FOXO Proteins Regulate Tumor Necrosis Factor-related Apoptosis Inducing Ligand Expression

IMPLICATIONS FOR PTEN MUTATION IN PROSTATE CANCER*

Mutations in PTEN occur in 60–80% of prostate cancers and lead to a constitutive activation of the phosphatidylinositol 3-kinase pathway and a resultant loss of activity of the FOXO family of forkhead transcription factors FKHRL1 and FKHR. To provide insight into the role of PTEN mutations in prostate cancer, we used microarrays to identify genes regulated by FKHRL1 and FKHR in LAPC4 prostate carcinoma cells. These studies revealed that adenoviral overexpression of FKHRL1 and FKHR in the LAPC4 prostate cancer cell line resulted in apoptosis and induced the expression and induced many genes that affect cellular proliferation or survival. The expression of one of these FOXO-regulated genes, TRAIL, a pro-apoptotic member of the tumor necrosis factor family, was decreased in human metastatic prostate tumors. The altered expression of TRAIL in these tumors correlated directly with decreased PTEN expression and the resultant loss of FKHRL1 and FKHR activity. Analysis of the effects of FOXO proteins on the TRAIL promoter localized the FKHRL1 responsive element of the TRAIL promoter to nucleotides −138 to −121 and demonstrated that TRAIL is a direct target of FKHRL1. These findings suggest that the decreased activity of FKHRL1 and FKHR in prostate cancers resulting from loss of PTEN leads to a decrease in TRAIL expression that may contribute to increased survival of the tumor cells.

The forkhead transcription factors are DNA-binding proteins characterized by the presence of a conserved 110-amino acid winged helix DNA binding domain (1). They play important roles in embryogenesis, tumorigenesis, and maintenance of differentiation status. In Caenorhabditis elegans the forkhead transcription factor DAF-16 is under control of the insulin receptor/PI 3-kinase pathway (2). The human orthologs of DAF-16 include FKHRL1, FKHR, and AFX and belong to the FOXO subfamily of forkhead transcription factors. The PI 3-kinase pathway, via activation of its downstream kinase Akt, phosphorylates each of the FOXO proteins at three different Ser/Thr residues (3). These phosphorylated FOXO proteins interact with 14-3-3 proteins and are subsequently sequestered in the cytoplasm where they are inactive. Inhibition of the PI 3-kinase pathway by PTEN overexpression or pharmacologic means leads to dephosphorylation and nuclear translocation of active FKHRL1, FKHR, and AFX, which in turn leads to cell cycle arrest and apoptosis (4). Conversely, loss of PTEN activity results in increased Akt activity leading to inhibition of FOXO protein activity through their phosphorylation and cytoplasmic sequestration. In addition, the expression of dominant negative FKHRL1 results in the inhibition of apoptosis, demonstrating that FOXO transcriptional activity controls cellular proliferation and apoptosis downstream of PTEN (5).

The PTEN gene was initially identified by its frequent loss in glioblastomas (6), but subsequent studies have shown that PTEN is commonly mutated in prostate cancer (7–12). In prostate tumors, the loss of PTEN occurs late in the tumorigenic process, suggesting that it is more important for tumor progression than tumor initiation (13, 14). Several lines of evidence indicate that PTEN is a tumor suppressor gene. First, PTEN overexpression in tumor cell lines results in cell cycle arrest and apoptosis (4, 15–20). Second, heterozygous deletion of the PTEN gene in mice leads to the spontaneous development of multiple neoplasias (21). Finally, germline mutation of PTEN in humans results in an increased incidence of endometrial, thyroid, and breast tumors (22, 23).

Recent studies indicate that cell cycle arrest downstream of PTEN occurs via the transcriptional induction of p27kip1 by AFX and FKHRL1 (24). The resulting increased level of p27kip1 inhibits cyclin E-cdk2 and causes G1/S-phase arrest. However, the mechanism of apoptosis induced by FOXO proteins is less well understood. In T-cells FKHRL1 has been shown to induce Fas ligand, but this phenomenon has not been reproduced in prostate cancer cell lines (25). In HeLa cells, AFX induces the expression of the anti-apoptotic BCL-XL and leads to apoptosis (26). Together, these results indicate that FOXO proteins are important downstream effectors of PTEN tumor suppressive activity; however, their molecular targets and mechanisms of action in prostate cancer are not understood.

To delineate the molecular events that are perturbed by PTEN mutation in prostate carcinoma cells, we focused on identifying genes that are regulated by FOXO proteins in the prostate. Overexpression of FKHRL1 and FKHR in LAPC4 prostate carcinoma cells using adenoviral vectors induced dramatic apoptosis. Furthermore, expression profiling of LAPC4...
TRAIL Is a Gene Target of FKHRL1

Quantitative RT-PCR—Quantitative RT-PCR (qRT-PCR) was performed with 1 μg of total RNA using the Taqman model 7700 sequence detection system (PerkinElmer Life Sciences) as described earlier (27–30). Primer sequences used are: FKHR1fwd, 5'-tcaagacagtctgcaccca-3'; FKHR1rev, 5'-ggactcactaagcccttgt-3'; FKHR2fwd, 5'-aacctgccagctcggc-3'; FKHRrev, 5'-aatacgagcagagtcatcctg-3'; AFXfwd, 5'-tttcttcgtgccagttagg-3'; AFXrev, 5'-tcacacgctgctcctc-3'; TRAILfwd, 5'-GACCTCGCTGCTGATCGTG-3'; and TRAILrev, 5'-GTGCTCTGATCTGGTCTCAGCT-3'.

Tissue Specimen—To quantify relative amounts of FKHRL1, FKHR, and AFX mRNA in the prostate, four specimens of normal prostate and six specimens of prostate cancer were obtained from the Alvin J. Siteman Cancer Center Tissue Procurement Core Facility, Washington University, St. Louis, MO. The prostate tumor samples had greater than 70% tumor tissue by histology. Fifty-μm sections of the tissues were prepared using a cryostat and total RNA was isolated using TRIzol reagent (Invitrogen) according to manufacturer’s instructions.

Adenovirus Infection—LAPC4 human prostate carcinoma cells (gift from C. Sawyers, UCLA, Los Angeles, CA) were cultured in Iscove’s medium supplemented with 7% fetal bovine serum. HEK293 cells (ATCC, Rockville MD) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% serum. Adenovirus recombinants expressing FKHR-TSSA and FKHRL1-TM were prepared as described below (31). FKHR-TSSA and HA-tagged FKHR-L1 TM were gifts from T. Unterman, University of Illinois College of Medicine, Chicago, IL, and M. Greenberg, Harvard Medical School, Boston, MA, respectively (25, 32). The cDNA fragments were subcloned into the AdtrackGFP vector and inserted into the adenoviral backbone by recombinant with AdEasy vector (31). The AdtrackGFP vector itself was recombined into the AdEasy vector and inserted into the adenoviral backbone by recombination with AdEasy vector (31). The AdtrackGFP vector itself was recombined into the AdEasy vector to produce a virus that only expresses GFP for use as a negative control in all experiments. Adenoviral recombinants were purified on a cesium chloride gradient, titrated, aliquoted, and stored at −80 °C until further use. For gene delivery, purified adenovirus was added to LAPC4 cells at a multiplicity of infection (m.o.i.) of 50–100 for 2 h and washed twice with medium. Thereafter, cells were cultured for the indicated time before harvest. For immunocytochemistry, the lysates were harvested from cells infected for the indicated times, resolved by polyacrylamide gel, and subjected to Western blotting. Antibodies used were anti-FKHR (New England Biolabs) or anti-HA (monoclonal 12CA5, Roche Molecular Biochemicals) for FKHRL1 or anti-TRAIL (R & D Systems Inc.).

Cell Survival and DNA Laddering—Cell survival assays were performed with the MTS assay (Promega Corp.) as per the manufacturer’s instructions using untreated LAPC4 cells and 2% formaldehyde-fixed cells as controls. DNA ladders were visualized as described (33).

Oligonucleotide Microarray Analysis—Ten μg of total RNA was isolated from LAPC4 cells infected with either AdGFP (negative control) or AdFKHR-TSSA or AdFKHRL1-TM for 1523 to 23 probe, 5'-AGGAGGACATGTGAGCCCTTGTCTGGTCAGTTGGG3'; 115 to −69 probe, 5'-GACATCTTTGAGAAGACTGCTCCTGCAGTGAAAGGGA-3'; −141 to −98 probe, 5'-TCTCTTCTTGCTTCTTTTCCCACAGACTCTTTTGAAGACAGA3'-; −165 to −130 probe, 5'-AGGCACGAAGGAGGAGGTCTCTGATCCAGTTCCCC-3'; FKHR binding element, 5'-GGCTCTTTTGTCTGGTCTGGTTATTTTCTTAAC-3'; and the TRAIL promoter GAS-like sequence, 5'-GACTTCTAGGACGAGA3'.

Reporter Assays—LAPC4 cells were transfected using Tfx-20 transfection reagent (Promega Corp.) and HEK293 cells were transfected using LipofectAMINE (Invitrogen Corp.) according to the manufacturer’s instructions. Transfections were performed in 12-well plates seeded with 3 × 105 cells/well using 250 ng of TRAIL promoter luciferase reporter, 100 ng of CMVLaZ reporter (control), 50 ng of plasmid expressing wild type or FKHRL1-TM expression vector (pDNA1.1), and 600 ng of Bluescript plasmid (Stratagene). The −1523 to −233 TRAIL promoter luciferase reporter deletion plasmids were described previously (37). Luciferase reporter assays were performed as described (38, 39). Mutation of the forkhead binding site in the −1523 to −233 TRAIL promoter luciferase construct was generated using the QuikChange kit (Stratagene Corp.) and complementary primers 5'-GAGGAGGCTTCAGACGAGACCCCTGCAGACACACCCG3' and 5'-GCTGGGATGGCTGCTGCTGCTGCTCC-3'. The sequence of the mutant promoter construct was verified prior to use.

RESULTS

FKHRL1 and FKHR Are the Predominantly Expressed Members of the FOXO Subfamily in the Prostate—The frequent loss of PTEN in human prostate tumors decreases the activity of the FOXO subfamily of forkhead transcription factors, as they become sequestered in the cytoplasm. To determine the members of the FOXO subfamily that are expressed in human prostate, we performed qRT-PCR on normal human prostate and prostate tumors as well as the prostate carcinoma cell lines LAPC4, LnCap, DU145, and PC3. Quantitative analysis demonstrated that FKHRL1 and FKHR were expressed at much higher levels than AFX in all the analyzed samples (Fig. 1). FKHR and FKHRL1 were expressed at similar levels in normal prostate and prostate tumors; however, FKHRL1 expression
FKHRL1 and FKHR are preferentially expressed in the prostate. Total RNA from normal human prostate, prostate tumors, and LAPC4, LnCaP, DU145, and PC3 prostate carcinoma cell lines were subjected to qRT-PCR for AFX, FKHRL1, and FKHR. Results are expressed as the mean -fold change ± S.D. relative to AFX expression in normal prostate.

Overexpression of FKHRL1 and FKHR in LAPC4 Cells Results in Apoptosis—The preferential expression of FKHRL1 and FKHR in the prostate suggested that they would be important in mediating transcriptional changes caused by the loss of PTEN activity. To better understand the biologic roles of FKHRL1 and FKHR in the prostate, it is important to identify genes that are regulated by these transcription factors. For this purpose, we monitored the changes in gene expression that occur in response to overexpression of these proteins using microarrays. LAPC4 cells infected with AdFKHRL1-TM, AdFKHR-TSSA, or AdGFP control virus for 12 or 15 h was subjected to gene expression profiling. Analysis of these data showed that gene expression was not altered after only 12 h of infection (i.e. profiles of the baseline AdGFP versus AdFKHRL1 or Ad FKHR microarrays were similar). In contrast at 15 h, infection of LAPC4 cells with AdFKHRL1-TM or AdFKHR-TSSA induced changes (>3-fold) in the expression of 26 and 21 genes, respectively, when compared with AdGFP-infected cells (Tables I and II).
TRAIL Is a Gene Target of FKHRL1

The identification of genes that are differentially expressed in LAPC4 cells overexpressing either FKHRL1 or FKHR provides clues to their biological actions. First, a significant number of the induced genes encode proteins with pro-apoptotic or tumor suppressive activity. These included TRAIL, Bcl2 adenovirus E1B interacting protein, DAP kinase 1, and SMAD4. Second, expression of a number of genes encoding proteins with roles in cellular signal transduction was increased, suggesting that FKH-R-TSSA and FKHRL1-TM overexpressing cells have alterations in cell signaling. These genes included Jak1, growth factor receptor bound protein 14, and p90 S6 kinase. Third, expression of proteins that modulate global transcription, such as Cbp300/p300, general transcription factor ii, human menopausal gonadotropin box-containing protein 1, and SATB1, was increased. Additionally, the list also included some genes whose functions are as yet unknown and that may be important for the biologic actions of FKHRL1 and FKHR.

Further analysis revealed that the expression of five target genes was increased significantly by both FKHRL1-TM and FKH-R-TSSA (Fig. 4A). These included: TRAIL, which is a member of the TNF family of ligands known to have potent pro-apoptotic activity against a wide range of tumors; SMAD4, a transcription factor downstream of the transforming growth factor-β signaling pathway that is a tumor suppressor mutated in human tumors; cyclinG2, a member of the cyclin family of CDK kinase regulators up-regulated in late S phase; ATPase, aminophospholipid transporter, which is involved in the transport of amphiphatic molecules like phosphatidylserine; and BCE-1, a protein of unknown function. Overall, the transcriptional profiles of LAPC4 cells overexpressing FKHRL1 and FKHR suggest that these proteins regulate a genetic program important in cellular proliferation and survival.

TRAIL Expression Is Aberrantly Regulated in Metastatic but Not Localized Prostate Tumors—To determine whether genes regulated by FKHRL1 or FKHR in LAPC4 play a role in prostate cancer in vivo, the expression of the target genes discussed above were analyzed in human prostate specimens. For this purpose, we utilized previously published expression profiles of prostate carcinoma specimens (35). This particular microarray study identified a number of genes that were differentially expressed between NAP, localized (PCA) or metastatic (MET) prostate carcinoma, including PTEN, which regulates indirectly the activity of FKHR and FKHRL1. A comparison of the prostate carcinoma expression profile with our profiles of genes regulated by FOXO proteins in LAPC4 cells revealed that TRAIL was aberrantly expressed in prostate tumors. Statistical analysis of differences in TRAIL expression between NAP, PCA, and MET showed that TRAIL expression was decreased in MET versus NAP (p = 0.009), but not statistically different in NAP versus PCA (Fig. 4B). Additionally, TRAIL expression was significantly decreased in MET versus PCA (p = 0.023), suggesting that a decrease in TRAIL expression occurs late in tumor progression. Interestingly, the expression of PTEN in these same tumors follows a similar pattern, consistent with the fact that loss of PTEN occurs in late stage prostate tumors (13, 35). Thus, the loss of PTEN decreases the activity of the FOXO proteins leading to a decrease in the expression of TRAIL.

TRAIL Is a Direct Target of FKHRL1 and FKHR—TRAIL activates signal transduction using the DR4 and DR5 TRAIL receptors and acts to induce apoptosis in transformed mammalian cells in a paracrine manner (41). This suggests that TRAIL could influence the survival of tumor cells and, as regulators of TRAIL expression, suggests that FOXO proteins could also play such a role. To confirm the FKHRL1- and FKHR-mediated regulation of TRAIL observed in our microarray experiments, we performed additional qRT-PCR experiments using RNA isolated from LAPC4 cells infected for 15 h with either Ad-FKHRL1-TM or Ad-FKH-R-TSSA. The qRT-PCR results demonstrated that FKHRL1 overexpressing cells have altered expression of TRAIL, consistent with the FOXO proteins leading to a decrease in the expression of TRAIL.

To determine whether TRAIL is a direct target of FKHRL1, a luciferase reporter containing −1523 to +23 nt of the TRAIL promoter was co-transfected with FKHRL1 wild-type or mutant (FKHRL1-TM) expression constructs. FKHRL1-TM induced TRAIL promoter activity 10.5-fold, whereas wild-type FKHRL1 increased expression of the TRAIL reporter only 1.9-fold over basal activity, supporting the contention that FKHRL1 increases the activity of FOXO proteins leading to a decrease in the expression of TRAIL.

To identify sequences in the TRAIL promoter essential for FKHRL1-mediated TRAIL induction, a series of 5’ deletions of the −1523 TRAIL promoter luciferase reporter were examined. These deletions range from −1523 to −35 nt upstream of the TRAIL gene transcription start site and were previously used by us to localize β-interferon responsive sequences in the TRAIL promoter (37). Co-transfection of these TRAIL promoter luciferase reporters with wild-type or FKHRL1-TM in HEK293 cells showed that all but the −35 TRAIL promoter reporter

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![Fig. 3. TRAIL Is a Gene Target of FKHRL1 and FKHR—TRAIL activates signal transduction using the DR4 and DR5 TRAIL receptors and acts to induce apoptosis in transformed mammalian cells in a paracrine manner (41). This suggests that TRAIL could influence the survival of tumor cells and, as regulators of TRAIL expression, suggests that FOXO proteins could also play such a role. To confirm the FKHRL1- and FKHR-mediated regulation of TRAIL observed in our microarray experiments, we performed additional qRT-PCR experiments using RNA isolated from LAPC4 cells infected for 15 h with either Ad-FKHRL1-TM or Ad-FKH-R-TSSA. The qRT-PCR results demonstrated that FKHRL1 overexpressing cells have altered expression of TRAIL, consistent with the FOXO proteins leading to a decrease in the expression of TRAIL.](http://www.jbc.org/Downloaded from)
Changes in LAPC4 prostate carcinoma cell gene expression after 15 h of infection with AdFKHRL1-TM were assessed by dChip analysis. The upper and lower bound columns show 90% confidence intervals of -fold change values. Unigene ID, gene symbol, and Unigene description were obtained from the NCBI Unigene project. Genes in common to the profiles of FKHR-TSSA and FKHRL1-TM are highlighted in bold.

| Unigene ID | Gene symbol | Unigene description | p value | Category |
|------------|-------------|---------------------|---------|----------|
| Hs.17839   | G2-1        | TNF-induced protein 3-like | 4.06    | 5.74     | 0.05 Anti-apoptosis |
| Hs.132955  | BNIP3L      | BCL2/adenovirus E1B 19 kDa-interacting protein 3-like | 4.57    | 5.65     | 0.03 Apoptosis |
| Hs.83429   | TNFSF10     | Tumor necrosis factor (ligand) superfamily, member 10 (TRAIL) | 9.72    | 14.85    | 0.03 Apoptosis |
| Hs.79069   | CCNG2       | Cyclin G2 | 4.82    | 6.25     | 0.04 Cell cycle |
| Hs.189689  | BPAG1       | Bullous pemphigoid antigen 1 (230/240 kDa) | 4.15    | 5.58     | 0.04 Extracellular matrix |
| Hs.1369    | DAF         | Decay accelerating factor for complement (CD55, Cromer blood group system) | 5.23    | 7.38     | 0.04 Membrane protein |
| Hs.77348   | HPGD        | Hydroxyprostaglandin dehydrogenase 15-(NAD) | 6.54    | 8.67     | 0.03 Metabolism |
| Hs.100293  | OGT         | O-Linked N-acetylglucosamine (GloNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase) | 4.44    | 6.47     | 0.04 Metabolism |
| Hs.28491   | SAT         | Serine/threonine N1-acetyltransferase | 5.92    | 6.67     | 0.03 Metabolism |
| Hs.79969   | PROL2       | Proline-rich protein with nuclear targeting signal | 9.01    | 16.29    | 0.04 Nuclear protein |
| Hs.3144    | CRLB        | Cas-Br-M (murine) ectropic retroviral transforming sequence | 8.72    | 13.25    | 0.04 Signaling |
| Hs.83070   | GRB14       | Growth factor receptor-bound protein 14 | 6.45    | 13.24    | 0.05 Signaling |
| Hs.50651   | JAK1        | Janus kinase 1 (a protein-tyrosine kinase) | 4.00    | 4.90     | 0.03 Signaling |
| Hs.197751  | DAAM1       | Disheveled-associated activator of morphogenesis 1 | 4.92    | 6.59     | 0.05 Signaling |
| Hs.278589  | GTF2I       | General transcription factor II, i | 4.89    | 7.85     | 0.04 Transcription factor |
| Hs.10882   | HBp1        | Human menopausal gonadotropin box-containing protein 1 | 4.63    | 6.22     | 0.03 Transcription factor |
| Hs.75862   | MADH4       | MAD (mothers against decapentaplegic, Drosophila) homolog 4 | 3.67    | 4.35     | 0.04 Transcription factor |
| Hs.74592   | SATB1       | Special AT-rich sequence binding protein 1 (binds to nucleases) | 4.27    | 5.48     | 0.05 Transcription factor |
| Hs.170133  | FOXO1A      | Forkhead box O1A (rhabdomyosarcoma) (FKHR) | 4.53    | 6.51     | 0.05 Transcription factor |
| Hs.144931  | ATP8A1      | ATPase, aminophospholipid transporter (APLT) Class I, type | 15.03   | 28.00    | 0.03 Transport |
| Hs.301350  | FXD3        | FXD domain-containing ion transport regulator 3 | 4.27    | 5.90     | 0.05 Transport |
| Hs.278428  | DGS4        | Progestin-induced protein | 3.63    | 4.50     | 0.05 Ubiquitin |
| Hs.93975   | DEPP        | Decidurop protein induced by progesterone | 506.10  | 100,000.00 | 0.03 Unknown |
| Hs.99824   | BCE-1       | BCE-1 protein | 6.01    | 7.60     | 0.02 Unknown |
| Hs.180737  | Homo sapiens clone 23664 and 23905 mRNA sequence | 7.70    | 10.32    | 0.03 Unknown |
| Hs.12702   | H. sapiens mRNA; cDNA DKFZp586N012 (from clone DKFZp586N012) | 3.99    | 4.69     | 0.03 Unknown |
Changes in LAPC4 prostate carcinoma cell gene expression after 15 h of infection with AdFKHR-TSSA were assessed by dChip analysis. The upper and lower bound columns show 90% confidence intervals of fold change values. Unigene ID, gene symbol, and Unigene description were obtained from the NCBI Unigene project. Genes in common to the profiles of FKHR-TSSA and FKHRL1-TM are highlighted in bold.

| Unigene ID | Gene symbol | Unigene description | -Fold change | Lower bound of FC | Upper bound of FC | p value | Category      |
|------------|-------------|---------------------|--------------|-------------------|-------------------|---------|---------------|
| Hs.153924  | DAPK1       | Death-associated protein kinase 1 | 4.45         | 3.61              | 5.71              | 0.05    | Apoptosis     |
| Hs.83429   | TNFSF10     | Tumor necrosis factor (ligand) superfamily, member 10 (TRAIL) | 7.56         | 5.56              | 11.56             | 0.04    | Apoptosis     |
| Hs.79069   | CCNG2       | Cyclin G2           | 4.05         | 3.28              | 5.24              | 0.04    | Cell cycle    |
| Hs.182285  | KRT19       | Keratin 19          | -5.06        | -4.08             | -6.50             | 0.05    | Cytoskeletal  |
| Hs.8769    | BCMP1       | Brain cell membrane protein 1 | 5.85         | 4.46              | 8.34              | 0.04    | Membrane protein |
| Hs.21201   | DKFZP566B0846 | Nectin 3         | 6.03         | 4.73              | 8.26              | 0.03    | Membrane protein |
| Hs.81934   | ACADSB      | Acyl-coenzyme A dehydrogenase, short/branched chain | 3.48         | 3.02              | 4.05              | 0.04    | Metabolism    |
| Hs.6986    | Human glucose transporter pseudogene | 4.71 | 3.39 | 7.68 | 0.05 | Pseudogene |
| Hs.2359    | DUSP4       | Dual specificity phosphatase 4 | -4.50        | -3.84             | -5.42             | 0.03    | Signaling     |
| Hs.173965  | RPS6KA3     | Ribosomal protein S6 kinase, 90 kDa, polypeptide 3 | 3.65         | 3.01              | 4.61              | 0.04    | Signaling     |
| Hs.197751  | DAAM1       | Dishevelled associated activator of morphogenesis 1 | 4.26         | 3.38              | 5.68              | 0.05    | Signaling     |
| Hs.82071   | CITED2      | Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxyl-terminal domain, 2 | 5.14         | 3.94              | 7.29              | 0.04    | Transcription factor |
| Hs.26703   | CNOT8       | CCR4-NOT transcription complex, subunit 8 | 4.24         | 3.16              | 6.37              | 0.05    | Transcription factor |
| Hs.75862   | MADH4       | MAD (mothers against decapentaplegic, Drosophila) homolog 4 | 3.75         | 3.23              | 4.42              | 0.04    | Transcription factor |
| Hs.144931  | ATP8A1      | ATPase, aminophospholipid transporter (APLT), Class I, type | 12.95        | 8.83              | 24.09             | 0.02    | Transport     |
| Hs.75231   | SLC16A1     | Solute carrier family 16 (monocarboxylic acid transporters) | 5.73         | 4.51              | 7.68              | 0.05    | Transport     |
| Hs.99824   | BCE-1       | BCE-1 protein       | 4.35         | 3.53              | 5.56              | 0.05    | Unknown       |
| Hs.79844   | DKFZP564M1416 protein | 4.01 | 3.26 | 5.17 | 0.04 | Unknown |
| Hs.11449   | DKFZP564O123 protein | 3.88 | 3.13 | 5.04 | 0.05 | Unknown |
| Hs.25159   | H. sapiens cDNA FLJ10784 fis, clone NT2RP4000448, highly similar to H. sapiens mRNA; cDNA DKFZp566G0746 | 6.72 | 5.22 | -9.30 | 0.04 | Unknown |
| Hs.107479  | KIAA0738    | KIAA0738 gene product | 3.84         | 3.28              | 4.57              | 0.04    | Unknown       |
construct recapitulated the FKHRL1-TM-mediated induction observed when using the /H11002
1523
TRAIL
promoter (Fig. 6
A
).

Through this analysis the sequences important for TRAIL induction by FKHRL1 were localized to a region between 165 and 35 nt upstream of the transcriptional start site. To further delimit the region responsible for FKHRL1-mediated induction of
TRAIL
transcription, we obtained four overlapping probes that spanned the /H11002
165 to
/H11002
35-nt region of the
TRAIL
promoter. We performed EMSA using these oligonucleotide probes along with nuclear extracts prepared from HEK293 cells infected for 24 h with either AdFKHRL1-TM or AdGFP control. The sequence spanning /H11002
141 to
/H11002
98 nucleotides showed a unique gel shift band with FKHRL1 nuclear extracts that was not present in AdGFP-infected cells (Fig. 6
B
).

The specificity of this interaction was confirmed by addition of 100-fold excess unlabeled /H11002
141 to
/H11002
98 probe, which completely inhibited the formation of this complex. In contrast, gel shift patterns with the other three oligonucleotide probes showed no differences between AdFKHRL1-TM- or AdGFP-infected cells, indicating that FKHRL1 did not recognize any sequence elements in this region of the promoter. To determine whether FKHRL1 directly binds the /H11002
141 to
/H11002
98 sequence, a supershift experiment using anti-FKHRL1 antibodies was performed. The addition of anti-FKHRL1 but not an irrelevant antibody caused a shift in the mobility of the FKHRL1-specific band (Fig. 6
C
). To further support the idea that FKHRL1 binds the /H11002
141 to
/H11002
98 sequence, a 100-fold excess of an oligonucleotide probe containing the FKHR binding element from the glucose-6-phosphatase gene was added to the binding reaction and this also completely abrogated the formation of the FKHRL1-DNA complex (Fig. 6
C
). Thus, a cis-acting element in the
TRAIL
promoter located between 141 and 98 nt upstream of the transcriptional start site serves as a recognition site for FKHRL1.

We next examined whether this FKHRL1 element is important for transcriptional activation of the
TRAIL
promoter by FKHRL1. For this purpose, the location of the crucial FKHRL1

Fig. 4. The FOXO target,
TRAIL,
is aberrantly regulated in prostate cancer metastases. A, Venn diagram displays the intersection of the subsets of FKHRL1- and FKHR-regulated target genes. B, meta-analysis of prostate tumor microarray data to show
TRAIL
expression in NAP, PCA, and MET. A horizontal line indicates the median of the
TRAIL
expression level in each category. The median expression of
TRAIL
in MET was significantly different from NAP (p = 0.009) and PCA (p = 0.023) by the Mann-Whitney Rank sum test.

Fig. 5. 
TRAIL
is a direct target of FKHRL1. A, quantitation of
TRAIL
expression by microarray analysis (solid bars) and qRT-PCR (hatched bars). The microarray analysis showed AdFKHR-TSSA induced
TRAIL
7.56-fold (90% confidence interval 5.56 to 11.56) and AdFKHRL1-TM induced
TRAIL
9.72-fold (90% confidence interval 7.17 to 14.85). The mean ± S.E. of
TRAIL
induction measured by qRT-PCR for AdFKHR-TSSA is 12.5 ± 2.6 and AdFKHRL1-TM is 15.3 ± 3.2. B, Western blot analysis of lysates from LaPC4 cells infected with AdGFP, AdFKHRL1-TM, or AdFKHRL1-TSSA for 24 h using anti-TRAIL antibodies. C, −1523 to +23
TRAIL
promoter luciferase reporter was transfected into LaPC4 cells alone (TRAIL) or with either wild type (+WT) or mutant FKHRL1 (+TM). The solid bars represent -fold change relative to the luciferase activity of the
TRAIL
promoter alone. All values are ± S.E. of the mean of triplicate measurements and the data presented are from one of three experiments with similar results.
FKHRL1 binds to a sequence element in the TRAIL promoter. A, 5’ deletion constructs of the TRAIL promoter luciferase reporter construct (TRAIL) were transfected into HEK293 cells along with either wild type FKHRL1 (hatched bars) or FKHRL1-TM (solid bars) expression vectors. The fold change in luciferase activity relative to that obtained using the wild-type promoter sequence was first narrowed by further EMSA and competition experiments (data not shown). For example, whereas the −141 to −98 sequence interacted with FKHRL1, probes containing the flanking sequences −165 to −130 or −115 to −69 of the TRAIL promoter did not interact with FKHRL1. Thus, we were able to delimit the FKHRL1 binding site in the TRAIL promoter to the region between these two flanking sequences (i.e. −138 to −121 nt). Inspection of this region showed that it shares commonality with known FOXO protein DNA binding sites in that it contains multiple stretches of 5’-TTT-3’ (25, 32, 42). To disrupt FKHRL1 binding and consequent transcription of the TRAIL promoter, the three TTT motifs between −138 and −121 were mutated to AGA within the −165 to +23 TRAIL promoter reporter construct (Fig. 7A). Co-transfection of FKHRL1-TM with this mutated TRAIL promoter luciferase reporter construct completely abrogated FKHRL1-TM-induced luciferase expression (Fig. 7B). This data indicates that a cis-acting element located between −138 and −121 nt of the TRAIL promoter is important for FKHRL1-mediated transcription of TRAIL.

Fig. 6. FKHRL1 binds to a sequence element in the TRAIL promoter. A, 5’ deletion constructs of the TRAIL promoter luciferase reporter construct (TRAIL) were transfected into HEK293 cells along with either wild type FKHRL1 (hatched bars) or FKHRL1-TM (solid bars) expression vectors. The fold change in luciferase activity relative to that obtained using the −35 to +23 construct is shown. All values are triplicate measurements of mean ± S.E. and the data presented are from one of two experiments with similar results. B, nuclear extracts from HEK293 cells infected with AdGFP (G) or AdFKHRL1-TM (F) were incubated with 32P-end-labeled double-stranded oligonucleotides corresponding to TRAIL promoter sequences −165 to −130, −141 to −98, −115 to −69, or −79 to −35. The binding reactions were electrophoresed through an 8% polyacrylamide gel and autoradiographed. Lane C depicts addition of 100-fold excess unlabeled oligonucleotide in each case. An arrow indicates the specific FKHRL1 gel shift band detected only with the −141 to −98 probe. C, nuclear extracts from cells infected with AdGFP (lane 1) or AdFKHRL1-TM (lanes 2–6) were incubated with 32P-end-labeled double-stranded oligonucleotide corresponding to −141 to −98 of the TRAIL promoter sequence. Also shown is the effect of preincubation of binding reaction with a 100-fold excess unlabeled −141 to −98 oligonucleotide (lane 3), 100-fold excess of known FKHR binding sequence from the glucose-6-phosphatase gene (lane 4), 100-fold excess of TRAIL promoter − interferon activated sequence-like sequence (lane 5), 1 μg of anti-FKHR1 antibody (lane 6), and 1 μg of nonimmune rabbit IgG (lane 7). The reaction mixture was electrophoresed on a 6% polyacrylamide gel and autoradiographed. The data presented are from one of two experiments with similar results.

Fig. 7. FKHRL1-activated transcription of TRAIL promoter requires an element between −138 and −121 nt. A, schematic of the TRAIL promoter highlighting the FKHRL1 binding region. B, TRAIL promoter luciferase reporter constructs −35 to +23 (−35), wildtype (−165), and mutated −165 (−165m) were co-transfected into HEK293 cells alone (control) or with FKHRL1-TM expressing plasmid. The fold change in luciferase activity relative to that obtained using the −35 to +23 construct is shown. All values are triplicate measurements with mean ± S.E. The data presented are from one of two experiments with similar results.
PTEN is one of the most commonly mutated genes in human tumors (7, 12). In prostate cancer, PTEN mutations are rarely detected in early stage tumors, but are commonly found in late stage carcinomas and metastases, suggesting that PTEN loss aids tumor progression rather than tumor initiation (13). The molecular basis for PTEN action in vitro is associated with its ability to negatively regulate the PI 3-kinase pathway by enzymatic hydrolysis of a phosphate group from the 3-position of phosphatidylinositol phosphate, an intracellular second messenger (20). In vitro experiments have demonstrated that PTEN overexpression can induce G1 to S-phase arrest associated with apoptosis (19, 43, 44). This cell-cycle arrest occurs by the induction of p27kip, a potent cell cycle inhibitor (21). Many studies have shown that important targets of PTEN are the FOXO proteins. FOXO proteins are sequestered in the cytoplasm when the PI 3-kinase pathway is active, however, the inhibition of this pathway by PTEN results in dephosphorylation and consequent nuclear translocation of active FOXO proteins leading to decreased cellular proliferation or apoptosis (4). Loss of PTEN activity leads to decreased FOXO activity leading to inappropriate cell survival thus contributing to tumor progression.

Experiments with the C. elegans forkhead protein DAF-16 have demonstrated its importance in growth factor signal transduction and longevity (2). The FOXO proteins are the DAF-16 mammalian homologs and are downstream effectors of the PI 3-kinase pathway, whose activity is decreased by the loss of PTEN in tumor cells. To help understand the biological roles of the FOXO proteins in prostate cancer, we identified a number of FOXO-regulated genes using microarray analysis. These experiments were performed in LAPC4 prostate carcinoma cells (40) that have low PTEN expression and therefore contain mostly phosphorylated forms of FOXO proteins. Because the endogenous FOXO proteins are sequestered in the cytoplasm by phosphorylation, these cells have minimal basal FKHL1 or FKHR activity and are sensitive to overexpression of constitutively active mutant forms of these transcription factors (FKHL1-TM and FKHR-TSSA). Using adenoviral delivery of these transcription factors, we identified a number of FOXO protein target genes in prostate carcinoma cells, including many that function in proliferation and cell survival. Furthermore, FKHL1 was found to induce the expression of FKHR, further linking the expression and function of these two transcription factors. This finding may have implications for FKHL1 and FKHR actions in vivo as it has been found that they have largely overlapping tissue expression profiles (45).

Finally, the identification of FKHL1 and FKHR target genes suggest that despite some differences in the transcriptional profiles of cells expressing these two proteins, both of them appear to regulate programs that lead to decreased cell survival and proliferation.

We examined the expression pattern of FOXO protein-regulated genes identified in vitro in human prostate specimens. For this analysis, we exploited the vast amounts of gene expression data now being accumulated on a wide variety of normal and diseased human tissues. For example, there are a number of studies detailing the expression patterns of prostate tumors and one of these was selected for detailed analysis (35). These prostate tissue gene expression profiles were compared with the profiles we obtained in LAPC4 cells overexpressing FKHL1 and FKHR. By this analysis, we found that TRAIL expression was significantly decreased in metastatic prostate tumors. In this same set of tumors, Dhanasekaran et al. (35) reported that PTEN expression was decreased, a finding that is in accord with previous studies showing that PTEN loss is a late manifestation of prostate tumorigenesis (13, 40, 46). The correlation of decreased PTEN and TRAIL expression in metastatic prostate tumors, but not in localized prostate carcinomas, strongly supports our in vitro data indicating that TRAIL expression is regulated by FOXO proteins. Thus, tumors that have decreased PTEN activity have decreased expression of TRAIL because of decreased FOXO activity. The re-analysis of publicly available gene expression profiles has enabled us to highlight a potentially biologically important phenomenon in vivo. Such a pooling of resources will enhance the power of future investigations and provide additional insight into understanding the pathogenesis of disease.

TRAIL is a pro-apoptotic member of the TNF family of proteins with the ability to induce apoptosis in a wide variety of tumor cells while being nontoxic to nontransformed cells, a property that is being exploited for therapeutic purposes (47). In prostate cancer cells, it is known that the inhibition of PI 3-kinase by PTEN sensitizes them to TRAIL-mediated apoptosis (48). The decreased activity of the PI 3-kinase pathway also leads to nuclear translocation of FKHL1 and FKHR and results in TRAIL transcription. Hence, TRAIL induction by FKHL1 and FKHR, which lie downstream of PTEN and are effectors of the PI 3-kinase pathway, is a potentially important mechanism for inhibiting cell growth.

Despite the knowledge that TRAIL is an important pro-apoptotic factor, very little is known about its transcriptional regulation. We have previously shown that TRAIL is induced by β-interferons (37) and here we show that both FKHL1 and FKHR can regulate TRAIL transcription. Furthermore, by promoter analysis, the sequence required for TRAIL induction by FKHL1 was localized to −138 to −121 nt of the TRAIL promoter. Mutation of this sequence completely abrogated TRAIL promoter induction by FKHL1, indicating that FKHL1 can directly regulate TRAIL gene transcription. Comparison of the FKHL1 binding site in the TRAIL promoter with known forkhead sites revealed that these sequences constitute a novel forkhead protein binding site. Furthermore, this site is in close proximity to a γ-interferon activated sequence-like sequence in the TRAIL promoter, which raises the possibility that FKHL1 may modulate the ability of interferons to regulate TRAIL transcription under certain circumstances, leading to cross-talk between the Jak-STAT and the PI 3-kinase pathways. This is especially relevant in the light of the fact that interferons also stimulate apoptosis of prostate cancer cells (49). Such cross-talk may be important for prostate homoeostasis and could potentially be exploited for therapeutic purposes.

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