Senescence is a cellular stress response characterized by persistent cell growth arrest under various stress conditions, including oncogene activation or tumor suppressor loss, which functions as a critical barrier that must be overcome to allow the progression from a precancerous or preinvasive lesion to a malignant tumor. Trefoil factor 1 (TFF1) is a secreted protein involved in maintaining the gastrointestinal epithelium by serving a tumor-suppressive role; however, TFF1 is overexpressed in several types of cancers. Here we report that TFF1 acts as a promoter of tumorigenesis in the context of prostate and pancreatic cancers by suppressing oncogene-induced senescence (OIS). Expression of TFF1 allows human prostate epithelial cells to escape OIS caused by the activated Ras oncogene or by reduced expression of the tumor suppressor PTEN, in part by the involvement of the EGF receptor-mediated pathway and inhibition of the expression of the cell cycle regulator p21. Without intrinsic promitogenic activity TFF1 may act in both autocrine and paracrine manners to enable cells to undergo the initial transformation and expansion against the restrictive microenvironment during early stage tumorigenesis. Taken together, our findings identify TFF1 as a soluble factor designed to act mainly to antagonize the OIS process to accelerate tumorigenesis.

As a member of the trefoil factor family (TFF) of proteins, TFF1 (pS2) is a 9-kDa mucin-associated secreted protein expressed in the gastrointestinal (GI) tract mucosa that is characterized by a 38- to 39-amino-acid trefoil domain containing six cysteine residues that form a cloverleaf disulfide structure (14). This structure renders the protein acid- and protease-resistant, allowing it to function as a tumor suppressor in the harsh environment of the GI tract (15, 16) by stimulating restitution of damaged tissue associated with ulceration and inflammation to reduce tumor formation (17, 18). Interestingly, TFF1 was shown to be overexpressed in tumor tissues from various organs where it is not normally expressed, including the pancreas (Table 1) and the prostate (Table 2), although its functional significance remains unclear. To address this, we investigated the function of TFF1 in the context of prostate and pancreatic tumorigenesis and found that this secreted protein acts to suppress OIS. Although the signaling pathway by which TFF1 functions to counter the senescence process remains largely unclear, EGF receptor (EGFR) activity and the cell cycle regulator p21 appear to play a critical role. Taken together, our results indicate that in prostate and pancreatic cancer, the previously defined tumor suppressor TFF1 promotes tumorigenesis by enabling precancerous lesions to overcome the barrier of OIS.

**Results**

**TFF1 Expression Is Up-Regulated in Pancreatic and Prostate Cancer.** A large number of studies indicate that TFF1 expression is absent or occurs at low levels in nonmalignant pancreatic and prostate tissues. Elevated expression levels of TFF1 are frequently observed in pancreatic and prostate malignancies (19–31), suggesting that TFF1 may be involved in the tumorigenesis of these cancers. These data are not, however, sufficiently convincing because of the lack of strict statistical analysis in some studies and the reduced statistical power in others with small sample sizes. We performed a systematic review of all published studies analyzing expression of TFF1 in nonmalignant or malignant tissues of the pancreas and prostate. In pancreatic cancer, positive expression of TFF1, detected by immunohistochemistry (IHC), Northern blot, or RT-PCR, was consistently reported by independent studies to occur more frequently in malignant pancreatic tissues compared with nonmalignant tissues. Compared with only 7.3% of normal tissues, 74.1% of the tumor samples tested positive for TFF1 expression ($P < 0.001$, Table 1). In prostate cancer, differences in the overall relative frequencies of positive TFF1
expression between nonmalignant and malignant tissues were not as large as those in pancreatic cancer (24.5% vs. 60.8%); however, this difference was still found to be highly statistically significant ($\chi^2 = 65.2, P < 0.001$, Table 2). Additionally, a previously reported microarray analysis indicated that TFF1 was upregulated ~5.62-fold in pancreatic intraepithelial neoplasia (PanIN) lesions compared with normal duct epithelium (27). These findings provide quantitative confirmation to our qualitative results. Together, these data indicate that TFF1 expression is elevated in both prostate and pancreatic cancers.

To investigate if TFF1 overexpression in prostate tumors was correlated with specific stages of cancer development, we performed IHC analysis on eight normal prostate tissue samples, two prostatic intraepithelial neoplasia (PIN) tissue samples that represent preinvasive lesions, and eight prostatic carcinoma tissue samples (Fig. S1 and B). Consistent with our meta-analysis, the proportion of TFF1-positive samples in those with malignancy (including PIN and carcinoma) was significantly higher compared with that in normal prostate tissue samples (70.0% vs. 0%, $P = 0.004$). Significant differences in TFF1 expression were also observed when comparing normal prostate to either PIN (0% vs. 100%, $P = 0.022$) or carcinoma (0% vs. 62.5%, $P = 0.013$). Although a slight decrease in the proportion of TFF1-positive cells was observed in the carcinoma samples (62.5%) compared with PIN samples (100%), this difference was not significant ($P = 0.467$). We also failed to find any correlation between TFF1 expression and Gleason score among the carcinoma samples ($P = 1.00$). We concluded that TFF1 is upregulated in prostatic cancer samples, but overexpression is not correlated with a specific cancer stage.

Knockdown of TFF1 Results in Decreased Viability of Prostate and Pancreatic Cancer Cells In Vitro and In Reduced Tumor Growth In Vivo.

Having confirmed the expression status of TFF1 in prostate cancer, we determined if TFF1 plays a role in tumorigenesis in the context of xenograft tumor formation. We screened a group of established human prostate and pancreatic cancer cell lines and found that many expressed various levels of TFF1 protein. We chose the PC3 prostate cancer cell line and the HS766T pancreatic cancer cell line for analysis by stably reducing the expression of TFF1 by shRNA-mediated knockdown. To avoid an off-target effect, we used two independent TFF1 shRNA constructs. In both cell lines, the shRNA-1 construct reduced TFF1 levels more efficiently than the shRNA-2 construct (Fig. 1 A and B). In direct correlation with the extent of TFF1 suppression, the number of viable cells from both cell lines was decreased proportionally to the degree of TFF1 knockdown (Fig. 1 C and D). We then determined the effect of TFF1 reduction on the formation of xenograft tumors when these cells were implanted s.c. in nude mice. There was a dramatic reduction in the size of tumors formed by the TFF1 knockdown cells compared with controls, indicating that TFF1 is essential for tumor growth (Fig. 1 E and F).

To determine if this phenotype could occur in cancer cells derived from GI tissues where this protein is normally expressed, we used our TFF1 shRNA constructs to reduce TFF1 levels in the human gastric cancer cell line Kato III (Fig. S3 A). In contrast to PC3 and HS766T cells, there was no significant change in cell viability between vector control (VC) and knockdown cells as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Fig. S3 B and C). These data suggest that the observed effects are specific for prostate and pancreatic tumors.

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### Table 1. Meta-analysis of previous reports examining TFF1 expression in pancreatic tumor samples compared with normal samples

<table>
<thead>
<tr>
<th>Literature</th>
<th>Method</th>
<th>Positive</th>
<th>Negative</th>
<th>Positive</th>
<th>Negative</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piggott et al. (26)</td>
<td>IHC</td>
<td>(0.0%)</td>
<td>1 (100.0%)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Henry et al. (24)</td>
<td>IHC</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Luqmani et al. (25)</td>
<td>IHC</td>
<td>—</td>
<td>—</td>
<td>(46.7%)</td>
<td>(53.3%)</td>
<td>—</td>
</tr>
<tr>
<td>Sagol et al. (26)</td>
<td>IHC</td>
<td>—</td>
<td>—</td>
<td>(77.8%)</td>
<td>(22.2%)</td>
<td>—</td>
</tr>
<tr>
<td>Yeh et al. (31)</td>
<td>IHC</td>
<td>—</td>
<td>—</td>
<td>(80.0%)</td>
<td>(20.0%)</td>
<td>—</td>
</tr>
<tr>
<td>Collier et al. (22)</td>
<td>IHC</td>
<td>(20.0%)</td>
<td>(80.0%)</td>
<td>(54.8%)</td>
<td>(45.2%)</td>
<td>0.161</td>
</tr>
<tr>
<td>Singh et al. (29)</td>
<td>Northern blot</td>
<td>(16.7%)</td>
<td>(83.3%)</td>
<td>(75.0%)</td>
<td>(25.0%)</td>
<td>0.119</td>
</tr>
<tr>
<td>Prasad et al. (27)</td>
<td>RT-PCR</td>
<td>(20.0%)</td>
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<td>(84.2%)</td>
<td>(15.8%)</td>
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<tr>
<td>Welter et al. (30)</td>
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<td>(100.0%)</td>
<td>(73.9%)</td>
<td>(26.1%)</td>
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<tr>
<td>Combined</td>
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<td>(7.3%)</td>
<td>(92.7%)</td>
<td>(74.1%)</td>
<td>(25.9%)</td>
<td>&lt;0.001</td>
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</table>

Meta-analysis reveals elevated TFF1 expression levels in malignant pancreatic and prostatic lesions that are not observed in the normal samples. IHC, immunohistochemistry; RT-PCR, reverse transcriptase PCR.

### Table 2. Meta-analysis of previous reports examining TFF1 expression in prostate tumor samples compared with normal samples

<table>
<thead>
<tr>
<th>Literature</th>
<th>Method</th>
<th>Positive</th>
<th>Negative</th>
<th>Positive</th>
<th>Negative</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piggott et al. (26)</td>
<td>IHC</td>
<td>(0.0%)</td>
<td>1 (100.0%)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Henry et al. (24)</td>
<td>IHC</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Luqmani et al. (25)</td>
<td>IHC</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Bonkhoff et al. (21)</td>
<td>IHC</td>
<td>(48.2%)</td>
<td>(51.8%)</td>
<td>(76.7%)</td>
<td>(23.3%)</td>
<td>—</td>
</tr>
<tr>
<td>Colombel et al. (23)</td>
<td>RT-PCR</td>
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<td>(82.2%)</td>
<td>(92.6%)</td>
<td>(7.4%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Abdou et al. (19)</td>
<td>IHC</td>
<td>(80.0%)</td>
<td>(20.0%)</td>
<td>(91.5%)</td>
<td>(8.5%)</td>
<td>0.217</td>
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<td>Ather et al. (20)</td>
<td>IHC</td>
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<td>(95.8%)</td>
<td>(86.0%)</td>
<td>(14.0%)</td>
<td>0.125</td>
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<tr>
<td>Combined</td>
<td>—</td>
<td>(24.5%)</td>
<td>(75.5%)</td>
<td>(39.2%)</td>
<td>(60.8%)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Meta-analysis reveals elevated TFF1 expression levels in malignant prostate tumor samples that are not observed in the normal samples. IHC, immunohistochemistry; RT-PCR, reverse transcriptase PCR.
Knockdown of TFF1 Results in Increased Senescence in Pancreatic and Prostate Cancer Cells. The observed decreases in cell viability and reduced tumor growth in mice. (A) TFF1 knockdown in PC3 or HS766T cells. (B) MTT absorbance in PC3. (C) HS766T cells with or without TFF1 knockdown. (D) Representative results showing the formation of s.c. tumors in nude mice injected as indicated. Two independent experiments were performed. **P < 0.002; *P < 0.05 (n = 10). (E and F) Representative results showing the formation of s.c. tumors in nude mice injected with PC3 (E) or HS766T (F) cells. NT, no tumor. Two independent experiments were performed. **P < 0.002; *P < 0.05 (n = 10).

Knockdown of TFF1 Results in Increased Senescence in Pancreatic and Prostate Cancer Cells. The observed decreases in cell viability in vitro and tumor formation in vivo in the PC3 and HS766T cells could be the result of reduced cell proliferation, increased apoptosis, or senescence. To address this question, we performed cell cycle analysis on PC3 cells stably expressing the shRNA-2 construct or VC. Compared with the control cell line, there was an observable increase in the cell population in the sub-G1 phase of the cell cycle when TFF1 expression was decreased, with only a slight decrease in S-phase population detected (Fig. 2A). Similar results were obtained using HS766T cells stably expressing the shRNA constructs (Fig. 2B). We next tested if TFF1 knockdown resulted in increased apoptosis. Using annexin-V staining, we detected lower levels of apoptosis in TFF1 knockdown PC3 cells compared with control (Fig. 2C), suggesting that apoptosis was not likely the underlying mechanism for the observed phenotype in cancer cells with reduced TFF1 expression.

While conducting cell cycle analysis, we noted that the morphology of many of the TFF1 knockdown PC3 and HS766T cells became greatly enlarged and flattened, resembling that of senescent cells. To determine if TFF1 reduction caused those cells to undergo senescence, we used β-galactosidase (β-gal) staining to quantify the differences in the control and knockdown cells under both normal and serum-starved conditions. A dramatic increase in the percentage of β-gal-positive cells was observed after TFF1 knockdown in both cell lines (Fig. 3A and B). To confirm TFF1 knockdown results in senescence, we used another pancreatic cancer cell line (Capan-1) stably infected with a different set of lentiviral-based shRNA constructs and observed a similar senescence phenotype (Fig. S4A). Interestingly, it appears that senescence observed after TFF1 knockdown is specific for pancreatic and prostate cancer, as there was no difference in the level of β-gal staining between the control and the Kato III gastric cancer cells with decreased TFF1 expression (Fig. S4B), again demonstrating the tissue-specific impact of TFF1 reduction.

To determine if the senescence phenotype observed in prostate and pancreatic cancer cells was primarily a consequence of reduced TFF1 expression, we added TFF1-conditioned media from PC3 cells to the PC3 TFF1 knockdown cells. We observed a partial rescue of the senescence phenotype in the TFF1 knockdown cells by the addition of conditioned media, suggesting that TFF1...
was likely the secreted factor preventing the cancer cells from senescing (Fig. S5).

**TFF1 Suppresses Cellular Senescence Induced by Oncogenic H-Ras or Reduced PTEN Expression.** In the context of tumorigenesis, senescence caused by oncogenic activation must frequently be overcome for the tumorigenic process to continue. To test the hypothesis that secreted TFF1 could help normal cells overcome OIS during early stage tumorigenesis, we used a model system where cellular senescence is induced in prostate epithelial cells (PrEC) by either ectopically expressing oncogenic Ras or knocking down the expression of the tumor suppressor PTEN. To validate the suitability of the system for this purpose, we showed that OIS could be induced in normal human PrEC by the introduction of oncogenic H-Ras (H-RasG12V) by retroviral delivery that resulted in a flattened and enlarged cell morphology associated with senescence, as well as a large increase in β-gal staining (Fig. S6). To avoid the potential complication of using conditioned media from established cancer cell lines that may contain unknown factors, we collected conditioned media from immortalized human PrEC infected with a retroviral vector with or without TFF1 and added the media to normal human PrEC infected with oncogenic H-Ras or VC. Addition of the conditioned media with or without TFF1 did not affect the status of those cells infected with the control virus in terms of the percentage of cells defined as senescent by β-gal staining (Fig. 4A). There was, however, a significant reduction in the number of β-gal-positive cells in the culture infected with H-Ras retrovirus when TFF1 is present compared with those without TFF1, suggesting that TFF1 could have a potent effect in preventing a large percentage of normal PrEC from entering OIS (Fig. 4A).

Although the Ras-mediated signaling pathway is activated in many cancer types, activation mutations in the Ras gene are rare in prostate cancer. In contrast, the tumor suppressor PTEN is frequently lost in prostate cancer (32). To mimic the biological process during which reduction in PTEN expression could lead to OIS in normal PrEC, we used an inducible lentiviral system to target the expression of PTEN. Doxycycline-induced reduction in PTEN expression led to a significant increase in cellular senescence of those cells as indicated by an increase in β-gal-positive cells (Fig. 4B). Incubation of those cells with conditioned media containing TFF1 reduced this level of senescence to the basal state, again demonstrating the potent inhibitory effect of TFF1 on the ability of PTEN inactivation to induce senescence in normal PrEC (Fig. 4B). To rule out the possibility that some unknown factor(s) other than TFF1 in the conditioned media could be responsible for the observed phenotype, we subsequently used recombinant TFF1 protein to repeat these experiments and confirmed that TFF1 is indeed the primary factor that could act to rescue the OIS phenotype (Fig. S7).

**EGFR Activity Is Required for TFF1 to Overcome OIS and Loss of TFF1 Expression Results in Increased Expression of the OIS Marker p21, Required for Senescence Induced by TFF1 Knockdown.** TFF1 is a secreted protein that likely exerts its effects through protein(s) anchored on the cell surface, but the molecular nature of its receptor remains elusive. To gain insight into this question, we took a candidate approach by examining the possible involvement of known signaling pathway(s) initiated by plasma membrane-bound receptors for growth factors for mediation of TFF1 activity in the context of overcoming OIS. In this regard, previous reports indicated that TFF1 and EGFR are co-overexpressed in certain malignant tumors and that TFF1 might exert its activity through EGFR, although no definitive signaling pathways have been elucidated (31). To test if EGFR activity contributes to the ability of TFF1 to suppress OIS, we repeated the experiment using PrEC with Ras-induced OIS and recombinant TFF1 in the presence or absence of an EGFR inhibitor. As shown in Fig. 4A, TFF1 is unable to suppress the OIS phenotype in the presence of the EGFR inhibitor, indicating that EGFR activity is required for TFF1 to exert its antisenescence effect.

To further explore the molecular nature of the TFF1-signaling pathway in suppressing OIS, we examined changes in the expression status of genes that are likely involved in this process. We used an RT-PCR array to assess the expression profile of 84 genes involved in the regulation of the cell cycle using PC3 cells with or without the stable knockdown of TFF1 (Fig. S8A). Among the candidate genes whose expression profile displayed significant changes due to the difference in TFF1 expression status (a partial list is shown in Fig. S8B), we chose the cyclin-dependent kinase inhibitor p21 (CDKN1A or CIP1) for further analysis. We selected this candidate gene primarily because of its sevenfold increase at the mRNA level in TFF1 knockdown PC3 cells (Fig. S8B and Fig. S8B) and its known function in maintaining senescence under conditions of oncogenic stress (33). The observed change in p21 mRNA level was confirmed at the protein level using PC3 cells expressing either TFF1-targeting shRNA construct (Fig. 5C). Additionally, cells transduced with

**Fig. 4.** TFF1 add-back rescues cells from Ras- or PTEN-induced senescence. (A) Prostate epithelial cells were infected with constructs containing VC or H-Ras G12V and then grown in normal or TFF1-conditioned media. After 10 d the cells were fixed and then analyzed by light microscopy for β-gal staining (*P = 0.0025). (B) Prostate epithelial cells were infected with inducible VC shRNA or PTEN shRNA constructs and cultured in the presence or absence of dox for 10 d. β-Gal staining was then analyzed by light microscopy. Errors bars are representative of three independent experiments (*P = 0.002). Dox, doxycycline.
shRNA constructs targeting both TFF1 and p21 displayed a significant reduction in senescence compared with PC3 cells transduced with TFF1 shRNA alone (Fig. 5D). These results demonstrate a potent ability of TFF1 to suppress expression of p21, providing a potentially unique mechanism whereby TFF1 may allow cells to escape from OIS.

In contrast to the status of p21, the protein expression profile of other well-defined senescence markers, such as p14 and p15, did not show any significant change (Fig. 5C). Surprisingly, the protein level of p16, a cell cycle regulator often associated with cellular senescence, was dramatically decreased in the cells with reduced TFF1 expression (Fig. 5C), a profile consistent with that of the RT-PCR results showing an approximately 2.7-fold decrease in p16 mRNA expression (Fig. S8B). A possible explanation for these findings is that p21- and p16-mediated senescence pathways can function independently, and p21 may play a role in early stage senescence with p16 functioning at a later stage (34). Another possible explanation for our experimental results is that TFF1 also directly influences Ras-mediated cell growth and apoptosis pathways. To confirm that our results are specific to p21 function, we assayed HS766T cells that were transduced with scrambled control or TFF1 shRNA constructs for activation of common Ras pathway components. We observed no significant changes in the phosphorylation status of MEK, ERK, or AKT, indicating that Ras signaling is not altered by changes in TFF1 expression status (Fig S9).

Discussion

Tumorigenesis in mammals is a complex multistep process consisting of genetic and epigenetic alterations that allow normal cells to progress into malignant variants that eventually become highly invasive tumors (35). One of the barriers that inhibit the initial phase of the tumorigenic process is the induction of senescence in response to oncogene activation or loss of tumor suppressors. The notion that OIS is intimately associated with the tumorigenic process is strongly supported by a large body of in vivo evidence as senescence markers are detected in various types of precancerous lesions (11); however, the mechanisms associated with suppression of OIS and subsequent successful clonal expansion of cells that harbor oncogenic mutations remain largely unknown. In this regard, identification of TFF1 acting to counter the barrier of OIS represents a major step toward a better understanding of the molecular basis of the antisenescence process, because very few soluble factors have been reported to be functionally defined to act in a similar manner as TFF1 as an anti-senescence factor. Previous studies have shown that certain promitogenic growth factors and cytokines may display activities to counter the senescence process, primarily through promoting cell proliferation; however, several cytokines, such as interleukin 6, appear to play an opposite role because they are required for the execution and maintenance of OIS when they are produced by senescent fibroblasts in a cell-autonomous manner (11). In contrast to those promitogenic growth factors, TFF1 does not exhibit a mitogenic activity when it is applied to normal prostate epithelial cells despite displaying a potent suppressive effect on OIS. In this sense, TFF1 may serve as a biomarker for the transition between the senescent state and precancerous/cancerous lesions because its presence would signal an initial breach of the OIS barrier during early tumorigenesis. This notion is supported by our IHC data showing that high TFF1 expression can be detected in early stage prostate carcinoma samples. Thus, TFF1 may be considered to be an example of an antisenescence factor that could play a critical role in overcoming the OIS barrier.

As a secreted protein, TFF1 may act in both autocrine and paracrine manners to enable the TFF1-producing and neighboring cells to undergo the genetic change-induced initial transformation and expansion process against the restrictive microenvironment. Although the mechanism by which TFF1 expression is aberrantly induced during the initiation of prostate or pancreatic lesions remains to be determined, it is likely that TFF1 may allow at least a subset of premalignant cells to avoid senescence and begin to acquire additional genetic and epigenetic changes to fully evolve into a neoplastic state. To further test this, we transplanted genetically modified transgenic mice, which enabled the inducible expression of TFF1 in a tissue-specific manner to determine if the presence of TFF1 may accelerate tumorigenesis induced by activated Ras or deletion of PTEN. Using the same mouse model systems, we determined if TFF1 could additionally exert a positive effect on late-stage tumorigenesis because our results indicated that even the metastatic prostate and pancreatic cancer cells examined in our study appear to require the production of TFF1 for xenograft tumor formation. This suggests that some tumors may become “addicted” to the presence of TFF1 to facilitate the protumorigenic functions of activated oncogenes by allowing the cells to bypass antitumorigenic mechanisms (36, 37).

The physiological function of TFF1 in the lumen of the digestive tract is postulated to be that of a positive regulator for the restitution process following tissue injury such as ulceration (16–18). TFF1 is secreted into the lumen in large quantity to help wound repair, and its activity may even be associated with inflammation. Reduced TFF1 expression or loss of the TFF1 gene leads to increased tumor formation, likely due to ineffective wound healing and chronic inflammation in the epithelial tissue exposed to the harsh external environment of the gastrointestinal tract (16–18). In such a tissue context, TFF1 is functionally defined as a tumor suppressor; however, when TFF1 is abnormally overproduced in epithelial tissues of glandular organs, such as prostate and pancreas, it acts as a promoter of tumorigenesis, likely through its antisenescence activity. Thus, TFF1 represents another example of genes whose complex roles in tumorigenesis are manifested in a tissue-specific manner, although it remains to be determined if different intrinsic activities at the cellular and molecular level underlie the opposing activities of TFF1.

Although the signaling pathway by which TFF1 may exert its antisenescence effect remains to be fully elucidated, the activity of the EGFR-mediated pathway appears to be required by TFF1 to overcome the barrier of OIS, an observation consistent with a previous report that aberrant EGFR activity promotes tumor cell...
escape from OIS (38). It remains to be determined, however, if TFF1 acts to suppress OIS through direct or indirect activation of EGFR and if the EGFR-signaling cascade represents a parallel pathway, the activity of which is required for TFF1 to function as an antioncogene factor. Regardless of the upstream signaling components, it is clear that TFF1 appears to inhibit the onset of OIS by potently repressing the expression of p21, one of several cyclin-dependent kinase CDK inhibitors that are known to be involved in the initiation of cellular senescence. This idea is further supported by our finding that knockdown of TFF1 does not result in high levels of senescence in cells where p21 is also knocked down. Interestingly, the expression of p16, a factor considered to be more uniformly involved in the maintenance of senescence, is increased by the presence of TFF1, whereas the expression of two other CDK inhibitors, p14 and p15, remains unchanged, suggesting complex roles played by this group of cell cycle regulators under specific cellular contexts. Further study will be required to delineate this pathway and provide insight into the mechanisms regulating tumor progression and maintenance in the presence of oncogenic stress.

Materials and Methods

Meta-analysis. Studies investigating the expression of TFF1 (pS2, pNR-2, HP52, BCFI) in pancreatic and prostatic cancers were obtained from the PubMed database. Only reports studying TFF1 expression in the nonmalignant/malignant tissues of pancreas/prostate were analyzed. For ideal representation, reports studying extremely rare malignancies, such as neuroendocrine cancer, ampullary carcinoma, and pancreatic cystic neoplasm that together compose only <5% of all pancreatic cancer cases, were excluded from the analysis, and only studies of PanIN or PIN and adenocarcinomas were included. For the meta-analysis, only two categories were considered: nonmalignant (normal, benign, or non-neoplastic) and malignant (neoplastic and carcino-
matus). Tissues from each category were compared with each other in the analysis of the immunohistochemistry of the prostate tissue samples. This work was supported by National Institutes of Health Grants CA122998 and CA151541 (to X.-F.W.), National Institutes of Health Grant GM081650 (to E.S.), and Department of Defense Congressionally Directed Medical Research Program Prostate Cancer Fellowship W81XWH-07-10019 (to D.R.R.).

Plasmids. TFF1 cDNA was cloned from HS766T cells and cloned into a pBluescript-PI vector (39). Retroviral vectors expressing TFF1 shRNAs were generated by designing oligonucleotide selection markers into the FREETER retro-puro vector (40). Coding sequences and other vectors are listed in SI Materials and Methods.

Supporting Information

Radillof et al. 10.1073/pnas.1017269108

SI Materials and Methods

Immunohistochemical Staining. Paraffin-embedded human prostate cancer tissue was processed for antigen retrieval by heating in 10 mM sodium citrate (pH 6.0) for 20 min at 95 °C. Sections were immunostained with a monoclonal antibody against human trefoil factor 1 (TFF1) (Zymed Laboratories). Immunostaining was performed with an avidin-biotin complex staining system (Vector Laboratories) using the avidin-biotinylated-peroxidase detection method.

Plasmids. Coding sequences of the TFF1 shRNAs are AGACA-
GAGACGTGTACAGT (TFF1 shRNA-1) and GTGCTTCTAT-
CCTAACCT (TFF1 shRNA-2). pLKO.1 lentiviral nontargeting shRNA clone (NT) and TFF1 shRNA clones (TFF1 shRNA 4–7) were purchased from Sigma (MISSION shRNA bacterial stock). pBabe-HRasG12V puro vector was a kind gift from Christopher Counter (Department of Pharmacology and Cancer Biology, Duke University, Durham, NC). TRIPZ Lentiviral Inducible shRNA mir for PTEN and control vector were purchased from Open Biosystems.

Cell Culture, Transfection, and EGFR Inhibitor Treatment. HS766T, 293T, and Capan-1 cells were cultured in DMEM (Mediatech) supplemented with 10% or 20% (Capan-1) FBS. PC3 cells were cultured in RPMI (Mediatech) supplemented with 10% FBS. Kato III cells were cultured in IMDM (Gibco) supplemented with 20% FBS. PrEC, PrEC-LHS (a gift from Phillip Febbo, Institute for Genome Sciences and Policy, Duke University, Durham, NC), and PrEC-LHS-TFF1 cells were cultured in prostate epithelial basal medium (PrEBM) supplemented with PrEGM SingleQuots (Lonza). Retroviruses expressing pBabe-TFF1-puro, TFF1 shRNA 120, and TFF1 shRNA 241 were produced from a 293T packaging line. Transfections were performed using FuGene 6 reagent (Roche). Viruses were harvested 48 and 72 h after infection, filtered, and used to infect cells in the presence of 5 μg/mL polybrene. Infected cells were selected for puromycin resistance. The EGFR receptor inhibitor GW2974 (a gift from Tso-Pang Yao, Duke University, Durham, NC) was dissolved in DMSO, and the cells were exposed to the indicated doses.

Subcutaneous Injection. Cancer cells were harvested from subconfluent cell culture plates, washed with PBS, and resuspended in DMEM or RPMI with 10% FBS, and 2 × 10^6 HS766T cells or 4 × 10^6 PC3 cells in a final volume 0.2 mL were injected s.c. into 5-wk-old male Balb/C AnNcr-Nu/Nu (Nude) mice (National Cancer Institute). Mice were killed 90 d post injection, and tumors were excised and weighed.

Western Blot Analysis and Antibodies. Protein for Western blot analysis was isolated using lysis buffer pH 7.5 (50 mM Tris, 150 mM NaCl, 50 mM NaF, 0.5% Nonidet P-40). TFF1 protein levels were determined by immunoblot with an anti-TFF1 (pS2) polyclonal antibody (Santa Cruz). MEK1/2, phospho-MEK1/2, ERK, phosphor-ERK, AKT, phospho-AKT p21, p14, p15, and p16 protein levels were determined by immunoblot with specific polyclonal antibodies (Cell Signaling). Equal loading was confirmed with anti-γ-tubulin antibody (Sigma).

Real Time PCR. mRNA was isolated with the RNeasy Plus Mini Kit (Qiagen), and cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Following reverse transcription, quantitative real-time PCR was performed using iQ SYBER Green Supermix (Bio-Rad), and results were normalized to β-2-microglobulin levels. Primers for TFF1 and p21 were purchased from SABiosciences.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) Assay. Cells were plated at a density of 20 × 10^5 cells/well in a 24-well plate and grown in culture for 48 h at which time MTT reagent was added to each well and assayed according to the manufacturer's protocols. MTT absorbance readings were taken at 570 nM, and background absorbance readings were taken at 690 nM on a Beckman DU640 Spectrophotometer. Background absorbance at 690 nM was subtracted from readings taken at 570 nM for final absorbance of MTT.

Cell Cycle Analysis. PC3 and HS766T cells stably expressing either vector control or shRNA TFF1 constructs were seeded into 10-cm dishes at a density of 1.0 × 10^6 and allowed to grow overnight. Cells were then harvested and fixed with 70% ethanol overnight at −20 °C. Cells were then washed with PBS and stained with 25 μg/mL propidium iodide containing RNase A (0.1 mg/mL) for 20 min at room temperature. Cell cycle profiles were determined using a FACSscan analyzer, and the data were analyzed using CellQuest software.

Cell Cycle RT-PCR Array. Human cell cycle PCR arrays were performed according to the manufacturer's instructions (RT² Profile PCR Array Human Cell Cycle; SABiosciences).

Senescence Assays. PC3, HS766T, or Kato-III cells stably expressing either scrambled control or shRNA constructs targeting TFF1 were seeded into 35-mm dishes at a density of 1.0 × 10^5 and allowed to grow overnight. These cells were then fixed for β-gal staining (Calbiochem) or were subjected to serum starvation in media containing 0.5% FBS for the times indicated and then fixed. Cells were stained with X-gal reagent and visualized using an Olympus CK40 light microscope equipped with an Olympus DP20 digital camera. For immunofluorescence, PC3 cells were plated as described above and then fixed in 2% paraformaldehyde for 15 min, washed twice with PBS, and permeabilized with 0.2% Triton-X 100 in PBS for 5 min on ice. Cells were then blocked with 1% BSA in PBS for 30 min and incubated with primary β-gal antibody diluted 1:100 (Abcam) for 1 h at room temperature. Cells were then washed with 1% BSA in PBS and incubated with secondary antibody conjugated to FITC (Jackson Immunoresearch) for 30 min at 37 °C. Following incubation the cells were washed with PBS and coverslips were mounted (Trevenig). β-Gal fluorescence was visualized using a Zeiss Axios Imager widefield fluorescence microscope equipped with a Hamamatsu Orca ER monochrome cooled-CCD camera with IEEE. Analysis was carried out using MetaMorph software.
Fig. S1. Elevated TFF1 expression is prevalent in early stage prostatic intraepithelial neoplasia (PIN) and high-grade prostatic carcinoma but absent in normal prostate epithelium. (A) Immunohistochemistry analysis of TFF1 expression in prostate cancer. TFF1 staining is shown in brown. (a) Normal prostate epithelium, (b) PIN, (c) prostatic carcinoma Gleason score 8 (4 + 4), and (d) higher magnification of prostatic carcinoma Gleason score 8 (4 + 4). (B) Pattern of TFF1 expression in normal prostate epithelium, PIN, and prostatic carcinoma. Arrows indicate elevated TFF1 staining in the cytoplasm of prostate carcinoma cells.

Fig. S2. TFF1 mRNA levels are reduced after transduction with TFF1 shRNA.

Fig. S3. The human gastric cancer cell line Kato III does not exhibit loss of viability after TFF1 knockdown. (A) Transduction with TFF1 shRNA reduces TFF1 protein levels. (B) MTT absorbance is not affected by TFF1 knockdown in Kato III cells. (C) MTT absorbance is not reduced in Kato III cells under serum starvation conditions in the presence of TFF1 knockdown.
Fig. S4. TFF1 knockdown in Capan-1, but not in Kato III, results in increased senescence. (A) Capan-1 cells were stably transduced with four independent shRNA constructs targeting TFF1, and cellular senescence was determined after 72 h by β-gal staining (*P < 0.0001). (B) Kato III cells were transduced with two independent shRNA constructs targeting TFF1, and β-gal staining was used to quantify senescence.

Fig. S5. Addition of TFF1-conditioned media rescues the senescence in TFF1-depleted PC3 cells. (A) Senescence was measured in the presence or absence of TFF1 add-back in TFF1-depleted PC3 cells by β-gal staining or immunofluorescence using an antibody targeting β-gal. (B) β-Gal staining was quantified using light microscopy, and our results demonstrate that TFF1 add-back can overcome the senescence phenotype in TFF1-depleted cells (*P = 0.0053).

Fig. S6. Oncogene-induced senescence can be induced in normal human prostate epithelial cells by the introduction of oncogenic H-Ras. PC3 cells were transduced with a construct expressing H-Ras<sup>G12V</sup>, and β-gal staining was analyzed by light microscopy. (A) Light microscopy analysis of senescence in prostate epithelial cells transduced with oncogenic Ras or vector control expression constructs. (B) Quantification of beta-galactosidase staining in the cells analyzed in A. (C) Western blot analysis of TFF1 protein expression in prostate epithelial cells transduced with a vector control or TFF1 expression construct.
Fig. S7. Add-back of recombinant TFF1 to TFF1-depleted PC3 cells can overcome the senescence phenotype. Senescence was measured in the presence or absence of rTFF1 add-back in TFF1-depleted PC3 cells by β-gal staining, and our results demonstrate that concentrations of 10 ng/mL (*P = 0.0078) or 20 ng/mL (*P = 0.0002) of rTFF1 are sufficient to overcome the senescence phenotype.

Fig. S8. Microarray analysis and partial list of gene expression profiles measured by RT-PCR that are influenced by TFF1 depletion. (A) Scatterplot of genes whose expression levels are elevated or downregulated following TFF1 knockdown in PC3 cells. (B) A table summarizing genes whose expression levels are significantly altered following TFF1 knockdown.
Fig. S9. Ras and PTEN signaling is not significantly altered by TFF1 knockdown. Phosphorylation of MEK1/2, ERK, and AKT were determined in the presence and absence of TFF1 knockdown.