Suppression of Tak1 Promotes Prostate Tumorigenesis

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Abstract

More than 30% of primary prostate cancers contain a consensus deletion of an approximately 800 kb locus on chromosome 6q15.1. The MAP3K7 gene, which encodes TGF-β activated kinase-1 (Tak1), is a putative prostate tumor suppressor gene within this region whose precise function remains obscure. In this study, we investigated the role of Tak1 in human and murine prostate cancers. In 50 well-characterized human cancer specimens, we found that Tak1 expression was progressively lost with increasing Gleason grade, both within each cancer and across all cancers. In murine prostate stem cells and Tak1-deficient prostatic epithelial cells, Tak1 loss increased proliferation, migration, and invasion. When prostate stem cells attenuated for Tak1 were engrafted with fetal urogenital mesenchyme, the histopathology of the grafts reflected the natural history of prostate cancer leading from prostatic intraepithelial neoplasia to invasive carcinoma. In the grafts containing Tak1-suppressed prostate stem cells, p38 and c-jun-NH2-kinase activity was attenuated and proliferation was increased. Together, our findings functionally validate the proposed tumor suppressor role of Tak1 in prostate cancer. Cancer Res; 1–11. ©2012 AACR.

Introduction

Chromosome 6q14-21 is frequently deleted in the genome of prostate cancer (1), although specific prostate tumor suppressor genes have not been identified in this region. Recently, Liu and colleagues using single-nucleotide polymorphism arrays identified an 870 kb consensus deletion located on chromosome 6q15 (2). The frequency of 6q15 deletion was significantly higher in tumors with Gleason score 8 or more (12 of 17 tumors, 71%) than in those of Gleason scores 7 or less (9 of 38 tumors, 24%). The association between 6q15 copy number changes and Gleason score was stronger than the association of any other common recurrent DNA copy number change identified, including PTEN, which had a similar prevalence of deletion but lower association with Gleason score. The majority of 6q15 deletions were hemizygous.

Five genes are deleted in the 6q15 consensus region; MDN1, CASP8AP2, CX62, BACH2, and MAP3K7, as well as one pseudogene (LOC644269). Four of these 5 genes are either required for cell viability [MDN1 (3) and CASP8AP2 (4, 5)] or are expressed in a tissue-specific manner that does not include the prostate [CX62 (6) and BACH2 (7)]. Therefore, these 4 genes are not good candidate prostate tumor suppressor genes.

The MAP3K7 gene encodes TGF-β activated kinase-1 (Tak1), a member of the MEKK family, which was identified for its role in noncanonical TGF-β receptor signaling through the mitogen-activated protein (MAP) kinase pathway leading to c-jun-NH2-kinase (JNK) and p38 activation (8). Tak1 is also a downstream signaling molecule of numerous other pathways, including interleukin (IL)-1 (9), TNF (10), Wnt/β-catenin (11), and Toll receptor (12) pathways. Cytokines such as TNFα and IL-1 activate signaling cascades in inflammatory cells that involve MAP kinase signaling and lead to activation of NFκB and/or JNK (13). Tak1 has been found to be a central mediator of these signaling pathways (10, 12). Recent work has shown a role for Tak1 in the activation of Snf1/AMP-activated protein kinase (14, 15) and in the activation of LKB1 (16). These studies show an important regulatory role of Tak1 in integrating multiple signaling pathways and suggest that MAP3K7 could function as a tumor suppressor.

Together, our previous genetic data that showed frequent hemizygous deletion of MAP3K7 in prostate cancer and the known roles of Tak1 in integrating and regulating multiple signaling pathways important for prostate growth and differentiation support a role for Tak1 as a prostate tumor suppressor. However, the functional role of Tak1 as a prostate tumor suppressor has not been reported.

Materials and Methods

Human samples

Formalin-fixed paraffin-embedded prostate tissues were from men who underwent radical prostatectomy at Stanford
University Medical Center between 1983 and 1998. There is no information on race in this cohort. Historically during this period greater than 95% of men who underwent prostatectomy at Stanford University were Caucasian. This study received Institutional Review Board approval. Fifty prostate cancers were sectioned at Stanford University. The detailed information of staining and scoring TAK1 protein is described in Supplementary Materials and Methods and Supplementary Table S1.

Cell culture

The isolation and characterization of the adult mouse prostatic stem/progenitor cell line WFU3 was described previously in detail (17, 18). Tak1\(^{lox/lox}\) mouse prostatic epithelial cells (MPEC) were isolated from the prostate of 8-week Tak1\(^{lox/lox}\) male mice as previously described (18). Generation and genotyping of Tak1\(^{lox/lox}\) mice were described previously in detail (12). To generate Tak1\(^{−/−}\) cells, Tak1\(^{lox/lox}\) MPECs were infected with self-deleting Cre-recombinase lentivirus (19). Individual clones from Cre or Mock-infected cells were isolated by limiting dilution. Both types of cells were maintained in 50:50 Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F-12 Medium (F12) with supplements as previously described (17, 18).

Reverse transcription PCR

Total RNA of WFU cells was isolated with TRIzol Reagent (Invitrogen). RNA was quantified and treated with RQ1 RNase-free DNase (Promega) to remove DNA contaminations. The detailed materials, methods, and sequences of each primer used in PCR reactions are described in Supplementary Materials and Methods and Supplementary Table S2.

Lentivirus-mediated gene knockdown

The short hairpin RNA (shRNA) plasmids, pBS/U6/Map3k7 (target sequence GGCGATGTTGAAACAACATT) and pBS/U6/shControl (GGGCATGGCACGTAC GGCAAG) were constructed based on previously published work (20). The shRNA targeting sequences were cloned into a modified Lent2lox3.7 lentivirus vector (21, 22). Methods to generate lentivirus were described previously (23). Infection efficiency was detected by EGFP expression. Individual clones were isolated using cloning cylinders after cells were grown at clonogenic density.

Cell growth assay

Mouse shTak1 or shControl cells were inoculated onto 6-well culture dishes at 1 \(\times\) 10\(^4\) cells per well \((n = 3)\). At the indicated time points, the cells were labeled with trypan blue and counted using Vi-CELL Series Cell Viability Analyzers (Beckman Coulter) following the manufacturer’s instructions. Clonogenic assays were carried out by inoculation of serial 0.5-fold dilutions (from 125–2,000 cells per dish) of viable cells onto 60-mm dishes \((n = 3)\). After 10 days growth in culture, cells were fixed in 10% formalin and stained with 0.1% crystal violet/95% ethanol solution for 10 minutes. Colonies were counted manually.

Immunoblots

Protein lysates from cells or tissues were collected and quantified by following standard protocols. Approximately 25 \(\mu\)g of lysate were used for immunoblots. Protein resolving, membrane transferring, and blocking were carried out following standard protocols. The membranes were incubated with primary antibodies for Tak1 (1:1,000), P-JNK (1:1,000), JNK (1:2,000), P-p65 (1:1,000), p65 (1:2,000), P-p38 (1:12,000), p38 (1:1,000), α-tubulin (1:4,000; all from Cell Signaling), and β-actin (1:100,000; Sigma-Aldrich) at 4°C, overnight. Following washing, membranes were incubated for 1 hour at room temperature with rocking with a 1:2,000 dilution of horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology). The HRP signal was detected using the ECL Plus Kit (Amersham Pharmacia Biotech).

Immunofluorescence

Cells were inoculated at clonogenic densities in 6-well dishes containing glass coverslips (Carolina Biological). Twenty-four to 48 hours after inoculation, the cells attached to coverslips were washed with 1× PBS, fixed in 3% formaldehyde/PBS (Electron Microscopy Sciences) and permeabilized in 0.1% Triton-x-100/PBS (Fisher Scientific). F-Actin was labeled with Alexa Fluor 594 phallolidin conjugate (1:250 in 5% donkey serum/PBS; Molecular Probes). Nuclei were labeled with Hoechst 33258 (1:10,000 dilution in PBS for 5 minutes; Molecular Probes). All coverslips were mounted in ProLong Gold (Invitrogen).

Migration assay

Cells were inoculated onto 60-mm dishes \((1 \times 10^6\) cells per dish) and allowed to grow for 24 hours. After the incubation period, a wound was made in the monolayer by scratching the dish with a 1-ml pipette tip. Cells were washed with HEPES/HBS twice and cultured in complete growth medium. At time zero specific loci were marked for future reference, and the closing of the scratches was observed at subsequent time points. Digital images of each indicated locus were taken every 10 hours. The distance of cell migration at a particular time point was measured with Photoshop CS3 (Adobe Systems Incorporated).

Invasion assay

Invasion assays were done using BD BioCoat Matrigel invasion chambers (BD Biosciences) following the manufacturer’s instructions. The detailed material and methods were described in Supplementary Materials and Methods.

Tissue recombination

All procedures with animals were approved by the Wake Forest University Institutional Animal Care and Use Committee. Prostate tissue recombination was carried out as described previously (17, 24–26). The detailed materials and methods are described in Supplementary Material and Methods.

Immunohistochemistry

Immunohistochemistry was carried out by using SensiTek HRP (Anti-Polynalent) Ready-To-Use Lab Pack (ScyTek Laboratories).
Laboratory) by following the manufacturer’s instructions. Sections were incubated at 4°C with primary antibodies: androgen receptor (AR; 1:500; Santa Cruz Biotechnology), p63 (1:500; Santa Cruz Biotechnology), CK5 (1:1,000; Covance), CK14 (1:500; Covance), CK8 (1:100; Abcam), CK18 (1:200; Abcam), and Ki67 (1:2,000; Abcam). Detailed methods were described in Supplementary Materials and Methods.

Statistical methods
Repeated measures ANOVA was carried out that accounted for the within-clone correlation of repeated measures due to replicates or time points using a mixed effects regression modeling approach to test for differences by group and clone. ANOVA and t-tests were used for analysis of cross-sectional data. More detailed information is described in Supplementary Materials and Methods. A 2-sided P value less than 0.05 was considered statistically significant. All analyses were carried out using SAS v9.2 (SAS Institute Inc.) and Stata v10.1 (StataCorp).

Results
Tak1 expression is lost in high-grade human prostate cancer
Figure 1A shows serial sections of prostate cancers of the indicated Gleason grades with hematoxylin and eosin (H&E) staining (top panels) and Tak1 immunohistochemistry (bottom panels). The numbers within images indicate the deduced score of staining. Note that the benign sample also contains a focus of cancer in the image presented, which is also scored for Tak1. Most slides contained some proportion of benign glands as well as multiple grades of tumor due to heterogeneity (Supplementary Table S1). Each slide was evaluated for Tak1 score for each Gleason grade of cancer in the section by 2 board-certified human prostate pathologists, J.S.S and A.C (Supplementary Fig. S1 and Supplementary Table S1). Tak1 expression was progressively lost with increasing Gleason grade, both within each cancer and across cancers (Supplementary Table S1 and Fig. 1B).

Gene copy number analysis showed that only one copy of the MAP3K7 gene is retained in 3 prostate cancer cell lines (LnCaP, DU145, and PC3; Supplementary Fig. S2A). No mutations were detected in the retained copy of the MAP3K7 gene in any of the 3 cell lines (Supplementary Fig. S2A). We analyzed the methylation of MAP3K7 promoter region by using bisulfite sequencing method. We found CpG islands in the promoter regions of DU145 were hypermethylated (Supplementary Fig. S2A and S2B), but not in LnCaP or PC3 cells.

Deficiency of Tak1 altered growth of mouse prostate cells in vitro
To model the role of Tak1 in prostate growth and development both in vitro and in vivo, we used a novel mouse prostate stem cell developmental model that we described (17). In this model, a stem cell line we developed (WFU3; ref. 18) can be

![Figure 1. Expression of Tak1 is lost in high-grade human prostate cancer. A, H&E and immunohistochemistry for Tak1 in representative prostate specimens. The numbers within images are the deduced expression score based on the expression matrix depicted in Supplementary Fig. S1 for Tak1 expression. Note: The benign section contains a small focus of carcinoma with reduced expression of Tak1 relative to the benign tissue in the same section. B, the expression intensity of Tak1 was scored in each section based on the matrix presented in Supplementary Fig. S1 and categorized based on tissue pathology. Each bar represents the mean ± SEM. Numbers in parentheses represent the sample size for that specific pathology that was scored. Bars with the same letter above them are not statistically significantly different from each other. Statistical significance was determined by ANOVA with post hoc analysis by Sheffe multiple comparison test, P < 0.05 was considered significant.](cancerres.aacrjournals.org)
grown and manipulated in vitro and subsequently used for in vivo analysis of prostate glandular development. We explored the expression of the 5 genes, Map3k7, Casp8ap2, Gja10, Bach2, and Mdn1, whose homologs are deleted in prostate cancer at the human 6q15 locus, in the syntenic region of the mouse genome (Chr4A4; Supplementary Table S2). Reverse transcription PCR of total RNA isolated from semiconfluent WFU3 cells is shown in Fig. 2A. Negligible gene expression was detected for Bach2, Gja10 (the mouse CX22 homolog), and Mdn1. Both Map3k7 and Casp8ap2 exhibited robust expression. Given the recent literature that indicates an essential role of Casp8ap2 in histone gene expression (4, 5), we reasoned that the possibility of Casp8ap2 being a tumor suppressor gene was less likely than for Map3k7. Therefore, the role of Tak1 as a tumor suppressor was explored further.

We knocked down the expression of Tak1 in WFU3 cells by infection with Tak1 shRNA lentivirus (shTak1). shRNA lentiviruses (shControl) that do not significantly recognize the sequence of any known gene was used as control. To remove the uninfected cells from the pooled cell populations, we isolated individual clonal populations with high E-GFP expression. Immunoblots probed for Tak1 expression showed that shTak1 shut down the expression of Tak1 protein to nearly undetectable levels (Fig. 2B). We assessed cell growth in monolayer and clonogenic growth assays (Fig. 2C and D). Tak1 deletion induced cell proliferation in vitro (Fig. 2F).

Figure 2. Deiciency of Tak1 in mouse prostate stem cells promotes growth. A, expression in normal WFU3 prostate stem cells of the 5 genes that are deleted at 6q15.1 in human prostate cancer. Note: GJA10 is the mouse homolog of CX22. B, immunoblot of Tak1 expression in individual clones from WFU3 cells infected with virus expressing control shRNA (shControl) or Tak1 shRNA (shTak1). C, kinetics of cell viability of shControl or shTak1 clones in monolayer growth. D, clonogenic growth assay of shTak1 clones or shControl clones over a 10-day growth period. Results are mean ± SEM, n = 3. A and B are significantly different at each time point, P < 0.05. Two group t tests and repeated ANOVA were used to compare individual clones. E, deletion of Tak1 in MPECs. Top 2 panels, PCR results from DNA isolated from Cre lentivirus-infected cells and Mock-infected Tak1lox/lox cells. Bottom 2 panels, immunoblot of Tak1 in individual Tak1lox/lox or Tak1−/− clones. F, loss of Tak1 increases cell proliferation in vitro. Trypan blue exclusion assay of Tak1lox/lox (n = 3) or Tak1−/− (n = 3) clones over 140-hour growth. Results are mean ± SEM. A and B are significantly different at each time point, P < 0.05. Two group t tests were used to compare individual clones.
presence or absence of Tak1. Interestingly, we found that shTak1 WFU3 cells exhibited faster kinetics of growth under monolayer conditions (Fig. 2C) and greater clonogenic survival relative to shControl cells (Fig. 2D). In parallel to these studies with WFU3 progenitor cells, we also generated MPECs from Tak1lox/lox mice (12) and deleted the floxed allele in vitro with self-deleting Cre lentivirus (ref. 19; Fig. 2E). Clonal populations were isolated from Cre lentivirus- and mock-infected cells.

Similar to what we found with shTak1 WFU3 cells, loss of Tak1 increased cell viability under monolayer growth conditions (Fig. 2F).

**Deficiency of Tak1 expression promotes cell motility and invasion in vitro**

During the course of generation and characterization of WFU3 cells with knockdown of Tak1, we noted a distinct morphologic phenotype in shTak1 cells relative to shControl cells. We found that shControl cells grew in clusters with extensive cell–cell junctions and presented a polygonal cobblestone-like shape (Fig. 3A). This pattern of growth is a normal phenotype for WFU3 cells grown under these conditions. In contrast, shTak1 WFU3 cells grew in a loosely scattered pattern, with very few cell–cell junctions and an elongated spindle-like shape (Fig. 3A). Cell scatter assay showed that more shTak1 cells underwent scattering and cell–cell disassociation compared with shControl cells (Supplementary Fig. S3A and B). To further evaluate the morphologic difference between shTak1 and shControl cells, we labeled the cells with fluorescent phalloidin to visualize F-actin in cells growing in monolayer. shControl cells grew in clusters with F-actin staining concentrated at the cell interfaces, whereas shTak1 clones exhibited a reorganization of the F-actin away from the membrane into stress fibers with fan-shaped protruding edges (Fig. 3A). This morphologic change was suggestive of a more motile phenotype. To test the motility of the cells, we carried out wound healing, or scratch assays, a scratch was made in a confluent monolayer and repeat images were captured from specific locations every 10 hours. The rate of cell migration into the "wound" was determined after calculation of the distance migrated at each time point. We observed enhanced migration of shTak1 cells at every time point (Fig. 3B). The migratory enhancement was evident for multiple clones and was reproduced in Tak1-null MPECs, relative to their floxed counterparts (Fig. 3C). We next measured the ability of shTak1 and shControl cells to migrate through an extracellular matrix using a Matrigel Boyden Chamber assay. Knockdown of Tak1 enhanced migration through Matrigel approximately 4-fold (Fig. 3D). In response to TNFα, Tak1 can activate JNK/p38 or NFκB, which regulate the transcription of genes involved in apoptosis, survival, motility, or inflammatory response (10, 12, 13). We found that activation of both JNK and NFκB (p65) was attenuated in Tak1 null prostate epithelial cells upon the treatment of TNFα (Fig. 3E).

**Suppression of Tak1 in prostate stem cells promotes prostate tumorigenesis in vivo**

We hypothesized that if the in vitro alterations we observed with shTak1 cells were manifested in vivo, then hyperplastic and possibly invasive developmental abnormalities would ensue. Three clones each of shTak1 cells and shControl cells were independently combined with UGM and grafted under the renal capsules of nude mice. For each clone, 4 independent grafts were prepared. After 10 weeks of in vivo growth, the grafts were harvested, fixed, and sectioned for histology. The histologic evaluation was done by a board-certified veterinary pathologist, J.M.C and by S.D.C. Grafts containing UGM alone exhibited small mesenchymal masses with no epithelium evident (Fig. 4A). Grafts from shControl cells only formed benign structures (Fig. 4A and B) with complete lineage differentiation into androgen receptor (AR)-, CK8-, and CK18-positive luminal cells, p63-, CK5-, and CK14-positive basal cells, and secretion of mouse dorsolateral secretory protein (mDLP) into the lumens (Fig. 4C). Three of the grafts from the shControl cells had small foci of benign hyperplasia (p63 and AR positive) with no evidence of nuclear atypia (Supplementary Fig. S4). These results are consistent with the benign phenotype of the parental WFU3 cells that we reported previously (17). In contrast, although all of the grafts formed from shTak1 cells had the presence of some benign glands, 80% showed dysplasia and/or high-grade prostatic intraepithelial neoplasia (PIN; Fig. 4A and B), and one had evidence of invasive carcinoma (Fig. 4A and B). The average weight of grafts formed either by shControl cells or shTak1 cells was not significantly different (Fig. 4D). To confirm the histopathologic diagnosis, we stained sections with lineage-specific markers AR, CK8, CK18 (luminal cell markers), p63, CK5, and CK14 (basal cell markers; Fig. 5; ref. 27). Areas with morphologic features of high-grade PIN were confirmed by abundant luminal cell crowding, nuclear atypia, prominent nucleoli (arrow head in Fig. 5) and with retention of an intact p63, CK5, or CK14-positive basal cell layer (arrow in Fig. 5. We also observed basal cell hyperplasia characterized with crowding of basal cells and association with inflammatory cells (28), suggesting the histologic transition to more proliferative phenotypes related to normal and nodular structures (circles in CK5 and CK14 staining, Fig. 5). Areas of invasive carcinoma were primarily composed of tightly packed small tubular structures either forming well-formed glands with lumens or poorly formed glands without lumina. Immunohistochemically, invasive carcinoma showed strong AR, CK8, and CK18 positivity (arrow head in Fig. 5), with invasion confirmed by absence of p63-, CK5-, and CK14-positive basal cells (arrow in Fig. 5). Focal areas of p63-positive clusters were also observed, which may indicate areas of basal cell hyperplasia or stem cell clusters. We also evaluated expression of Tak1 in all grafts by using immunohistochemistry and immunoblot probed with Tak1. As expected, expression of Tak1 was diminished in grafts formed by shTak1 cells compared with grafts formed by shControl cells (Fig. 6A and B). Moreover, activation of p38 and JNK was shown to be attenuated in the grafts containing shTak1 cells compared with shControl grafts, which was consistent with the in vitro results that activation of JNK was decreased in shTak1 cells when treated with TNFα (Fig. 3E).
Figure 3. Suppression of Tak1 promotes an invasive phenotype in vitro. A, suppression of Tak1 alters cell morphology and arrangement of F-actin. Top panels: Representative phase contrast images of shControl and shTak1 clones. These morphologic differences were consistent across multiple clones for each
We measured \textit{in vivo} cell proliferation in shControl or shTak1 grafts by Ki67 staining. We found that cell proliferation was increased between areas of benign glands, PIN, and invasive carcinoma in shTak1 grafts (Fig. 7A and B). 

**In vitro**, we observed that suppression or loss of Tak1 increased cell proliferation in both monolayer and clonogenic growth conditions (Fig 2C, D, and F). Therefore we compared cell proliferation by Ki67 staining in all benign regions from shControl and shTak1 grafts. Ki67 staining showed a significant increase in proliferation \textit{in vivo} by suppression of Tak1 (Fig. 7C and D). These data showed that suppression of Tak1 in prostate stem cells leads to a high proliferation index \textit{in vivo}.

**Discussion**

This study investigated the tumor suppressor roles of Tak1 in prostate cancer. We showed progressive loss of Tak1 expression in human prostate cancer with increasing Gleason grade. We also found promoter methylation in the retained copy of \textit{MAP3K7} in DU145 cells. The data support previous background (not shown). Middle and bottom panels, immunofluorescent visualization of F-actin. Red, Alexa Fluor 594 phalloidin labeling. Blue, Hoechst. B, suppression of Tak1 promotes migration. Scratch assay with shControl or shTak1 clones to assess migration as a function of Tak1 status. Top panel: Representative phase contrast images at the indicated time points. Black lines on images depict the location of the cell front. Bottom panel: Quantification of migration. Results are mean ± SEM. A and B are significantly different; \(P < 0.05\). Two group t tests were used to compare individual clones. C, loss of Tak1 promotes migration. Scratch assay with Tak1\textsuperscript{lox/lox} or Tak1\textsuperscript{−/−} clone to assess migration. Top panel: Representative phase contrast images at the indicated time points. Black lines on images depict the location of the cell front. Bottom panel: Quantification of migration as described in (B). D, Boyden chamber assay for evaluation of the invasive ability of shControl and shTak1 cells. Top panel: Images from crystal violet-stained chambers after invasion. Bottom panel: Quantification of invasive cell number. Bars represent the mean ± SEM, \(n = 3\) wells for each group. Statistics were described in (A). E, immunoblots show that loss of Tak1 attenuates activation of JNK and NF-\(\kappa\)B in response to TNF\(\alpha\) \textit{in vitro}. Cells were starved by depletion of all supplements for 16 hours before treatment with TNF\(\alpha\) (10 ng/mL). Lysates were collected at indicated time points.
genetic data that showed deletion of the MAP3K7 locus. We used a novel developmental model, which has mouse prostate stem cells at its core, to assess the functional role of Tak1 expression on proliferation, motility, invasion, and morphologic changes in vitro and in vivo. We found that loss of Tak1 in the prostate stem cell increased proliferation and invasion both in vitro and in vivo. In vivo, loss of Tak1 recapitulated developmental stages of human prostate cancer, with shTak1 cells producing benign glands, abundant PIN, and frankly invasive carcinoma. These studies are important because they establish a causal role for loss of Tak1 expression in prostate tumor development. Importantly, these studies establish a paradigm to functionally model putative prostate cancer genes using an in vitro/in vivo prostate developmental model.

Figure 5. Suppression of Tak1 in Prp/SCs leads to heterogeneous phenotypes including benign prostate, PIN, and carcinoma in vivo. Immunohistochemistry for AR, p63, CK8, CK18, CK5, or CK14 of representative areas of benign prostate, PIN, and carcinoma from shTak1 grafts. PIN areas show retention of intact basal layer (arrow), pleomorphic nuclei, prominent nucleoli, and hyperplastic filling of lumen (arrow head). Carcinoma areas show lacking of intact p63, Ck5, or CK14 positive basal layer (arrow) and elevated AR, CK8, or CK18 expression (arrow head). All images were taken at 400× magnification. Scale bar = 50 μm.
Previous work by our group showed somatic loss of 6q15.1 in human prostate cancer at rates approaching 40% (2). The loss of 6q15.1 was the most prevalent deletion in the genome. In addition, approximately 70% of high-grade cancers showed loss of one copy. These studies suggested the presence of a tumor suppressor at this locus. Of the 5 genes in the locus of the consensus deletion, \textit{Map3k7} and \textit{Casp8ap2} were the only ones expressed in our mouse prostate stem cells. The lack of expression of the other 3 is consistent with previous literature.

\textit{CASP8AP2}, caspase 8–associated protein 2 (also known as FLASH), was identified as a homolog of the \textit{Caenorhabditis elegans} CED-4 protein (the mammalian ortholog of CED-4 is Apaf-1). \textit{CASP8AP2} was originally described as a proapoptotic activator of caspase 8 (29). However, subsequent work has raised doubts about whether this protein is proapoptotic (30, 31). Given the contradictory findings on the role of FLASH in apoptosis, our studies focused on Tak1, encoded by \textit{MAP3K7}, as the most likely tumor suppressor at this locus.

Tak1 is a prominent and important component of multiple signaling pathways including IL-1 and Toll receptor signaling, TRAIL, and TNF signaling as well as modulation of the Wnt/\&beta; -catenin pathway through phosphorylation of nemo-like kinase. Here we show that loss of Tak1 attenuated TNF\&alp;mediated phosphorylation of p38 and p65. In the liver, loss of Tak1 leads to fibrosis, inflammation, and carcinogenesis (32).

Recent work has attributed the tumor suppressive role of Tak1 in liver cancer to its role in TNF-induced NFkB activation and modulation of NEMO activity (33). Further work is needed to elucidate the role of Tak1 loss in these processes in prostate cancer.

Interestingly, loss of Tak1 expression in and of itself is sufficient to promote changes in morphology and invasion \textit{in vitro}. These observed functions of Tak1 are not previously described and imply a basal role of Tak1 in the maintenance of a differentiated epithelial phenotype. In addition, this study shows the usefulness of stem cell models to functionally validate prostate tumor suppressors and, by extension, prostate oncogenes. Until recently, previous models relied on intact transgenic or knockout mice for specific target evaluation. These studies have been extremely informative and have validated such targets as c-myc (34), Pten (35), Nkx3.1 (36), and p27 (37). However, these models are time consuming, expensive, and become exponentially cumbersome when interrogating gene–gene and epithelial/mesenchymal interactions. The model described here is (i) rapid approximately 6 months from target identification to \textit{in vitro} gene manipulation and \textit{in vitro/in vivo} functional analysis; (ii) comparatively inexpensive; and (iii) uniquely suited for interrogating interactions between genes and tissue compartments. Primary isolation of mouse prostate stem cells by cell sorting has recently been used to evaluate the functional

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\caption{Suppression of Tak1 attenuates activation of p38 and JNK \textit{in vivo}. A, expression of Tak1 is decreased in grafts containing shTak1 cells. Immunohistochemistry for Tak1 in benign, PIN, or carcinoma areas from grafts containing shControl or shTak1 cells. B, suppression of Tak1 attenuates activation of p38 and JNK in grafts. Protein lysates were collected from grafts containing shControl or shTak1 cells. Immunoblots for phospho-p38 (P-p38) and phospho-JNK (P-JNK) were carried out to evaluate activation of p38 and JNK, respectively.}
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roles of stem cells in prostate tumor progression (38). Recently prostate stem cell models have been used as powerful screening tools to interrogate the functional role of putative prostate cancer genes (39–41).

In summary, we describe MAP3K7 as a prostate cancer tumor suppressor gene whose encoded protein, Tak1, has important roles in proliferation and invasion. We also describe the usefulness of a prostate developmental model for validation of prostate cancer genes.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

Figure 7. Suppression of Tak1 increases cell proliferation in vivo. A, immunohistochemistry for Ki67 of representative areas of benign prostate, PIN, and carcinoma from ShTak1 grafts. B, quantification of Ki67+ cells in representative areas of benign prostate, PIN, and carcinoma from grafts containing ShTak1 cells. Bar chart shows the significant increase of percentage of Ki67-positive cells in PIN and tumor areas. Expression of Ki67 was quantified with percentage of Ki67-positive cells to the total cells in each area (n = 10). Results are mean ± SEM. A, B, and C are significantly different; P < 0.05, 2-sided. Nonparametric analysis was used. C, immunohistochemistry for Ki67 in benign areas from ShControl or ShTak1 recombinants. D, quantification of Ki67+ cells from C. Quantification methods and statistics were described in B.

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