eIF4E antisense		7	11

KEY RESEARCH ACCOMPLISHMENTS

- ❑ Made retroviral, episomal, and morpholine antisense eIF4E RNA vectors
- ❑ Found eIF4E to be expressed in a significant number of prostate tumors; similar to breast, bladder, colon, and squamous cell tumors of the head and neck
- ❑ Established that eIF4E expression in prostate cells could modulate VEGF, a growth factor key to controlling tumor angiogenesis
- ❑ Established that p53 status may have little effect on the ability of eIF4E to modulate VEGF, making an anti-eIF4E therapeutic approach even more attractive
- ❑ Successfully tested eIF4E suppression *in vitro* and *in vivo* as a viable therapeutic modality for further development

REPORTABLE OUTCOMES

Additional Funding

From these experiments, I was successful in obtaining funding from the New York Academy of Medicine for salary support as an awardee of the 1999-2001 Edwin Beer Fellowship in Urology. The Beer Fellowship in Urology is a highly competitive and prestigious award in the prostate research community. The studies outlined in the Beer application significantly expanded the scope of the present DOD proposal, but were derived from the preliminary study of eIF4E in our unique patient population. In our study of over 100 prostate tumors, there were significantly more blood vessels in the tumors from African-American men and especially in regions with high levels of eIF4E. While eIF4E levels did not appear to be expressed differently between ethnic groups, some other factor that was affected by eIF4E levels appeared to be. These studies have shaped the direction of my laboratory for the next several years. The American Society for Cell Biology (ASCB) accepted an abstract for presentation at their annual meeting in 2000, and included our work in their Press Book and their website (www.ascb.org). As a result, several print and web publications have written articles on our work.

Through these studies and others, my laboratory has identified other candidate genes controlled by eIF4E, or candidates that play a role in eIF4E-mediated tumor angiogenesis. The direction of the future studies is considerably divergent from the present application, but the fundamental goals are the same; to understand more fully the molecular mechanisms behind prostate tumor angiogenesis and progression. Two RO1 applications to the NCI have been submitted. One has been submitted and revised, and will hopefully be funded shortly. The second is in revision. A new DOD application has been sent in for review in the 2001 PCRP cycle. I chose not to ask for Phase II funds to continue the present line of research. Continued support for eIF4E antisense RNA development for human trials will be particularly expensive and is beyond the scope of Phase II funds. The next steps in my opinion are to further develop *in vivo* studies and to work to develop a delivery system to take eIF4E antisense to clinical trial. The last experiments using a p53 reporter construct to evaluate eIF4E translational regulation are still underway, but was not strictly part of this present application.

Training

The present DOD grant enabled me to support at least in part, training of several students and post-doctoral fellows. While they did not all receive direct (salary) support from the DOD application, they did participate in the studies. The following is a listing of those students and the role they played.

Students:

- Christopher Stage, LSUHSC-S Medical Student, summer American Foundation for Urological Disease Fellow, animal studies and immunohistochemical staining of sponge experiment.
- Nathan Goodyear, LSUHSC-S Medical Student, immunohistochemical staining of sponge experiment.
- Stephanie Cross, Graduate Student (MS August 2000), In vitro assays of eIF4E modulation

Post-doctoral Fellows:

- Peggy S. Carter, PhD, in vitro assays of eIF4E modulation, in vitro and animal studies of morpholine antisense vector
- Raymond K. Yeh, PhD, development of p53 luciferase construct for measuring effect of eIF4E on translation of p53 (future experiments derived from this application)

Research Associates:

- Elizabeth V. Mire, MS, *in vitro* and *in vivo* studies of endothelial cell proliferation and CLS formation.

Manuscripts:

The manuscripts listed below are submitted and a pre-print and/or reprint will be sent to the DOD as soon as they are accepted for publication. All contain the appropriate acknowledgment to the DOD. Another manuscript is planned once the p53-UTR luciferase experiments are finished, as well as a manuscript covering the use of the eIF4E adenovirus on pre-formed and orthotopic tumors.

1. Eastham JA, Acree TD, Tyler K, Stage AC, Goodyear N, Carter PS, Mire EV, Williams BJ (submitted) The influence of eIF4E on prostate tumor angiogenesis in African-American men.
2. Williams BJ, Carter PS, Mire EV, Walls SE, Eastham JA (submitted) Suppression of the translation initiation factor eIF4E lowers VEGF levels and inhibits prostate tumor growth.
3. Williams BJ, Gao M, Rhoads RE (submitted) Assignment of the human translation initiation factor EIF4E1 gene to 4q21-q22 and the intronless EIF4E2 gene to 17q21.

Meetings attended/presentations:

Dr. Williams attended several meetings presenting the work supported by this application. Many of those abstracts were published and all are listed below.

Published abstracts:

- American Society for Gene Therapy, podium talk, Seattle, WA, 1998, Novel Regulation of Prostate Tumor Angiogenesis, presented by Jill Williams
- American Urological Association, poster, Dallas, TX, 1999, B. Jill Williams, Keith Tyler, Chris Stage, Elizabeth V. Mire, James A. Eastham, The Influence of eIF4E on Angiogenesis in Prostate Tumors from African-American Men, presented by Jill Williams

Unpublished abstracts:

- Society of Basic Urologic Research, poster, Asilomar, CA, 1997, B. Jill Williams, Arrigo DeBenedetti, Tim Acree, Dennis Venable, James Eastham, Overexpression of the Translation Initiation Factor eIF4E Correlates with Increased Angiogenesis in Prostate Cancer
- American Association for Cancer Research (special topic meeting) "Angiogenesis of Cancer", Orlando, FL, 1998, B. Jill Williams, James A. Eastham, Dennis D. Venable, Arrigo DeBenedetti, D. Timothy Acree, The Effect of the Translation Initiation Factor eIF4E on VEGF and Angiogenesis in Prostate Cancer
- Keystone Symposium, "Molecular Biology of Breast and Prostate Cancer", Lake Tahoe, NV, 2000, Williams, J., Suppression of eIF4E by a morpholino antisense oligonucleotide decreases expression of VEGF in the PC-3 prostate cancer cell line. Keystone Symposium, Molecular Mechanisms of Breast and Prostate Cancer, Lake Tahoe, NV, March 2000.

Invited talks:

- Novel Regulation of Angiogenic Genes in Prostate Cancer, The Prostate Centre at Vancouver General Hospital, Vancouver, BC, September 1999 [host: Paul Rennie, Ph.D.].
- A Role for TIMP-1 in Regulation of Angiogenesis in Prostate Cancer, MD Anderson Cancer Center, Houston, TX, March 2000 [host: Menashe Bar Eli, Ph.D.].
- Regulation of Prostate Cancer Angiogenesis, LSU-S Biology Program, Shreveport, LA, October 1999 [host: Leonard Seelig, Ph.D.].
- Angiogenic Gene Expression in Prostate Cancer, LSUHSC, Department of Pathology, Shreveport, LA, December 1999 [host: Jay Russel, Ph.D.].

Other career development resulting directly or indirectly from this grant:

- DOD PCRP Scientist Reviewer, 1999, 2000, 2001
- Editorial Board member, *The Prostate*, 1998-present
- Invited Participant, "Workshop on Minorities and Prostate Cancer", Richmond, VA, June 2000

CONCLUSIONS

Importance or implications: Tumor angiogenesis has been identified as a fundamental contributing factor to early tumor progression as well as to the metastatic process. A great deal of attention is being given methods of turning down or turning off the production of angiogenic growth factors expressed by tumor cells. The purpose of our study was to investigate a poorly understood level of gene regulation applicable to tumor angiogenic growth factors.

Translational regulation of these growth factors may provide another step that can be modulated to achieve a reduction in the tumor's blood supply. The translation initiation factor, eIF4E, is capable of modulating expression of FGF-2 and VEGF, the two most potent angiogenic growth factors in model cell lines such as CHO and in other tumor types. Our studies illustrate that eIF4E is overexpressed in a majority of prostate tumors and metastatic lesions and overexpression of eIF4E can increase expression of VEGF protein levels and resultant microvessel density of the surrounding tissue. Overall, the studies confirm that modulation of eIF4E will have an effect on the primary angiogenic growth factors, VEGF and FGF-2. Our studies also illustrated that not all outcomes in non-cancer cell line models are applicable to cancerous cells. For example, it was not too surprising to us that prostate cancer cells did not use the same mechanism as CHO cells that switch mRNA isoforms to affect modulation of FGF-2. The experiments reported here support translational repression as another mechanism cells use to modulate gene expression. Our in vivo studies suggest that decreasing eIF4E levels

using an antisense RNA that blocks translation of eIF4E message might be of benefit in reducing the primary tumor growth and invasion of blood vessels. Future studies will be directed at development of anti-eIF4E delivery systems and combination therapies that might be even more beneficial.

Future studies: We had intended to use a second experimental model where a tumor would be allowed to grow to a certain size and then treat it with the retroviral eIF4E antisense vector. The retrovirus was difficult to produce to a sufficiently high titer to be useful to directly inject into pre-formed tumors in animals. We hope to be able to produce higher titers of the adenovirus that is under construction for such studies. This is an important experiment as it more accurately reflects the utility of a gene therapy used once a patient is diagnosed with the tumor. The previous experiments where the tumors are grown from cells expressing either more or less eIF4E than the parental cells were useful in determining the biological role eIF4E might play. The experiments answer two different and important questions.

REFERENCES

Kevil C, Carter P, Hu B, DeBenedetti A (1995) Translational enhancement of FGF-2 by eIF-4 factors, and alternate utilization of CUG and AUG codons for translation initiation. *Oncogene* 11: 2339-2348.



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REPLY TO
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