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13. ABSTRACT (Maximum 200 Words)

This annual report contains data demonstrating the effectiveness of a new adenovirus based optical imaging vector. The vector was injected into human tumors implanted into SCID mice. Animals were injected with D-luciferin and imaged in a Xenogen IVIS device. The vector was able to detect enhanced MAPK levels stimulated by Epidermal Growth Factor in prostate cancer tumors of xenograft animals. The vector was also able to detect inhibition by the small molecule EGF receptor inhibitor Iressa. The data suggest the possibility that gene expression based imaging may be able to detect augmented MAPK levels predictive of disease progression as well as the efficacy of cancer therapeutics.

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Introduction: The goal of our study is to analyze the mechanism of androgen-receptor (AR)-mediated gene activation in androgen-independent (AI) prostate cancer using powerful new technologies: Noninvasive optical imaging, chromatin immunoprecipitation and immobilized template analysis. Our previous studies have demonstrated our ability to track metastatic lesions and illuminate prostate cancer using gene expression-based imaging cassettes in combination with a charge coupled device (CCD) optical imaging system. Our current imaging cassette is termed TSTA or two-step transcriptional activation. In TSTA, a modified PSA enhancer is employed to synthesize GAL4-VP16, which activates luciferase expression to very high levels. An emerging benchmark of advanced prostate cancer progression is elevated mitogen activated protein kinase (MAPK) activity initiated by receptor tyrosine kinase (RTK) signaling (Figure 1). This directly or indirectly leads to augmented transcription complex assembly and expression of AR-regulated genes *in vivo*. The mechanism of this effect is largely unknown but probably underlies the development of AI cancer. Our hypothesis is that the MAPK signaling induces modifications of AR function that permit AR to act in a ligand-depleted environment. Our goal was to develop a prostate cancer imaging system to detect augmented MAPK activity in prostate tumors of living animals and correlate it with enhanced AR function.

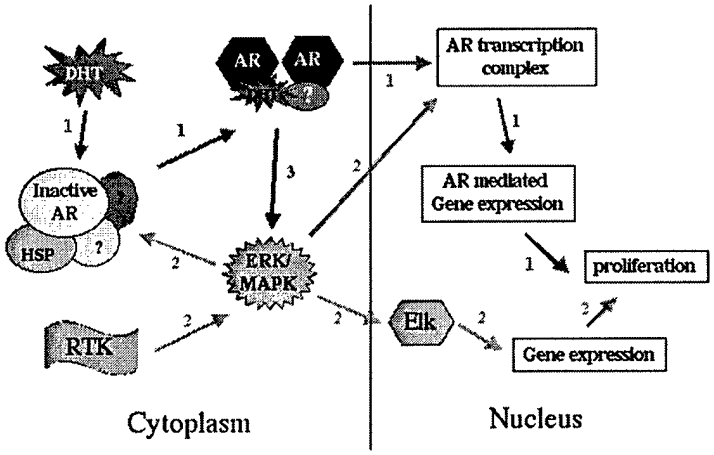

Figure 1 AR-mediated prostate cancer growth: A diagram of proposed mechanisms for AR-regulated gene expression and cell growth. In pathway 1, DHT binds AR, causing dissociation of HSP chaperones, dimerization, nuclear localization, transcription complex assembly, gene activation and cell proliferation. In pathway 2, Receptor tyrosine kinase (RTK)-linked MAPK cascades converge at ERK, which activates Elk-1 and possibly some components of the AR transcription complex. This allows growth of prostate cancer at castrate levels of ligand. An alternative pathway is that MAPKs facilitate response to castrate levels of ligand by acting on the chaperone complex and the nuclear localization of AR. Finally, pathway 3 illustrates a non-genotropic mechanism by which AR activates the MAPK pathway directly

Statement of Work and Results

Task 1: Develop the MAPK-TSTA expression system.

The initial goal of the study was to develop an imaging system that could detect elevated MAPK levels during the transition between the androgen dependent (AD) and independent (AI) forms of prostate cancer. The basic hypothesis as stated in Figure 1 is that elevated MAPK facilitates the conversion to the AI state. To test the idea that MAPK is elevated in AI cancer we developed a plasmid-based imaging system founded on a concept being developed in the lab termed two step transcriptional activation or TSTA.

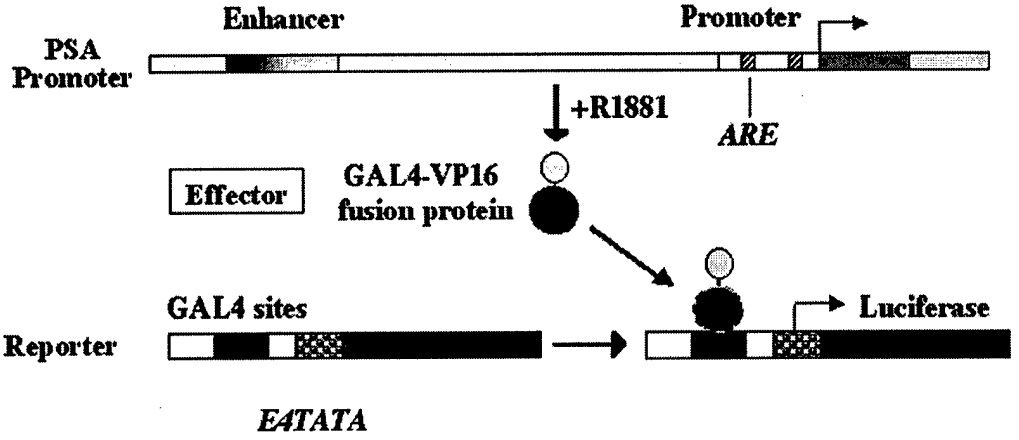

Figure 2. The Rationale of Two Step Transcription Activation (TSTA) system. In the first step, the "effector" GAL4-VP16 derivatives (oval circles) are expressed in prostate cancer cells in the presence of androgen (R1881), which activates the PSA enhancer, PSE. In the second step, GAL4-VP16 binds to a GAL4-responsive promoter, and activates expression of the "reporter" firefly luciferase. GAL4: GAL4 DNA Binding Domain. VP16: VP16 activation domain. E4TATA contains the adenovirus E4

In the TSTA system (Figure 2) we use an androgen receptor (AR)-responsive prostate specific antigen (PSA) enhancer to drive GAL4-VP16. GAL4-VP16 is a potent transcriptional activator and binds to GAL4 recognition sites upstream of a Firefly luciferase reporter gene. When AR is active then GAL4-VP16 is synthesized, binds DNA, and luciferase is expressed. The amount of luciferase is proportional to the amount of AR activity in the cell. We measure luciferase in cell culture by adding D-luciferin and ATP to cell extracts and light is quantitated using standard luminometry. In live animals, we inject D-luciferin into the animal and detect luciferase activity using a Xenogen charge coupled device camera, which quantifies the photons emitted from tissues expressing luciferase in the live animal. The process is not toxic and animals can be imaged repetitively over weeks or months. The system is described in several previous publications from my lab with my collaborators, Drs. Sam Gambhir and Lily Wu. These are listed under Reportables below.

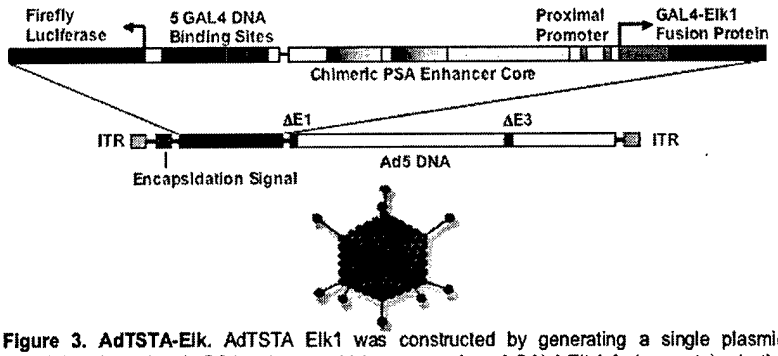

Figure 3. AdTSTA-Elk. AdTSTA Elk1 was constructed by generating a single plasmid containing the chimeric PSA enhancer driving expression of GAL4-Elk1 fusion protein. In the presence of active androgen receptor GAL4-Elk1 is synthesized and binds to the 5 GAL4 sites upstream of the firefly luciferase reporter gene. GAL4-Elk1 is inactive however until it is phosphorylated by active MAPK (ERK1 and 2). Thus, as described in our proposal, the system will only synthesize luciferase in prostate tissue, when AR is active and when MAPK levels are elevated. The TSTA-Elk1 cassette was cloned into pShuttle and recombined into the adenovirus genome in bacteria using AdEASY. Genomic DNA was isolated and transfected into 293A cells. The virus was plaque purified, scaled up and used for injection studies into LAPC9 xenografts in SCID mice.

After having established the activity of AR in the original transcription based TSTA imaging system we next modified TSTA to measure MAPK activity. We took advantage of the fact that MAPK activates the transcription factor Elk-1 by phosphorylation as stated in Figure 1. We replaced the VP16 activation domain in our TSTA cloning vectors with that of Elk-1. We described how this was done and the successful outcome of initial cell culture tests in last year's report. Over the past year the single plasmid TSTA-Elk system was cloned into an adenovirus shuttle vector. We isolated individual plaques and purified the virus. Figure 3 shows a schematic of the final viral construct and describes the steps in its synthesis.

Task 2: Testing the MAPK-TSTA system in cell based assays.

The goal of this task was to test for prostate specificity of the TSTA-Elk system in cell lines and to test for response to androgens, MAPK stimuli and pathway inhibitors. This phase of the testing was completed and described in last year's report.

Task 3: Testing the MAPK-TSTA system in live animals.

The goal of task3 was to generate systems that could detect elevated MAPK signaling in prostate tumors using gene expression-based optical imaging. My student Ms. Myla Ilagan and my technician Ms. Jill Pottratz generated a TSTA-Elk1 adenovirus (AdTSTA-Elk1) expressing firefly luciferase (Figure 3). Our cell culture data presented in last year's report suggested that TSTA-Elk1 could image MAPK activity in a tumor. However, the critical preliminary experiments with viruses in animals were not presented. The data below are the first example of gene expression-based imaging of MAPK in xenograft tumors.

Previous studies have shown that EGF activates MAPK levels in LAPC9 tumors implanted into SCID mice. Figure 4 shows an experiment demonstrating the ability of the virus to respond to EGF, a known MAPK stimulant, in the context of an LAPC9 AI tumor. We utilized four mice all of whose tumors were injected with low doses of AdTSTA-Elk1. Three days after virus injection we acquired baseline images using a Xenogen IVIS CCD camera (column 1). Two mice were then injected intraperitoneally with EGF (EGF1 and EGF2 rows) and two mice with vehicle (veh). Signals were measured 20 h and 72 h later (columns 2 and 3). At this point, we observed a small increase in luciferase activity in the EGF-treated but not in the vehicle-treated animals.

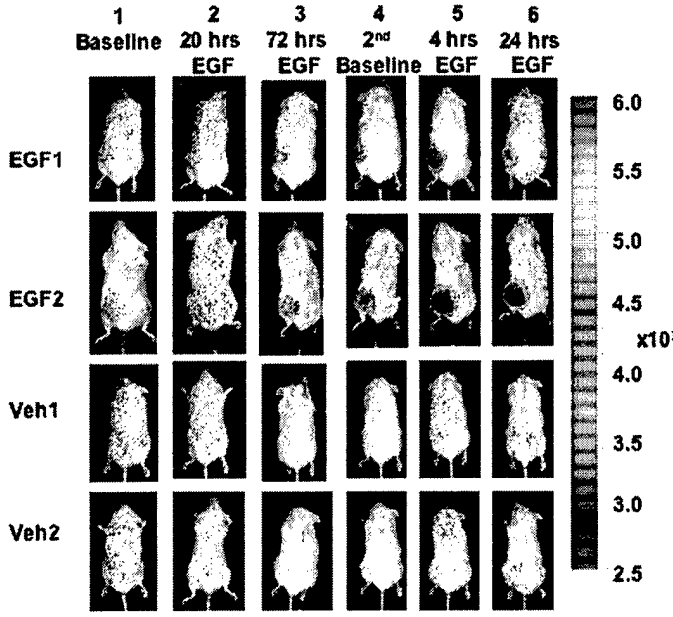

Figure 4. EGF Stimulates AdTSTA-Elk in Tumors. AdTSTA-Elk1 was injected at a low dose intratumorally into 4 animals bearing LAPC9 AI tumors. A baseline image was taken 3 days later and two mice were stimulated with IP injection of EGF and two with vehicle (Veh). The mice were imaged 20 and 72 hours later. Another two days later a second baseline image was taken and the mice were re-injected with EGF. Images were then taken 4 and 24 hours after injection. Note the increase in activity during the second EGF stimulation. Signal is in p/s/cm^2/sr.

We acquired another baseline image two days later (column 4) and re-stimulated the animals with vehicle and EGF. We then measured luciferase levels 4 and 24 hours later (columns 5 and 6). In both of the EGF-treated animals a significant increase in signal was observed but not in vehicle-treated animals. We believe that the first EGF-treatment sensitized the animals to further stimulation. This is an interesting phenomenon that we will continue to monitor. Similar EGF-stimulated increases were observed in mice bearing CWR22R tumors (data not shown). The experiment established proof of principle that we can measure MAPK activity in live animals using optical imaging technologies. We have repeated the experiment once and are in the process of more detailed analyses.

These experiments were performed with very low doses of virus as our intention was to measure an effect of EGF. Current studies with higher doses have begun to allow us to measure the basal unstimulated MAPK activities of the tumors. The goal in the next year will be to determine basal MAPK activities in AD vs. AI mice in the LAPC9 and CWR22 xenograft lines. Current efforts are also designed to perform the experiment enough times to get solid standard deviations upon graphing.

Task 4: Analyze the AR-MAPK pathway using the TSTA-Elk *in vivo* imaging system.

There are several subtasks that relate to the ability of TSTA-Elk to respond to anti-androgens like flutamide and anti-MAPK drugs such as PKI166 and Iressa in live animals. We initially performed these experiments with the TSTA system to evaluate the drug effects on AR in vivo in the context of tumors. This was an essential control for determining the effects of the drugs on TSTA-Elk since AR function is necessary to activate the Elk system. These experiments were discussed in last year's report.

Our current studies are focused on evaluating the effects of Iressa and other EGF receptor tyrosine kinase inhibitors on the AD-AI transition. We have recently been testing the effect of Iressa on growth of CWR22 AI tumors to demonstrate its efficacy.

We chose CWR22 because the AdTSTA-Elk infection experiments and plasmid TSTA-Elk transfection experiments suggested that the endogenous MAPK signals were higher than in other cell lines (data not shown). It was plausible that this elevated signal was driven by EGFR. We therefore treated tumor-bearing animals with Iressa, a small molecule EGFR inhibitor, as described in the legend. Figure 5 shows one such experiment with 5 representative mice in which the baseline signals were similar. In vehicle treated mice (on the right) the signal continues to increase from the baseline value on day 3 (post injection of virus) until day 7 and then further on day 10. Iressa was added to the treated cohort on day 3 and continued up to day 7 after which it was discontinued. The data show that Iressa slows the increase in MAPK signal observed during tumor growth (compare the two Iressa-treated animals on the left to the three vehicle treated animals on the right). The tumors displayed a small reduction in size due to Iressa but the decrease in tumor size was small relative to the inhibition with Iressa. We are currently trying to increase the size of the cohort and repeat the experiment to obtain statistically significant values. This work is in the early stages and will be continued throughout next year.

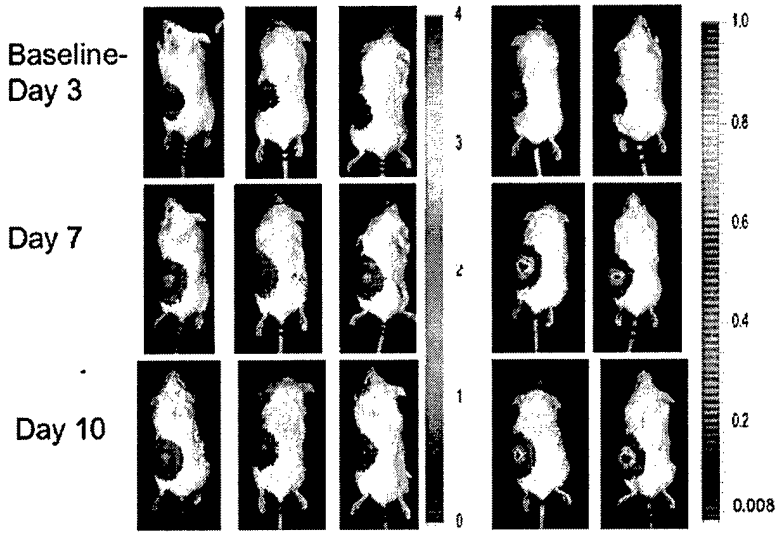

Figure 5. Drugs. Castrated SCID mice bearing bearing 0.4-cm CWR22 AI tumors were injected intratumorally with AdTSTA-Elk at $4.7x10^6$ pfu/tumor at Day 0 and baseline imaged at Day 3. Iressa treatment (three mice on left) or vehicle treatment (two animals on right) began after the baseline measurements. Treatment was by gastric gavage at 150 mg/kg for 5 days after which images were taken (day 7). Iressa withdrawal occurred at Day 8 and images were taken at Day 10.

Task 5: Perform nuclear localization tests of AR (months 11-36)

One of the key questions regarding AR function during androgen independent prostate cancer is whether it has the ability to localize the nucleus in the presence of castrate levels of ligand. We described that AR is in the nucleus of AI tumors in last years report. The data are published in Zhang et al. 2003.

Task 6: Perform chromatin immunoprecipitation on AD and AI tumors.

We found that AR and pol II bound to promoters of the PSA gene in AI tumors and described this in last years report. The data are published in Zhang et al. 2003.

Task 7: Perform immobilized template assay.

Our attempts to employ the immobilized template in crude tumor extracts have been unsuccessful. These extracts are significantly more proteolyzed than ones from cell culture and contain significant hemoglobin due to contamination with blood from the

animal. While we continue to be interested in performing this experiment it may not be technically feasible.

Key Research Accomplishments:

- Creating the AdTSTA-Elk imaging vector.
- Testing and validating activity of AdTSTA-Elk in cell culture.
- Demonstration that AdTSTA-Elk responds to MAPK signaling in live animals.
- Demonstration that AdTSTA-Elk can monitor the effects of a MAPK pathway inhibitor Iressa in live animals.

Reportable Outcomes:

The work funded in part by this grant has now been described in 4 publications:
1. Iyer M, Salazar FB, Lewis X, Zhang L, **Carey M**, Wu L, Gambhir SS. Noninvasive imaging of enhanced prostate-specific gene expression using a two-step transcriptional amplification-based lentivirus vector.
Mol Ther. 2004 Sep;10(3):545-52.
PMID: 15336654
2. Ray S, Paulmurugan R, Hildebrandt I, Iyer M, Wu L, **Carey M,** Gambhir SS. Novel bidirectional vector strategy for amplification of therapeutic and reporter gene expression.
Hum Gene Ther. 2004 Jul;15(7):681-90.
PMID: 15242528
3. Sato M, Johnson M, Zhang L, Zhang B, Le K, Gambhir SS, **Carey M**, Wu L. Optimization of adenoviral vectors to direct highly amplified prostate-specific expression for imaging and gene therapy.
Mol Ther. 2003 Nov;8(5):726-37.
PMID: 14599805
4. Zhang L, Johnson M, Le KH, Sato M, Ilagan R, Iyer M, Gambhir SS, Wu L, **Carey M**. Interrogating androgen receptor function in recurrent prostate cancer.
Cancer Res. 2003 Aug 1;63(15):4552-60.
PMID: 12907631

Over the past year the work has been described in three posters at national meetings by Dr. Carey's students and in three platform presentations by Dr. Carey.
Posters:
1. Abstract for 95th Annual AACR Meeting, March 27-31, 2004
Control/Tracking #: 04-AB-6363-AACR
Interrogating Androgen Receptor and MAPK Function in Androgen Independent Prostate Cancer by Optical Imaging with Two-Step Transcription Amplification Systems
Romyla Ilagan, Liqun Joann Zhang, Kim Le and Michael Carey
2. Abstract for 3rd Annual Society for Molecular Imaging, Sept.9-13, 2004
Tracking number #04-A-430-SMI

New Transcription-Based Vectors for Bioluminescence Imaging of AR and MAPK Activity in Recurrent Prostate Cancer
Romyla Ilagan, Liqun Joann Zhang, Jill Pottratz, Kim Le and Michael Carey
3. Abstract for 94th Annual AACR Meeting, July 11-14, 2003
Poster #R4333
Study of Androgen Receptor-Mediated Gene Regulation in Prostate Cancer by Molecular Imaging.
Liqun Joann Zhang, Myla Ilagan, Kim H. Le, Andrea Smallwood, Sanjiv. S. Gambhir, Lily Wu, and Michael Carey.

Talks:
1. Invited Speaker: Third Annual Meeting of the Society for Molecular Imaging
St. Louis, MO
Sept 9-12, 2004
"Two Step Transcriptional Imaging Vectors and Optical Imaging Applications"
2. Invited Speaker: Merck Pharmaceuticals
West Point, PA
July 12, 2004
"Molecular Imaging of Androgen Receptor Function in Prostate Cancer Xenografts"
3. Invited Speaker
NCI Symposium: Androgen Action in Prostate Cancer
Keystone Resort, Colorado
March 4-6, 2004.
"Optical Imaging of AR Function in Prostate Cancer
4. Invited Speaker,
Molecular Imaging of Prostate Cancer Workshop
February 1-2, 2004
Washington D.C.
"Optical Imaging of AR and MAPK Genetic Pathways in Prostate Cancer Xenografts"

Conclusions:

The current work continues to provide support for the concept that gene expression based optical imaging can be applied to study cancer progression in animal models. In last year's report we detailed the AR-responsive readout system and showed that it functioned as an accurate indicator of AR activity in live animals (Zhang et al., 2003) as compared to numerous molecular and cellular benchmarks. In last year's report we also discussed the initial cell culture and ex vivo transfection data that supported the transition of the TSTA-Elk system to an adenovirus. This past year we have synthesized the adenovirus and begun in vivo testing to determine if it can measure MAPK robustly within a xenograft tumor. We tested proof of principle by injecting a MAPK stimulant, EGF, directly into mice and measured a response. A robust increase in optical signal was observed within the tumor. We extended these studies to

test the effect of small molecule pharmaceuticals in an AI model that displayed high TSTA-Elk activity in cell culture and was inhibited by Iressa. Xenografts of this model also displayed inhibition of AdTSTA-Elk signal by Iressa. Our results demonstrate the ability to measure signaling environments of a tumor repetitively and non-invasively. The data suggest the possibility that the technology could be used clinically if we can extend the method to employ PET reporter genes. Such studies are in progress.

References: None
Appendicies: None