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in Androgen-Responsive Prostate Cancers

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13. ABSTRACT (Maximum 200 Words) TMEFF2 is a novel transmembrane protein-containing two follistatin domains and an epidermal growth factor-like motif-that is mainly expressed in the prostate and brain. TMEFF2 is highly expressed in the AD prostate cancer cell line, LNCaP, and is controlled by androgen. TMEFF2 is also upregulated by estrogen in a dose- and time-dependent manner. Ectopic expression of TMEFF2 can inhibit prostate cancer cell growth. I cloned the 5'-flanking region of the human TMEFF2 gene and using a luciferase reporter assay showed that it contains a functional promoter. The 0.7-kb region upstream to the TMEFF2 transcription start site encompasses the minimal promoter required for TMEFF2 expression. Sequence analysis of the TMEFF2 promoter revealed potential binding sites for several transcription factors including Sp1 and an E-box that could be recognized by c-Myc. An inverse correlation between TMEFF2 and c-Myc expression was found in CWR22 prostate xenografts. Reporter gene and mobility shift assays demonstrated that c-Myc could repress TMEFF2 gene expression through its cognate site. In light of the probable role of TMEFF2 in inhibiting cell growth, its suppression may contribute to the oncogenic properties of c-Myc.				
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TMEFF2: A novel gene expressed selectively in androgen-responsive prostate cancer

Introduction:

Prostate cancer, the most frequent solid cancer in older men, is a leading cause of cancer deaths. Although proliferation and differentiation of normal prostate epithelia and the initial growth of prostate cancer cells are androgen-dependent (AD), prostate cancers ultimately become androgen-independent (AI) and refractory to hormone therapy. Using subtractive hybridization, we initially searched for genes that were differentially expressed between AD and AI prostate cancers, and identified a novel gene TMEFF2 (also known as tomoregulin, TPEF and HPP1). The purpose of this research was to explore the role of TMEFF2 in the proliferation of prostate cancer cells.

Results:

TMEFF2 is Highly Expressed Almost Exclusively in the Prostate.

TMEFF2 expression was measured in cell lines from several tissues (e.g. lung, liver, breast, pancreas, bone and hematopoietic cells, **Fig. 1**). The results showed that TMEFF2 is expressed only in AD LNCaP cells. Analysis of TMEFF2 expression in normal human tissues demonstrated that TMEFF2 is expressed specifically in normal brain and prostate.

Factors that Modulate TMEFF2 Expression in Prostate Cancer Cells.

As TMEFF2 is expressed in AD LNCaP cells but not in AI PC3 and DU145 cells, we tested whether TMEFF2 is regulated by androgens. LNCaP cells were grown in media supplemented with charcoal stripped (CS) FBS (to remove steroid hormones) and treated with dihydrotestosterone (DHT). As shown in **Fig. 2**, levels of TMEFF2 mRNA were low in cells grown in CS FBS but increased in a time- and dose-dependent manner upon treatment with DHT.

Genes regulated by androgens can often be induced by ligands of other nuclear hormone receptors. Therefore, the effect of estrogen (E2) and 1,25-dihydroxyvitamin D₃ (VD₃) on TMEFF2 expression was examined. TMEFF2 mRNA levels were up regulated in LNCaP cells by E2 and VD₃ in a dose- and time-dependent manner (**Fig. 3**).

Overexpression of TMEFF2 Inhibits Proliferation of Prostate Cancer Cells.

We generated DU145 stable cell lines (DU145/TMEFF2) that overexpressed TMEFF2 and measured their proliferative rates. We found that the growth rate of DU145/TMEFF2 clones in culture was reduced by 43-66 % as compared with DU145/neo control cell lines (transfected with the empty vector, **Fig. 4**). The effect of TMEFF2 on cell proliferation was also examined by clonogenic assays. PC3 cells were transfected with either an empty pcDNA3.1 vector or a pcDNA3.1 vector expressing TMEFF2 and these cells were cultured in the presence of G418. Expression of TMEFF2 resulted in a 34-65 % decrease in the number of G418-resistant colonies (**Fig. 4**).

Cloning and Characterizing TMEFF2 Regulatory Region.

We cloned the 5'-flanking region of the human TMEFF2 gene and using a luciferase reporter assay showed that it contains a functional promoter (**Fig. 5**). Sequence analysis of the TMEFF2 promoter revealed potential binding sites for several transcription factors including Sp1 and an E-box that could be recognized by c-Myc. Reporter assays (**Fig. 6**) and mobility shift assays (**Fig. 7**) demonstrated that c-Myc could repress TMEFF2 gene expression through its cognate site. In light of the probable role of TMEFF2 in inhibiting cell growth, its suppression may contribute to the oncogenic properties of c-Myc.

TMEFF1

TMEFF1 is a gene that displays a high degree of homology with TMEFF2. We expanded our study and investigated whether TMEFF1 plays a role in prostate cancer. Our data suggest that TMEFF1 is not involved in prostate cancer. However, we found that TMEFF1 may have a suppressive role in brain carcinogenesis. Complete details of this study can be found in the manuscript entitled, '*TMEFF1 and brain tumors*' in the appendix.

Reportable Outcomes:

Repression of the TMEFF2 promoter by c-myc. J Mol Biol. 2003 328(5):977-83.

TMEFF1 and brain tumors. Oncogene. 2003 22(18):2723-27.

Conclusions:

Taken together, our results suggest that TMEFF2 may act as a tumor suppressor gene in the prostate and its inactivation is associated with androgen-independent disease progression. Despite recent advances in detection and treatment of prostate cancer, the overall mortality rate has not fallen. Hopefully, identifying tumor suppressor genes like TMEFF2 may provide novel therapeutic approaches to this disease.

Figure Legends

Fig. 1. Tissue specific expression of TMEFF2. *A*, TMEFF2 expression in cancer cell lines from various tissues. RNA was extracted from different cell lines and Northern blot was performed using radiolabeled TMEFF2 probe. The blot was rehybridized with a control $\alpha^{32}\text{P}$ -dATP-labeled GAPDH probe. *B*, Examination of TMEFF2 expression in multiple normal tissues. Multiple tissue Northern blots were hybridized with radiolabeled TMEFF2.

Fig. 2. TMEFF2 is regulated by androgen. *A*, Dose-dependent effect of dihydrotestosterone (DHT) on TMEFF2 expression. LNCaP cells were plated in media supplemented with charcoal-stripped FBS for two days, and then incubated for an additional two days with different concentrations of DHT. *B*, Time-course of TMEFF2 induction by DHT. LNCaP cells were plated as described in panel A and incubated with 0.01 μM DHT for the times indicated. *C*, TMEFF2 expression in response to androgen withdrawal. CWR22 xenografts were propagated in male mice that were then castrated. Tumors were removed at days 0, 1, 2, 3, 4 and 10-post castration. Northern blots were hybridized with $\alpha^{32}\text{P}$ -dATP-labeled TMEFF2 and reprobed with radiolabeled cDNA probes for PSA (*C*) and GAPDH (*A-C*).

Fig. 3. Effect of 17 β -estradiol (E2) and 1,25-dihydroxyvitamin D₃ (VD₃) on TMEFF2 expression in LNCaP cells. *A*, Dose-dependent effect of E2 on TMEFF2 expression. *B*, Time course of TMEFF2 induction by E2. *C*, Dose-dependent effect of VD₃ on TMEFF2 expression. *D*, Time course of TMEFF2 induction by VD₃. LNCaP cells were treated with either E2 or VD₃ as described in Fig 3. Northern blots were hybridized with $\alpha^{32}\text{P}$ -dATP-labeled TMEFF2, and reprobed with radiolabeled GAPDH.

Fig. 4. Effect of TMEFF2 on growth of prostate cancer cells. *A*, MTT assay. Two DU145/neo sublines (n1, n2) and four DU145/TMEFF2 sublines (TM1-4) were plated in 96-well plates at a concentration of 1000 cells per well. MTT assays were performed after four days. Data represent mean absorbance reading at OD 590 \pm S.D. Results are from quadruplicate samples (representative experiment from three independent experiments). *B*, Colony formation assay. PC3 cells were transfected with either an empty pcDNA3.1 vector or a pcDNA3.1 expressing TMEFF2 (pcTMEFF2) and cultured in the presence of G418 (500 $\mu\text{g}/\text{ml}$). The values are the mean \pm SD of three plates (representative experiment from two independent experiments).

Fig. 5. Promoter activity of the 5'-flanking region of the human TMEFF2 gene. *A*, The 5'-flanking 3.5-kb (-3500 to +31) DNA fragment of TMEFF2 was subcloned into the luciferase reporter gene in the pGL3 vector. DU145 and LNCaP prostate cancer cells were cotransfected with either 1 μg of the pGL3 (-3500 to +31) promoter-luciferase vector or the pGL3 empty vector, along with 0.1 μg of the pRL-SV40 vector that served as internal control for transfection efficiency. *B*, Schematic presentation of the 5' region of the TMEFF2 gene. The thick line represents the minimal promoter. *C*, Deletions of the 5'-flanking 3.5-kb fragment of TMEFF2 were made and subcloned into the luciferase reporter gene in the pGL3 vector. DU145 cells were cotransfected with 1 μg of either one of the various pGL3 promoter-luciferase reporter constructs or an empty pGL3 vector, together with 0.1 μg of the pRL-SV40 vector. After two days, cells were harvested and luciferase activity was measured. Results represent the mean \pm SD of triplicate transfections. The experiments were repeated three times with triplicate plates per experiment point.

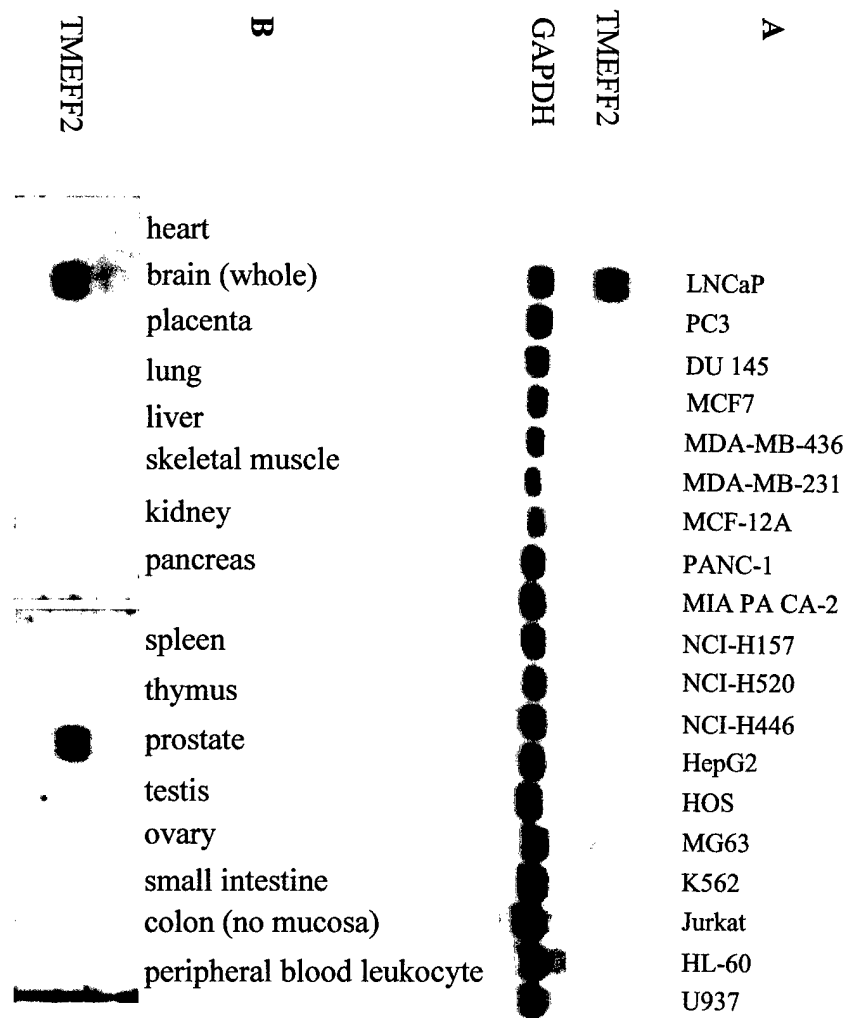
Fig. 6. c-Myc represses expression of the TMEFF2 promoter. DU145 cells were cotransfected with the 0.7-kb (-690 to +31) fragment of the TMEFF2 promoter-luciferase and either a pMV6/c-Myc expression vector or an empty pMV6 vector and 0.1 μg of the pRL-SV40 vector. Cells were harvested

and luciferase activity was measured as described in Fig. 1. Results represent the mean \pm SD of triplicate transfections. The experiments were repeated three times with triplicate plates per experimental point.

Fig. 7. c-Myc binds to the c-Myc binding site in the TMEFF2 promoter. EMSA was performed using 10 μ g of nuclear extract proteins from LNCaP cells. Extracts were incubated with 32 P-labeled wild type oligonucleotide containing the E-box site (CACGTG) from the TMEFF2 promoter (-100 to -78). Unlabeled wild type competitor (wt competitor), mutant c-Myc competitor (containing a mutation in the E-box; CACGTG \rightarrow CAGTTG, mut competitor) or a c-Myc antibody were added to the reactions as indicated.

Appendices: Copy of manuscripts, Repression of the TMEFF2 promoter by c-myc, and TMEFF1 and brain tumors.

Fig. 1



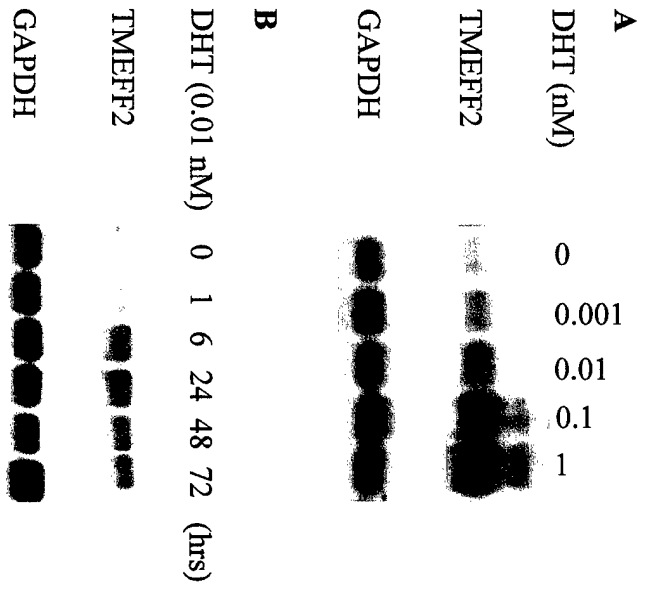


Fig. 2

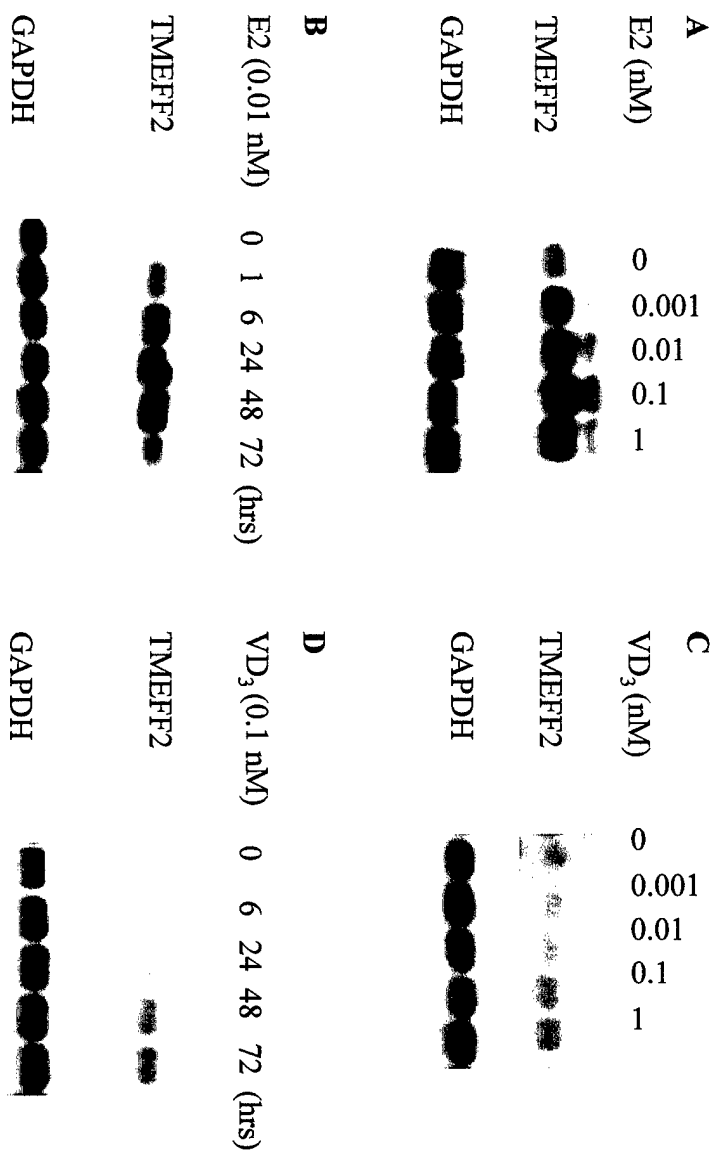


Fig. 3

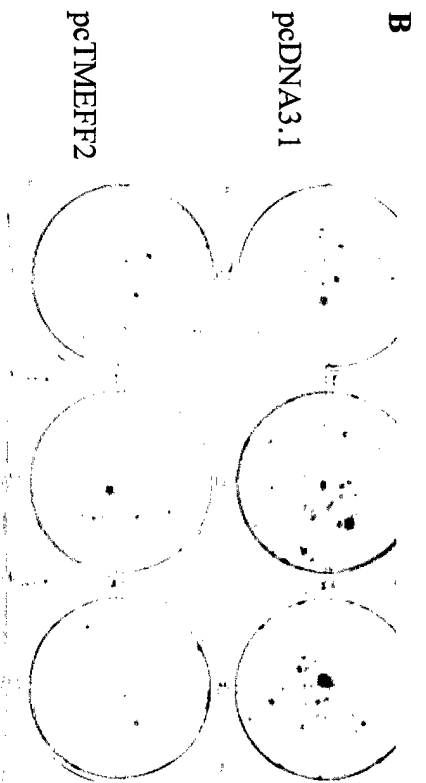
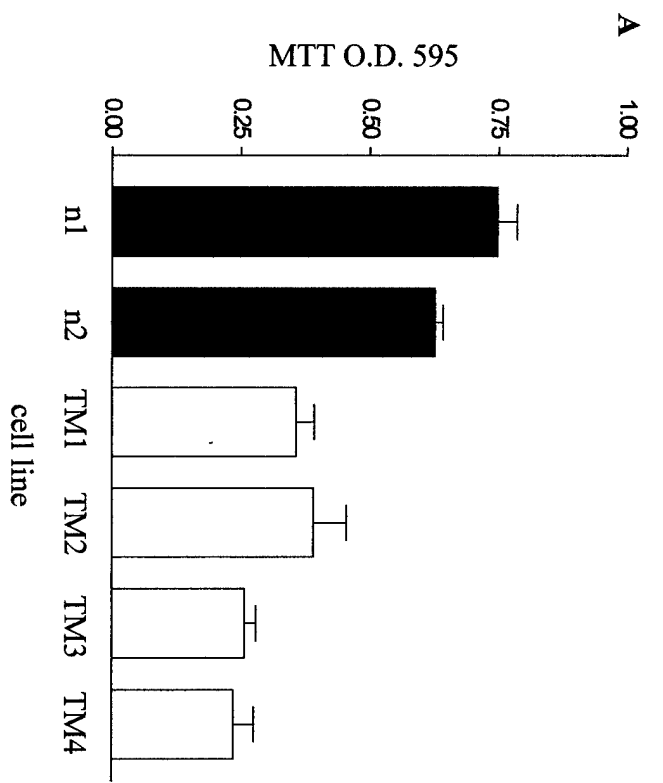
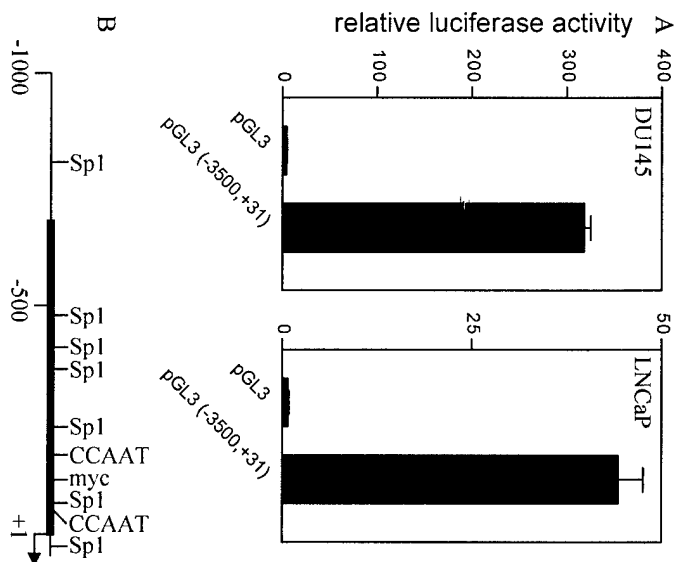


Fig. 4

Fig. 5



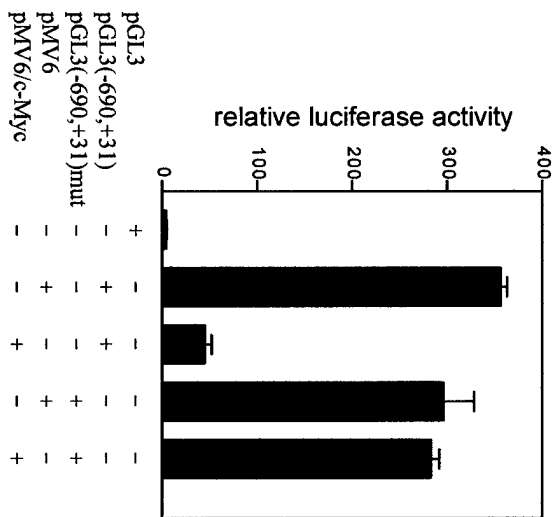


Fig. 6

wt competitor 10-fold
 wt competitor 100-fold
 mut competitor 10-fold
 mut competitor 100-fold
 anti c-Myc

c-Myc →

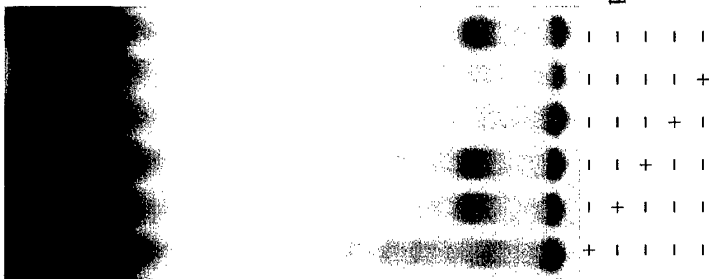


Fig. 7

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Repression of the TMEFF2 Promoter by c-Myc

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TMEFF2 is a novel transmembrane protein, containing two follistatin domains and an epidermal growth factor-like motif that is mainly expressed in the prostate and brain. Recently, we showed that expression of TMEFF2 could inhibit prostate cancer cell growth. In addition, the TMEFF2 gene is frequently hypermethylated in human tumor cells, suggesting that it might be a tumor suppressor gene. We cloned the 5'-flanking region of the human TMEFF2 gene and using a luciferase reporter assay showed that it contains a functional promoter. The 0.7 kb region upstream to the TMEFF2 transcription start site encompasses the minimal promoter required for TMEFF2 expression. Sequence analysis of the TMEFF2 promoter revealed potential binding sites for several transcription factors including Sp1 and an E-box that could be recognized by c-Myc. An inverse correlation between TMEFF2 and c-Myc expression was found in CWR22 prostate xenografts. Reporter gene and mobility shift assays demonstrated that c-Myc could repress TMEFF2 gene expression through its cognate site. In light of the probable role of TMEFF2 in inhibiting cell growth, its suppression may contribute to the oncogenic properties of c-Myc.

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Keywords: TMEFF2; c-Myc; promoter; transcriptional-suppression; growth inhibition

*Corresponding author

Introduction

TMEFF2 (tomoregulin, TPEF, HPP, TENB2) is a recently cloned transmembrane protein containing an altered epidermal growth factor (EGF)-like motif and two follistatin domains, that is predominantly expressed in prostate and brain.^{1–5} The function of TMEFF2 is largely unknown, but its structural domains suggest that it may play a role in signaling by growth factors. We have recently shown that forced expression of TMEFF2 can inhibit growth of human prostate cancer cells.⁶ In addition, the 5'-region of the TMEFF2 gene is frequently hypermethylated in human tumor cells, suggesting that it might be a tumor suppressor gene.^{3,5}

The c-Myc proto-oncogene has been implicated in a variety of cellular processes including proliferation, differentiation and apoptosis, and is involved in many chromosomal abnormalities that play a role in tumorigenesis (reviewed in Refs. 7 and 8). Overexpression of c-Myc may contribute to the onset of some cancers including prostate cancer. Myc is a transcription factor, and its role in

transformation has been mainly ascribed to the regulation of downstream target genes. c-Myc forms heterodimers with Max that can bind to the E-box sequence CACGTG (as well as related non-canonical sites) and activates transcription of target genes. Among c-Myc inducible genes, ornithine decarboxylase⁹ and cdc25A¹⁰ are of interest in this respect because of their involvement in cell cycle progression. Max also forms alternative dimers with the Mad network of proteins that compete with the Myc–Max heterodimers resulting in transcriptional repression. Recent reports have demonstrated that c-Myc repression results not from direct binding to DNA by Myc/Max/Mad dimers, but rather by its interactions with other transcription factors (reviewed in Refs. 11 and 12). Interestingly, many of the genes reported to be down-regulated by Myc, like gadd45,¹³ gas1,¹⁴ p21^(WAF1/CIP1),¹⁵ p15^(INK4b),¹⁶ and p27^(kip1)¹⁷ are involved in growth arrest.

Here, we cloned the 5'-flanking region of the human TMEFF2 gene and showed that it contains a functional promoter. We also demonstrated that c-Myc could repress TMEFF2 transcription in prostate cells. Given the relevance of TMEFF2 in slowing cell growth, its suppression may contribute to the oncogenic properties of c-Myc.

Abbreviations used: EPF, epidermal growth factor.
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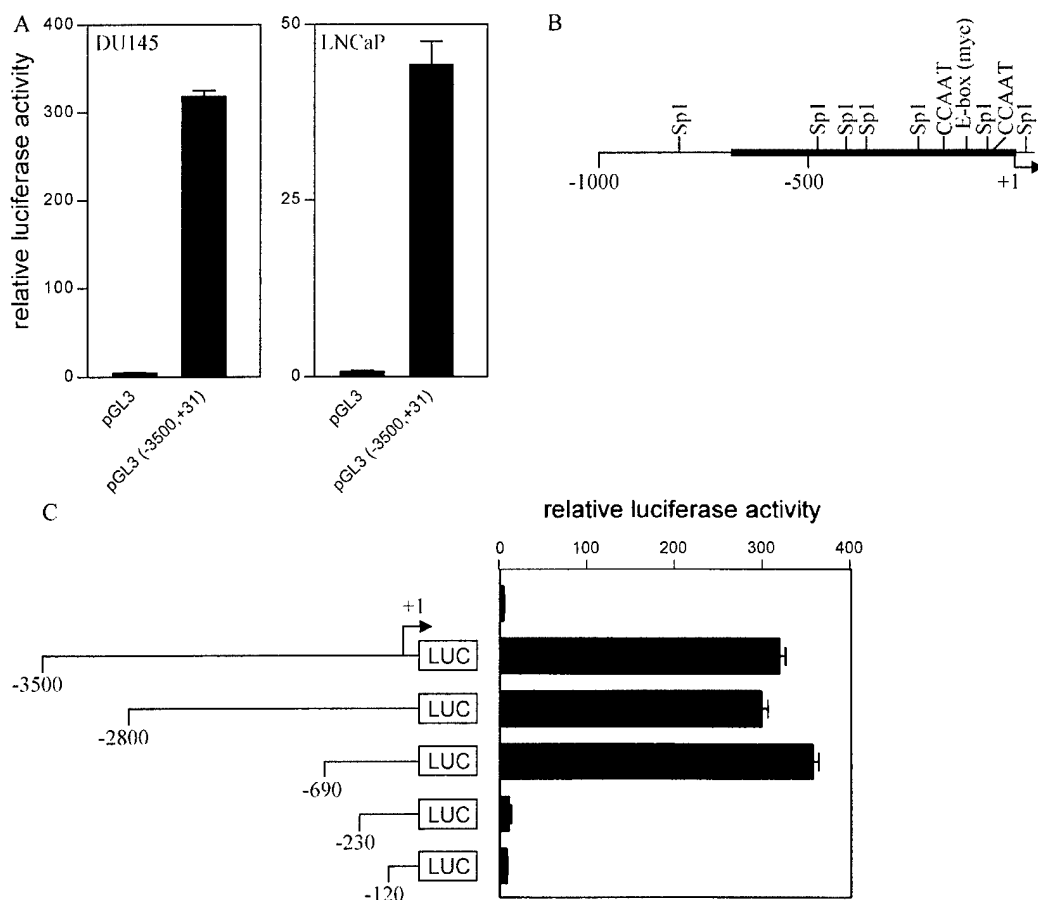


Figure 1. Promoter activity of the 5'-flanking region of the human TMEFF2 gene. *A*, the 5'-flanking 3.5 kb (-3500 to +31) DNA fragment of TMEFF2 was subcloned into the luciferase reporter gene in the pGL3 vector. DU145 and LNCaP prostate cancer cells were cotransfected with either 1 μ g of the pGL3 (-3500 to +31) promoter-luciferase vector or the pGL3 empty vector, along with 0.1 μ g of the pRL-SV40 vector that served as internal control for transfection efficiency. *B*, Schematic presentation of the 5' region of the TMEFF2 gene. The thick line represents the minimal promoter. *C*, Deletions of the 5'-flanking 3.5 kb fragment of TMEFF2 were made and subcloned into the luciferase reporter gene in the pGL3 vector. DU145 cells were cotransfected with 1 μ g of either one of the various pGL3 promoter-luciferase reporter constructs or an empty pGL3 vector, together with 0.1 μ g of the pRL-SV40 vector. After two days, cells were harvested and luciferase activity was measured. Results represent the mean \pm SD of triplicate transfections. The experiments were repeated three times with triplicate plates per experiment point.

Results

Cloning the TMEFF2 promoter region

We have mapped the genomic locus of TMEFF2 to the human chromosome 2q32.1.⁶ Searching the Genbank database, we found a human BAC clone from chromosome 2 (PR11-394A2) that was partly homologous to TMEFF2 cDNA. From this BAC, we cloned a 12 kb *Eco*RI fragment that encompassed the 5' genomic region of TMEFF2. To test whether this region has a functional promoter, a 3.5 kb fragment containing upstream TMEFF2 sequences, from -3500 to +31 relative to the TMEFF2 transcription start site,³ was placed in front of a luciferase reporter gene in the pGL3 basic vector. A significant increase in luciferase

activity was observed in the prostate cancer cell lines DU145 (80-fold) and LNCaP (60-fold), transfected with the TMEFF2 promoter-luciferase vector compared to cells transfected with the empty reporter vector (Figure 1(A)). Sequence analysis of this region revealed that the TMEFF2 5'-flanking region had potential binding sites for several transcription factors including Sp1 and Myc (E-box), as well as 2 CCAAT-boxes, but no TATA-box was present (Figure 1(B)). Similar results were reported in an earlier study where the TMEFF2 promoter had been cloned from a λ genomic library and subjected to sequence analysis. The results from that study also showed that the 5'-region of TMEFF2 contained a CpG island.³

Next, to define DNA regions important for gene expression, deletions of the 5'-flanking sequences

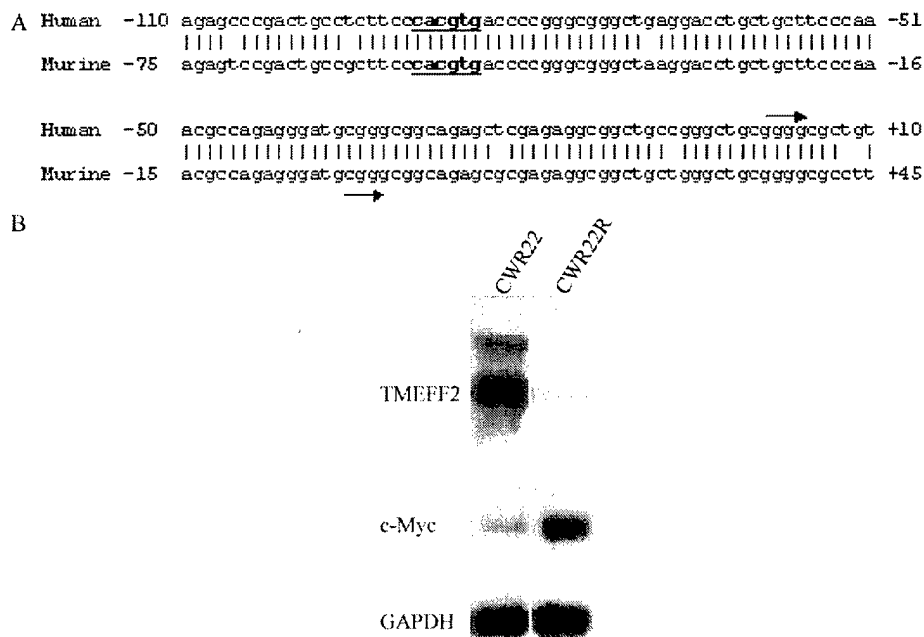


Figure 2. TMEFF2 is a potential target gene for c-Myc. (A) Comparison between the human and murine potential c-Myc binding site (E-box) in the TMEFF2 promoter. The E-box sequence is underlined and is in bold letters; the upper arrow represents the human (GenBank accession number AC092644) transcription starting point; the lower arrow displays the murine (GenBank accession number AC098743) transcription-starting site. (B) TMEFF2 expression inversely correlates with c-Myc expression in CWR22 xenografts. RNA from androgen-dependent (CWR22) and androgen-independent (CWR22R) xenografts was electrophoresed, and Northern blot was performed using radio-labeled TMEFF2 cDNA probe. The blots were rehybridized with a control GAPDH probe. The blot was stripped and reprobed with [α^{32} P]dATP-labeled cDNA probes for c-Myc and GAPDH.

were constructed and used in reporter gene assays. Deletion to -690 resulted in the highest luciferase activity (85-fold compared to the empty vector, Figure 1(C)). Further deletion to -230 and -120 resulted in a lower level of activity. These results suggest that the 0.7 kb region upstream to the TMEFF2 transcription start site contains the minimal promoter of TMEFF2.

TMEFF2 is a possible target gene for c-Myc

A potential c-Myc binding site, 5'-CACGTG-3' (E-box), was identified at position -90 relative to the TMEFF2 transcription start site (+1). Comparison of the murine and human TMEFF2 genes revealed that the murine TMEFF2 genomic upstream region (-75 to the transcription start site) is 92% homologous to the human TMEFF2 promoter, and that the murine gene also contains an E-box in a similar position (Figure 2(A)). We have previously shown that TMEFF2 is expressed in the androgen-dependent CWR22 prostate cancer xenografts and levels are downregulated in the androgen-independent CWR22R xenografts.⁶ Northern blot analysis showed that a negative correlation existed between expression of TMEFF2 and c-Myc in the CWR22 xenograft samples

(Figure 2(B)). These findings suggest that TMEFF2 might be a downstream target for c-Myc.

c-Myc negatively regulates TMEFF2 through the E-box sequence

The effect of c-Myc expression on the TMEFF2 promoter was examined in a reporter assay. DU145 cells, that express endogenous c-Myc, were cotransfected with the 0.7 kb (-690 to $+31$) TMEFF2 promoter-luciferase construct along with a c-Myc expression plasmid. The results showed that overexpression of c-Myc inhibited luciferase activity by tenfold compared to cells transfected with an empty expression vector (Figure 3). Mutations of the E-box (CACGTG \rightarrow CAGTTG) in the TMEFF2 promoter abolished the inhibitory effect of c-Myc, indicating that the site is essential for negative regulation of TMEFF2 by c-Myc.

The ability of c-Myc to bind specifically to its respective consensus sequence in the TMEFF2 promoter was determined by EMSA using nuclear extracts from LNCaP cells, which express endogenous c-Myc. The results show that a complex from LNCaP cells could bind the oligonucleotide probe that contained the E-box from the TMEFF2 promoter (Figure 4, lane 1). The binding was competed by unlabeled homologous oligonucleotides

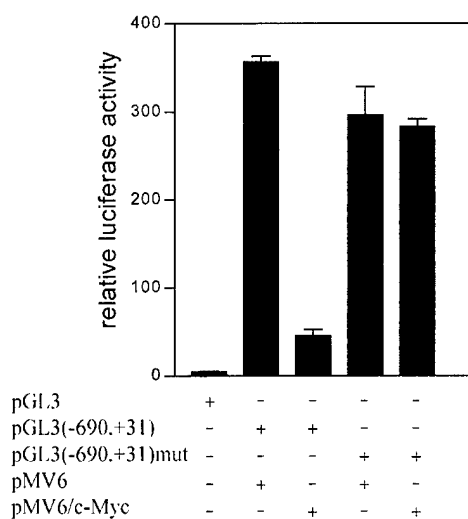


Figure 3. c-Myc represses expression of the TMEFF2 promoter. DU145 cells were cotransfected with the 0.7 kb (-690 to +31) fragment of the TMEFF2 promoter-luciferase and either a pMV6/c-Myc expression vector or an empty pMV6 vector and 0.1 μ g of the pRL-SV40 vector. Cells were harvested and luciferase activity was measured as described in the legend to Figure 1. Results represent the mean \pm SD of triplicate transfections. The experiments were repeated three times with triplicate plates per experimental point.

(lanes 2 and 3) but not by mutated oligonucleotides in which the E-box sequences were altered (lanes 4 and 5). The band was also disrupted by addition of antibodies against c-Myc (lane 6). These results indicate that c-Myc can bind to its cognate site in the TMEFF2 promoter.

Discussion

Here, we cloned the 5'-flanking region of the human TMEFF2 gene, and analyzed the ability of the TMEFF2 promoter to drive expression of a reporter gene in prostate cancer cells. We demonstrated that the 5'-flanking region contained a functional promoter and c-Myc could repress TMEFF2 promoter activity.

A typical TATA box was not present in the 5'-flanking sequence upstream to the TMEFF2 transcriptional start site. One group of genes that lack the TATA box consists of GC-rich promoters with several Sp1 binding sites.¹⁸ The 5'-region of TMEFF2 is a CpG island and contains several potential Sp1 binding sites.^{3,5} TMEFF2 may, therefore, belong to this class of genes.

TMEFF2 is expressed almost exclusively in the prostate and brain.¹⁻⁶ The present study did not identify factors responsible for this high tissue-specific expression. Moreover, we have shown that TMEFF2 is an androgen-regulated gene. A

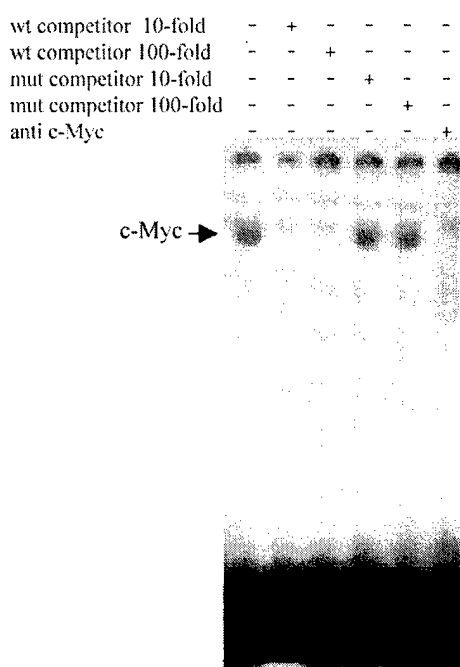


Figure 4. c-Myc binds to the c-Myc binding site in the TMEFF2 promoter. EMSA was performed using 10 μ g of nuclear extract proteins from LNCaP cells. Extracts were incubated with ³²P-labeled wild-type oligonucleotide containing the E-box site (CACGTG) from the TMEFF2 promoter (-100 to -78). Unlabeled wild-type competitor (wt competitor), mutant c-Myc competitor (containing a mutation in the E-box; CACGTG \rightarrow CAGTTG, mut competitor) or a c-Myc antibody were added to the reactions as indicated.

sequence with significant homology to the consensus androgen response element (ARE) was not identified within the 3.5 kb region upstream to the TMEFF2 transcriptional start site. An ARE might be present in a further upstream regulatory region. Alternatively, up-regulation of TMEFF2 by androgen could be a secondary event. Further work needs to be done to understand the molecular mechanism leading to the tissue-specific and androgen-regulated expression of TMEFF2.

Using a reporter assay, we identified a 0.7 kb fragment (-690 to +1) that consists of the TMEFF2 minimal promoter. This region contained several putative transcription factor-binding sites, including the E-box sequence -CACGTG- that could be recognized by Myc. The human and murine TMEFF2 promoters both contain the E-box sequence in a similar position. In addition, an inverse correlation between TMEFF2 and c-Myc expression was found in CWR22 prostate xenografts. Reporter assays using TMEFF2 minimal promoter and a c-Myc expression vector demonstrated that c-Myc could repress TMEFF2 promoter activity. Moreover, mutation in the E-box abolished

c-Myc-mediated repression. Furthermore, gel shift experiments showed that c-Myc could bind to the E-box sequence of TMEFF2 and that antibodies against c-Myc could disrupt the binding. These results suggest that c-Myc could inhibit TMEFF2 expression through its cognate site. Results from reporter assays showed that the basal levels of two smaller promoter constructs (-230 to +31 and -120 to +31) were lower than the minimal promoter level (-690 to +31) in the presence of the c-Myc expression vector. This suggests that the region between -690 and -230 may contain sequences that are targets for transcriptional repression. Further studies are required to determine the identity of these sequences and the factors that bind them.

Multiple pathways of c-Myc-mediated gene repression have been elucidated.^{11,12} In several promoters, gene repression by c-Myc has been mapped to the initiator (Inr) element, indicating that factors that bind at the start site of transcription may be targets for c-Myc activity.^{19,20} For example, c-Myc repression of the cyclin-dependent kinase inhibitor (CDKI) p15^{INK4b} is mediated through association of Myc to the Miz-1 transcription factor that binds to the Inr element, and this does not require Max.¹⁶ Down-regulation of another CDKI, p27^{Kip1}, by c-Myc involves binding of the Myc/Max complex to the Inr element of the p27^{Kip1} promoter.¹⁷ In a third case of Myc-mediated repression of a CDKI, p21^(WAF1/CIP1), the mechanism is not mediated *via* the Inr element or Myc/Max interaction but involves sequestering of the Sp1/Sp3 transcription factors by c-Myc.¹⁵ Here, we show that c-Myc down-regulates TMEFF2 through the E-box site in the TMEFF2 promoter. We did not identify which protein partner binds with c-Myc to facilitate this repression. Since c-Myc cannot bind DNA alone, further studies will need to be done to identify the other protein(s) that interact with c-Myc to inhibit TMEFF2 expression.

Although TMEFF2 function is unknown, its structural domains (two follistatin domains and an EGF-like motif) suggest that it may have a role in the regulation of growth factor signaling either as a ligand precursor, a membrane-bound receptor or as a binding protein for growth factors. In the EGF-like domain of TMEFF2, a critical arginine residue at position 41 is replaced by a histidine. Replacement of this arginine, results in a drastic decrease of EGF affinity to its receptor.²¹ Follistatin domains have been identified in other proteins such as Agrin and Osteonectin, where they were shown to bind and neutralize different growth factors including members of the transforming growth factor- β family, platelet-derived growth factor and vascular endothelial growth factor.²²⁻²⁴ We have previously shown that forced expression of TMEFF2 in prostate cancer cells inhibited their proliferation, suggesting that TMEFF2 has a negative effect on growth.⁵ Also, two independent studies suggest that TMEFF2 might be a tumor suppressor gene because it is frequently hyper-

methylated in human tumor cells.^{3,5} However, in contrast to these findings, TMEFF2 was shown to promote the survival of specific neurons in primary culture,² and a recent report associated TMEFF2 (TENB2) expression with prostate cancer progression.¹ More detailed studies need to be undertaken to understand better the function of TMEFF2 in normal cells and its potential role in cancer.

c-Myc is a proto-oncogene that plays a prominent role in various types of cancer including prostate cancer. Overexpression of c-Myc in quiescent cells induces cell cycle entry and deregulated expression of c-Myc induces tumorigenesis. Recent studies suggest that gene repression is essential for c-Myc-induced cell cycle progression and cellular transformation. Repression of cell cycle inhibitory genes, as well as induction of genes with growth stimulating functions, could provide the mechanism by which c-Myc promotes cell growth. We suggest that inhibition of TMEFF2 expression by c-Myc may play a similar role in prostate cells. Further studies will determine the relevance of TMEFF2 suppression to oncogenic transformation.

Materials and Methods

Cloning of the 5'-flanking region of the human TMEFF2 gene

The human BAC clone PR11-394A2 (GenBank accession number AC092644, Research Genetics) was digested with *EcoRI* and the resulting products were cloned into the pGEM-3Z vector (Promega). The colonies were screened by hybridization to the TMEFF2 probe. A single clone was isolated and a 12 kb insert was characterized by restriction enzyme analysis and partial sequencing. Various size promoter fragments were amplified by PCR and cloned into the promoterless luciferase reporter vector, pGL3 basic (Promega). Mutations in the c-Myc binding site were introduced using the GeneEditor In Vitro Site-Directed Mutagenesis System (Promega).

Prostate xenografts and cell culture

The prostate cancer xenograft CWR22 has been described.²⁵ The prostate cancer cell lines LNCaP, and DU145 were obtained from the American Type Culture Collection and grown in the recommended medium and conditions.

Transfections and luciferase assay

Cells were transfected using the GenePORTER Transfection Reagent (GTS Inc.) with 1 μ g of each promoter-luciferase construct. In co-transfection experiments, 1 μ g of c-Myc expression vector²⁶ (pMV6/c-Myc, which contains the human c-Myc inserted between *EcoRI* and *HindIII* sites in the pMV6 vector) was transfected with the promoter-luciferase construct. Lysates were harvested 48 hours post-transfection and luciferase activity was measured with the Dual-Luciferase reporter 1000 assay system (Promega). Transfection efficiency was normalized using 0.1 μ g pRL-SV40.

Northern blot analysis

Total RNA was isolated from prostate cancer xenografts using TRIzol (Life Technologies, Inc.). For Northern analysis, 10 µg of total RNA was fractionated on 1.2% (w/v) agarose denaturing gels and transferred to nylon membranes (Amersham). Probes were labeled with the Strip-EZ DNA kit (Ambion) and hybridizations were performed in the ULTRAhyb buffer (Ambion) according to the manufacturer's instructions.

Electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotides were end-labeled with [γ -³²P]ATP by T4 polynucleotide kinase. The oligonucleotide sequences were as follow: wild-type oligonucleotide: 5'-TGCCTCTCCACGTGACCCCGG CG-3'; mutant oligonucleotide (used in competition experiments): 5'-TGCCTCTCCAGTTGACCCGGG CG-3'. Nuclear extracts were prepared from LNCaP cells with the CellLytic Nuclear extraction Kit (Sigma). Nuclear extract proteins (10 µg) were incubated with 20,000 cpm of labeled wild-type probe. Binding reactions were incubated for 30 minutes on ice and then analyzed on 4% (w/v) polyacrylamide gel. When cold competitor or a c-Myc antibody (sc-764X, Santa Cruz Biotechnology) were used, they were added to the reactions 20 minutes prior to the labeled probe.

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TMEFF1 and brain tumors

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TMEFF1 is a novel transmembrane protein, containing two follistatin domains and an epidermal growth factor-like region. These structural domains suggest a role for TMEFF1 in growth factor signaling. TMEFF1 fused to enhanced green fluorescent protein revealed that TMEFF1 is expressed on the cell membrane. Northern analysis of normal human tissue showed that TMEFF1 is predominantly expressed in the brain. Study of cancer cell lines from different tissues including the brain, demonstrated moderate to low levels of TMEFF1 in most of these transformed cell lines. Furthermore, quantitative real-time RT-PCR analysis of 54 brain tumors showed that most of these tumors (96%) had lower levels of TMEFF1 expression than normal brain tissue. Interestingly, ectopic expression of TMEFF1 in brain cancer cells resulted in their growth inhibition. These data suggest that TMEFF1 may behave as a tumor suppressor gene in brain cancers.

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Keywords: brain cancer; epidermal growth factor and follistatin

Introduction

Glioblastomas are among the most malignant tumors for which no curative treatment exists. Growth factors and their signal transduction elements have been widely implicated in the molecular pathogenesis of brain tumors (Westermarck and Westermarck, 1995). TMEFF1 is a novel transmembrane protein, composed of two follistatin domains and a unique epidermal growth factor (EGF)-like region. These structural domains suggest a role for TMEFF1 in the regulation of growth factor signaling either as a ligand precursor, a membrane-bound receptor or as a binding protein for growth factors. Previous studies characterized TMEFF1 expression in *Xenopus laevis* (Eib and Martens, 1996) and mice (Eib *et al.*, 2000), and found that it was predominantly expressed in the brain. Although a human ortholog has been cloned (Eib *et al.*, 1998), where it is expressed has not been studied.

In this report, we used Northern blot analysis and quantitative real-time PCR to assess TMEFF1 expression in normal human tissues, clinical brain cancer samples and cancer cell lines. We found that TMEFF1 was highly expressed in normal brain and poorly expressed in brain tumors. Moreover, forced expression of TMEFF1 resulted in inhibition of cell growth. These results suggest that TMEFF1 may have a suppressive role in brain carcinogenesis.

Results

TMEFF1 expression in normal human tissue

Northern blot analysis of human tissues showed that TMEFF1 mRNA (3 kb) is expressed predominantly in the brain (Figure 1). Moderate levels were detected in heart, placenta and skeletal muscle, and weak expression was observed in the liver, kidney and pancreas.

TMEFF1 is localized to the cell membrane

The TMEFF1 coding region contains a single putative C-terminal transmembrane domain at residues 319–342. To determine where the protein is localized TMEFF1 cDNA was fused to enhanced green fluorescent protein (EGFP) in a mammalian expression vector. Fluorescent microscopy of transiently transfected U118 and NIH 3T3 cells demonstrated that TMEFF1 is expressed on the cell membrane (Figure 2).

TMEFF1 expression in cancer cell lines

We further tested TMEFF1 expression in cancer cell lines from different tissues (Figure 3). Four brain cancer cell lines showed moderate (U343 and T98G) to low (U118 and U183) expression of TMEFF1. In the androgen-independent prostate cell lines DU145 and PC3, TMEFF1 was expressed at high and moderately low levels, respectively; and it was undetectable in the androgen-dependent prostate cell line LNCaP. Similarly, TMEFF1 was highly expressed in two estrogen-independent breast cancer cell lines MDA-MB-436 and MDA-MB-231, but low levels of TMEFF1 were found in the estrogen-responsive breast cancer cell line MCF7 and the normal breast cell line MCF-12A. TMEFF1 was highly expressed in two small lung cancer cell lines NCI-H446 and NCI-H526. In five other nonsmall lung cancer

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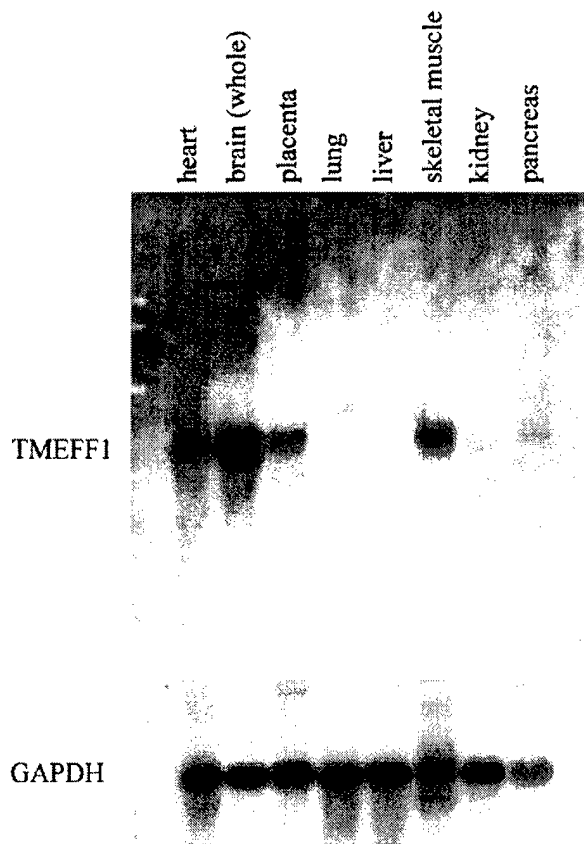


Figure 1 TMEFF1 expression in normal tissues. A multiple tissue Northern blot was hybridized with radiolabeled TMEFF1 cDNA. The blot was stripped and rehybridized with a control α -³²P-dATP-labeled GAPDH

cell lines, TMEFF1 expression was moderate (NCI-H125) to low (NCI-H1299, NCI-H157, NCI-H520 and NCI-H157). In cell lines from a pancreatic cancer (PANC-1), hepatoma (HepG2) and leukemia (U937), TMEFF1 levels were in the middle range. Dihydrotestosterone and 17β -estradiol (10^{-8} M, 2 days) were added

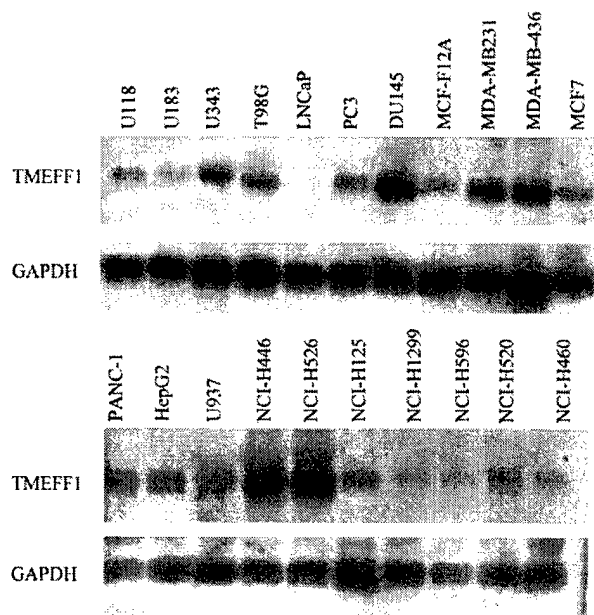


Figure 3 TMEFF1 expression in cancer cell lines from various tissues. RNA was extracted from different cell lines and Northern blots were performed using radiolabeled TMEFF1 cDNA probe. The blots were rehybridized with GAPDH probe

to the androgen-responsive LNCaP prostate cancer cell line, and the estrogen-responsive MCF7 breast cancer cell line, respectively. No change in expression of TMEFF1 occurred (data not shown).

Forced expression of TMEFF1 inhibited proliferation of brain cancer cells

To test the effect of TMEFF1 on cell growth, we generated U118 glioblastoma cells that stably expressed prominent levels of TMEFF1. The growth rate of U118 cells that overexpressed TMEFF1 (U118 TM1) was slowed by approximately 41% compared to control cells (U118 N, stably transfected with the empty vector,

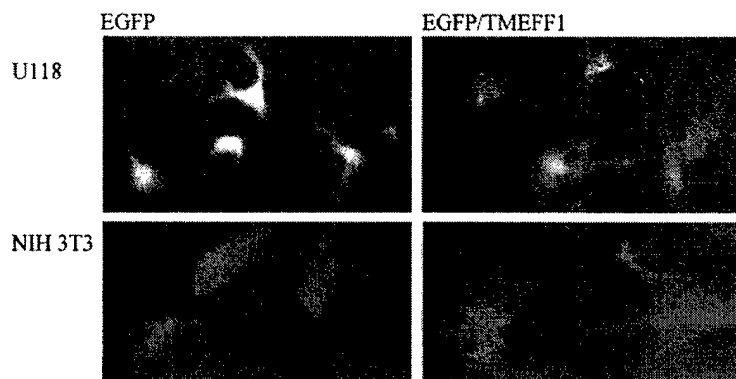


Figure 2 TMEFF1 is expressed on the cell membrane. U118 brain cancer cells and NIH 3T3 cells transfected with a vector expressing either EGFP or EGFP/TMEFF1 were grown on cover slips and analysed by fluorescent microscopy ($\times 500$ magnification and $\times 1000$ magnification, respectively)

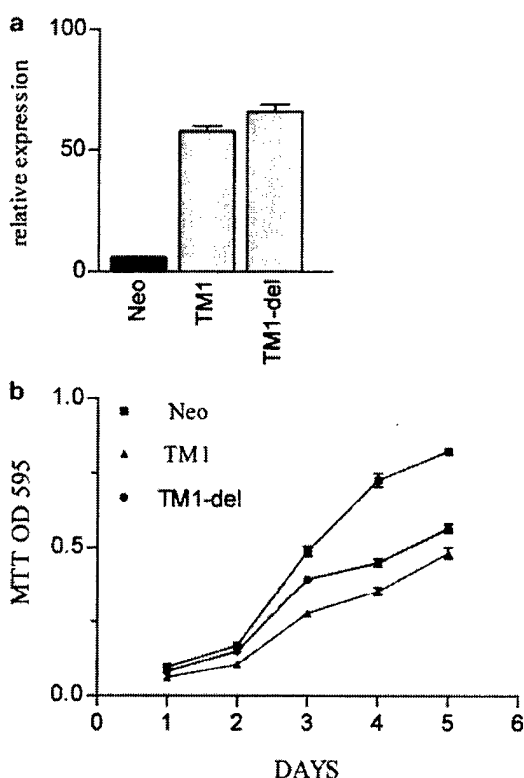


Figure 4 TMEFF1 expression decreases cell proliferation. (a) Expression of TMEFF1 in stably transfected cell lines. U118 brain cancer cells were stably transfected with either empty vector (Neo), TMEFF1 (TM1) or TMEFF1 deletion mutant (TM1-del). The expression level was measured by real-time RT-PCR with TMEFF1 specific primers and probe. The results are expressed in arbitrary units as a ratio of TMEFF1 transcripts/18S transcripts (each value represent the mean of three measurements of the sample). (b) Effect of TMEFF1 on growth rate of U118 brain cancer cells. The cells were plated in 96-well plates at 1000 cells per well and MTT assays were performed at days 1–5. Data represent mean absorbance reading at OD 590 \pm s.d. Results are from quadruplicate samples

Figure 4). To test whether the EGF-like motif is involved in the growth inhibition, a TMEFF1 deletion mutant of the EGF-like motif was made leaving the transmembrane domain intact. This mutant gene (TM1-del) was stably introduced into U118 cells. The growth rate of U118 cells overexpressing this mutant (U118 TM1-del) was reduced 33% compared to the control cells (Figure 4). These results suggest that the EGF-like motif is not essential for the growth inhibition mediated by TMEFF1.

TMEFF1 expression was low in a large collection of primary brain cancer samples

In normal brain, TMEFF1 is expressed at high levels (Figure 1). In contrast, TMEFF1 expression was low to moderate in the four brain cancer cell lines that we studied (Figure 3). To establish whether levels of TMEFF1 expression were altered in brain cancers, real-time reverse transcription-PCR was used to quan-

tify TMEFF1 levels in 54 primary brain tumors and four normal brain samples (Figure 5). The mean expression of TMEFF1 in the four normal samples (456 relative expression units by real-time PCR) was considered as the normal level of expression in the brain. In 54% of the tumor samples TMEFF1 expression was extremely low (less than 50 relative expression units), 37% of tumor samples had low levels of TMEFF1 (between 50 and 300 relative expression units) and 6% of the tumor samples had high TMEFF1 expression (more than 600 relative units). Notably 60% of the extremely malignant glioblastoma (GBM) samples had very low TMEFF1 levels (mean 12 relative expression units), compared to only 12% of the astrocytoma samples that had very low TMEFF1 levels (mean 13 relative expression units). In 90% of the meningioma samples, TMEFF1 levels were also very low (mean 11 relative expression units). Overall, more than 96% of the brain tumor samples had lower levels of TMEFF1 than the average expression level in the normal brain samples.

Discussion

In this report, we describe TMEFF1 expression in human cells. We demonstrated that an EGFP/TMEFF1 fusion protein was expressed on the cell membrane of brain cells. Northern analysis of normal human tissue showed that TMEFF1 is expressed predominantly in the brain and at lower levels in other tissues. High expression of TMEFF1 was detected in only several cancer cell lines from different origins including prostate, breast and lung. This gene displays a high degree of homology with TMEFF2 (Horie *et al.*, 2000; Glynn-Jones *et al.*, 2001). Both TMEFF1 and TMEFF2 are expressed at high levels in the brain, implicating their importance in the function of the normal central nervous system. However, whereas TMEFF2 is highly tissue specific and is expressed only in normal brain and prostate (Glynn-Jones *et al.*, 2001; Gery *et al.*, 2002). TMEFF1 has a wider range of tissue expression.

Although TMEFF1 function is largely unknown, its structural domains suggest that it may act as an inhibitor of growth factors. Follistatin domains have been identified in other proteins where they were shown to bind and neutralize different growth factors (Patthy and Nikolics, 1993; Kupprion *et al.*, 1998; Patel, 1998). In the EGF-like domain of TMEFF1, a critical arginine residue is replaced by a histidine. Replacement of this arginine results in a drastic decrease of EGF affinity to its receptor (Engler *et al.*, 1990). Overexpression of TMEFF1 in U118 cells resulted in their growth inhibition, suggesting that TMEFF1 acts as a negative regulator of growth in brain cells. A deletion in the EGF-like domain did not abolish the growth inhibitory phenotype suggesting that the follistatin domains are sufficient in causing growth inhibition. The function of the EGF-like domain requires further analysis. The question of whether TMEFF1 regulates either the cell

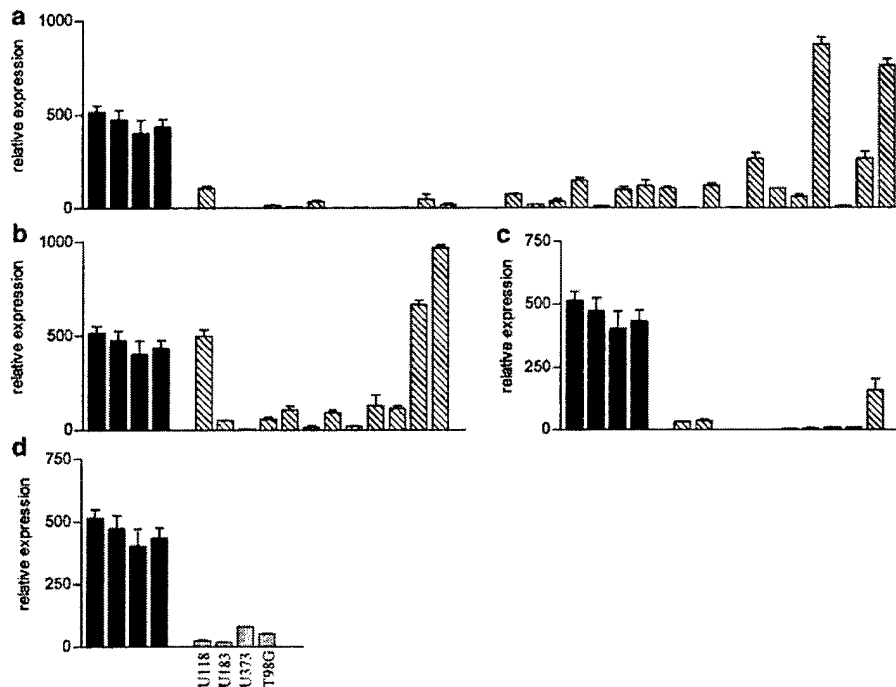


Figure 5 Quantitative real-time RT-PCR analysis of TMEFF1 expression. cDNAs from normal tissues (solid columns), primary tumors (stripped columns) and cell lines (dotted columns) were subjected to real-time RT-PCR with TMEFF1 specific primers and probe. The results are expressed in arbitrary units as a ratio of TMEFF1 transcripts/18S transcripts (each value represent the mean of three measurements of the sample). The samples of panel a, glioblastoma multiforme; panel b, astrocytoma; panel c, meningioma; panel d, glioma cell lines. Each panel shows the expression of the normal brain tissues

cycle or contributes to cell loss, perhaps by apoptosis, is also unanswered by the current study.

To determine the relation of TMEFF1 expression to brain cancer, we measured TMEFF1 levels in a large series of brain tumors. All of the brain tumors in this study were derived from nonneuronal cells. In the vast majority of them (96%), as well as in four brain cancer cell lines TMEFF1 expression was low compared to its expression in normal brain cells. This was especially true for the highly proliferative, malignant GBM. These results suggest that TMEFF1 may behave as a tumor suppressor gene, and diminished expression of this protein may provide brain cancers with a growth advantage over normal cells in a significant number of individuals. This is consistent with our findings that forced expression of this protein in brain cancer cells decreases their growth. Two cancer cell lines originating from lung, which normally do not express TMEFF1, show high level of TMEFF1 expression. TMEFF1 expression was also moderately present in a pancreas-derived tumor cell line, whereas in normal pancreas, TMEFF1 is weakly expressed. This suggests that while TMEFF1 has a growth suppressive effect in brain cancer cells, it might not behave in a similar fashion in lung and pancreas. More detailed studies need to be undertaken to understand more clearly the function of TMEFF1 in normal cells and its potential role in cancer.

Taken together, the results presented in this study suggest that deregulation of expression of TMEFF1 is associated with the transformed phenotype of brain

cancers. As TMEFF1 is expressed on the cell membrane and is potentially involved with growth factor signaling, it could be considered as a drug target and for monitoring the development and treatment of brain cancers.

Materials and methods

Brain samples

The following samples were studied: four normal brains, 32 glioblastoma multiformes (GBMs), 12 astrocytomas and 10 meningiomas. All samples were snap-frozen in liquid nitrogen and stored at -70°C until processing.

Cell culture

The following cell lines were used: glioma (U118, U183, U343, T98G), prostate cancer (LNCaP, PC3, DU145), breast cancer (MCF7, MDA-MB-436, MDA-MB-231), normal breast (MCF-12A), lung cancer (NCI-H446, NCI-H526, NCI-H125, NCI-H1299, NCI-H596, NCI-H520, NCI-H460), pancreas cancer (PANC-1), hepatoma (HepG2), acute myeloid leukemia (U937) and murine fibroblasts (NIH 3T3), which were obtained from the American Type Culture Collection and grown in the recommended medium and conditions.

Northern blot analysis and quantitative real-time RT-PCR

Total RNA was isolated from human brain samples and cell lines using TRIzol (Life Technologies, Inc.). For Northern analysis, 10 μg of total RNA was fractionated on 1.2% agarose

denaturing gels and transferred to nylon membranes (Amersham). The human multiple tissue blot was obtained from Clontech (Laboratories, Inc.). Probes were labeled with the Strip-EZ DNA kit (Ambion) and hybridizations were performed in the ULTRAhyb buffer (Ambion) according to the manufacture's instructions. For quantitative real-time RT-PCR, 2 µg of total RNA from human brain samples or cell lines was converted into cDNA using MMLV reverse transcriptase (Life Technologies, Inc.) according to the manufacture's instructions. The following primers and probes were used: TMEFF1 primers, 5'-TTGTTGGGAAAGAAA-GATGATGGA-3' and 5'-GATGCAGTAACCATTGAGGT TTT-3'; 18S primers, 5'-AAACGGCTACCACATCCAAG-3' and 5'-CCTCCAATGGATCCTCGTTA-3'; TMEFF1 probe 5'-TGGAAACCACATGCCTTGCCC-3'; 18S probe 5'-AG-CAGGCGCAAATTACCC-3'. Primers were synthesized by Life Technologies Inc. Probes were purchased from Applied Biosystems (Foster City, CA, USA) and were labeled with the reporter dye FAM in the 5' end and the quencher dye TAMRA in the 3' end. Amplification reactions were performed with the Universal Taqman PCR mastermix (Applied Biosystems) in triplicates in an iCycler iQ™ system (Biorad, Hercules, CA, USA). The thermal cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. To determine the relative expression level of each sample, additional reactions with four serial five-fold dilutions of DU145 cDNA were performed to generate a standard curve, which related the threshold cycle to the log input amount of template.

Transfections and cell proliferation assays

cDNA coding for TMEFF1 was amplified from DU145 and cloned into the pCDNA3 expression vector (TM1, Invitrogen). A deletion mutation in the EGF-like domain (amino acids 290–317) was generated by PCR and cloned into the same vector (TM1-del). U118 cells were transfected using the

GenePORTER™ Transfection Reagent (GTS Inc.) with 10 µg of either an empty pCDNA3 vector, TM1 or TM1-del. Stable U118 clones were obtained by selection in G418 (500 µg/ml). Real-Time RT-PCR confirmed expression of wild type and mutated TMEFF1. For MTT assays, cells were seeded in quadruplicate in 96-well plates at 1000 cells per well. Cell proliferation was assayed using the Cell Proliferation Kit I (MTT, Boehringer, Mannheim, Germany) according to the manufacturer's instructions.

Fluorescent microscopy

cDNA coding for TMEFF1 was amplified from DU145 cells and cloned into the pEGFP expression vector (Clontech). Either EGFP or EGFP/TMEFF1 plasmids were transfected into U118 brain cancer cells and NIH 3T3 cells as described above. Transfected cells were plated on cover slips and analysed 2 days after transfection by fluorescent microscopy using a 510 nm filter.

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