Imaging Mitogen-Activated Protein Kinase Function in Xenograft Models of Prostate Cancer

Romyla Ilagan, Jill Potratz, Kim Le, Liqun Zhang, Steven G. Wong, Raul Ayala, Meera Iyer, Lily Wu, Sanjiv S. Gambhir, and Michael Carey

Abstract
Mitogen-activated protein kinases (MAPK) play important roles in malignancy. The ability to detect and quantify MAPKs in live animal models of cancer will facilitate an understanding of disease progression. We have developed a gene expression-based imaging system that detects and quantifies MAPK activity in prostate cancer tumors implanted into severe combined immunodeficient mice. The imaging technology uses a modified version of two-step transcriptional amplification (TSTA). The tissue specificity of gene expression is imparted by an enhanced version of the prostate-specific antigen regulatory region that expresses GAL4-ELK1. GAL4-ELK1 confers MAPK specificity by activating a firefly luciferase (FLuc) reporter gene when the Ets-like transcription factor (ELK) 1 activation domain is phosphorylated by MAPK. FLuc activity in live animals was detected using the Xenogen In vivo Imaging System. We validated the TSTA-ELK1 system by analyzing its response to epidermal growth factor treatment in transfected tissue culture cells and in adenovirus (AdTSTA-ELK1)–injected prostate cancer xenograft tumors. We measured MAPK activity in two well-characterized xenograft models, CWR22 and LAPC9. Although no significant differences in MAPK levels were detected between androgen-dependent and androgen-independent xenografts, the CWR22 models display significantly higher levels of AdTSTA-ELK1 activity versus LAPC9. Western blots of tumor extracts showed that the elevated imaging signal in CWR22 xenografts correlated with elevated levels of phosphorylated extracellular signal-regulated kinase 1/2 but not p38 or c-Jun NH2-terminal kinase. We conclude that a gene expression-based optical imaging system can accurately detect and quantify MAPK activity in live animals. (Cancer Res 2006; 66(22): 10778-85)

Introduction
Elevated mitogen-activated protein kinase (MAPK) activity is a hallmark of many malignancies (1). Of the three branches of the MAPK pathways, extracellular signal-regulated kinase (ERK), p38, and c-Jun NH2-terminal kinase (JNK), the ERK1/2 stands out. Cytokine receptors, such as epidermal growth factor (EGF) receptor (EGFR), stimulate cell growth by causing induction of the Ras/MAPK signaling cascade that ultimately leads to phosphorylation of ERK by MAPK/ERK kinase (MEK) 1 (2). ERK phosphorylates the transcription factor Ets-like transcription factor (ELK) 1 and other targets to stimulate cell proliferation (3). The MAPK pathway has been hypothesized to play a role in prostate cancer (4).

The initial androgen-dependent phase of prostate cancer relies on androgen receptor (AR) function (5). AR is a 110-kDa steroid receptor, which is sequestered in the cytoplasm in the absence of its ligand. In the presence of dihydrotestosterone, AR dimerizes, enters the nucleus, binds to androgen response elements, and activates transcription of responsive genes (6). In the recurrent or androgen-independent phase of cancer, AR activity is resuscitated despite the lowering of androgen levels by pharmacological intervention (7).

The androgen-independent phase of prostate cancer has been associated with AR overexpression and gene amplification (8). However, hyperactivation of various growth factor pathways that intersect MAPK has also been reported (9). Additionally, drugs that interfere with receptor tyrosine kinases (RTK) inhibit tumor growth (10). The mechanism by which AR is reactivated is unknown but may involve cross-talk with specific growth factor pathways.

To further understand the role of MAPK in prostate cancer, our groups have been attempting to decipher the activity of the AR and MAPK pathways in live animals using gene expression-based bioluminescence imaging. Our studies use xenograft models that recreate prostate cancer progression from an androgen-dependent to an androgen-independent phase (11). In gene expression-based bioluminescence imaging, a promoter is placed upstream of a bioluminescence reporter gene (12). The reporter cassette is introduced into tumor cells in an animal and the promoter activity is imaged using a Xenogen In vivo Imaging System (IVIS; Alameda, CA) following injection of the animals with d-luciferin (13).

A challenge in bioluminescence imaging is that cellular promoters are typically weak and detection of optical signals in dense tissues is hampered by light attenuation and scattering (14). We developed an approach to augment cellular promoter activity and light output based on a concept termed two-step transcriptional amplification (TSTA; refs. 15, 16). In the original TSTA scheme, a cellular promoter expresses a potent chimeric activator, GAL4-VP16 (17, 18). GAL4-VP16 binds a GAL4-responsive reporter gene and generates high levels of firefly luciferase (FLuc). Our prostate cancer–specific version of the TSTA system uses a modified prostate-specific antigen (PSA) promoter, which responds more robustly than the native promoter to the AR (19). It also contains a more potent derivative of GAL4-VP16, where the VP16 activation domain is dimerized (GAL4-VP2). We have used this AR-responsive TSTA vector to study AR function in xenograft models of prostate cancer and in transgenic mice (16, 20).
To detect MAPK in a prostate-specific manner, we took advantage of the binary design of TSTA to craft an imaging cassette that simultaneously measures AR and MAPK function. The principle is shown in Fig. 1A. In the first step, a modified PSA regulatory region expresses GAL4-ELK1 rather than GAL4-VP16. GAL4-ELK1 contains the GAL4 DNA binding domain fused to the activation domain of ELK1 (21). Phosphorylation of the ELK1 activation domain by ERK1/2 occurs at several serines and threonines (22). Phosphorylated ELK1 stimulates transcription from a GAL4-responsive reporter gene expressing Fluc. The Fluc bioluminescent activity is detected using the Xenogen IVIS. Our previous studies have shown that the amount of luciferase activity is proportional to the activity of AR both in vitro and in animals (23). Further, the AdTSTA approach was highly successful in quantifying AR function in response to androgen deprivation and anti-androgen therapy (23, 24). The system is tissue specific and detects Fluc only in prostate when inserted into transgenic animals (20). Based on our previous results showing that TSTA successfully monitored AR activity and function, we surmised that the TSTA-ELK1 system would be able to detect combined effect of AR and MAPK on transcription in living animals.

Our study was designed to address three key questions. (a) Can the TSTA-ELK1 system image MAPK signals in cell culture and in live animals? (b) Are there differences in MAPK activity between androgen-dependent and androgen-independent tumors? (c) Do different xenograft models display different levels of MAPK?

Materials and Methods

Plasmid and adenoviral constructs. The TSTA-ELK1 construct was generated from the optimal TSTA plasmid. A XhoI-NotI GAL4-ELK1 fragment derived from pFA2-ELK1 (Stratagene, La Jolla, CA) was subcloned into the TSTA XhoI-NotI parental plasmid generating a single 8.7-Kb vector termed TSTA-ELK1. The NotI-SalI fragment from TSTA-ELK1 was subsequently inserted into the NotI-SalI site of pShuttle (Qbiogene, Carlsbad, CA). The TSTA-ELK1 fragment was then incorporated into the adenovirus vector AdEasy (Qbiogene) through homologous recombination.

Figure 1. The TSTA-ELK1 prostate- and MAPK-specific imaging construct. A, in the first step, the modified PSA enhancer present within the effector cassette of the imaging construct activates expression of the GAL4-ELK1 derivatives (ovals) in prostate cancer cells. In the second-step, the GAL4-ELK1 fusion protein is activated by phosphorylation of MAPK (ERK1/2). The GAL4-ELK1 fusion protein binds to a GAL4-responsive promoter in the reporter cassette and activates expression of Fluc. B, validation of TSTA-ELK1 response to AR and MAPK pathways in cultured cells. LNCaP cells grown in six-well plates were transiently transfected with TSTA-ELK1 plasmid. We added 10 nmol/L R1881 (synthetic androgen) and dominant-active MEKK (DA-MEKK), together or separately, to samples 1 hour after transfection and measured the luciferase activity 48 hours after stimulation. Columns, average values of a representative experiment done in triplicate; bars, SD. Y axis, relative light unit reading from the luminometer. C, immunoblot (WB) showing LNCaP cell extracts probed with GAL4 antibodies to detect GAL4-ELK1 expression. D, validation of TSTA-ELK specificity for the Ras/MAPK/ERK pathway. CWR22V1 cells were grown in 12-well plates and transiently transfected with TSTA-ELK plasmid and cotransfected with 200 ng MAPK activators: MEK1 (ERK), MEK3 (p38), or MEKK (JNK). E, inhibitors to p38 and JNK do not efficiently block TSTA-ELK activity. CWR22V1 cells were transfected with TSTA-ELK plasmid. Cells were treated with R1881 and EGF to simultaneously stimulate both AR and MAPK/ERK pathways. Inhibitors to MEK1 (PD98059) significantly blocked activity (P = 0.002), whereas inhibitors to p38 (SB202190) and JNK (SP600125) did not block TSTA-ELK activity.
The virus was scaled up, purified via a CsCl gradient, and titered using the Adeno-X Rapid Titer kit (Clonetech) following infection of 293 monolayers [infectious units = plaque forming units (pfu)]. Virus was stored at −80°C at 10^10 pfus/ml in 10 mmol/L Tris-HCl, 1 mmol/L MgCl2, and 10% glycerol.

**Cell culture transfection experiments.** The human prostate cancer cell line lymph node metastatic prostate cancer cell line (LNCaP) was grown in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution (Life Technologies, Gaithersburg, MD and Mediatech, Herndon, VA). Before transfection, the cells were transferred into medium containing 5% charcoal-stripped serum (Omega Science, Tarzana, CA) for 24 hours. Transfection experiments were as described previously (16). Each transfection mixture contained 0.5 μg of the imaging construct and 0.5 μg of carrier DNA pgL3 (Promega, Madison, WI). Stimulants used in the experiments were methylentrienedione (R881, NEN Life Science Products, Boston, MA), a synthetic androgen, and dominant-active mutated form of MAPK kinase kinase (MEKK), DA-MEKK [provided by Dr. Charles L. Sawyers, University of California at Los Angeles, Los Angeles, CA], or EGF (Sigma, St. Louis, MO). R881 was added to the medium at concentrations noted in the figure legends. Depending on the experiment, either 200 ng DA-MEKK or 100 ng EGF (Sigma) was added to the medium for 1 hour. Experiments using inhibitors PKI-166 (provided by Charles L. Sawyers), a RTK inhibitor, and Casodex, an anti-androgen drug, were added along with R881 and EGF to determine inhibitory effect on the TSTA and TSTA-ELK1 systems. PKI-166 was given at 5 μmol/L and Casodex at 10 μmol/L. Cells were incubated at 37°C for 48 hours. For the MAPK activator experiments, 500 ng TSTA-ELK was transiently transfected into CWR22V1 cells with or without 200 ng plasmids expressing MAPK activators: MEK1, MEK3, and MEKK (Stratagene); they activate ERK1/2, MAPK. CWR22V1 cells were seeded and cultured in charcoal-stripped medium overnight. The cells were transfected with the TSTA-ELK plasmid for 1 hour and treated with 1 mmol/L R881 and 100 ng EGF with or without the inhibitors to MEK1 PD98059 (Cell Signaling, Danvers, MA) at 50 μmol/L, p38α/β SB202190 (Sigma) at 50 mmol/L, and JNK SP600125 (A.G. Scientific, San Diego, CA) at 40 mmol/L. Cells were harvested and lysed using the passive lysis buffer and measured FLuc activities (Dual-Lucerase Assay System, Promega). FLuc activities of 5% of the cell lysates with 100 μL of substrate α-luciferin were measured using a luminometer (Lumat 9507, Berthod, Germany) with an integration time of 20 seconds.

**Animal CDD experiments.** The imaging was done as described previously (16, 23). Animal care and euthanasia were done with full approval of the University of California Animal Research Committee. For LNCaP and CWR22 xenografts, a total of 10^6 pfu of AdTSTA-ELK1 in 20 μL Dulbecco’s PBS (Life Technologies) was injected into a tumor measuring 0.5 cm in diameter at two locations. The virus was allowed to express the encoded genes and distribute throughout the tumor for 3 days before imaging. On the day of imaging, mice were anesthetized with ketamine-xylazine mix (4:1). Imaging was done using a Xenogen IVIS 100 cooled CCD camera (Xenogen). The mice were injected with 200 μL of 15 mg/mL α-luciferin i.p. for 15 minutes before imaging after which they were placed in a light-tight chamber. A gray-scale reference image was obtained followed by the acquisition of a bioluminescent image. The acquisition time ranged from 1 to 5 minutes. The images shown are pseudomages of the emitted light in photons/s/cm²/steradian superimposed over the gray-scale photographs of the animal. Time courses were done where mice were imaged every 3 days. Following the baseline image, a subset of mice were treated with 500 μL of 100 ng/mL of EGF that was dissolved in 10 mmol/L acetic acid containing 0.1% bovine serum albumin. EGF-treated mice were then imaged 4 hours later to determine AdTSTA-ELK1 activity. The data were analyzed statistically using the Student’s t-test.

**Immunobots.** For cell culture analysis, LNCaP cells were grown in six-well plates, transfected with TSTA-ELK1, and treated with mitogens as indicated in the figures. We harvested and lysed the treated cells using radioimmunoprecipitation assay (RIPA) lysis buffer (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% sodium deoxycholate, 1 mmol/L EDTA, 1% NP40). We normalized extracts by protein concentration [bicinchoninic acid (BCA) protein assay kit, Pierce, Rockford, IL] and the samples were fractionated on 4% to 15% gradient acrylamide gels (Bio-Rad, Hercules, CA) and subjected to immunoblot analysis with rabbit polyclonal antibodies generated against GAL4 (25, 26). For whole-tumor analysis, tumors were harvested from mice via surgical resection at the imaging end points and immediately frozen in liquid N₂. Frozen tumors were homogenized using a mortar and pestle in the presence of liquid N₂. Samples were then resuspended in 400 to 600 μL RIPA buffer (10 mmol/L Tris-HCl, 1% NP40, 1% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.2% SDS, 1 mmol/L PMSF, 1 μg/mL leupeptin and pepstatin, 1 mmol/L Na₃VO₄, 1 mmol/L NaF). Lysates were passed through 255/8-gauge needles to shear genomic DNA followed by heating at 65°C and centrifugation at 14 K at 4°C for 20 minutes to remove insoluble debris. Samples were assayed for total protein concentration using the Pierce BCA protein assay kit. Extracts were fractionated on 4% to 15% Tris-HCl Ready Gels (Bio-Rad) and immunoblotted. After a second round of normalization using β-actin (Sigma A5441) and ERK (SC-93; Santa Cruz Biotechnology, Santa Cruz, CA), blots were probed with antibodies from Cell Signaling against phosphorylated p44/p42 ERK, p38, phosphorylated p38, and phosphorylated JNK. Antibody to JNK (SC-474) was obtained from Santa Cruz Biotechnology. Positive control extracts for p38 and JNK were obtained from Cell Signaling.

**Results**

**TSTA-ELK1 detects MAPK in cell culture.** Figure 1B shows that the TSTA-ELK1 system functions in a transfection experiment done in LNCaP cells. A plasmid bearing the TSTA-ELK1 system was transfected with combinations of R881, an androgen agonist, and another plasmid encoding a mutated, dominant-active form of MEKK (DA-MEKK). Modest increases in FLuc reporter gene activity were observed with DA-MEKK and R881 alone. The combination led to a synergistic increase in FLuc expression. The fact that low level expression is observed with R881 and DA-MEKK alone is consistent with previous reports showing that androgens can activate the MAPK pathway through a non-genotropic signaling mechanism (27, 28) and DA-MEKK can facilitate AR function in the presence of only trace, castrate levels of androgen (29). Figure 1C shows an immunoblot confirming that GAL4-ELK1 expression is detected at a low level in the absence of R881. This low level expression is likely due to the potency of the modified PSA enhancer (19). We reported previously that the TSTA vector can use trace amounts of dihydrotestosterone remaining in charcoal-depleted serum (16). Nevertheless, GAL4-ELK1 expression is increased significantly by R881 and by the combination of R881 and DA-MEKK.

**Next**, we tested whether activators of p38 and JNK MAPKs could stimulate TSTA-ELK1 transcriptional activity (Fig. 1D). For this assay, we used the CWR22V1 cell line as it is derived from xenografts we will use in later figures. The combination of R881 and MEK1, an ERK1/2 activator, stimulates transcription 52-fold. In contrast, MEK3, an activator of p38, and MEKK, an activator of JNK, stimulated transcription <10-fold. We conclude that the TSTA-ELK system is particularly sensitive to ERK1/2 in CWR22V1 cells.

To determine whether a natural MAPK pathway stimulant would increase TSTA-ELK reporter activity in CWR22V1 cells, we tested the effect of EGF (Fig. 1E). EGF and R881 stimulated transcription 10-fold. EGF is known to affect multiple branches of the MAPK pathway (2, 30). We therefore compared whether inhibitors of the ERK1/2 upstream kinase MEK1/2 (PD98059) or inhibitors of p38 (SB202190) and JNK (SP600125) would affect EGF stimulation. Figure 1E shows that, in CWR22V1 cells, TSTA-ELK1 activity is significantly blocked by the MEK1/2 inhibitor PD98059. In contrast,
inhibitors to p38 and JNK, SB202190 and SP600125, respectively, had only small effects (P > 0.05). These data support the idea that the prostate cancer cell line CWR22rV1 responds to EGF largely by activation of the ERK1/2 pathway.

Many studies support some form of cross-talk between the AR and MAPK pathways (31). MAPK has been shown to stimulate AR activity in a variety of different contexts (32–35). To further validate and characterize the TSTA-ELK1 system, which measures the combined action of MAPK and AR, we compared it with the original TSTA system, which is primarily AR responsive. Figure 2A shows that the TSTA-ELK1 reporter response is synergistic in LNCaP cells treated with a combination of R1881 and EGF. Furthermore, specific inhibitors of the EGFR tyrosine kinase and AR, PKI-166 (10) and Casodex (also called bicalutamide), respectively, eliminate the synergistic response. Figure 2B shows a similar experiment with the TSTA system. In this example, EGF alone stimulates a mild increase in AR activity but R1881 strongly stimulates activity. The TSTA reporter response is inhibited by Casodex but only marginally affected by PKI-166 (Figure 2B columns 5-10). The inhibition by PKI-166 of basal AR activity confirms previous reports that EGF or HER-2 affects AR activity, although the effect under the conditions used here is not as dramatic as optimized conditions reported by others (33). Although the TSTA system responds to EGF and its inhibitors, the effects are <2-fold and significantly less than the strong synergy observed with R1881 and EGF in the TSTA-ELK1 system. The difference between R1881 alone and R1881 plus EGF is only 1.5-fold with TSTA versus 25-fold with TSTA-ELK1. We conclude that the TSTA-ELK1 system is more specific for studying the MAPK response in prostate cancer than the original TSTA system.

To evaluate functional MAPK levels in xenograft models of prostate cancer, we generated a replication-defective adenovirus bearing the TSTA-ELK1 cassette. Figure 3A shows the structure of AdTSTA-ELK1, where a duplicated PSA enhancer, attached to the PSA promoter, expresses GAL4-ELK1 in the rightward direction, whereas a GAL4-responsive FLuc reporter is oriented head-to-head in the leftward direction in an E1/E3-deleted Ad5 vector (36).

In Figure 3B, we show a time course of FLuc expression 3 to 4 days after injection of AdTSTA-ELK1 into 0.5-cm tumors in the CWR22r-2524 (CWR22-AI) prostate xenograft model (37). CWR22-AI is a subclone of the CWR22 xenograft series, a human prostatic carcinoma grown on castrated male severe combined immunodeficient (SCID) mice (38). CWR22-AI expresses PSA and a mutant AR (39). A series of prior optimization experiments revealed that FLuc expression peaked within 4 hours of EGF injection. This point is emphasized in the figure, where FLuc activity increases 4 hours after injection, versus a control animal, and remains high for at least 24 hours. We observe a minor elevation in activity when a second baseline measurement is taken 72 hours later followed by a dose of EGF for 4 hours.

We observed similar stimulations 4 hours after injection in the LAPC9 model. LAPC9 is derived from a bone metastasis and expresses wild-type AR and PSA (11, 40). Representative images are shown for the LAPC9 androgen-dependent and androgen-independent xenograft models in Fig. 3C. Figure 3D is a bar graph averaging data from cohorts of eight LAPC9 androgen-dependent and androgen-independent animals. The blue columns represent the fold change in the vehicle- or control-treated animals and the red columns represent EGF-treated animals 4 hours after taking baseline measurements. A consistent 2- to 3.5-fold induction of FLuc expression was detected in the EGF-treated animals versus controls. We conclude that the AdTSTA-ELK1 can detect the systemic effect of EGF injection on MAPK-mediated activity in tumors of live animals.

Differences between MAPK activity in androgen-dependent and androgen-independent tumors. One of the hypotheses about the transition of prostate cancer from an androgen-dependent to androgen-independent state is that it is driven by RTK and downstream MAPK activity (4). One prediction of that hypothesis is that the basal MAPK levels of androgen-independent tumors will greatly exceed that of androgen-dependent tumors. We therefore used the AdTSTA-ELK1 system to examine the basal levels of functional MAPK activity within xenograft tumors of live animals. We compared the levels of MAPK in both CWR22 and
LAPC9 xenografts. Figure 4A shows the optical CCD signals from representative androgen-dependent and androgen-independent animals 3 days after viral injection and Fig. 4B graphs the results from cohorts (n = 10, androgen dependent; n = 8, androgen independent) of animals. We show the animals using the same photonic scale to show the large difference between models as described below.

Two important observations emerged from this analysis. First, we found only marginal differences between the androgen-dependent and androgen-independent tumors within each xenograft model. This result suggests that MAPK levels do not change significantly when the tumor transitions into the androgen-independent state in the xenografts. The second observation was that the AdTSTA-ELK1 activity in the CWR22 models is over 100-fold higher than the signal observed in the LAPC9 models. This latter observation suggested the possibility that the xenograft models display vastly different levels of functional MAPK. This explanation, however, required further validation.

We considered the possibility that the CWR22 androgen-dependent and androgen-independent tumors grew at a significantly different rate versus LAPC9. MAPK levels are elevated in highly proliferating cells (41). However, when the growth rates (n = 5) of LAPC9 and CWR22 androgen-dependent and androgen-independent xenografts were measured, we found no correlation between functional MAPK and the growth (data not shown).

Another possibility was that the differences in AdTSTA-ELK1 activity derive from differences in the functional AR activity or infectivity of the adenovirus in the different xenograft models. To address these issues, we injected cohorts of LAPC9 and CWR22 androgen-dependent and androgen-independent xenografts with low levels of AdTSTA. As described above, AdTSTA expresses GAL4-VP16 from a modified PSA enhancer and measures the AR responsiveness of a prostate tumor. The bar graph in Fig. 5 compares the ratio of the AdTSTA activity in LAPC9 versus CWR22 androgen-dependent and androgen-independent xenografts with low levels of AdTSTA. We allowed 4 hours of incubation for EGF to stimulate the MAPK pathway in the tumor and then imaged the in vivo signal. The acquisition time of imaging was 3 to 5 minutes. Following 72 hours of rest, the mice were again baseline imaged and then received EGF as noted above. Representative control- and EGF-treated mice. C, imaging AR and MAPK signaling in the LAPC9 prostate cancer xenograft model. Castrated [androgen independent (AI)] or intact [androgen dependent (AD)] male SCID mice implanted s.c. with LAPC9 tumors (0.5 cm) and were injected intratumorally with 2e7 plus of AdTSTA-ELK1. After 3 days, mice were injected i.p. with EGF or vehicle control and then imaged 4 hours later. Representative mice. D, bar graph summarizes several experiments with cohorts of n = 8 of LAPC9 androgen-dependent independent xenografts and n = 8 of LAPC9 androgen-independent xenografts injected with AdTSTA-ELK1. Fold change of signal of AdTSTA-ELK1 activity post-EGF treatment. Columns, fold change signal; bars, SD.
AdTSTA values, there remains a 20-fold difference between the AdTSTA-ELK1 and AdTSTA activity in androgen-dependent tumors and a 10-fold difference in androgen-independent tumors that cannot be accounted for based on AR activity or differential infectivity. We hypothesized that this difference is due to the MAPK activity in the xenografts.

**Comparison of MAPK levels in the xenografts by immunoblotting.** To determine which of the MAPK pathways was being detected by the AdTSTA-ELK system in the xenografts, we did immunoblotting on tumor extracts (Fig. 6A and B). The overall ERK1 levels are similar between the LAPC9 and CWR22 androgen-dependent and androgen-independent xenografts. However, phosphorylated ERK levels are >6-fold higher in CWR22 versus the LAPC9 models. Thus, we surmise that the enhanced AdTSTA-ELK1 imaging activity is due to enhanced phosphorylated ERK environment in the CWR22 xenografts.

To eliminate the possibility that the TSTA-ELK system was also detecting p38 or JNK, we measured their levels in the xenograft extracts. The immunoblots in Fig. 6B show that, although both CWR22 and LAPC9 both express p38 and JNK, in neither case do we observe measurable levels of phosphorylated kinase. We conclude that the elevated AdTSTA-ELK imaging signal in CWR22 xenografts is most likely due to elevated levels of phosphorylated ERK1/2.

**Discussion**

The use of bioluminescence imaging of live animals is an emerging tool for tumor studies in preclinical models of cancer. The advantage of this technology is the ability to noninvasively, quantitatively, and reproducibly image targeted molecules and biological processes in living organisms (42).
The earliest applications of bioluminescent reporters used a luciferase reporter driven by a viral promoter to image transfected HeLa cells that were injected into SCID mice (44). Another application involves genetically modified tumors that stably express luciferase (45). In this case, the effect of pharmaceuticals on tumor growth can be easily measured.

Bioluminescent imaging probes have also been designed to monitor cell signaling pathways. One such example was monitoring the response of nuclear factor-κB promoter to tumor necrosis factor-α ex vivo (46). Other researchers have developed bioluminescent imaging reporters to study circadian rhythms, proteasomal regulation, IκB kinase activation, and transforming growth factor-β/SMAD pathways (47, 48). Additionally, tetra-ubiquitin and IκBα fused to FLuc reporters were used to study total and IκBα-specific proteasomal activity in xenografts and the effect of inhibitors (49).

We have taken a different approach to studying signaling pathways in xenograft tumors that do not grow as cell lines in culture. These tumors are interrogated with adenoviral-based luciferase reporters (16, 24). Our data showed that the adenoviral TSTA system could monitor AR signaling and effects of pharmaceuticals on AR activity in vivo. We therefore applied the adenovirus approach to evaluate signaling pathways in a tissue-specific manner within a living subject. The ERK1/2 branch of the MAPK pathway is one of the major signaling pathways hypothesized to be operational in cancer. The goal of our study was to develop an imaging system that could measure MAPK signaling in tumors.

To image MAPK activity, the TSTA paradigm that we developed several years ago was modified (15, 19). A potent version of the AR-responsive and prostate-specific PSA gene enhancer was used to express GAL4-ELK1. GAL4-ELK1 then activates FLuc expression only when it is phosphorylated by MAPK. Thus, the system is specific to prostate tissue and MAPK simultaneously.

Our cell culture data showed that the TSTA-ELK system responds synergistically to the combined action of AR and MAPK using either artificial (DA-MEKK and MEK) or natural stimulants (EGF) of the Ras/MAPK pathway. We further showed that the system, when transferred into an adenovirus, could reproducibly measure the response to systemic EGF stimulation in a tumor in vivo. We found that the ability to respond to EGF is a general property of androgen-dependent and androgen-independent tumors from the LAPC9 and CWR22 xenograft models.

We then used the system to address whether MAPK activity is typically elevated on transition of a tumor from the androgen-dependent to androgen-independent states (50). This issue was investigated in two widely studied animal xenograft models of human prostate cancer, CWR22 and LAPC9. We chose two models to evaluate the possibility that different xenograft models might display differential responses. These data strongly suggest that CWR22 exhibited greater MAPK activity than LAPC9 as shown by in vivo imaging and confirmed by immunoblotting of tumor extracts. However, within each xenograft model, the androgen-dependent and androgen-independent tumors displayed nearly identical functional MAPK activities.

One surprising difference between the CWR22 and LAPC9 models is the significant increase in functional MAPK in CWR22 as measured by imaging and whole-tumor phosphorylated ERK levels. The phosphorylated ERK levels did not correlate with tumor growth rates. Numerous studies have shown that AR activity can be up-regulated by stimulants of the MAPK pathway, including EGF. Elevated MAPK may therefore facilitate AR function in the CWR22 versus the LAPC9 model. If this was the case, then the imaging system could be used to test drugs that inhibit MAPK activity.

The results from our gene expression-based imaging vector TSTA-ELK suggest that functional AR and MAPK activity can be monitored in live animals. Imaging data correlate with the biochemical data showing that functional activated ERK is present in both androgen-dependent and androgen-independent CWR22 tumors. However, CWR22 and LAPC9 xenografts represent only a snapshot of the entire spectrum of prostate cancer genotypes and phenotypes. We envision that this system could be used to interrogate many different cell lines and xenografts to give a more comprehensive picture of MAPK levels in prostate cancer. Binary gene expression imaging systems, such as the TSTA and TSTA-ELK models, may facilitate the future analysis of signaling pathways during cancer progression and response of tumors to cancer treatments.

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**Figure 6.** Molecular differences in the LAPC9 and CWR22 xenograft models. Western blot analysis of phosphorylated ERK1/2 (p-ERK; p42/44) and ERK1 (p44) expression in xenografts. Total protein was extracted from tumors and immunoblotting was done using specific antibodies against each protein. p-Actin and ERK were used to normalize loading of the extracts onto the gels for the final immunoblot. Lanes 1, 2, 5, and 6, independent isolates of androgen-dependent tumor samples from LAPC9 (lanes 1 and 2) and CWR22 tumors (lanes 5 and 6). Lanes 3, 4, 7, and 8, separate androgen-independent tumor samples from LAPC9 (lanes 3 and 4) and CWR22 (lanes 7 and 8) tumors. B, Western blot analysis of p38 and JNK activity in xenograft tumors. Total protein was extracted from tumors and p-actin was used to normalize samples. Far left lane, positive controls of phosphorylated p38 and JNK from cell extracts. JNK and p38 are present in both CWR22 and LAPC9 models but show no measurable amount of the activated forms of the kinases.
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